AUTHOR: ADEYILEKA-TRACZ, B.A.

TITLE: The effect of single nucleotide polymorphisms and metabolic substrates on the cellular distribution of mammalian BK channels.

YEAR: 2017

This work was submitted to- and approved by Robert Gordon University in partial fulfilment of the following degree:

Doctor of Philosophy, School of Pharmacy and Life Sciences

The Effect of Single Nucleotide Polymorphisms and Metabolic Substrates on the Cellular Distribution of Mammalian BK Channels

Bernadette Ayokunumi Adeyileka-Tracz

A thesis submitted in partial fulfilment of the requirements of the Robert Gordon University for the degree of Doctor of Philosophy

September 2017

Declaration

I declare this thesis for degree of Doctor of Philosophy has been composed entirely by myself. The experimental work which is documented was conducted by myself. Verbatim extracts and information contained within this thesis which have not arisen from the results generated have been specifically acknowledged and referenced.

Contents

Acknowledgement i

Abstract iii

1. General Introduction 1

1.1 Introduction Synopsis 1

1.2 Genetic variation in humans 1

1.3 Projects investigating human single nucleotide polymorphisms 4

1.4 Linkage of single nucleotide polymorphisms with disease 6

1.4.1 Exome screening and Genome Wide Association Studies (GWAS) 6

1.4.2 Diseases associated with SNP 7

1.5 SNPs in the context of disease treatment 8

1.6 Bioinformatics 10

1.6.1 Bioinformatics Databases 10

1.6.2 Biocuration 13

1.6.3 Bioinformatics Prediction Software 14

1.7 A focus on the BK channel 19

1.7.1 BK Channels 22

1.7.2 Complexity of the BK channel 26

1.7.3 External Environment and the BK channel 33

1.7.4 Cell membrane expression of BK channel trafficking 36

1.8 BK channel as a personalised medicine target 41

1.8.1 Pharmacological agents targeting BK channels 41

1.8.2 Disease causing SNP in BK channels 41

1.9 Thesis Aims 44

1.9.1 Rationale for Study 44

1.9.2 Aims 44

2 General Methods 46

2.1 Bioinformatics 46

2.1.1 Identification of the Human KCNMA1 Gene and Transcripts 46

2.1.2 Identification of the amino acid locations of KCNMA1 protein structural and functional sites 52

2.1.3 Identification of nsSNPs on KCNMA1 transcripts 52

2.1.4 The predicted damaging potential of BK channels nsSNP calculated by SIFT and PolyPhen-2 55

2.1.5 Assessment of previous site directed mutagenesis studies of the BK channel 55

2.1.6 Identification of PTM sites on the KCNMA1 canonical transcript 59

2.1.7 Assessment of BK channel nsSNP and Key site co-localisation 61
2.1.8 Prioritisation of BK channel nsSNP 61
2.2 Lab Based Methods 62
2.2.1 Addition of FLAG and HA antigen tags to the BK channel protein 62
2.2.2 Site Directed Mutagenesis to create BK channel mutants 62
2.3 Cell Lines 71
2.3.1 Standard Cell Culture Passage Protocol 71
2.3.2 Transfection of Cells using Lipofectamine2000 71
2.3.3 HEK293 Cell Treatments 72
2.4 Immunofluorescent Protein Labelling in HEK 293 cells transfected with BK channel constructs 74
2.5 Imaging Protocols 77
2.5.1 Confirmation of Fluorescence-Labelling of HEK Cells 77
2.5.2 Confocal Imaging of fluorescently-labelled cells 77
2.6 Quantification of Immunofluorescent labelling 80
2.7 Critical appraisal of key methodologies 82
3 Bioinformatics 87
3.1 Introduction 87
3.2 Results 90
3.2.1 The location of the human BK channel gene 90
3.2.2 BK channel transcripts 90
3.2.3 The amino acid composition of the BK channel structural and functional regions 92
3.2.4 Identification of post-translational modification sites on the BK channel 95
3.2.5 Identification of DNA variations sites on the BK channel gene 116
3.2.6 BK channel nsSNP were scored by damage prediction software 117
3.2.7 Site-directed mutagenesis of BK channel amino acids 129
3.2.8 SNP Deep Dive 132
3.3 Discussion 148
3.3.1 Creation of an ‘at a glance’ resource for the BK channel 148
3.3.2 Prioritised BK channel genetic variations may affect channel function via altered post-translational modification. 149
3.3.3 Additional Findings 151
3.3.4 Study Challenges 152
3.3.5 Implications and practical application 157
3.3.6 Future Work 159
3.3.7 In summary 160

4 Lab-Based Chapter 162

4.1 Introduction 162

4.1.1 Amino acid residues of the BK channel S0-S1 linker and modulation of channel cell-membrane expression 162

4.1.2 The potential for metabolic substrates to modulate the cell membrane expression of BK channel constructs. 163

4.2 Results 169

4.2.1 Assessment of the cellular distribution of the full-length wild-type BK channel construct (ZERO) in HEK293 cells cultured in 25mM glucose. 169

4.2.2 Assessment of the distribution of the SNP-mimetic BK channels constructs H55Q, G57A and H55Q:G57A in HEK293 cells cultured in 25mM glucose. 170

4.2.3 Assessment of the effect of glucose on the cellular distribution of the SNP mimetic and cysteine mutant BK channels in HEK293 cells 179

4.2.4 Assessment of the effect of palmitate on the cellular distribution of the SNP mimetic BK channels in HEK293 cells 181

4.2.5 Assessment of the effect of palmitate on the cellular distribution of the cysteine mutant BK channels in HEK293 cells 185

4.2.6 Assessment of the effect of oleate on the cellular distribution of the SNP mimetic BK channels in HEK293 cells 187

4.2.7 Assessment of the effect of oleate on the cellular distribution of the cysteine mutants BK channels in HEK293 cells 188

4.2.8 Assessment of the differential effect of fatty acids on the membrane expression of ZERO and the BK channel mutants. 188

4.3 Discussion 193

4.3.1 The cell membrane expression of the BK ‘wild-type’ ZERO channel 193

4.3.2 The cellular distribution of BK 'SNP-mimetic channels' and the predicted palmitoylation status of the C56 cysteine residue of the S0-S1 linker 195

4.3.3 The cellular distribution of the cysteine mutant channels C53:54A and C53:54:56A .196

4.3.4 The cellular distribution of the cysteine mutant channels C53:54A and C53:54:56A and the predicted palmitoylation status of C56 197

4.3.5 Low glucose concentration affected BK channel membrane expression in a manner related to C56 palmitoylation status 198

4.3.6 The addition of extracellular palmitate, but not oleate, increases the cell membrane expression of BK channels in HEK293 cells 199

4.3.7 The effect of palmitate and oleate, in combination with changes in glucose concentration, on BK channel distribution 200

4.3.8 The metabolic substrates glucose, palmitate and oleate may be moderating BK channel membrane expression in HEK293 cells via multiple mechanisms 201

4.4 Limitations and Further Work 205

Future Work 205

5 General discussion and Further Work 209
5.1 Thesis aims 209
5.2 Main Themes 209
5.2.1 Bioinformatics outputs require lab-based verification 209
5.2.2 The S0-S1 linker cysteine residues as cell membrane expression modulators 213
5.2.3 The potential physiological effects of H120Q and G122A mutation in humans 213
5.3 Future Work 215
5.3.1 Further investigation of the effects of H120Q and G122A mutation on the BK channel 215
5.3.2 The effect of a combination of fatty acids on BK channel cellular distribution 215
5.3.3 Additional sites for potential lab based research 215
5.4 Conclusion 217
6 References 219

List of Figures

Figure 1.1- Illustration of the DNA sequence consequence of single nucleotide polymorphisms. 3
Figure 1.2- Representation of the voltage-gated ion channel superfamily. 20
Figure 1.3- The potassium channel family. 21
Figure 1.4- Schematic of BK channel α-subunit. 25
Figure 1.5- Reversible process of palmitoylation. 31
Figure 1.6- BK channel trafficking pathway. 40
Figure 1.7- Schematic of proposed thesis workflow. 45
Figure 2.1-Locating the BK channel gene on the Ensembl database. 47
Figure 2.2- Selection of BK channel transcripts on the Ensembl database. 48
Figure 2.3- Transcript Summary and Protein Amino Acid Sequence output in Ensembl. 49
Figure 2.4- Locating KCNMA1 protein information using the UniProt database. 50
Figure 2.5- UniProt Summary of human KCNMA1 protein isoforms. 51
Figure 2.6- Uniprot output: Protein Regions and Secondary Structure. 53
Figure 2.7- Ensembl Domains and Features output. 54
Figure 2.8- Ensemble Variation Table and sample 'Missense Variant' output. 56
Figure 2.9- Ensembl KCNMA1 protein Variation Image Output. 57
Figure 2.10- UniProt KCNMA1 Natural variations and Experimental Information output. 58
Figure 2.11- Post-translational modification predictive software.. 60
Figure 2.12- Schematic of FLAG and HA tagging of BK channel constructs. 64
Figure 2.13- QuickChange II Site-Directed mutagenesis overview. 66
Figure 2.14- Amino acid sequence for mutated BK channel proteins. 68
Figure 2.15- Schematic of DNA replication by PCR. 69
Figure 2.16- Schematic of Cell Culture and Treatment Protocol. 73
Figure 2.17- Schematic of double FLAG fluorescent tagging methodology using primary and secondary antibodies. 76
Figure 2.18- Schematic of Confocal Z-Stack Image Acquisition. 78
Figure 2.19- Single slice confocal image of HEK293 cells transiently expressing the BK channel ZERO construct.. 79
Figure 3.1- Schematic of BK Channel Gene Location and Transcript Overview. 91
Figure 3.2- BK channel canonical protein structure. 93
Figure 3.3- BK channel functional domains. 96
Figure 3.4- Phosphorylation Sites on the BK Channel. 99

Figure 3.5- Percentage of total potential BK channel phosphorylation sites predicted to be phosphorylated per region (schematic view). 101
Figure 3.6- BK Channel palmitoylation scores for cysteine residues calculated by CSS-Palm 4.0 predictive software. 104
Figure 3.7- Palmitoylation sites on the BK Channel 107
Figure 3.8- BK channel S-nitrosylation scores for cysteine residues calculated by GPS-SNO predictive software. 109
Figure 3.9- BK channel predicted SUMOylation scores for lysine residues calculated by GPS-SUMO predictive software. 110
Figure 3.10- BK channel predicted N-glycosylation scores for asparagine residues calculated by NetNGlyc 1.0 predictive software.. 112
Figure 3.11- BK channel post-translational modifications: S-nitrosylation, SUMOylation, N-glycosylation 113
Figure 3.12- Summary of post-translational modifications on the BK channel 115
Figure 3.13- DNA variations on the BK channel 118
Figure 3.14- Assessment of the predicted damage of nsSNP on the BK channel 119
Figure 3.15- Comparison of nsSNP damage scoring between SIFT and PolyPhen-2. 121
Figure 3.16- SNP location and level of predicted damage in relation to structural BK channel domains. 122
Figure 3.17- SNP location and predicted damage score in relation to BK channel functional domains. 123
Figure 3.18- Summary of SNP co-localisation with BK channel functional and structural sites. 124
Figure 3.19- Summary of SNP co-localisation with BK channel linker regions. 125
Figure 3.20- Post-translational modification sites and nsSNP on the BK channel: SIFT and PolyPhen-2 damage scoring 127
Figure 3.21- Summary of post-translational modification sites and nsSNP on the BK channel. 128
Figure 3.22- Site-directed mutagenesis sites and nsSNP on the BK channel: SIFT and PolyPhen-2 damage scoring 131
Figure 3.23- Investigation into nsSNPs flanking C121 residue (H120Q and G122A) 134
Figure 3.24- Investigation into nsSNP Y359N in the BK channel pore domain 137
Figure 3.25-- Investigation into nsSNPs G694C and G694V in the RCK1-RCK2 linker 140
Figure 3.26- Investigation into nsSNP S722L in the STREX splice insert 143
Figure 3.27- Investigation into nsSNP: R1222G and R1222M 146
Figure 3.28- Schematic of thesis workflow: Summary of chapter 3. 161

Figure 4.1- The cellular distribution of ZERO channels expressed in HEK293 cells cultured in 25mM glucose. 171
Figure 4.2- Analysis of the distribution of ZERO channels expressed in HEK293 cells cultured in 25mM glucose: Raw ratio and normalised values. 172
Figure 4.3- Confocal images of BK channel SNP mimetic mutants H55Q, G57A and H55Q:G57A: channel cellular distribution in HEK293 cells cultured in 25mM glucose. 174
Figure 4.4- ZERO and SNP mimetic BK channels H55Q, G57A and H55Q:G57A distribution in HEK293 cells cultured in 25mM glucose.. 175
Figure 4.5- Normalised predicted palmitoylation scores for the S0-S1 loop cysteines for ZERO and the SNP-mimetic mutants H55Q, G57A and H55A:G57A. 177
Figure 4.6- Correlation between predicted palmitoylation score and normalised raw ratio for BK channel constructs. 178
Figure 4.7- ZERO, C53:54A and C53:54:56A cysteine mutant channel distribution in HEK293 cells cultured in 25mM glucose. 180
Figure 4.8- ZERO and SNP mimetic BK channels H55Q, G57A and H55Q:G57A cell membrane expression in HEK293 cells cultured in 5mM glucose. 182
Figure 4.9- ZERO and BK channel SNP mimetic mutants channel distribution in HEK293 cells cultured in 25mM glucose and 0.4mM Palmitate. 183
Figure 4.10- ZERO and BK channel SNP mimetic mutants channel distribution in HEK293 cells cultured in 5mM glucose and 0.4mM Palmitate. 184
Figure 4.11- ZERO, C53:54A and C53:54:56A cysteine mutant channel distribution in HEK293 cells cultured in 5mM glucose and 0.4mM palmitate. 186
Figure 4.12- ZERO and BK channel SNP mimetic mutants channel distribution in HEK293 cells cultured in 25mM glucose with and without 0.4mM oleate. 190
Figure 4.13- ZERO and BK channel SNP mimetic mutants channel distribution in HEK293 cells cultured in 5mM glucose with and without 0.4mM oleate. 191
Figure 4.14- The effect of fatty acid on the cell membrane expression of ZERO and SNP-mimetic channels cultured in 25mM or 5mM glucose. 192
Figure 4.15- AMPK activation as a mechanism of glucose control of BK channel cell-membrane expression. 204
Figure 4.16- Schematic of thesis workflow: Summary of chapter 4. 208
Figure 5.1- Sites of tyrosine phosphorylation of interest for future investigation. 216
Figure 5.2- Schematic of thesis workflow: Thesis summary and future work. 218

List of Tables

Table 1.1- Summary of the main aspects of primary and secondary bioinformatics databases.. 11

Table 2.1- BK Channel Site Directed Mutagenesis Primer Sequences. 67

Table 3.1- Details of the reference KCNMA1 transcript (canonical transcript). 92

Table 3.2- Experimentally verified phosphorylation sites on the BK channel. 103

Table 3.3- Experimentally verified palmitoylation sites on the BK channel.. 106

Table 3.4-Examples of Site Directed Mutagenesis of the BK channel. 130

Table 4.1- Conversion of hSlo amino acid numbering to mSlo equivalent over residues of investigation in the S0-S1 linker 162

Abbreviations

ACC: acetyl-CoA carboxylase

AMPK: 5' AMP-activated protein kinase

APT: acyl-protein thioesterase

BK channel: large conductance calcium- and voltage-activated potassium channel

BSA: bovine serum albumin

CFTR: cystic fibrosis transmembrane conductance regulator

CO: carbon monoxide

CNV: copy number variation

cDNA: coding DNA

CSS-Palm: clustering and scoring strategy- palmitoylation

DMEM: Dulbecco's Modified Eagle Medium

DNA: deoxyribonucleic acid

EIV: entrance to the intracellular vestibule

ENCODE: Encyclopaedia of DNA Elements

ER: endoplasmic reticulum

GA: Golgi apparatus

GEPD: generalized epilepsy and paroxysmal dyskinesia

GFP: green fluorescent protein

GLUT: glucose transporter

GPCR: G protein-coupled receptors

GWAS: genome wide association studies

HA: hemagglutinin

HBSS: Hanks Balanced Salt Solution

HEK: Human embryonic kidney

HIF-1a: hypoxia inducible factor 1 a

hSlo: human BK channel

KCa channel: calcium-activated potassium channel

Kv channel: voltage-gated potassium channel

LQTS: Long QT Syndrome

mSlo: murine BK channel

NAR: nucleic acid research

Nav: voltage-gated sodium channels

NCBI: National Centre for Biotechnology Information

nsSNP: non-synonymous single nucleotide polymorphism

PAT: palmitoyl acyl transferases

PCC: Pearson's correlation coefficient

PFD: pore-forming domain

PKA: protein kinase A

PKC: protein kinase C

PKG: protein kinase G

PolyPhen: polymorphism phenotyping

PP1: protein phosphatase 1

PPARγ: peroxisome proliferator-activated receptor gamma

PPT: palmitoyl-protein thioesterases

PTM: post-translational modification

RCK: regulator of the conductance of potassium

ROI: region of interest

RR: raw ratio

SDM: site directed mutagenesis

SNP: single nucleotide polymorphism

SIFT: Sorts Intolerant From Tolerant

STREX insert: stress axis regulated insert

TG: triglyceride

TMD: transmembrane domain

UniProt: Universal Protein Resource

UTR: untranslated region

VSD: voltage sensing domain

WTSI: Wellcome Trust Sanger Institute

YFP: yellow fluorescent protein

zDHHC: aspartate-histidine-histidine-cysteine motif with a zinc finger

Acknowledgements

Undertaking this PhD has been a character building experience for me and it would not have been possible without the support and guidance that I received from many people.

Firstly, I would like to thank the Robert Gordon University (IHWR) for funding my three-year studentship, without this, it would not have been possible for me to return to study.

Importantly, I would like to thank my principal supervisor and academic mentor Dr Iain Rowe, for his varied and vast technical expertise, attention to detail, patience, guidance and creative ideas. Thank you for always keeping a sense of optimism, and for being able to find a practical solution for every challenge. I cannot express how much your holistic view on life and research has benefited me and my work during my time as your PhD student. Once again, thank you.

I would also like to thank my co-supervisory team of Dr Stuart Cruickshank and Dr Helene Widmer for providing support, suggestions, feedback and comment on my work, and importantly for the data discussion sessions that prepared me for life as an academic! A special thank you again to Helene for the hours spent with me at the patch-clamp rig, mixing physics, chemistry, biology and French.

Thanks also go to Prof Alison Strath, not only for enabling my data collection period in Edinburgh, but for providing me with professional mentorship at the highest level. You have supplied an example of what can be achieved professionally with extremely hard work, focus and perseverance. Thank you for every opportunity that you afforded me.

I would like to thank Prof Mike Shipston at The University of Edinburgh for allowing me access to the facilities in the lab and for your guidance on the data and research direction. I'd also like to thank Dr Lie Chen, Dr Lijun Tian, Dr Peter Duncan and Dr Danlei Bi for their technical support and advice whilst I was in Edinburgh.

Special thanks go to Heather McClafferty for not only for patiently guiding me with her outstanding technical expertise, but for her genuine interest in my project. Our many discussion sessions in the lab were invaluable to me, and form some of the fondest memories I have of my time as a PhD student. Finally, thank you for never giving up on me and teaching me how to use my strengths to complete this project.

I would like to thank Georgi Hristov for being a friend and debating partner for life and also for giving me the hard to hear feedback when I needed it most. Without this, I would not have managed to complete my thesis on time.

To the wonderful Amy, thank you for the many laughs in the lab, and for your encouraging words and wisdom during the write-up period. Thank you, Vin, for your guidance and encouragement when times were at their most challenging.

Thank you to my family for their love and support; and for answering, with love, the millions of questions that I have asked you during my lifetime! I'd like to start by thanking my dad for helping me to believe that my wildest dreams are possible, and for giving me the upbringing and tools to achieve my goals. Thank you for working hard to support me financially through my studies, and for doing everything in your power to make sure that I do the best that I can. I'd like to thank my mum for always being there for me at any time of day or night, for her prayers and gentle but 'tough-love' way of getting me through the difficult times. I'm sorry for the worry that I have caused you over the years, but know that I couldn't have gotten this far without you. I'd like to thank my little brothers Daniel and Samuel, who are way too wise for their age, and who remind me constantly to work hard but to have fun at the same time. I also would like to thank my dearest uncle, Pastor Olumide for his mentorship, prayers and advice.

I would like to thank my husband and fellow ENTP Greg. I could not have hoped for a better person to spend my life with. Thank you for being my soul mate, counsellor, debating partner, travel buddy and graphics designer. Without you, I wouldn't have had the strength to complete this PhD! Our life will never be boring and I love you.

Thanks are also due to Greg's parents Michel and Danuta for their unconditional support, especially during the write-up period. Merci beaucoup pour tout!

Abstract

Humans are approximately 99% similar with inter-individual differences caused in part by single-nucleotide polymorphisms (SNPs), which poses a challenge for the effective treatment of disease. Bioinformatics resources can help to store and analyse gene and protein information to address this challenge, however these resources have limitations, so the collation and biocuration of gene and protein information is required. Using the large conductance calcium- and voltage-activated potassium channel, also known as the Big Potassium (BK) channel as an example, due to its ubiquitous expression and widespread varied role in human physiology, this study aimed to prioritise SNPs with the potential to affect the function of the channel. Using a BK channel resource created with bioinformatics tools and published literature, mSlo SNPs H55Q and G57A, located in the S0-S1 linker, were prioritised and selected for lab-based verification. These SNPs flank three cysteine residues proven to modulate channel cellular distribution via palmitoylation, a reversible process shown to increase protein association with the cell membrane. The SNPs alter the predicted palmitoylation status of C56, one of the cysteine residues located in the S0-S1 linker. The cellular distribution of BK channels incorporating the SNPs was assessed using confocal microscopy and revealed that the direction and magnitude of SNP mimetic cell membrane expression was closely related to the C56 predicted palmitoylation score; a 'C56 palmitoylation pattern' was observed. It was shown that exposure to metabolic substrates glucose, palmitate and oleate modulated SNP-mimetic cellular distribution and could invert the 'C56 palmitoylation pattern', indicating that there is interplay between the metabolic status of the cell and the amino-acid composition of the channel via palmitoylation. The creation of a novel BK channel resource in this thesis highlighted the limitations, and inter-dependency of bioinformatics and lab based experimentation, whilst SNP verification experiments solidified the link between S0-S1 cysteine residues and BK cellular distribution. BK channel function is linked with a number of physiological processes; thus, the potential clinical consequences of the SNPs prioritised in this thesis require further research.

Keywords: SNPs, BK channel, cell membrane expression, palmitoylation, glucose, palmitate, oleate, biocuration, cysteine, bioinformatics

1. General Introduction

1.1 Introduction Synopsis

Every human has a unique genetic composition, with DNA variations contributing to genotypic and phenotypic differences existing between individuals (Levy *et al.*, 2007); in some cases DNA variations contribute to the development of disease (as reviewed by Goldstein, 2009; Hindorff *et al.*, 2009; Albert and Kruglyak, 2015). These inter-individual variations pose a challenge for the effective treatment of disease, as any one therapy may not be equally effective for all. Personalised medicine aims to take into account these inter-individual variations, and seeks to determine the individual's risk for disease and probable response to treatment (Guttmacher and Collins, 2003; Burke and Psaty, 2007). This process aims to target and optimise disease screening and treatment therapies at the individual level, rather than at the 'average' of the population, to increase treatment efficacy. Personalised medicine utilises bioinformatics resources to facilitate the storage and analysis of the vast amount of biological information produced with the aim of identifying new genetic targets in the treatment of disease (Fernald *et al.*, 2011; Overby and Tarczy-Hornoch, 2013; Alyass *et al.*, 2015). The number of genetic variations across the whole genome is too vast to assess as part of any one thesis. Consequently, for the purpose of this work, a single gene was selected to investigate the prioritisation of potentially harmful single nucleotide polymorphisms, on the basis of existing information available in bioinformatics resources. The BK channel, a potassium channel found ubiquitously in the human body, and involved in processes such as neuronal function and the regulation of smooth muscle tone was selected. Genetic variations occurring in the BK channel gene have been shown to result in disease (Du *et al.*, 2005; Díez-Sampedro *et al.*, 2006; Wang, Rothberg and Brenner, 2009; Plante, 2016); proving that mutations in the BK channel gene can affect normal physiology, making the channel a worthy protein for research.

1.2 Genetic variation in humans

Every human has a unique genetic code, comprised of approximately 3 billion base pairs, the building blocks of DNA (Lander *et al.*, 2001; Venter *et al.*, 2001). The first large scale multinational investigation into the base pair composition of the complete human genome was undertaken by the Human Genome Project. The consortium comprising 20 international research centres began the investigation in 1990 and concluded with the announcement of the near-complete (99%) human DNA sequence in 2003 (Human Genome Sequencing Consortium, 2004). This created the first reference map of the

human genome, and facilitated research into the genetic differences between individuals not only by providing increased detail and understanding of genomic composition, but by releasing study findings to open access resources within 24 hours of discovery (Guyer and Guyer, 1998). Research has estimated that there is approximately 95% to 99.9% sequence homology between humans (Collins and Mansoura, 2001; Human Genome Sequencing Consortium, 2004; Levy *et al.*, 2007), with the remaining percentage of mismatch associated with genetic variation. Genetic variation is responsible for the visible phenotypic differences observed between individuals, such as eye colour, height and the ability to roll the tongue, but also for genotype differences that are not immediately apparent, such as blood type and the shape of the red blood cells. Genetic variation is multi-level and serves to increase the diversity between individuals, such as during meiosis occurring in the process of normal human reproduction (as reviewed by Thompson, 2013).

Human variation can occur out with the DNA, such as in the case of epigenetics, which involves the expression or silencing of genes without alterations to the DNA base pair sequence (as reviewed by Mazzio and Soliman, 2012). Epigenetics has been associated with many disease states, with evidence reviews conducted for diabetes (Sommese *et al.*, 2017), cardiovascular disease (Muka *et al.*, 2016), cancer (Biswas and Mallikarjuna Rao, 2017), autism (Grafodatskaya *et al.*, 2010) and mental retardation (Nageshwaran and Festenstein, 2015). There is also evidence of variations in the number of copies and order of genes in the DNA, or alterations to large sections of DNA creating 'copy number variations' (CNVs) which add to inter-human variation (as reviewed by Zhang *et al.*, 2009; Zarrei *et al.*, 2015). CNVs have been associated with diseases such as Crohn's disease (Fellermann *et al.*, 2006; McCarroll *et al.*, 2008), psoriasis (Hollox *et al.*, 2008) and Alzheimer disease (Rovelet-Lecrux *et al.*, 2006). Whilst variation occurs via a number of mechanisms, it is thought that the most frequent type of sequence variation is the single nucleotide polymorphism (SNP) (Human Genome Sequencing Consortium, 2004).

Single nucleotide polymorphisms

The human DNA sequence is composed of a chain of four nucleotide bases: Adenine (A), Thymine (T), Guanine (G) and Cytosine (C), structured in two complementary DNA strands within a chromosome. A single nucleotide polymorphism or a SNP is a variation or 'point mutation' of a single nucleotide within the DNA sequence. The convention is that the frequency of a DNA point mutation must be above 1% in the population to qualify as a SNP (Cavalli-Sforz and Bodmer, 1971; Arias, Jorge and Barrantes, 1991).

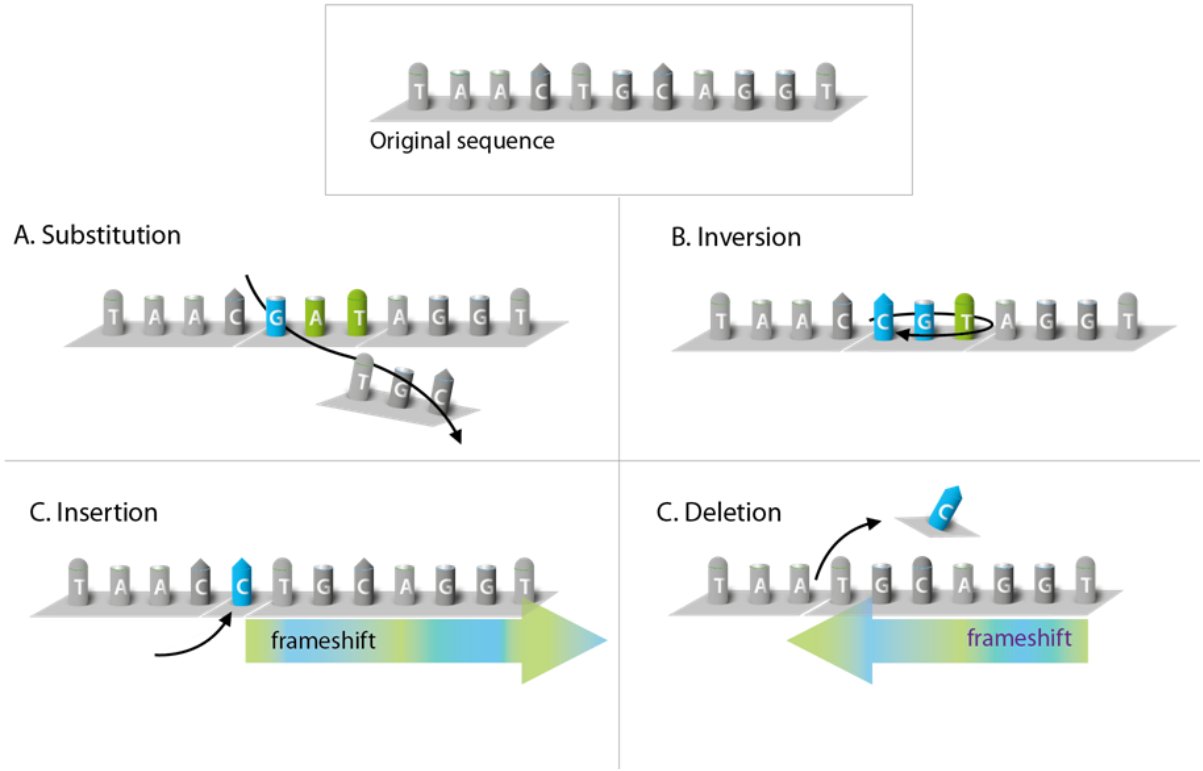


Figure 1.1- Illustration of the DNA sequence consequence of single nucleotide polymorphisms. Detailed in this figure are the four different categories of single nucleotide polymorphism consequence on the DNA base pair sequence. Illustrated is A) substitution, B) inversion, C) insertion and D) deletion. The top row of letters shows the original base pair sequence, whilst each of the quadrants illustrates the consequence of the DNA mutation. Adapted from Genome Research Limited, 2017.

However, DNA variations with a lower frequency have also been referred to as SNP (Wong *et al.*, 2003; Bush and Moore, 2012; Yoo, Sun and Bull, 2013), and have been shown to have downstream effects. Consequently, revision of the criteria has been suggested (Karki *et al.*, 2015). As shown in Figure 1.1, there are four types of DNA mutation: substitution, inversion, insertion and deletion. In addition, there is further categorisation of SNP depending on their location in the gene, and their downstream consequence. SNPs located within the exonic (protein coding) region of the DNA in which a base pair substitution results in a change in the translated amino acid sequence of the protein are called 'missense' or non-synonymous SNP (nsSNP). Synonymous SNP (sSNP) cause a change in base pair sequence, however do not result in a change in amino acid.

Non-protein coding areas of the DNA include 5' and 3' untranslated (UTR), intronic and regulatory regions. SNPs in the non-coding regions of DNA have no direct effect on the amino acid composition of a protein, but may augment the expression of proteins produced by affecting areas in the DNA associated with regulation of transcription and translation (as reviewed by Shastry, 2009; Zhang and Lupski, 2015). DNA mutations can change the amino acid sequence of a protein, or affect the amount of protein expressed, therefore having the potential to disrupt physiological processes and result in disease. As a result of this potential, the identification of the number, location and consequence of SNP has been the focus of large scale investigative projects. A selection of the projects most relevant to the thesis work are introduced below.

1.3 Projects investigating human single nucleotide polymorphisms

The ENCODE project

The Encyclopaedia of DNA Elements (ENCODE) project was launched in 2003 by the US National Human Genome Research Institute (NHGRI) and aimed to develop and apply high-throughput approaches to detect DNA sequence elements that confer biological function (The ENCODE Project Consortium, 2004). In several publications spanning over a decade, the ENCODE project determined that over 80% of the human genome was 'active' and associated with biological functions, such as controlling coding DNA (cDNA) levels, and thus protein expression levels (The ENCODE Project Consortium, 2004, 2007; Ecker *et al.*, 2012; ENCODE Project Consortium and The ENCODE Project, 2012; Kellis *et al.*, 2014). To encourage use of the ENCODE project outputs, rapid data release to the scientific community was agreed (The Wellcome Trust, 2003). The ENCODE project added to the reference genome mapped by the Human Genome Project, by taking steps towards the accurate annotation of the function of DNA elements in the genome; this facilitated the classification of SNP potential damage as more was known about the regions in which they occurred (Bryzgalov *et al.*, 2013).

The 1000 genomes and the 100,000 genomes projects

In 2001, the International SNP Map Working Group identified the location of 1.42 million SNPs (Sachidanandam *et al.*, 2001), comprised of data from the Human Genome Project (Venter *et al.*, 2001) and the SNP Consortium (Lander *et al.*, 2000). As technology advanced and the number of genomes sequenced increased, the estimated number of SNPs also increased; the approximate value in a recent publication by the 1000 Genomes Project Consortium was 84.7 million (Auton *et al.*, 2015). The 1000 genomes project aimed to identify 95% of 'common' SNPs (those occurring above a frequency of 1%) in the genome. At the completion of the study in 2015, the project had successfully sequenced the genomes of 2,504 individuals from 26 populations (Auton *et al.*, 2015; Sudmant *et al.*, 2015). In 2012, the Genomics England initiative, the '100,000 genomes project' was announced (Cameron, 2012; Torjesen, 2013). The project focuses on the identification of SNPs associated with rare disease and cancer by sequencing the genomes of individuals effected by disease, their family members and the genetic material contained within the tumours of those with cancer. The aim is to facilitate the identification and treatment of patients suffering from rare diseases and cancer by the creation of a comprehensive database of genomic material and SNPs, which can be used by clinicians to sub-group and stratify patients to the most effective treatments.

The International HapMap Project

The International HapMap Project commenced in 2002 and aimed to create a haplotype (group of genes inherited together) map (HapMap) of the human genome to facilitate the discovery of genes and genetic variations that affect health, disease and response to drug treatments (Goldstein and Weale, 2001; The International HapMap Consortium *et al.*, 2003; Thorisson *et al.*, 2005; International HapMap Consortium, 2007). A goal was that the resource would reduce the number of SNPs required to be investigated in order to establish association with a phenotype, and utilised linkage disequilibrium (the non-random association of alleles and thus SNP at different sites in the DNA) to achieve this goal. The project data release in 2007 provided the location and base pair consequence of approximately 3 million SNPs from the genome of 1400 individuals from 11 populations (International HapMap Consortium, 2007). This information was used to create technologies containing SNP information that could reduce the time required to conduct a basic screen of the genome, such as Chip-based genotyping assays (Edenberg and Liu, 2009). The popularity of The 1000 Genomes Project resulted in traffic to the HapMap website decreasing dramatically; this alongside issues with security resulted in it being closed down in June 2016 (Buchanan *et al.*, 2012; National Center for Biotechnology Information, 2016).

Progress made by the international collaboration of large research organisations has enabled an exponential expansion in the understanding of the composition of the human genome, the role and location of genes, as well as a more accurate estimation of the number of single nucleotide polymorphisms. Investigations now focus on linking SNP with disease; these include global collaborations to complete genome wide association studies and large-scale exome screening. These genome investigation, SNP identification and SNP-disease association projects work alongside one and other and inform each other to advance upon current understanding.

1.4 Linkage of single nucleotide polymorphisms with disease

1.4.1 Exome screening and Genome Wide Association Studies (GWAS)

SNPs occurring in non-coding DNA regions are associated with disease (Greenwood and Kelsoe, 2003; Yasuda *et al.*, 2008; Nascimbeni *et al.*, 2010; Castaman *et al.*, 2011), however, nsSNP continue to be studied extensively due to their direct effect on the amino acids residues comprising proteins. It is proposed that 83% of disease-causing mutations affect the stability of proteins (Wang and Moult, 2001). In addition, estimates suggest that nsSNP in exonic regions constitute approximately 80-90% of inherited-disease causing mutations (Cooper, Krawczak and Antonorakis, 1995), and approximately 50% of the entries of disease causing genetic changes in the human gene mutation database (HGMD) (Stenson *et al.*, 2014). Exome screening (also known as exome sequencing) is a method used to investigate the occurrence of SNP in the genome and link genetic variations with disease. Exome screening is efficient at detecting low frequency genomic differences resulting in rare disease, due to low cohort number requirements (Gilissen *et al.*, 2011). Exome screening focuses on only the exonic regions of the DNA, which comprises less than 2% of the whole genome; although advances in technology are reducing the cost of whole genome screening, exome screening can produce outputs in a much shorter timeframe and a reduced cost in comparison to whole genome screening (Belkadi *et al.*, 2015; Meienberg *et al.*, 2016). As a result, exome screening is more commonly used in a clinical setting (as reviewed by Majewski *et al.*, 2011; Retterer *et al.*, 2016).

A genome wide association study (GWAS) is the examination of thousands to millions of genetic variants in large cohorts of individuals to assess if a mutation is associated with a phenotype or disease trait, (as reviewed by Pearson and Manolio, 2008; Wang and Wang, 2009; Visscher *et al.*, 2012). The principles of GWAS were first proposed in 1996 (Risch and Merikangas, 1996), and the first major analysis results by large research groups were published a decade later (Klein *et al.*, 2005; DeWan *et al.*, 2006; Wellcome Trust Case Control Consortium, 2007; Manolio and Collins, 2009). GWAS investigations are conducted across the whole genome and are hypothesis generating rather than

hypothesis driven, as a hypothesis is not formed in advance of undertaking the study (Hirschhorn and Daly, 2005). Variant association is determined by powered statistical analysis, which assesses the frequency of SNPs in participants sharing a phenotypic trait or disease straits; those SNPs are then taken forward for further analysis and verification. GWAS are well suited to associate genetic variations with disease in SNP with a relatively high frequency, above 1%. This guideline cut-off is because current technology limits the number of variant sites that can be incorporated into analysis arrays to approximately 5 million, and an ever increasing number of sample genomes will be required to provide statistical power to accurately detect associations with low frequency SNP (Lam *et al.*, 2006; Tabangin, Woo and Martin, 2009; Corvin, Craddock and Sullivan, 2010).

As described in the review articles, a challenge of GWAS is that the location of SNP in the genome of interest must have already been established to compare SNP frequency between subjects; there is also the requirement to have a large number of genome samples with detailed subject information, such as ancestry and phenotype description, from which to compare. Advantages of GWAS are that a) pre-selection of target regions of the gene to be screened is not required, b) it is not necessary to have prior understanding of the function of genes in the genome to associate them with disease and c) a large number of SNPs, in the order of millions, can be investigated simultaneously. However, exome screening in contrast to GWAS, is not reliant on previous database of SNP, and can be used to identify undiscovered SNP in exome in regions of DNA (as reviewed by Lee *et al*. 2014).

1.4.2 Diseases associated with SNP

SNPs are being directly attributed to the causation and development of many diseases. GWAS in combination with exome screening have successfully led to the identification of genes previously unknown to be associated with disease, such as peroxisome proliferator-activated receptor gamma (PPARγ) in relation to type II diabetes (Yen *et al.*, 1997; Ruchat *et al.*, 2010) and hypothalamus located melanocortin 4 receptor (MC4R) associated with body mass index (Loos *et al.*, 2008; Wang *et al.*, 2011) to name a few. However, there are diseases, especially inherited diseases, where a causal link to genetic mutations, rather than an association, has been established. A well-known example is sickle-cell anaemia, were a nsSNP substituting glutamic acid with valine in the β-globin gene coding for haemoglobin results in 'sickle' shaped red blood cells. These abnormally shaped blood cells are more likely to clump together and result in haemolytic anaemia (Herrick, 1910; Taliaferro and Huck, 1923; Ingram, 1959).

Another example is Cystic Fibrosis, where multiple mutations on the CFTR gene cause Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) protein malfunction, (as reviewed by Moskowitz *et al*.

2008). CFTR is a cAMP-dependent chloride channel located in epithelial cells which regulates salt transport and fluid flow; malfunction of the CFTR protein results in airway disease, pancreatic failure, meconium ileus and male infertility (as reviewed by Hull 2012). Phenylketonuria (PKU), where a series of damaging nsSNPs affecting the phenylalanine hydroxylase (PAH) gene results in impaired hydroxylation of phenylalanine to tyrosine, causing hyperphenylalaninemia and if untreated, intellectual disability and neurological abnormalities, (discussed by Gámez *et al.*, 2000; Blau *et al.*, 2010). Further examples of mutations causative of disease are the BRCA1 and BRCA2 gene mutations, which are proposed to result in a 5 to 30-times increased risk of breast cancer and ovarian cancer; however the exact percentage of increased risk is yet to be determined (Antoniou *et al.*, 2003; Pal *et al.*, 2005; Chen and Parmigiani, 2007; Levy-Lahad and Friedman, 2007). Huntington's disease, identified in 1872 (Huntington, 1872) is caused by mutations in the HTT gene responsible for the production of the protein huntingtin. In patients suffering from Huntington's disease, the CAG segment within the gene is repeated 36 to 120 times, rather than the 10 to 35 in people without the disease, as discussed by (The Huntington's Disease Collaborative Research Group, 1993; Petersén A *et al.*, 2001; Zheng and Diamond, 2012). The specific role of the huntingtin protein is not yet known, however base pair repeats in the HTT gene have been shown to cause chorea, dystonia, cognitive and psychological disorders.

Thus far, global collaborative research efforts such as The Human Genome Project, HapMap and 1000 genomes project have advanced knowledge of the composition of the human genome, the physiological role of genes, the location of SNPs, and the role of SNP in disease. Increasing knowledge, and the improvement of disease diagnosis and treatment were long-term goals of the global research projects. To realise these goals, a new field of medicine has emerged that seeks to make use of the huge volume of insights and data produced from these studies to better target disease therapies to individuals.

1.5 SNPs in the context of disease treatment

Each individual has a unique genetic composition; when treating complex diseases, unlike those affecting a single gene, inter-individual genetic variations pose a challenge for healthcare professionals as diseases with a similar phenotypic presentation may be of differing aetiology and thus no one treatment may be suitable for all. An example of this would be the case of diabetes type II (which presents as deranged glucose and lipid levels) as the disease is one of linked, however heterogeneous factors of aetiology and progression. Contributing factors include peripheral insulin resistance, reduced pancreatic beta-cell insulin secretion, increased secretion of glucagon consequently increasing hepatic gluconeogenesis, and enhanced glucose re-uptake in the kidneys (as reviewed in

Cerf 2013; Farber, Berger and Earle 1951; Moon and Won 2015; Taylor 2012). As a result, it is unlikely that a standard treatment would be sophisticated enough to account for the inter-individual differences.

Personalised medicine (sometimes referred to as precision medicine, stratified medicine or individualised medicine) seeks to take into account inter-individual variations, and aims to determine the individual's risk for disease and probable response to therapy in order to achieve optimum treatment outcomes (Guttmacher and Collins, 2003; Burke and Psaty, 2007). This is achieved by sub-grouping individuals according to the biological or risk characteristics, also known as 'stratifying' individuals (Trusheim, Berndt and Douglas, 2007), which represents a paradigm shift in the thought process behind patient treatment, and is a step away from treating the 'average' patient.

Personalised medicine has been implemented successfully in a number of cases; advance testing is with vemurafenib (brand name Zelboraf®), a drug used to treat advanced malignant melanoma (Chapman *et al.*, 2011), which is only effective in those with a V600X mutation in the gene 'BRAF' which codes for the oncogenic protein kinase 'B-Raf'. Vemurafenib is a potent inhibitor of mutated B-Raf and has marked anti-tumour effects against melanoma cells expressing the BRAF V600E mutation (Tsai *et al.*, 2008). Another example of disease stratification in personalised medicine is in the prediction of 6-mercaptopurine efficacy by assessment of gene deficiency and SNP occurring on the thiopurine methyltransferase (TPMT) gene (Karas-Kuzelicki and Mlinaric-Rascan, 2009). Treatment regimen and 6-mercaptopurine dosage can then be amended to the individual patient.

The field of personalised medicine seeks to utilise information regarding the molecular and genetic mechanisms of disease to improve treatment; therefore it is informed by genomics, proteomics, metabolomics and pharmacogenomics (Chen and Snyder, 2013; Glauber, Rishe and Karnieli, 2014). The volume of biological information associated with these 'omics' is vast and varied, therefore personalised medicine utilises bioinformatics to store, analyse and manipulate this data (Bao *et al.*, 2014).

1.6 Bioinformatics

1.6.1 Bioinformatics Databases

Bioinformatics is a combination of biology, informatics, computing and statistics and is used to store and perform powerful analysis of biological data (as reviewed by Luscombe, Greenbaum and Gerstein, 2001; Pabinger *et al.*, 2014). This storage and analysis of biological data is achieved through the utilisation of databases and software programs. The number and type of bioinformatics databases is vast and varied, comprising of primary databases that store basic, experimentally derived biological data such as nucleotide and protein sequences; whilst secondary databases summarise and further process the data from primary databases and data contained within the literature (Luscombe, Greenbaum and Gerstein, 2001; The European Bioinformatics Institute, 2017). A summary of the main aspects of primary and secondary bioinformatics databases, and examples of each are contained within Table 1.1.

The latest release of the database issue of the journal Nucleic Acid Research (NAR), which is the key source for a comprehensive list of curated databases, states there are 1,685 live biological databases available online (Rigden, Fernández-Suárez and Galperin, 2016). The team at NAR ascertained the number of 'live' databases using measures such as the number of data downloads, literature citations and web links from outside sources (Cochrane and Galperin, 2010; Rigden, Fernández-Suárez and Galperin, 2016). The 1,685 databases are comprised of many broad categories, often developed to cater to a specific need. Examples include organism specific databases such as FlyBase and WormBase (Howe *et al.*, 2016; Millburn *et al.*, 2016), disease specific databases such as the cancer database COSMIC (Forbes *et al.*, 2011), databases for protein families (Finn *et al.*, 2010) and of protein nomenclature, such as ExPASy for enzymes (Gasteiger *et al.*, 2003). There is evidence however that the databases are not accessed with equal frequency; a recent assessment of bioinformatics database and software usage discovered that 5% of resources accounted for 47% of total database usage (Duck *et al.*, 2016). This implies that the vast majority of databases are not commonly frequented; this finding was in line with other articles that discussed the issues associated with unused databases, such as under-utilisation of relevant data and the waste of financial investment (Cannata *et al.*, 2005; Wren and Bateman, 2008). Due to the overwhelming number and categories of databases available, only the databases used in the thesis will be introduced below.

	Primary database	Secondary database
Synonyms	Archival database	Curated database, knowledgebase
Source of data	Direct submission of experimentally-derived	Results of analysis, literature research and interpretation, often of data in primary databases
Examples	• ENA, GenBank, and DDBJ (nucleotide sequence) • ArrayExpress Archive and GEO (functional genomics data) • Protein Data Bank (PDB coordinates of three-dimensional macromolecular structures)	• InterPro (protein familites, motifs and domains) • UniProt Knowledgebase (sequence and functional information on proteins) • Ensembl (variation, function, regulation and-more layered onto whole genome sequences)

Table 1.1- Summary of the main aspects of primary and secondary bioinformatics databases. Included in the table are the synonyms, source of data and examples of primary and secondary bioinformatics databases. Adapted from The European Bioinformatics Institute, 2017.

NCBI

The National Center for Biotechnology Information (NCBI) was established in 1988 as a national resource for molecular biology information, which aimed to develop new technologies to facilitate understanding of the molecular and genetic origins of health and disease (Woodsmall and Benson, 1993). This was achieved in the creation of automated systems for storing and analysing data produced by molecular biology, biochemistry and genetics, and is accessible from www.ncbi.nlm.nih.gov . NCBI is host to other established databases such as PubMed, the sequence alignment tool BLAST and the short genetic variations database dbSNP (Sherry *et al.*, 2001).

Ensembl

The Ensembl Project is a collaboration between the European Bioinformatics Institute (EMBI-EBI) and the Wellcome Trust Sanger Institute (WTSI) and began in 1999 (Hubbard *et al.*, 2002). The aim of the project was to automatically annotate the genome and integrate this annotation with biological data generated from data generation projects such as the Human Genome Project. In line with the Human Genome Project, another aim of Ensembl was to enable open access to the data produced via the web (Hubbard *et al.*, 2002). To this end, the Ensembl database produced a free to access database in July 2000 (The European Bioinformatics Institute, 2000) and is accessible from http://www.ensembl.org/index.html. Although categorised as a secondary database (see Table 1.1), the database includes primary data (nucleotide sequence), in addition to comparative genomics, variation data and regulatory data.

UniProt

The Universal Protein Resource (UniProt) is a collaboration between the European Bioinformatics Institute (EMBL-EBI), the SIB Swiss Institute of Bioinformatics and the Protein Information Resource (PIR) and is accessible from http://www.uniprot.org/. The UniProt Knowledgebase (UniProtKB) database (accessible from http://www.uniprot.org/uniprot/) is a secondary database and contains functional information on proteins with annotation conducted manually by Swiss-Prot (Bairoch and Boeckmann, 1991), and computationally by TrEMBL (Bairoch and Apweiler, 1996). Swiss-Prot entries are reviewed and manually annotated, integrating results from lab-based experimentation and scientific conclusions (Bairoch and Boeckmann, 1991; Boeckmann *et al.*, 2003). However, as the number of sequence data submissions to the UniProt database for inclusion increased, the capacity for timely review was exceeded. Therefore TrEMBL (Translated EMBL Nucleotide Sequence Data Library) was created to annotate computationally and increase the speed of review. It is the aim of

UniProt to manually annotate all sequences; therefore, sequences annotated computationally by TrEMBL may later be reviewed Swiss-Prot. Although categorised as a secondary database (see Table 1.1), the database includes primary data (nucleotide sequence), in addition to comparative genomics, regulatory data and variation data from sources such as dbSNP and the 1000 genomes project.

1.6.2 Biocuration

Biocuration is the processes of collecting, interpreting, organising and validating biological information. This process is used by individuals and large scale projects such as CCDS and Swiss-Prot and serves as a form of 'quality control' to improve the accuracy and quality of data, for instance the information held in bioinformatics databases (Boeckmann *et al.*, 2005; Pruitt *et al.*, 2009). Key to this process is the integration of information from multiple sources such as scientific literature and large datasets produced by next-generation sequencing, and the clarification of discrepancies in this information such as protein name, sequence, structure, function and interactions (Altman, 1998). The International Society for Biocuration (ISB) encourages the profession to facilitate the linking of researchers to biocurated data in an effort to ensure the data is in the ideal form to support further investigation and computational analysis (Bateman, 2010). As databases are increasingly becoming a mainstay in biological research, biocuration of the data has moved from a niche to something that is essential; however, the 'gold standard' of manual biocuration is 'time-expensive' and cannot match the speed of data generation. Automated methods such as TrEMBL aim to tackle this challenge (Boeckmann *et al.*, 2003); however, often a large amount of information associated with a protein will remain un-curated and can result in protein records that are not comprehensive.

Consensus CDS (CCDS)

The annotation of genes is achieved by combining information from multiple sources (such as journal articles, raw data from high throughput screens and annotation via Ensembl and NCBI); this can result in information that is similar but not necessarily identical. Thus, the Consensus CDS (CCDS) project, a collaboration between projects such as NCBI, EBI WTSI and HUGO Gene Nomenclature Committee (HGNC), was established to provide consistent high-quality annotation. CCDS aimed to facilitate the creation of a 'gold standard' set of gene annotations for the murine and human genome , and define "Consensus" as "protein-coding regions that agree at the start codon, stop codon, and splice junctions, and for which the prediction meets quality assurance benchmarks" (Pruitt *et al.*, 2009). This consensus is achieved by mandating agreement on annotation by multiple collaborators before changes can be made (Harte *et al.*, 2012). In order to achieve the standard annotation across databases, the concept

of a 'canonical' transcript and protein was created, so that protein annotation could be uniform, independent of the website being used. Ensembl details the canonical transcript for a gene according to the following hierarchy: 1) Longest CCDS translation with no stop codons, 2) the longest Ensembl/Havana merged translation with no stop codons, 3) the longest translation with no stop codons or 4) the longest non-protein-coding transcript.

1.6.3 Bioinformatics Prediction Software

1.6.3.1 SNP damage prediction software

SNP information is stored in a number of databases such as Ensembl and EBI, which collate SNP data from specialised SNP databases such as dbSNP (Sherry *et al.*, 2001). These specialised SNP databases are populated with SNP information from high-throughput sequencing from projects such as The Human Genome Project and the 1000 genomes project. Due to the increased density and quality of genome annotation, and ever increasing richness SNP location and consequence data, computational methods of SNP damage prediction have been developed (as reviewed by Wu and Jiang, 2013). These software programs, with the aid of algorithms, aim to assess the likelihood that a SNP is damaging to function. It is important to note that 'damaging' is the terminology used in the field of bioinformatics to describe SNPs that have 'an effect' on protein function, rather than necessarily causing 'damage' to a protein or organism in actual terms. However, the term will be used in this thesis to maintain consistency of terminology with other publications in the field. Damaging potential is assessed based upon factors such as: a) the level of sequence conservation at the SNP location across orthologs, b) the functional role of the region (such as a splice site), c) alignment of orthologs to indicate if the SNP lies on a key domain such an enzyme binding site and d) 'supervised machine learning' of experimentally verified protein specific data (Tavtigian *et al.*, 2008; Geurts *et al.*, 2009; Dukka, 2013). Algorithms associated with damage scoring software use differing sources of information to compile algorithm training data sets (Frousios *et al.*, 2013; Nan Zhao *et al.*, 2014). Consequently, each has its own strengths and weaknesses and therefore it has been suggested that multiple software using different algorithms and training data are used to achieve a more accurate prediction of SNP damage (Frousios *et al.*, 2013).

The database issue of the journal Nucleic Acid Research (NAR) does not provide a comprehensive list of SNP prediction software, however it is estimated that there are approximately 90 tools available online (Martin, 2016). Commonly utilised are; SIFT, PolyPhen-2, MutationTaster, Mutation Assessor, PANTHER and FATHMM (Ng and Henikoff, 2001; Thomas *et al.*, 2003; Reva, Antipin and Sander, 2007; Adzhubei *et al.*, 2010; Schwarz *et al.*, 2010; Adzhubei, Jordan and Sunyaev, 2013; Shihab *et al.*, 2013).

However, SIFT and PolyPhen-2 are two of the most widely used because of their differing but complementary programming (Flanagan, Patch and Ellard, 2010) to this effect, they are imbedded into the Ensembl variation table output.

SIFT

The SIFT (Sorts Intolerant From Tolerant) software program is available from http://sift.jcvi.org/, and is a SNP damage prediction tool that aims to reduce the number of functional assays required to determine phenotypes affected by SNP (Ng and Henikoff, 2001). SIFT predicts whether an amino acid substitution is likely to affect protein function based purely on residue conservation score from multiple alignment sequence homology and the physicochemical similarity between the alternate amino acids. SIFT does not use supervised machine-learning methods and is therefore not continually trained using experimentally verified SNP consequence data. SIFT classifies the predicted damage into two categories; 'tolerated' and 'deleterious'. The potential scores for SIFT outputs range from 1 to 0, with lower score indicative of a higher probability of damage. The thresholds for each of the damaging categories are 1 to 0.05 for tolerated and 0.05 to 0 for deleterious. SIFT has been utilised in a number of previous publications to prioritise damaging SNP (Stead *et al.*, 2011; Teng *et al.*, 2012; Alipoor *et al.*, 2016; Kalia *et al.*, 2016).

PolyPhen-2

The PolyPhen-2 (Polymorphism Phenotyping v2) software program is available from http://genetics.bwh.harvard.edu/pph2/ and is a tool used for the classification of nsSNPs (Adzhubei *et al.*, 2010). Unlike SIFT, the damage score can be calculated from the amino acid or nucleic acid sequence. Machine learning of PolyPhen-2 is based upon a set of eight sequence, and three structure-based features (Adzhubei *et al.*, 2010), that include multiple sequence alignment sequence conservation and Swiss-Prot annotation, taking into account any structural and functional annotation associated with the protein to predict nsSNP damage. The software can also use information from an available homologous protein 3D structure to assess the effect to protein stability (Adzhubei, Jordan and Sunyaev, 2013). If no 3D structure is available, the structural features are prediction based. PolyPhen-2 is trained using data sets with nsSNP of known consequence, and has been shown to be one of the most sensitive tools for the prediction of SNP functional consequence (Frousios *et al.*, 2013). The potential scores for PolyPhen-2 outputs range from 0 to 1, with higher score indicative of a higher probability that the nsSNP is damaging to protein function. Poly-Phen-2 classifies predicted damage into three categories; 'benign', 'possibly damaging' and 'probably damaging, with thresholds cut-offs

at 0.15 and 0.85. PolyPhen-2 has been used in a number of previous publications to prioritise damaging SNP (Flanagan, Patch and Ellard, 2010; Hepp, Gonçalves and de Freitas, 2015; Kalia *et al.*, 2016).

It is not uncommon for the tools to be unable to classify certain variants if the information required to do so is unavailable, such as appropriate ortholog alignment for that amino acid location (Frousios *et al.*, 2013); this is true for PolyPhen-2 where the outcome is reported as unknown if there is insufficient data.

1.6.3.2 Post-Translational Modification Prediction Software

The modification of a protein during or after translation (post-translational modification) includes processes such as phosphorylation (the addition of phosphate groups), N-glycosylation (the addition of carbohydrate moieties) and lipidic modifications such as palmitoylation and myristoylation. Post-translational modifications (PTMs) play a crucial role in the regulation of protein function, stability and targeting (as reviewed by Walsh 2006). Additional detail regarding the process of PTMs and examples of their role in proteins will be provided later in the chapter.

PTMs are crucial for the proper function and regulation of proteins and this has encouraged investigations to better understand their occurrence and location. PTM frequency and location information is also of value to determine potential protein function perturbation as a result of SNPs occurring on PTM sites. Lab-based experimentation to identify and verify sites of PTM are costly and time consuming, therefore PTM prediction software tools were developed to tackle this challenge. The software aims to reduce the number of functional assays required to determine the location of a PTM, and focus experimentation the amino acids most likely to undergo a PTM (Chen *et al.*, 2016). PTM prediction tools work by utilising algorithms, much in the same way as nsSNP damage prediction software, and are trained using datasets containing the locations of experimentally verified sites of the respective PTM.

The prediction of the location of PTMs that do not have a conserved motif is challenging and requires sophisticated algorithms. As a result, many PTM prediction software employ an 'artificial neural-network' method which utilises a network of artificial neurons that function in a manner comparable to a biological brain. The principles and methods underpinning this process are complex; however, the aim of the neural network method in PTM prediction software is to improve the accuracy of predictions. This is achieved by allowing the algorithm to 'learn' from the training dataset, and establish the requirements for an amino acid residue to qualify as a PTM site. The software can then use these requirements to identify new potential PTM sites.

The score thresholds in PTM prediction software represent the cut-offs set based upon performance evaluation and are related to the accuracy and rate of false positive results from the training dataset of experimentally verified sites. The threshold value for each individual amino acid prediction indicates the minimum score required to provide confidence that the site is involved in a true PTM. This threshold value is informed by conservation at that site, as well as results from previous datasets. With this information, it confirms that the location of the amino acid is not the only determinant of the predicted PTM score. A wide variety of PTM predictive tools are available, however a brief introduction will be provided only for the predictive software utilised in this thesis, which includes NetPhos 2.0 for phosphorylation, CSS Palm for palmitoylation, GPS-SNO for S-Nitrosylation, GPS–SUMO for SUMOylation and NetNGlyc for N-glycosylation.

NetPhos 2.0 and NetNGlyc 1.0

NetPhos 2.0 (available from http://www.cbs.dtu.dk/services/NetPhos-2.0/) is provided by the Centre for Biological Sequence Analysis (CBS), and is an artificial neural-network based software package designed to predict sites of phosphorylation on serine (S), threonine (T) and tyrosine (Y) amino acid residues (Blom, Gammeltoft and Brunak, 1999). The NetPhos 2.0 neural-network was trained using a dataset of known phosphorylation sites (584 serine, 108 threonine and 210 tyrosine) and takes into account the tertiary structure of the protein, sourced from the structure database Protein Data Bank (Bernstein *et al.*, 1977) where available. The NetPhos 2.0 output score ranges from 0 to 1, with higher values indicating greater confidence in the prediction and greater similarity to one or more of the phosphorylation sites in the training dataset. The NetPhos 2.0 prediction threshold score is 0.5. NetPhos software has been used in a number of publications to identify predicted sites of phosphorylation (Eklund and Edqvist, 2003; Savas and Ozcelik, 2005; Jianxi Liu *et al.*, 2006; Chen *et al.*, 2009; Dos Santos, Nunez-Castilla and Siltberg-Liberles, 2016). NetNGlyc 1.0 (available from http://www.cbs.dtu.dk/services/NetNGlyc/) is provided by CBS and like NetPhos 2.0, and is an artificial neural-network based software package. NetNGlyc 1.0 is designed to predict sites of N-glycosylation on asparagine (N) amino acid residues (Gupta and Brunak 2002). Training dataset information was not available for this software. The NetNGlyc 1.0 prediction threshold score is 0.5. NetNGlyc 1.0 software has been used in other studies to identify predicted sites of N-glycosylation (Rafiee *et al.*, 2012; Sheta *et al.*, 2016).

CSS-Palm 4.0

CSS-Palm 4.0 (available from http://csspalm.biocuckoo.org/online.php) is a software tool designed to predict sites of palmitoylation on cysteine (C) amino acid residues (Zhou *et al.*, 2006; Ren *et al.*, 2008). CSS-Palm 4.0 utilises a complex computational method called Group-based Prediction System (GPS) (Xue, Liu, Cao, *et al.*, 2010), formally known as Group Based Phosphorylation Scoring, to increase the accuracy of palmitoylation site prediction and scoring (Zhou *et al.*, 2004). In summary, GPS works by incorporating the biochemical characteristics of neighbouring amino acids and the predicted 3D structure conservation of the local amino acid environment, in addition to scoring based on verified palmitoylation sites to estimate the likelihood of palmitoylation. CSS-Palm 4.0 was developed by taking 510 experimentally verified palmitoylation sites from 232 distinct proteins and using them as training data to improve the accuracy of prediction (Xue and Ren, 2013). Prediction threshold values of 95%, 90% and 85% (high, medium and low), were set on the percentage specificity calculated after performance evaluation assessments. The prediction threshold for each cysteine residue is calculated individually, however details of the criteria for this are not detailed specifically. The CSS-Palm 4.0 output score does not have a fixed range and is based upon the degree of conservation at each site, with a higher score indicating a greater likelihood of palmitoylation. Examples of threshold and scoring will be provided in chapter 3. CSS-Palm has been used in a number of publications to identify predicted sites of palmitoylation (Jeffries, 2010; Zoltewicz *et al.*, 2012; Oku *et al.*, 2013; Bi, 2014).

GPS-SNO 1.0 and GPS–SUMO

GPS-SNO 1.0 (available from http://sno.biocuckoo.org/index.php) is a software tool designed to predict sites of S-nitrosylation on cysteine (C) amino acid residues (Xue, Liu, Gao, *et al.*, 2010). GPS–SUMO (available from http://sumosp.biocuckoo.org/) is a tool designed to predict sites of SUMOylation on lysine (K) residues (Ren *et al.*, 2009; Zhao *et al.*, 2014). Both software tools utilise the same GPS method of PTM prediction scoring as previously described for CSS-Palm 4.0. However, GPS-SNO 1.0 prediction threshold values of approximately 90%, 85% and 80% (high, medium and low) were set based on the percentage specificity calculated after performance evaluation assessments. GPS-SNO 1.0 was developed using a training dataset comprising 504 experimentally verified S-nitrosylation sites from 327 unique proteins, whilst GPS-SUMO was developed using 983 SUMOylation sites from 545 proteins. A number of studies have utilised GPS-SNO 1.0 (Gould *et al.*, 2013; Chaki *et al.*, 2014; Zahid *et al.*, 2014; Kashyap, Sehrawat and Deswal, 2015) and GPS–SUMO (Aguilar-Martinez *et al.*, 2015; Nepveu-Traversy *et al.*, 2016) tools for PTM site identification.

The number of genetic variations and post-translational modification sites across the whole genome is too vast to assess as part of any one thesis. Consequently, for the purpose of this work, a single gene, KCNMA1 coding for the BK channel α-subunit, was selected to investigate the prioritisation of potentially harmful nsSNPs on the basis of existing information available in bioinformatics resources and the literature.

1.7 A focus on the BK channel

Ion channels are membrane spanning proteins that allow for the selective and rapid movement of ions through a pore, across an otherwise impermeable lipid membrane and are found in cells throughout the body. These membrane proteins are integral for homeostasis of intracellular ion concentrations within physiological limits and for the changes in concentration gradient associated with membrane depolarisation and repolarisation. They differ from many other proteins due to the presence of hydrophobic membrane-spanning domains, which allow insertion of the protein across lipidic membranes. There are many forms of ion channel family, including voltage-gated ion channels (Figure 1.2), extracellular ligand-gated ion channels and intracellular ligand-gated ion channels. Ions such as sodium, calcium, magnesium, chloride and potassium have specialised channels that allow for their passage.

The potassium channel family (Figure 1.3) is the largest and most diverse group of ion channels with over 70 loci found in the mammalian genome (Gutman, 2005). The DNA motifs that code for the protein that forms the potassium channel is highly conserved across species (Butler *et al.*, 1993). Potassium channels allow the selective passage of potassium ions across lipidic membranes regulating membrane potential and cellular function. The resting membrane potential of many cells is related to the potassium channel equilibrium potential and contributes to charge regulation (Brayden and Nelson, 1992; Nelson and Quayle, 1995; Nelson *et al.*, 1995; Salkoff *et al.*, 2006). Potassium channels can be grouped into three main structural classes encoding 2 transmembrane (2 TMD), 4 transmembrane (4 TMD) and 6 transmembrane (6 TMD) domains. Various subfamilies of potassium channels are known and generally correspond to the physiological signal by which pore opening is activated; for example, voltage-gated and calcium-gated (Miller, 2000). Potassium channels are also grouped based on their configuration and conductance, such as inward rectifying, outward rectifying, small conductance (SK), and big conductance (BK) (Köhler *et al.*, 1996; Sah, 1996; Vergara *et al.*, 1998; Peña and Rane, 1999; Shipston *et al.*, 1999; Diness *et al.*, 2010).

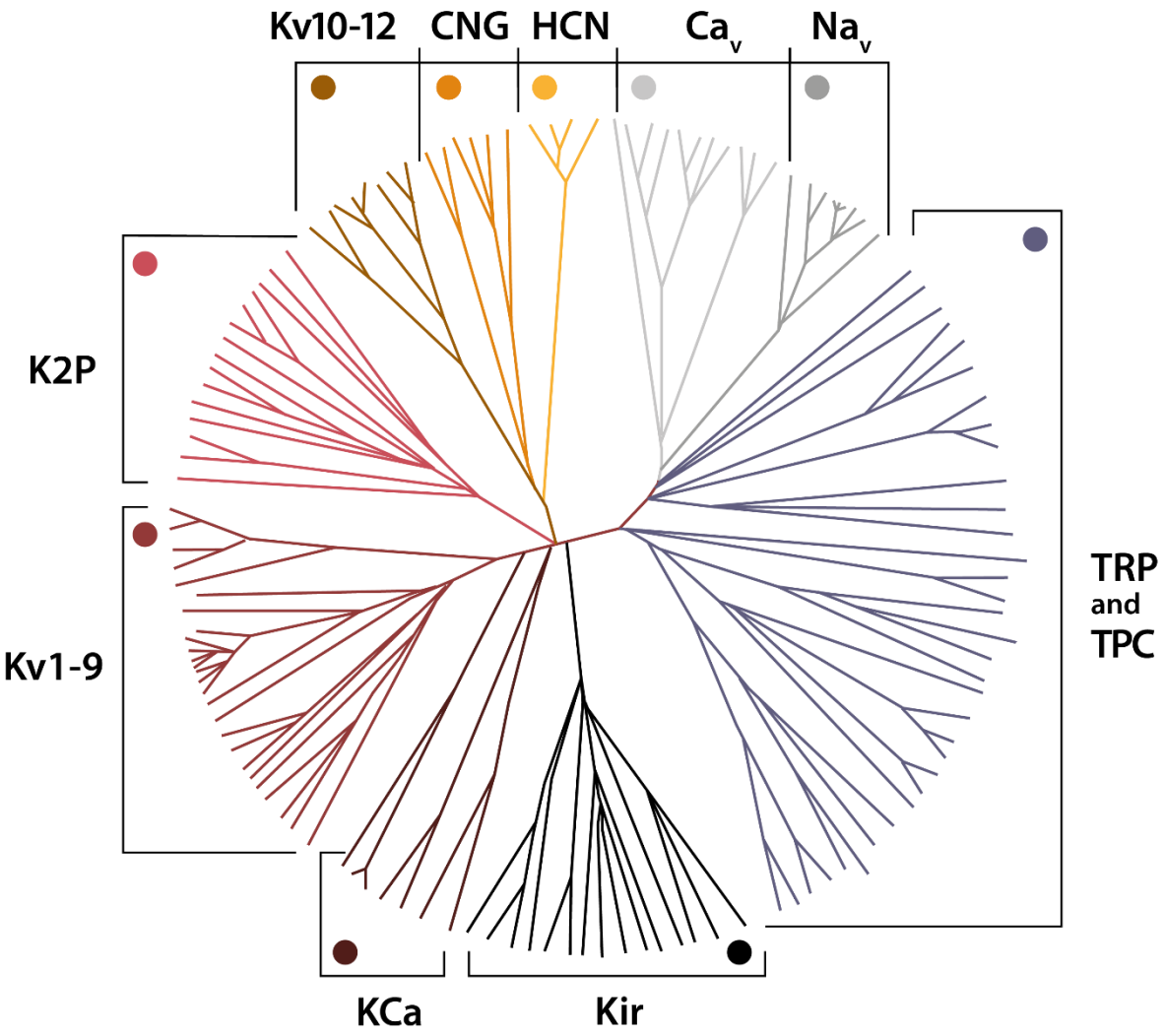


Figure 1.2- Representation of the voltage-gated ion channel superfamily. Phylogenetic tree illustrating the diversity of the voltage-gated ion channel superfamily comprising 143 members of related ion channel genes. The voltage-gated calcium (CaV) and sodium (NaV) channels are denoted by grey branches, cyclic nucleotide-gated channels (CNG) and hyperpolarization-activated cyclic nucleotide–gated (HCN) are denoted by orange branches, the large voltage-gated potassium (Kv) family is separated into two, with Kv1-9 denoted by red branches and Kv10-12 by brown branches. The two-pore potassium (K2P) channels are denoted by pink branches, the calcium activated potassium (KCa) channel family denoted by dark red branches, and the inward rectifier (Kir) channels denoted by black branches. Transient receptor potential (TRP) and two-pore channels (TPC) channels are denoted by purple branches. Figure adapted from (Yu *et al.*, 2005).

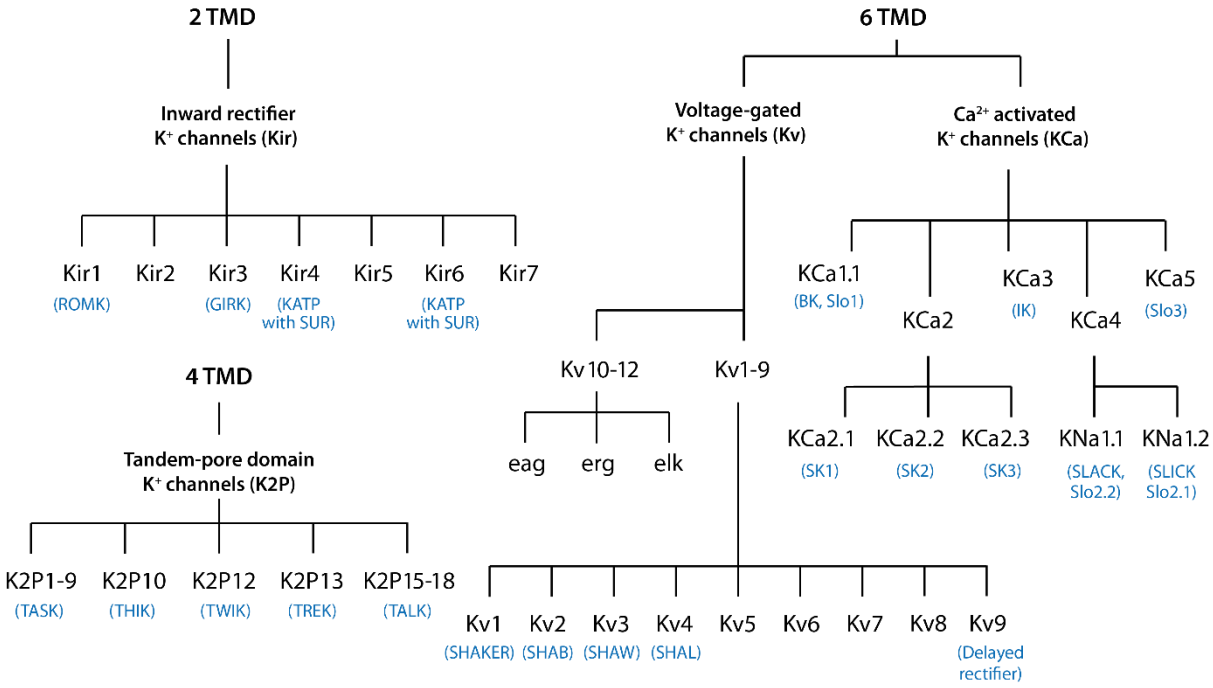


Figure 1.3- The potassium channel family. A hierarchical classification of potassium channels. Potassium channels can be grouped into three main structural classes encoding 2 transmembrane (2 TMD), 4 transmembrane (4 TMD) and 6 transmembrane (6 TMD) domains. Grouping is also based upon the physiological signal by which pore opening is activated; for example, voltage and calcium gated. Potassium channels are also grouped based upon their configuration and conductance, such as inward rectifying, small conductance (SK) and big conductance (BK). Adapted from Wei, 2005 and Zhong *et al.*, 2013.

Calcium- and Voltage-gated Potassium channels

There are two groups of calcium-activated potassium channels providing 8 α-subunit types (Figure 1.3) (as reviewed by Wei 2005). The first group are voltage insensitive and are sensitive to calcium concentration via binding of the cation to calmodulin; members include KCa2.1-2.3 (the SK1-3 channels) and the KCa3 (the IK channel). The second group are 'Slo' gene members and include KCa1.1 (Slo, the BK channel), KCa4.1 and KCa4.2 (Slo2.2 Slack and Slo2.1 Slick) and KCa5 (Slo3). Each channel differs in conductance, with the BK channel having the largest at approximately 250pS in symmetrical 150 mM KCl (Marty, 1981; Latorre, Vergara and Hidalgo, 1982; McManus and Magleby, 1991).

1.7.1 BK Channels

BK channels are large conductance calcium- and voltage-gated potassium channels also known as Slo or 'MaxiK' and are members of the voltage gated potassium channel family. Calcium- and Voltage-gated Potassium channels can be activated by both depolarisation and a rise in intracellular calcium and therefore can integrate the response to intracellular calcium and cell membrane potential (Adelman *et al.*, 1992; Cui, Cox and Aldrich, 1997; Horrigan, Cui and Aldrich, 1999). Calcium and voltage sensitivity of the channel are related (Schreiber and Salkoff, 1997) and this characteristic is vital for their role as a feedback mechanism to regulate the activity of voltage gated calcium channels (Sah and Louise Faber, 2002). BK channels, then known as 'Gardos channels', were first 'described' in erythrocytes in the late 1950's when Hungarian haematologist George Gardos described a change in potassium permeability across the plasma membrane that responded to minor increases in intracellular calcium (Gardos, 1958). It was noted that when calcium was buffered using EDTA as a chelating agent, potassium movement across the membrane was substantially reduced. However, there was a substantial delay before an ionic current activated by a rise in cytosolic calcium was first identified (Meech, 1972). Studies conducted in 1981 and 1985 using cultured rat skeletal muscle served to prove the BK channel can be activated by calcium and membrane potential independently and enhance the channels opening probability (Pallotta, Magleby and Barrett, 1981; Pallotta, 1985).

1.7.1.1 BK channel structure

The BK channel is comprised of four pore-forming α-subunits assembled into a tetramer to form the channel pore; a recent publication details the full-length BK channel 3D structure as determined by cryo-electron microscopy at a resolution of 3.5 Å (Tao, Hite and MacKinnon, 2016). Each α-subunit within the tetramer is comprised of seven transmembrane domains (TMDs) spanning from S0 to S6, with an extracellular N-terminus (NH_2) and an intracellular C-terminus (COOH) comprising

approximately two thirds of the channel length and containing four additional, but non-membrane inserted, hydrophobic domains (S7-S10) (Figure 1.4).

The S0 TMD is unique to the BK channel and is not shared by members of the related voltage activated potassium channel family, who unlike the BK channel have six TMDs (S1-S6) (Wallner, Meera and Toro, 1996; Meera *et al.*, 1997). Consequences of this additional TMD are the relocation of the N-terminus to the extracellular space and the creation of an S0-S1 TMD linker region; these additions are highly conserved among vertebrates suggesting that they play an important role in the structure or function of the channel (Koval, Fan and Rothberg, 2007). Experimentation has provided further evidence of this crucial role; BK channels mutated to omit the S0 TMD were found not to be active, and were not expressed in the cell membrane (Wallner, Meera and Toro, 1996; Morrow *et al.*, 2006; Guoxia Liu *et al.*, 2008).

The α-subunit contains key regions of functional importance, such as the voltage sensing domain (VSD) (spanning TMD S1-S4). This region is responsible for sensing changes in membrane potential and link depolarisation with opening of the channel pore. The channel is dually activated by calcium and depolarisation however activation can occur in the absence of calcium (Horrigan, Cui and Aldrich, 1999). The VSD contains charged amino acid residues involved in voltage-dependent gating of the channel; three arginine residues (Ma, Lou and Horrigan, 2006; Sakai, Harvey and Sokolowski, 2011) within the S4 TMD are proposed to move up and out towards the extracellular space, 'tightening' the linker between S4 and the pore region, facilitating pore opening (Jensen *et al.*, 2012). Charged residues in TMDs S2 and S3 (Ma, Lou and Horrigan, 2006) have also been shown to be involved in voltage activation of the channel. Mutations in the S0 TMD have been shown to alter activation curves and thus may be involved in voltage-gating (Koval, Fan and Rothberg, 2007). Site directed mutations of amino acids in this domain result in reduced coupling of calcium with channel opening (Díaz *et al.*, 1998).

The pore-forming domain (PFD) of the channel is comprised of TMD S5, the P-loop and TMD S6, and forms the channel pore situated in the centre of the BK channel tetramer. Within the PFD is also the potassium selectivity filter containing the GYG sequence, which is conserved in potassium channels across species from *Drosophila* to humans (Heginbotham *et al.*, 1994). Mutations to this sequence result in alterations to potassium channel ion selectivity (Yool and Schwarz, 1991; Chapman, Krovetz and VanDongen, 2001). The entrance to the intracellular vestibule (EIV) is located in the PFD and spans the entirety of the S6 TMD. The structure of the EIV is larger than other potassium channels and this is thought to allow for the large potassium conductance associated with the channel (Li and Aldrich, 2004; Brelidze and Magleby, 2005). The EIV has a negatively charged ring structure, composed of eight

negatively charged glutamate residues. This structure creates an environment that facilitates the targeting of cations such as potassium from the intracellular domain to the selectivity filter (Brelidze and Magleby, 2005). Mutation to this region results in a 50% reduction in single channel conductance and conversion of the channel to inwardly rectifying, where potassium ions flow preferentially in the inward direction (into the cell) than in the outward direction (out of the cell) (Brelidze, Niu and Magleby, 2003). The intracellular C-terminal region comprises approximately two thirds of the total channel length and is involved in many aspects of channel function. The C-terminus is involved in α-subunit tetramerization; mutation of an association domain in this region prevented the correct assembly of the channel (Quirk and Reinhart, 2001). Within the C-terminus are the regulators of potassium conductance (RCK) domains RCK1 and RCK2 (Jiang *et al.*, 2001) comprised of S7-S8 and S9-S10 respectively. These regions confer calcium sensitivity to the channel; the addition of calcium to the intracellular face of the channel results in a left-shift in activation voltage to a more physiological range.

Within RCK2 is the calcium bowl (Schreiber and Salkoff, 1997) with a conserved sequence of aspartic acid residues (DQDDDDDPD), conserved from nematode to human (Wei, Jegla and Salkoff, 1996). Mutation of the calcium bowl has been shown to reduce calcium mediated channel activation (Schreiber and Salkoff, 1997). Two aspartic acid residues in the region have been shown to be essential for calcium sensing (Bao *et al.*, 2002, 2004), however mutations to those residues did not completely abolish calcium sensitivity; therefore, the presence of additional calcium binding sites was postulated. A low affinity binding site for divalent cations in RCK1, that can bind with calcium and magnesium in millimolar concentrations was identified (Zhang, Solaro and Lingle, 2001). The calcium sensing ability means that the BK channel can act as a feedback mechanism for intracellular calcium concentration from local intracellular increases in calcium (calcium sparks) and to the activity of voltage gated calcium channels for example.

1.7.1.2 Physiological role of BK channels

BK channels are ubiquitously expressed in the body with the exception of the cell membrane of cardiomyocytes; however there is contrary evidence which supports their presence in rodent cardiomyocytes (Ko *et al.*, 2009; Imlach *et al.*, 2010). Functioning BK channels have also been identified in the inner mitochondrial membrane (as reviewed by Szabo and Zoratti, 2014), with the first discovery made in 1999 when a single channel current of 295pS was recorded from mitoplasts (mitochondrion with the outer membrane removed) of the human glioma cell line LN229 (Siemen *et al.*, 1999).

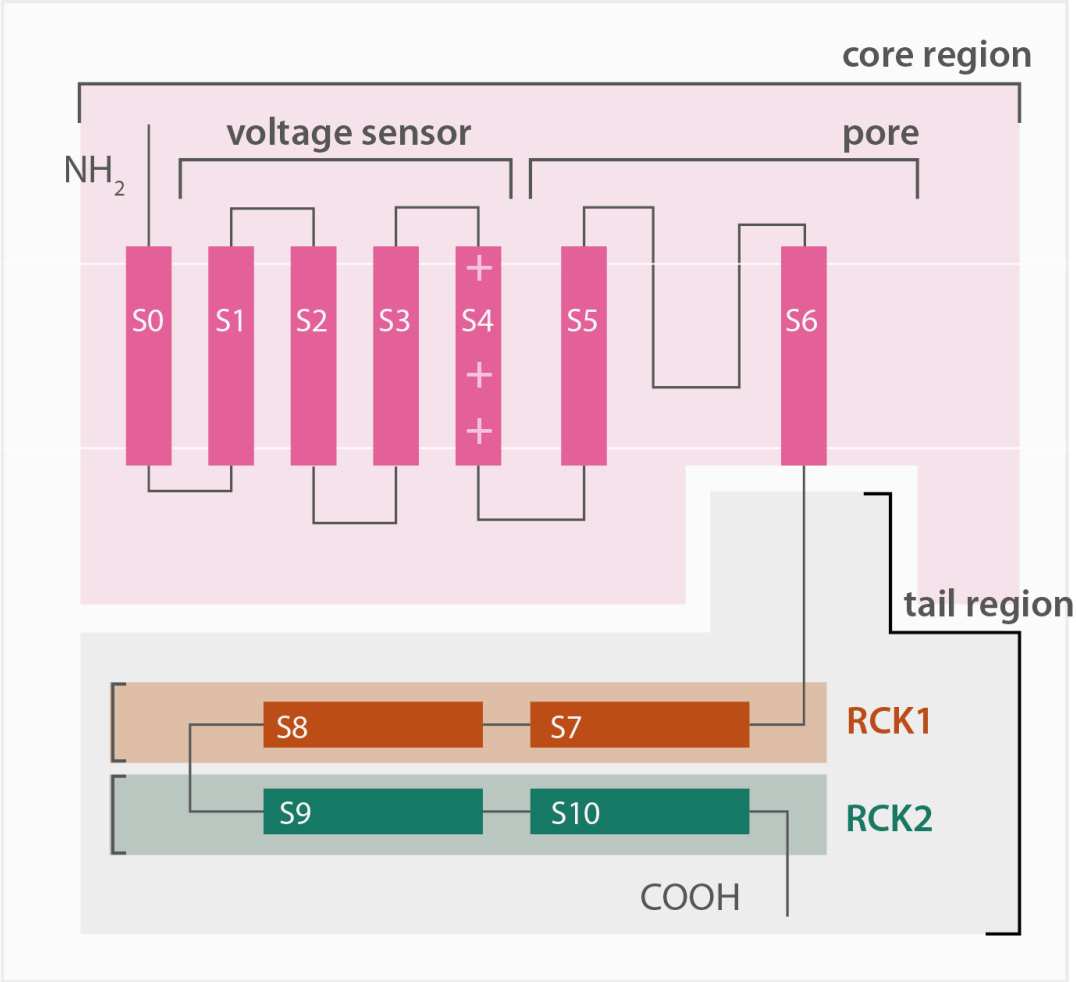


Figure 1.4- Schematic of BK channel α-subunit. The schematic illustrates the BK channel α-subunit formed of seven transmembrane domains (S0-S6) in the core region (pink shading) and four hydrophobic domains (S7-S10) in the c-terminal tail region (green shading). The core region contains the voltage sensing domain spanning from (S1-S4) and the pore-forming domain and p-loop spanning from S5 to S6. The intracellular C-terminal tail region contains the regulators of the conductance of potassium (RCK1 and RCK2 in orange and green respectively). Schematic not drawn to scale. Adapted from Orio *et al.*, 2002.

BK channels are implicated in a wide range of processes, many relating to their effect on smooth muscle contractility. An example is in the physiological regulation of vascular tone (Aldrich *et al.*, 2000; Sausbier *et al.*, 2005) where BK channel activation results in vascular smooth muscle cell relaxation, vasodilation and thus a reduction in blood pressure. BK channels have also been implicated in bladder control (Meredith *et al.*, 2004); channel activation results in relaxation of urinary bladder smooth muscle cells and prevents incontinence as a result of an overactive bladder. BK channel function is linked to uterine contractility via myometrial smooth muscle (as reviewed by Khan *et al.*, 2001), where channel activation supresses myometrial activity during pregnancy. This suppression is lost during labour due an uncoupling of calcium and BK channel activation.

In the brain, BK channels are found on the plasma membrane and presynaptic terminals of neurons and are implicated in the regulation of neuronal cell function and neurotransmitter release. For example, presynaptic BK channels have been shown to serve as negative regulators of neurotransmitter release by indirectly regulating calcium entry and modulating cell repolarisation (Robitaille and Charlton, 1992; Raffaelli *et al.*, 2004; Wang, 2008). The hyperpolarising effect of BK channels can either reduce neuronal activity or increase it and in cases is associated with autism, mental retardation and epilepsy (Hu *et al.*, 2001; Sausbier *et al.*, 2004; Du *et al.*, 2005; Laumonnier *et al.*, 2006; Ghezzi *et al.*, 2010). BK channel activity also play a role in hearing (Rüttiger *et al.*, 2004; Föller *et al.*, 2012).

Publications detailing the varied role of the BK channel on physiological processes led to speculation about the organism wide effect of the channel, and investigation into the effect a lack of BK channels on mammalian physiology *in vivo* was of interest. The development of a knockout model was accomplished in 2004 when BK channel α-subunit deficient mice (Kcnma1-/-) were created (Meredith *et al.*, 2004; Sausbier *et al.*, 2004). These mice were viable, however presented with minor abnormalities linked to BK channel roles, such as hypertension, over-active bladder, ataxia, abnormal gait, erectile dysfunction and hearing loss (Meredith *et al.*, 2004; Rüttiger *et al.*, 2004; Sausbier *et al.*, 2004; Werner *et al.*, 2005; Pyott *et al.*, 2007).

1.7.2 Complexity of the BK channel

1.7.2.1 Splicing

The BK channel is coded for by a single gene (KCNMA1), however there is a high degree of diversity in channel function; this is in part because KCNMA1 displays extensive pre-mRNA splicing at multiple sites throughout the channel (Adelman *et al.*, 1992; Butler *et al.*, 1993; Xie and McCobb, 1998). These variants have been shown to influence the calcium and voltage sensitivity of the channel (Adelman *et*

al., 1992; Lagrutta *et al.*, 1994; Tseng-Crank *et al.*, 1994; Saito *et al.*, 1997; Shipston *et al.*, 1999), regulate phosphorylation (Tian, Duncan, *et al.*, 2001; Tian *et al.*, 2004) and control cell surface expression (Zarei *et al.*, 2001, 2004; Kwon and Guggino, 2004; Chen *et al.*, 2010). A number of inserts may be accomplished at each splice site. For example, there are five splice variants originating at the C2 site; e20, STress Regulated EXon (STREX) e21, e22(exon 22), Δe23 and ZERO (no insert) (Chen *et al.*, 2005). A study was conducted with the purpose of characterising C2 BK splice variants STREX, e22 and ZERO by their sensitivity to elevations in intracellular free calcium level mediated by inomycin and the extent of inhibition by BK channel blocker paxilline (Saleem, Rowe and Shipston, 2009). Results showed a calcium response potency in order STREX>e22>ZERO and relatively similar paxilline inhibition, proving that splicing at a single site can dramatically alter calcium sensitivity in this case.

1.7.2.2 Auxiliary Subunits

The BK channel is associated with two types of auxiliary unit; the β-subunit and γ-units (as reviewed by Li and Yan, 2016). The gating characteristics of the BK channel can be modulated by β-subunits, two TMD units which associate with the channel in a 1:1 ratio via the S0 TMD (Wallner, Meera and Toro, 1996) and are coded for by genes KCNMB1-4. The β-subunits have wide ranging effects on the α-subunit, including altering the calcium and voltage sensitivity of the channel, gating kinetics, and sensitivity to extracellular modulators, as comprehensively reviewed in a 2014 article (Torres, Granados and Latorre, 2014). There are four subtypes of the β-subunit and each confers differing properties to the channel (Petrik and Brenner 2007). The β_1- subunit is primarily located in smooth muscle cells where it shifts BK activation to more negative voltages for a given calcium concentration and confers slower current activation and increased open probability to the channel (Dworetzky *et al.*, 1996); the β_2-subunit is prominent in the kidney, brain, lung and heart and causes rapid channel inactivation. This effect is thought to be due to the 'ball and chain' NH_2 region of the beta subunit blocking the channel opening (Xia, Ding and Lingle, 1999; Bentrop *et al.*, 2001). The β_3- subunit is found in organs such as the spleen, placenta, heart and liver and is shown to cause extremely rapid but incomplete inactivation of the BK channel, reducing, but not abolishing channel activity after initial opening (Xia *et al.*, 2000; King *et al.*, 2006). The β_4 subunit is extremely prevalent in brain and neuronal tissue, as with β_1, β_4 increases calcium sensitivity and slows BK activation (Brenner *et al.*, 2000; King *et al.*, 2006). β_4 knockout mice have been shown to experience temporal lobe seizures indicating that the β_4 subunit is necessary for control of neuronal firing rate (Brenner *et al.*, 2005).

The auxiliary γ-units were discovered comparatively recently in 2005 in BK channels with an uncharacteristically low half-activation voltage of approximately 30 mV in the absence of intracellular calcium (Gessner *et al.*, 2005). Since then three additional subtypes have been identified (Yan and

Aldrich, 2010, 2012) found throughout the body and are implicated in the modulation of aspects such as vasodilation and male fertility (Yang *et al.*, 2011; Evanson *et al.*, 2014).

1.7.2.3 PTM of the BK channel

Post translational modification (PTM) is the chemical modification of a protein after translation; this process serves to increase the diversity and complexity of proteins that can be produced from a relatively basic set of genes (Burge at al 1999, Hastings and Krainer 2001, Shipston 2011) . There are a number of post translational modifications currently known; some involve the addition of hydrophobic/hydrophilic groups or modifications which change the nature of the amino acids within the proteins themselves (Walsh and Jefferis, 2006). The modifications that are relevant to this project are listed and explained below.

Phosphorylation

Phosphorylation is believed to be the most abundant form of PTM affecting a multitude of signalling pathways and is the addition of a phosphate chemical group to a serine, tyrosine, threonine or histidine amino acid residue of a protein or other organic molecule. Phosphorylation was first described in 1927 (Rimington, 1927). Phosphate groups are large, negatively charged and bulky, thus the addition of a phosphate group may cause a conformational change potentially leading to alterations in binding affinity and thus changing the functional properties of the protein (Swope *et al.*, 1992). The process of phosphorylation is reversible and is undertaken by protein kinase enzymes which add phosphate groups; phosphatase enzymes remove phosphate groups (Swope *et al.* 1992). The phosphorylation and de-phosphorylation of enzymes, receptors and ion channels are crucial for regulation of their activity and adds to functional diversity (Levitan, 1994; Tian *et al.*, 2004). BK channels are regulated by a range of kinase enzymes (such as PKA, PKC and PKG) and phosphatases (such as PP1 and PP2A) (White *et al.*, 2000; Tian, Duncan, *et al.*, 2001; Widmer, Rowe and Shipston, 2003; Tian *et al.*, 2004; Zhou *et al.*, 2012). The kinase AMPK has been reported to increase BK channel trafficking to the cell membrane in *Xenopus oocytes* (Föller *et al.*, 2012). The precise molecular mechanism of the effect has not yet been defined, and it is unclear whether direct phosphorylation of the BK channel or phosphorylation of another component involved in channel trafficking is required. Phosphorylation effect has been shown to be variable depending on splice variant; in the ZERO form of the channel PKA and PKG phosphorylation cause activation and PKC causes inhibition, however in STREX, PKA phosphorylation results in channel inhibition (Tian, Duncan, *et al.*, 2001).

Lipidation

Lipidation is defined as the covalent binding of a lipid group to a peptide chain; this may affect intracellular trafficking of the protein and therefore the cellular location, or alter its activity in the case of a channel or an enzyme (Resh 1999). Several categories of lipid are included in this process such as fatty acids, isoprenoids and cholesterol (Salaun, Greaves and Chamberlain 2010). Lipidation can be divided into two broad categories; those that occur in the cytoplasm or the cytoplasmic side of the plasma membrane and those that occur within the cell membrane (Nadolski and Linder 2007). Myristoylation and palmitoylation (the addition of a 14 and 16-carbon fatty acid respectively), are two of the most common forms of protein lipidation. The role of palmitoylation in BK channels will be briefly introduced below.

Palmitoylation

Palmitoylation is the addition of a 16-carbon saturated fatty acid molecule (palmitate) to a cysteine amino acid residue (Figure 1.5). The addition of palmitate increases the hydrophobicity of a protein, and facilitates protein biosynthesis, membrane trafficking and anchorage in the cell membrane (Linder and Deschenes, 2007). Investigation of palmitoylated proteins by mass-spectroscopy has shown that other long chain fatty acids such as stearate (C18:0), palmitoleate (C16:1) and oleate (C18:1) can be attached to cysteine residues in place of palmitate. This has been shown in a number of proteins such as Src family kinases (Liang *et al.*, 2001, 2004), hemagglutinating glycoproteins of influenza viruses (Kordyukova *et al.*, 2008), viral spike proteins (Kordyukova *et al.*, 2010), human platelets (Muszbek *et al.*, 1999), synaptosomal-associated protein 25 (SNAP-25) (Greaves *et al.*, 2017), and the G-protein-coupled receptor (GPCR) (O'Brien and Zatz, 1984).

Two forms of palmitoylation have been identified; N-palmitoylation and S-palmitoylation that differ in their method of protein linkage and reversibility. N-palmitoylation is the attachment of palmitate to an N-terminal cysteine residue via amide linkage; this process is irreversible (Iwanaga *et al.*, 2009). This form of palmitoylation offers a stable, permanent form of lipidation; a limited number of proteins such as the secreted morphogen 'Hedgehog' undergo this modification (Pepinsky *et al.*, 1998; Buglino and Resh, 2008, 2012). S-palmitoylation is the reversible thioester linkage of palmitate to a cytoplasmic cysteine residue. The most commonly described function of palmitoylation is to increase the affinity of proteins to the cell membrane, which can affect its recognition and transport by the cell cytoskeleton or vesicular transport (Salaun, Greaves and Chamberlain 2010). The reversible nature of palmitoylation presents a dynamic regulatory mechanism for protein location and function (Mumby 1997, Conibear and Davis 2010, Shipston 2011). It is speculated that palmitoylation serves to enable

clustering of the protein, binding to specific cytosolic lipid and protein domains, to encourage orderly folding and to prevent aggregation (Salaun, Greaves and Chamberlain, 2010). It is also speculated that initial protein palmitoylation is necessary for assembly of signalling complexes (Resh, 2006).

The BK channel has been shown to be palmitoylated; palmitoylation can occur at specific cysteine residues in the S0-S1 linker (Jeffries, 2010; Jeffries *et al.*, 2010) and STREX splice insert (Tian *et al.*, 2008; Jeffries, 2010), and this palmitoylation allows the protein to reversibly locate from the cytoplasm to the cell membrane, thus determining its overall function. Palmitoylation of STREX has been shown to abolish PKA mediated inhibition of the BK channel (Tian *et al.*, 2008).

Palmitoylation Enzymes

As more is revealed about the role of palmitoylation and de-palmitoylation in cell mechanisms and pathways, it was of interest to investigate the enzymes that undertake the process of palmitoylation. There are 23 known palmitoylation enzymes all of which share a cysteine rich domain, a 51 amino acid sequence Asp-His-His-Cys (DHHC motif) with a $CX_2CX_9HCX_2CX_2CH_4DHHCCX_5CX_4NX_3FX_4$ consensus sequence (Mitchell *et al.*, 2006; Korycka *et al.*, 2012), and thus are often referred to as DHHC palmitoyl acyl transferases (PATs) (Xie and McCobb, 1998; Tsutsumi, Fukata and Fukata, 2008; Tian *et al.*, 2010). Many proteins share this specific motif, however not all have been shown to palmitoylate cysteine residues and therefore cannot be included as palmitoylation enzymes (Xie and McCobb 1998). The exact location of active palmitoylation enzymes within cells has not been fully elucidated; some theorise that they are situated in cell membranes such as the Golgi apparatus and endoplasmic reticulum whilst others suggest that they can be found in the cytosol. It is postulated that PATs are transmembrane proteins found in the plasma, Golgi and ER membranes (Baekkeskov and Kanaani, 2009). In a study to test the localisation and distribution of human DHHC proteins in HEK293 cells, eight were restricted to the ER, six in the Golgi apparatus and four in both the ER and Golgi (Ohno *et al.*, 2006). The specific role that each of the palmitoylation enzymes has on protein trafficking/function has not been fully elucidated- though the importance of specific enzymes in the regulation of proteins involved in neuronal function and specific disease states has recently been reviewed (Salaun, Greaves and Chamberlain 2010).

It would be expected due to the relatively large number of PATs in the mammalian genome, that there would be a similar number of enzymes that remove palmitate from cysteine residues; however, this seems not to be the case. To date two palmitoyl-protein thioesterases (PPTs) have been discovered in mammals. PPT1 has been shown to de-palmitoylate proteins undergoing degradation in lysosomes (Camp and Hofmann, 1993; Hellsten *et al.*, 1996).

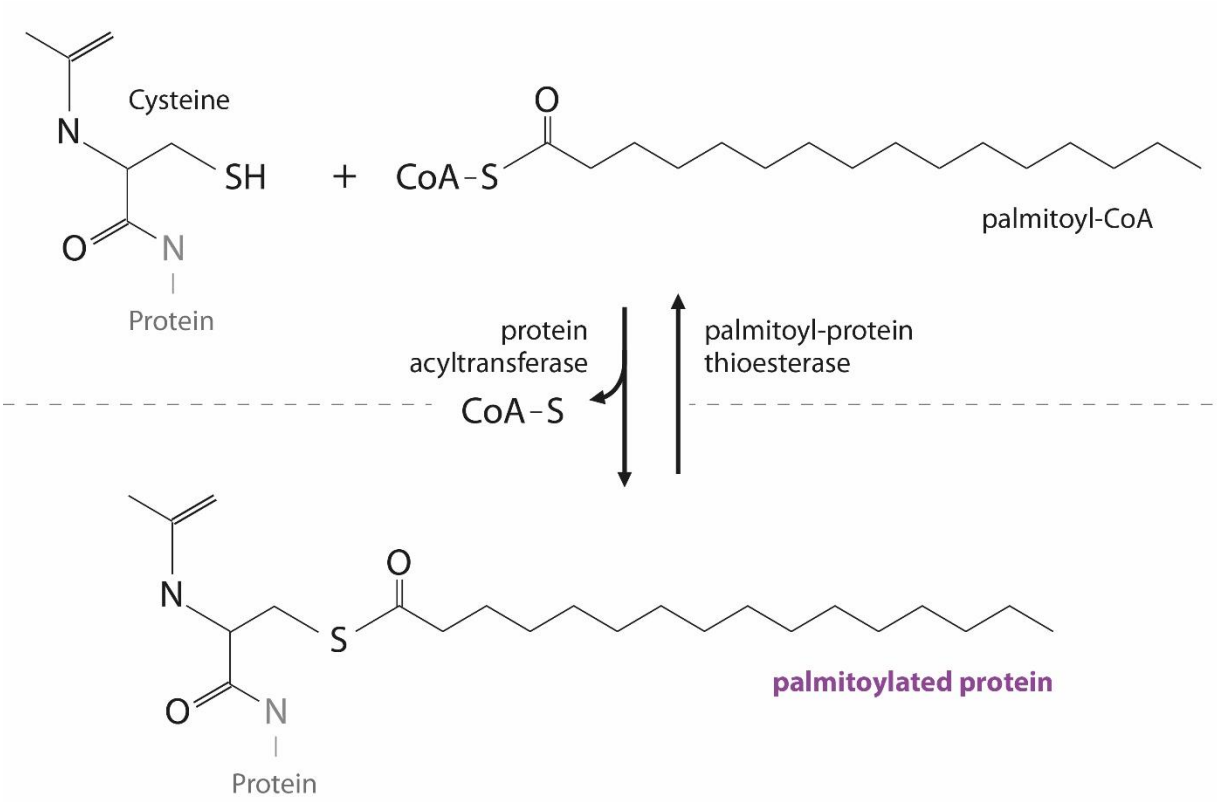


Figure 1.5- Reversible process of palmitoylation. Schematic illustrating the reversible addition to, and removal of, palmitate (C16) from a cysteine amino acid by protein acetyltransferases (PATs) and palmitoyl-protein thioesterases (PPT). A cysteine containing protein and a palmitate molecule esterified to Coenzyme A (Palmitoyl-CoA) are shown initially. A representation of a palmitoylated protein is also shown. Adapted from Christie, 2014.

PPT1 is also involved in the in the de-palmitoylation of H-Ras, N-Ras, GAD65 and endothelial nitric oxide synthase, and the trafficking of SNAP25 in neuronal synaptic vesicles (Duncan and Gilman, 1998; Baekkeskov and Kanaani, 2009). PPT2 shares 18% homology with PPT1 and is also found in lysosomes where it hydrolyses long-chain fatty acyl CoAs (Baekkeskov and Kanaani, 2009). Acyl-protein thioesterases (APTs) APT1 and APT2, have been identified as palmitoyl thioesterases that de-palmitoylate cytosolic cysteine residues of the BK channel (Tian *et al.*, 2012). An APT1 homolog, APT1-like thioesterase, coded for by the gene LYPLAL1, has been identified that is involved in de-palmitoylation of the BK channel (Tian *et al.*, 2012). Interestingly however, the enzyme was shown to contain a narrow substrate-binding pocket unable to accommodate long-chain fatty acids (Bürger *et al.*, 2012). It is worth noting that it can be challenging to differentiate between the de-palmitoylation enzymes in the literature, as publications use the terms APT and PPT inconsistently, possibly due to the lack of an identifying motif and increasing understanding of the proteins function over time (Dunphy and Linder, 1998; Munday and López, 2007; Tsutsumi, Fukata and Fukata, 2008; Iwanaga *et al.*, 2009; Edmonds and Morgan, 2014).

Additional PTMs of interest

Additional PTMs have been linked with the BK channel, such as S-nitrosylation, N-glycosylation and SUMOylation. S-nitrosylation is the covalent modification of a cysteine amino acid residue by a nitrous oxide group to generate S-nitrosocysteine (CySNO), which facilitates regulation of protein function, as reviewed in 2013 (Gould *et al.*, 2013). It is generally accepted that the BK channel activating effects of nitric oxide can be mediated via elevation of intracellular cGMP (Feil, 2003; Kyle, Mishra and Braun, 2017), however nitric oxide can exert BK activating effects independent of cGMP for instance via S-nitrosylation (Ahern, 2002; Tjong *et al.*, 2008).

N-glycosylation is the addition of a carbohydrate moiety to the amino acid residue asparagine (N) and is implicated in the control of ion channel folding, stability, trafficking and function (Schwarz and Aebi, 2011; Moharir *et al.*, 2013). An N-glycosylation site has been detected in the N-terminal of Drosophila BK channels, however this site is not conserved in mammalian BK channels (Meera *et al.*, 1997). Mutation of an N-glycosylation site identified in the human BK channel had no effect on cell membrane expression but resulted in a significant left-shift in voltage for half-maximal activation (Bravo-Zehnder *et al.*, 2000).

SUMOylation is the addition of SUMOs (small ubiquitin-like modifiers) to proteins via the lysine (K) amino acid residue, and is categorised as a post-translational modification that tags proteins for

ubiquitin-dependent degradation (as reviewed by Geoffroy and Hay, 2009; Wang and Prelich, 2009; Miteva *et al.*, 2010). C-terminal ubiquitination of BK channels in rodent brain has been shown to cause channel retention in the ER, and inhibition of channel surface expression (Liu *et al.*, 2014).

1.7.3 External Environment and the BK channel

There are a number of metabolic factors that influence BK channel function and distribution, such as oxygen concentration (Liu, Moczydlowski and Haddad, 1999; Williams *et al.*, 2004; McCartney *et al.*, 2005; Cheng *et al.*, 2008), pH levels (Avdonin, Tang and Hoshi, 2003) and exposure to compounds such as alcohol (Dopico, Lemos and Treistman, 1996; Jakab, Weiger and Hermann, 1997). However, the external factors most relevant to the thesis will be briefly introduced below.

Glucose

Glucose is a tightly regulated energy substrate required for metabolic homeostasis and plays a role in a plethora of cellular processes. Alteration of cell glucose concentration has been shown to affect the BK channel and its associated auxiliary subunits. Exposure of murine podocytes to high glucose at 36.1mM reduced BK channel steady-state plasma membrane expression by 70% (Kim and Dryer, 2011). The hyperglycaemia associated with diabetes type 2 has been shown to down-regulate β1 subunit expression in retinal arterial smooth muscle of rats (McGahon *et al.*, 2007). A review article written in 2011 describes impaired vascular BK channel function associated with type 2 diabetes (Lu and Lee, 2010) and highlights that studies collectively show a down regulation of vascular BK channel β1 subunit expression (McGahon *et al.*, 2007; Zhang *et al.*, 2010). The β1 subunit of the BK channel is responsible for the modulation of calcium and voltage activation, it is speculated that that is a mechanism by which high glucose causes channel deregulation. High glucose concentration has also been shown to increase BK channel activity in retinal pericytes (Berweck *et al.*, 1994). In human podocytes, high glucose causes an increase in BK channels at the plasma membrane and increased channel activity; this was hypothesised to be due to increased oxidative stress, increasing intracellular calcium and leading to BK channel activation (Huang and Ma, 2010). The relationship between oxidative stress, intracellular calcium and the BK channel is varied and complex, and is described in a comprehensive 2015 review article (Hermann, Sitdikova and Weiger, 2015).

Although low glucose concentration causes cell death, as comprehensively reviewed in 2008 (Zhao *et al.*, 2008), exposure of cells to high glucose concentrations can result in apoptosis (Ho *et al.*, 2000; Maedler *et al.*, 2003; Samikkannu *et al.*, 2006; Jiaqi Liu *et al.*, 2016). It has been proposed that, in some cases, BK channel activation and up-regulation can play a role in cell apoptosis, such as was shown

when HEK293 cells were exposed to high (25mM) glucose concentrations (Chang et al. 2011). The increased cell apoptosis was thought to be caused by increased potassium loss (due to BK channel activation), which is associated with apoptotic cell shrinkage (Dallaporta *et al.*, 1998), and activation of apoptotic pathways involving protease enzyme caspases family and members of the apoptosis regulator Bcl-2 family amongst others (Elmore, 2007).

Lipids

The effects of lipid on BK channel activity has been discussed in a review by Dopico and Bukiya (2014). Cholesterol is one of the most widely studied lipids and there is mounting evidence that cholesterol has direct effects on BK channel function (Lam, Shaw and Duszyk, 2004; Bukiya *et al.*, 2011). There are a number of potential mechanisms to explain the effects; for example, the incorporation of cholesterol into the plasma membrane increases the lateral steric pressure on the channel, increasing the mechanical energy required to overcome this and open the channel, reducing the open probability (Po) of the channel (Lam, Shaw and Duszyk, 2004). Cholesterol is reported to cause destabilisation of the BK channel open state via steric bilayer lateral stress, decreasing open probability in rat cerebral artery myocytes (Bukiya *et al.* 2011). BK channels may be involved in the secretion of trans-epithelial anions through a cholesterol-dependant mechanism in colonic epithelial cells of mice (Lam, Shaw and Duszyk 2004). Another hypothesis is that when a cell is loaded with cholesterol, that this altered protein:lipid ratio causes a change in the formation of lipid rafts and altered translocation of the channel into the cell membrane. This may be seen as a change in the number of channels in the cell membrane, or altered cycling of the channel.

Several studies have shown that an increased extracellular concentration of palmitate elicits a change in cellular processes. Such examples include altered autophagy regulation in human aortic endothelial cells mediated by diminished AMPK activity, and increased phosphorylation of previously inactive kinase enzymes (Weikel *et al.*, 2014), induction of apoptosis in hepatocytes via activation of protein kinase C (Cai *et al.*, 2014), increased expression of interleukin-6, a cytokine with an inflammation modulating role, in human myotubules (Staiger *et al.*, 2004; Weigert *et al.*, 2004) and to increase the protein expression of the pseudo-kinase Tribbles homolog 3 (TRB3) (Morse *et al.*, 2010; Yan *et al.*, 2014). Palmitate has also been shown to affect the cell membrane expression of proteins; when THP-1 cells were exposed to palmitate at 0.3mM, the cell membrane expression of the integrins CD11b and CD36 was increased by over 60% (Pararasa, 2013). Palmitate also increased by more than 2-fold, tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor 5 (DR5) cell surface expression in hepatocarcinoma (Huh-7) cells exposed to 0.4mM palmitate for 8 hours (Cazanave *et al.*, 2011). There is limited evidence of a direct effect of palmitate on BK channels (Pipili, 2012), however

there has been extensive investigation of the effects of palmitoylation on BK channel function (as reviewed by Shipston 2011).

Oleate (18:1) has been used in laboratory experiments as a fatty acid control for saturated lipids such as palmitate, to determine if any of the measured variables are affected by the degree of fatty acid saturation (Yuzefovych, Wilson and Rachek, 2010). Experiments frequently compare and contrast the effects of the lipids on mitochondrial function, markers of ER stress, apoptosis, insulin signalling, insulin resistance and inflammation (Gaster, Rustan and Beck-Nielsen, 2005; Coll *et al.*, 2008; Yuzefovych, Wilson and Rachek, 2010; Kwon, Lee and Querfurth, 2014; Hetherington *et al.*, 2016). The majority of studies demonstrate that oleate has no significant effect on study endpoints in comparison to control, normally culture medium supplemented with bovine serum albumin (BSA). However, a small number of studies have shown that excess oleate can influence cellular processes. For example, incubation of the neural cell culture line N2a with 0.3mM oleate decreased mitochondrial superoxide levels, which are associated with oxidative damage and inflammation (Kwon, Lee and Querfurth, 2014), exposure to oleate at 1mM for 48 hours resulted in cell death of hepatic stellate cells (Hetherington *et al.*, 2016) and oleate exposure at 0.1mM increased the phosphorylation of Akt protein kinase (Hardy *et al.*, 2005). No literature describing the effect of oleate on BK channel or ion channel cellular distribution was found, however there are publications describing the activating effects of oleate on BK channel activity (Denson *et al.*, 2000; Clarke *et al.*, 2002; Sun *et al.*, 2007).

Hypoxia

Chronic hypoxia has been shown to cause increased production of calcium transport systems (Jurkovicova *et al.* 2008) and inhibition of BK channel opening (Hartness *et al.* 2003). In a study conducted in 2005, the STREX splice variant was used to demonstrate that splice variants exhibit differing sensitivities to oxygen tension (McCartney *et al.*, 2005). In this case, hypoxia caused the BK channel with STREX insert to be inhibited, with the inhibition being calcium sensitive and reversible and not mediated by redox regulation. Hypoxia also increases the expression of the β1 regulatory subunit (Ahn *et al.*, 2012) and through monitoring of KCNMB1 and hypoxia inducible factor 1 a (HIF-1a) expression it was discovered that HIF-1a is responsible for the increased expression of the β1 subunit through binding to the KCNMB1 promoter. Hypoxia causes a 300% increase in BK a-subunit mRNA expression and a 200% increase in β1 subunit expression in both cultured ovine pulmonary smooth muscle cells (PSMC) and Sprague-Dawley rats (Resnik *et al.*, 2006). Williams et al provide evidence that the BK channel complex contains the enzyme hemoxygenase-2, and that this, through the production of carbon monoxide (CO) enhances channel activity in normoxia and causes channel

inhibition in hypoxia (Williams *et al.*, 2004). Native BK channel responses to hypoxia are extremely variable depending on the tissue they are expressed in (Park *et al.*, 1995). The general consensus is that hypoxia causes BK channel inhibition through direct mechanisms such as oxygen and carbon monoxide binding, indirect mechanisms such as changes in splice variant and auxiliary β-subunit expression and via secondary signalling such as changes in calcium, haemoxygenase or AMPK (Tang *et al.*, 2003; Laderoute *et al.*, 2006; Hou *et al.*, 2008; Yi, Morgan and Ragsdale, 2010).

REDOX

REDOX is the term for reversible oxidation and reduction reactions. The sulphur containing amino acids cysteine and methionine are sensitive to REDOX reactions due to the presence of highly reactive disulphide bonds. Cysteine residues can be oxidized at their thiol groups where two cysteine molecules link to form disulfide bonds internally within the protein or with other proteins. Disulfide bonds can be readily reduced by agents such as dithiothretiol (DTT), and oxidised by agents such as dithiodipyridine (DTDP) and hydrogen peroxide. A 2015 review comprehensively details the effect of REDOX on BK channels (Hermann, Sitdikova and Weiger, 2015). As an example, DTT shifted the voltage activation of the channels to more negative potentials and increased current activation as well as channel open probability human BK channels expressed in HEK293 cells, whilst oxidation using hydrogen peroxide had the opposite effect (DiChiara and Reinhart, 1997).

1.7.4 Cell membrane expression of BK channel trafficking

The BK channel must be inserted into a membrane to function; therefore, the channel needs to be transported from the location of synthesis in the endoplasmic reticulum (ER) -via its processing site the Golgi apparatus-, to the cell membrane. The cell must also have the ability to rapidly alter the number and location of BK channels; therefore, transport vesicles, endosomes and lysosomes are important parts of the cell machinery. Figure 1.6 shows a schematic of the current understanding of BK channel trafficking within the cell, highlighting the main steps in channel movement from creation in the ER to degradation in the lysosome. Many factors have been shown to modulate BK channel trafficking and enable the fine-tuning of channel location and distribution in the cell; however, relatively little is known about the specific spatiotemporal dynamics of BK channel control (Shipston, 2014; Shipston and Tian, 2016).

Palmitoylation

Palmitoylation has been shown to occur in a wide range of proteins (Yang *et al.*, 2009), however evidence that palmitoylation plays a role in protein trafficking in each case is lacking. Notwithstanding, a relationship between palmitoylation and protein trafficking is increasingly being shown. For example, evidence suggests that the small GTPase enzymes H-Ras and N-Ras cycle between the cell membrane and the Golgi apparatus, regulated by palmitoylation (Goodwin *et al.*, 2005; Rocks *et al.*, 2005; Iwanaga *et al.*, 2009; Lynch *et al.*, 2015). The post-synaptic molecular scaffold protein PSD-95 has been shown to undergo dual palmitoylation on cysteine residues adjacent to the N-terminus which aid association with synaptic AMPA receptors at the synaptic membrane (Craven, El-Husseini and Bredt, 1999; El-Husseini *et al.*, 2002; Elias and Nicoll, 2007). Palmitoylation can also modulate the trafficking of BK channels; the cysteine residues of the S0-S1 TMD linker (mSlo C53, C56 and C56) have been shown to modulate channel localisation to the cell membrane via palmitoylation. This relationship was demonstrated in work published in 2010 (Jeffries, 2010; Jeffries *et al.*, 2010), in 2012 (Tian *et al.*, 2012) and in 2014 (Bi, 2014; Kim *et al.*, 2014); here de-palmitoylation of the S0-S1 cysteine residues suppresses BK channel cell membrane expression by approximately 50%.

Fundamental to the process of BK channel palmitoylation are the palmitoylation and de-palmitoylation enzymes. PATs zDHHC22 and zDHHC23 have been identified as primarily responsible for the palmitoylation of the cysteine residues of the S0-S1 linker (Tian *et al.*, 2012); knockdown of the PATs resulted in trapping of the channel in trafficking vesicles, recycling endosomes or lysosomes. The cellular location of zDHHC22 was not determined, as overexpression of the PAT was not tolerated by the HEK293 cells. However, it was determined that zDHHC23 was located in the Golgi network and is facilitates BK channel exit from the ER. There is evidence of compensatory feedback between the PAT enzymes; knockdown of zDHHC5, zDHHC7, and zDHHC17 resulted in a paradoxical increase in BK channel membrane expression (Tian *et al.*, 2012), however, this occurred alongside a significant up-regulation of zDHHC22 and zDHHC23 expression. APT 1 is located in the Golgi apparatus; activity is associated with trapping of the channel in the GA. Overexpression of de-palmitoylation enzymes APT1 and APTL1 reduced surface expression of ZERO channels (Tian *et al.*, 2012).

Splice Variants

The addition or removal of coding exons (resulting in splice variants) add to the diversity of the BK channel and have been shown to modulate the channel trafficking pathway. Splice variant SV1, found in the S0-S1 linker, creates an ER retention signal that prevents efficient export of the channel from

the ER (Zarei *et al.*, 2004), whilst splice variant mk44, also found in the S0-S1 linker, results in N-terminal endoproteolytic cleaving and trapping of the remainder of the channel in the ER (Korovkina *et al.*, 2001). Cysteine residues located in the STREX splice insert in the C-terminus (Tian *et al.*, 2008; Jeffries, 2010) have been linked with increased cell membrane localisation. In addition, an acidic cluster-like motif upstream of the STREX splice insert site was found to be essential for channel exit from the endoplasmic reticulum and subsequent cell surface expression (Chen *et al.*, 2010). However, differences in splice variant cell membrane expression have also been noted. Splice variant Δe23 in the C-terminus, located at the same site of splicing as STREX, results in a frameshift, and introduction of a premature stop codon (Chen *et al.*, 2005) and produces channels lacking a C-terminus past RCK1 which act as a dominant negative of cell membrane expression.

β -subunits

Interaction of auxiliary subunits with the BK channel further increases the functional diversity of the channel and have been shown to modulate BK channel trafficking. For example, the β4-subunit may play an important role in trafficking of the BK channel to the cell membrane; it has been shown that assembly of β4-subunits with α-subunits reduces the surface expression of the channel complex in HEK293 cells (Shruti *et al.*, 2012). Interestingly, the effect of β4-subunit and α-unit co-expression on channel surface expression is splice variant dependent; the surface expression of α-subunits containing the REVEDEC motif (normally associated with reduced membrane expression (Kim *et al.*, 2007) is upregulated by β4-subunit co-expression (Chiu *et al.*, 2010; Chen *et al.*, 2013).

Phosphorylation

A level of cross-talk between ion channel palmitoylation and phosphorylation has been demonstrated. PKC has been shown to inhibit the BK ZERO channel by approximately 50%, however, inclusion of a palmitoylated STREX insert prevents PKC inhibition by preventing the phosphorylation of serine 695, which is required for PKC inhibition (Zhou *et al.*, 2012); in the same study, removal of STREX palmitoylation at C645 and C646 restored PKC inhibition. However, palmitoylation of the STREX insert is required for BK channel inhibition by PKA (Tian *et al.*, 2008). There have been few publications to date detailing a direct role of phosphorylation on BK channel membrane expression. However, in a 2012 study, overexpression of 5' AMP-activated protein kinase (AMPK) was shown to increase the surface expression of BK channels expressed in *Xenopus oocytes* (Föller *et al.*, 2012). The study also

showed that AMPK deficient mice had reduced BK-channel expression in the inner ear in comparison to wild-type.

Cytoskeleton

Cytoskeletal networks and the trafficking machinery controlling movement of the BK channel within the cell are integral to the positioning of the BK channel within the cell membrane(Kim and Oh, 2016). For example, an actin-binding domain situated in the C-terminus of the BK channel was shown to promote trafficking of the channel to the plasma membrane in HEK293 cells (Zou *et al.*, 2008); a point mutation of this domain at L1020, reduced membrane association by 75%. The cell cytoskeleton has also been implicated in the control of rat BK channels in the cell membrane; lateral movement of BK channels in COS-7 cells was increased by point mutagenesis of the actin-binding domain (Won, Lee and Park, 2011). Overexpression of synaptopodin, an actin-associated protein with a role in actin-based cell shape and motility regulation, with the BK channel resulted in increased channel surface expression in the HEK293T cell line of approximately 100% (Won, Lee and Park, 2011). Filamin A, an actin-binding protein that forms perpendicular actin cross-links to modulate cell shape, is required for BK channel membrane expression in M2 melanoma cells (Kim, Ridgway and Dryer, 2007). Contrary to actin and filamin-A, co-expression of membrane-associated guanylate kinase with inverted orientation protein-1 (MAGI-1), a cell scaffolding protein, with the BK channel inhibits channel membrane expression by approximately 50% in HEK293T cells (Ridgway, Kim and Dryer, 2009). Cereblon, a protein which forms part of the E3 ubiquitin ligase complex, suppresses the surface expression of rat BK channels (Jo *et al.*, 2005) and reduces trafficking of the BK channel from the ER.

Other cytoskeleton proteins such as microtubule-associated protein 1A (MAP1A) (Park *et al.*, 2004) have been shown to bind to the BK channel, however there has been limited evidence of their role in membrane trafficking.

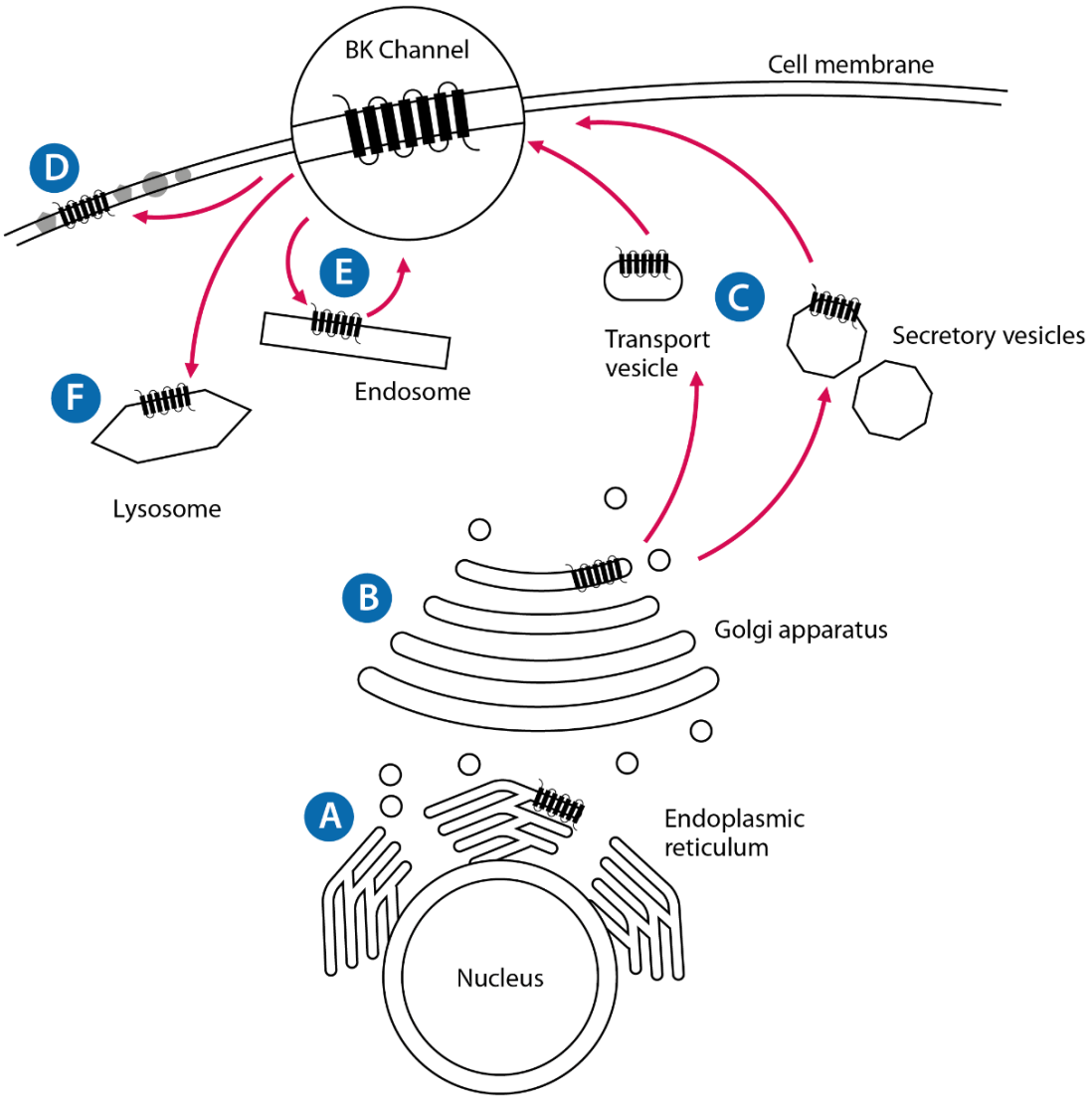


Figure 1.6- BK channel trafficking pathway. A) BK channel protein synthesis in the endoplasmic reticulum (ER). **B)** BK channel processing in the Golgi apparatus (GA). **C)** BK channel sorting and trafficking to target membranes via transport and secretory vesicles. **D)** Localisation of BK channels to specific cell membrane microdomains. **E)** BK channel recycling between the cytosol and the cell membrane via endosomes. **F)** BK channel degradation via lysosomes. Figure adapted from (Jeffries, 2010; Shipston, 2014).

1.8 BK channel as a personalised medicine target

1.8.1 Pharmacological agents targeting BK channels

Due to the wide and varied effects of the BK channel in physiology, it has been investigated as a pharmacological target (Gribkoff, Starrett and Dworetzky, 2001). Agents have been formulated to tackle pathophysiologies associated with channel dysfunction or take advantage of the hyperpolarising effect of the channel. An example of this is in the treatment of urinary incontinence; the BK channel activating agent NS11021 was designed to inhibit bladder spasms whilst leaving voluntary bladder contraction intact (Layne *et al.*, 2010). Compounds NS-8, TA-1702 and NS19504 have also been investigated as BK channel targets for overactive bladder, however efficacy in patients has not yet been conclusively established (Tanaka *et al.*, 2003; Tanabe, 2007; Nausch *et al.*, 2014).

BK channel opener BMS-204352 was designed to prevent the toxic increase in intracellular calcium concentration observed in neurones during ischaemic stroke (Jensen, 2002). Whilst initial phase I and phase II clinical trial results were positive, a phase III trial found no significant difference in stroke outcomes compared to placebo and so the agent was not developed further (Jensen, 2006). The lack of effect is proposed to be due to delay in administration of the compound to patients due to logistical issues (Wulff and Zhorov, 2008). The BK channel blocker GAL-021 was developed to treat respiratory insufficiency in an acute care setting; GAL-021 increased respiratory drive and stimulated ventilation by blocking BK channels in the hypoxia sensitive carotid body (McLeod *et al.*, 2014). BK channels are also a target of interest for asthma with several agents in development (as reviewed by Rusiecka and Kocic, 2012).

However, due to the lack of widespread therapeutic success of compounds targeting BK channels to date (Wulff and Zhorov, 2008; dela Peña and Cheong, 2011), another approach may be warranted. This approach would be to look for inter-individual difference in the BK channel gene, and investigate the potential effects this could have on channel function and thus disease. This would enable the stratification of patients for more precise treatment targeting with existing therapies. To this end, SNP occurring on the BK channel are of interest.

1.8.2 Disease causing SNP in BK channels

Genome wide association (GWA) studies have provided evidence that DNA mutations occurring in the intronic regions of the BK channel gene are associated phenotypic traits related to diseases. The BK channel gene (KCNMA1) was identified as an obesity suceptability gene; in a GWAS using a morbidly obese case group (n=4214) and control cohort (n=5417) a significant association between SNP

rs2116830 and obesity was identified (Jiao *et al.*, 2011). Further investigation uncovered increased KCNMA1 mRNA expression in adipose tissue and fat cells. As stimulation of BK channels in fat cells has led to pre-adipocyte proliferation (Hu *et al.*, 2009), the study proposed that changes in BK channel expression levels could result in obesity by increasing the number of adipocytes. Further evidence to support this theory was provided in a 2016 study where it was shown that BK channel knockout mice had reduced body weight and overall fat mass in comparison to wild-type (Illison *et al.*, 2016).

The BK channel gene has also been associated with all cause mortality in those with heart failure (Morrison *et al.*, 2010). In this 2010 GWAS, the genomes of 2,992 individuals who suffered a heart failure event were screened and the SNP rs4979906 was found to be associated with increased all cause mortality. Whole exome sequencing identified KCNMA1 SNP rs16934182 and rs1131824 associated with severe hypertension and myocardial infarction (Tomás *et al.*, 2008). SNP rs7071206 was identified during a meta-analysis of GWAS that assessed SNP occurring in the genome of 133,460 individuals; rs7071206 is associated with risk of low-trauma fracture, however the nature of the realtionship between fractures and the BK channel has not yet been established (Estrada *et al.*, 2012).

The nsSNP D434G (rs137853333), characterised in both the human and murine form of the channel (Du *et al.*, 2005; Wang, Rothberg and Brenner, 2009; Yang *et al.*, 2010) is responsible for the genetic disorder Generalized Epilepsy and Paroxysmal Dyskinesia (GEPD). GEPD occurs as a result of the substitution of an aspartic acid amino acid residue for a glycine residue in the conserved cytosolic linker region in the C-terminus, downstream from the S6 transmembrane domain (Yang *et al.*, 2010). The mutation results in reduced activation time and enhanced channel calcium sensitivity as a result of increased calcium affinity (Du *et al.*, 2005; Díez-Sampedro *et al.*, 2006; Lee and Cui, 2009; N'Gouemo, 2014). The mechanism behind this increased calcium sensitivity is proposed to be reduced flexibility of the AC region in the RCK1 domain (Yang *et al.*, 2010).

The BK channel nsSNP A138V (rs144215383) is located in the intracellular S0-S1 linker has been associated with a loss of function of the channel; in this case, the substitution of valine for alanine is associated with autism and mental retardation (Laumonnier *et al.*, 2006). The biomolecular cause of the effect has yet to be elucidated, however it is postulated that the mutation creates a 'cryptic' splice site, a disadvantageous splice site that is dormant or used only at low levels unless activated by mutation, that results in impaired channel expression (Guglielmi *et al.*, 2015).

Disease causing nsSNP in ion channels

Disease causing SNPs have been identified in other ion channels. The voltage-gated sodium channel $Na_v1.7$ plays a role in the generation of action potentials in peripheral neurons and thus is an important step in the pathway for the generation of pain signals. This channel is coded for by the SCN9A gene and variations in this channel that cause either gain or loss of function mutations (Drenth and Waxman, 2007). Gain of function mutations in this channel, such as L858H, I848T (Yang *et al.*, 2004), L858F (Han *et al.*, 2006) and N395K (Drenth *et al.*, 2005), are responsible for the disease Primary Erythermalgia, a rare, hereditary syndrome of hyperaemic and inflamed extremities, which in all cases has been attributed to mutations in the highly-conserved regions of the channel. The majority of perturbing mutations are located in the cytoplasmic transmembrane domain linkers, and cause a hyperpolarizing shift in the voltage dependence of channel activation. This allows the channel to be activated by smaller than normal depolarizations whilst also slowing the deactivation of the channel, resulting in the open-state being present for longer. Loss of function mutations of the $Na_v1.7$ channel are causative of Channelopathy-associated Insensitivity to Pain (CIP); nsSNP S459X, I767X and W897X (Cox *et al.*, 2006) result in nonsense mutations that code for a triplet sequence that does not code for an amino acid residue, resulting in a truncated, non-functioning ion channel protein.

The voltage-gated calcium channel, CaV3.2, coded for by the CACNA1H gene, is present in the adrenal glomerulosa. Gain of function mutations in this channel, result in early-onset hypertension as a result of primary aldosteronism (Scholl *et al.*, 2015). Exome sequencing was conducted for 40 subjects suffering from the condition and the root cause identified as nsSNP M1549V. This mutation occurring in a highly-conserved domain causes channel activation at more hyperpolarised membrane potentials and impaired channel inactivation. This results in a significant and chronic increase in intracellular calcium concentrations, leading to increased aldosterone production. As aldosterone is responsible for increasing the retention of sodium, which is coupled with water, an increase in this hormone lead to an inappropriate increase in blood volume and thus blood pressure.

1.9 Thesis Aims

1.9.1 Rationale for Study

Bioinformatics informs personalised medicine and facilitates the discovery of SNPs with the potential to cause disease. Due to the volume of SNPs associated with the human genome (over 84 million (Auton *et al.*, 2015)), it is not practical in any one thesis to assess those most likely to cause harm across the whole genome using a protein map; thus, the selection of a single gene would allow a more in-depth study and enable the prioritisation of those SNP likely to alter BK channel function. The BK channel was selected as the model gene of investigation due to its ubiquitous nature and role in a diverse range of physiological processes. The SNP D434G is causative of generalised epilepsy and paroxysmal dyskinesia (GEPD) (Du *et al.*, 2005); this provided evidence that genetic mutations of that gene can result in disease. Before the prioritisation of SNP can commence, biocuration of BK channel data contained in bioinformatics databases will be undertaken. Due to the inherent limitations of bioinformatics, the SNPs categorised as having a high potential to cause a change in BK channel function would need to be validated experimentally to confirm effect. Thus, the SNP will be taken to the lab for verification.

1.9.2 Aims

To capture and curate the key aspects of data relating to the human BK channel (such as the locations of structural, functional, PTM, site-directed mutagenesis sites and single-nucleotide polymorphisms (SNPs)) stored within bioinformatics databases. This will be undertaken in steps 1-4 of the thesis workflow, shown in Figure 1.7.

- To use the collated and curated BK channel information to create a novel 'at a glance' resource for the BK channel with the synthesised information. This will be undertaken in step 5 of the thesis workflow.
- To use the BK channel resource to facilitate the prioritisation of SNPs predicted to affect BK channel function. This will be undertaken in step 6 of the thesis workflow.
- To assess the effect of prioritised S0-S1 linker SNPs (hSlo H120Q and G122A) on the cellular distribution of BK channels expressed in HEK293 cells cultured in combinations of the metabolic substrates glucose, palmitate and oleate. This will be undertaken in step 7 of the thesis workflow.

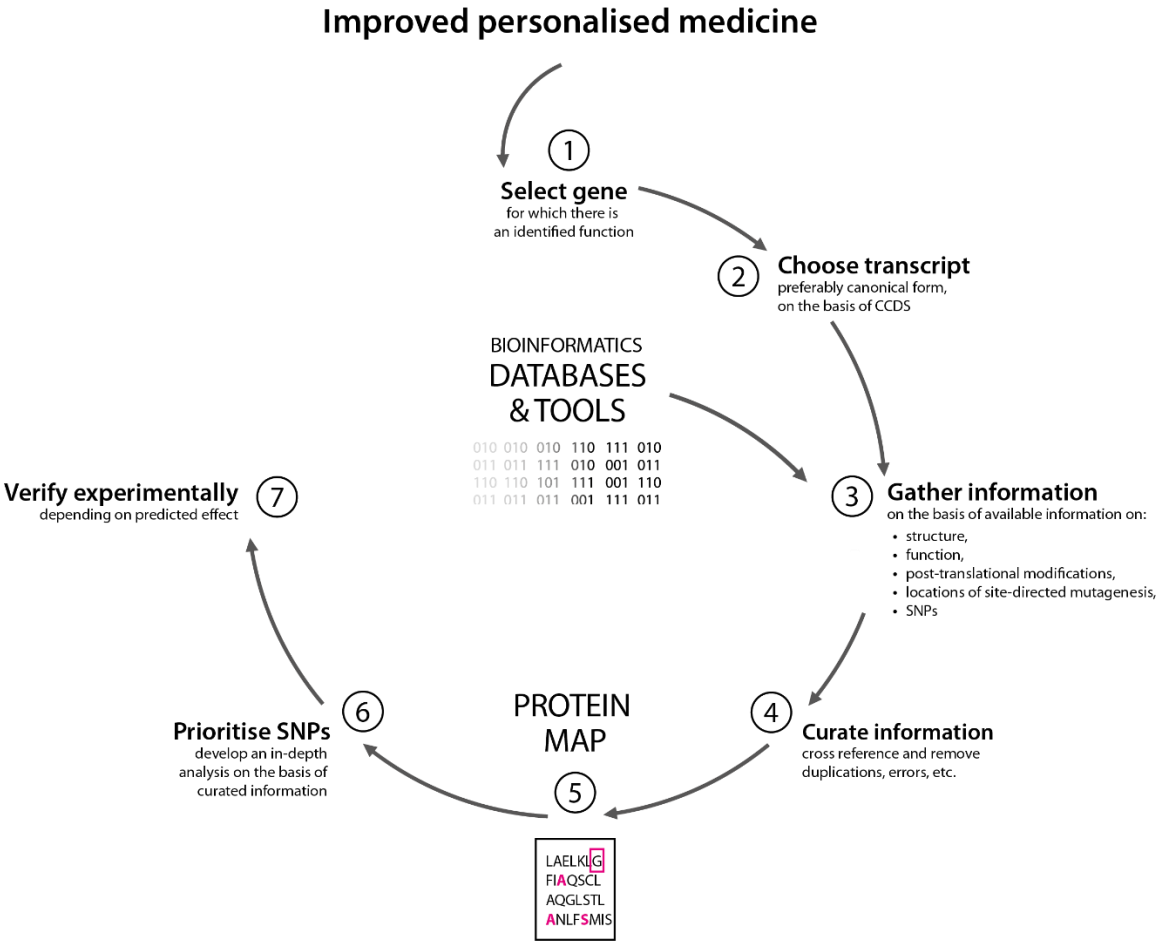


Figure 1.7- Schematic of proposed thesis workflow. Described are the steps to be undertaken in this thesis work to identify new potential genetic targets for personalised medicine. Step 1: the BK channel gene has been selected for investigation as it is coded for by a single gene, plays a diverse role in physiology and has prior evidence of SNP mediated dysfunction. Steps 2-6 will be undertaken in chapter 3, whilst step 7 will be addressed in chapter 4.

2 General Methods

2.1 Bioinformatics

2.1.1 Identification of the Human KCNMA1 Gene and Transcripts

BK channel genomic information contained in bioinformatics databases was investigated for the human form of the KCNMA1 gene using a range of databases and software tools including Ensembl (release 72, accessible via www.ensembl.org/index.html). Some of the key steps and procedures have been captured in Figure 2.1. For example, the first steps in procedure for accessing the appropriate gene are shown in Figure 2.1. The molecular location for the BK channel gene, as well as the transcript data was accessed by selecting the 'Result in Detail' option and the 'Gene:KCNMA1' tab respectively (see Figure 2.2). The cytogenic location of the BK channel gene was also accessed via the Ensembl database.

Selection of the associated 'Transcript ID' link for a specific transcript in the 'transcript ID' column presents additional transcript information, including the number of exons associated with each transcript and identifiers from other databases as can be seen in Figure 2.3A. This option must be selected before searching for information for differing transcripts to ensure that splice variant specific information is gathered. Ensembl contains amino acid sequence information for all transcript entries in FASTA format; this can be accessed by selection of the 'Protein' tab in 'Transcript-based displays'. A sample FASTA output for one of the KCNMA1 transcripts can be seen in Figure 2.3B.

In addition to Ensembl, data held on the UniProt bioinformatics database (available from www.uniprot.com) was used to determine the canonical transcript for the KCNMA1 gene and retrieve additional protein information. Figure 2.4 shows the steps taken to retrieve BK channel transcript information using UniProt. After selection of the KCMA1_HUMAN protein family, a large amount of information becomes available on a single page including an 'Alternative Products' section near to the bottom of the page. This section provides details of transcripts (here identified as isoforms) associated with the KCNMA1 gene. Selection of a transcript from the 'Alternative products' page produces an output as shown in Figure 2.5. 'Isoform 1' in the list is the canonical transcript of the BK channel. In order to illustrate the nature of the process, data from the canonical transcript is shown in Figure 2.5B, which displays the amino acid sequence for the canonical protein and the link to the amino acid sequence in the FASTA format. The rational for selection of the canonical protein will be examined and discussed in the following chapters.

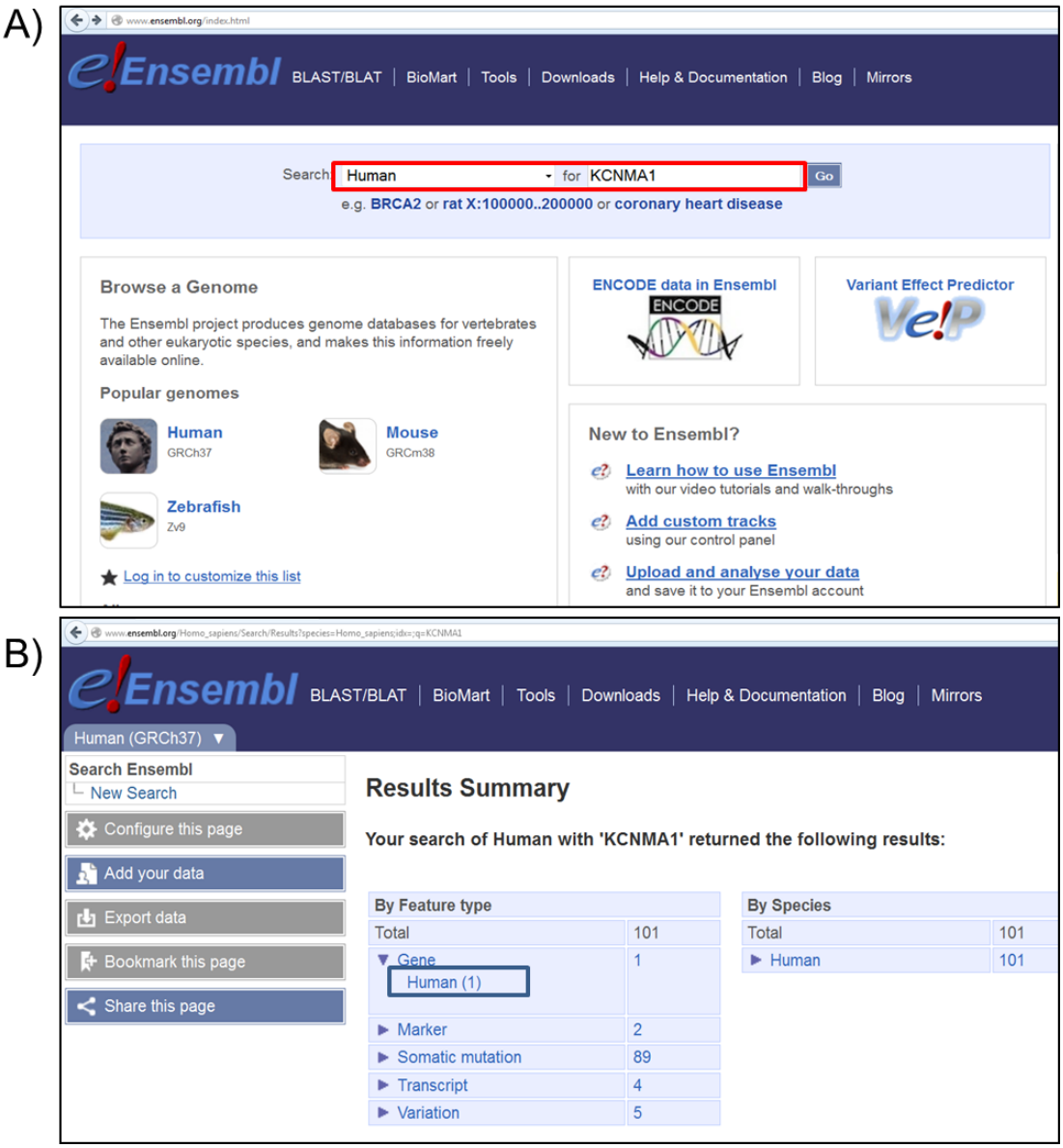
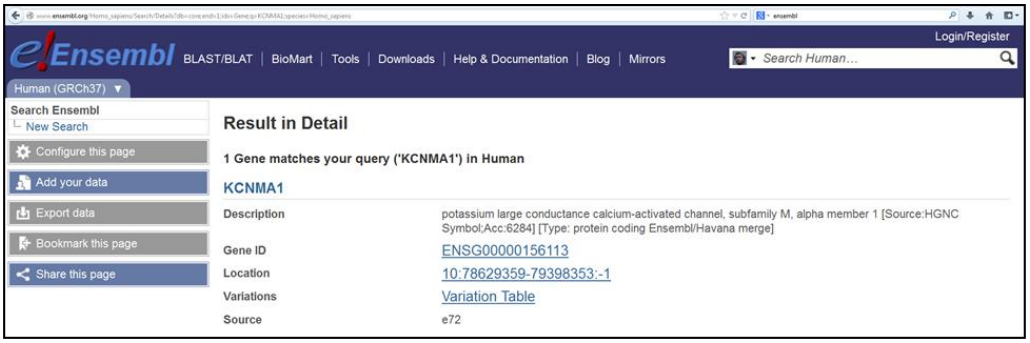


Figure 2.1-Locating the BK channel gene on the Ensembl database. A) Ensemble gene search from release 72. Search boxes containing search terms highlighted in red. The human genome was selected from the species dropdown menu and 'KCNMA1' was entered into the search box. After pressing enter the search result page is produced. **B)** Ensembl search result for Human KCNMA1 gene. This shows the total number of entries for the gene and a breakdown of entry category**.** The human form of the gene was selected from the menu as highlighted in blue.

A)



B)

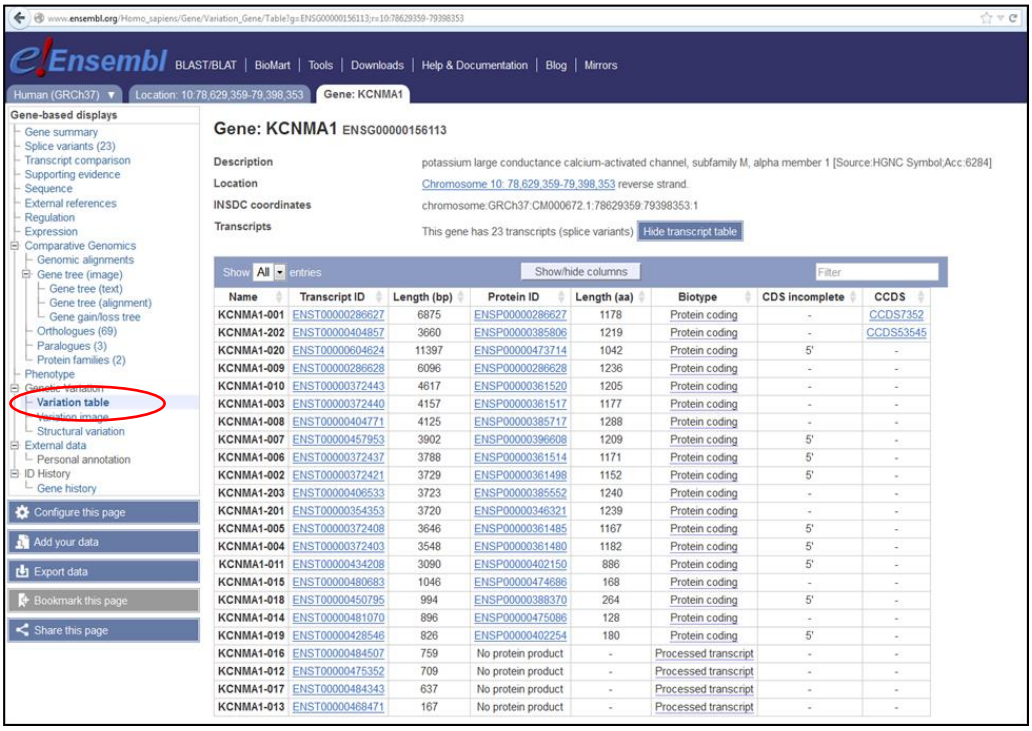


Figure 2.2- Selection of BK channel transcripts on the Ensembl database. A) Expanded Ensembl search result for Human KCNMA1 Gene. Selection of the 'variation table' link under the 'variations' subheading and opening up the 'show transcript table' produces a list of all transcripts associated with the KCNMA1 gene. In this section gene description and links to the gene ID, location, variation and source are included. **B)** Ensembl summary of all KCNMA1 associated transcripts. The chromosome location and number of splice variants are included in this output, as well as splice variant name, Ensembl ID, length in base pairs (bp), the ID of the resulting protein, the protein length in amino acids (AA) and the biotype of the splice variant. Highlighted in red to the left is the 'Variation Table' option which is selected to view SNPs associated with each transcript.

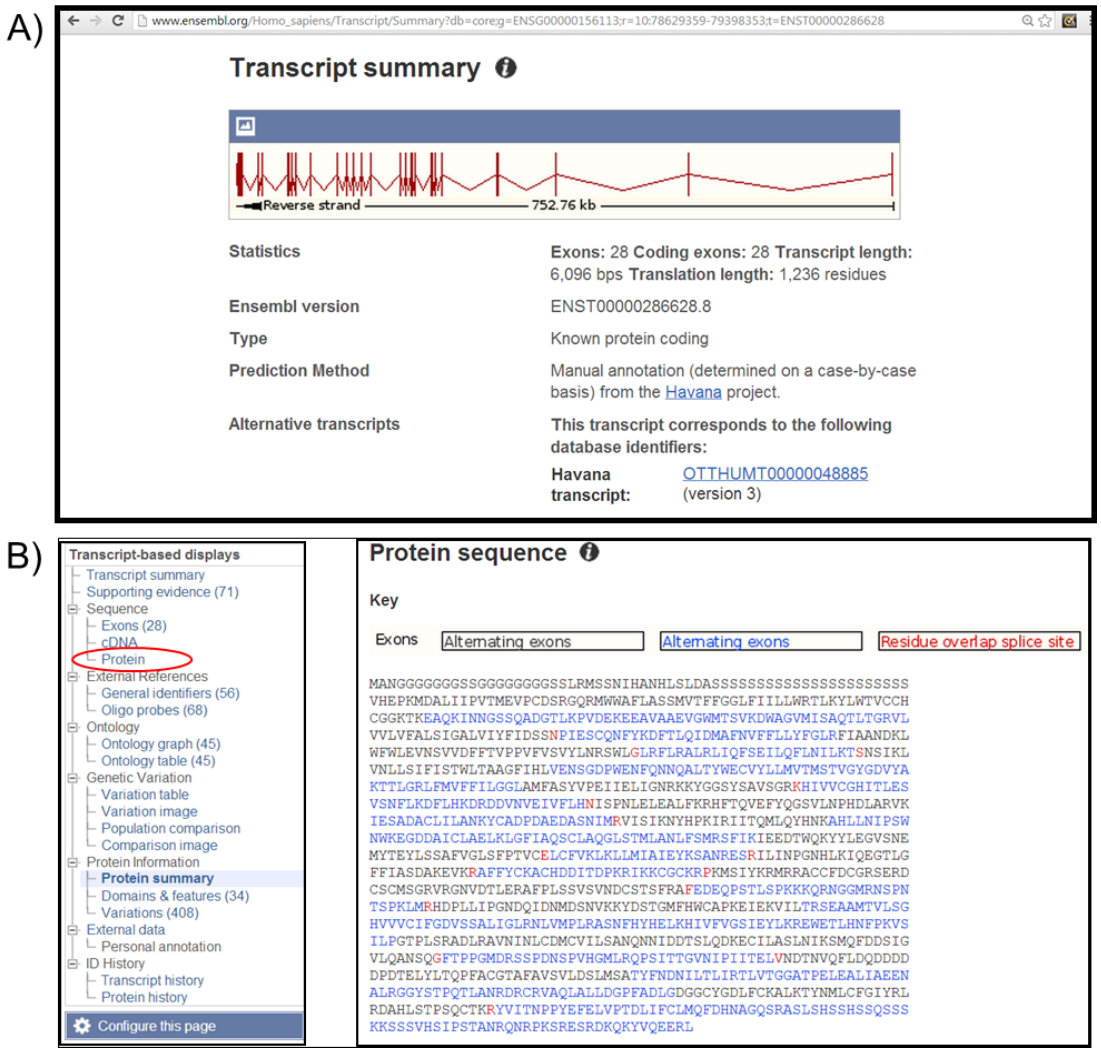
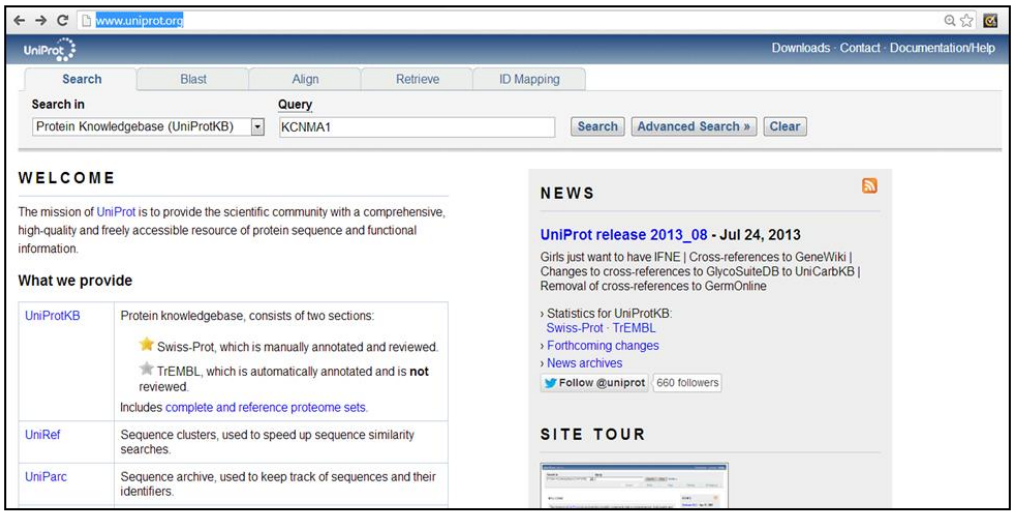


Figure 2.3- Transcript Summary and Protein Amino Acid Sequence output in Ensembl. A) Ensembl transcript summary output. Presented here is a schematic highlighting the number of exons that make up the selected transcript; exons are represented by vertical red bars, separated by introns represented by horizontal red lines. The direction in which the chromosome strand is read is shown at the bottom of the schematic, with additional information about the protein such as transcript length in base pairs (bps) and in amino acid residues and its identifier in other databases. **B**) Ensembl amino acid sequence output for exemplar transcript. After selection of the 'protein' link circled in red, the amino acid (AA) sequence for the protein is displayed. Alternate exons are differentiated by alternating blue and black segments; red letters indicate residue overlap splice sites.

A)



B)

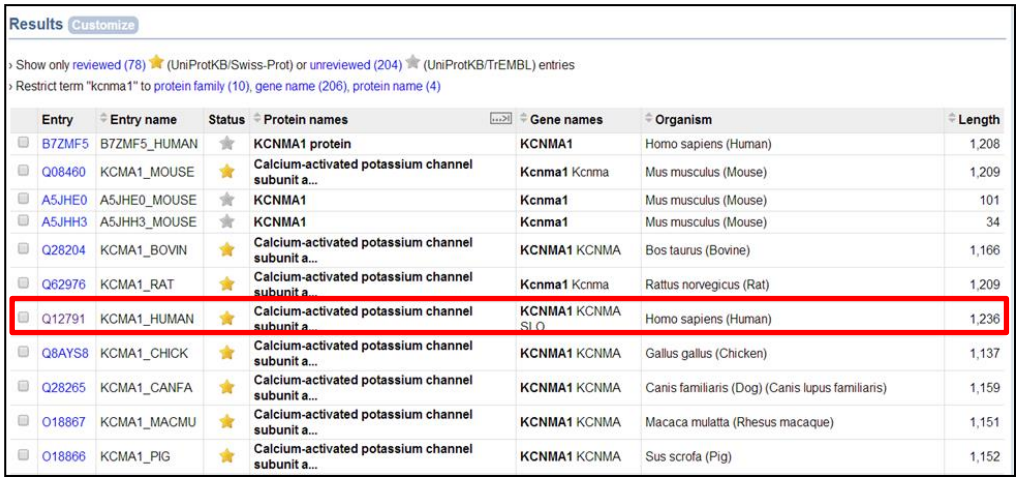


Figure 2.4- Locating KCNMA1 protein information using the UniProt database. A) UniProt Protein Search from Homepage. KCNMA1 was entered into the 'query'; search bar on the UniProt homepage and the search option selected. **B)** Selection of the human KCNMA1 protein family from the UniProt database. This option displays results of the search query; in this output all entries containing KCNMA1 as a keyword have been shown. Highlighted in red is the human canonical KCNMA1 protein, which is listed on UniProt as KCMA1_HUMAN.

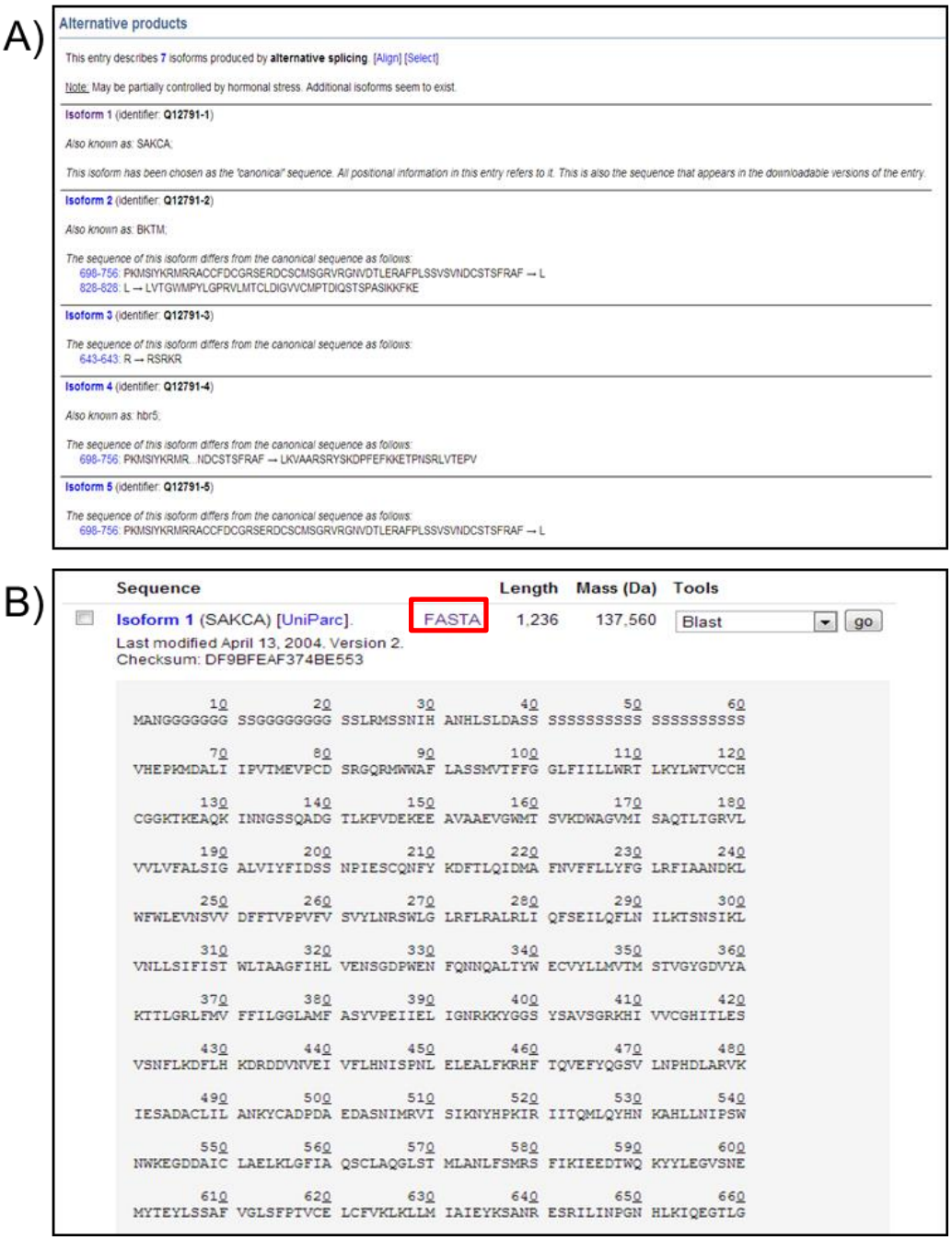


Figure 2.5- UniProt Summary of human KCNMA1 protein isoforms. A) UniProt Summary of all human KCNMA1 protein isoforms held on the UniProtKB database. In this output, a comprehensive list of documented transcripts is displayed with a brief annotation on how each isoform deviates from the canonical isoform. **B) UniProt transcript-amino acid sequence output.** Amino acid sequences are listed in groups of ten. The link that leads to the amino acid sequence in FASTA format is highlighted in red.

2.1.2 Identification of the amino acid locations of KCNMA1 protein structural and functional sites

UniProt and Ensembl databases were used to identify the amino acid location of structural and functional areas on the KCNMA1 canonical transcript. In the UniProt database, the name of the structural and functional sites or 'Regions' on the channel were listed alongside the position of the site on the channel, and the site length in amino acid residues. A simplified schematic indicating where these sites are located on the channel is displayed in Figure 2.6A. UniProt gives users the option to view areas of the protein involved in secondary structure, illustrated in Figure 2.6B. The secondary structure information can be used in conjunction with the 'Regions' subsection to locate areas involved in protein folding. The Ensembl outputs for BK channel structure and functional regions are accessed by selecting the 'Domains and features section' tab on the left-hand side of the page as indicated in Figure 2.7; here the functional regions are described.

2.1.3 Identification of nsSNPs on KCNMA1 transcripts

Collation of the location and nature of single nucleotide polymorphisms (SNP) on the BK channel was undertaken prior to the implementation of a bioinformatics methodology to allow for prioritisation of damaging SNP. Therefore, SNP data for the BK channel was accessed using the Ensembl database. Upon selection of a transcript from the 'variation table' option shown in Figure 2.3B, a table containing a summary of variants, their nature and description could be obtained (Figure 2.8). This option enables the user to retrieve variation information associated with the gene as a whole or for a particular transcript. Such variations include 'in-frame insertions', 'intron variants' and 'synonymous variants'. The number of variations of each type is included in the output, alongside a brief description of the type and the nature of variations that are included in the category. Selection of a category of variants provides comprehensive information about the nature of each variant within that category. In Figure 2.8B, a sample of information contained in the database for 'missense' (non-synonymous) variations of the BK channel are listed. This section is used to find specific information about a variation such as its amino acid location within the protein, the specifics of the amino acid substitution (such as the base pair alteration) or its molecular location in the gene.

By selecting the 'Variation Image' option either in Gene view or after selecting a specific transcript, an image with SNP data is produced with locations in respect to the associated transcript and its composite exons (Figure 2.9).

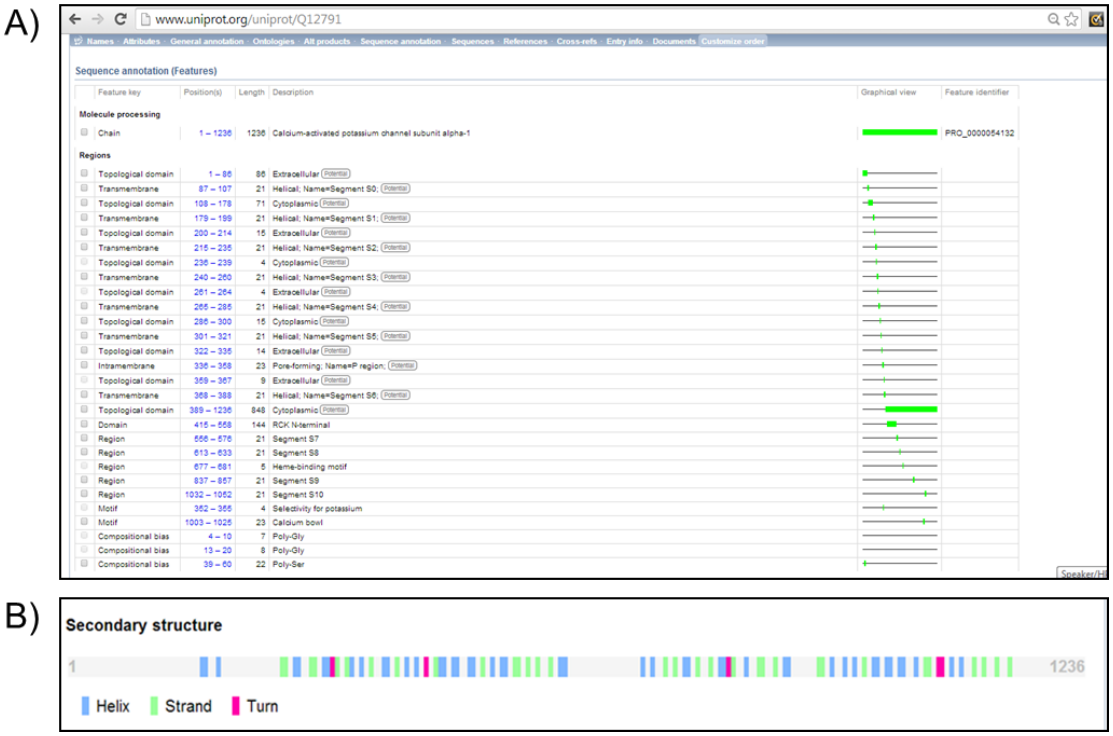


Figure 2.6- Uniprot output: Protein Regions and Secondary Structure. **A)** Uniprot 'Regions' subsection output. Shows the amino acid locations of structural and functional regions of the canonical KCNMA1 protein. This output details the region type, location in terms of amino acids and a basic graphic denoting rough location and proportion of the total protein that the region occupies. **B)** UniProt Output for KCNMA1 Protein Secondary Structure. Colour coded as per the key, the bar above is a representation of the areas within the protein that are involved in the formation of secondary structure.

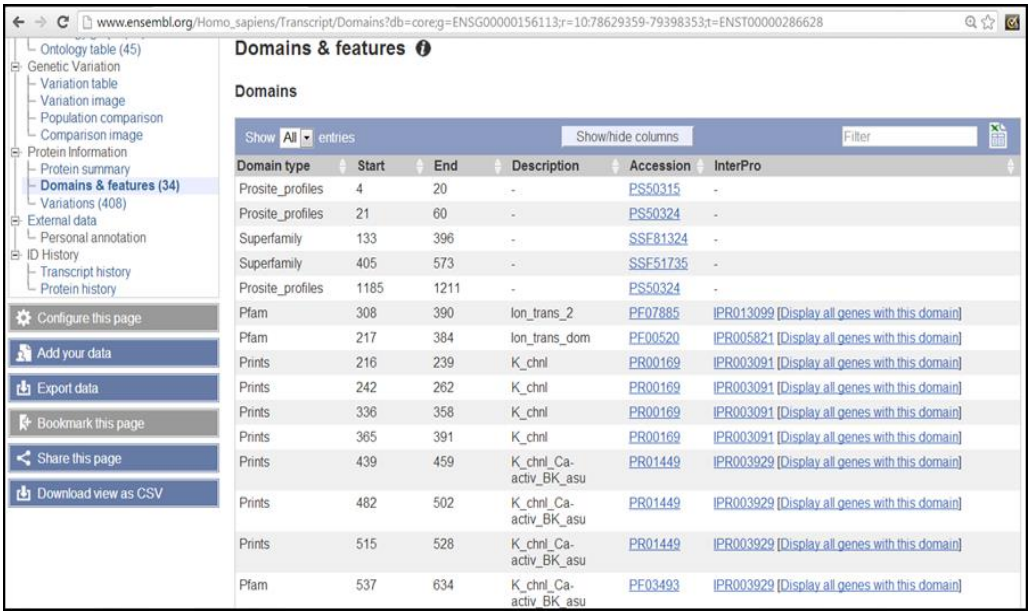


Figure 2.7- Ensembl Domains and Features output. Detailing the domains associated with the KCNMA1 protein, with location listed in amino acid residues via numbering the start and end of the domain. Also included is a brief description of the domain.

This output aids SNP location visualisation and complements the information presented in the 'variation table' output. More detailed information on each variation can be accessed by clicking on a filled box; a pop-up will be created containing the same information, found in the 'variation table'. An alternative method of viewing the SNPs associated with a protein coding transcript is to select the 'Protein summary' option. This option, like the 'variation image', allows SNPs to be presented as colour coded boxes and in relative position to the protein and its functional regions. As with the 'variation image', clicking on one of the coloured boxes presents the user with expanded variation information.

BK channel variation information is also contained in the UniProt database and can be found under the 'Natural Variations' subheading on the KCNMA1 protein family homepage (Figure 2.10A). Here, naturally occurring variations are compared with the canonical transcript of the gene. A key to the right of the page indicates the location of the variation on the transcript, whilst another column explains the nature of the variation.

2.1.4 The predicted damaging potential of BK channels nsSNP calculated by SIFT and PolyPhen-2

Damage prediction software outputs were used to facilitate the prioritisation of nsSNP on the BK channel. The predicted damage score of BK channel nsSNPs was calculated by SIFT and PolyPhen-2 software. The Ensembl variation output includes the predicted damage category and score for nsSNPs which is integrated into the output, therefore a query sequence does not need to be separately submitted manually for each variation. SIFT and PolyPhen-2 software can be accessed via http://sift.jcvi.org/ and http://genetics.bwh.harvard.edu/pph2/ respectively.

2.1.5 Assessment of previous site directed mutagenesis studies of the BK channel

Site directed mutagenesis studies are useful for studying the functional effects of nsSNPs, and facilitate the prioritisation of BK channel nsSNPs, as they can indicate the role that a region plays in the structure and function of the channel. As a result, the occurrence of SNP at those same sites could predict the consequence; thus, knowledge of the locations of site directed mutagenesis on the channel is desirable. The UniProt database holds information on mutagenesis studies, an example of which is shown in Figure 2.10B. This section combines the results of studies where the effects of KCNMA1 mutations have been tested. The list of mutagenesis sites in the UniProt database was not extensive, therefore a literature search was also conducted to establish the location, nature and consequence of mutagenesis sites of the BK channel.

A)

Show All entries Filter

Number of variant consequences		Type	Description
0	-	Transcript ablation	A feature ablation whereby the deleted region includes a transcript feature (SO:0001893)
6	Show	Splice donor variant	A splice variant that changes the 2 base region at the 5' end of an intron (SO:0001575)
5	Show	Splice acceptor variant	A splice variant that changes the 2 base region at the 3' end of an intron (SO:0001574)
19	Show	Stop gained	A sequence variant whereby at least one base of a codon is changed, resulting in a premature stop codon, leading to a shortened transcript (SO:0001587)
15	Show	Frameshift variant	A sequence variant which causes a disruption of the translational reading frame, because the number of nucleotides inserted or deleted is not a multiple of three (SO:0001589)
0	-	Stop lost	A sequence variant where at least one base of the terminator codon (stop) is changed, resulting in an elongated transcript (SO:0001578)
0	-	Initiator codon variant	A codon variant that changes at least one base of the first codon of a transcript (SO:0001582)
0	-	Transcript amplification	A feature amplification of a region containing a transcript (SO:0001889)
2	Show	Inframe insertion	An inframe non synonymous variant that inserts bases into in the coding sequence (SO:0001821)
9	Show	Inframe deletion	An inframe non synonymous variant that deletes bases from the coding sequence (SO:0001822)
320	Show	Missense variant	A sequence variant, that changes one or more bases, resulting in a different amino acid sequence but where the length is preserved (SO:0001583)
55	Show	Splice region variant	A sequence variant in which a change has occurred within the region of the splice site, either within 1-3 bases of the exon or 3-8 bases of the intron (SO:0001630)
0	-	Incomplete terminal codon variant	A sequence variant where at least one base of the final codon of an incompletely annotated transcript is changed (SO:0001626)
194	Show	Synonymous variant	A sequence variant where there is no resulting change to the encoded amino acid (SO:0001819)
0	-	Stop retained variant	A sequence variant where at least one base in the terminator codon is changed, but the terminator remains (SO:0001567)
1	Show	Coding sequence variant	A sequence variant that changes the coding sequence (SO:0001580)
0	-	Mature miRNA variant	A transcript variant located with the sequence of the mature miRNA (SO:0001620)
0	-	5 prime UTR variant	A UTR variant of the 5' UTR (SO:0001623)
168	Show	3 prime UTR variant	A UTR variant of the 3' UTR (SO:0001624)
0	-	Non coding exon variant	A sequence variant that changes non-coding exon sequence (SO:0001792)
15669	Show	Intron variant	A transcript variant occurring within an intron (SO:0001627) (WARNING: table may not load for this number of variants!) View list in BioMart
0	-	NMD transcript variant	A variant in a transcript that is the target of NMD (SO:0001621)
0	-	NC transcript variant	A transcript variant of a non coding RNA (SO:0001619)
82	Show	Upstream gene variant	A sequence variant located 5' of a gene (SO:0001631)
119	Show	Downstream gene variant	A sequence variant located 3' of a gene (SO:0001632)
16609	Show	ALL	All variations (WARNING: table may not load for this number of variants!) View list in BioMart

B)

Missense variant consequences

Show All entries Show/hide columns Filter

ID	Chr: bp	Alleles	Global MAF	Class	Source	Evidence	Clin-sig	Type	AA	AA co-ord
rs75843969	10:78647031	C/T	-	SNP	dbSNP	-	-	Missense variant	R/Q	1235
rs139370249	10:78647032	G/A	-	SNP	dbSNP		-	Missense variant	R/W	1235
rs150023686	10:78647054	T/G	-	SNP	dbSNP		-	Missense variant	K/N	1227
rs200410171	10:78647061	C/T	-	SNP	dbSNP	-	-	Missense variant	R/Q	1225
rs148648986	10:78647062	G/A	-	SNP	dbSNP		-	Missense variant	R/W	1225
rs199766376	10:78647071	T/C	-	SNP	dbSNP	-	-	Missense variant	R/G	1222
rs200723995	10:78647082	C/T	-	SNP	dbSNP	-	-	Missense variant	R/Q	1218
rs201703516	10:78647083	G/C	-	SNP	dbSNP	-	-	Missense variant	R/G	1218
rs199678989	10:78647084	G/C	-	SNP	dbSNP	-	-	Missense variant	N/K	1217
rs144301574	10:78647097	G/A	-	SNP	dbSNP		-	Missense variant	A/V	1213
rs200773083	10:78647100	G/A	-	SNP	dbSNP	-	-	Missense variant	T/I	1212
rs202024249	10:78647151	G/C	-	SNP	dbSNP	-	-	Missense variant	S/W	1195
rs200141207	10:78647178	C/T	-	SNP	dbSNP	-	-	Missense variant	R/Q	1186
rs200284976	10:78647229	G/A	-	SNP	dbSNP	-	-	Missense variant	T/M	1169

Figure 2.8- Ensemble Variation Table and sample 'Missense Variant' output. A) Truncated sample of Ensembl 'Variation Table' output for protein of transcript KCNMA1-009. Selection of the 'variation table' option results in all recorded variants for the selected transcript (in this case KCNMA1-009) to be displayed. Variations are categorised by the location of the variant on the transcript, for example 5 prime UTR .... or intron variant .... or on the resulting alteration to normal translation such as in-frame deletion .... or missense variant .....The number of variants in each category is displayed on the left of each category, and a description of each variant consequence is included in the right-hand column. **B)** Sample 'Missense Variant' output for KCNMA1 transcript. Details of variant ID, molecular location, alleles involved in the variation, class of change, the source of information, type of variation and amino acid coordinates provided in this output.

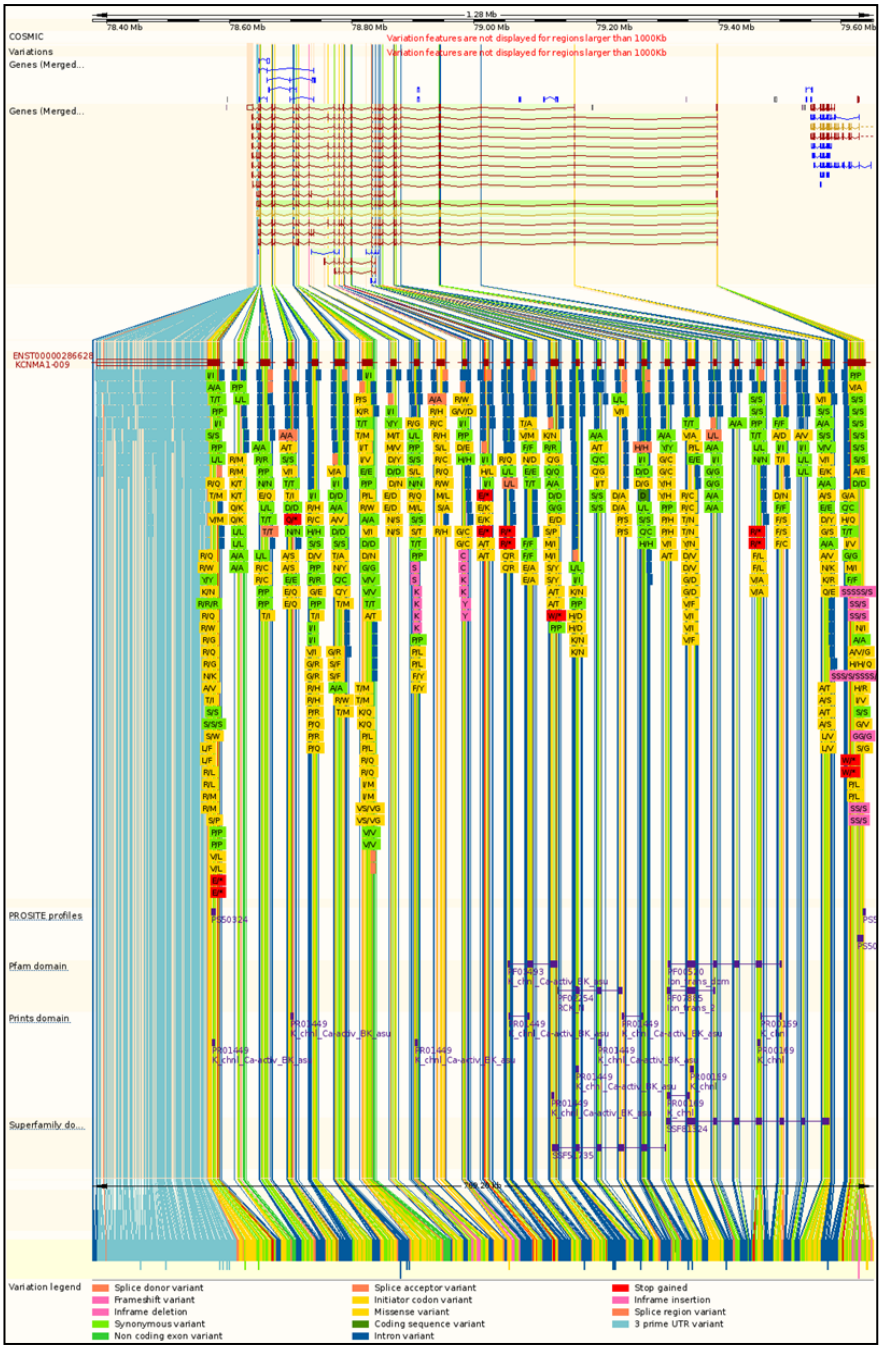


Figure 2.9- Ensembl KCNMA1 protein Variation Image Output. A copy of a page for a specific transcript, its composite introns and exons, notable functional regions in the protein, and variations. These variations are colour coded into categories as seen in the key at the bottom of the figure. The transcript is at the top of the figure in brown; exons are represented as filled boxed with the thinner lines on either side representing introns. The hollow box to the left of the first exon represents the three prime untranslated region (3' UTR region). Boxes below the transcript help to decipher the position and category of variants; the letters contained within the boxes inform of any change in amino acid residue as a result of the variation in question. A colour key for the category of variations is included at the bottom of the figure.

A)

Natural variations

Alternative sequence	127 – 168	42	EAQKI...DWAGV → ATHFGSPEMPPAARSWSGSP PEAAVLRGASSLALEVARCR RL in isoform 6.		VSP_009952
Alternative sequence	169 – 1236	1068	Missing in isoform 6.		VSP_009953
Alternative sequence	643	1	R → RSRKR in isoform 3.		VSP_009954
Alternative sequence	698 – 756	59	PKMSI...SFRAF → L in isoform 2 and isoform 5.		VSP_009955
Alternative sequence	698 – 756	59	PKMSI...SFRAF → LKVAARSRYSKDPFEFKKET PNSRLVTEPV in isoform 4.		VSP_009956
Alternative sequence	698 – 756	59	PKMSI...SFRAF → RWEEHCSLWRLESKGNVRRL NYCRGQQTSFVKVKVAARSR YSKDPFEFKKETPNSRLVTE PV in isoform 7.		VSP_009957
Alternative sequence	828	1	L → LVTGWMPYLGPRVLMTCLDI GVVCMPTDIQSTSPASIKKF KE in isoform 2.		VSP_009958
Natural variant	434	1	D → G in GEPD; may have a synergistic effect with ethanol in the triggering of symptoms. Ref.30		VAR_023821

B)

Experimental info

Mutagenesis	118	1	C → A: Decreased localization to the plasma membrane. Abolishes localization to the plasma membrane; when associated with A-119 and A-121. Ref.24 Ref.25	
Mutagenesis	119	1	C → A: Decreased localization to the plasma membrane. Abolishes localization to the plasma membrane; when associated with A-118 and A-121. Ref.24 Ref.25	
Mutagenesis	121	1	C → A: Decreased localization to the plasma membrane. Abolishes localization to the plasma membrane; when associated with A-119 and A-121. Ref.24 Ref.25	
Mutagenesis	269	1	L → R or H: No effect in the coupling between calcium and channel opening. Ref.17	
Mutagenesis	272	1	R → E: Induces reduction in the coupling between calcium and channel opening. Ref.17	
Mutagenesis	275	1	R → N: Induces reduction in the coupling between calcium and channel opening. Ref.17	
Mutagenesis	278	1	R → Q: Induces reduction in the coupling between calcium and channel opening. Ref.17	
Mutagenesis	281	1	Q → R: No effect in the coupling between calcium and channel opening. Ref.17	

Figure 2.10- UniProt KCNMA1 Natural variations and Experimental Information output. A) UniProt Natural variations output. Natural variations in transcript composition are compared with the canonical transcript for the gene. The amino acid location of the variation is shown in the second column, then the length and nature of the variation. **B**) UniProt experimental information output. Results from mutagenesis studies are presented; with columns stating the amino acid location, length, nature and position of the mutation in the transcript.

2.1.6 Identification of PTM sites on the KCNMA1 canonical transcript

A range of software tools were utilised to identify predicted sites of post translational modification on the human KCNMA1 transcript. Post-translational modification is important for the function of the BK channel, as reviewed by (Shipston and Tian, 2016). Identifying the location of post-translational modification sites on the BK channel will aid prioritisation of potentially damaging nsSNP; as nsSNP co-localised with post-translational sites may perturb channel function.

Phosphorylation prediction software site, NetPhos 2.0 is (available at (http://www.cbs.dtu.dk/services/NetPhos/), was used to determine serine (S), tyrosine (Y) and threonine (T) phosphorylation sites on the BK channel (Figure 2.11A). The FASTA sequence of the BK channel was entered; and the resultant serine, tyrosine and threonine sites were selected for investigation. A table and a graphic of the predicted phosphorylated amino acids, the threshold and the predicted score is produced as an output of the software.

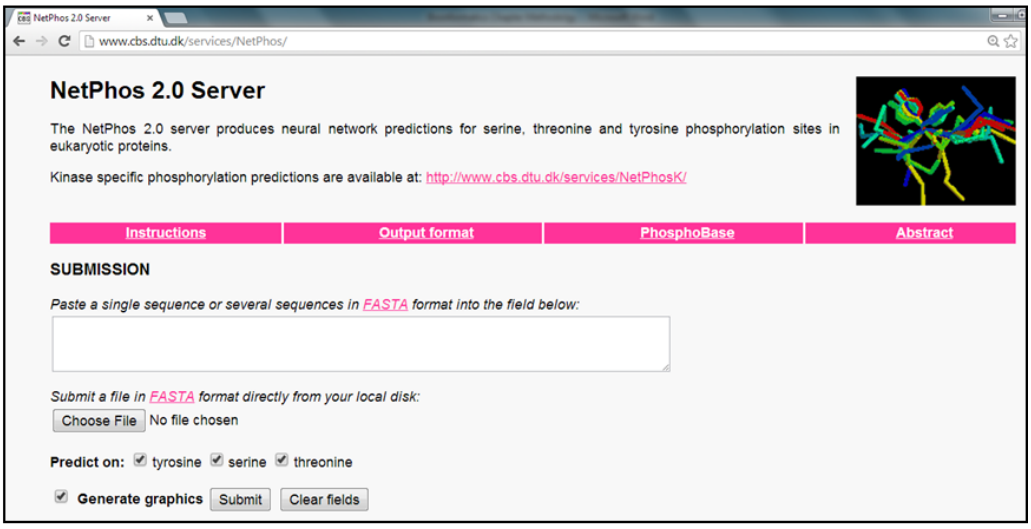
Palmitoylation prediction software site CSS-Palm 4.0 (available at http://csspalm.biocuckoo.org/) pinpoints the location of potentially palmitoylated cysteine residues in the amino acid sequence entered. To determine the number of cysteine residues on the BK channel that were predicted to be palmitoylated, the FASTA sequence was entered, as shown in Figure 2.11B, producing a list of cysteine residues predicated to be palmitoylated, along with the threshold for each residue and the predicted score. A high threshold for detection was selected as supported by previous publications utilising the software (Jeffries, 2010; Shipston, 2011; Feng *et al.*, 2013; Aguilar-Martinez *et al.*, 2015).

Using a link on the left-hand side of the CSS-Palm 4.0 website, the online server homepage for S-nitrosylation (http://sno.biocuckoo.org/online.php), and SUMOylation (http://sumosp.biocuckoo.org/) were accessed. The predictive software packages for S-nitrosylation and SUMOylation have the same controls as CSS-Palm 4.0, and thus predicted sites were accessed using the same procedure.

Predicted sites of N-glycosylation on the BK channel were predicted using NetNGlyc 1.0, accessible via http://www.cbs.dtu.dk/services/NetNGlyc/. The BK channel FASTA sequence was entered into the software at default detection threshold. The output produces an amino acid sequence with N-glycosylated sights highlighted, as well as a table listing the amino acid location of the predicted site, as well as the predicted score.

Sites of verified post-translational modification of the BK channel were identified using a literature search.

A)



B)

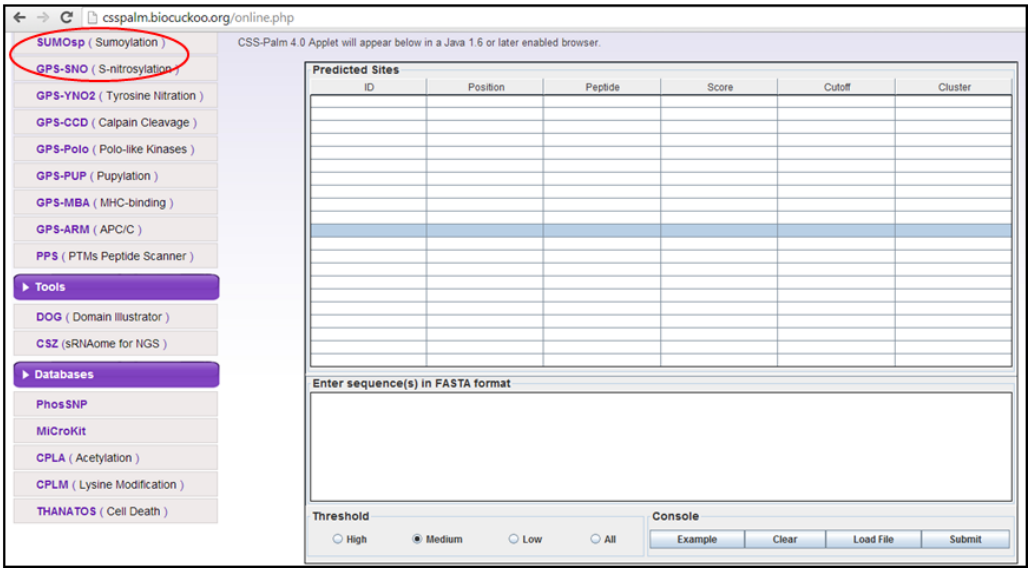


Figure 2.11- Post-translational modification predictive software. A) NetPhos 2.0 homepage. The FASTA sequence of the particular transcript is entered into the search box and the appropriate phosphorylated amino acid type (tyrosine, serine or threonine) is selected via tick box. The default setting is to predict for all three possible phosphorylated amino acid residues. **B)** CSS-Palm 3.0 online server homepage. The FASTA sequence of the transcript in question is entered into the search box and the threshold of the results is selected at the bottom of the webpage. Homepage accessible via http://csspalm.biocuckoo.org/online.php. The links to access the online server homepages to predict sumoylation and S-nitrosylation sites within the transcript are highlighted in red. Predictive software for N-glycosylation (NetNGlyc) is available via http://www.cbs.dtu.dk/services/NetNGlyc/.

2.1.7 Assessment of BK channel nsSNP and Key site co-localisation

The co-localisation of nsSNP and key sites on the BK channel was assessed by cross-referencing the amino acid locations. Key sites include, structural, functional, post-translational modification and those previously examined by site-directed mutagenesis. For the initial assessment, only sites with a direct match (occurring at the same amino acid location) were included; during nsSNP prioritisation, sites that lay within 3 amino acid residues of a nsSNP were included.

2.1.8 Prioritisation of BK channel nsSNP

SNPs predicted to result in alterations in protein function and possibly cause disease have been prioritised in previous studies with the aid of algorithms (Lee, 2009; Stead *et al.*, 2011; Teng *et al.*, 2012; Ryan *et al.*, 2014). In the studies referenced above, this method has been proven to be effective, resulting in a reduction in the number of nsSNPs in comparison to the original number, based upon the criteria set in each study. However, algorithms rely exclusively on previous machine learning of key sites on the channel, which is often not comprehensive and can contain ambiguous labelling, creating challenges for accuracy (Huang *et al.*, 2017). In this study, nsSNP prioritisation was conducted manually by the investigator. Priority was given to those SNPs that were; a) co-localised with, or were less than four amino acids away from a crucial functional site, such as the voltage sensor, b) proximal to crucial sites demonstrated by previous site-directed mutagenesis experiments to have a profound effect on channel function, such as mutations of the potassium selectivity motif, c) nsSNP with a significant effect on the local post-translational modification environment, for example a SNP that results in the creation or removal of a cysteine rich domain or d) proximal to another nsSNP shown to have physiological effects, such as D434G (Díez-Sampedro *et al.*, 2006; Yang *et al.*, 2010). The detail of this process is shown in the results section with a range of individual examples that help illustrate the key aspects of this process.

2.2 Lab Based Methods

Functional assessment of the nsSNP prioritised following the procedure detailed in the bioinformatics section will be investigated *in vitro*. This lab based approach involves the production of appropriate BK channel constructs via site directed mutagenesis, their expression in HEK293 cells and assessment via confocal microscopy. The lab-based methods used in previous studies (Chen *et al.*, 2005; Jeffries, 2010; Bi, 2014), for the investigation of the cell membrane expression of BK channels in HEK293 cells have served as a basis of the method selection for the lab based experiments.

2.2.1 Addition of FLAG and HA antigen tags to the BK channel protein

To assess the proportion of BK channels in the membrane, both membrane-bound and cytosolic channels must be tagged in a manner that allows for quantitative analysis. This study utilises confocal microscopy and thus a method producing fluorescently tagged channels was employed. FLAG and hemagglutinin (HA) tagging allows for the attachment of antibodies with fluorescent moieties to the BK channel. Expression of full length murine BK ZERO channel constructs with N-Terminal FLAG and C-terminal HA epitope tags were created as previously described using pcDNA 3.1 vectors (Chen *et al.*, 2005). Briefly, synthetic double-stranded FLAG and HA- oligonucleotides were blunt end ligated in frame with the initiator methionine (*DYKDDDDK*DALI…) codon for the FLAG tag and C-terminal cysteine (...REVEDEC*YPYDVPDYA*) HA tag using forward and reverse oligonucleotides (Figure 2.12).

2.2.2 Site Directed Mutagenesis to create BK channel mutants

To determine whether amino acid mutation occurring in the S0-S1 linker of the BK channel affects cell membrane distribution in HEK293 cells, BK channels containing the cysteine mutants (C53:C54A and C53:54:56A) and the SNP mimetic mutants (H55Q, G57A and H55Q:G57A) first needed to be created. This was achieved using site directed mutagenesis using the QuickChange II site-directed mutagenesis kit. An overview of the QuickChange II site-directed mutagenesis process is illustrated in Figure 2.13.

Design of oligonucleotides for site directed mutagenesis

Normally, when designing primers for site directed mutagenesis, a 20-30 base oligonucleotide with the mismatch located in the centre is sufficient for a single base mutation and 40-45 bases for a double mutation. However due to the high G/C content of the primer sequences, extended sequences were required to allow for additional perfectly matched nucleotides on either side of the mismatch. Oligonucleotides were ordered from Eurofins MWG Operon, (Ebersberg, Germany). The final primer design for each mutant is detailed in Table 2.1. The DNA template used for amplification was the murine ZERO form of the BK channel with Flag and HA epitope tags as described previously (Chen *et al.*, 2005).

Codon oligonucleotide substitution for the H55Q mutation on the forward strand was 'CAC' (H) to 'CAG' (Q) and for the G57A mutation was 'GGG' (G) to 'GCG' (A); the substitution of a cysteine amino acid residue to an alanine residue, as was conducted for the C53:54A mutant and C53:54:56A mutant, the codon sequence was changed from 'TGC' (C) to 'GCC' (A). The C53:54:56A mutant was made using a 2-step process; initially the cysteine residues at amino acid locations 53 and 54 were mutated to alanine residues, then this transcript was further mutated at cysteine 56 to produce the final product. A schematic of the amino acid sequence of mutant proteins is displayed in Figure 2.14.

Site Directed Mutagenesis

Site directed mutagenesis of amino acid residues was carried out using PCR in 50µl reactions with the QuikChange II XL Site-directed Mutagenesis Kit (Stratagene #200522). Each reaction mixture contained, 1µl *Pfu* turbo polymerase, 5µl of 10x reaction buffer, 3µl Quiksolution (Stratagene), 1µl of dNTP mix (NE BioLabs), 125ng of oligonucleotide sense primer, 125ng of antisense primer, 1.5µl of $MgCl_2$ (50 mM), 50ng template DNA, and DEPC water to make up to a 50µl final volume. Samples were heated to 95^{O}C for 1 minute to denature template DNA. Reactions were generally run for 18 cycles with 50 seconds at 95^{O}C, annealing temperature was set at 60 ^{O}C. Extension phases were conducted at 68 ^{O}C with times set at 1 minute per kb of plasmid length. This was followed by 10 minutes at 68^{O}C after the final cycle for the final extension. The PCR product then underwent *Dpn* I (10U/µl) digestion for 1 hour at 37 ^{O}C to eliminate any parental methylated DNA template.

Figure 2.12- Schematic of FLAG and HA tagging of BK channel constructs. FLAG and HA tag amino acid sequence shown alongside N-terminal and C-terminal BK channel amino acid sequence. The initiator methionine is removed to allow ligation of the FLAG antigen, and moved to the front of the FLAG tag giving sequence MDYK.

A)

$$Tm = \frac{81.5 + 0.41\ (\%\ GC) - 675}{N - (\%\ mismatch)}$$

B)

$$Tm = 81.5 + 0.41\ (\%\ GC) - \frac{675}{N}$$

Equation 2.1-A) Stratagene Primer Melting Temperature Formulas. N= Primer length in base pairs **B) Stratagene Primer Melting Temperature Calculation for Insertions and Deletions.** N=Primer length in base pairs, excluding bases being inserted or deleted.

Transformation of Chemically Competent *E. coli*

Transformation of newly synthesized plasmid DNA was carried out using chemically competent XL10-Gold® ultra-competent *E. coli* cells (Stratagene). The process of transformation was undertaken to cause the uptake of the plasmid vector containing the DNA mutations into the competent cells, with the aim of sealing up the nicks in the backbone from the overlapping primer PCR method and generate more copies of the plasmid containing the mutated sequence. 100µl aliquots were stored at -80^{O}C and subsequently thawed on ice. They were then split into 50µl aliquots. 10ng of plasmid DNA was added and the mixture was incubated on ice for 20 minutes. Subsequently, cells were heat-shocked at 42^{O}C for 45 seconds and 500µl of Luria Broth (LB) medium was added. The mixture was incubated for 30 minutes to 1 hour at 37^{O}C in a shaking incubator, at 200 rpm and then 200µl of cells were plated onto selective agar with ampicillin at a working concentration of 100ug/ml and incubated overnight at 37^{O}C.

PCR Screening of Bacterial Colonies

PCR screening of bacterial colonies was undertaken to confirm the successful incorporation of DNA within the plasmid vector. Individual colonies grown on selective agar plates were selected using a sterile pipette tip, then transferred into a 50µl PCR reaction tube containing primers specific to the region of interest. A standard PCR reaction amplified the region of interest (Figure 2.15), which could then be run out on a 1% (w/v) agarose gel. Those colonies shown by PCR screening to be the correct size (or if using double digest plasmid DNA, those that contained the correct size of insert), were again picked from the same plate using a sterile pipette tip and inoculated into either 5ml or 250 ml of LB medium with 100µg/ml ampicillin. This was incubated overnight at 37^{O}C in a shaking incubator, at 200 rpm, in order to set up either a mini- or maxi- prep culture.

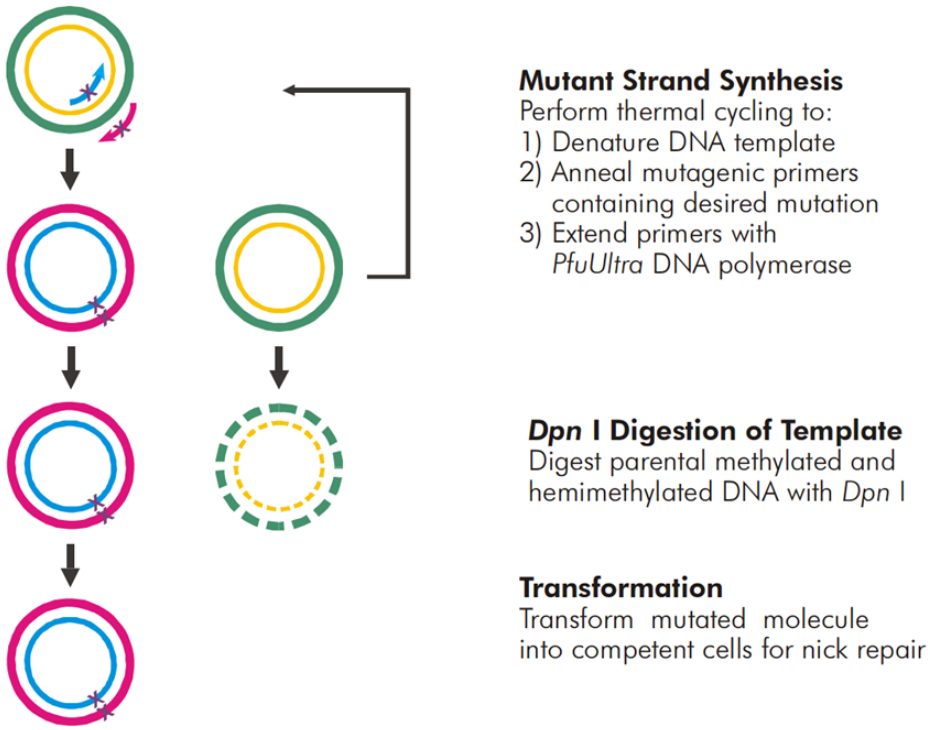


Figure 2.13- QuickChange II Site-Directed mutagenesis overview. An overview of the procedure to conduct site directed mutagenesis of the BK channel using QuickChange II is illustrated. The green and yellow strands denote template DNA strands, whilst pink and blue strands denote the newly synthesised DNA strands incorporating base pair substitutions, denoted by the purple X. After sufficient replication of the DNA strands incorporating the base pair substitutions, template DNA is removed by *Dpn* I digestion. Figure was adapted from QuickChange II Site-Directed mutagenesis protocol.

Mutation	Direction	Sequence (5'-3')	
H55Q	FWD 5'-	ACC TGT GGA CCG TTT GCT GCC AGT GCG GGG GCA AGA CGA AGG AG	-3'
	REV 5'-	CCT CCT TCG TCT TGC CCC CGC ACT GGC AGC AAA CGG TCC ACA GG	-3'
G57A	FWD 5'-	GTG GAC CGT TTG CTG CCA CTG CGC GGG CAA GAC GAA GGA GGC CCA G	-3'
	REV 5'-	CTG GGG CTC CTT CGT CTT GCC CGC GCA GTG GCA GCA AAC GGT CCA C	-3'
H55Q:G57A	FWD 5'-	CTG TGG ACC GTT TGC TGC CAG TGC GCG GGC AAG ACG AAG GAG GC	-3'
	REV 5'-	GCC TCC TTC GTC TTG CCC GCG CAC TGG CAG CAA ACG GTC CAC AG	-3'
C53:54A	FWD 5'-	GTA CCT GTG GAC CGT TGC CGC CCA CTG CGG GGG CAA	-3'
	REV 5'-	CTT GCC CCC GCA GTG GGC GGC AAC GGT CCA CAG GTA	-3'
C56A	FWD 5'-	CCG TTG CCG CCC ACG CCG GGG GCA AGA CGA AG	-3'
	REV 5'-	CTT CGT CTT GCC CCC GGC GTG GGC GGC AAC GG	-3'

Table 2.1- BK Channel Site Directed Mutagenesis Primer Sequences. Mutagenesis primer sequences for the SNP mimetic mutants (H55Q, G57A and H55Q:G57A) and the cysteine mutant channels (C53:54A and C53:54:56A) are detailed. Numbering starts from initiator methionine MDALI.. in the murine BK channel (accession number NM_010610).

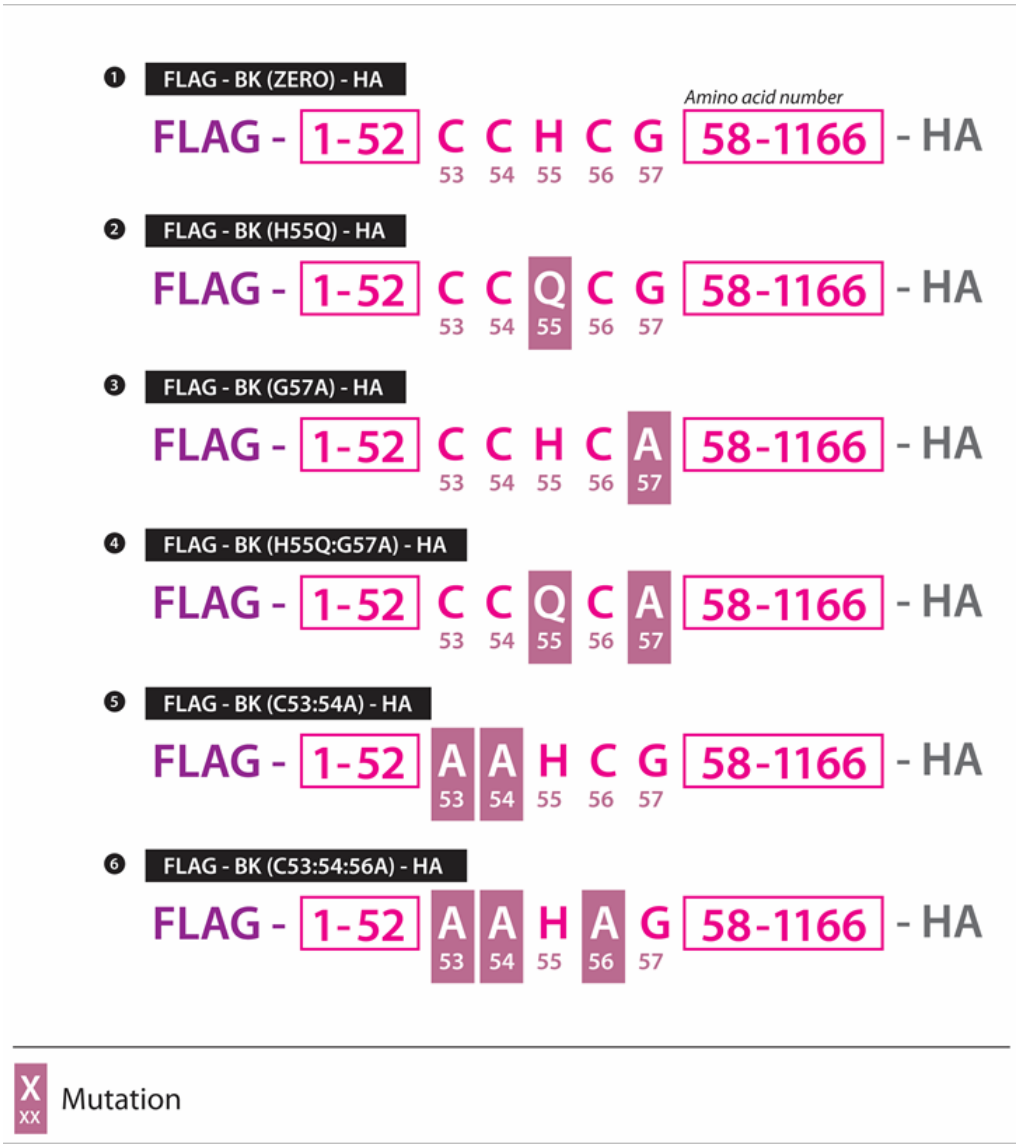


Figure 2.14- Amino acid sequence for mutated BK channel proteins. The mutant name in the format FLAG-BK(X)-HA is annotated alongside the amino acid sequence of interest, with numbering, starting from the initiator methionine, detailed below the sequence. Sequence 1 is the ZERO channel with the original amino acid sequence; amino acid mutations are indicated by a box, as per the key. Details of the codon alterations for each of the mutated amino acids is detailed in the section 'Design of oligonucleotides for site directed mutagenesis' earlier in this chapter.

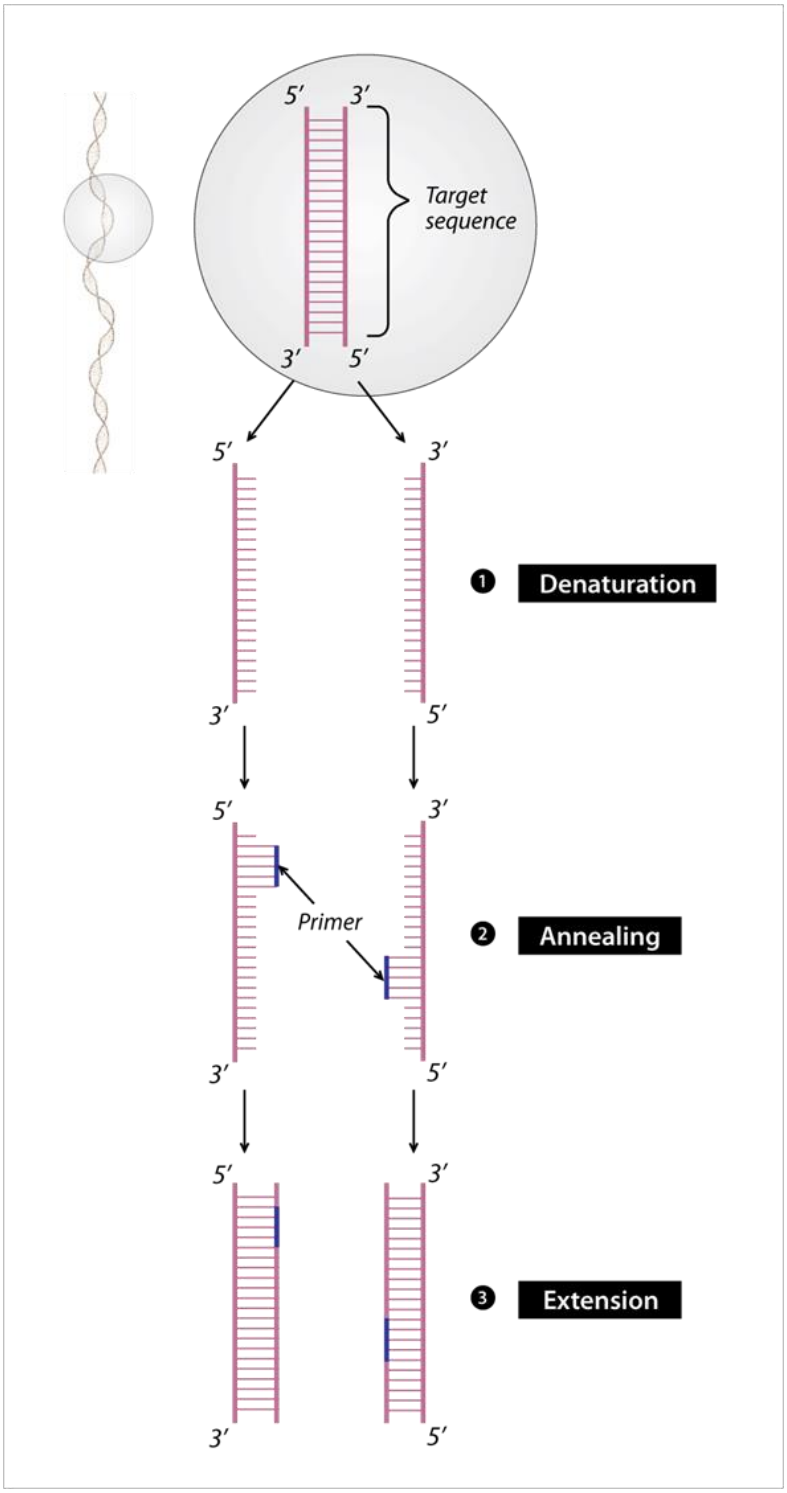


Figure 2.15- Schematic of DNA replication by PCR. 1) The target DNA sequence is first heated to 95ºC to melt double stranded DNA to single strands. 2) The temperature is lowered to allow primers to anneal to the newly exposed single stranded DNA. Typically, the annealing temperature is about 3–5 °C below the Tm of the primers, to allow for greatest binding and alignment accuracy 3) Temperature is increased to 72°C and DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding dNTPs that are complementary to the template in 5' to 3' direction. Modified from (Lawley, 2009)**.**

Sub cloning

Double Restriction Digest

Sequencing of the BK channel oligonucleotide sequence over the mutated region was conducted by Eurofins MWG Operon, Germany, to confirm incorporation of point mutations into the DNA sequence. On confirmation of the incorporation of the desired mutations, double restriction digestion was used to isolate the sequenced region and to then insert it into a new vector to assure a clean vector backbone without possible additional erroneous mutations in regions not submitted for sequence screening. Double restriction digests were prepared in 20µl reactions, each containing 2-10 U of restriction enzyme in the appropriate buffer diluted to 1x with DEPC water and 0.2-1µg of DNA sample. These reaction mixtures were then incubated at 37 ^{O}C for 1 hour.

DNA Agarose Gel Electrophoresis and Gel Purification

Gel Electrophoresis, to separate the DNA by molecular weight to confirm sizes of digested fragments, was carried out using a 1% agarose (w/v) gel prepared in 50ml or 100ml of 1xTBE buffer (45 mM Tris-base, 45 mM Boric acid, 2 mM EDTA, pH 8.0), depending on the number of samples, and heated to allow dissolution. If the expected size of the DNA fragment was less than 300 base pairs, the percentage of agarose was increased up to 2% to increase band definition when imaging under UV transillumination. SYBR Safe DNA gel stain (Invitrogen Molecular Probes) was added at a 1x final concentration and mixed by swirling. The gel was then poured onto a plate to set. DNA samples were mixed with 5µl of loading dye (10x formula for 100 ml: 60% glycerol (v/v), 0.25% bromophenol blue (w/v), 33% 150 mM Tris (pH7.6) (v/v) in H_20), loaded and run for 20-30 minutes at 130 V using a Bio-Rad model 200/2.0 power supply and Bio-Rad wide min-sub cell GT gel electrophoresis apparatus. The appropriate DNA fragments could then be cut out and purified from the agarose gel.

Ligation of Plasmid Vector and Insert DNA

Ligation of the vector backbone and newly generated site directed mutant insert was undertaken using T4 DNA ligase (Fermentas), which catalyses the formation of covalent phosphodiester linkages between the free 5’ phosphate and 3’ hydroxyl ends of opposing double stranded DNA. DNA ligase joins blunt end and sticky end termini as well as repairing single gaps in double stranded DNA. Transformation of newly ligated DNA was carried out using chemically competent *E. coli* cells as described previously.

Standard PCR Conditions

In order to create a sufficient amount and concentration of DNA to enable successful HEK293 cell transfection, PCR was used to amplify selected regions of DNA. The PCR was carried out in 50µl reactions containing 0.25µl of *Taq* DNA polymerase (Fermentas), 5µl of 10x reaction buffer (Fermentas), 1µl of 10mM dNTPs at (NE BioLabs), 1.25µl each of forward and reverse primers (10uM), 1.5µl of $MgCl_2$ (50 mM), 5µl of DNA solution at 10ng/ µl and DEPC water to make a final volume of 50µl. Reactions were then run for 1 minute at 95 ^{O}C as a DNA denaturing step, followed by 25 cycles using annealing temperatures appropriate for the primers. The Tm was calculated using the Stratagene formula (Hogrefe *et al.*, 2002) (Equation 2.1) and 72 ^{O}C extension phases according to the length of the amplicon, approximately 1 minute per kb, followed by 7 minutes at 72 ^{O}C after the final cycle.

2.3 Cell Lines

2.3.1 Standard Cell Culture Passage Protocol

The HEK293 cell line was maintained in Dulbecco's Modified Eagle Medium (DMEM) high (25mM) glucose (GIBCO®, Invitrogen, 41965-039) + L-glutamine and Phenol Red supplemented with 10% (v/v) foetal calf serum (FCS) (GIBCO®, Invitrogen, 16000). Cells were maintained in 25 cm^2 flasks, at 37 ^{0}C and passaged every 3-4 days at 70-90% confluency. All cell incubation at 37^{0}C was undertaken in 95% (v/v) air, 5% (v/v) CO_2 unless otherwise stated. When deemed ready for passage, the medium was removed and discarded. Cells were then washed with 5ml of Hanks Balanced Salt Solution (HBSS) (GIBCO®, Invitrogen, 14175) and 0.5 ml of Trypsin-EDTA x1 (GIBCO®, Invitrogen, 25300) was added and cells incubated at 37 ^{O}C for 1-2 minutes. Cells detached when gently tapped and were re-suspended in 1 ml of DMEM + 10% FCS by trituration using a sterile p1000 pipette. Once cells were re-suspended, 150µl of cell suspension was added to 5mls of fresh DMEM + 10% FCS and transferred to a new 25 cm^2 flask. HEK293 cells were utilised between passage 1 and 30 to conserve native phenotype and transfection efficiency (Thomas & Smart, 2005). The HEK293 cell line was cultured in growth medium without the addition of antibiotics.

2.3.2 Transfection of Cells using Lipofectamine2000

Briefly, transfection is the process of incorporating exogenous nucleic acids containing genes into cells to enable the study of the effect of that gene or the product of the gene, which in many cases is a protein. Transfection of the HEK293 cell line with DNA coding for the BK channel constructs was undertaken to investigate the effects of amino acid mutation on channel function, as transfection

would result in the over-expression of the transfected BK channel construct. Cells were cultured on glass coverslips (Warner Instruments, No 1 Glass Coverslips, 15mm, round, 0.15mm ± 0.02mm thickness. CS-15R, 64-0703) in sterile 6 well tissue culture plates with 2 ml DMEM + L-glutamine with 10% (v/v) FCS at 37^{O}C until 40% confluent. For each transfection, 2µg of DNA and 5µl Lipofectamine^{TM} 2000 (Invitrogen, 11668-019) were diluted separately in 50µl DMEM + L-glutamine without serum per well, and incubated for 5 minutes at room temperature. The DNA and Lipofectamine2000 reactions were then combined to create 100µl of DNA-Lipofectamine mix per well, with a final concentration of 1µg/ml cDNA and 2.5µl/ml of Lipofectamine2000 and incubated for 20 minutes. 100µl of the DNA-lipofectamine2000 complex in DMEM was added directly into the culture medium in each well. Cells were subsequently incubated for 24-48 hours at 37^{O}C in preparation for experimental study. This protocol is in line with manufacturer recommendation (Ciccarone 1999; Invitrogen Life Technologies 2014) and previous work on BK channel expression in HEK cells (Tian, Duncan, *et al.*, 2001; Chen *et al.*, 2005; Tian *et al.*, 2006).

2.3.3 HEK293 Cell Treatments

Cells were maintained in 25mM glucose growth medium; 24 hours after transfection, cell medium was replaced as necessary to achieve treatment conditions. For glucose concentration experiments, cell medium was removed and replaced with fresh 25mM glucose or changed to 5mM glucose growth medium (Figure 2.16). For alteration of fatty acid content, 641mg palmitic acid (Sigma, P0500) was added to 10ml of 100% (v/v) ethanol to create a 250mM stock solution; 200µl of this solution was then added to 10ml growth medium (no BSA) heated to 37^{O}C being vigorously swirled, thus creating a 5mM solution. Subsequently, 80µl of solution was added per ml of media to give a final concentration of 0.4mM and 0.16% ethanol content. During dissolution, growth medium was maintained at 37 ^{O}C to avoid palmitate precipitation. Oleate treatment was conducted in the same manner as for palmitate; however, for creation of the 250mM stock solution, 793µL (0.706g) of oleic acid (Sigma-Aldrich, O1008) was added to 10mls of 100% (v/v) ethanol. It has been shown in the literature that HEK 293 cells can be exposed to ethanol at concentrations of up to 0.2% (v/v) without detriment (Hannah *et al.*, 2001; Hallak and Rubin, 2004; Diaz *et al.*, 2011). Comparison of BK channel expressing HEK293 cells, cultured in a) 25mM glucose growth medium and b) 25mM glucose growth medium containing ethanol at 0.16% (v/v) within this study did not result in a significant difference in channel cell membrane expression.

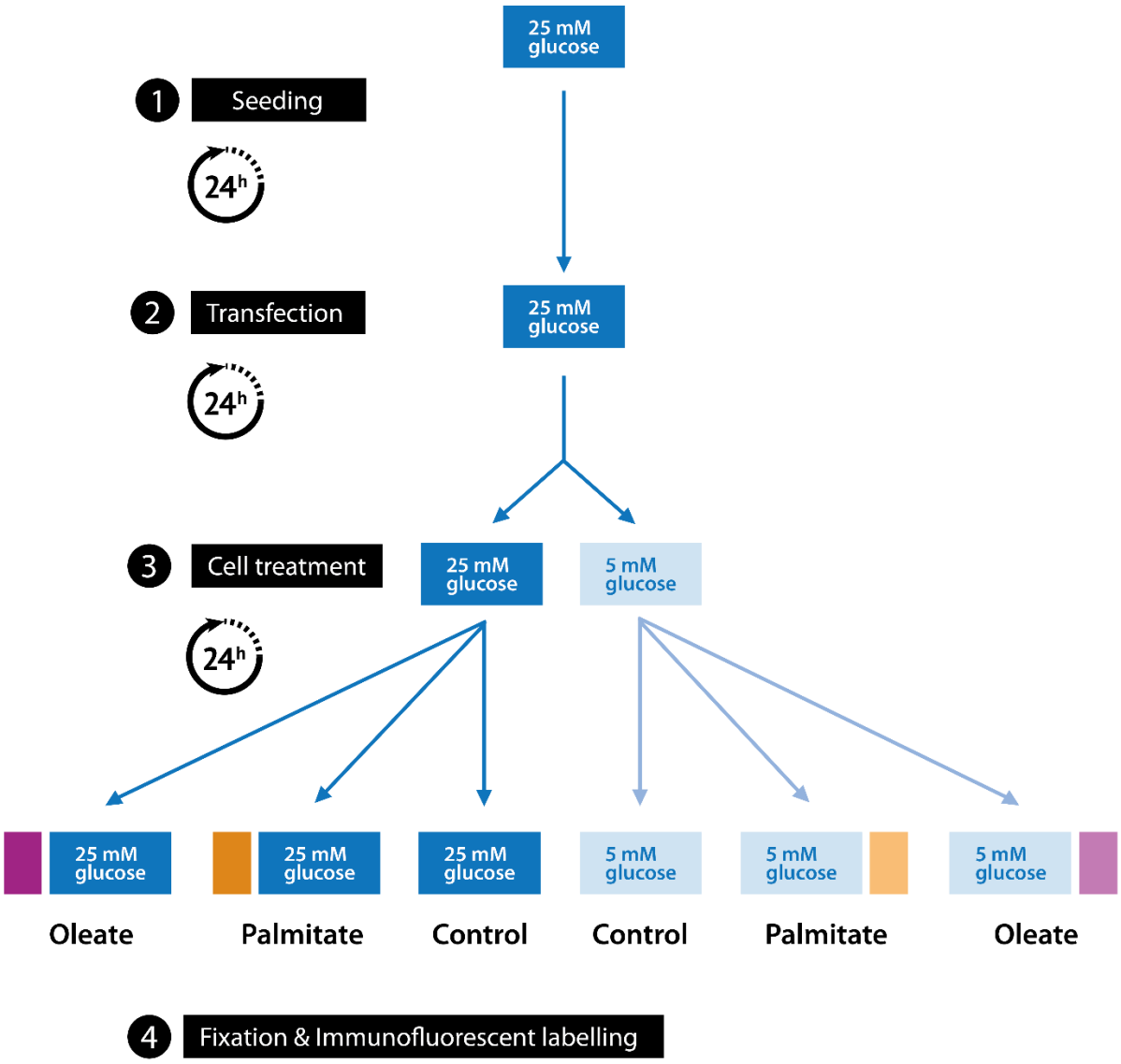


Figure 2.16- Schematic of Cell Culture and Treatment Protocol. 1). Cells cultured in 25mM glucose are seeded onto coverslips in 6 well plates and incubated for 24 hours. **2)** Cells were transfected in 25mM glucose and incubated for a further 24 hours. **3)** Cells were split into two groups, 25mM glucose and 5mM glucose, with one third of each group being supplemented with palmitate, or oleate, to a final concentration of 0.4mM. Cells were incubated for a further 24 hours. **4)** Cells were then fixed and immunofluorescently labelled.

2.4 Immunofluorescent Protein Labelling in HEK 293 cells transfected with BK channel constructs

Transfected HEK293 cells were prepared for immuno-fluorescent protein labelling 48 hours after transfection; the major steps in the procedure are highlighted in figure 2.17. In order to label BK channel constructs that were at cell surface and those located in the intracellular compartments, the following procedure was undertaken.

Labelling of BK channels located at cell membrane of HEK 293 cells. Existing medium was removed from each well of a 6-well plate and HEK 293 cells washed with fresh DMEM L-glutamine + 10% (v/v) FCS to remove any remaining transfection mixture. The primary antibody, (Monoclonal ANTI-FLAG® M2 antibody produced in mouse, Sigma, F1804) was diluted 1:100 in DMEM + L-glutamine + 10% (v/v) FCS and 1ml was added to each well. Cells were then incubated for 120 minutes at 4ºC to label the FLAG antigen and to inhibit protein recycling at the cell membrane. This step resulted in the binding of the anti-FLAG antibody to the FLAG antigen tag on the N-terminal of BK channels located at the cell membrane, as is shown in Figure 2.17 step 3. The medium was then removed and cells were washed once again with fresh DMEM L-glutamine + 10% (v/v) FCS.

The secondary antibody, Alexa Fluor® 546 (red) conjugated donkey, anti-mouse IgG antibody (Invitrogen / Molecular Probes A11036) was diluted 1:1000 in DMEM + L-glutamine + 10% (v/v) FCS and 1ml added to each well, this was then incubated at 4˚C for a further 60 minutes in the dark, in order to add the fluorophore to the BK channels at the cell membrane. This step resulted in the binding of the secondary antibody (Alexa Fluor® 546) to the anti-FLAG primary antibody, located at the N-terminus of BK channels located at the cell membrane, as is shown in Figure 2.17 step 4. Successfully labelled channels emit red fluorescence when imaged using confocal microscopy, and are representative of membrane bound BK channels.

Labelling of BK channels located intracellularly in HEK 293 cells. Cells were then washed, gently, 3 times with 1ml of PBS to remove any remaining antibody, in preparation for fixing with 4% (w/v) paraformaldehyde in PBS solution with 2ml added to each well; this was left for 30 minutes in the dark, at room temperature to fix the cells, before cells were washed with 1x PBS buffer. Cell membrane permeabilisation was accomplished by treating cells with 0.3% (v/v) Triton X-100 (Sigma-Aldrich, X100) in PBS at room temperature for 10 minutes (Figure 2.17 step 5). The reaction was blocked with 1x PBS buffer, then 3% (w/v) Bovine Serum Albumin (BSA) (Sigma-Aldrich, 05470) in 1x PBS buffer and 0.05% (v/v) Triton X-100 for 60 minutes. The cells were then rinsed with PBS. For the double FLAG tag methodology, cells were then incubated either overnight at 4ºC, or for 1 hour at room temperature with 1ml of primary antibody, diluted 1:100 in DMEM + L-glutamine + 10% (v/v) FCS. As the cell

membrane of the HEK 293 cells had been permeabilised in the previous step, this allowed access of larger molecules, in this case antibodies, into the intracellular space. Therefore, exposure of the cells to the anti-FLAG antibody post-permeabilisation resulted in the binding of the antibody to the FLAG antigen tag on the N-terminus of BK channels located within the cells, as is shown in Figure 2.17 step 6. The BK channels located intracellularly were not tagged during initial exposure to the anti-FLAG antibody (step 3) as the cell membrane was not permeable to the antibody.

Cells were then washed once again with 1 ml PBS and incubated with an alternate secondary antibody, Alexa Fluor® 488 (green) Goat Anti-Mouse IgG (H+L) Antibody (Invitrogen / Molecular Probes A11017) in a 1:000 dilution for 60 minutes, in the dark, at room temperature. This step resulted in the binding of the secondary antibody (Alexa Fluor® 488) to the anti-FLAG primary antibody, located at the N-terminus of BK channels located intracellularly, as is shown in Figure 2.17 step 7. Successfully labelled channels emit green fluorescence when imaged using confocal microscopy, and are representative of intracellular BK channels. Figure 2.17 step 8 provides an illustration of a HEK 293 cell with a labelled and fluorescing BK channel located at the cell membrane (red) and a BK channel located within the intracellular space (green). Although the BK channels expressed contain a C-terminal HA antigen tag, this wa not utilised as a tagging site; a double FLAG tagging method is utilised.

The coverslips were then washed with 1x PBS buffer, followed by distilled water to prevent crystal formation, and two coverslips mounted onto one labelled 26mmx76mmx1mm glass slide (Thermo Scientific, MNJ-150-030U) with ProLong Gold Antifade Mountant (Invitrogen / Molecular Probes, P36930). Slides were stored in the dark to minimise fade.

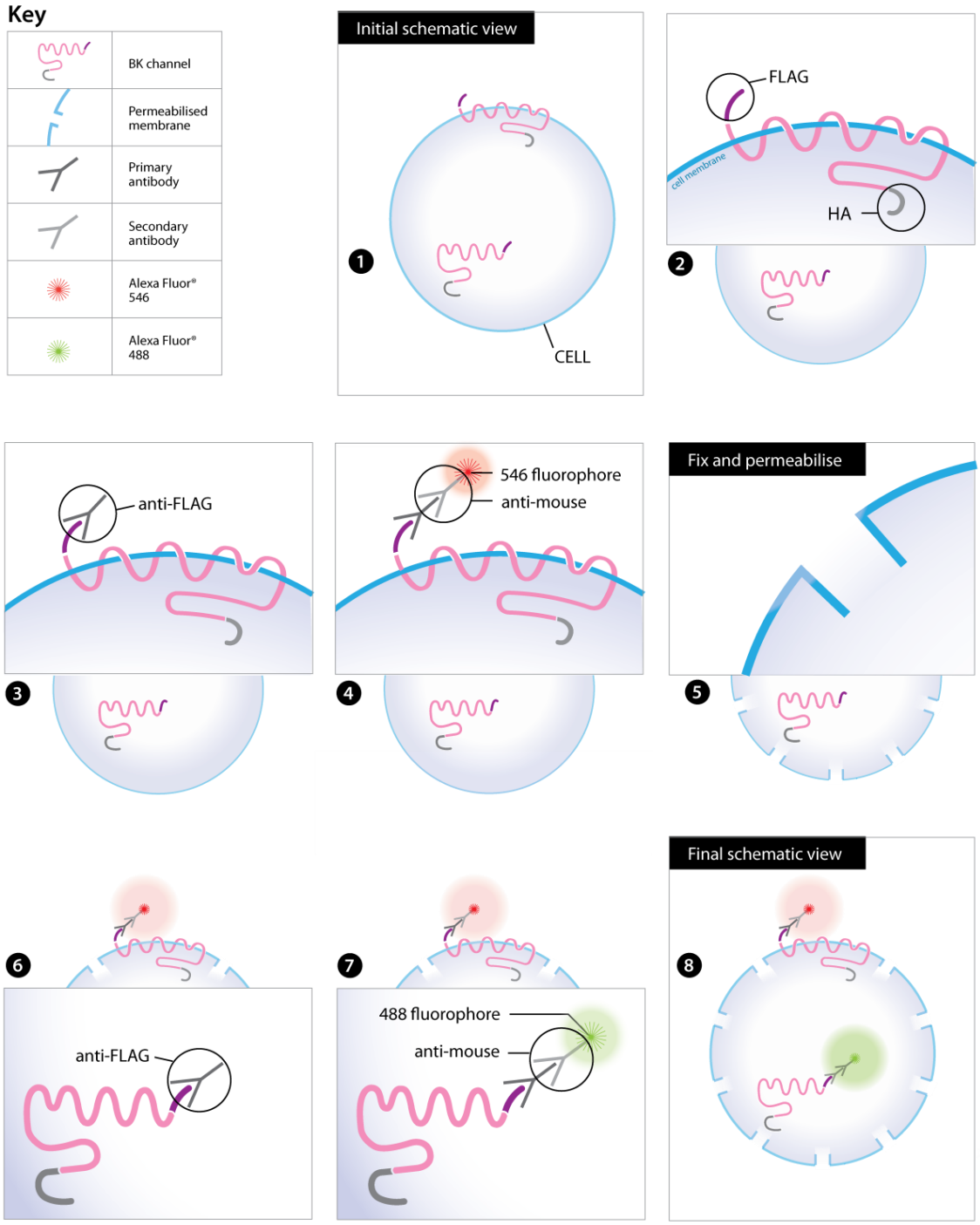


Figure 2.17- Schematic of double FLAG fluorescent tagging methodology using primary and secondary antibodies. 1) Diagram of an idealised HEK 293cell showing membrane bound and intracellular BK channels 2) FLAG and HA antigen tags highlighted 3) Mouse Anti-FLAG primary antibody binds to extracellular FLAG antigen. 4) Anti-mouse secondary antibody with attached Alexa Fluor 546 (red) binds to the mouse primary antibody. 5) Cells are fixed using 4% paraformaldehyde, cell membrane permeabilized using 0.3% (v/v) Triton X. 6) Cells are re-incubated with Mouse Anti-FLAG primary antibody which now can access intracellular FLAG antigen. 7)Anti-mouse secondary antibody with attached Alexa Fluor 488 (green) binds to the mouse primary antibody attached to intracellular BK channels 8) Overview of cell showing permeabilized cell membrane and fluorescently tagged membrane bound and cytoplasmic BK channels. Concept adapted from (Mattanovich and Borth, 2006).

2.5 Imaging Protocols

2.5.1 Confirmation of Fluorescence-Labelling of HEK Cells

HEK293 cells transiently expressing BK channel constructs due to successful transfection, can be traced as a result of fluorescent labelling of FLAG antigen tags. Therefore, to confirm the presence of cells labelled with fluorescent probes a preliminary screen was undertaken using a standard epifluorescent Nikon ECLIPSE TE200 microscope. Coverslips were viewed under white light to detect areas with adherent cells. These cells were then examined for fluorescence under a 100x/1.3 Planar Fluor lens at excitation wavelengths 488 and 532 nm; coverslips containing fluorescent cells were marked and retained for confocal imaging.

2.5.2 Confocal Imaging of fluorescently-labelled cells

Once cells had been shown to be fluorescently labelled, indicating that they were successfully expressing tagged BK channels, images were acquired using a Zeiss Axiovert confocal laser scanning microscope (LSM510) operating with a 25mW argon laser tuned to 488 and 532 nm. Coverslips were examined initially under 20x magnification to locate fluorescing cells, then a Zeiss plan-apochromat 1.4 NA x63 oil immersion objective lens was used to obtain confocal images. In order to obtain images that would allow for analysis of labelled BK channel distribution, Z-stacks comprising z-sections collected at 0.34-0.5µm intervals were acquired (Figure 2.18). HEK cells with intact membranes within a particular FOV were imaged in multi-tracking mode as has been used commonly in other studies (Barden *et al.*, 2003; Ishiwata *et al.*, 2004; Sun *et al.*, 2010; Sheng and Acquaah-Mensah, 2011; Li *et al.*, 2013). Areas with pixel saturation were avoided by using the 'range indicator' function of the LSM510 software package. Saturated areas in the FOV identified during image analysis were excluded using the 'region of interest' (ROI) function available in the ImageJ software package. Gain and offset settings were optimised to the first sample of the control group as to utilise as much of the detector dynamic range as possible; these settings were constant and were used to image the remainder of the control group samples and treatment group samples from the same experiment set.

An exemplar single slice confocal image of HEK293 cells transiently expressing the BK channel ZERO construct, fluorescently tagged via double FLAG tagging with Alexa Fluor 546 (red) indicating BK channels at the cell membrane and Alexa Fluor 488 (green) indicating BK channels intracellularly is shown in Figure 2.19.

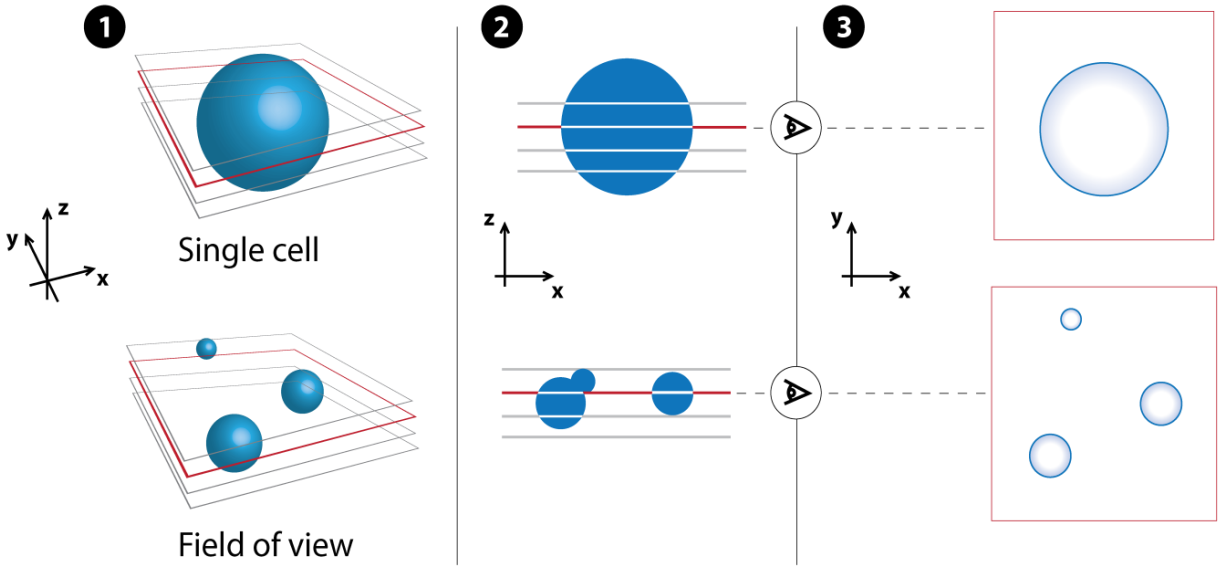


Figure 2.18- Schematic of Confocal Z-Stack Image Acquisition. 1) Cell/cells in the FOV with prospective locations of optical slices. 2) Cross section of cell/cells, indicating that there is a tangible distance between slices and that each slice has a thickness. 3) The slice highlighted in red has been removed and presents a cross section of the cell/cells.

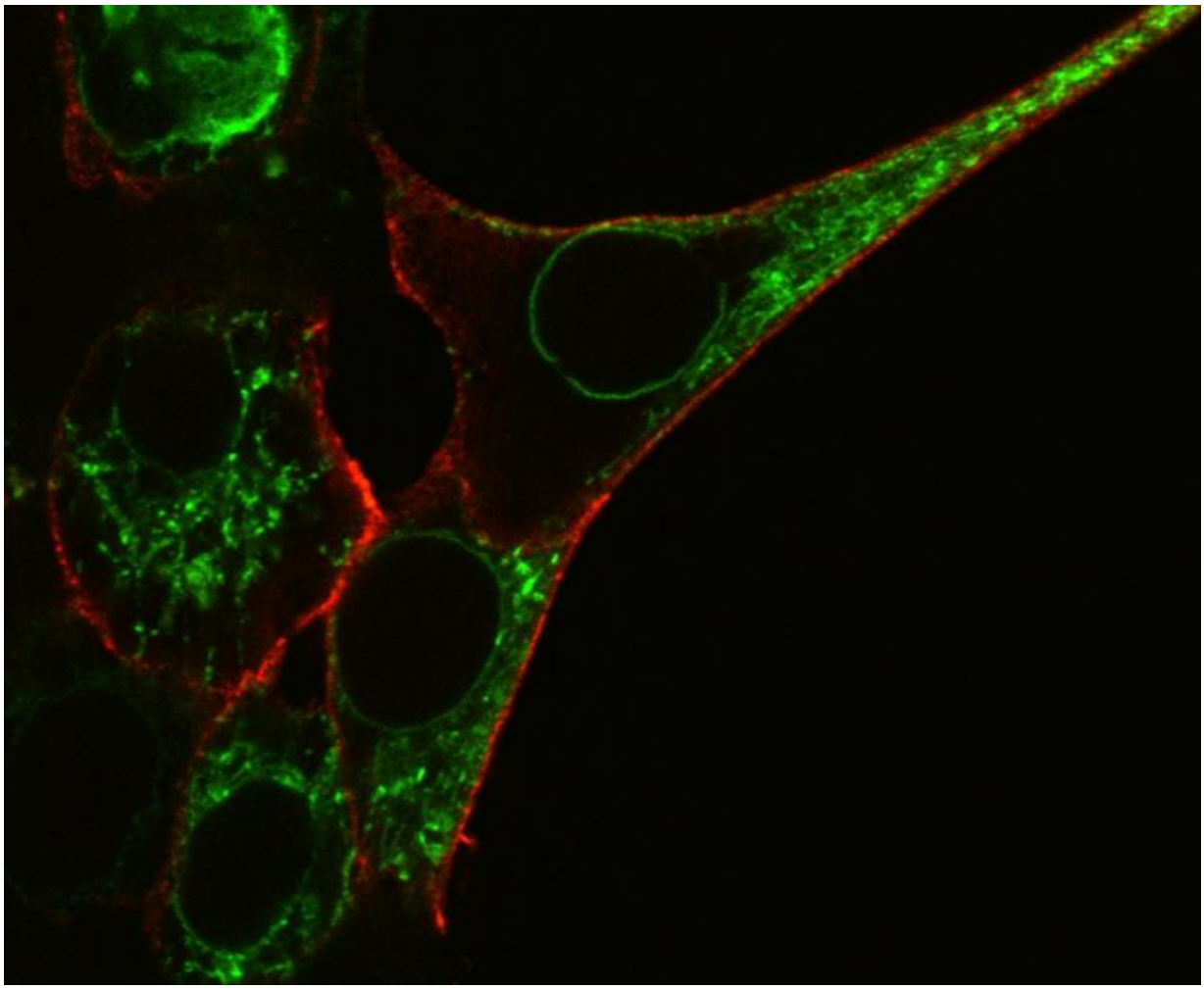


Figure 2.19- Single slice confocal image of HEK293 cells transiently expressing the BK channel ZERO construct. BK channels were fluorescently tagged via double FLAG tagging with Alexa Fluor 546 (red) indicating BK channels at the cell membrane and Alexa Fluor 488 (green) indicating BK channels intracellularly. Image corresponds to the schematic in (Figure 2.17, 8) and in (Figure 2.18, 3).

2.6 Quantification of Immunofluorescent labelling

Quantification of FLAG surface expression was undertaken using a double FLAG tagging methodology. As demonstrated in Figure 2.17, the exposed FLAG-tagged N-terminus of the BK channel, situated outside the cell membrane was tagged with Alexa Fluor® 546 red fluorophores. Red fluorescence was taken as representative of all of the BK channels located in the membrane at the time prior to permeabilization. After fixing, the cell membrane was permeabilized allowing previously inaccessible intracellular Flag tags to be tagged with Alexa Fluor® 488; as a result, green fluorescence was therefore attributed to BK channels located intracellularly.

Confocal images were stored as LSM files which were then exported to the image processing software ImageJ for analysis (Abràmoff, Magalhães and Ram, 2004; Rasband, 2012; Schindelin *et al.*, 2012) where the signal was converted to a 16-bit data file (8- bit image for red and green channels). The red and green channels were split, a region of interest (ROI) selected and background fluorescence removed using the 'Remove Background' function inbuilt into the software. Using 'Stack Interlever' and 'Multi-Measure' functions, the average red and green fluorescence for each confocal slice was calculated.

To determine the proportion of BK channel located in the membrane in comparison to the total amount of BK channel produced, the 'Raw Ratio' (RR), which represents the cell membrane expression of the BK channel, was calculated. This was achieved by dividing the fluorescence from the red channel by the total of the red plus green channel. Results were also normalised to those of a selected group, resulting in that group being represented as 100% or 1, and the test group being expressed as plus or minus that value (Equation 2.2).

$$\text{Raw Ratio (RR)} = \frac{\text{Red}}{\text{Red} + \text{Green}}$$

$$\text{Normalised Raw Ratio} = \frac{\text{FOV average value}}{\text{Mean RR of selected group}}$$

Equation 2.2- Formula to Calculate Raw Ratio and Normalised Raw Ratio for the BK channel.

Normalisation of CSS-Palm 4.0 score for BK channel cysteine residues

To enable comparison of the pattern of cell membrane expression of the SNP-mimetic mutants H55Q, G57A and H55Q:G57A to the pattern of C56 predicted palmitoylation for each of the mutants, the CSS-Palm 4.0 scores were normalised. This was achieved by dividing the predicted C56 palmitoylation score for each of the mutants by the C56 palmitoylation detection threshold. The value for each was then normalised to the value of the wild type channel (ZERO), to a value of 1, representing 100%.

Statistical Analysis

Statistical analysis was performed using the GraphPad Prism® software package. All results are expressed as mean values ± standard deviation (SD). Experiments containing two groups were assessed using a Students T-Test with Welch's correction to account for the possibility that groups have unequal variances. Experiments containing three or more groups were assessed using one-way ANOVA, assuming Gaussian distribution of sample data. Tukey's post-hoc analysis was undertaken to compare the means of each test group. P-values equal to or below 0.05 were considered significant ($P \leq 0.05$).

To measure the strength of the association between the cell membrane expression of BK channel constructs ZERO and the SNP-mimetic mutant channels (expressed as normalised raw ratio) and the corresponding normalised CSS-Palm 4.0 score for the constructs, the Pearson's correlation coefficient (PCC) was calculated. This was achieved by analysing the values using the 'Correlation' function contained within the 'analyse' option within the GraphPad Prism® software package. The Pearson's correlation coefficient was also calculated to assess the extent of co-localisation between red and green fluorescence emitted from HEK293 cells expressing FLAG tagged BK channels (red fluorescence is representative of cell membrane located BK channels and green representative of intracellular BK channels). This was achieved by utilising the 'Colocalization' plugin contained within the ImageJ software package to analyse confocal images of the transfected HEK293 cells. Coefficient values for PCC range from 1 to -1. A value of 1 indicates a complete positive correlation between the two channels (as red increases, green increases), whereas -1 indicates a negative correlation (as one increases, the other decreases). A score of 0 indicates no correlation.

2.7 Critical appraisal of key methodologies

Selection of bioinformatics methodology

There are a number of studies in the literature that support the utilisation of a bioinformatics methodology to prioritise SNPs (Lee, 2009; George Priya Doss *et al.*, 2010; Cline and Karchin, 2011; Liu, Jian and Boerwinkle, 2011; Teng *et al.*, 2012; Bryzgalov *et al.*, 2013; Ghaedi *et al.*, 2015; Hepp, Gonçalves and de Freitas, 2015; Alipoor *et al.*, 2016; Kalia *et al.*, 2016). Significantly less investigation has focussed on SNP prioritisation in potassium channels; however, an interesting example is a 2011 publication that aimed to prioritise SNP occurring on the Kv channel (Stead *et al.*, 2011), due to the occurrence of clinically significant nsSNP occurring on the channel that cause cardiac arrhythmogenesis, sudden cardiac death, Long QT Syndrome, and finally epilepsy, which is also linked with BK channel nsSNP (Du *et al.*, 2005).

Stead *et al* utilised a machine learning bioinformatics methodology with a training dataset that assessed 14 factors to prioritise SNPs, and focussed on the 5 most accurate of the 14 which were; a) amino acid conservation score, b) change in hydrophobicity, c) change in structural stability, d) change in Hidden Markov Models (HMM) score (the probability that the amino acid sequence is a member of the protein family) and e) subfamily membership. As part of this current investigation, 4 different parameters were investigated in the process of SNP prioritisation that focus on functional and experimental data specific to the BK channel; a) site directed mutagenesis (SDM) data, b) the functional role of the SNP site, c) PTM data and d) SNP sites with clinical evidence. Selection of the most optimal parameters to predict the effect of a SNP is challenging; the majority of tools utilise assessment parameters based solely upon ortholog sequence and structure (Sim *et al.*, 2012; Martin, 2016); however the inclusion of additional protein specific information has been shown to increase SNP damage prediction accuracy (Stead *et al.*, 2011; Teng *et al.*, 2012). This supports the use of BK channel specific information to improve the accuracy of SNP prioritisation, as was conducted in this investigation.

The machine learning method is argued to be an advancement on manual methods due to an increase in the number of sites that can be analysed, a reduction in analysis time, and increased complexity of parameters tested (Huang *et al.*, 2017). However, there are limitations as an automated method relies on algorithms developed with training data produced by lab-based experimentation; these datasets may be incomplete or not be up to date, potentially resulting in inaccurate results. Thus, a method that includes the use of machine learning bioinformatics tools, such as CSS-Palm 4.0 and NetPhos 2.0, alongside manual annotation with data extracted from the literature, and lab-based verification of prioritised SNPs serves to minimise inaccurate results.

Selection of a suitable mammalian expression system for BK channel expression

For ion channel research, a recombinant expression method that allows for the study of the structure, function, localisation and interactions of channels in mammalian cells that construct, process and fold proteins using their endogenous machinery is the most appropriate approach. The Human Embryonic Kidney 293 (HEK 293) cell line was used as it expresses the biochemical machinery to conduct the post-translational folding and processing required to produce functional proteins from a wide range of mammalian nucleic acids (Hames and Higgins, 1999; Thomas and Smart, 2005; European Molecular Biology Laboratory, 2014; Thermo Scientific, 2014). HEK293 cells do not endogenously express BK channels (Tian, Hammond, *et al.*, 2001), therefore only the BK channel constructs introduced via transfection will be manufactured by the cells and thus will be the only BK channels involved in analysis. In addition, expression systems provide an environment where assessing the effects of amino-acid substitution in proteins via site-directed mutagenesis is possible (Braun and Sy, 2001; McCartney *et al.*, 2005; Jianxi Liu *et al.*, 2008; Jeffries *et al.*, 2010; Bi, 2014).

Although there is value in conducting investigations in cells that endogenously express the BK channel such as smooth muscle cells (Brayden and Nelson, 1992) or neuronal cells (Knaus *et al.*, 1996), HEK293 cells are ideal cell candidates to achieve the thesis research objectives as BK channel DNA mutation was required. HEK293 cells have been successfully utilised as an expression system for the BK channel for over a decade (Ahring *et al.*, 1997; Shipston *et al.*, 1999; Shipston, 2001; McCartney *et al.*, 2005; Jeffries, 2010; Bi, 2014); however, other mammalian expression systems have been used for experiments using the BK channel, such as the 'COS' cell line, a fibroblast-like cell line derived from monkey kidney tissue (Won *et al.*, 2010; Won, Lee and Park, 2011; Kim *et al.*, 2014).

Selection of a suitable BK channel cellular distribution assessment method

The BK channel has been investigated extensively using patch-clamp electrophysiology (Berweck *et al.*, 1994; Ahring *et al.*, 1997; Raffaelli *et al.*, 2004; Pyott *et al.*, 2007; Naruse, Tang and Sokabe, 2009; Jeffries, 2010; Kim *et al.*, 2014); this has been essential for characterisation of the channel and understanding the effects of different environments, exposure to ion channel modulators and the role of conserved regions of the channel. Whilst an electrophysiology approach can provide details of BK channel function and kinetics, it cannot provide information on the location and proportional distribution of total BK channels inside the cell and in the cell membrane, which are the objectives of the study. For proper function, the BK channel must insert into the membrane; therefore, factors that

affect this are worthy of investigation. Therefore, this study utilised an imaging based screen in preference to electrophysiology.

Confocal microscopy in combination with immunofluorescent protein labelling; used to determine the location of BK channels in HEK293 cells (as either in the cell membrane or in the intracellular space) and to quantify the ratio of BK channels in the cell membrane in comparison to total. This method has been utilised successfully to investigate BK channel cellular expression (Williams *et al.*, 2004; Rüttiger *et al.*, 2004; Samaranayake *et al.*, 2004; Chen *et al.*, 2005, 2010, Tian *et al.*, 2006, 2008, 2010; Pyott *et al.*, 2007; Kim, Alvarez-Baron and Dryer, 2009; Ridgway, Kim and Dryer, 2009; Chiu *et al.*, 2010; Jeffries *et al.*, 2010; Tajima *et al.*, 2011; Föller *et al.*, 2012).

To successfully use confocal microscopy the protein of interest must be fluorescently tagged. As shown in Figure 2.17, the BK channels used in this study were N-terminally tagged with the FLAG antigen tag, and C-terminally tagged with the HA tag. To attach fluorophores to the BK channel, a FLAG-FLAG antibody tagging methodology in combination with secondary tagging with Alexa Fluor® fluorophores was utilised, where fluorophores were attached to N-terminal the FLAG tag only, on both membrane bound and cytosolic BK channels using surface and permeabilised labelling, as was undertaken in a 2013 study (Chen *et al.*, 2013). Use of the same antibody removes the potential challenge of differing antibody sensitivities affecting results, however FLAG-HA tagging has been used in other BK investigations (Erxleben *et al.*, 2002; Tian *et al.*, 2006; Tong *et al.*, 2006; Huang and Ma, 2010; Jeffries, 2010; Jeffries *et al.*, 2010; Chang *et al.*, 2011; Kim and Dryer, 2011). There was a theoretical possibility that surface BK channels could be tagged during the permeabilised tagging steps, leading to an overassessment of the number of cytosolic channels; therefore, a co-localisation assessment was undertaken. Other fluorophores have been used in BK channel investigations, such as yellow fluorescent protein (YFP) and green fluorescent protein (GFP) (Chen *et al.*, 2005; Kim, Choi and Dryer, 2008; Tian *et al.*, 2008, 2010; Li *et al.*, 2009; Jeffries *et al.*, 2010).

Another method of assessing the cellular distribution of BK channels in would be to grow, transfect and fluorescently tag the HEK293 cells on a 96- well plate, with fluorescence intensity measured using a micro plate reader (Chiu *et al.*, 2010; Hei *et al.*, 2016). This would dramatically reduce the time taken to capture BK channel membrane expression in comparison to confocal microscopy, and enable the capture of fluorescence from the total sample, completely removing any selection bias. In addition, thousands of cells could be analysed per experiment, however less detailed information would be collected in comparison to confocal microscopy, and the location of BK channels within the cell could not be assessed with the same accuracy.

Flow cytometry can be used for cell counting and to measure multiple physical characteristics of a single cell such as size, granularity and biomarker detection simultaneously as the cell flows in suspension (Adan *et al.*, 2016). Like confocal microscopy, flow cytometry utilises lasers to detect fluorescently tagged proteins within or on the surface of a cell. As cells are in a flow, reducing the time in contact with the laser in comparison to confocal microscopy, fluorophores with the same excitation wavelength, but different emission wavelength are desired, such as. Alexa 488 and R-PE. Flow cytometry has been used to quantify BK channel expression (Ge *et al.*, 2012; Tao *et al.*, 2015; Warsi *et al.*, 2015), and an advantage of flow cytometry is it can achieve multi-factorial analysis, however it cannot provide visualisation of the location of the BK channel in HEK293 cells, as required to fulfil the objectives of the study.

Western blotting has been used to quantify the absolute amount of BK channel protein in the cell (Ling *et al.*, 2000; Meredith *et al.*, 2004; Chen *et al.*, 2005, 2010; Tong *et al.*, 2006; Naruse, Tang and Sokabe, 2009; Jeffries *et al.*, 2010; Tajima *et al.*, 2011; Föller *et al.*, 2012); this function is an advantage in comparison to confocal microscopy. Like confocal microscopy, Western blotting can determine the ratio of BK channels in the membrane in comparison to total, and uses of antibodies to isolate the channel from other proteins. This technique was used in a 2011 study (Kim and Dryer, 2011), where immunoblot cell surface labelling was utilised prior to cell lysing and anti-Slo tagging. Despite the many positives of Western blotting for protein quantification, it was not the optimum method to achieve the objectives of the thesis, because the location of the BK channel within the cell cannot be visualised *in situ*.

Selection of HEK293 cell treatment conditions

A reduction in extracellular glucose from 25mM to 5mM was chosen to assess the effect of glucose on BK channel membrane expression; this choice was driven by; a) 5mM being within the normal blood glucose range for humans (4mM to 6mM), and b) the fact that a considerable number of studies have used this concentration to test the effect glucose on different processes (Nutt and O'Neil, 2000; Moritz *et al.*, 2001; Lin *et al.*, 2005; Smith *et al.*, 2006; Daoyan Liu *et al.*, 2008; Chang *et al.*, 2011; Sun *et al.*, 2013; Viskupicova *et al.*, 2015), thus making the data outputs from this study comparable.

Initially, the glucose concentration of the HEK293 cells was reduced from 25mM to 5mM at the seeding stage (Figure 2.16), however, the cells would tend to lift off the glass coverslips 48 hours later, during the process of immunofluorescent tagging (due to frequent coverslip washing steps), thus preventing further cell analysis. To combat this, the coverslips were coated in poly-L-lysine (Sigma-Aldrich P9155)

in an attempt to increase cell adhesion; this was however unsuccessful. The method was then optimised for a glucose concentration change at transfection stage which was also unsuccessful. Finally, the glucose concentration was altered at the cell treatment stage, which dramatically improved cell adhesion during cell washing, allowing immunofluorescent tagging and completion of cell analysis via confocal microscopy.

The concentration of palmitate to be used in the investigation was 0.4mM as it is within the range found endogenously in human plasma (0.3mM to 4.1mM) (Abdelmagid *et al.*, 2015), and is within the palmitate concentration range commonly used in research publications (0.2mM to 0.5mM) (Luiken *et al.*, 1997; Staiger *et al.*, 2004; Weigert *et al.*, 2004; Bunn *et al.*, 2010; Oberbach *et al.*, 2012; Schilling *et al.*, 2013; Weikel *et al.*, 2014). The endogenous human plasma concentration of oleate is in the range of 0.03mM and 3.2mM (Abdelmagid *et al.*, 2015). In addition, it was important that the concentration of palmitate was the same as oleate, to remove fatty acid concentration as a potential cause of membrane expression differences. Therefore, it was decided that a concentration of 0.4mM oleate would be used to investigate the impact of an unsaturated fatty acid on BK channel construct cellular distribution.

There were initially some challenges in the production of growth media containing 0.4mM palmitate, as the fatty acid would precipitate out when added to the media from the ethanol stock solution. In order to combat this, the creation of BSA conjugated-palmitate was attempted, as has been used in publications (Haim *et al.*, 2010; Lee *et al.*, 2013; Weikel *et al.*, 2014). Unfortunately, the protocol was not successful due to denaturing of the BSA during the conjugation process. A palmitate-BSA conjugate formulation was available for purchase (Agilent 102720-100), however it was not economically feasible to use this product for the study. Therefore, another method of palmitate dissolution was attempted, where the concentration gradients between dissolution steps were halved and the temperature of the growth medium was increased from room temperature to 37 ^{O}C to aid palmitate dissolution; this method was successful. The duration of metabolic substrate treatment was set at 24 hours, this time-scale is in line with a number of publications that demonstrated a substrate mediated effect (Peiró *et al.*, 2001; Chang *et al.*, 2011; Kim and Dryer, 2011; Su *et al.*, 2013).

3 Bioinformatics

3.1 Introduction

In this chapter, the focus is twofold: to capture key aspects of data relating to BK channels held on bioinformatics databases, and to use this information to prioritise potentially damaging single-nucleotide polymorphisms (SNPs) for lab-based experimentation.

SNPs have a proven effect on BK channel function; current evidence shows that BK channel SNPs associated with disease cause either a gain or loss of function and are found within the exonic, protein coding region of the gene. An example of a gain of function mutation of the BK channel is the non-synonymous SNP (nsSNP) D434G, characterised in both the human and murine form of the channel (Du *et al.*, 2005; Wang, Rothberg and Brenner, 2009; Yang *et al.*, 2010). This mutation is responsible for the genetic disorder Generalized Epilepsy and Paroxysmal Dyskinesia (GEPD), and arises as a result of the substitution of an aspartic acid amino acid residue for a glycine residue in the conserved cytosolic linker region in the C-terminus, downstream from the S6 transmembrane domain (Yang *et al.*, 2010). The mutation results in reduced activation time and enhanced channel calcium sensitivity as a result of increased calcium affinity (Du *et al.*, 2005; Díez-Sampedro *et al.*, 2006; Lee and Cui, 2009; N'Gouemo, 2014). The mechanism behind this increased calcium sensitivity is proposed to be reduced flexibility of the AC region in the RCK1 domain (Yang *et al.*, 2010). Conversely, the BK channel nsSNP A138V located in the intracellular S0-S1 linker has been associated with a loss of function of the channel; in this case, the substitution of valine for alanine is associated with autism and mental retardation (Laumonnier *et al.*, 2006). The biomolecular activity that is associated with this change in channel sequence is yet to be elucidated, however it is postulated that the mutation creates a 'cryptic' splice site, a disadvantageous splice site that is dormant or used only at low levels unless activated by mutation, that results in impaired channel expression (Guglielmi *et al.*, 2015).

Genome wide association (GWA) studies have provided evidence that DNA mutations occurring outside of exonic regions, in the intronic regions of the BK channel gene are associated with diseases such as diabetes, obesity and increased blood pressure (Tomás *et al.*, 2008; Jiao *et al.*, 2011). However, unlike mutations D434G and A138V, there is no published evidence of direct effects of these intronic region mutations on BK channel function. However, obesity was linked to increased RNA expression of the BK channel in adipose tissue (Jiao *et al.*, 2011).

Some gene mutations associated with or proven to cause disease, such as D434G, have been identified by exome screening patients presenting with the signs and symptoms of disease, and determining the common genetic variations in those patients. This method, whilst informative and effective, is costly and time consuming (Sastre, 2014). In addition, the challenge with the method is that the number of

SNPs occurring across even single genes, such as the BK channel, is overwhelming (in the region of tens of thousands), making it difficult to identify common genetic variations or patterns of genetic variation between the subjects with the disease (Schrijver *et al.*, 2012; Bertier, Joly and Hétu, 2016; Petersen *et al.*, 2017). Thus, a screen of genetic information associated with the BK channel gene, incorporating a prioritisation system '*in silico*', to focus on the variations with a high probability of affecting channel function, may help overcome these time and cost barriers. This would involve the analysis of available free to access information as an alternative to the costly collection of additional data. This approach has been utilised in other studies, for example for the prioritisation of a) SNPs perturbing microRNA regulation (Ghaedi *et al.*, 2015), b) SNPs occurring in Toll-like receptors (Alipoor *et al.*, 2016), and c) SNPs involved in gene regulation (Teng *et al.*, 2012).

To effectively prioritise BK channel SNPs, there are key aspects of the channel that need to be mapped, as SNP proximity to key structural and functional regions may be an indicator of potential effect. The amino acid composition and amino acid location of the foundation structural units and functional domains of the human BK channel have previously been established (Adelman *et al.*, 1992; Dworetzky, Trojnacki and Gribkoff, 1994; Pallanck and Ganetzky, 1994; Tseng-Crank *et al.*, 1994; McCobb *et al.*, 1995; Meera *et al.*, 1997). However, this information is yet to be collated into a resource where the relative locations of the regions can be easily cross referenced. Therefore, one aim of this chapter is to synthesise and present structural and functional information in a format that allows the locations of regions to be compared at a glance. This is particularly important for the bioinformatics approach to SNP prioritisation as it enables SNP proximity to functional and modulatory motifs, and the potential impact of this proximity on BK channel function to be established. The 3D structure of the BK channel has been resolved at 3.5 angstrom using crystallography and cryo-electron microscopy (Jiang *et al.*, 2002; Wang and Sigworth, 2009; Yuan *et al.*, 2010; Hite, Tao and MacKinnon, 2016; Tao, Hite and MacKinnon, 2016). Whilst the 3D structure of the BK channel is important for understanding the spatial interactions between amino acids and chemical groups, the effect of mutagenesis and SNPs on the 3D structure of the BK channel will not be included in this thesis work.

Post translational modifications (PTMs) have been established as having regulatory and modulatory effects on BK channel function (see reviews by Kyle & Braun 2014; Shipston & Tian 2016). Identifying the location of sites of PTM on the BK channel is important for interpreting the possible effects of SNPs. The proximity of SNPs to key PTM sites of channel activity or cell membrane translocation (Tian *et al.*, 2004; Jeffries *et al.*, 2010) may disrupt these functions. Sites of PTM can be predicted using bioinformatics software (Blom, Gammeltoft and Brunak, 1999; Gupta, Jung and Brunak, 2002; Ren *et al.*, 2008; Xue, Liu, Gao, *et al.*, 2010; Q. Zhao *et al.*, 2014) or verified experimentally using techniques such as mass spectroscopy or site directed mutagenesis.

Site directed mutagenesis (SDM) is a synthetically mediated change in the amino acid composition of a protein at a specific location, which can affect single or multiple amino acid residues. The mutated protein is then assessed for effect on a single function (such as membrane translocation) or multiple factors (such as membrane translocation and ion channel activity). The results of SDM are published in peer reviewed articles and held on databases containing secondary protein information, such as UniProt. The software to compile and classify the results of SDM and add them to bioinformatics databases exists, however have not been widely applied.

Several nsSNPs have been proven to cause perturbations to BK channel function (Du *et al.*, 2005; Díez-Sampedro *et al.*, 2006; Wang, Rothberg and Brenner, 2009; Yang *et al.*, 2010; N'Gouemo, 2014) and it is plausible that there are other function-altering nsSNPs on the channel that have not yet been identified or associated with a functional change or disease. Therefore, in an effort to identify potentially damaging nsSNPs, this work will collate the predicted damage score for each SNP using SIFT and PolyPhen-2 predictive software programs (Kumar, Henikoff and Ng, 2009; Adzhubei *et al.*, 2010) and this information will then be added to the annotated BK channel amino acid sequence.

Chapter Aims

The aims in this chapter will be achieved by completing the following objectives;

1. Map the locations of structural and functional sites on the BK channel amino acid sequence
2. Identify the amino acid locations of predicted and verified post-translational modification sites and map them on the BK channel sequence
3. Identify the locations and consequences of site-directed mutagenesis investigations of the BK channel
4. Identify the number and locations of non-synonymous SNPs, and map them on to the BK channel amino acid sequence.
5. Establish the extent of co-localisation between nsSNP and of structure, function, PTM and SDM sites on the BK channel
6. Prioritise BK channel nsSNP for further in-depth bioinformatics analysis, by investigating aspects such as nsSNP frequency, background information of the individual/s and effect on local PTM environment
7. Select SNPs for lab based investigation as part of this thesis.

3.2 Results

3.2.1 The location of the human BK channel gene

A database search was undertaken to determine the molecular location and cytogenic location of the human KCNMA1 gene. The 'molecular location' refers to a gene in terms of the chromosome and base pair number; in this format, the initial number is the chromosome number and the succeeding number is the location given as a base pair coordinate. The Ensembl database listed the human KCNMA1 gene to be 768,994 base pairs in length, spanning from 10: 78,629,359 to 10: 79,398,353 on the reverse strand (Figure 3.1A). The cytogenic reference for the human KCNMA1 gene (which is based upon the gene location on the chromosome relative to the chromosome structure and banded regions that appear on the chromosome after staining) was found to be 10q22.3; the initial number refers to the chromosome number (**10**q22.3), whilst the letter indicates on which arm of a chromosome the gene is found, using the centromere as a reference point (p=short or **q**=long). A distinctive set of bands appear when the chromosome is Giemsa stained (Seabright, 1971) and the final set of numbers (10q**22.3**) denote in which band the gene is located. Once the location of the gene was established, a database search was conducted to determine the number and nature of transcripts associated with the gene.

3.2.2 BK channel transcripts

The process of transcription and translation of a single gene can lead to the production of multiple forms of a protein, referred to (often ambiguously) as transcripts, protein isoforms or splice variants. For this reason, a search of Ensembl and UniProtKB databases was undertaken to determine to what extent alternative splicing occurs with the human KCNMA1 gene. There were 23 transcripts identified; 19 of these transcripts code for a protein and are successfully translated into a BK α-subunit (protein coding) and 4 of these are processed transcripts that form a long, non-coding RNA sequence not translated into an α-subunit protein (Figure 3.1B). The 19 BK α-subunit transcripts, coded for by the KCNMA1 gene, varied greatly in length, ranging from 128 amino acids (ENSP00000475086) to 1288 amino acids (ENSP00000385717). Of the 19 protein coding isoforms for the KCNMA1 gene, 5 were members of the human consensus coding sequence CCDS (Pruitt *et al.*, 2009); (ENSP00000385806, ENSP00000286628, ENSP00000474686, ENSP00000475086 and ENSP00000286627).

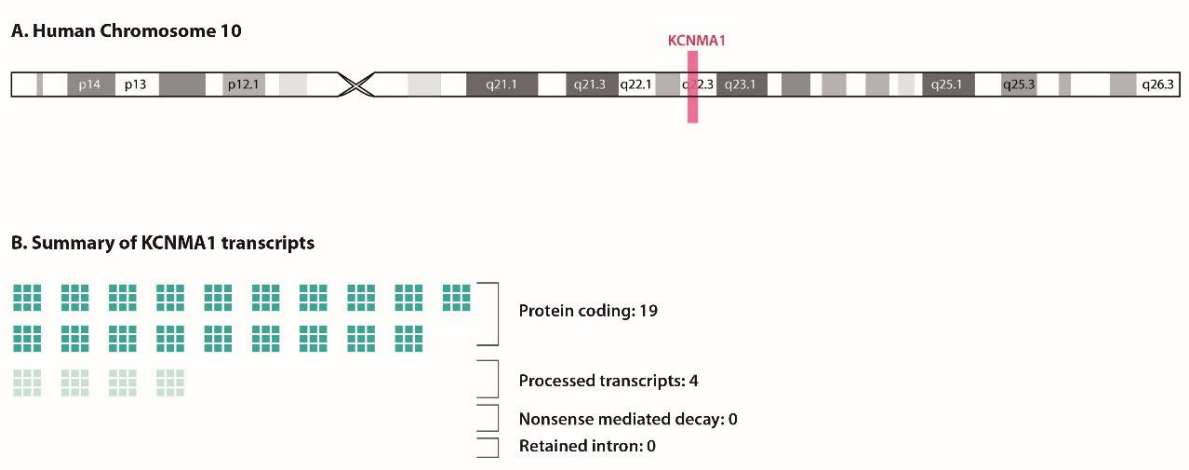


Figure 3.1- Schematic of BK Channel Gene Location and Transcript Overview. A) A schematic of human chromosome 10 is shown annotated at position 10q22.3 with the location of the BK channel α-subunit gene, KCNMA1, highlighted with a pink box. The molecular location of KCNMA1 is 10:78,629,359 to 10:79,398,353 and is 768,994 base pairs in length. Data was extracted from Ensembl Database webpage as a result of a search for the human BK channel gene, KCNMA1. Figure adapted from 'Region in detail' tab of gene ENSG00000156113 on the Ensembl database. **B)** Summary of KCNMA1 transcripts. There were 23 BK channel transcripts identified in total, 19 of which are protein coding and 4 of which are processed transcripts. Nonsense mediated decay (NMD) and retained intron transcripts were not identified as a product of the KCNMA1 gene. Transcripts are represented by the turquoise grid boxes. Data was extracted from 'transcript summary table' of the Ensembl database.

The 'canonical' protein isoform of the KCNMA1 gene

Given the variety of pore-forming α-subunit isoforms available, it was necessary to choose a form that would serve as the reference protein isoform for the study. The protein chosen was the CCDS protein with the longest amino acid sequence, which is accepted to be 'canonical'. This protein was identified by the UniProtKB database as being 1,236 amino acids in length, with the identifiers ENSP00000286628 and Q12791-1. The equivalent transcript is named as ENST00000286628 in the Ensembl database. This transcript includes the STREX splice insert, a 58 amino acid insert at the C2 splice site, responsible for stretch sensitivity and enhanced calcium sensitivity (Saito *et al.*, 1997; Xie and McCobb, 1998; Chen *et al.*, 2005; Naruse, Tang and Sokabe, 2009; Saleem, Rowe and Shipston, 2009; Jeffries, 2010), hypoxia sensitivity (McCartney *et al.*, 2005) and increased cell membrane affinity (Jeffries, 2010). Details of the canonical BK channel protein isoform are listed in Table 3.1.

Database	Identifier	Length (BP)	Length (AA)	Mass (Da)	Coding Exons
Ensembl	ENSP00000286628	6,096	1,236	137,560	28
	ENST00000286628				
	KCNMA1-009				
UniProt	Q12791-1				
Havana	OTTHUMT00000048885				

Table 3.1- Details of the reference KCNMA1 transcript (canonical transcript). The table contains details the BK channel canonical transcript, including its transcript identifier in bioinformatics databases Ensembl, UniProt and Havana, the protein coding transcript length in base pairs (BP), the protein length in amino acids (AA), protein mass in Daltons (Da) and the number of coding exons in the transcript.

3.2.3 The amino acid composition of the BK channel structural and functional regions

Mapping the structural and functional domains of the BK channel onto its amino acid sequence was a necessary initial step, as a compilation of this information did not already exist in the desired format and detail required for the aims of this thesis. A single figure, where the functional and structural domains of the BK channel are overlaid onto the amino acid sequence was produced to provide a visual summary of the location and amino acid composition of essential elements of the BK channel. Identification of the amino acid location of BK channel structural and functional domains was undertaken using the UniProt and Ensembl databases. A schematic of the canonical BK channel protein and the amino acid FASTA sequence, annotated with structural domains, is shown in Figure 3.2.

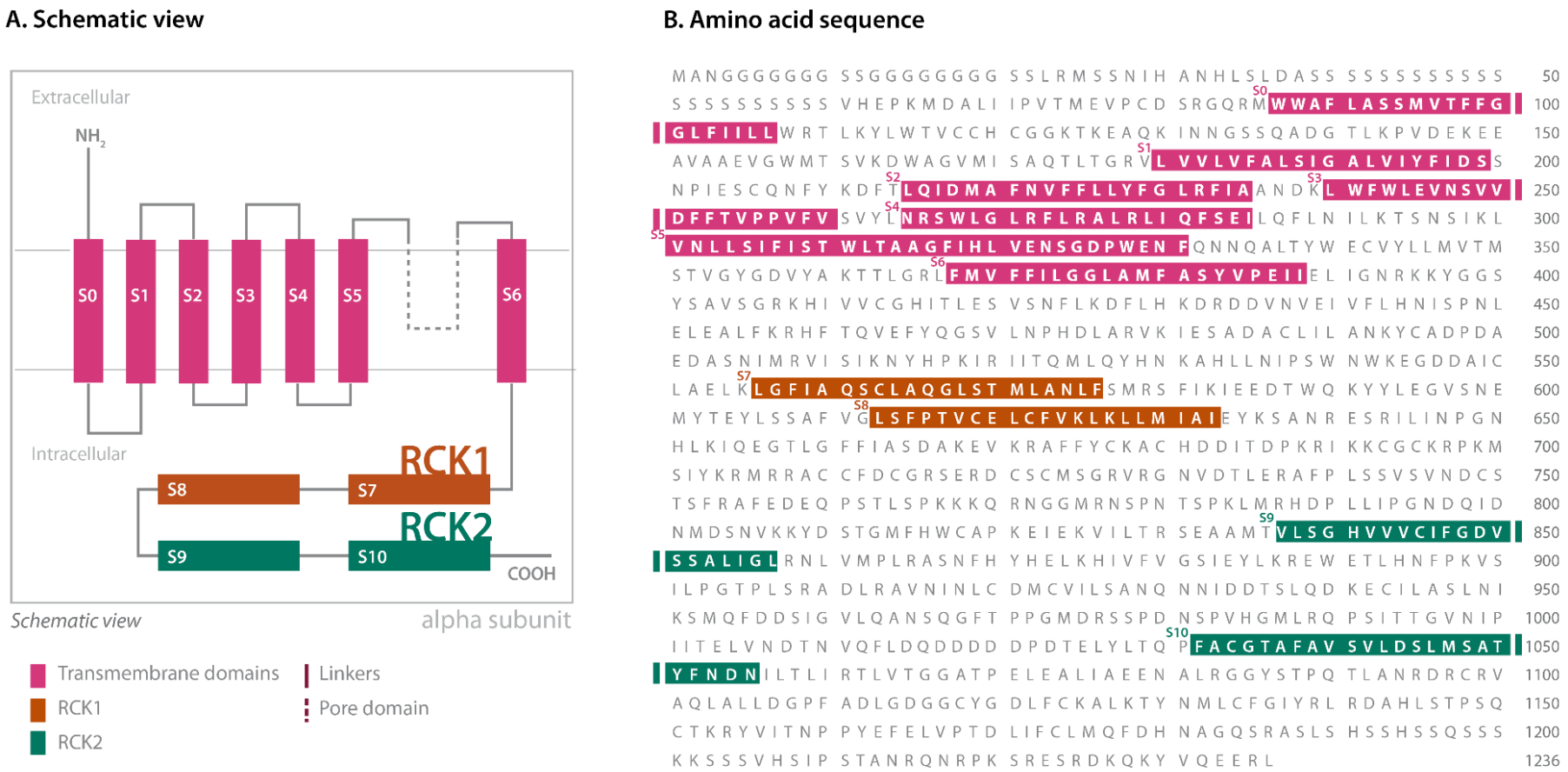


Figure 3.2- BK channel canonical protein structure.

Figure 3.2- BK channel canonical protein structure. A) Schematic of the BK channel α-subunit canonical protein, annotated with structures of: transmembrane domains (TMD) S0-S6 (pink blocks), linker region, the pore region and the C-terminus beginning at the end of the S6 TMD, containing the regulator of the conductance of potassium (RCK) domains (RCK1 in orange and RCK2 in green blocks). Schematic not drawn to scale.

B) The amino acid sequence of the BK channel α-subunit canonical protein annotated with structural domains and regions, colour coded to correspond with the locations in Figure 3.2A. The total length of the channel is 1,236 amino acids. Domain amino acids locations extracted from UniProt database (identifier Q12791-1).

The BK channel is shown to be formed of 7 transmembrane domains, named S0-S6. The length of the transmembrane domains was consistent at 20 to 21 amino acids, whilst the length of the linker regions varied greatly from 4 amino acids between S2-S3 and S3-S4, to 72 amino acids between S0 and S1. The cytoplasmic (C-terminus) end of the channel contains the regulators of the conductance of potassium (RCK) domains, RCK1 and RCK2. These, alongside the associated linker regions comprise approximately two thirds of the channel sequence. The search of the UniProt and Ensembl databases also identified the amino acid location of 14 domains of functional importance, including the potassium selectivity filter, the voltage sensing domain and calcium binding sites that are central to the definition of a BK channel (Figure 3.3). Other key components that alter or modify channel function, such as the haeme binding site, the STREX insert, and poly serine and glycine sites are identified. These domains have been chosen for inclusion based on their crucial impact on BK channel activity, thus allowing the identification of key functional and structural components and the proximity of SNPs at a later stage in the investigation.

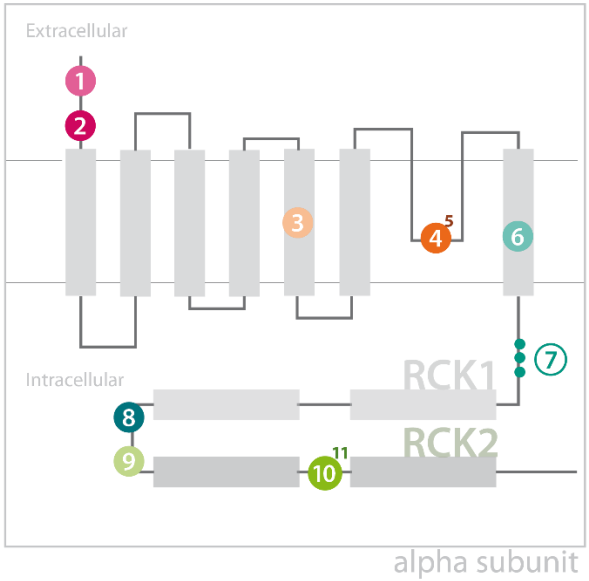
3.2.4 Identification of post-translational modification sites on the BK channel

The data on the structural and functional domains within the BK α-subunit provides a backbone on which to build further layers of complexity. For example, the functional domains identified key regulatory features that influence channel activity in isolation, such as the intrinsic voltage and calcium sensitivity of the channel, and the STREX insert further modifies these properties. However, this does not capture a further layer of control and modulation that can occur with BK channels, namely that of post-translational modification.

Phosphorylation

Protein phosphorylation is the reversible addition of a phosphate group to an amino acid residue, a process of considerable importance in mammals where the regulated addition and removal of phosphate to serine, threonine and tyrosine residues can lead to changes in protein function (as reviewed by Nishi, Hashimoto & Panchenko 2011). More recently, studies have established that the phosphorylation of histidine residues can occur in mammals (Besant, Tan and Attwood, 2003; Zu, Besant and Attwood, 2008; Wieland *et al.*, 2010; Wieland and Attwood, 2015). In an earlier publication, histidine phosphorylation was hypothesised to account for approximately 6% of total phosphorylation in eukaryotes (Matthews, 1995).

A. Schematic view



B. Location and length of functional sites

Name	Location	Length (AA)
1. Poly-glycine sites	4-10, 13-20	7, 8
2. Poly-serine sites	39-60	22
3. Voltage sensing domain	265-285	21
4. Pore forming domain	336-358	23
5. Potassium selectivity motif	352-355	4
6. Entrance to the intracellular vestibule	368-388	21
7. Magnesium binding site	439, 462, 464	1, 1, 1
8. Haeme binding motif	677-681, 693-695	5, 3
9. STREX insert	699-756	58
10. Calcium bowl	1003-1025	23
11. Calcium binding site	1012, 1015, 1018, 1020	1, 1, 1, 1

C. Amino acid sequence

Figure 3.3- BK channel functional domains.

Figure 3.3- BK channel functional domains. A) BK channel structure schematic annotated with functional domains identified using bioinformatics databases. The structural domains (from Figure 3.2) are shown as grey blocks and 11 different domains of functional importance numbered and coloured as listed in the figure key (B).

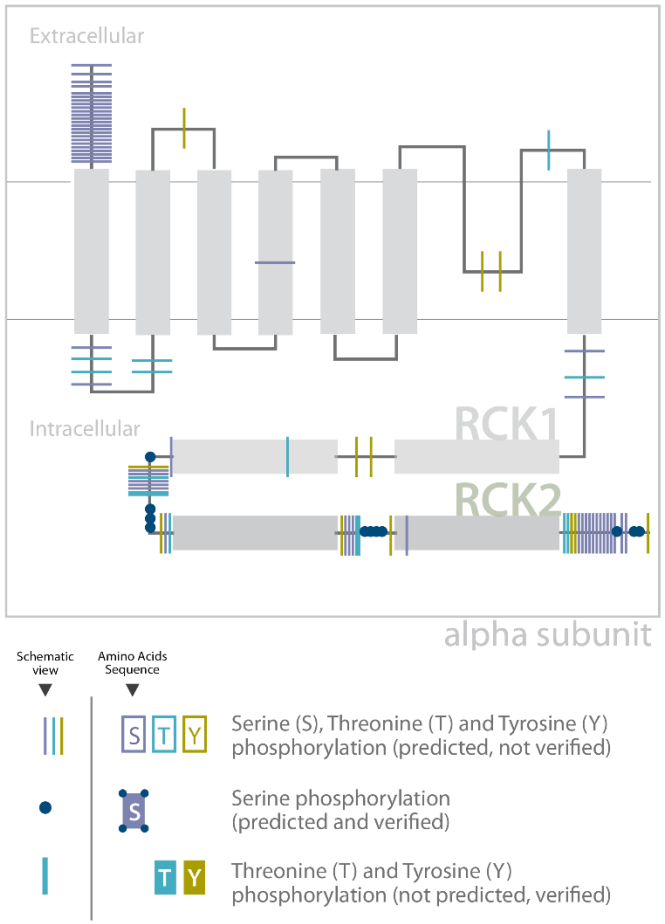
B) Figure Key: provides the name of the functional domain, location in terms of the amino acid number and the length of the domain in amino acids. **C)** BK channel amino acid sequence annotated with the structural components in grey blocks and the various coloured functional motifs corresponding to the schematic (A) and figure key (B). Additional functional sites not annotated on the amino acid sequence are the hypoxia motif (C721-C723), REDOX sensitive cysteine residues (C495, C680, C1034) and carbon monoxide sensitive amino acids (H430, H459, D432).

To identify the potential phosphorylation sites on the BK channel, the KCNMA1 protein FASTA sequence was exported from UniProt and submitted to the phosphorylation prediction software NetPhos 2.0, which uses a neural-network based assessment method. The output showed that 87 amino acid residues were predicted to be phosphorylated; 63 serines (S), 11 threonines (T) and 13 tyrosines (Y). Figure 3.4 shows the location of these residues on the BK channel structural schematic and on the BK channel amino acid sequence.

The investigation revealed a density of predicted serine phosphorylation sites at both the N- and C-termini. In the extracellular portion of the channel (at the N-terminus), 22 of 28 serine residues were predicted to be phosphorylated. At the C-terminus, 15 of the 20 serine residues in the terminal region were predicted to be phosphorylated. There was a secondary area of increased predicted phosphorylation of amino acids in the region linking transmembrane domains S0 and S1 (S0-S1 loop), where 4 of the 7 threonine residues in the linker were predicted to be phosphorylated. As there were only 11 threonine residues predicted to be phosphorylated across the whole channel, the 4 predicted phosphorylation sites in the S0-S1 loop accounts for a large proportion of the total number of predicted threonine phosphorylation sites. In addition, there were two predicted sites of serine phosphorylation in the S0-S1 loop (S136 and S161), identifying this region as one of high potential regulation by phosphorylation. This region has already been associated with membrane translocation (Jeffries, 2010; Jeffries *et al.*, 2010), and thus a high density of potential phosphorylation may be an indicator of a further level of regulation of membrane translocation. There are two predicted tyrosine phosphorylation sites in the pore-forming domain between S5 and S6 (Y355 and Y359); this region has been shown to be important for channel function as discussed by (MacKinnon, 2003; Hoshi, Pantazis and Olcese, 2013; Kuang, Purhonen and Hebert, 2015). Further downstream in the amino acid sequence, phosphorylation was less commonly predicted in the transmembrane and RCK domains, with 3 predicted phosphorylation sites in total (S248, T617 and S1041). There was also serine residue predicted to be phosphorylated within the hypoxic sensing region of the channel (S722), and a threonine residue predicted to be phosphorylated next to a magnesium binding site (T461).

Figure 3.5 illustrates the percentage of total serine, threonine and tyrosine residues that were predicted to be phosphorylated per transmembrane domain and linker region. As existing evidence links phosphorylation to key mechanisms, such as BK channel activation or inhibition (Schubert and Nelson, 2001; Tian, Duncan, *et al.*, 2001; Widmer, Rowe and Shipston, 2003; Jianxi Liu *et al.*, 2006; Zhou *et al.*, 2010), the existence of regions of high predicted phosphorylation of the BK channel signpost areas of interest to look for potential SNP co-localisation.

A. Schematic view



B. Amino acid sequence

Figure 3.4- Phosphorylation Sites on the BK Channel

Figure 3.4- Phosphorylation Sites on the BK Channel A) BK channel schematic annotated with the locations of predicted and experimentally verified sites of phosphorylation on serine (S), threonine (T) and tyrosine (Y) amino acid residues. Sites of predicted phosphorylation were identified using the NetPhos-2 algorithm, available as online software at http://www.cbs.dtu.dk/services/NetPhos/. In total 87 predicted sites of phosphorylation were identified; 63 (S), 11 (T) and 13 (Y). Of these, 13 sites of phosphorylation have been verified experimentally and were identified via literature and database search.

B) BK channel amino acid sequence annotated with the locations of predicted and verified sites of phosphorylation. The figure key provides the symbol for serine, threonine and tyrosine predicted and verified phosphorylation in the schematic and amino acid sequence. There are instances of threonine and tyrosine residues that are verified but were NOT predicted (T763, Y885 and T970).

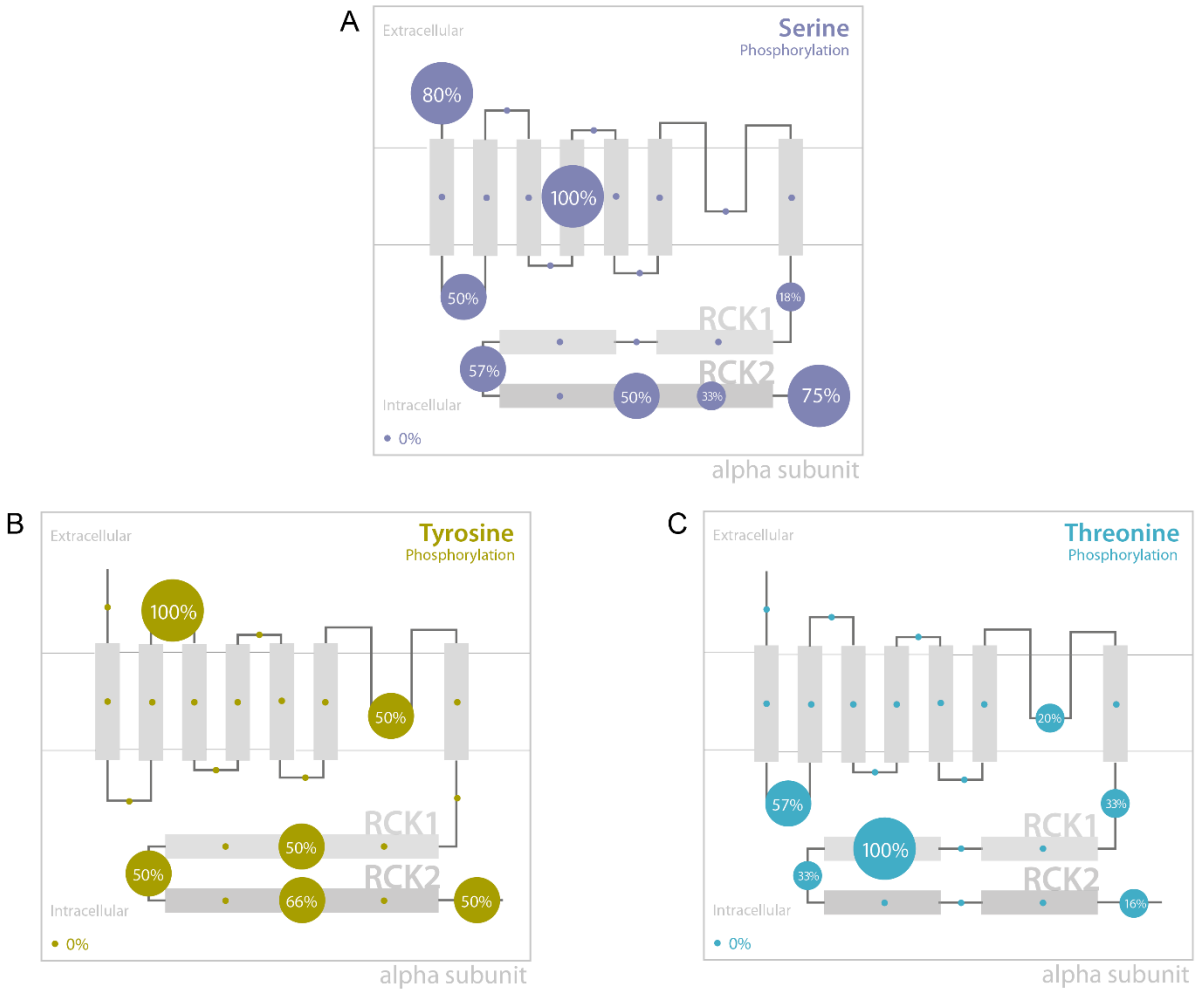


Figure 3.5- Percentage of total potential BK channel phosphorylation sites predicted to be phosphorylated per region (schematic view). A schematic annotated with the percentage of total **A)** serine (S), **B)** tyrosine (Y) and **C)** threonine (T) amino acid residues predicted to be phosphorylated per region of the BK channel. The percentages highlighted in the schematics show that predicted phosphorylation most commonly occurred in the linker regions of the channel.

The STREX splice insert, spanning from K699 to F756, has been shown to be a region where phosphorylation is important for channel function (Tian, Duncan, *et al.*, 2001; Tian *et al.*, 2004). The bioinformatics search identified 7 predicted sites of phosphorylation on the STREX splice insert, 5 of which were associated with serine amino acids, 1 with tyrosine and 1 with threonine: S701, Y703, S717, S722, T734, S745 and S752. The predicted phosphorylation rate of linker regions from S1 to S6 plus the C-terminal linker regions was approximately 1 in 3, with 10 of 31 and 29 of 89 total serine, threonine and tyrosine sites predicted to be phosphorylated. In contrast, the predicted phosphorylation for the whole channel sequence was much lower at approximately 1 in 14 amino acids.

Verified sites of BK channel phosphorylation

A search of the literature, combined with the data held on the UniProt database identified 13 sites of experimentally verified phosphorylation of the BK channel, which are listed in Table 3.2 and plotted in Figure 3.4. The verified sites were located in the cytosolic C-terminus downstream of the RCK1 domain; 10 were associated with serine residues (S701, S765, S778, S782, S978, S982, S992, S1204, S1221 and S1224), 2 with threonine residues (T763 and T970) and 1 with tyrosine (Y885). Of these 13 verified sites of phosphorylation, 10 serine residues were predicted to be phosphorylated by NetPhos 2.0.

Only experimental results detailing the specific amino acid residue proven to be phosphorylated by means such as mutation or phosphorylation assay, were considered when listing verified sites. Furthermore, to be considered, the results had to provide an identifier for the transcript used in the experiments or provide sufficient flanking amino acid sequence to ensure accurate identification of the residue in question; this enabled the specific amino acid to be accurately plotted onto the BK channel amino acid sequence 'map'. In instances where the amino acid verified to undergo phosphorylation was not conserved in the human BK channel sequence, the residue could not be included in the list of verified sites. For example, the threonine phosphorylation site T107 on bovine BK channels was not included (Jianxi Liu *et al.*, 2006), as the site is not conserved in the human form of the channel, and therefore could not be plotted on the human BK channel amino acid sequence.

Human ref.	Species	Location	Reference
S701	Murine	S3 of STREX insert	Tian et al. (2008)
S765	Bovine	S695	Zhou et al. (2010)
S778	Murine	S724	Huttlin et al. (2010)
S782	Murine	S728	Huttlin et al. (2010)
Y885	Murine	Y766	Ling et al. (2000)
S978	Murine	S924	Huttlin et al. (2010)
S982	Murine	S928	Huttlin et al. (2010)
S992	Murine	S899	Tian et al. (2003)
S1204	Canine	S1072	Fukao et al. (1999)
S1221	Bovine	S1151	Zhou et al. (2001)
S1224	Bovine	S1154	Zhou et al. (2001)
T763	Chicken	T847	Bai et al. (2012)
T970	Murine	T916	Huttlin et al. (2010)

Table 3.2- Experimentally verified phosphorylation sites on the BK channel. This table lists the details for experimentally verified phosphorylation sites, including the species of BK channel construct that was used for verification, the amino acid location in that species of BK channel, the equivalent amino acid reference on the canonical human BK channel and the reference for the publication that the data was documented in. The majority of (8 of the 13) verified phosphorylation sites listed in the table were first characterised in the murine form of the BK channel. Threonine phosphorylation site T107 on the bovine BK channel was not included (Jianxi Liu *et al.*, 2006), as the site is not conserved in the human form of the channel.

Palmitoylation

Palmitoylation, a form of s-acylation, is the reversible addition of a palmitate moiety to an amino acid residue, via a thioester bond, resulting in a wide range of actions in the BK channel, such as protein-membrane targeting and modulation of other post-translational modifications, such as phosphorylation (Widmer, Rowe and Shipston, 2003; Tian *et al.*, 2008; Jeffries, 2010; Jeffries *et al.*, 2010; Bi, 2014). To ascertain the number of predicted palmitoylated amino acid residues for the BK channel, the FASTA amino acid sequence for the KCNMA1 canonical protein transcript was exported from the UniProt bioinformatics database and submitted to the CSS-Palm 4.0 palmitoylation prediction software. In total, 8 sites of palmitoylation were predicted with C118, C119 and C121 located in the S0-S1 linker, C206 in the S1-S2 linker, C710 and C711 in the STREX splice insert located between RCK1 and RCK2, and C1124 and C1134 at the extreme C-terminal end of the channel. The amino acid location of the identified sites as well as the scores from the predictive software are detailed in Figure 3.6 and Figure 3.7.

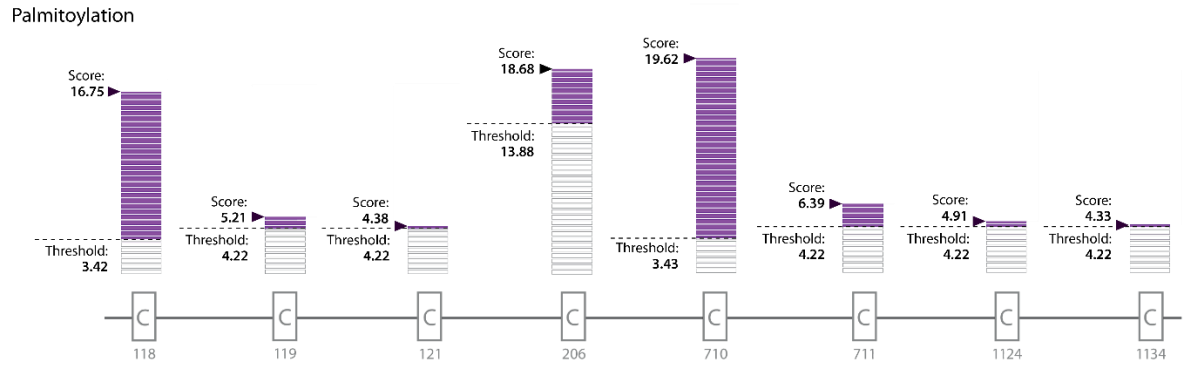


Figure 3.6- BK Channel palmitoylation scores for cysteine residues calculated by CSS-Palm 4.0 predictive software. Figure displaying palmitoylation scores for cysteine residues predicted to be palmitoylated; included is the CSS-Palm 4.0 calculated palmitoylation prediction threshold indicated by the grey section of each column. The prediction score for each of the cysteine residues indicated by the purple section of each column.

All the predicted palmitoylation sites are located in intracellular linker regions, with the exception of C206 which is located in an extracellular linker region. In comparison to protein phosphorylation, predicted palmitoylation has a much lower occurrence. Phosphorylation is predicted to occur at a rate of 1 in 14 of the total channel amino acid residues, whilst palmitoylation is predicted to occur at a rate of 1 in 155 of the total amino acids residues. When considering only serine, threonine and tyrosine amino acid residues for phosphorylation and only cysteine residues for palmitoylation, the rates become similar due to the lower number of cysteine residues (35 in total) in the BK channel, with approximately 1 in 3 serine/threonine/tyrosine residues and 1 in 4 cysteine residues predicted to be phosphorylated and palmitoylated respectively.

As has been shown previously with cysteine residues involved in post-translational modification, there was grouping of the residues into 'cysteine rich domains' (McCartney *et al.*, 2005; Jeffries, 2010; Jeffries *et al.*, 2010; Zhou *et al.*, 2012). For example, all 3 of the available cysteine residues in the S0-S1 linker are predicted to be palmitoylated, the same is true for the S1-S2 linker where the only cysteine in that sequence is predicted to be palmitoylated. Therefore, those domains had a predicted palmitoylation frequency of 100% (1 in 1) of cysteine residues. In other regions, the density of palmitoylation was lower; for example, the S8-S9 linker, containing the STREX insert, had a predicted palmitoylation rate of 1 per 6 cysteine residues.

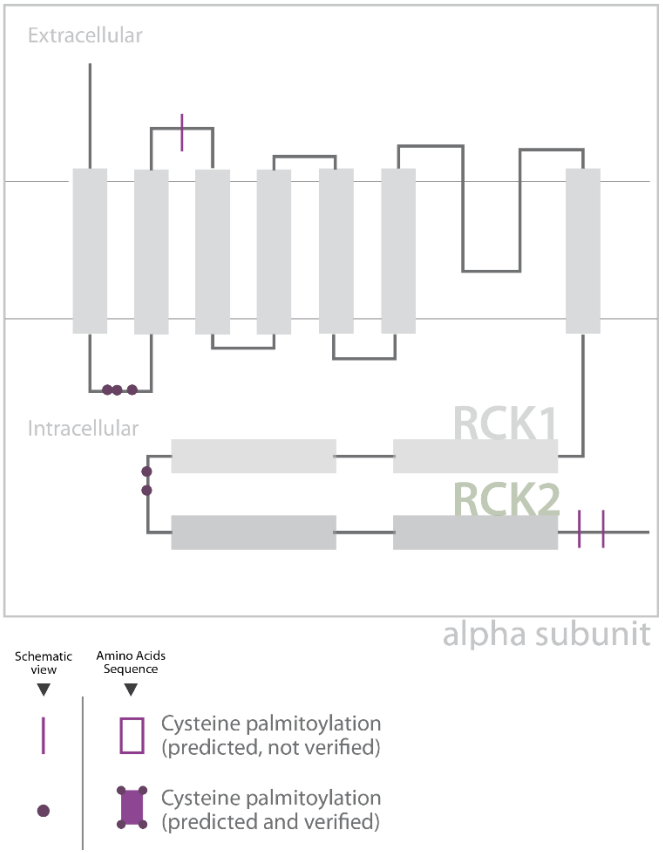
Verification of palmitoylation sites on the human BK channel.

To ascertain the extent of established palmitoylation of the BK channel, a literature and database search was conducted to identify the locations of cysteine residues that had been verified to undergo palmitoylation by lab- based experimentation. This search identified 5 verified sites, all of which were predicted by CSS-Palm 4.0 software. C118, C119 and C121 are located in the S0-S1 loop and have been linked with channel-membrane targeting (Jeffries, 2010; Jeffries *et al.*, 2010), whilst C710 and C711 are located in the C-terminal STREX splice insert are associated with cell-membrane targeting as well as regulating the effect of phosphorylation on BK channel activity (Tian *et al.*, 2008; Jeffries, 2010). All of the sites listed in Table 3.3 were verified in the murine form of the BK channel, however the cysteine residues are conserved in the human form of the channel and therefore could be included.

Human ref.	Species	Location	Reference
C118	Murine	C53	Jeffries et al. (2010)
C119	Murine	C54	Jeffries et al. (2010)
C121	Murine	C56	Jeffries et al. (2010)
C710	Murine	C645	Tian et al. (2008)
C711	Murine	C646	Tian et al. (2008)

Table 3.3- Experimentally verified palmitoylation sites on the BK channel. This table lists the details for experimentally verified palmitoylation sites, including the species of BK channel construct that was used for verification, the amino acid location in that species of BK channel, the equivalent amino acid reference on the canonical human BK channel and the reference for the publication that the data. All experimentally verified publication sites were identified in the murine form of BK channel.

A. Schematic view



B. Amino acid sequence

```
MANGGGGGGG SSGGGGGGGG SSLRMSSNIH ANHLSLDASS SSSSSSSSSS   50
SSSSSSSSSS VHEPKMDALI IPVTMEVPCD SRGQRMWWAF LASSMVTFFG  100
GLFIILLWRT LKYLWTVCCH CGGKTKEAQK INNGSSQADG TLKPVDEKEE  150
AVAAEVGWMT SVKDWAGVMI SAQTLTGRVL VVLVFALSIG ALVIYFIDSS  200
NPIESCQNFY KDFTLQIDMA FNVFFLLYFG LRFIAANDKL WFWLEVNSVV  250
DFFTVPPVFV SVYLNRSWLG LRFLRALRLI QFSEILQFLN ILKTSNSIKL  300
VNLLSIFIST WLTAAGFIHL VENSGDPWEN FQNNQALTYW ECVYLLMVTM  350
STVGYGDVYA KTTLGRLFMV FFILGGLAMF ASYVPEIIEL IGNRKKYGGS  400
YSAVSGRKHI VVCGHITLES VSNFLKDFLH KDRDDVNV I VFLHNISPNL  450
ELEALFKRHF TQVEFYQGSV LNPHDLARVK IESADACLIL ANKYCADPDA  500
EDASNIMRVI SIKNYHPKIR IITQMLQYHN KAHLLNIPSW NWKEGDDAIC  550
LAELKLGFIA QSCLAQGLST MLANLFSMRS FIKIEEDTWQ KYYLEGVSNE  600
MYTEYLSSAF VGLSFPTVCE LCFVKLKLLM IAIEYKSANR ESRILINPGN  650
HLKIQEGTLG FFIASDAKEV KRAFFYCKAC HDDITDPKRI KKCGCKRPKM  700
SIYKRMRRACCFDCGRSERD CSCMSGRVRG NVDTLERAFP LSSVSVNDCS  750
TSFRAFEDEQ PSTLSPKKKQ RNGGMRNSPN TSPKLMRHDP LLIPGNDQID  800
NMDSNVKKYD STGMFHWCAP KEIEKVILTR SEAAMTVLSG HVVVCIFGDV  850
SSALIGLRNL VMPLRASNFH YHELKHIVFV GSIEYLKREW ETLHNFPKVS  900
ILPGTPLSRA DLRAVNINLC DMCVILSANQ NNIDDTSLQD KECILASLNI  950
KSMQFDDSIG VLQANSQGFT PPGMDRSSPD NSPVHGMLRQ PSITTGVNIP 1000
IITELVNDTN VQFLDQDDDD DPDTELYLTQ PFACGTAFAV SVLDSLMSAT 1050
YFNDNILTLI RTLVTGGATP ELEALIAEEN ALRGGYSTPQ TLANRDRCRV 1100
AQLALLDGPF ADLGDGGCYG DLFCKALKTY NMLCFGIYRL RDAHLSTPSQ 1150
CTKRYVITNP PYEFELVPTD LIFCLMQFDH NAGQSRASLS HSSHSSQSSS 1200
KKSSSVHSIP STANRQNRPK SRESRDKQKY VQEERL                1236
```

Figure 3.7- Palmitoylation sites on the BK Channel

Figure 3.7- Palmitoylation sites on the BK Channel A) The BK channel α-subunit schematic annotated with verified and predicted sites of palmitoylation; 8 cysteine amino acid residues (C) were predicted to be palmitoylated, 5 of which have been verified experimentally (C118, C118, C121, C710 and C711). Cysteine residues annotated as predicted were identified using CSS-Palm 4.0 software. The FASTA amino acid sequence for the BK channel canonical protein isoform was entered. Verified sites were identified by literature search.

B) BK channel amino acid sequence annotated with predicted and verified palmitoylated cysteine residues, as plotted in Figure 3.7A. The figure key provides the symbols for cysteine predicted and verified palmitoylation in the schematic and amino acid sequence.

S-Nitrosylation

S-nitrosylation is the covalent modification of a cysteine amino acid residue by a nitrous oxide group to generate S-nitrosocysteine (CySNO), which facilitates REDOX-dependent signalling and regulation of protein function (as reviewed by Gould *et al.*, 2013). The extent of S-nitrosylation of the BK channel was assessed using the predictive software GPS-SNO 1.0; the amino acid sequence for the canonical BK channel protein isoform was submitted to the software, this resulted in the identification of 5 predicted sites of S-nitrosylation. The amino acid location of the identified sites as well as the scores from the predictive software are detailed in Figure 3.8. The 5 predicted sites, C487, C680, C845, C943 and C1098, are located in the cytosolic C-terminus of the channel (Figure 3.11). Only C845 is located within a hydrophobic domain (S9), whilst the remainder lie within the intracellular linker regions. There is a predicted site of S-nitrosylation at cysteine C680, which is part of the haeme binding motif and a REDOX sensitive site, spanning from C677 to H681. There is no overlap between the cysteine residues predicted to undergo palmitoylation and those predicted to undergo S-nitrosylation. Experimentally verified S-nitrosylation sites on the BK channel were not identified during a literature search. The overall incidence of predicted S-nitrosylation is 1 per 247 total amino acid residues, and 1 in 7 based upon the total number of cysteine residues in the BK channel.

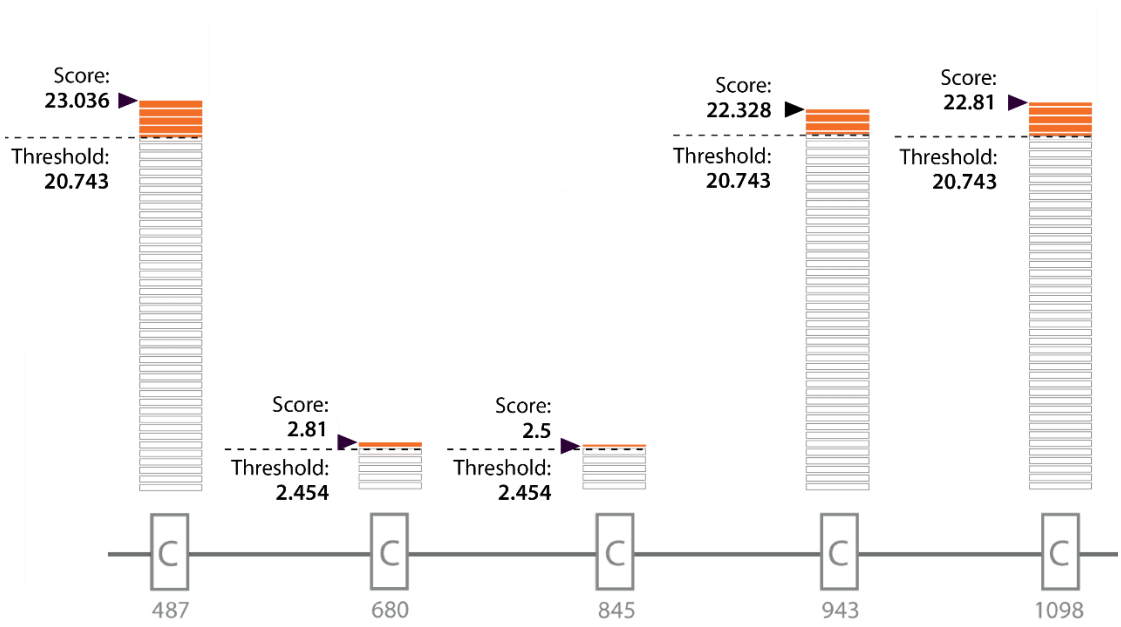


Figure 3.8- BK channel S-nitrosylation scores for cysteine residues calculated by GPS-SNO predictive software. Figure displaying GPS-SNO 1.0 scores for cysteine residues predicted to be S-nitrosylated. Included is the GPS-SNO 1.0 calculated S-nitrosylation prediction threshold for each residue indicated by the grey section of each column. The prediction score for each of the cysteine residues indicated by the orange section of each column.

SUMOylation

SUMOylation is the addition of SUMOs (small ubiquitin-like modifiers) to proteins via the lysine (K) amino acid residue, and is categorised as a post-translational modification that tags proteins for ubiquitin-dependent degradation (Geoffroy and Hay, 2009; Wang and Prelich, 2009; Miteva *et al.*, 2010). Sites of predicted SUMOylation were identified by submitting the canonical BK channel protein isoform amino acid sequence to the GPS-SUMO predictive software. This process identified 4 sites of predicted SUMOylation, K65, K480, K583 and K887. The amino acid location of the identified sites as well as the scores from the predictive software are detailed in Figure 3.9. Three of the predicted sites are found in the intracellular linker regions, with the exception of K65 which is located on the extracellular N-terminal region of the channel (Figure 3.11). The overall frequency of predicted SUMOylation is 1 per 309 total amino acid residues, and the frequency related to the total number of lysine residues in the BK channel is 1 in 16. SUMOylation predicted sites were not located within three amino acids of key structural, or functional sites, nor near other predicted post-translational modification sites. None of the predicted sites had been experimentally verified for BK channels.

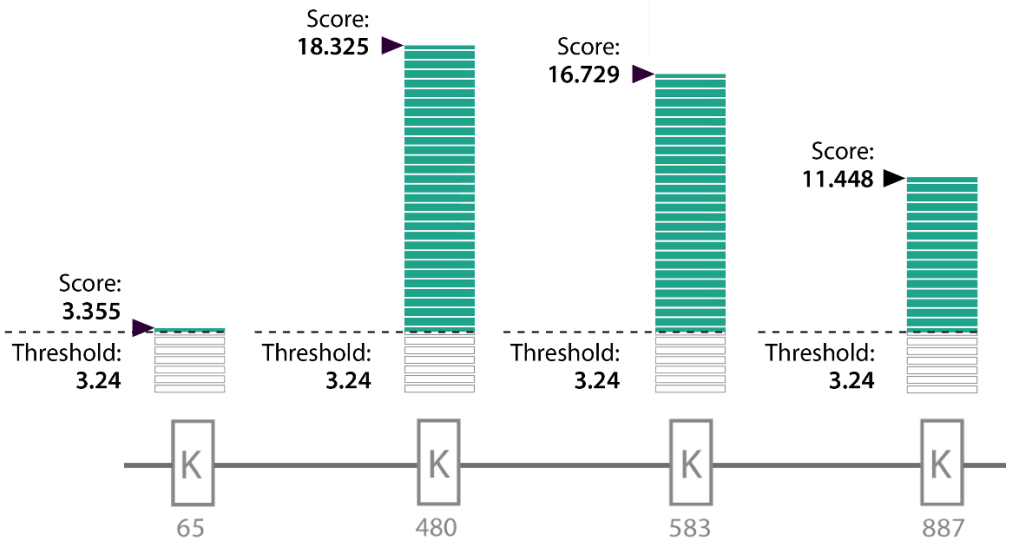


Figure 3.9- BK channel predicted SUMOylation scores for lysine residues calculated by GPS-SUMO predictive software. Bar chart displaying GPS-SUMO scores for lysine residues predicted to be SUMOylated; included is the GPS-SUMO calculated SUMOylation prediction threshold for each lysine residue indicated by the grey section of each column. The prediction score for each of the lysine residues indicated by the green section of each column.

N-Glycosylation

N-glycosylation is the addition of a carbohydrate moiety to the amino acid residue asparagine (N) and is implicated in the control of ion channel folding, stability, trafficking and function (Schwarz and Aebi, 2011; Moharir *et al.*, 2013). Using the prediction software NetNGlyc 1.0, the predicted number of N-glycosylated residues on the BK canonical protein isoform was identified. Three sites were predicted, N133, N265 and N1007, details of which are shown in Figure 3.10. N133 and N1007 are located in the intracellular linker regions between S0–S1 and S9–S10 respectively (Figure 3.11). N265 is located on the first amino acid of the S4 trans-membrane domain, which is the voltage-sensing region that spans the length of the domain. Also of significance is N1007, which is located within the calcium bowl, spanning from T1003 to E1025. The overall frequency of predicted N-glycosylation is 1 per 412 amino acid residues, and the frequency related to the total number of asparagine residues in the BK channel is 1 in 22.

When a literature search was conducted to identify N-glycosylation sites that had been verified experimentally, only one site was found (N200). Interestingly, the N-glycosylation site identified and experimentally verified in 2000, was not predicted by NetNGlyc 1.0 software (Bravo-Zehnder *et al.*, 2000). The publication places the asparagine residue between transmembrane domains S3 and S4 of the human BK channel with the GenBank accession number U11058. In the BK channel isoform used in this investigation, there is no asparagine amino acid residue between transmembrane domains S3 and S4, and given the location of N200, it is plausible that the location is the asparagine N201 found between S1 and S2. Alignment of the amino acid sequence of known human BK channel isoforms indicate that there is no asparagine amino acid at N200. In a recent review (Shipston and Tian, 2016), there was debate as to whether this site was truly modified by N-glycosylation, as mutation of this site to alanine had no effect on channel localization, but the mutation resulted in a change to activation voltage. Figure 3.12 summarises the number of software program predicted post-translational modification sites on the BK channel, and illustrating the number of experimentally verified sites associated with each.

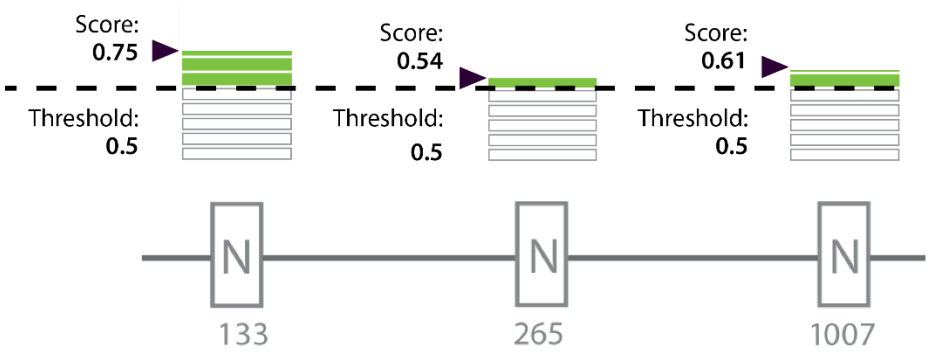


Figure 3.10- BK channel predicted N-glycosylation scores for asparagine residues calculated by NetNGlyc 1.0 predictive software. Bar chart displaying N-glycosylation scores for asparagine residues predicted to be N-glycosylated; included is the NetNGlyc 1.0 calculated N-glycosylation prediction threshold for each asparagine residue indicated by the grey section of each column. The prediction score for each of the asparagine residues indicated by the green section of each column.

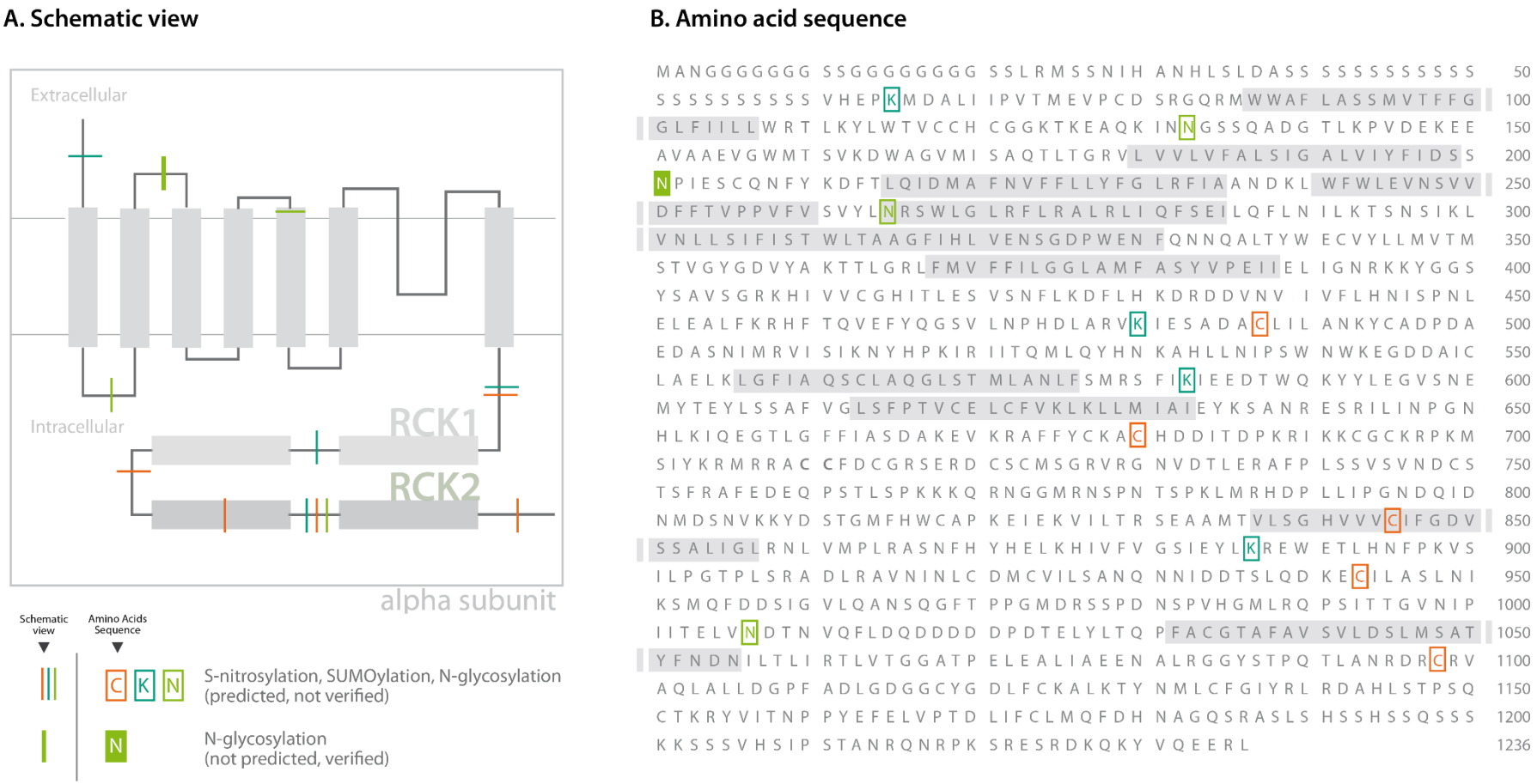


Figure 3.11- BK channel post-translational modifications: S-nitrosylation, SUMOylation, N-glycosylation

Figure 3.11- BK channel post-translational modifications: S-nitrosylation, SUMOylation, N-glycosylation. A)-The BK channel α-subunit schematic is annotated with verified and predicted sites of S-nitrosylation, SUMOylation and N-glycosylation. The FASTA amino acid sequence for the BK channel canonical protein isoform was entered into the relevant predictive software for each PTM. Verified sites were identified by literature and database search.

B) BK channel amino acid sequence annotated with predicted and verified palmitoylated amino acid residues, as plotted in Figure 3.11A. The figure key provides the symbols for amino acid predicted and verified to undergo S-nitrosylation, SUMOylation and N-glycosylation in the schematic and amino acid sequence.

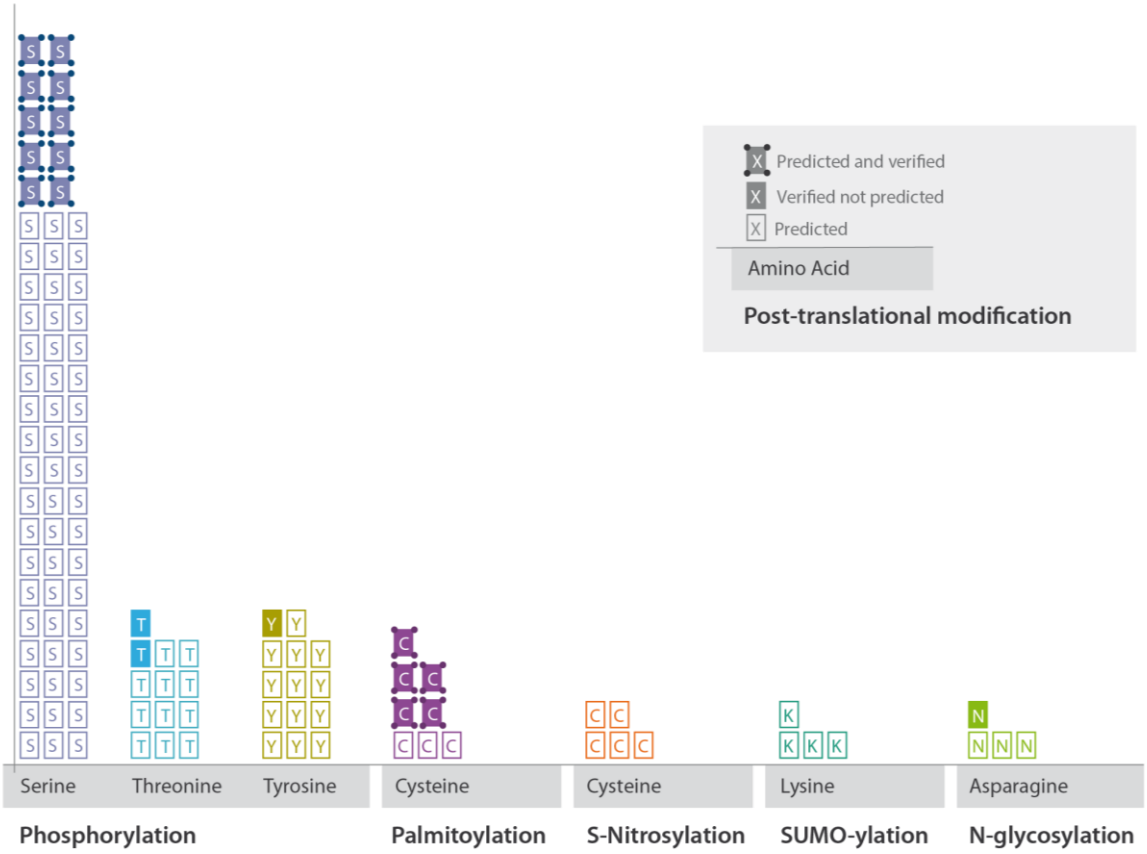


Figure 3.12- Summary of post-translational modifications on the BK channel. Presented is an illustration of the number of phosphorylation, palmitoylation, S-nitrosylation, SUMOylation and N-glycosylation sites on the BK channel, which were identified using predictive software tools. This is shown alongside those post-translational modification sites that have been verified by lab-based experimentation and were identified as a result of a literature and database search (filled boxes). The figure key provides the symbols for the amino acids predicted and verified to undergo post-translational modifications.

3.2.5 Identification of DNA variations sites on the BK channel gene

In the quest for a better understanding of the origins of disease and the most effective way to treat those diseases, there has been a significant shift of opinion on the best way to proceed, moving from treatments based on generic population characteristics, to understanding that the future lies in fully individualised treatment. To achieve this, new research has sought to uncover, down to the molecular level, what differentiates one human from another, and if there are any changes to an individual person's DNA that may make them more susceptible to disease or perhaps more resistant to treatment. Thus far in this chapter, the investigation has focused on mapping the amino acid locations of structural and functional regions of importance onto the human BK channel amino acid 'reference map'. In addition, the locations of post-translational modifications, that add a further layer of control and modulation to the BK channel, have been identified using predictive software and literature searches, have been added the BK channel amino acid 'reference map'. One way to make use of this map is to incorporate it into an attempt to prioritise BK channel single nucleotide polymorphisms based upon their proximity to key domains, and thus the number, type and location of SNPs needs to be established. For this reason, this study attempts to determine the extent of genetic variation of the human BK channel using bioinformatics databases, and map the locations of the variations in relation to domains of structural, functional and post-translational modification.

The BK Channel canonical protein isoform is subject to extensive DNA variation.

To determine the extent of genetic variation of the BK channel, the KCNAM1 canonical protein isoform (ENSP00000286628) was selected from the list of isoforms on the Ensembl database. Selection of the 'variation table' option for this isoform presented a table containing known variations associated with the gene as a whole. In total, there were 14,132 variations (representing 1.8% of the total gene base pairs), with the majority (94.7%) located in the intronic regions of the transcript, as shown in Figure 3.13A. The 3'UTR region contained 162 variations comprising 1.1% overall, whilst the 5'UTR region was not associated with any variations. The 'Others' category contains promoter and enhancer regions, and contained 203 variations comprising 1.4% overall.

Non-synonymous SNP were selected for further investigation.

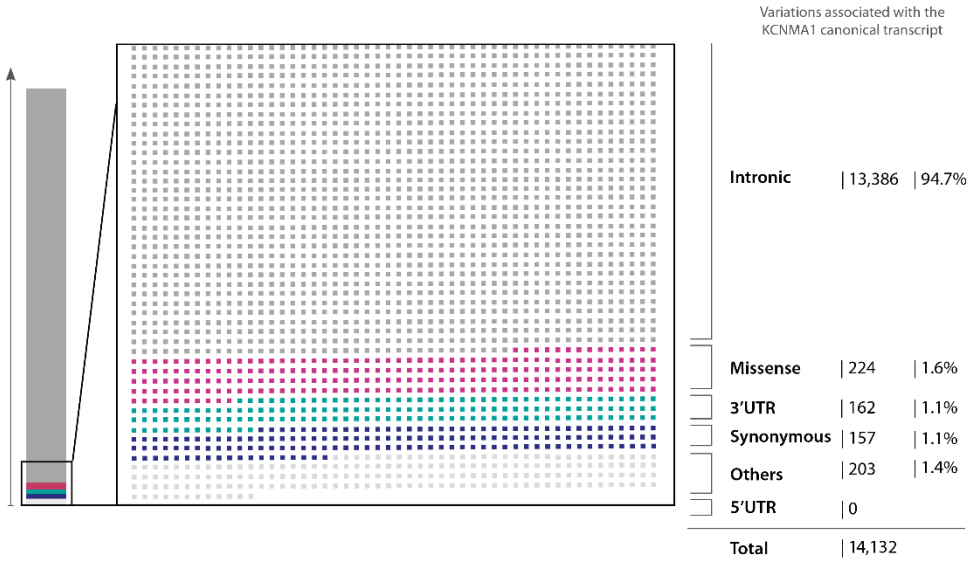
There were 157 synonymous variations (sSNP) in the exonic regions, where there is a change in codon composition but no change to the resulting amino acid, comprising 1.1% of total variations. Missense or non-synonymous SNP (nsSNP) variations located in exonic regions of the KCNMA1 gene were the focus for the next stage of investigations as, these changes modify protein sequence and have been shown to impact on BK channel function (Du *et al.*, 2005; Laumonnier *et al.*, 2006). There were 224 variations of this type, comprising 1.6% of the total number of SNPs. After correction for duplicates, 167 unique nsSNP were identified and these are plotted on the BK channel schematic in Figure 3.13B, and highlighted on the BK channel FASTA sequence as shown in Figure 3.14.

3.2.6 BK channel nsSNP were scored by damage prediction software

The 'damaging potential' of the nsSNPs has been analysed to aid in the prioritisation of targets for further investigation. Illustrated in Figure 3.14 is the location of the 167 nsSNP associated with the BK channel, alongside the damage category allocated to each SNP by the prediction software programs SIFT and PolyPhen-2. SIFT places nsSNP into two different categories: tolerated; and deleterious; whilst PolyPhen-2 has 3 categories: benign; possibly damaging; and probably damaging. SIFT allocated 54% of nsSNP to the 'tolerated' category and 45% into 'deleterious', with a score for the remaining 1% of nsSNP unable to be calculated. PolyPhen-2 placed 39% percent of nsSNP into the most harmful category of 'probably damaging' and 35% in the least damaging category of 'benign'. In addition, PolyPhen-2 contains the category of 'possibly damaging' in which 20% of nsSNP were categorised and 5% of nsSNPs were not scored.

Each program uses a slightly different method or criteria of scoring the damaging potential of a DNA variation. Nevertheless, there was 66% agreement between SIFT and PolyPhen-2 for 'tolerated' and 'benign', and 87% percent agreement between the programs for deleterious and probably damaging. The extra category used by PolyPhen-2 of 'possibly damaging' covered the approximately 20% SNPs that lay at the border between benign and deleterious in SIFT and can be seen in Figure 3.15.

A. Summary of variations on the BK channel canonical transcript



B. Schematic of nsSNPs (missense) on the BK channel canonical transcript

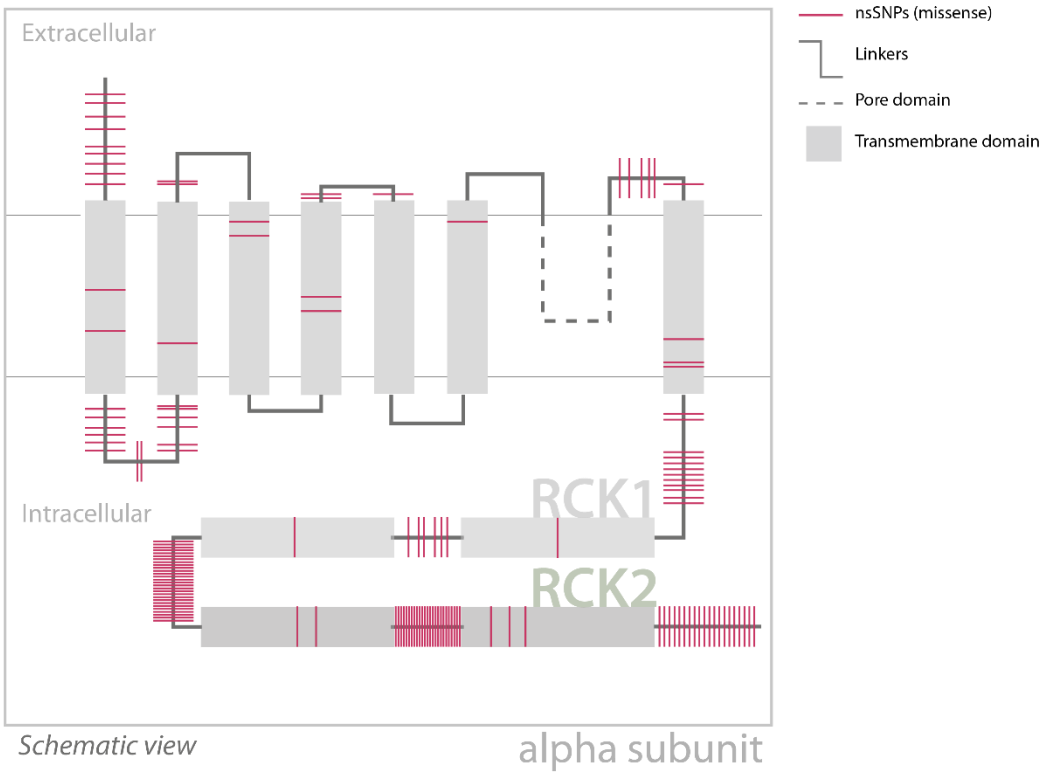


Figure 3.13- DNA variations on the BK channel. A) Illustration of the variations associated with the BK channel canonical protein DNA. The majority of variations are located in the intronic regions (94.7%). 3'UTR, 5'UTR as well as the exonic synonymous and missense (nsSNP) variations accounted for the remaining 5.3%. Data was extracted from the Ensembl database. **B)** Schematic of the BK channel structure annotated with the location of nsSNPs, represented by pink bars, and the transmembrane domains by grey columns.

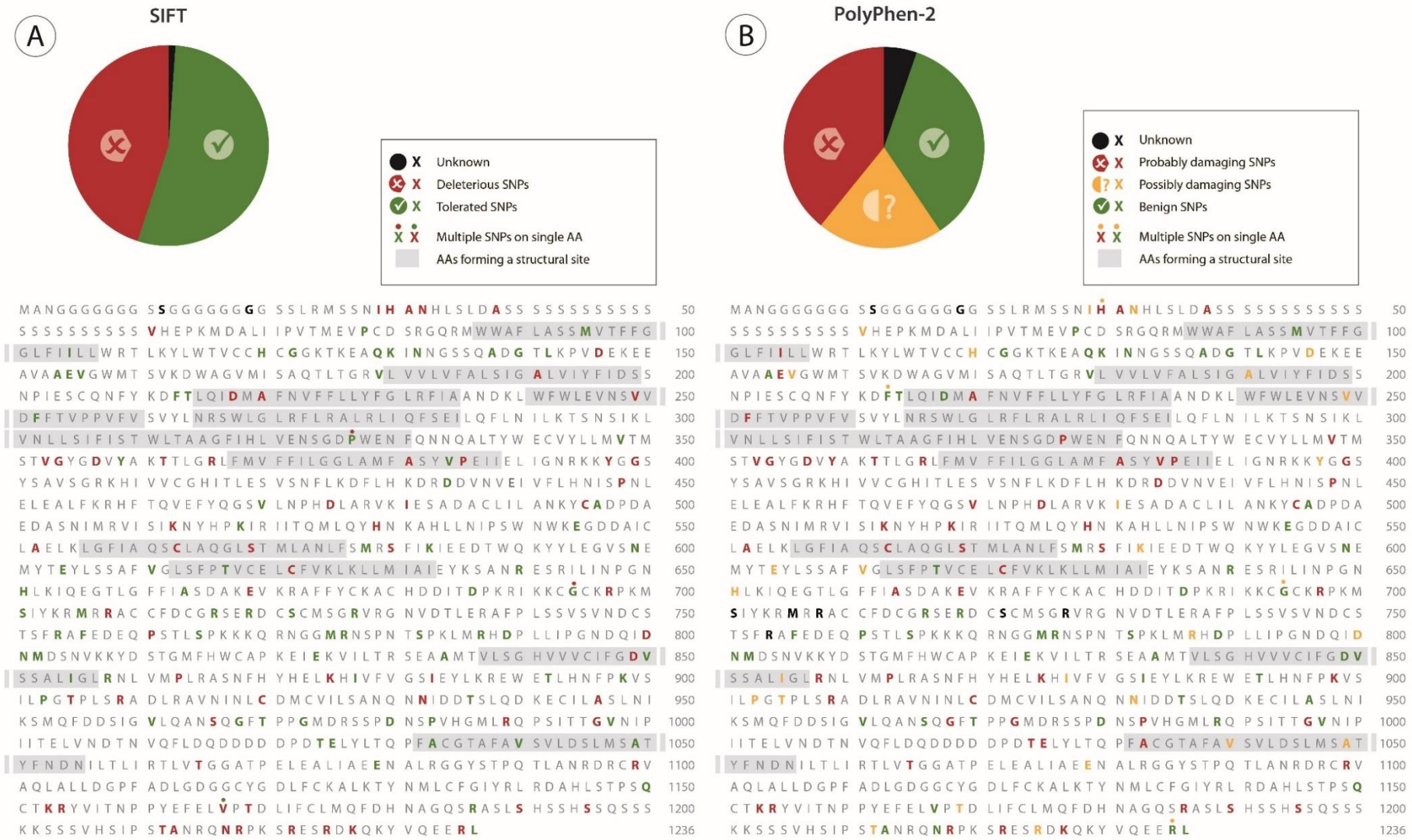


Figure 3.14- Assessment of the predicted damage of nsSNP on the BK channel

Figure 3.14- Assessment of the predicted damage of nsSNP on the BK channel. The damage prediction scores generated by **A)** SIFT and **B)** PolyPhen-2 for the 167 unique nsSNP associated with the BK channel are shown as a proportion of total in the pie chart, and with the corresponding colour code on the BK channel amino acid sequence. SIFT placed 54% of nsSNP into the 'tolerated' category and 45% into 'deleterious. PolyPhen-2 predicted that 39% are 'probably damaging', 20% are 'possibly damaging' and that 35% are 'benign'.

Figure 3.15- Comparison of nsSNP damage scoring between SIFT and PolyPhen-2. This figure illustrates the extent of agreement in BK channel nsSNP damage category between damage prediction software programs SIFT and PolyPhen-2.

3.2.6.1 nsSNP co-localisation with BK channel structural sites

The BK channel amino acid sequence has been annotated with channel a) structural regions, b) functional domains and c) nsSNP location and the damage categories allocated by SIFT and PolyPhen-2 (Figure 3.16 and Figure 3.17). Results indicate that the frequency of genetic variation within the transmembrane regions S0 to S6 and hydrophobic domains S7 to S10 is approximately 13% of the total number of nsSNPs (Figure 3.18). The prediction software is not designed to take into account the structural and functional motifs of a specific protein and so may provide a predictive score that does not correlate with location within the BK channel. Thus, although an assumption would be that highly conserved regions would consistently obtain a higher score, this does not appear to be the case. Of the 21 nsSNP in these regions, SIFT scored 11 of them as 'tolerated' and PolyPhen-2 scored 5 as benign and 5 as possibly damaging. The linker with the highest incidence of nsSNP was the S7-S8 linker which was associated with 19% co-localisation (Figure 3.19).

3.2.6.2 nsSNP co-localisation with BK channel functional sites

BK channel nsSNP have been plotted over areas of functional importance to highlight co-localisation. As shown in Figure 3.18, nsSNP co-localisation with areas of functional importance was variable; 17% within the pore-forming domain, 14% within the entrance to the intracellular vestibule and 11% within the calcium bowl. There was also co-localisation at REDOX sensitive cysteine C495, hypoxia sensitive site S722 and G694, a haeme binding site. Transmembrane domain S4, which duals as the voltage sensing domain was not associated with any variation, as was the serine motif and the magnesium binding sites.

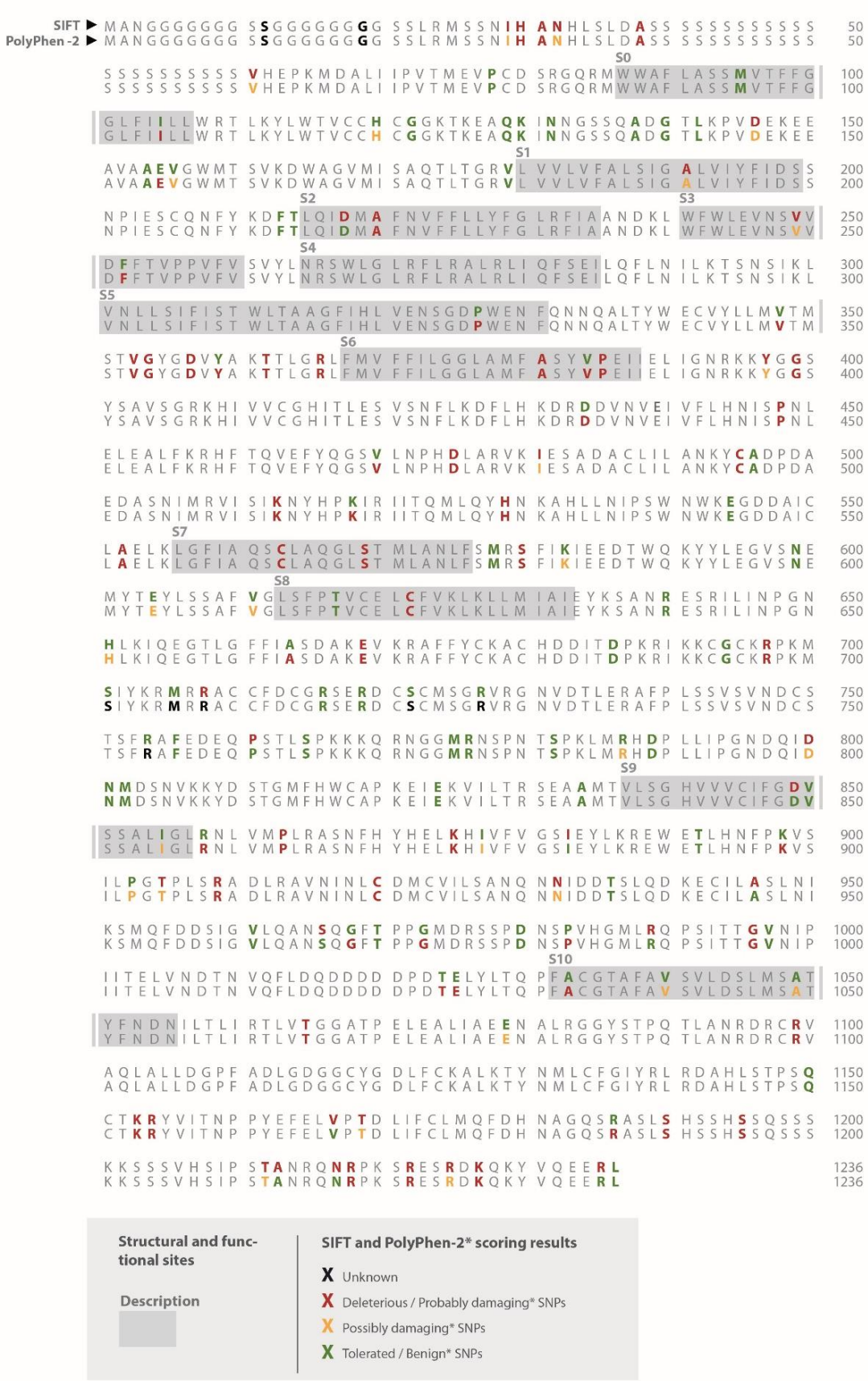


Figure 3.16- SNP location and level of predicted damage in relation to structural BK channel domains. The BK channel amino acid sequence was annotated with structural domains and SNP damage score calculated by SIFT and PolyPhen-2.

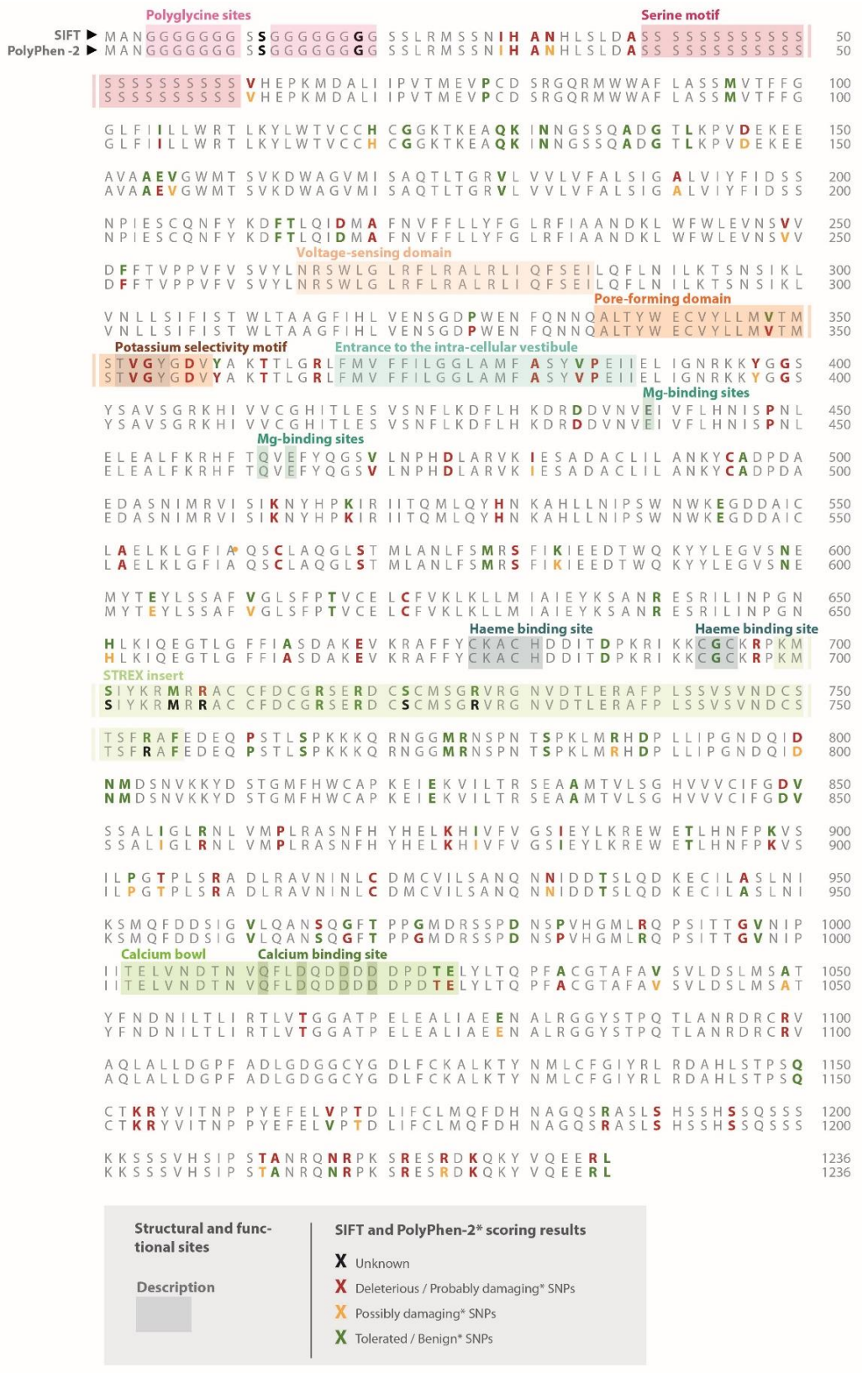


Figure 3.17- SNP location and predicted damage score in relation to BK channel functional domains. The BK channel amino acid sequence was annotated with functional domains and the nsSNP location and damage prediction categorisation allocated by SIFT and PolyPhen-2. Additional functional sites not annotated are hypoxia motif (C721-C723), REDOX sensitive cysteine (C495, C680, C1034), CO sensitive amino acids (H430, H459, D432).

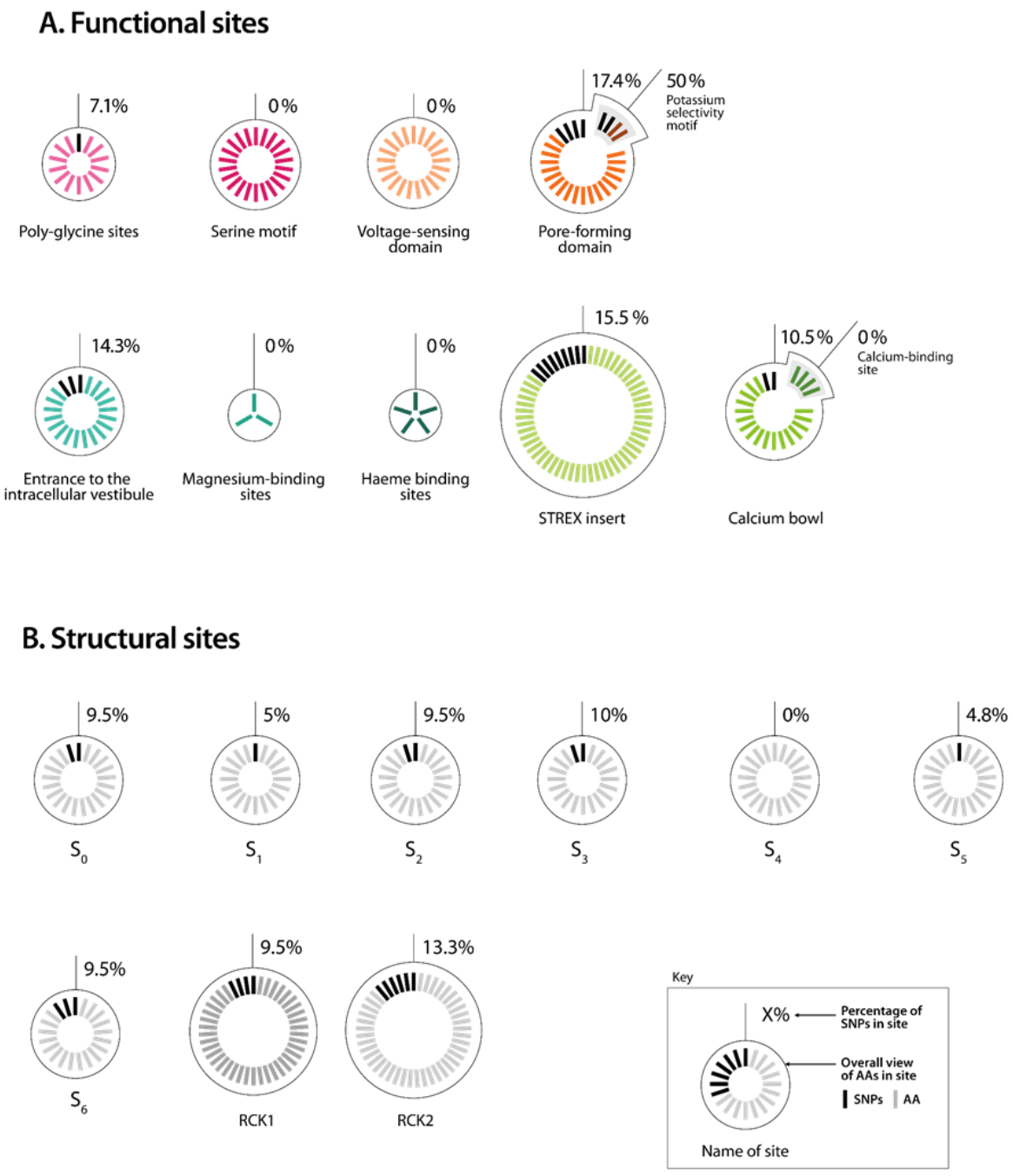


Figure 3.18- Summary of SNP co-localisation with BK channel functional and structural sites. An illustration of co-localisation of nsSNP with A) functional sites and B) structural sites of the BK channel. The black bars indicate the number of amino acids within a domain that are co-localised with nsSNP, the colour bars indicate the number of unaltered amino acids within the site. Structural domains S0-S10 are shown as light grey bars. In each case, the proportion of amino acids within each domain that are co-localised with nsSNP is expressed as a percentage. Additional functional sites not annotated: hypoxia motif (C721-C723) and REDOX sensitive cysteines (C495, C680, C1034) each have 1 nsSNP co-localisation (33%), whilst there was no co-localisation with CO sensitive amino acids (H430, H459, D432).

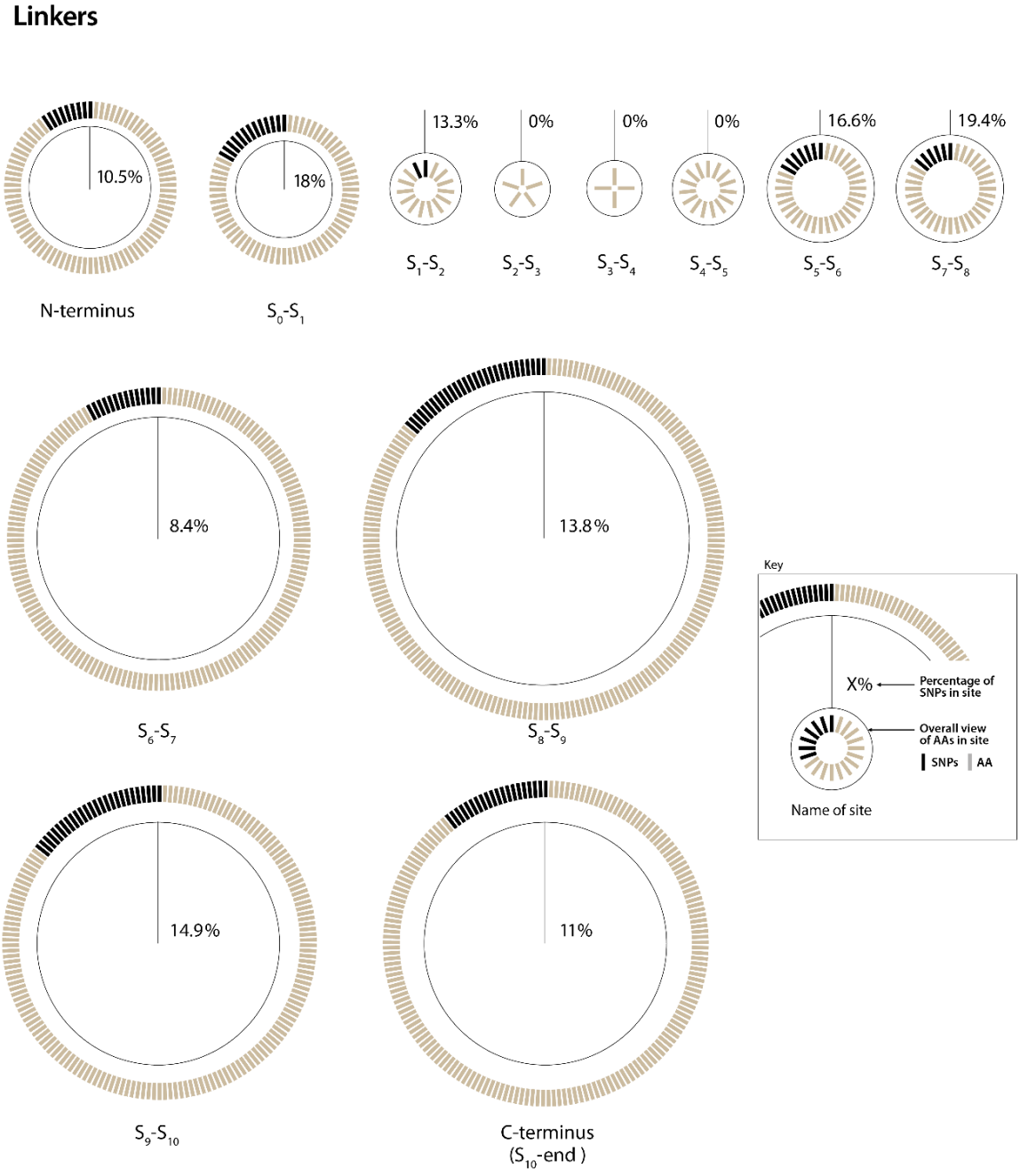


Figure 3.19- Summary of SNP co-localisation with BK channel linker regions. An illustration of co-localisation of nsSNP with BK channel linker regions. The black bars indicate the number of amino acids within a domain that are co-localised with nsSNP, the brown bars indicate the number of unaffected amino acids within the linker region. In each case, the proportion of amino acids within each domain that are co-localised with nsSNP is expressed as a percentage.

3.2.6.3 nsSNP co-localisation with BK channel post-translational modification sites

The structural and functional domains of the BK channel, the location of nsSNP and the nsSNP predicted damage score category taken from SIFT and PolyPhen-2 have been compiled into a single figure to enable the prioritisation of SNPs based upon their proximity to key regions on the BK channel. However, as post-translational modifications play a role in further amending BK channel function, such as channel activity and membrane localisation it was important to assess the extent of co-localisation of nsSNP and post-translational modifications. Thus, sites of post-translational modification of the BK channel have been combined with the other key channel domains to create a comprehensive resource (Figure 3.20). To assess the extent of co-localisation at a glance, Figure 3.21 summarises the number of nsSNP per type of post-translational modification.

There was a 1 in 18 incidence of co-localisation of nsSNP with serine phosphorylation sites that had been predicted but not verified (S722, S1188 and S1195), but a 1 in 3 incidence of nsSNP co-localisation with serine phosphorylation sites that had been predicted but also verified by experimentation (S701, S765 and S782). For threonine phosphorylation, for those sites predicted, there was a 1 in 11 incidence of co-localisation, with the site T617. However, of the two verified sites discovered by literature search, one of those, T970, was co-localised with a nsSNP, resulting in a 50% overlap. Tyrosine phosphorylation sites that were predicted co-localised with nsSNP at one site, Y359. One site that was verified but not predicted was Y885; this site was not associated with a nsSNP.

There was no co-localisation of nsSNP with sites of palmitoylation, whether predicted or verified. This may be due to the low occurrence of predicted palmitoylation across the channel, with 8 predicted sites associated with the modification in comparison with 87 for phosphorylation. The same was true for predicted sites of S-Nitrosylation and N-Glycosylation. However, there was one site of predicted SUMOylation, K583, that was co-localised with an nsSNP.

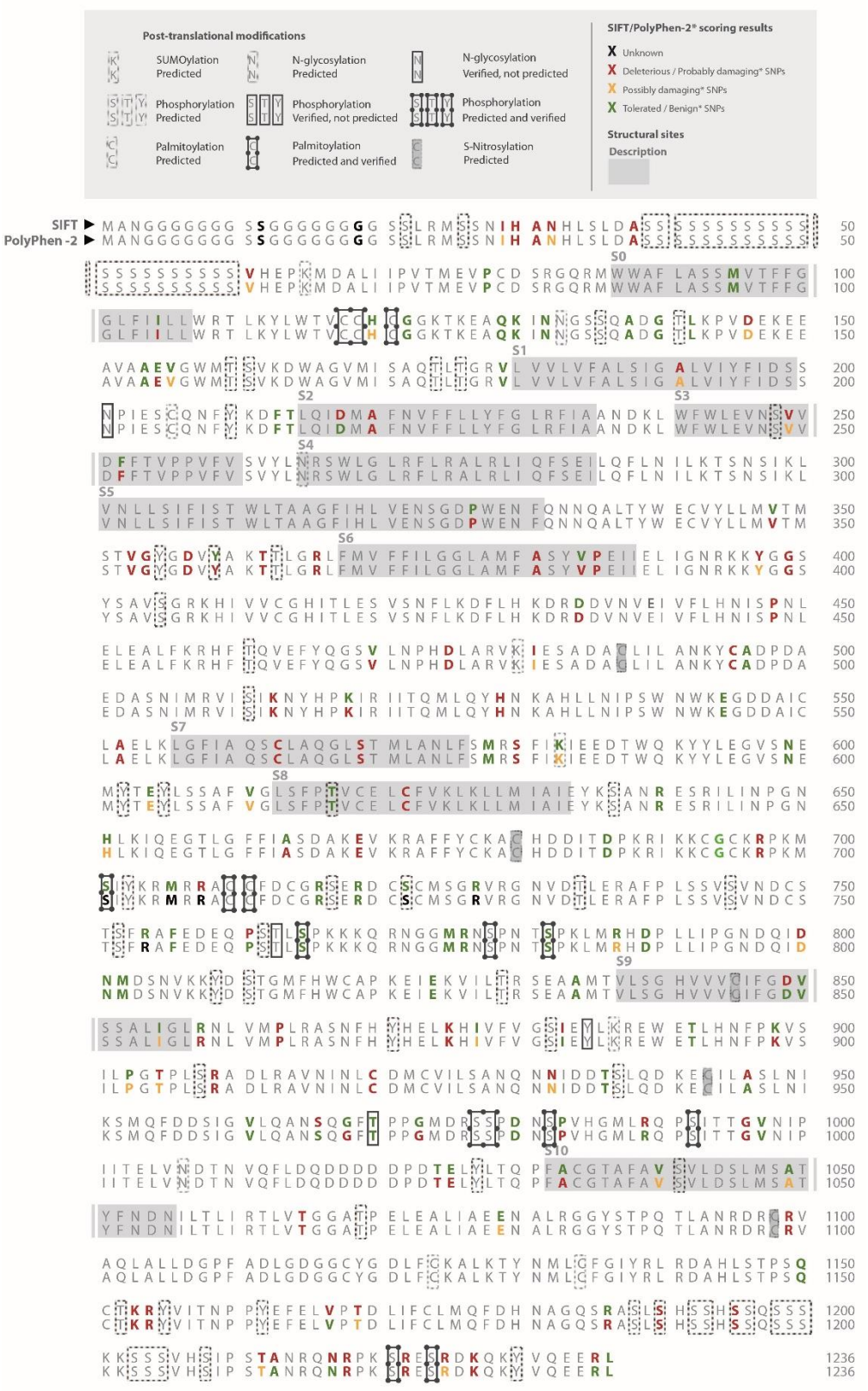


Figure 3.20- Post-translational modification sites and nsSNP on the BK channel: SIFT and PolyPhen-2 damage scoring. The BK channel amino acid sequence, annotated with post-translational modification sites (phosphorylation, palmitoylation, SUMOylation, S-nitrosylation and N-glycosylation) is overlaid with the location and predicted damage score for nsSNP generated by SIFT and PolyPhen-2.

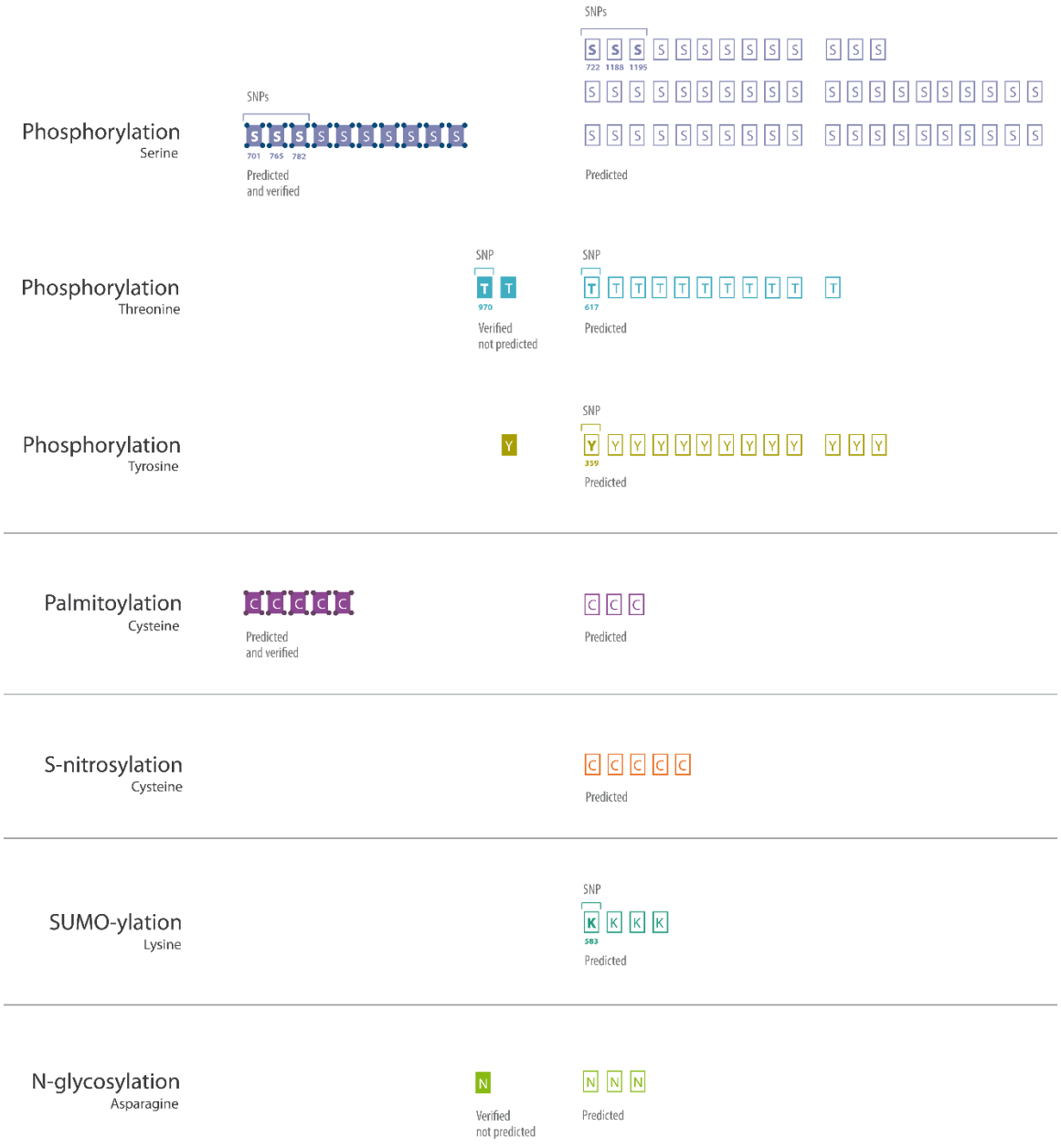


Figure 3.21- Summary of post-translational modification sites and nsSNP on the BK channel. Illustration of the number of post-translational modification sites that have been i) predicted but not verified experimentally, ii) predicted and verified and iii) verified but not predicted by the software programs. Overall, the incidence of co-localisation between nsSNP and predicted sites of post-translational modification was 1 in 15. In predicted and verified sites, this was 1 in 5, and in those verified but not predicted, 1 in 4.

3.2.7 Site-directed mutagenesis of BK channel amino acids

Research into BK channel function and regulation over the past three decades has included the application of site-directed mutagenesis as a means of investigating the role of amino acids on cellular processes, such as membrane translocation (Jeffries, 2010; Jeffries *et al.*, 2010), phosphorylation (Tian *et al.*, 2008, 2012) and channel activity (Schreiber and Salkoff, 1997). The location of these designed mutations and their relationship with sites and regions of structural and functional impact was investigated as a final step to complete the BK channel "map" (Figure 3.22). A literature search highlighted 43 sites of site directed mutagenesis on the BK channel; the amino acid locations of these sites are summarised in Table 3.4, and lists the amino acid location, the substitution and any effect noted. The list of site-directed mutagenesis is not exhaustive; the table provides an indication of the location and result of mutagenesis most relevant to the study. There is currently no bioinformatics database or software that captures all the SDM associated with the BK channel.

3.2.7.1 Co-localisation of nsSNP and AA involved in lab based mutagenesis

After assessing the co-localisation of nsSNP and sites of lab based mutagenesis, 8 sites were identified; D146, G354, A381, V384, D434, C495, S722 and S765. These sites are naturally occurring genetic variations that have also been investigated in a wet lab setting, and accounted for 5% of all nsSNP of the BK channel and 18% of identified site directed mutagenesis sites. The consequence of the lab based mutagenesis sites is already known and are detailed in Table 3.4, however it was of interest to see if any information on the physiological consequence of the naturally occurring nsSNP co-localised existed. Information was only available for nsSNP D434; as described in the chapter introduction, this mutation has been found in humans and is causative of the disease 'Generalized Epilepsy and Paroxysmal Dyskinesia' (GEPD). This is a 'gain of function' mutation that causes the channel to have an increased sensitivity to calcium, proposed to be reduced flexibility of the AC region in the RCK1 domain (Yang *et al.*, 2010).

AA Location	Species tested	AA Substitute	Effect on protein	Reference
C118	Murine	A	Decreased localisation to the plasma membrane	Jeffries et al. 2010 Tian et al. 2012
C119	Murine	A		
C121	Murine	A		
L269	Human	R	No effect on coupling between calcium and channel opening	Diaz et al. 1998
L269	Human	H		
R272	Human	E	Reduces coupling between calcium and channel opening	Diaz et al. 1998
R275	Human	N		
R278	Human	Q		
Q281	Human	R	No effect on coupling between calcium and channel opening	Diaz et al. 1998
E284	Human	K		
T352	Human	S	Activated at more negative voltages. Slower rate of inactivation	Gordon et al. 2010
354-356	Human	GYG-AAA	Loss of function	Quirk, 2001
F380	Human	A	Loss of function	Gordon et al. 2010
A381	Human	S	Activated at more negative voltages	Gordon et al. 2010
V384	Human	I	No effect on activation voltage	Gordon et al. 2010
E464	Human	A	No effect on carbon monoxide sensitivity	Hou et al 2008
E439	Human	A		
H415	Human	A		
H430	Human	R/A	Abolished carbon monoxide sensitivity	Hou et al 2008
H459	Human	R		
D432	Human	A		
D434	Human, Murine	G	Increased calcium sensitivity	Tang et al. 2010 Du et al. 2005 Wang et al. 2009
C495	Human	A	Altered response to cysteine modifying agent	Zhang and Horrigan 2005
C413	Human	A	No change in response to REDOX agents vs WT	Zhang and Horrigan 2005
C487	Human	A		
C693	Human	S		
C695	Human	S		
F675	Human	W	No effect on Haeme or carbon monoxide binding	Yi, Morgan and Ragsdale 2010
C677	Human	S	Reduction in carbon monoxide affinity to the Haeme binding domain	Yi, Morgan and Ragsdale 2010
C680	Human	S		
C680	Human	S	Decreased response to REDOX agents	Zhang and Horrigan 2005
C680	Human	S	Loss of Haeme induced channel inhibition	Tang et al. 2003
H681	Human	R		
H681	Human	A	Reduce Haeme binding	Yi, Morgan and Ragsdale 2010
C710	Murine	A	Loss of cell membrane targetting. Modulates phosphorylation	Tian et al. 2008
C711	Murine	A		
C721	Murine	A	Lack of hypoxia inhibition	McCartney et al 2005
C722	Murine	A		
C723	Murine	A		
D1017	Murine	A	Decreased calcium sensitivity	Schreiber and Salkoff 1997
C1034	Human	A	Decreased response to REDOX agents	Tang et al 2004

Table 3.4-Examples of Site Directed Mutagenesis of the BK channel. This table lists the details of site-directed mutagenesis of the BK channel, including the amino acid reference of the site in the human BK channel, the species of BK channel in which the mutation was made, the nature of the amino acid substitution and the resulting effect of the mutation on the BK channel. Also included is the reference for the mutagenesis publication. D146N and D1015N associated with reduced calcium sensitivity is not included in the table (Braun and Sy, 2001).

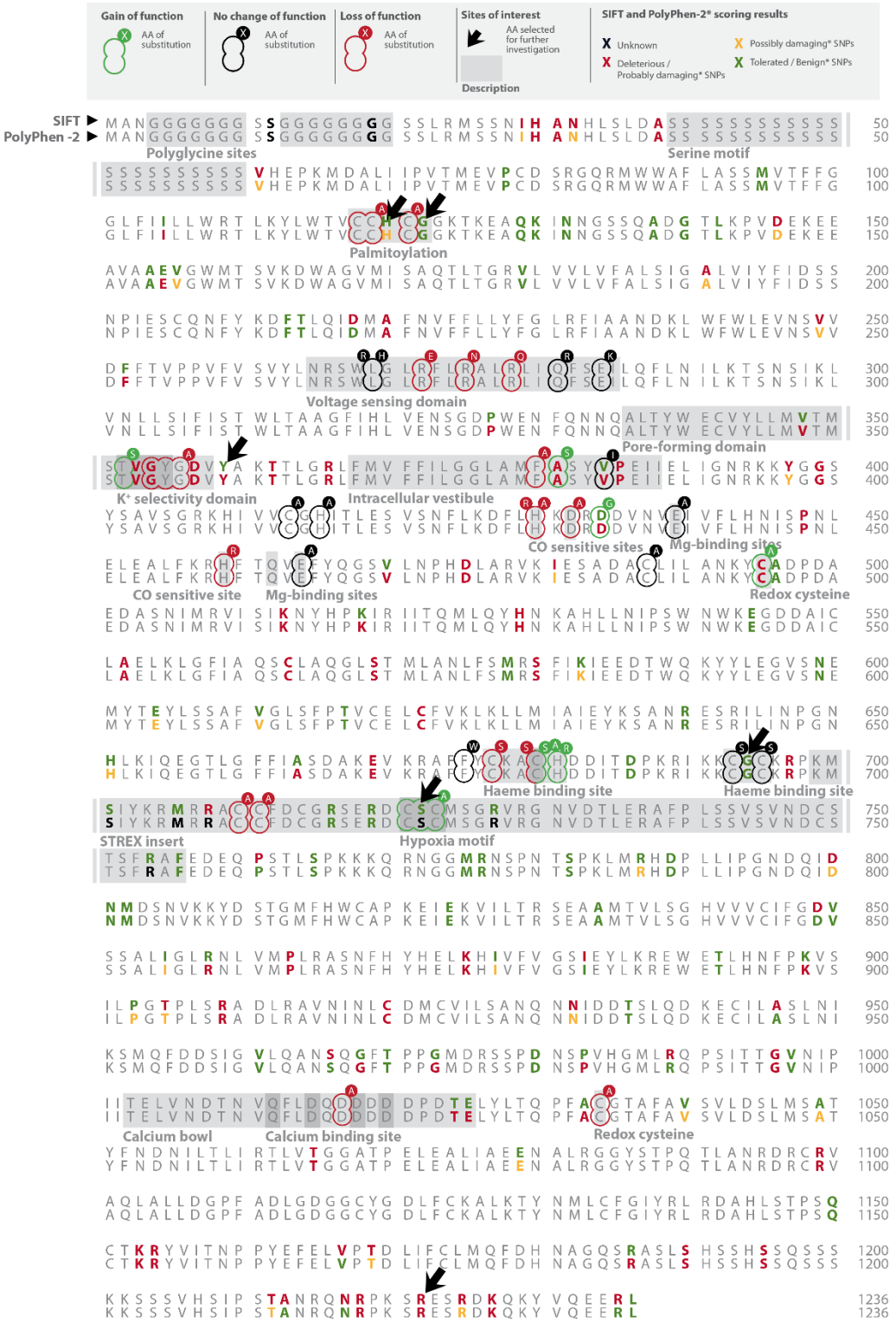


Figure 3.22- Site-directed mutagenesis sites and nsSNP on the BK channel: SIFT and PolyPhen-2 damage scoring. Shown are nsSNP sites with SIFT and PolyPhen-2 scoring category, alongside the location and result of amino acid mutagenesis. Black arrows indicate the amino acid residues selected for further investion in the 'deep dive' section.

3.2.8 SNP Deep Dive

One of the key aims of this work was to prioritise single nucleotide polymorphisms for further research. To achieve this, information on the structural, functional, post-translational modification and site-directed mutagenesis of the BK channel has been collated and mapped onto the BK channel canonical amino acid sequence. In addition, the location and nature of single nucleotide polymorphisms of BK channel amino acids has been collated and mapped onto the channel sequence and schematic to create a novel BK channel 'reference material'.

This reference material has enabled the extent of co-localisation between single nucleotide polymorphisms and the key domains of the channel to be viewed at 'at glance', and demonstrates that this co-localisation occurs in areas of structural, functional, post-translational modification and site-directed mutagenesis. However, in order to prioritise a single nucleotide polymorphism for lab-based research, more detailed information, such as the nsSNP effect on the local PTM environment, was required. Therefore, a search was undertaken for the single nucleotide polymorphisms that are most likely to have an effect on BK channel function. This was determined by a) co-localisation with, or being less than three amino acids away from a crucial functional site, such as the potassium selectivity domain, b) proximity to crucial sites proven by site-directed mutagenesis to have a profound effect on channel function, c) a SNP with a dramatic effect on the local post-translational modification environment, for example a SNP that results in the introduction or removal of a cysteine rich domain or d) proximity to another SNP shown to have physiological effects, such as D434G.

The search identified 8 single nucleotide polymorphisms as potential candidates for this more detailed analysis, based upon the aforementioned criteria and diverse potential effect on channel function. The following nsSNP were selected; H120Q and G122A located in the S0-S1 linker region and adjacent to the cysteine residues mediating channel cell membrane localisation, Y359N located in the S5-S6 intramembrane linker and three amino acids downstream of the potassium selectivity domain, and G694C and G694V located in the region between the RCK1 and RCK2 domains associated with haeme binding and REDOX sensitivity. Additionally, S722L in the hypoxia sensitivity domain, and R1222G and R1222M which are possible disruptors of a serine phosphorylation rich domain in the C terminus of the channel were selected.

3.2.8.1 H120Q and G122A and S0-S1 linker palmitoylation

H120Q and G122A mutations are located in the S0-S1 linker region, flanking cysteine residues C118, C119 and C121 that are sites for palmitoylation and have been shown to modulate cell-membrane association of the channel (Jeffries, 2010; Jeffries *et al.*, 2010; Bi, 2014; Kim *et al.*, 2014). Mutagenesis of C118, C119 and C121 to alanine residues reduced the cell membrane localisation of a truncated channel lacking the C-terminus by over 80%, and the full-length channel by approximately 50%. In addition, C121 has been shown to be the cysteine residue with the greatest single effect on channel cell membrane localisation, reducing membrane expression to 25% of wild-type. As a result, it was of interest to investigate the nsSNPs (H120Q and G122A) that are located within this key region.

H120Q (rs147098205) is the substitution of the positively charged histidine (H) amino acid for the neutrally charged glutamine (Q) amino acid. This mutation was detected in an unknown subject of European American decent, and uploaded to the Ensembl database by the NHLBI-ESP project. The mutation has a genotype frequency of 1 in 4299, indicating that based upon the population assessed, 1 in 4299 people will have this mutation. As shown in Figure 3.23, this mutation causes a small increase in the predicted palmitoylation score for cysteines C118 and C119, (score from 16.75 to 16.84 and 5.21 to 5.56 respectively) but is predicted to reduce predicted C121 palmitoylation below threshold. SIFT and PolyPhen-2 mutation damage prediction software allocated the mutation to different damage categories; SIFT as tolerated (0.81) and PolyPhen-2 as 'possibly damaging' (0.64). The H120Q mutation did not result in a change in the frequency, or change in predicted score for the other post-translational modifications assessed in this thesis, such as phosphorylation, N-glycosylation, S-nitrosylation or SUMOylation.

The G122A mutation is a substitution of the hydrophilic, neutrally charged glycine (G) amino acid for the hydrophobic alanine (A) amino acid. This mutation was detected in a male subject of Utah-European origin, and was submitted to Ensembl via the 1000 genomes project database. The mutation has a genotype count of 1:84 in Utah-Europeans and 1:378 in Europeans.

This variation is predicted to have a differing effect on palmitoylation than H120Q; the score for C118 was reduced from 16.75 to 15.72, whilst C119 and C121 scores are increased from 5.21 and 4.38 in the wild type, to 5.22 and 6.14 respectively in the mutant. There was a lack of agreement between SNP damage scoring software as to the damaging potential of the mutation, with SIFT scoring it as tolerated (0.92) and PolyPhen-2 as 'possibly damaging' (0.38). As with H120Q, the G122A mutation did not result in a change to the frequency or predicted score of other post-translational modifications on the channel. When comparing the H120Q and G122A mutations, the main difference is the predicted effect on C121 palmitoylation.

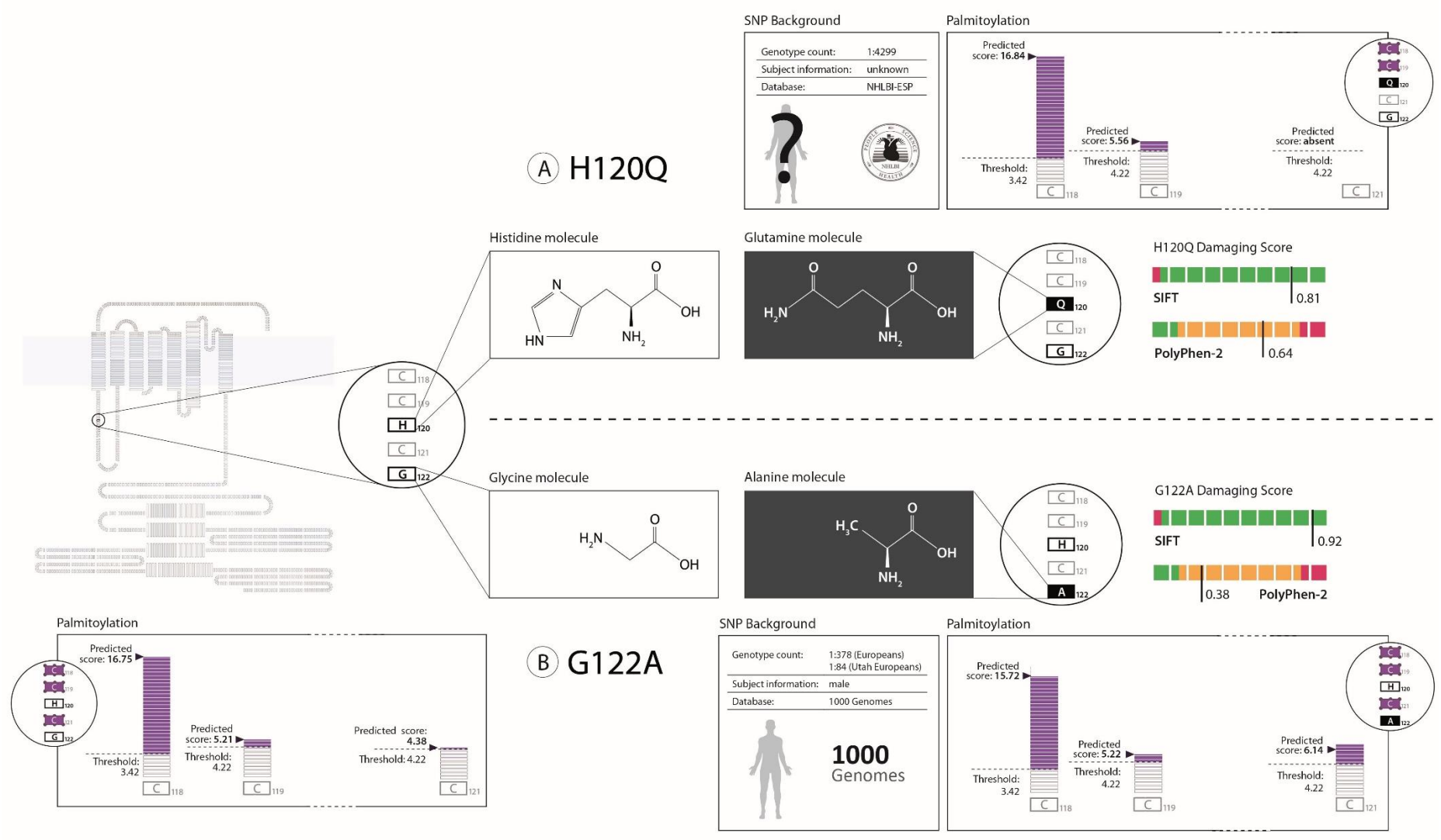


Figure 3.23- Investigation into nsSNPs flanking C121 residue (H120Q and G122A)

Figure 3.23- Investigation into nsSNPs flanking C121 residue (H120Q and G122A). This figure presents information held on bioinformatics databases for the **A)** H120Q and **B)** G122A mutants. Included is a schematic indicating the location of the mutations in the BK channel, a summary of the flanking amino acid sequence and an illustration of chemical structure of histidine (H), glycine (G), glutamine (Q) and alanine (A) amino acid residues. Also included are the background details of the mutation, such as the program and individual in which it was detected, and the genotype count of the mutation. The CSS-Palm 4.0 predicted palmitoylation score for the wild-type channel and the two mutants are plotted, as is the damaging score allocated to each of the mutants by SIFT and PolyPhen-2. The H120Q mutation results in a reduction in the predicted palmitoylation score for of C121 to below threshold, while in contrast the G122A mutation increases the predicted palmitoylation score.

3.2.8.2 Y359N in the BK channel pore domain

The Y359N mutation involves the substitution of the neutral, polar tyrosine (Y) amino acid residue with the neutral and polar asparagine (N) amino acid residue. The mutation was detected via exome screening of a tissue biopsy taken from carcinoma of the larynx of a 36-year-old smoker and drinker of unknown gender. Details of the mutation were submitted to the Ensembl database via the COSMIC database, which collates genetic variation data from those suffering from cancer. The genotype count for this mutation in the population studied was 1 in 74.

The mutation is located in the pore domain of the BK channel, and is three amino acids downstream of the GYG motif of the potassium selectivity domain spanning from G354 to G356. In addition to the mutation occurring near a crucial functional motif, the tyrosine amino acid involved is also a predicted site of phosphorylation. The mutation resulted in the loss of the predicted tyrosine phosphorylation site, and also had an effect on the phosphorylation status of nearby residues (Figure 3.24). The predicted phosphorylation score for tyrosine amino acid residue upstream of the mutation (Y355) increased from 0.938 to 0.955, whilst the score for the threonine amino acid residue downstream of the mutation (T363) reduced from 0.656 to 0.529.

Although this mutation adds a potential additional site for N-glycosylation as a result of asparagine amino acid addition, when the modified FASTA sequence containing the mutation was submitted to NetNGlyc 1.0 there was no difference in the number of predicted N-glycosylation sites, and there was no change to the scores of those N-glycosylation sites already predicted in comparison to wild type. There was a lack of consensus between SNP damage prediction software, as SIFT categorises the mutation as being tolerated and PolyPhen-2 as probably damaging (scores of 0.1 and 0.99 respectively).

Due to the proximity of the mutation to the potassium selectivity motif, an area where site-directed mutagenesis to alanine caused loss of channel function, and the alteration in predicted phosphorylation of amino acids in the region, it would be of interest to assess the effect of this mutation on channel function assessed by electrophysiology.

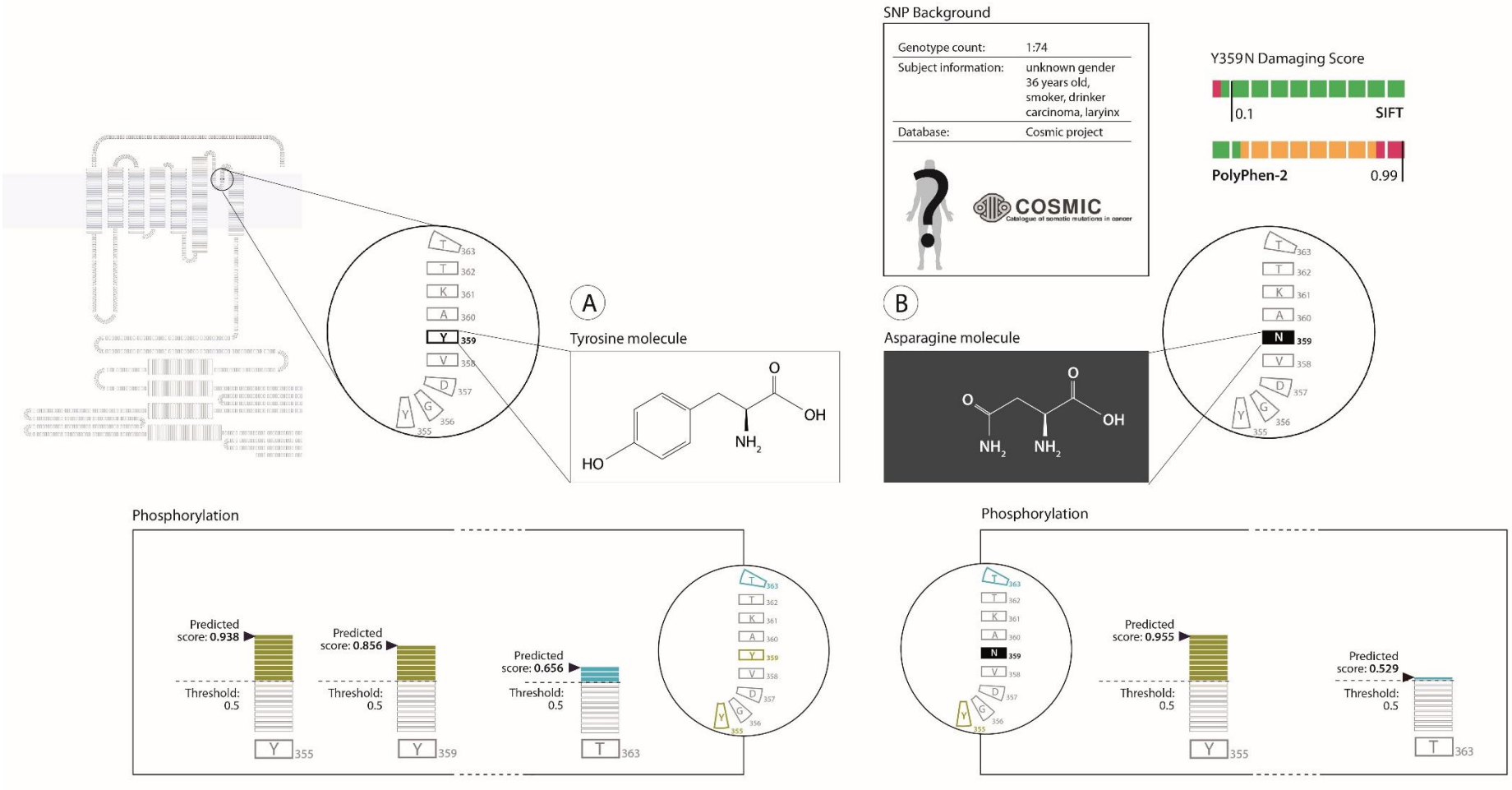


Figure 3.24- Investigation into nsSNP Y359N in the BK channel pore domain

Figure 3.24- Investigation into nsSNP Y359N in the BK channel pore domain. Summary of bioinformatics data for **A)** wild type Y359 and the Y359N mutant **B)**. A schematic indicating the location of the mutation in the BK channel, the flanking amino acid sequence and chemical structure of tyrosine (Y) and asparagine (N) amino acid residues. Details of the mutation with the program and individual in which the mutation was detected and the genotype count of the mutation. The NetPhos 2.0 predicted phosphorylation scores for the wild-type channel and the Y359N mutant are plotted and the damaging score allocated to each of the mutants by SIFT and PolyPhen-2. The mutation results in the removal of a tyrosine amino acid and therefore the loss of a predicted phosphorylation site. The inclusion of an arginine amino acid does not create a predicted N glycosylation site.

3.2.8.3 G694C and G694V in the RCK1-RCK2 linker

G694 is located in the region between the RCK1 and RCK2 domains in the C-terminus of the BK channel, and specifically in a region that has been identified as important for haeme binding and REDOX agent sensitivity (Tang *et al.*, 2003; Zhang and Horrigan, 2005; Yi, Morgan and Ragsdale, 2010). There are two mutations occurring on the same glycine amino acid residue (G694). The first mutation, G694C, substitutes the hydrophilic, neutrally charged glycine (G) amino acid for a cysteine (C) amino acid with similar characteristics, and was detected as a result of an exome screen of a biopsy taken from carcinoma of the lung of a 61-year-old female subject. The mutation was submitted to Ensembl via the COSMIC project database; unfortunately, there is no information on the genotype frequency of the mutant. The G694C variation introduced an additional cysteine residue into a region with an already high density of cysteine residues (9 in 50 amino acids residues); this had a significant impact on the palmitoylation status of the region. Whilst C693 and C695 are not predicted to be palmitoylated in the wild type channel, in the G694C mutant channel all three cysteines are predicted to be palmitoylated with scores of 15.73, 12.20 and 6.00 for C693, C694 and C695 respectively (Figure 3.25). This mutation however reduces the palmitoylation score of the STREX cysteine residue C710 from 19.62 to 19.10 and increases the palmitoylation score for C711 from 6.39 to 6.58. The mutation did not result in a change to the number or score of other post-translational modifications of the channel. As has been observed in the other 'deep dive' nsSNP, there was no agreement between the nsSNP damage prediction software regarding the damaging potential of the mutation; SIFT categorised the mutation as deleterious with a score of 0.02, whilst PolyPhen-2 categorised it as 'possibly damaging' with a score of 0.73.

The G694V mutation introduces a non-polar and hydrophobic valine (V) amino acid to the region, and was detected in a subject of unknown gender and of African origin. This was submitted to the Ensembl database by the 1000 genomes project, and the genotype frequency of this mutant is 1:660. The mutation had no effect on the predicted palmitoylation status of C693 and C695 which remained undetectable, however the mutation reduced the C710 score from 19.62 to 18.94, and increased C711 from 6.39 to 6.60. The mutation did not result in a change to the number or score of other post-translational modifications of the channel. SIFT and PolyPhen-2 were not in agreement that this nsSNP was not damaging, with categories of tolerated and possibly damaging allocated (scores of 0.09 and 0.23 respectively). Although the damage prediction software SIFT and PolyPhen-2 do not unanimously predict mutation at G694 to be harmful to the protein, there is evidence to support lab based investigation of the mutant as it is located in a region of the channel associated with haeme binding and sensitivity to REDOX agents, (site-directed mutagenesis has been shown to perturb these processes), and the G694C mutant results in the creation of a cysteine rich domain.

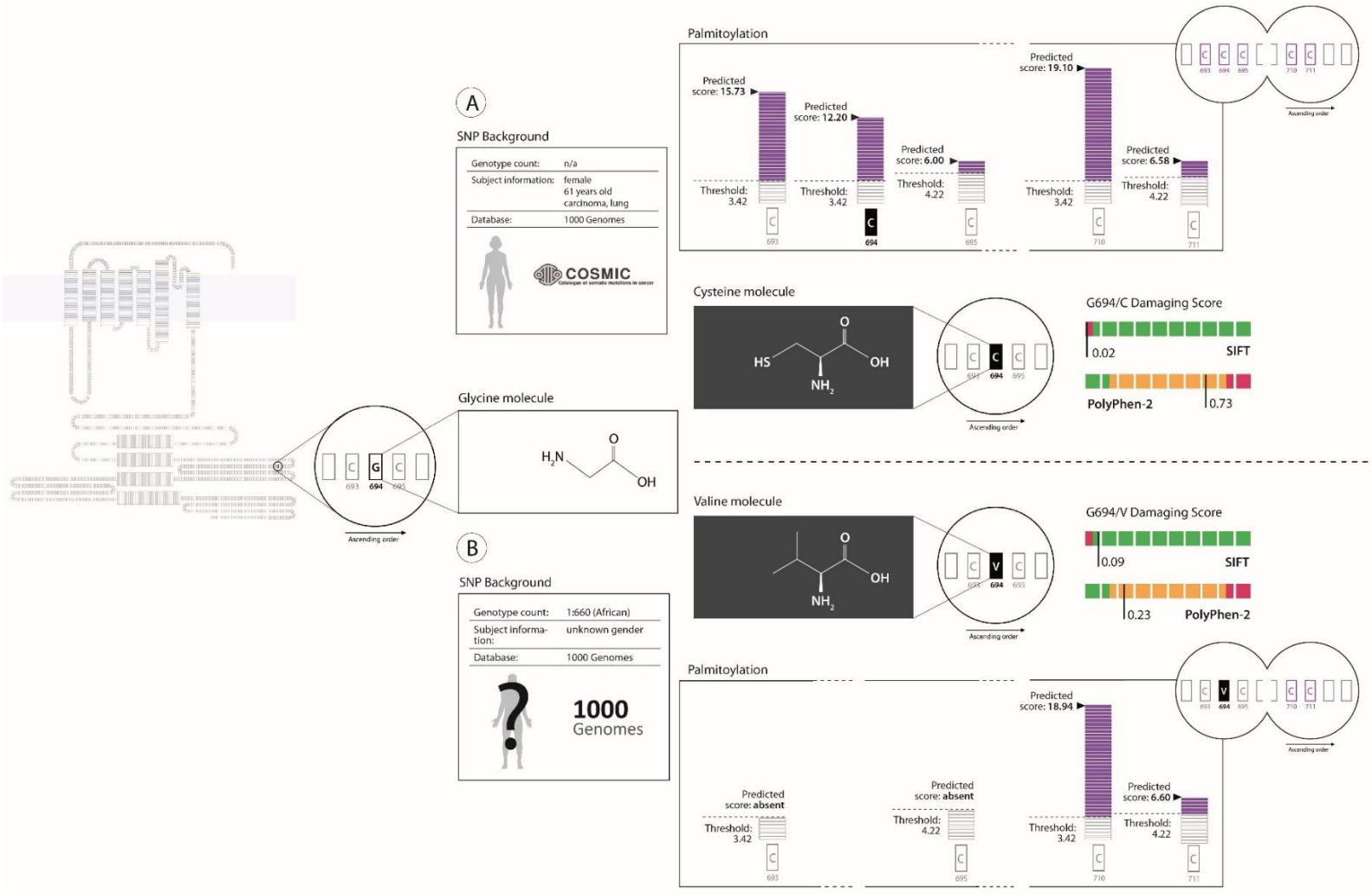


Figure 3.25-- Investigation into nsSNPs G694C and G694V in the RCK1-RCK2 linker

Figure 3.25- Investigation into nsSNPs G694C and G694V in the RCK1-RCK2 linker. Summary of bioinformatics data for the **A)** G694C and **B)** G694V nsSNPs. A schematic indicating the location of the mutations in the BK channel, the flanking amino acid sequence and the chemical structure of glycine (G), cysteine (C) and valine (V) amino acid residues. Also included are details of the program and individual/s that the mutation was discovered in. The CSS-Palm 4.0 predicted palmitoylation score for the two mutants are plotted, as well as the damaging score allocated to each of the mutants by SIFT and PolyPhen-2. The G694C mutation results in the creation of a cysteine rich domain, the G694V mutation has a limited effect on predicted palmitoylation in the region.

3.2.8.4 S722L in the STREX splice insert

Serine residue S722 is located between RCK1 and RCK2 in the STREX splice insert, and is situated adjacent to the cysteine residues crucial to the function of the hypoxia sensitivity motif; this serine residue is also predicted to be phosphorylated by NetPhos 2.0. The S722L mutation (rs373620901) involves the substitution of the polar, uncharged, hydrophilic serine amino acid residue (S) for the non-polar, hydrophobic amino acid residue leucine (L). The mutation was detected in an individual of unknown description and was submitted to Ensembl by the ESP database via the ExAc project, which holds large-scale sequencing data from 60,706 unrelated individuals (Figure 3.26).

The S722L mutation results in the removal of a predicted serine phosphorylation site. Though the mutation did not result in any significant change the phosphorylation score of other amino acids in the region, nor the palmitoylation score for the cysteine residues in STREX. The mutation was classified as tolerated by damage prediction software SIFT, with a score of 0.3, whilst PolyPhen-2 could not assign a score to the mutant.

Although the bioinformatics investigation did not detect a significant impact of the mutation on the local environment, due to the location of the mutation within the hypoxia motif and the fact that the mutation results in the removal of a predicted phosphorylation site, there is evidence to support the progression of this mutation forward to lab based investigation, particularly investigating the effect of the mutation on hypoxia sensitivity, and the effect of protein kinases on channel activity.

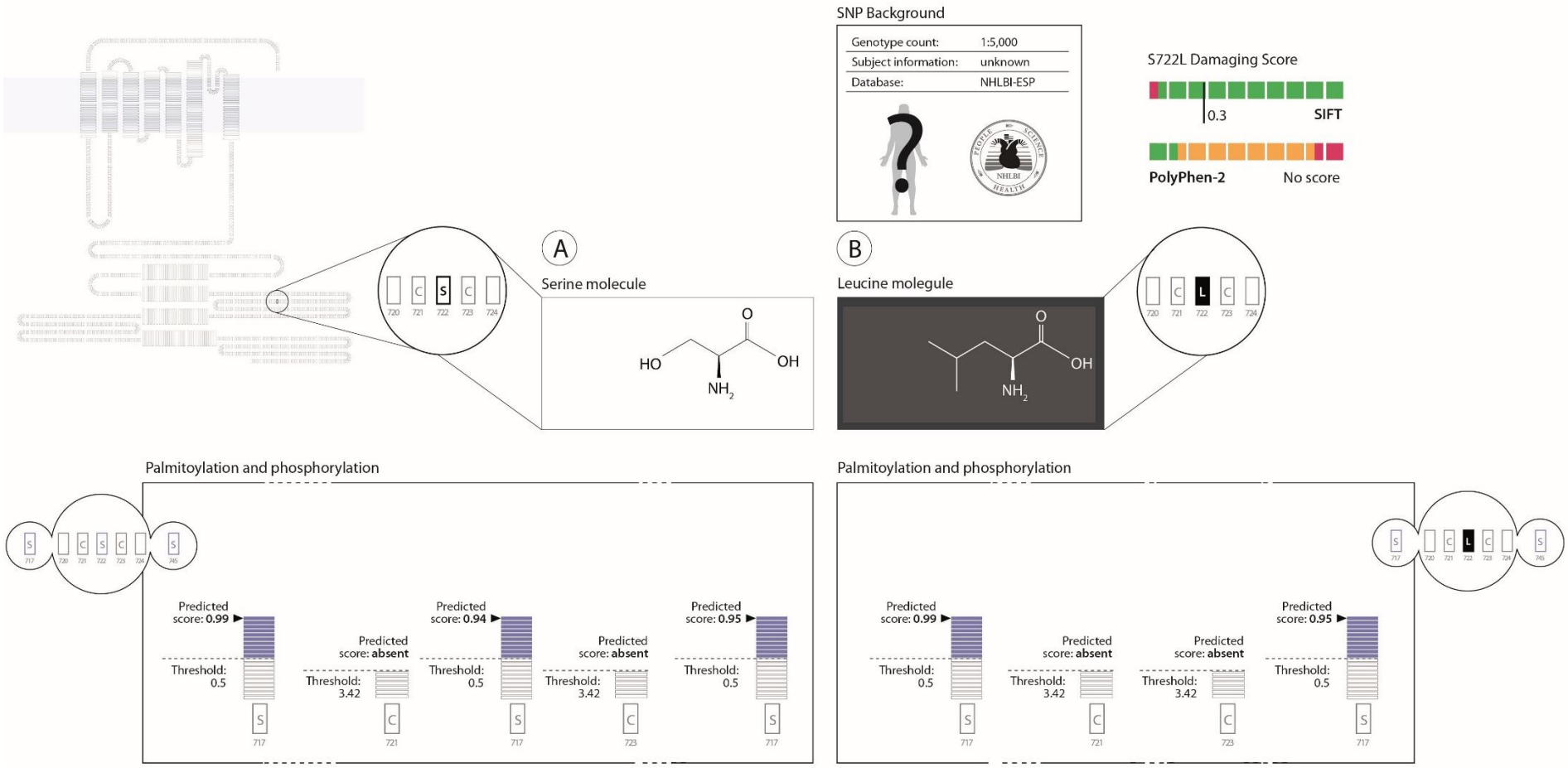


Figure 3.26- Investigation into nsSNP S722L in the STREX splice insert

Figure 3.26- Investigation into nsSNP S722L in the STREX splice insert. Summary of bioinformatics data for the wild type **A)** and S722L mutant **B)**. A schematic indicating the location of the mutation in the BK channel, the flanking amino acid sequence and chemical structure of serine (S) and leucine (L) amino acid residues. Details of the mutation with the program and individual in which the mutation was detected and the genotype count of the mutation. The NetPhos 2.0 predicted phosphorylation scores and CSS-Palm 4.0 predicted palmitoylation scores for the wild-type channel and the S722L mutant are plotted alongside the damaging score allocated to the mutant by SIFT and PolyPhen-2. The mutation results in the removal of a serine amino acid and therefore the loss of a predicted phosphorylation site. There is no change to the palmitoylation score of cysteine residues C721 and C723.

3.2.8.5 R1222G and R1222M in the C-terminus of the BK channel

R1222 is a charged arginine amino acid residue located in the extreme C-terminus of the channel, downstream from the S10 hydrophobic domain. This region is one of dense phosphorylation with 19 predicted sites in the 100 amino acids making up the intracellular C-terminal tail. There are two mutations identified to occur at the same amino acid location: R1222G and R1222M (Figure 3.27).

The R1222G mutation involves the substitution of arginine for a glycine residue; this moves the local environment from being positively charged and polar to being hydrophobic and neutral. This mutation was detected in an unknown subject and was uploaded to Ensembl via the dbSNP database. Whilst the mutation appears to have a minimal effect on neighbouring phosphorylation, causing only a small reduction in predicted phosphorylation of S1224 from 0.997 to 0.982, both SIFT and PolyPhen-2 databases are in agreement that the mutation is highly damaging, scoring the nsSNP as deleterious and probably damaging (0.03 and 0.99 respectively). There appears to be no effect on other post-translational modifications in the area.

A similar pattern is presented for the R1222M mutation, where the arginine amino acid residue is substituted with the non-polar and hydrophobic methionine (M) amino acid. The mutation was detected as a result of an exome screen of the biopsy from a 65-year-old male suffering from carcinoma of the prostate. The mutation was submitted to the Ensembl database by the COSMIC project. Damage prediction software also categorised this mutation as being harmful with SIFT and PolyPhen-2 calculating scores of 0.01 (deleterious) and 0.99 (probably damaging).

In this case, there is a reduction in the predicted phosphorylation score for S1224, from 0.997 to 0.96, however no other difference in post-translational modification of the channel was observed. Methionine residues are sensitive to redox agents, and it is possible that the R1222M mutation introduces redox sensitivity to the region, thus a lab based investigation into REDOX agent sensitivity in this region would of interest.

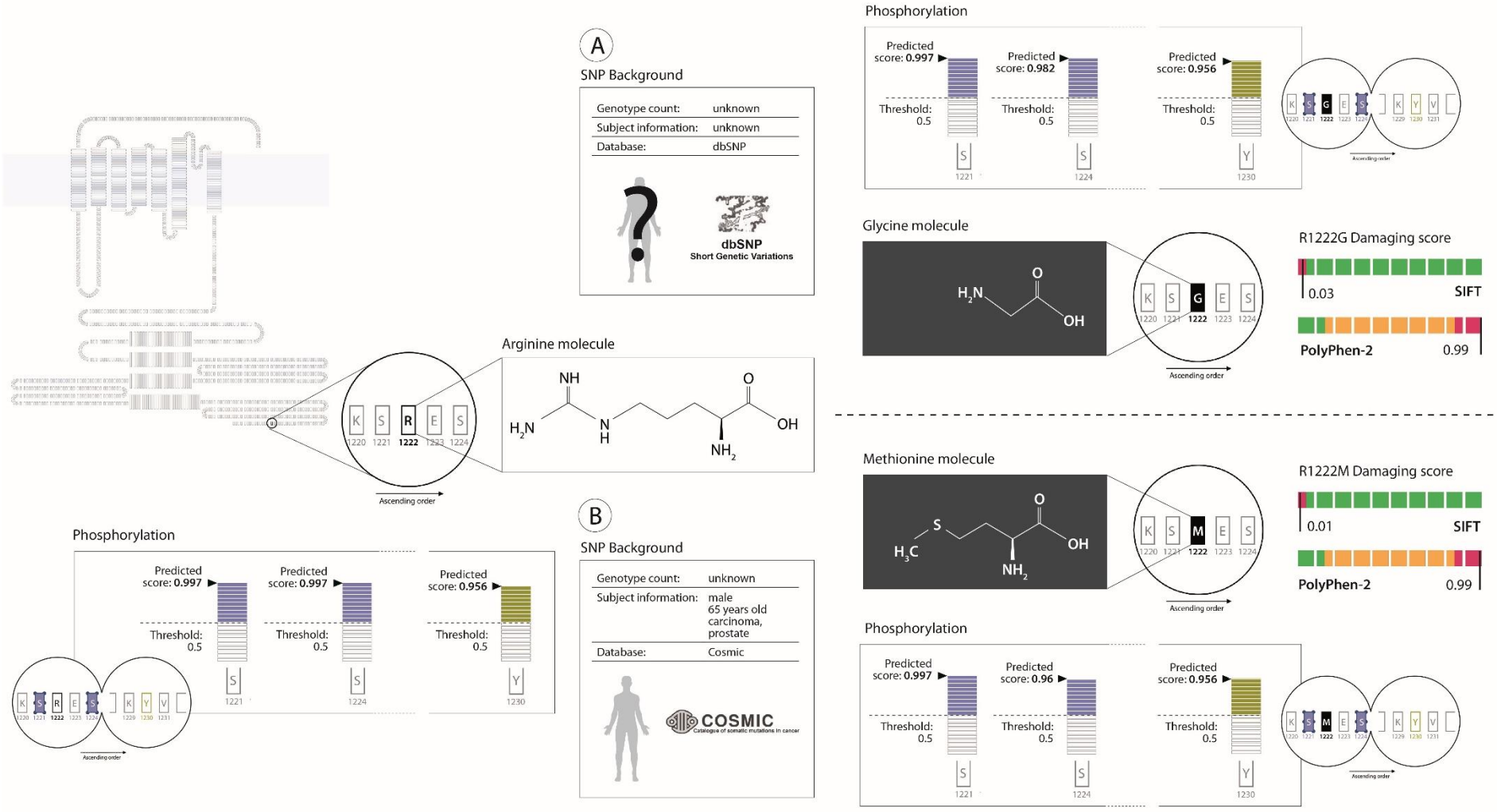


Figure 3.27- Investigation into nsSNP: R1222G and R1222M

Figure 3.27- Investigation into nsSNP: R1222G and R1222M. Summary of bioinformatics data for the **A)** R1222G and **B)** R1222M nsSNPs. A schematic indicating the location of the mutations in the BK channel, the flanking amino acid sequence and the chemical structure of arginine (R), glycine (G) and methionine (M) amino acid residues. Also included are details of the program and individual/s that the mutation was discovered in. The NetPhos 2.0 predicted phosphorylation score for the wild-type channel and the two mutants are plotted, as well as the damaging score allocated to each of the mutants by SIFT and PolyPhen-2. Damage scoring software SIFT and PolyPhen-2 predicted this mutation to be damaging.

3.3 Discussion

The aim of the work in bioinformatics chapter was firstly to capture key aspects of data relating to the BK channel contained within databases and to compile this information into a BK channel 'map', a resource in which the key structural and functional information, plus post-translational modification and site-directed mutagenesis sites on the BK channel could be assessed 'at a glance'. Subsequently, the aim was to use this resource to prioritise potentially damaging single-nucleotide polymorphisms for lab-based experimentation by investigating the location of SNPs in relation to the key features of the channel.

3.3.1 Creation of an 'at a glance' resource for the BK channel

The primary aim was achieved, as it was possible to create an 'at a glance' resource for the BK channel containing key domains of importance, which is the first of its kind. This resource contains information analysed and synthesised from a variety of databases and incorporates information from a range of software tools. The 'map' is based on the amino acid sequence of the BK channel canonical transcript and incorporates: the location of channel structural domains, the 167 unique nsSNP on the channel; and the location of 83 predicted sites of phosphorylation. The bioinformatics approach has also lead to the production of a list of the location and impact of site-directed mutagenesis on the BK channel, data that exists partially in databases and in publications, but has yet to be collated into a single resource.

The mining of the data has led to the creation of new insights into the BK channel such as, a) the relative proportions of predicted and experimentally verified post-translational modification sites, b) the extent of agreement between the damage score categories calculated by SIFT and PolyPhen-2 for BK channel nsSNP, and c) and the extent of co-localisation between nsSNP and structural, functional, post-translational modification and site-directed mutagenesis sites. These figures were constructed in collaboration with a graphic designer to ensure that the results were presented in a simple, aesthetic and informative way, with the aim that the key features and themes were easier to identify and appraise. Evidence of the aesthetic method of data presentation is displayed throughout the chapter and particularly in the figures displaying co-localisation of nsSNP with structural and functional sites, and the figures illustrating the results of a detailed nsSNP bioinformatics investigation. Previous studies have successfully assessed the occurrence and damaging potential of nsSNP in a gene using an algorithmic approach (Teng *et al.*, 2012; Ghaedi *et al.*, 2015; Liu *et al.*, 2015; Alipoor *et al.*, 2016), however there is no evidence of studies using a graphical and schematic approach such as used in this thesis to present results. Uniprot and Ensembl databases do provide basic gene and protein

schematics, as shown in the (general methods chapter) which do provide a good basis for understanding the positions of key sites on the gene/protein. Assessment of the effectiveness of a graphical data presentation method in comparison to an algorithmic method was not within the scope of this thesis; however, recent publications have discussed the importance of drawing compelling figures to aid scientific communication and ensure that data 'doesn't go unnoticed'; the use of a specialist graphic designer is also advocated (Rolandi, Cheng and Pérez-Kriz, 2011; Riccomini, 2013; Cheng and Rolandi, 2015; Rodríguez Estrada and Davis, 2015).

3.3.2 Prioritised BK channel genetic variations may affect channel function via altered post-translational modification.

This study demonstrated that it is possible to prioritise single nucleotide polymorphisms using the BK channel resource created in this research project, and this achieves the second main aim of the study. Cross-referencing of information within the resource highlighted the existence of nsSNP with the potential to affect BK channel function; 8 single nucleotide polymorphisms where prioritised for further bioinformatics investigation (H120Q, G122A, Y359N, G694C, G694V, S722L, R1222G and R1222M). Three of the mutations resulted in significant changes to the predicted palmitoylation status of neighbouring cysteine residues.

Firstly, predicted palmitoylation of the C121 residue in the S0-S1 loop, a residue proven to be important to the membrane translocation of the channel (Jeffries, 2010; Jeffries *et al.*, 2010), was abolished upon the introduction of the nsSNP H120Q, identified by the ESP project in the exome of an unidentified individual. On the other hand, nsSNP G122A, identified in the exome screen of a Utah European subject by the 1000 genomes project, resulted in an increase in the predicted palmitoylation score of C121. Introduction of the G694C mutant to the amino acid sequence coding for the haeme binding domain and REDOX sensitivity motif introduced a new cysteine rich domain. As palmitoylation modulates BK channel function (Tian *et al.*, 2008; Jeffries, 2010; Zhou *et al.*, 2012), the abolition or introduction of palmitoylation sites has the potential to affect the proper functioning of the channel. Due to the locations of the mutations, this may manifest as altered membrane translocation (H120Q and G122A) and changes to haeme binding affinity and sensitivity to REDOX agents (G694C).

Altered palmitoylation status of cysteine residues, as a result of endogenous genetic mutation, has previously been shown to affect ion channel function. Mutation C981F (rs199473591), was identified in cardiac voltage-gated sodium channels ($Na_v1.5$) as a result of an exome screening project in those suffering from Long QT Syndrome (LQTS) (Kapplinger *et al.*, 2009). Cysteine residue C981 was predicted to be palmitoylated by CSS-Palm 3.0, and site directed mutagenesis of this site from cysteine to

phenylalanine resulted in slowed inactivation of the channel, paradoxically accompanied by enhancement of closed-state inactivation leading to multiple openings of the channel during a single action potential. These factors combined to result in the increased duration of high intracellular sodium concentration, leading to extended depolarisation and LQTS. This demonstrates another potential link between mutations causing palmitoylation status alterations and disease. An Ensembl review of SIFT and PolyPhen-2 revealed that the databases were not in agreement; damaging category of the C981F mutation was of 0.36 (tolerated) and 0.99 (probably damaging) respectively.

Cysteine binding at the highly reactive sulfhydryl groups creates a dimer containing a disulphide bridge between two cysteines; as a result the effect of cysteine residues on protein structure and stability has been the focus of investigations (Tatara, Yoshida and Ichishima, 2005; Linder and Deschenes, 2007; Qiu *et al.*, 2015; Tao Liu *et al.*, 2016). This once again provides support for the prioritisation of nsSNPs that change the local cysteine environment.

Another nsSNP with an effect on the surrounding post-translational environment is Y359N, which was identified via exome screening of a 36-year-old by the COSMIC project, and resulted in the removal of a tyrosine residue predicted to be phosphorylated by NetPhos 2.0. This mutation is of importance due to its proximity to the pore forming domain containing the potassium selectivity domain, which sits immediately upstream of the mutation. Whilst this mutation introduced a potential N-glycosylation site, this was not predicted by the NetNGlyc software. Site directed mutagenesis of amino acids within and in the proximity of the pore-forming region and selectivity filter were associated with gain and loss of function changes. Examples such as the GYG354-356AAA mutation which resulted in loss of function of the channel (Quirk and Reinhart, 2001) and T352S which resulted in a channel activated at more negative voltages and with a slower rate of inactivation (Gordon *et al.*, 2010). There is no published experimental data verifying Y359 as a site of phosphorylation, and therefore the consequence of its removal from the sequence cannot be determined. However, as Y359 is situated within the membrane, it is unlikely the kinase or phosphatase enzymes would be able to gain access to the site and thus Y359 is not thought to be phosphorylated. Mutation of tyrosine in this highly-conserved domain may however result in a change of ion selectivity or conductance as was shown when residues 354, 355 and 356 were mutated; this potential effect could be tested using electrophysiology.

However, as the site-directed mutagenesis results suggest that mutations proximal to the pore forming and selectivity domains perturb channel function, the mutation was an ideal candidate for prioritisation.

3.3.3 Additional Findings

Despite differences in the number of predicted sites, the frequency of predicted phosphorylation and palmitoylation on the BK channel is similar.

Analysis of the data uncovered that although the number and frequency of predicted palmitoylation and phosphorylation sites on the BK channel were considerably different (8 vs 83 sites respectively, and frequency of 1 in 14 vs 1 in 155 respectively), when the frequency assessment was adjusted from incorporating all amino acid residues, to including only those amino acids that could participate in the post-translational modification, the rates of the two modification were similar (1 in 3 and 1 in 4 respectively). This was not the case for the other post-translational modifications investigated; S-nitrosylation had a frequency of 1 in 7 cysteine residues, SUMOylation 1 in 16 lysine residues and N-glycosylation 1 in 22 asparagine residues. This finding suggests that phosphorylation and palmitoylation are the most common post-translational modifications on the BK channel. Given the extensive research proving the effects of phosphorylation and palmitoylation on BK channel function as reviewed by (Nishi, Hashimoto and Panchenko, 2011; Contet, 2016), nsSNP affecting these processes would be of high priority for future investigation. As there is no known publication detailing the comparative occurrence of post-translational modifications per amino acid for the BK channel, the results obtained here cannot be compared. However, whilst the rate of respective PTMs have not been compared, publications exist that compile evidence of verified PTMs for proteins (Levitan, 1994; Dephoure *et al.*, 2013; Voolstra and Huber, 2014; Shipston and Tian, 2016).

BK channel linker regions are subject to greater nsSNP co-localisation than transmembrane domains

Linker regions have been associated with flexibility, variation and modulation of protein function across proteins via a range of protein-protein interactions and PTMs (as reviewed by Ma *et al.*, 2011), whilst TMDs are considered to be more conserved. One of the objectives of the work in this chapter was to determine the extent of co-localisation between nsSNP and key regions of the channel, such as structural domains. Compilation of the BK channel resource highlighted a small imbalance between nsSNP occurring in the transmembrane domains and in the linker regions, with a rate of 1 nsSNP per 11 amino acids and 1 nsSNP per 8 amino acids respectively. In support of this finding, a 2014 masters study investigating the occurrence of variations in exonic regions of DNA found that nsSNP occurrence in the transmembrane domains was significantly less than in linker regions (Petty, 2014). Therefore, the higher rate of nsSNP in BK channel linker regions is in line with existing evidence.

S0-S1 linker region is one of frequent and varied post-translational modification

The S0-S1 linker has been established as a region associated with channel translocation to the cell membrane via palmitoylation of cysteine residues (Jeffries, 2010; Jeffries *et al.*, 2010), and thus a link with this region and post-translational modification has already been established. However, the assessment highlighted that it is not only palmitoylation that is predicted to occur in this region; as well as the 3 palmitoylated cysteine residues, there were 6 sites of predicted phosphorylation and 1 site of predicted N-glycosylation. The predicted post-translational modification frequency of phosphorylation, palmitoylation and N-glycosylation is greater in the S0-S1 loop than in the rest of the linker regions; the frequency was 1 in 2, 1 in 1 and 1 in 2 respectively, in comparison to 1 in 3 and 1 in 4 and 1 in 19 respectively in the linker regions as a whole. Processes other than membrane translocation are associated with the S0-S1 linker, such as calcium sensitivity (Braun and Sy, 2001) and ethanol response (Jianxi Liu *et al.*, 2006); thus the high level of post-translational modification in the region may relate to the mechanisms for these processes, or may be an indicator of function that has yet to be determined. For this reason, the S0-S1 loop would make an ideal target for future investigation.

3.3.4 Study Challenges

A number of challenges arose in using a bioinformatics methodology as a tool in the creation of the BK channel resource and in the process of prioritisation of nsSNP for further research.

Bioinformatics prediction software outputs should be treated as hypothesis generating due to inherent limitations

Whilst the use of nsSNP damage prediction software is a useful tool to facilitate the prioritisation nsSNPs, findings should be treated as hypothesis generating, due to issues of accuracy of prediction. For example, SNPs that have been shown by site-directed mutagenesis to perturb BK channel function were classified by the damage prediction software as 'tolerated' or 'benign'. An example of this is single nucleotide polymorphism S722L, which occurs in the hypoxia sensing motif spanning from C721 to C723 in the STREX splice insert, and is also a predicted to be a phosphorylation site by NetPhos 2.0 software. Inclusion of this mutation into the BK channel amino acid sequence did not result in a change in the local predicted post-translational modification environment; however, site-directed mutagenesis experimentation showed that S722A mutation results in loss of hypoxia sensitivity (McCartney *et al.*, 2005), proving that mutation at that site had an effect on BK channel function.

However, this mutation was not predicted to be damaging to channel function by nsSNP damage software. This discrepancy between validated experimental results and the predictions of bioinformatics tools was also highlighted in instances of disagreement in the damage score allocated by SIFT and PolyPhen-2. The most notable example of this is D434G (the mutation associated with Generalized Epilepsy and Paroxysmal Dyskinesia) which was categorised as tolerated by SIFT, and probably damaging by PolyPhen-2.

The appraisal of nsSNP damage prediction software is challenging as the algorithms used to assign a damaging score to mutations are extremely complex, however the inconsistency between the lab based results and software programs may be due to a number of factors. Firstly, it could be related to the method of algorithm improvement and development; PolyPhen-2 predictive software is 'trained' with the location and consequences of nsSNP from previous lab based experimentation, whilst SIFT is not (Adzhubei, Jordan and Sunyaev, 2013). This may be the cause behind the PolyPhen-2 damage category allocation of 'probably damaging' for D434G, whilst SIFT having not being trained with this mutation has no information in the algorithm to indicate a change in BK channel function. Whilst training of prediction algorithms with verified results can be advantageous, the fact still remains that site directed mutagenesis investigations often can only assess for effect of the mutant on one or two variables. Thus, it is unlikely that the software has the complexity to accurately predict damage on a multifactorial basis; for example, the software may have difficulty accurately classifying a mutation that effects channel translocation to the membrane, but does not have an effect on channel kinetics once in the membrane.

Another explanation for the inconsistency between lab based results and software programs is the limitation of sequence-based characterisation and multiple sequence alignment used by the programs as methods of nsSNP damage estimation (Sim *et al.*, 2012; Adzhubei, Jordan and Sunyaev, 2013). This method aligns proteins with a similar amino acid sequence over the region of interest, and allocates a nsSNP damage score based upon information on structural and functional information (such as annotation of the region as a transmembrane domain or a functional site) from hundreds of proteins. This may mean that in areas crucial for effect in many proteins of the same type (such as voltage sensing domain and ion selectivity motif in voltage gated channels), mutations in these regions will be consistently classified as damaging, but in other regions such as the S6-RCK1 linker, where a functional relationship has not been widely established, these methods are not capable of predicting harm without the ability to incorporate experimentally verified data into the scoring system.

There also are challenges with the accuracy of post-translational modification prediction software; for example, there are instances where post-translational modification is predicted to occur in the

transmembrane domains (S248, N265, T617, C845 and S1041). There is limited experimental data to support this (Kordyukova *et al.*, 2008, 2010); therefore, it raises the question of whether the sites were predicted as a result of sequence alignment with other proteins which the sites are not within the transmembrane domain. Whilst it is known that PTM prediction software use a variety of methods and information types to determine the likelihood of PTM at a site, it is not possible as a tool user to ascertain the specific information used in the calculation of the score for a specific residue.

In the process of data gathering using bioinformatics resources another challenge arose with PTM identification. Phosphorylation can occur on histidine residues (Besant, Tan and Attwood, 2003; Zu, Besant and Attwood, 2008; Wieland *et al.*, 2010; Wieland and Attwood, 2015), however, the NetPhos 2.0 software has not been updated to reflect this evidence. This is another example to highlight that bioinformatics tools are reliant on algorithm updates to produce accurate results.

The bioinformatics tools discussed above have been developed to be generic to permit the assessment of any genome or protein; this has enabled the successful collation and examination of key areas on the BK channel without the need for experimental analysis. However, this 'wide net' approach means that when looking in detail at a specific gene or protein, discrepancies may come to light; this is likely because many of the algorithms (such as SIFT) do not incorporate BK specific experimental results comprehensively. As a result, the intention is not to rely solely on predictions but to prioritise nsSNP using a combination of bioinformatics tools and experimental evidence, and to verify experimentally the predicted effect of the selected nsSNP on BK channel function. Whilst a number of examples of bioinformatics tools and experimental result mismatch have been discussed, the tools provide a useful guide for further experimentation.

The reliability of bioinformatics investigation results may be limited by the quality of data input and the method of data validation

The accuracy and quality of the data included in bioinformatics database and software determines reliability of any assessment using a bioinformatics methodology. This begins with the accuracy of the protein or base sequence of a protein uploaded into the database. It is difficult to determine if the data entered is consistent and has it been verified against more than one source, however the advantage in using the canonical version of the BK channel is that it is part of the CCDS and thus sequence information associated with that transcript has been validated and is consistent across databases.

The level of certainty decreases when looking at the verification of post-translational modifications, which can be undertaken using several different methods, and are not included in a consensus project

to ensure quality and accuracy of results. For example, 5 post-translational modification sites on the BK channel, all of phosphorylation, were identified using mass spectroscopy (S778, S782, S978, S982 and T970); this method has advantages and disadvantages. Mass spectroscopy enables the analysis of complex mixtures of proteins in a high throughput fashion (Aebersold and Mann, 2003) and has been quoted as a 'hypothesis generating engine' for the identification of hundreds of different PTM types at multiple amino acid locations (Cravatt, Simon and Yates, 2007; Parker *et al.*, 2010; Doll and Burlingame, 2015). Over and above the high diversity of modifications that can be detected and high speed of analysis, the use of mass spectroscopy is advantageous because isolation of the specific region of analysis is not required, as is the case with site directed mutagenesis experimentation. In addition, the ability to quickly determine the nature and quantity of relative changes in PTM occupancy at specific amino acid sites is advantageous (Larsen *et al.*, 2006), as it increases the number of experiments that can be run in a set time, in comparison to techniques such as site-directed mutagenesis.

However, the technique is not without flaws; in a 2016 review article, the common errors associated with the detection of PTMs using mass spectroscopy are discussed (Kim, Zhong and Pandey, 2016). One of the errors addressed in the study is that the incorrect PTM can be associated with a protein because the of mass of two separate moieties being very similar; for example, phosphorylation (79.96633 Da) and O-sulfonation (79.95682 Da) are extremely similar in mass and occur on the same amino acid residues (i.e. serine, threonine and tyrosine) and thus the modifications can be inappropriately allocated. Another error that can occur using mass spectroscopy is that the PTM can be allocated to the incorrect amino acid residue, particularly when there are multiple residues of the same type neighbouring each other, such as in the serine rich domains in the N and C termini. Therefore, sites of interest identified using this method should ideally be verified using another method in addition. Phosphorylation site T763 is an example where this process was undertaken. The PTM site was initially identified in BK channels located in rat brain using mass spectroscopy (Yan *et al.*, 2008), but was then verified using SDM of the site in the BK channels of chicken cochlea (Bai *et al.*, 2012). Site directed mutagenesis was used to verify the majority of post-translational modification sites documented in this chapter, likely due to the ability to test the effect of the removal of a predicted site on factors such as channel activity.

BK channel post-translational modification and site-directed mutagenesis lists in bioinformatics databases are not comprehensive

Whilst bioinformatics databases such as UniProt contain information on the location and result of site-directed mutagenesis and experimentally verified sites of post-translational modification on the BK channel, there is currently no database which list the sites comprehensively. Consequently, the compilation of verified sites of post-translational modification and location of site-directed mutagenesis listed in this chapter was extremely challenging and time-consuming to produce. This is partly due to difficulty in gaining access to the publications themselves, and secondly, in locating the amino acid residue mutated in the publication. This was due to differences in the transcript used (species or transcript length) and the detail to which the location of the amino acid was described.

Experiments have taken place using BK channels constructs of differing amino acid composition or in species other than human. Consequently, time was spent determining the equivalent amino acid location on the human BK canonical transcript to produce the list of verified post-translational modification and site-directed mutagenesis sites. As a result, the sites compiled in this resource may not be comprehensive, but the lists serve as a current best estimate of the research conducted on the BK channel, and a basis for future study to compile a comprehensive list.

Bioinformatics rapidly evolves

The field of bioinformatics is a rapidly growing and evolving one, with tens of terabytes of new data uploaded to databases daily (Baker *et al.*, 2000; Marx, 2013). Projects such as ExAC and the 100,000 genomes progress knowledge by increasing the number of accurately sequenced exomes and genomes uploaded into the public domain via free to access databases. This constant upload of information, and the development and improvement of the methodologies means that information rarely remains static in the major bioinformatics databases. The work conducted in this thesis serves to provide a snapshot of BK channel information across several different bioinformatics databases and software, whilst compiling this with data in journal articles, still to be integrated into the databases.

3.3.5 Implications and practical application

The need for specialised BK channel biocuration

To create a resource containing the key domains associated with the BK channel, and achieve one of the primary aims of the chapter, information on the channel was taken from a number of sources such as bioinformatics databases, predictive software programs and published literature; this information was cross-referenced. This process required access to data from multiple disparate fields, ranging from basic genetics to mass spectroscopy and predictive algorithms and integration of this information with an understanding of BK channel structure and function. The cross-referencing and validation of biological information from multiple sources, with consideration for the functional properties of the protein, is also referred to as biocuration.

Examples of biocuration performed in this chapter were a) the removal of duplication from the 224 nsSNP initially associated with the BK channel by the Ensembl database down to 167 unique nsSNP, b) extracting the amino acid location of BK channel mutagenesis and PTM validation experiments from publications and locating the equivalent amino acid residue in the canonical human BK channel construct and c) compiling information on the amino acid locations of BK channel nsSNPs, structural and functional domains and creating a figure illustrating the extent of co-localisation.

Bioinformatics requires biocuration to increase the reliability and accuracy of the data held within the databases (Howe and Yon, 2008); however as described, this is a complex process which is time consuming and labour intensive. An example specific to this thesis was the collation of the location and consequence of sites of site-directed mutagenesis, which was extremely challenging. It has been quoted to take a minimum of five times longer to do the basic analysis of data than to upload it into databases (Mangubat 2016). Furthermore, the work of biocuration is rarely given recognition equal to projects that add to the amount of data in the databases (such as the ExAC, 1000 genomes and ESP studies) (Howe and Yon, 2008; Burge *et al.*, 2012; Mitchell *et al.*, 2015). Biocuration at this stage is unable to be fully automated due to the complexity of the task, as it requires knowledge of multiple areas of bioinformatics, such as PTM, SNP damage prediction software, and molecular science such as SDM and physical science such as mass spectroscopy. Thus, analysis and sorting of the data must be tailored and adjusted specifically to answer the specific question at hand. As a result of the complexity, high workload, restricted timelines and low funding in comparison to data generation projects, biocuration has been typically restricted to expert and PhD level analysis as a minimum level of qualification (Hirschman *et al.*, 2012; Mitchell *et al.*, 2015; Reiser *et al.*, 2016).

The creation of a dedicated BK channel bioinformatics database

The work in this chapter was successful in creating a paper-based resource (a thesis) for the BK channel and its key sites. The next step would be to process and map the steps taken to collate and curate the information, to enable the identification of the drivers and barriers and to minimise some of the challenges with a view to create an online BK channel specific database or software tool. One of the desired outcomes of a BK channel specific online resource would be to enable specialists in BK channel research, and a wider scientific audience, to access experimental data from a range of labs effectively and efficiently. This would be possible as the data would be collated at one site (likely a website or database) with instant access to the results rather than the delays associated drawing information from a range of sources such as peer review publication and other data resources. Another desired outcome would be the rapid and comprehensive validation of BK channel information contained within the database; this would be achieved by specialist ion channel researchers reviewing and assessing data uploaded into the system regularly. It is hoped that the time spent undertaking biocuration could be reduced, as the data within the database would be more comprehensive and constructs used in site directed mutagenesis or post-translational modification site verification could be instantly aligned, pinpointing the location of each mutation or PTM site for each isoform of the BK channel.

A BK channel specific resource does not currently exist, though dedicated bioinformatics resources have been developed for other ion channels. For example, KvSNP (available at http://www.bioinformatics.leeds.ac.uk/KvDB/KvSNP.html); is a database dedicated to predicting the damaging potential of genetic mutations occurring on voltage-gated potassium (Kv) channel genes (Stead *et al.*, 2011). 'VKCDB' (available at http://vkcdb.biology.ualberta.ca/index.php); is a multi-organism sequence database of Kv channels (Gallin and Boutet, 2010) and 'Channelpedia' an ion channel specific database (available at http://channelpedia.epfl.ch/), attempts to create an information management framework to collectively build ion channel knowledge base by accommodating both structured and unstructured data (Ranjan *et al.*, 2011). Whilst this latter database did have a sub-section for calcium gated potassium channels, it did not contain sufficient information on the BK channel and therefore could not be used as a reference source for data collection in this chapter.

Creation of specific bioinformatics databases and software programs has been extended to other proteins such as protein kinases (Torkamani and Schork, 2007; Lahiry *et al.*, 2010) available at http://sequoia.ucsf.edu/ksd/, where the prediction accuracy for damaging nsSNP was increased from 74% to 83% in comparison to whole genome prediction methods. A database has been created to store information on G protein-coupled receptors (GPCRs) (Elefsinioti *et al.*, 2004) available at

http://bioinformatics.biol.uoa.gr/gpDB/. These are a small number of examples where the need for protein specific resource was identified, and databases and tools were built to address this need.

To create a specific database software program for the BK channel a number of steps would need to take place; the publications listed above provide an example of what information and processes would be required. Examples include the collation of the most up-to-date structural information for the BK channel, available from databases as well as recent BK channel publications (Tao, Hite and MacKinnon, 2016), collation of SNP information from a number of sources both bioinformatics and literature and the selection of a machine learning model for the BK channel (Lin and Chen, 2015). Availability of open access databases and the formation of a consortium of researchers who specialise in the BK channel would likely be necessary to achieve this goal. Whilst challenges such as intellectual property, financial support for the project and the recruitment of a bioinformatician with a specialty in BK channels must be overcome. A pilot study was launched to evaluate whether a subscription-based membership model would provide a sustainable funding source for a *Arabidopsis thaliana* specific biocuration database and avoid reliance on short term grants (Reiser *et al.*, 2016).

3.3.6 Future Work

Selection of S0-S1 mutants for lab based experimentation

One of the aims of the work conducted in this chapter was to prioritise BK channel nsSNPs for lab-based investigation. This aim was achieved and 8 nsSNPs were prioritised and investigated in detail using information contained in the literature, in bioinformatics databases and through the use of bioinformatics software tools. Whilst all 8 mutations would be valid for future investigation, the mutants that will be taken forward for lab-based investigation in the next chapter will be H120Q and G122A located in the S0-S1 linker. As adjacent cysteine residue C121 is associated with modulating cell membrane translocation of the channel, lab-based investigations will focus on the effect of H120Q and G122A mutations on BK channel cellular distribution. Localisation to the cell membrane is required for BK channel function.

3.3.7 In summary

The aim of this chapter was to organise specified aspects of BK channel information already contained within bioinformatics databases and convert them into an accessible form that is easy to understand, so that others looking to conduct research on the BK channel have access to a resource of collated information. Secondary to this, the aim was to use this information to prioritise nsSNP with the potential to perturb channel function, and forward them for lab based experimentation. To achieve this, a process of biocuration, which involves the manual cross-referencing of biological information held in databases and lab-based research, was utilised. These aims were successfully achieved and whilst 8 nsSNP were put forward for detailed bioinformatics assessment, mutations H120Q and G122A, located in the S0-S1 linker, were prioritised and selected for lab-based investigation due to their varying effect on the CSS-Palm 4.0 predicted palmitoylation status of C121, a cysteine residue proven to modulate cell membrane expression of the channel. H120Q and G122A were selected also because there is existing detailed information relating to the flanking cysteine residues (C118, C119 and C121) and palmitoylation of the S0-S1 linker. There was also the availability of existing tools to study SNP effects- such as BK channels incorporating cysteine residue mutations and the facilities to conduct confocal microscopy. These factors made H120Q and G122A the “best” targets for further investigation. A summary of the thesis workflow is included in Figure 3.28.

There is no known published BK channel resource akin to the work produced in this chapter, making the resource a novel addition to the field. There is pressure to increase the speed of biocuration of bioinformatics data to ensure its usability and ability to positively influence lab based research. Therefore, future advancement avenues for this research would be to increase the extent of biocuration for the BK channel, and the creation of a dedicated online BK channel database software tool.

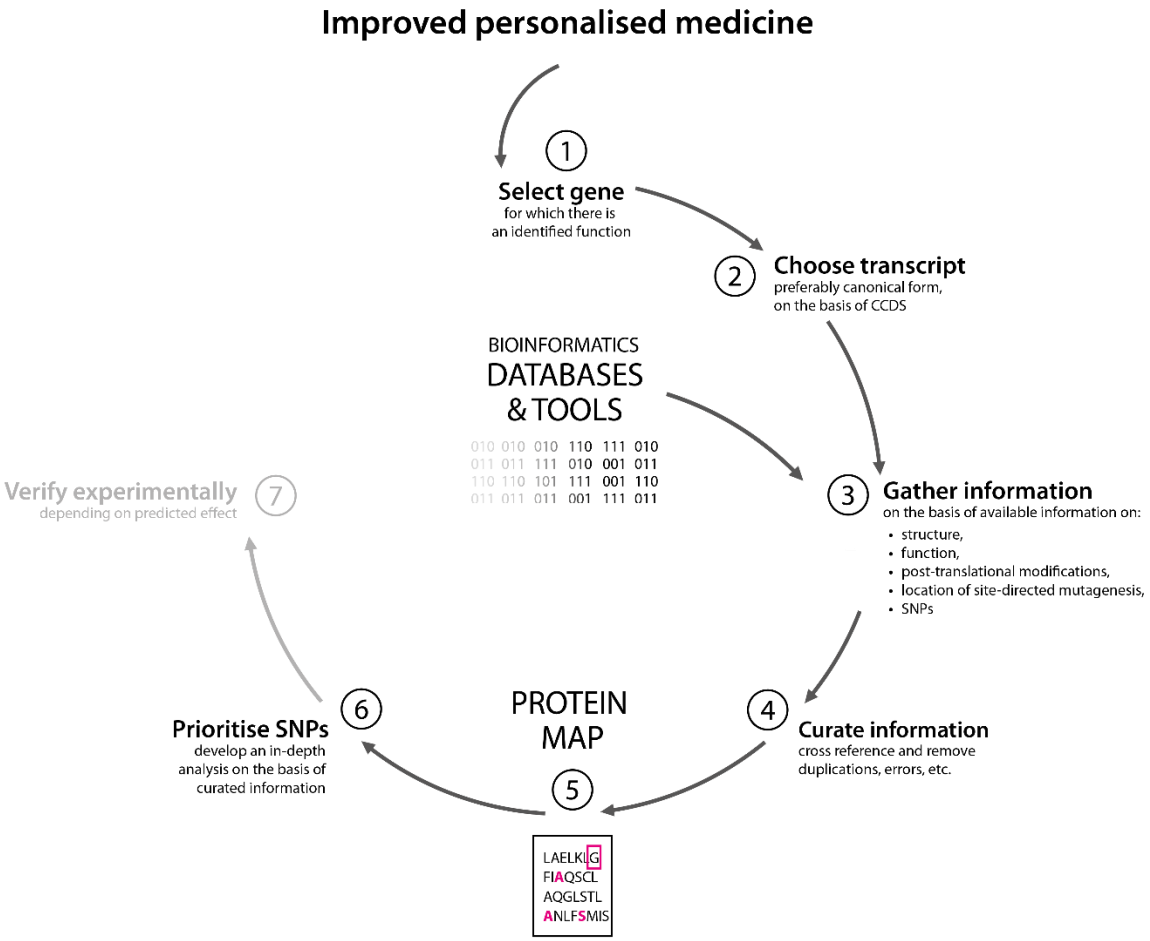


Figure 3.28- Schematic of thesis workflow: Summary of chapter 3. Described are the steps to be undertaken in this thesis work to identify new potential genetic targets for personalised medicine. Steps 1-6 have been successfully achieved in this chapter by the selection of the BK channel gene KCNMA1, the canonical BK channel transcript, the collation of available information, the biocuration of this work into a 'BK channel resource' and the prioritisation of nsSNP. Step 7 will be addressed in Chapter 4.

4 Lab-Based Chapter

4.1 Introduction

4.1.1 Amino acid residues of the BK channel S0-S1 linker and modulation of channel cell-membrane expression

In chapter 3, two endogenous single nucleotide polymorphisms (SNPs) in the S0-S1 loop of the BK channel (H120Q and G122A) were identified using a bioinformatics methodology and were prioritised for lab-based investigation. The decision was based on their proximity to, and predicted impact on palmitoylation of C121; a cysteine residue important for the cell membrane translocation of the BK channel. Introduction of the H120Q mutation into the canonical wild type BK channel sequence was predicted to result in the loss of C121 palmitoylation. On the other hand, the G122A mutation was predicted to increase the likelihood of C121 palmitoylation in comparison to wild type. The double mutant has a predicted palmitoylation score driven predominantly by G122A and thus has an increased likelihood of C121 palmitoylation in comparison to the wild type channel.

Previously, investigations on S0-S1 loop cysteine mutations were conducted using the murine version of the BK channel (mSlo) (Jeffries, 2010; Jeffries *et al.*, 2010). As the current lab-based investigation was carried out in a murine BK channel, the numbering of the human SNPs has been aligned with the murine channel sequence and numbering. Therefore, in this chapter the human SNPs H120Q and G122A will be referred to as the murine equivalents H55Q and G57A, and the potentially palmitoylated cysteine residues C118, C119 and C121, will be referred to as murine residues C53, C54 and C56 (Table 4.1). An illustration of the amino acid changes resulting from the DNA mutations is included in the general methods chapter (chapter 2). As the mutants H55Q, G57A and H55Q:G57A were designed to mimic the human SNPs, they will now be referred to at the SNP-mimetic mutants.

hSlo amino-acid numbering	C118	C119	H120	C121	G122
mSlo amino-acid numbering	C53	C54	H55	C56	G57

Table 4.1- Conversion of hSlo amino acid numbering to mSlo equivalent over residues of investigation in the S0-S1 linker

Palmitoylation of BK channel N-terminal cysteine residues C53, C54 and C56 located in the S0-S1 loop have been shown to modulate channel trafficking and surface expression in the HEK293 cell line (Jeffries, 2010; Jeffries *et al.*, 2010; Bi, 2014; Kim *et al.*, 2014). Mutation of these cysteine residues to alanine residues resulted in an almost complete cessation of channel palmitoylation and a two-fold reduction in membrane expression of the C53:54:56A mutant compared to the wild-type channel (ZERO) (Jeffries, 2010; Jeffries *et al.*, 2010). Palmitoylation of the S0-S1 C53:54A mutant channel was reduced by 50% and membrane expression of the C53:C54A-YFP fusion protein was reduced by 70% in comparison to the wild type S0-S1 ZERO construct (Jeffries 2010).

Of the three individual S0-S1 cysteine residues investigated, C56 had the greatest impact on channel membrane expression; with the S0-S1 C56A-YFP construct reducing cell membrane expression to 25% of control, in comparison to reductions to 31% and 64% for C53A-YFP and C54A-YFP respectively. The triple mutant (C53:54:56A-YFP) reduced expression to 13% of control, whilst expression of the triple mutation in the full-length channel reduced cell-membrane expression to 45% of wild-type (Jeffries, 2010; Jeffries *et al.*, 2010). A 50% reduction in BK channel membrane expression occurred when the C53:54:56A mutation was incorporated into rat BK channels (rSlo) transiently expressed in COS-7 cells (Kim *et al.*, 2014).

The SNP-mimetic mutants H55Q, G57A and H55Q:G57A have not previously been investigated for their impact on channel function or channel cellular distribution. However, incorporation of naturally occurring mutations into the BK channel and the subsequent expression of the channel in a mammalian cell model system has been successfully undertaken previously, as in the case of the D434 (Du *et al.*, 2005; Wang, Rothberg and Brenner, 2009; Yang *et al.*, 2010) and A138V (Plante, 2016) mutations. These studies showed mutation-dependent effects on channel activity identified using patch-clamp electrophysiology. The effects on the cell-membrane expression of the channel were not investigated, therefore SNP-mimetic mutant channels could be interesting constructs to investigate the mechanisms involved in BK channel trafficking to the cell membrane.

4.1.2 The potential for metabolic substrates to modulate the cell membrane expression of BK channel constructs.

A range of factors, in addition to channel amino acid sequence, such as the exposure to changes in composition and concentration of key signalling and metabolic agents has been shown to influence the function of BK channels. Examples include oxygen concentration (Liu, Moczydlowski and Haddad, 1999; Williams *et al.*, 2004; McCartney *et al.*, 2005; Cheng *et al.*, 2008), pH levels (Avdonin, Tang and Hoshi, 2003) and exposure to alcohol (Dopico, Lemos and Treistman, 1996; Jakab, Weiger and

Hermann, 1997). Studies on the impact of metabolic status on BK channel function and distribution have examined rapid reversible changes (10 to 30 minutes), longer acute changes (24-48 hours), and chronic changes (3 days or longer) in cell culture conditions. However, these studies have not specifically examined the impact of metabolic status through the prism of the BK channel's S0-S1 loop, nor investigated the influence of S0-S1 SNP–mimetic amino acid mutations in isolation nor in combination with alterations in metabolic status. Although an array of metabolic agents exists with the potential to alter cellular status, the decision was made to focus on three metabolic substrates: a) glucose, b) the C16 saturated fatty acid palmitate, and c) the C18:1 unsaturated fatty acid oleate.

Rationale for glucose as a metabolic agent for investigation

Glucose is a major substrate required for metabolic homeostasis and plays a role in a wide variety of cellular processes, enabled by activation of complex signalling cascades that link glucose with key processes, often via 'the master metabolic regulator' AMP-activated protein kinase (AMPK) (as reviewed by Hardie, Ross and Hawley, 2012) which is sensitive to the AMP-ADP-ATP ratio. Mitochondrial function and signalling is another major player in actioning the downstream effects of glucose concentration on metabolic homeostasis (Liesa and Shirihai, 2013; Pekkurnaz *et al.*, 2014; Mishra and Chan, 2016).

Glucose effects on cell metabolic status vary depending on the type and role of the cell. For example, research has shown that glucose concentration has modulatory and regulatory effects on signalling in the glucose sensitive pancreatic cells (Heimberg *et al.*, 1993; Schuit *et al.*, 1997; Jichun Yang *et al.*, 2005; Bagge *et al.*, 2012; Broca *et al.*, 2014), in insulin sensitive tissues such as skeletal muscle and adipocytes (Moore *et al.*, 2012; Bijland, Mancini and Salt, 2013; Koutny, 2013; Stanford *et al.*, 2013; Sakagami *et al.*, 2014; Tunduguru *et al.*, 2014; Yang, 2014; Sylow *et al.*, 2015) as well as endothelial cells (Wang *et al.*, 2012; Peiró *et al.*, 2016).

Some of the effects of glucose in mammalian cells are mediated by the BK channel; as it has been shown that high glucose concentrations can inhibit BK channels causing a significant reduction in cell apoptosis and increasing cell proliferation in HEK293 cells (Chang *et al.*, 2011). The reduction in apoptosis is thought to be mediated by a reduction in potassium loss; loss of intracellular potassium has been shown to cause apoptotic cell shrinkage (Dallaporta *et al.*, 1998), and activation of apoptotic pathways involving protease enzyme caspases family and members of the apoptosis regulator Bcl-2 family amongst others (Elmore, 2007).

There is evidence that both acute and chronic changes in glucose concentration can impact on BK channel expression and translocation to the cell membrane. For, example, mouse renal podocytes endogenously expressing BK channels were cultured in 11.1mM and 36.1mM glucose for 24 or 72 hours; western-blot assay revealed no significant difference in total BK channel expression with glucose concentration, though exposure to the higher glucose concentration resulted in a significant 70% decrease in BK channel cell membrane expression in the 24 and 72-hour exposure groups (Kim and Dryer, 2011). In the same publication, 25mM mannitol used in combination with 11.1mM glucose revealed that the changes were not due to alterations in osmotic pressure.

Investigation of BK channel cellular distribution in response to a reduction in glucose concentration may have implications for our understanding of the regulation of processes such as vascular tone, neuronal function and hormone release in the pancreas; dysregulation of these processes is implicated in the development and progression of diabetes and its associated co-morbidities. BK channels are required to be located in the cell membrane in order to function as modulators of membrane potential or calcium signals and integrators of phosphorylation and lipid signalling; thus, investigating if glucose concentration alters channel distribution would be a step closer to understanding the processes involved in the growing link between the BK channel and metabolic dysregulation. The specific culture medium adjustment chosen was a reduction in extracellular glucose from 25mM to 5mM; this choice was driven by; a) 5mM being within the normal blood glucose range for humans (4mM to 6mM), and b) the fact that a considerable number of studies have used this concentration to test the effect glucose on different processes (Nutt and O'Neil, 2000; Moritz *et al.*, 2001; Lin *et al.*, 2005; Smith *et al.*, 2006; Daoyan Liu *et al.*, 2008; Chang *et al.*, 2011; Sun *et al.*, 2013; Viskupicova *et al.*, 2015), thus making the data outputs from this study comparable.

Rationale for palmitate as a metabolic agent for investigation

The saturated fatty acid, palmitate (C16:0), is the principal product of the fatty acid synthase system in mammalian cells and is the precursor of other long-chain fatty acids such as stearate (C18:0) and arachidonate (C20:4). Palmitate is one of the most common fatty acids found in human serum and tissues, with large peaks in concentration observed in the post-prandial phase (Kingsbury *et al.*, 1961; Miles *et al.*, 2003; Sabin *et al.*, 2007; Abdelmagid *et al.*, 2015).

Several studies have shown that an increased extracellular concentration of palmitate elicits a change in cellular processes. Examples include altered autophagy regulation in human aortic endothelial cells mediated by diminished AMPK activity, and increased phosphorylation of previously inactive kinase enzymes (Weikel *et al.*, 2014), induction of apoptosis in hepatocytes via activation of protein kinase C

(Cai *et al.*, 2014), increased expression of interleukin-6, a cytokine with an inflammation modulating role, in human myotubules (Staiger *et al.*, 2004; Weigert *et al.*, 2004) and to increase the protein expression of the pseudo-kinase Tribbles homolog 3 (TRB3) (Morse *et al.*, 2010; Yan *et al.*, 2014).

Palmitate has also been shown to affect the cell membrane expression of proteins; when human monocytic (THP-1) cells were exposed to palmitate at 0.3mM, the cell membrane expression of the integrins CD11b and CD36 was increased by over 60% (Pararasa, 2013). While exposure to 0.4mM palmitate for 8 hours increased by more than 2-fold tumour necrosis factor-related apoptosis-inducing ligand (TRAIL) death receptor 5 (DR5) cell surface expression in Huh-7 cells (Cazanave *et al.*, 2011). Furthermore, high concentrations of extracellular palmitate (0.2-0.6mM) can impair stimulus-induced insulin secretion and induce beta cell apoptosis (Hagman *et al.*, 2005; Watson *et al.*, 2011; Broca *et al.*, 2014).

High glucose and palmitate concentrations are hypothesised to have a cumulative effect on a range of cellular processes including: increased autophagy in endothelial cells cultured in 40mM glucose and 0.5mM palmitate (Jiaqi Liu *et al.*, 2016) and in HUVEC cells cultured in 25mM glucose and 0.4mM palmitate (Yanxia Liu *et al.*, 2016); increased bone morphogenic protein (BMP4) expression in HUVEC cells cultured in 27.8mM glucose and 0.5mM palmitate for 24 hours (Hong *et al.*, 2016); and increased P2X7 and P2X4 expression in HUVEC cells cultured in 30mM glucose and 0.15mM palmitate for 24 hours (Sathanoori *et al.*, 2015).

There is also evidence to indicate that the combination of glucose and palmitate at high concentrations has a pronounced effect in pancreatic β-cells (Remizov *et al.*, 2003; Poitout and Robertson, 2008; Maris *et al.*, 2013), with some studies attributing this to a drastic increase in the formation of ceramide and associated increase in nitrous oxide (NO) production (Paumen *et al.*, 1997; Shimabukuro *et al.*, 1998; Maedler *et al.*, 2003), others attributing the effect to oxidative stress (Okuyama, Fujiwara and Ohsumi, 2003; Elsner, Gehrmann and Lenzen, 2011) or more specifically ER stress induced by calcium depletion in the ER (Cunha *et al.*, 2008; Cnop *et al.*, 2010). The nature of the more significant effect of 'glucolipotoxicity' on beta cell behaviour and the handling of cellular components is likely due to the acute sensitivity required for their role as energy sensor and regulator of energy homeostasis (Parker *et al.*, 2003; Remizov *et al.*, 2003; Thorn, 2010; Watson *et al.*, 2011; Martino *et al.*, 2012; Maris *et al.*, 2013).

The effects of palmitate toxicity have been shown to be mediated via ion channels; with apoptosis of HUVEC cells resulting from exposure to high concentrations of palmitate (0.25-8mM) linked to increased expression of the voltage activated potassium channel subtype Kv1.5 (Du *et al.*, 2017). Kv1.5 channel augmentation has been shown with palmitate addition to adult mouse ventricular myocytes

resulting in increased Kv1.5 peak outward current amplitude and is theorised to cause impairment of excitation-contraction coupling (Haim *et al.*, 2010). A literature search did not identify research papers describing the effects of palmitate, or palmitate in conjunction with high glucose, on BK channel function, protein expression or cell membrane expression, thus identifying a gap in knowledge. The concentration of palmitate to be used in the investigation was 0.4mM as it is within the range found endogenously in human plasma (0.3mM to 4.1mM) (Abdelmagid *et al.*, 2015), and within the range commonly used in research publications (0.2mM to 0.5mM) (Luiken *et al.*, 1997; Staiger *et al.*, 2004; Weigert *et al.*, 2004; Bunn *et al.*, 2010; Oberbach *et al.*, 2012; Schilling *et al.*, 2013; Weikel *et al.*, 2014).

Rationale for oleate as a metabolic agent for investigation

The monounsaturated fatty acid, oleate (C18:1), alongside palmitate (C16:0) and stearate (C18:0) is one of the most common fatty acids found in humans, and is the second most abundant fatty acid in human tissues, behind palmitate (Kingsbury *et al.*, 1961; Havel, Naimark and Borchgrevink, 1963; Miles *et al.*, 2003; Sabin *et al.*, 2007; Bergouignan *et al.*, 2009). Oleate has been linked with physiological effects, such as reductions in blood pressure, in the frequency of cardiovascular events, and in certain forms of cancer in humans who consume a diet rich in oleate containing foods such as olive oil (Martin-Moreno *et al.*, 1994; Perona *et al.*, 2004; Psaltopoulou *et al.*, 2004; Terés *et al.*, 2008).

Oleate is commonly used in laboratory experiments as a fatty acid control for saturated lipids such as palmitate. Experiments have compared and contrasted the effects of this lipid on mitochondrial function, markers of ER stress, apoptosis, insulin signalling, insulin resistance and inflammation (Gaster, Rustan and Beck-Nielsen, 2005; Coll *et al.*, 2008; Yuzefovych, Wilson and Rachek, 2010; Kwon, Lee and Querfurth, 2014; Hetherington *et al.*, 2016). The majority of studies demonstrate that oleate has no significant effect on study endpoints in comparison to control, normally culture medium supplemented with bovine serum albumin (BSA). However, a small number of studies have shown that raised oleate can influence cellular processes. For example, incubation of the neural cell culture line N2a with 0.3mM oleate decreases mitochondrial superoxide levels, which are associated with oxidative damage and inflammation (Kwon, Lee and Querfurth, 2014), exposure to oleate at 1mM for 48 hours resulted in cell death of hepatic stellate cells (Hetherington *et al.*, 2016) and oleate exposure at 0.1mM increased the phosphorylation of Akt protein kinase (Hardy *et al.*, 2005).

As this current study focussed on the potential effects of oleate on cell membrane expression of a channel protein, a literature search was conducted to determine if oleate had any effect on protein cellular distribution. No consistent pattern seems obvious; exposure of neutrophils to 0.08mM oleate for 5 minutes resulted in a 2.5 fold increase in CD11b cell membrane expression (Mastrangelo, Jeitner

and Eaton, 1998), whilst HEK293 exposure to oleate at 0.1mM for 16 hours reduced membrane expression of sterol regulatory element-binding proteins (SREBPs) to 40% of control (Hannah *et al.*, 2001). In another study oleate had no significant impact on protein cell membrane localization (Benoit *et al.*, 2009) but in others regulates the activity of adrenoceptor and G-protein activities, this effect has been theorised to be due to incorporation of oleate into the cell membrane, altering membrane structure (Funari, Barceló and Escribá, 2003; Prades *et al.*, 2003; Q. Yang *et al.*, 2005).

There is no data readily available in the literature describing the effect of oleate on BK channel or ion channel cellular distribution. However there are publications describing the effects of oleate on BK channel activity; oleate has been show to increase BK channel activity in rabbit pulmonary arteries (Clarke *et al.*, 2002), the rat pituitary tumour cell line (GH3) (Denson *et al.*, 2000) and in *Xenopus* oocytes (Sun *et al.*, 2007). In line with these studies, and results showing that the endogenous human plasma concentration of oleate can range between 0.03mM and 3.2mM (Abdelmagid *et al.*, 2015), it was decided that a concentration of 0.4mM oleate would be used to investigate the impact of an unsaturated fatty acid on BK channel construct cellular distribution.

Chapter Aims

1. To investigate the impact of SNP-mimetic changes in BK channel sequence, by determining the cellular distribution of S0-S1 loop SNP-mimetic BK channel constructs in HEK293 cells and comparing the distribution to the wild-type channel (ZERO).
2. To investigate the relationship between BK channel cellular distribution and the predicted palmitoylation scores of specific cysteine residues in the S0-S1 linker, by analysing the distribution of the wild-type BK channel, the SNP-mimetic and cysteine mutant BK channels in comparison with predicted palmitoylation software scoring.

To date, there is no known published data describing the effects of glucose, or fatty acid concentration on the cell membrane expression of BK channels containing endogenous SNPs of the S0-S1 loop. Thus, the next aim of the experiments conducted in this chapter is:

3. To investigate the impact of short term changes (24-48 hour) in metabolic substrates on BK channels through analysis of channel cellular distribution associated with changes in extracellular glucose, palmitate and oleate concentrations on SNP-mimetic, wild-type and cysteine mutant BK channels.

4.2 Results

4.2.1 Assessment of the cellular distribution of the full-length wild-type BK channel construct (ZERO) in HEK293 cells cultured in 25mM glucose.

One of the main aims of this research was to determine the impact of the SNP-mimetic mutants H55Q, G57A and H55Q:G57A on BK channel cellular distribution. In order to achieve this, it was necessary to first establish the baseline distribution of the wild-type 'control' isoform of the BK channel in the HEK293 cell line. The ZERO isoform of the BK channel was used as it forms functional units, and contains the S0-S1 loop, -the location of the SNP-mimetic mutations- as well as the key structural and functional regions used in the bioinformatics screen. The main difference between ZERO and the canonical form being the lack of the STREX splice insert in the intracellular linker between the RCK domains. Use of the BK channel isoform without the STREX insert was crucial, as the STREX variant contains cysteine residues (C12 and C13 in STREX amino acid numbering) shown to be palmitoylated and that contribute to the translocation of the channel to the cell membrane (Jeffries, 2010). These STREX located cysteine residues would complicate the interpretation of the impact of S0-S1 loop SNP-mimetic induced changes to cellular distribution. and for this reason, the ZERO form without STREX was utilised for all imaging experiments. The control condition for the baseline experiments was 25mM glucose culture medium; this culture environment has been utilised in other studies investigating the effect of BK channel amino acid mutation on cell membrane expression (Jeffries, 2010; Bi, 2014) and serves to facilitate comparison.

To estimate the proportion of BK channels in the cell membrane of HEK293 cells, the raw ratio was calculated as the total of red fluorescence – the amount of BK channel protein at the surface membrane- over the sum of red and green fluorescence -total BK channel protein in the HEK293 cells - with the green fluorescence capturing the intracellular distribution of channels). This proportion was calculated from analysis of field of view (FOV) images captured using confocal microscopy (Figure 4.1). The cell membrane-bound ratio of the ZERO channel (Figure 4.2A) was obtained from z-stacked 'fields of view' (FOV area 146μm^2) of cells from three separate transfections. The numbers of HEK293 cells in each stack varied from three to fifteen (average 9 cells per FOV) and the number of optical slices (slice thickness 0.34-0.5µm) within each stack varied from nine to nineteen depending on the distribution and structure of the cells within the FOV. Figure 4.2A shows the raw ratio values and normalised values for the six separate stacks as well as the mean and standard deviation (SD) for the group, which are plotted in Figure 4.2B.

Raw ratio values in this experiment set ranged from a minimum of 0.53 to a maximum of 0.68 for the stacks, with the average percentage of BK channels in the cell membrane of this group at the time of staining being 63 %, so a mean ratio of 0.63±0.05. This data is the product of a single experiment and

transfection (N=1) made up of six separate stacked fields of view (n=6); this nomenclature will be used throughout the rest of the manuscript. Figure 4.2C shows a plot of the raw ratio values from three separate experiments (N=1, n=6 for each), the results of which were combined to create the overall mean of the ZERO channel raw ratio in HEK293 cells cultured in 25mM glucose, which was 0.51 ± 0.11 (Figure 4.2D). This approach indicated that it was possible to calculate the cellular distribution of the BK channel ZERO construct based on the double FLAG tag (red/green) methodology.

When using red and green fluorescent markers to differentiate between membrane and intracellular distribution of the channel constructs, it was clear in the majority of slices and stacks that the two populations were clearly separate. However, when the red and green are adjacent or co-localised then there is the appearance of 'orange' as seen in slices 2 and 17 in Figure 4.1. These were taken from close to the top and bottom of the FOV stack comprised of 19 slices. To address the potential issue of membrane-bound and intracellular BK channel co-localisation, a co-localisation assessment of the image was undertaken using the image processing software Image J. The Pearson's correlation coefficient (PCC) value was 0.29 for the stack of cells; with the range from -1 to 1, with 1 indicating a total positive correlation (as red increases, green increases), 0 indicating no correlation and -1 indicating total negative correlation (as red increases, green decreases). The results thus imply a low level of co-localization between red and green fluorescence in the cells overall.

The double FLAG approach was therefore successful in determining that the membrane expression of ZERO in 25mM glucose was 51%. The next stage was to investigate the distribution of the SNP-mimetic mutants H55Q, G57A and H55Q:G57A, to determine their impact on the cellular distribution of BK channels.

4.2.2 Assessment of the distribution of the SNP-mimetic BK channels constructs H55Q, G57A and H55Q:G57A in HEK293 cells cultured in 25mM glucose.

The BK channel SNPs H55Q and G57A are of interest due to their potential effect on the probability of C56 cysteine residue palmitoylation in the intracellular S0-S1 region, as this cysteine residue has a modulatory role in channel cell membrane translocation. The naturally occurring H55Q and G57A mutations, and the synthetically produced combination of the two mutations, H55Q:G57A, were independently incorporated into the murine BK ZERO channel construct DNA and successfully transiently expressed in HEK293 cells. Exemplar confocal FOV images of the transfected cells with fluorescent channels labelling are shown in Figure 4.3.

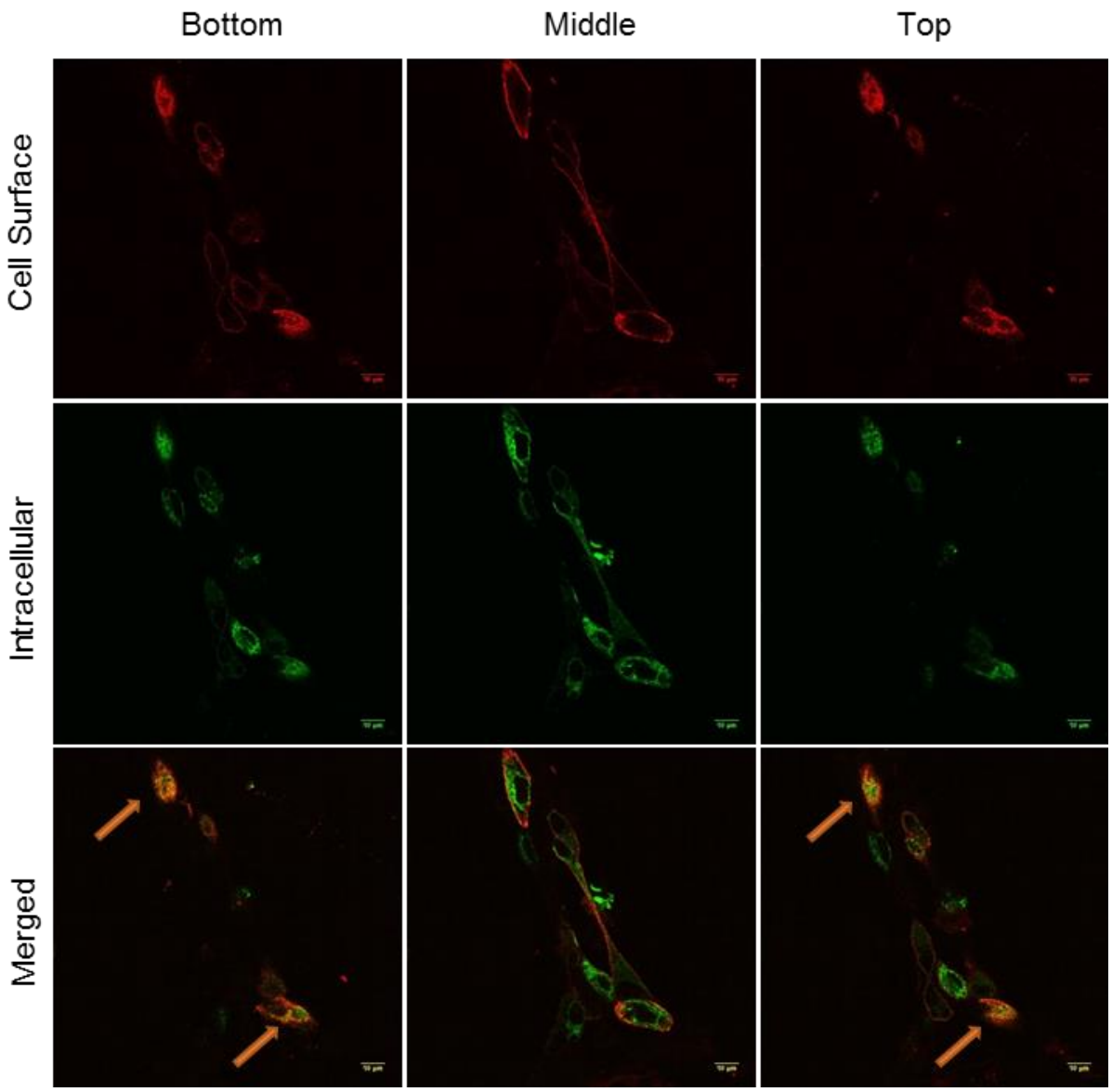


Figure 4.1- The cellular distribution of ZERO channels expressed in HEK293 cells cultured in 25mM glucose. A series of exemplar confocal images of HEK293 cells cultured in 25mM glucose and transfected with the BK channel construct ZERO. Each row shows three field of view (FOV) images taken from towards the bottom, middle and top (slices 2, 11 and 17) of a Z-stack comprised of 19 slices. The top row shows the Red (Alexa® 546) labelling that shows FLAG-tagged BK channels located at the cell membrane, with antibody and fluorophore labelling taken place prior to cell permeabilisation. The middle row contains Green (Alexa® 488) labelled intracellular FLAG-tagged BK channels. This labelling having taken place after cell permeabilisation. The red and green channel images were then merged as shown in the bottom row. Images show HEK293 cells from the control group, cultured in 25mM glucose. Orange arrows indicate areas of 'orange' in the FOV that are discussed in the chapter text. Image intensity settings were adjusted for illustrative purposes; intensity settings were not altered in images used for quantitative analysis. FOV area 146µm^2.

A

Group 5D1- Control 25mM Glucose	Average	Ratio to 25mM Control Average
Stack 1	0.68	1.07
Stack 2	0.53	0.84
Stack 3	0.60	0.96
Stack 4	0.64	1.01
Stack 5	0.67	1.06
Stack 6	0.67	1.06
AVERAGE	**0.63**	**1**
SD	**0.05**	**0.08**

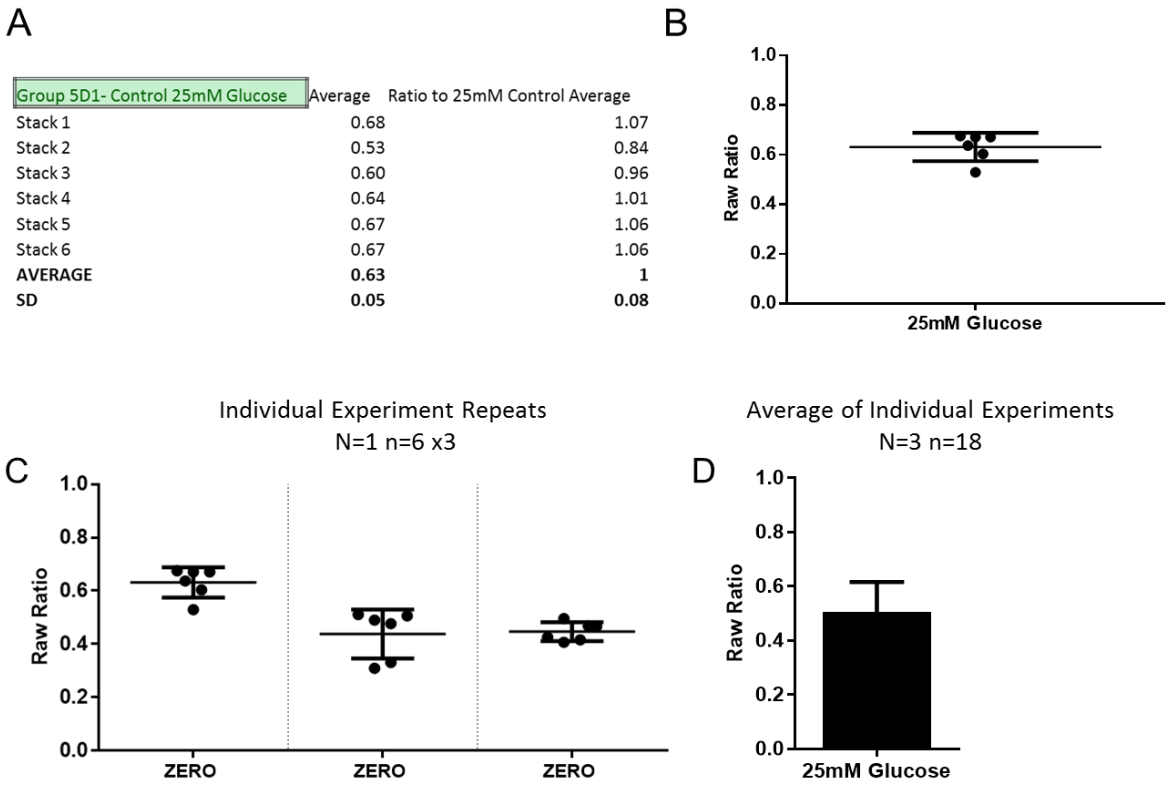


Figure 4.2- Analysis of the distribution of ZERO channels expressed in HEK293 cells cultured in 25mM glucose: Raw ratio and normalised values. A) The numerical values for the average raw ratio calculated for each of six individual stacked fields of view (n=6) from a single transfection/experiment (N=1), with the normalised value for each stack, which is a product of the individual raw ratio divided by the average for all six stacks (N=1, n=6). **B)** Scatter plot of the raw ratio values for the same 6 z-stack fields of view from a single transfection. Filled black circles indicate raw ratio values for each of the individual field of view stacks. The longer thinner horizontal line indicating the mean (0.63) for this group, and the thicker horizontal bars joined by a thin vertical line intersected by the mean indicates the range of a single standard deviation (SD): 0.05 in this case. **C)** Scatter plots of the raw ratio values for three separate experiments/transfections with the ZERO BK channel construct. The individual values of raw ratio for each stack, and the resulting means and SDs are shown (N=1, n=6 for each). **D)** Bar graph showing the mean raw ratio 0.51 ± 0.11 (N=3, n=18) that was calculated using the mean values from each FOV (n=18), rather than the average FOV value per experiment/transfection (N=3).

The raw ratio values for the SNP-mimetic mutant channels were determined, and ranged from 0.32 to 0.53 in H55Q transfected cells, 0.41 to 0.67 in G57A transfected cells and 0.44 to 0.60 in H55Q:G57A transfected cells with the mean raw ratio value of the H55Q mutant significantly lower than that of the G57A ($p<0.001$) and H55Q:G57A ($p<0.05$) mutants; though all three SNP mimetic mutants were not significantly different in mean raw ratio from ZERO (Figure 4.4). The H55Q mutant had a smaller proportion of total channels located in the membrane at 44% vs 51% and 57% for ZERO and H55Q:G57A respectively. This data may reflect a trend whereby the SNP-mimetics have an effect on membrane distribution but do not support the hypothesis that they significantly alter the baseline cellular distribution in comparison to the wild-type channel ZERO in 25mM glucose.

4.2.2.1 Relationship between cysteine residue predicted palmitoylation score and BK channel distribution

Nonetheless, the data may indicate that there is a relationship between palmitoylation of the S0-S1 loop cysteines and BK channel cell membrane insertion. If this is the case, then plotting the channel raw ratio against predicted palmitoylation score for the three cysteines should show a correlation. The first step was to plot the predicted palmitoylation scores for the three S0-S1 loop cysteines at C53, C54 and C56 for the wildtype ZERO and three SNP-mimetic mutants. One challenge was that when using a high threshold setting for the CSS Palm prediction software, the H55Q mutant returned a score for C56 of 0. This was overcome by setting a medium threshold in the CSS-Palm 4.0 software – that allowed the predicted score for the mutant to be calculated and did not alter the predicted scores returned for the other SNP mimetics.

Figure 4.5A shows the predicted palmitoylation scores of the cysteines C53, C54 and C56 for ZERO and each of the SNP-mimetic mutants, expressed as a normalised ratio of the palmitoylation score and the palmitoylation threshold value calculated by CSS-Palm 4.0 software (see methods). The normalised predicted palmitoylation score is similar across the mutants for C53 and C54 (0.9 to 1.0; and 1.0 to 1.1). In contrast, there are larger inter-mutant differences for C56 of 0.88 to 1.39 (Figure 4.5B), which has a similar in order/pattern to the normalised raw ratio values for the SNP-mimetics (Figure 4.4).

An assessment of the relationship between the predicted palmitoylation score of cysteine resides and the cell membrane expression of ZERO and SNP-mimetic mutant BK channels (Figure 4.6) showed no correlation between predicted palmitoylation score and SNP-mimetic raw ratio for C53 and C54. Pearson's correlation coefficient (PCC) value of +0.95 was obtained for C56, indicating a strong positive correlation between C56 predicted palmitoylation score and SNP-mimetic cell membrane expression.

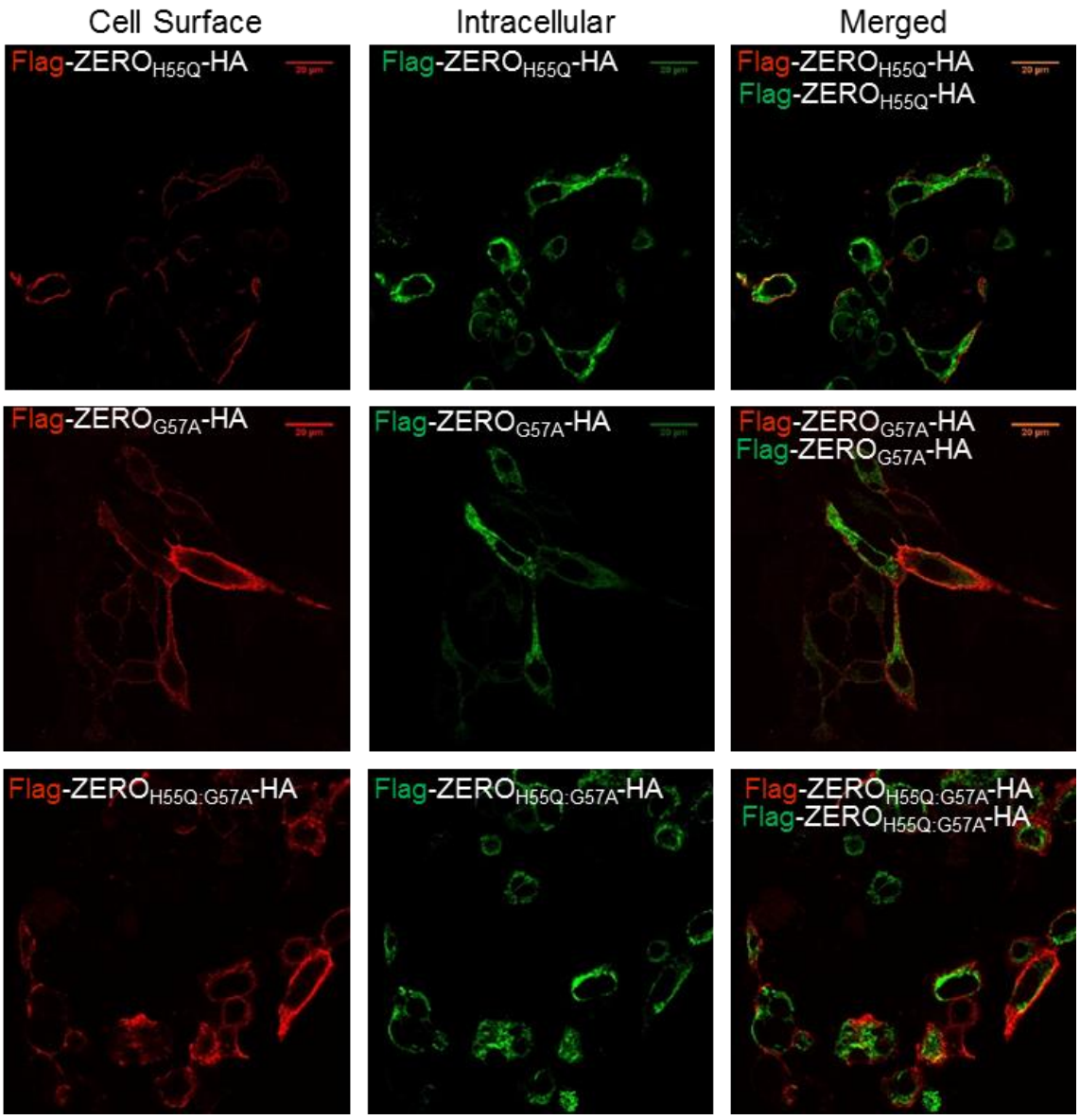


Figure 4.3- Confocal images of BK channel SNP mimetic mutants H55Q, G57A and H55Q:G57A: channel cellular distribution in HEK293 cells cultured in 25mM glucose. Exemplar single field of view, mid-stack, confocal images of HEK cells cultured in 25mM glucose and transfected with H55Q (top row), G57A (middle row) or H55Q:G57A (bottom row). The left column shows the **Red** (Alexa® 546) labelling that indicates BK channels located at the cell membrane, as the labelling had taken place prior to permeabilization. The middle column contains **Green** (Alexa® 488) labelled intracellular BK channels, as this labelling took place after cell permeabilisation. The red and green channel images were then merged in the right-hand column. Images show HEK293 cells cultured in 25mM glucose. Image intensity settings were adjusted to allow channel labelling to be clear for illustrative purposes; intensity settings were not altered in images used for quantitative analysis. FOV area 146µm^2.

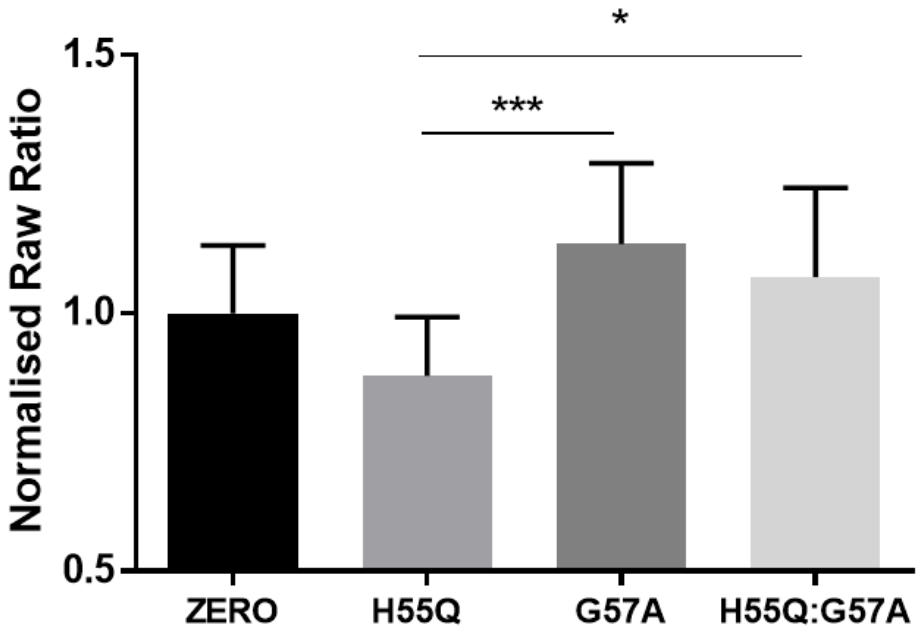


Figure 4.4- ZERO and SNP mimetic BK channels H55Q, G57A and H55Q:G57A distribution in HEK293 cells cultured in 25mM glucose. Normalised raw ratio values in 25mM glucose; the proportion of total H55Q channels at the membrane was significantly lower in comparison to G57A and H55Q:G57A. ZERO raw ratio= 0.51± 0.11, H55Q raw ratio= 0.44 ± 0.05, G57A= 0.57 ± 0.08 and H55Q:G57A= 0.54 ± 0.09. Normalised values are ZERO= 1, H55Q= 0.88, G57A= 1.13, H55Q:G57A= 1.07. Data expressed as mean ± SD. N=3, n=17-18 *=p<0.05 ***=p<0.001 one-way ANOVA with *post hoc* Tukey test analysis.

This supports the hypothesis that the proportion of BK channels in the cell membrane follows the CSS-Palm 4.0 calculated pattern of C56 palmitoylation probability, with H55Q mutant having the lowest cell membrane expression and G57A the highest.

As a result of this close relationship in the pattern of C56 predicted palmitoylation and cellular distribution of the SNP-mimetic mutants, it was of interest to investigate the cysteine mutant constructs, and establish if the same relationship and pattern was evident in their cellular distribution. In other studies, the C53:54:56A mutant had a reduced membrane expression in comparison to ZERO, and this was linked to a reduction in palmitoylation due to the removal of cysteines C53, C54 and C56. There is less information available for the C53:54A mutant in comparison to C53:54:56A mutant.

Assessment of the distribution of cysteine mutants C53:54A and C53:54:56A in HEK293 cells cultured in 25mM glucose.

The mean raw ratio values obtained from the ZERO BK channel construct were then compared with those containing double cysteine mutations (C53:54A) and triple cysteine mutations (C53:54:56A). Exemplar confocal field of view images are shown in Figure 4.7A and raw ratio values indicate that the double cysteine mutant had a significantly increased proportion of total BK channels in the cell membrane, with a 23% increase in comparison to ZERO; while the triple cysteine mutant showed no significant difference from ZERO but had 21% less channel in the membrane than the double mutant (Figure 4.7B).

The C56 predicted palmitoylation score for the cysteine triple mutant is null because the cysteine residue has been mutated out. Interestingly the C56 score for the cysteine double mutant is also null at all detection thresholds even though the cysteine residue is present in the amino acid sequence, and has been shown to be palmitoylated by experimentation with radiolabelled palmitate (Jeffries, 2010). The consequence of this is that there is no predicted palmitoylation pattern for C56 for the cysteine mutants. Nevertheless, the higher cell membrane expression of the C53:54A mutant in comparison with that of the triple mutant C53:54:56A, may support a role for the C56 residue as it is present in the double mutant and thus available for palmitoylation- despite the lack of predicted score from the software. The increase in the membrane expression of the double cysteine mutant in comparison with ZERO may reflect the balance between the role for C56 with the potentially negative correlation with membrane expression seen with C53 and C54 (Figure 4.6).

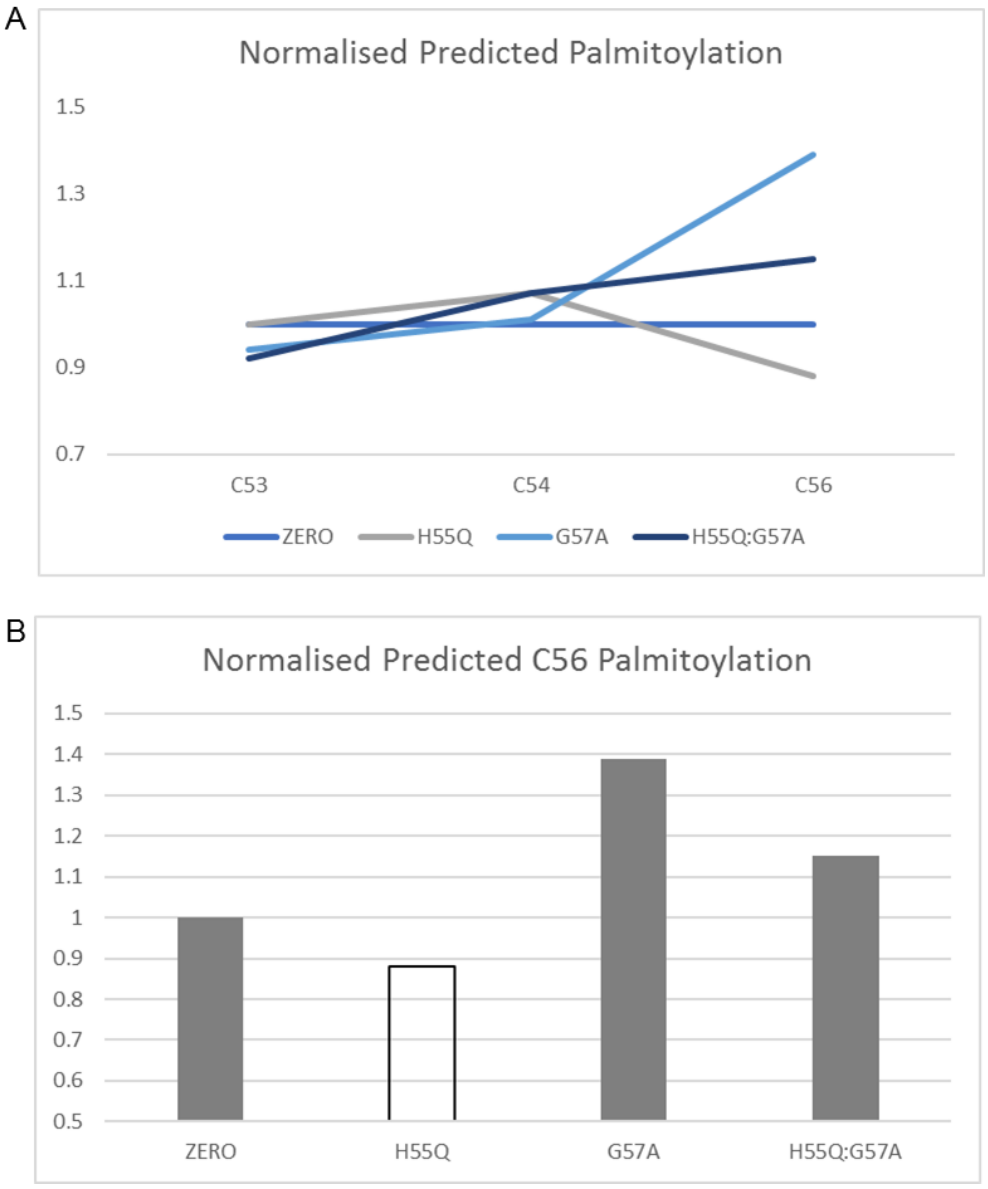


Figure 4.5- Normalised predicted palmitoylation scores for the S0-S1 loop cysteines for ZERO and the SNP-mimetic mutants H55Q, G57A and H55A:G57A. A) The predicted palmitoylation scores generated by the CSS-Palm 4.0 software have been normalised to ZERO with a score of 1.0 (100%); the deviation from ZERO is then shown for cysteines C53, C54 and C56. Mutation of the BK channel amino acid sequence to that of the SNP-mimetics H55Q, G57A and H55Q:G57A has little impact on predicted palmitoylation of C53 or C54, with greater inter-mutant differences present for C56. **B)** C56 normalised predicted palmitoylation scores show the raised G57A and H55Q:G57A palmitoylation scores in comparison to ZERO and the lower value for H55Q. CSS-Palm 4.0 scores are detailed in chapter 3.

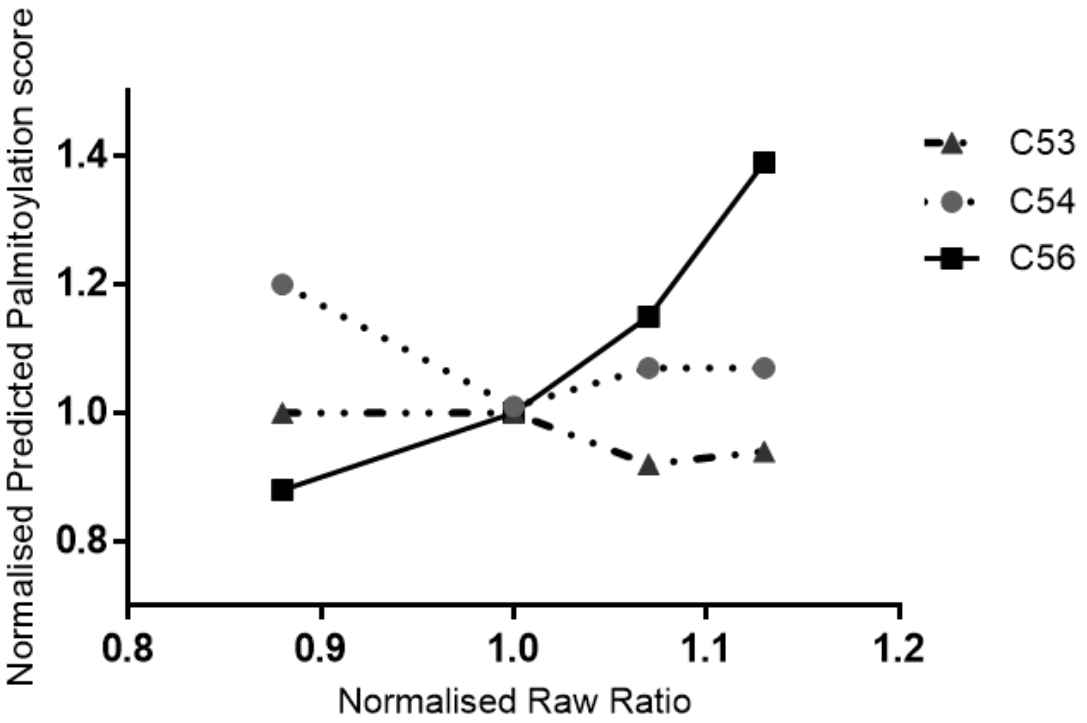


Figure 4.6- Correlation between predicted palmitoylation score and normalised raw ratio for BK channel constructs. The normalised predicted palmitoylation scores for cysteine residues C53, C54 and C56 for each of the SNP-mimetic mutants were plotted against the normalised raw ratio values for the SNP-mimetic mutants. A Pearson's correlation coefficient (PCC) value of +0.95 was obtained for C56, indicating a strong positive correlation between predicted palmitoylation score and cell membrane expression. Weaker negative correlation was observed for C53 and C54 with PCC values of -0.80 and -0.66.

Comparison of the cellular distribution of the cysteine mutants C53:54A and C53:54:56A and the SNP-mimetic mutants revealed a significant difference between H55Q and C53:54A (mean raw ratio 0.44 ± 0.05 vs 0.62 ± 0.10). The lack of predicted palmitoylation score for the C56 residue in the C53:54A mutant means that this difference with the lowest scoring SNP-mimetic mutant H55Q cannot currently be fitted to the C56 model and once again raises issues with the completeness of the algorithm and model used to calculate predicted palmitoylation and is discussed in more detail elsewhere.

4.2.3 Assessment of the effect of glucose on the cellular distribution of the SNP mimetic and cysteine mutant BK channels in HEK293 cells

Glucose is an important, tightly regulated, metabolic substrate with a vital, yet complex role in mammalian cellular systems and has an influence on ion channel distribution through a range of mechanisms including potential alterations in the balance of PTMs via phosphorylation and lipid metabolism. The simplest approach was to investigate the effect that a change in glucose concentration would have on the cellular distribution of the BK channel, and whether there were any patterns in this cellular distribution associated with S0-S1 linker mutations. This was achieved by seeding and culturing HEK293 cells in 25mM glucose for 48 hours prior to transfection with ZERO or the SNP mimetic mutants H55Q, G57A and H55Q:G57A. then maintaining the cells in a lower concentration of glucose (5mM) for 24 hours before the cells were fixed and imaged.

The 5mM glucose treatment resulted in an increase in membrane-bound ratio for both ZERO and H55Q channels in comparison to their distribution in 25mM glucose, and a decrease for both G57A and H55Q:G57A mutants (Figure 4.8) though only the H55Q mutant displayed a statistically significant change in membrane-bound ratio in comparison to its 25mM glucose equivalent ($P<0.05$). In addition, there was a significant inter-group difference between the SNP-mimetic mutants. The mean raw ratio for H55Q:G57A was 19% lower than that of the H55Q mutant ($p<0.05$). Furthermore, the pattern of inter-SNP-mimetic relationship based upon C56 predicted palmitoylation highlighted earlier in 25mM glucose was inverted. In 5mM glucose the mutant channel with the lowest C56 predicted palmitoylation (H55Q) has the highest cell membrane expression, and one of the channels with a C56 score higher than ZERO (H55Q:G57A) has the lowest cell membrane expression. This may indicate that the inter-SNP-mimetic cellular distribution pattern can modified by the glucose concentration of the cell culture medium.

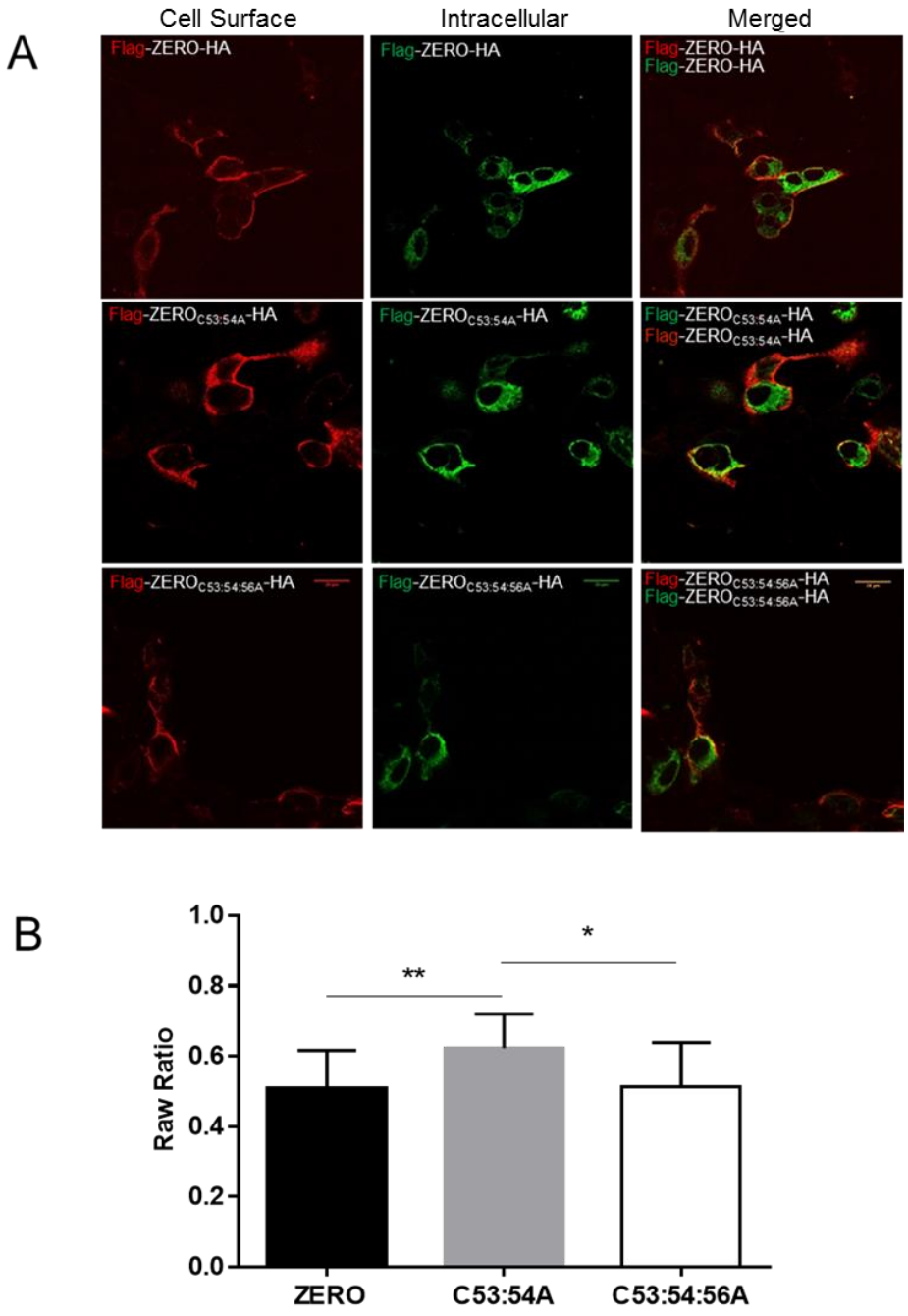


Figure 4.7- ZERO, C53:54A and C53:54:56A cysteine mutant channel distribution in HEK293 cells cultured in 25mM glucose. A) Exemplar confocal field of view images of HEK cells cultured in 25mM glucose and transfected with ZERO, C53:54A or C53:54:56A BK channel constructs. The left column shows the Red (Alexa® 546) labelling that indicates BK channels located at the cell membrane. The middle column contains Green (Alexa® 488) labelled intracellular BK channels. The red and green channel images were then merged in the right-hand column **B)** Comparison of BK channel construct mean raw ratio values; the proportion of C53:54A (raw ratio= 0.62 ± 0.10) channels at the membrane was significantly higher than ZERO (0.51 ± 0.11) and C53:54:56A (0.51 ± 0.13). Data expressed as mean ± SD. N=3 n=18. *=p<0.05 **=p<0.01, one-way ANOVA with *post hoc* Tukey test, FOV area 146µm^2

In 5mM glucose, there was no significant difference in cellular distribution between the C53:54A and C53:54:56A mutants (mean raw ratio value of 0.56 for both). There was also no difference found between ZERO (mean 0.56) and the cysteine mutants. The pattern of raised C53:54A mean raw ratio observed in 25mM glucose was absent in the 5mM glucose, and there was no also significant difference between the mean cellular distribution values of H55Q, C53:54A and C53:54:56A.

4.2.4 Assessment of the effect of palmitate on the cellular distribution of the SNP mimetic BK channels in HEK293 cells

Given the impact that a change in glucose concentration can have on BK channel distribution, the next step was to investigate the influence of palmitate concentration as it is also a metabolic substrate, and plays a role in several cellular signalling mechanisms and a link has been identified between the predicted palmitoylation score of the BK channel C56 cysteine residue and the membrane expression of the channel.

The impact of an increased concentration of palmitate in HEK293 cell growth medium on the proportion of total BK channels located in the cell membrane was undertaken by transiently expressing the BK channel ZERO or SNP mimetic mutants in HEK293 cells in 25mM glucose media containing 0.4mM palmitate. Exemplar confocal field of view images for H55Q and H55Q:G57A expressing cells cultured in 25mM glucose and 0.4mM palmitate are shown in Figure 4.9A. There was no statistically significant difference between the cell membrane expression of ZERO and the SNP mimetic mutants. However, when the individual SNP-mimetic mutants were assessed for variation, H55Q displayed a significant, 21% reduction in comparison with H55Q:G57A (Figure 4.9B). The 'C56 palmitoylation pattern' seen in 25mM glucose was maintained in 25mM glucose and raised palmitate concentration. In addition, culturing cells in 0.4mM palmitate resulted in a significantly increased mean cell membrane expression of ZERO to 64% (range 52% to 0.79%) from 51% (range 31% to 0.68%) in 25mM glucose control (Figure 4.9C). Palmitate addition lead to increased cell membrane expression across all the SNP-mimetic mutants in 25mM glucose, with significant increases for H55Q (from 44% to 56%) and H55Q:G57A (from 54% to 68%).

Earlier in the chapter, the concentration of glucose in the culture media was shown to affect the cellular distribution of BK channels. Therefore, to assess if glucose concentration changes the nature of the palmitate effects, an assessment of the cell membrane expression of BK channel constructs expressed in HEK293 cells cultured in 0.4mM palmitate was repeated, but this time with the lower glucose concentration of 5mM in the cell culture medium.

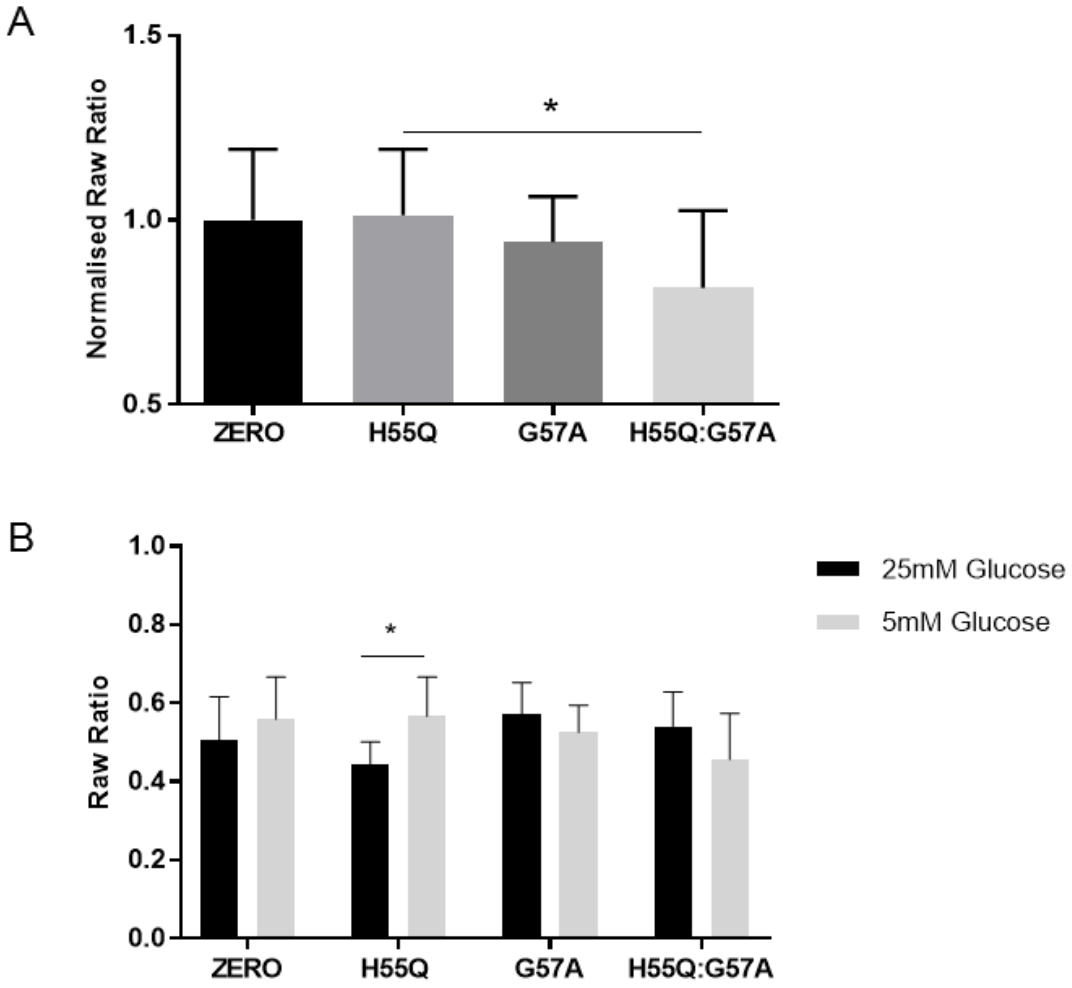


Figure 4.8- ZERO and SNP mimetic BK channels H55Q, G57A and H55Q:G57A cell membrane expression in HEK293 cells cultured in 5mM glucose. A) Comparison of BK channel construct raw ratio values in 5mM glucose, normalised to the ZERO mean raw ratio; the reduced raw ratio value of H55Q:G57A indicates that the proportion of total H55Q:G57A channels at the cell membrane was significantly reduced in comparison to H55Q alone. **B)** The raw ratio values for ZERO and the SNP-mimetic mutants in 5mM glucose are shown in comparison to the corresponding 25mM group; H55Q raw ratio is significantly reduced in 25mM glucose. Normalised values in 5mM glucose are ZERO= 1.0, H55Q= 1.01, G57A= 0.94, H55Q:G57A= 0.82. Mean raw ratio values in 5mM glucose are ZERO= 0.56, H55Q= 0.57 ± 0.10, G57A= 0.53 ± 0.07, H55Q:G57A= 0.46 ± 0.12. Data expressed as mean ± SD. N=3 n=18 *=p<0.05. One-way ANOVA with *post hoc* Tukey test.

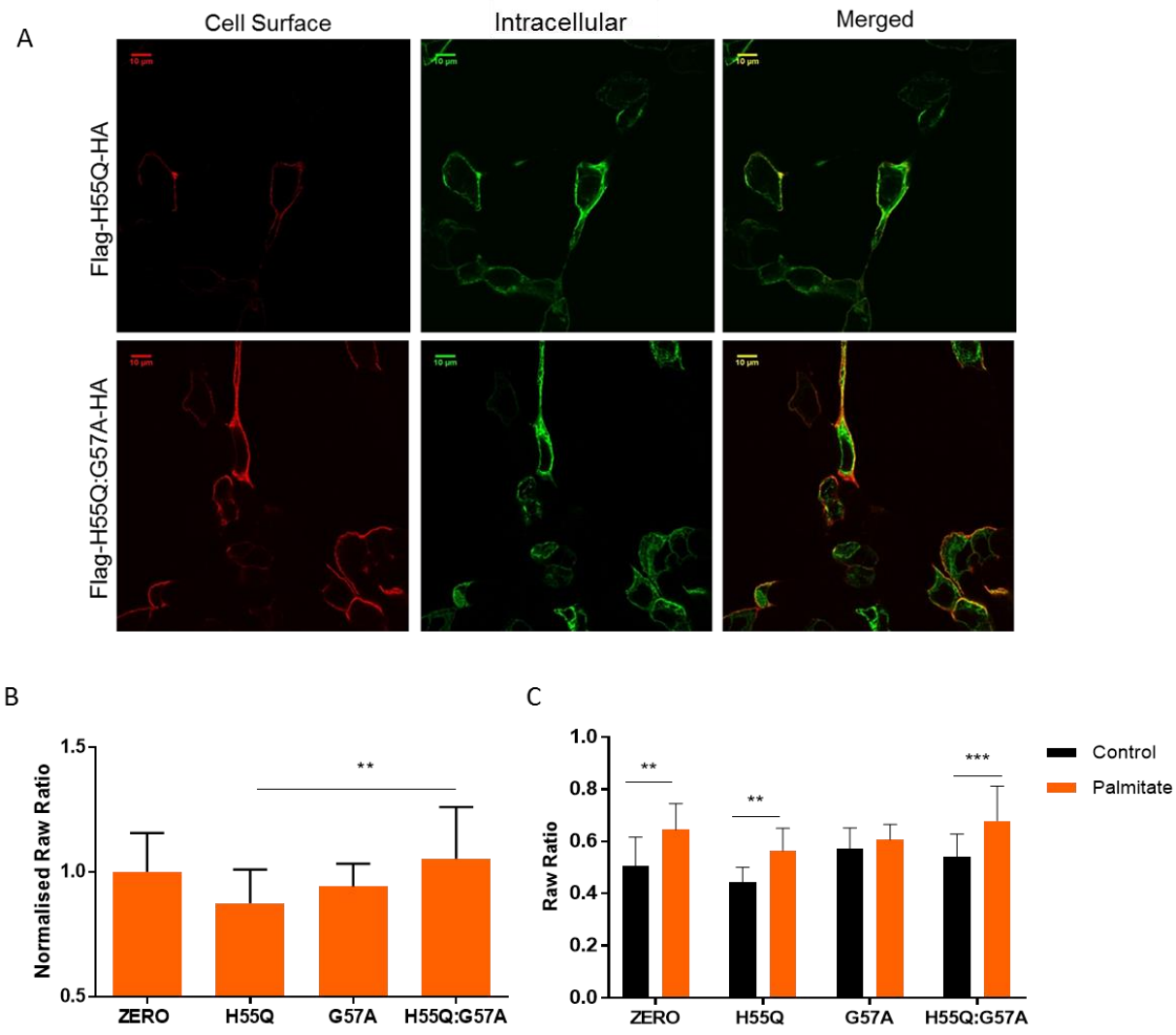


Figure 4.9- ZERO and BK channel SNP mimetic mutants channel distribution in HEK293 cells cultured in 25mM glucose and 0.4mM Palmitate. A) Exemplar FOV image of H55Q and H55Q:G57A transfected cells with non-permeabilized and permeabilized BK channel fluorescent tagging. The left column shows the **Red** (Alexa® 546) labelling that indicates BK channels located at the cell membrane, as the labelling had taken place prior to permeabilization. The middle column contains **Green** (Alexa® 488) labelled intracellular BK channels, as this labelling took place after cell permeabilisation. The red and green channel images were then merged in the right-hand column. Images show Image intensity settings were adjusted to allow channel labelling to be clear for illustrative purposes; intensity settings were not altered in images used for quantitative analysis. FOV area 146µm^2. **B)** The proportion of H55Q:G57A channels at the membrane was significantly greater than in the H55Q mutant. **C)** Comparison of BK membrane expression of cells cultured in 25mM glucose with and without palmitate. Palmitate significantly increased the membrane expression of ZERO, H55Q and H55Q:G57A. Raw ratio H55Q= 0.56 ± 0.09, G57A= 0.61 ± 0.06, H55Q:G57A= 0.68 ± 0.13. Data expressed as mean ± SD. N=3 n=12-18 **=p<0.01. One-way ANOVA with *post hoc* Tukey test.

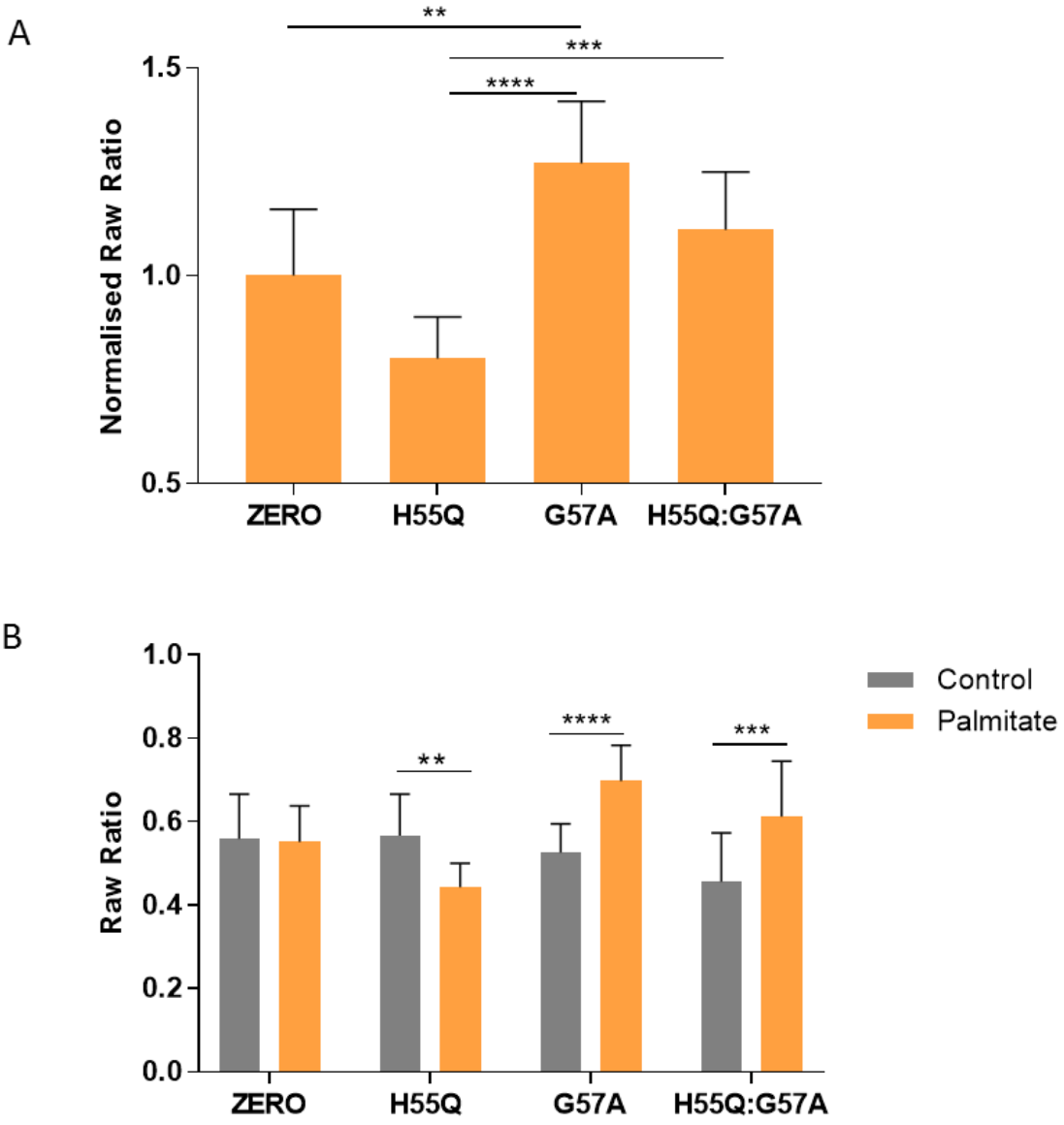


Figure 4.10- ZERO and BK channel SNP mimetic mutants channel distribution in HEK293 cells cultured in 5mM glucose and 0.4mM Palmitate. A) Reduced H55Q and increased G57A cell membrane expression results in multiple significant differences between BK channel mutants. G57A membrane expression alone was significantly different from ZERO; whilst the H55Q mutant was significantly different to G57A and H55Q:G57A. **B)** Comparison of BK membrane expression of cells cultured in 5mM glucose with and without palmitate. Palmitate significantly increased the membrane expression of G57A and H55Q:G57A, whilst reducing H55Q membrane expression. Raw ratio H55Q= 0.44 ± 0.06, G57A= 0.70 ± 0.08, H55Q:G57A= 0.61 ± 0.13. Data expressed as mean ± SD. N=3 n=16-18 **=p<0.01, ***=p<0.001, ****=p<0.0001. One-way ANOVA with post hoc Tukey test.

In 5mM glucose and 0.4mM palmitate the G57A mutant was the only SNP-mimetic mutant with a cell membrane expression that was statistically different to ZERO. As before there were significant differences in the cell membrane expressions of H55Q and G57A and H55Q:G57A (47% and 31% respectively). The 'C56 palmitoylation pattern' is apparent again under these condition in the cell membrane expression values of the SNP-mimetic mutants.

Palmitate addition did not significantly alter the cell membrane expression of ZERO in 5mM glucose (Figure 4.10B), though the cell membrane expression of H55Q was significantly lower (23%) than the corresponding 5mM control group. For mutants G57A and H55Q:G57A, palmitate addition significantly increased cell membrane distribution by 32% and 33% respectively. Thus, the addition of palmitate to the 5mM glucose media appears to result in the return of cellular distribution of the SNP-mimetic channels to fit with the C56 predicted palmitoylation scoring pattern seen initially with 25mM glucose alone.

4.2.5 Assessment of the effect of palmitate on the cellular distribution of the cysteine mutant BK channels in HEK293 cells

The effect of 0.4mM palmitate addition on BK channel cysteine mutants, C53:54A and C53:54:56A was also investigated. Results showed that there was no significant difference in mean raw ratio between the cysteine mutant channels, with variation from ZERO of 0% and 8% for C53:54A and C53:54:56A respectively. The membrane expression range was between 52% to 79% for ZERO; the double and triple cysteine mutants had a range from 52% to 80% and 43% to 72% respectively. The mean raw ratio of C53:54A and C53:54:56A was not significantly increased in cells cultured in 25mM glucose and 0.4mM palmitate when compared to equivalent cells cultured in the same glucose concentration in the absence of palmitate.

In the lower glucose concentration, C53:54A mean raw ratio was 21% higher than that of ZERO (Figure 4.11). There was no significant difference between 5mM glucose control and 5mM glucose and 0.4mM palmitate groups or 25mM glucose plus 0.4mM palmitate groups for ZERO, C53:54A or C53:54:56A. There was no significant difference in the mean raw ratio values of ZERO, the SNP-mimetic mutants and the cysteine mutants in 25mM glucose and 0.4mM palmitate.

A

BK Channel Construct	Raw Ratio	Standard Deviation	Number of FOV	Change from 5mM Control Equivalent	Change from 25mM Equivalent
Zero	0.551	0.088	17	-1%	-9%
C53:54A	0.664	0.167	18	+16%	+3%
C53:54:56A	0.582	0.115	17	+5%	-1%

B

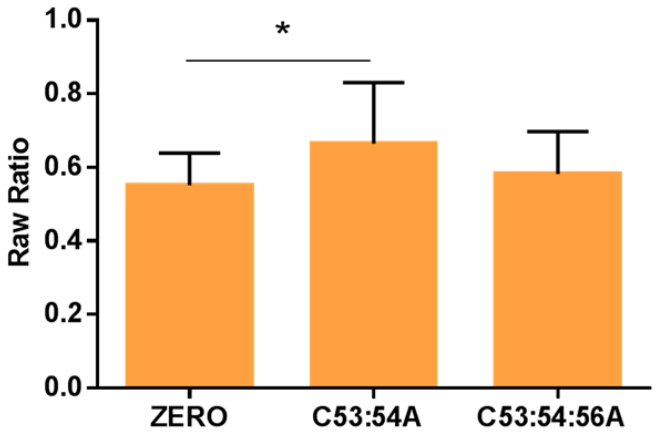


Figure 4.11- ZERO, C53:54A and C53:54:56A cysteine mutant channel distribution in HEK293 cells cultured in 5mM glucose and 0.4mM palmitate. A) Table detailing the raw ratio values, standard deviation, number of fields of view, and percentage difference in raw ratio between the equivalent 5mM glucose control and equivalent 25mM glucose plus 0.4mM palmitate group, for ZERO, C53:54A and C53:54:56A mutants. Raw ratio values were not significantly different between the control group and palmitate groups, as well as in comparison to the equivalent 25mM glucose + 0.4mM palmitate groups. **B)** C53:54A raw ratio was significantly increased in comparison to Zero when the 5mM glucose plus 0.4mM palmitate values were analysed and compared against each other using Tukeys *post hoc* analysis. One-way ANOVA for statistical analysis.

The results were more varied in 5mM glucose and 0.4mM palmitate, with the previously described high mean raw ratio of the G57A mutant that is significantly raised compared to ZERO and H55Q. H55Q is also significantly lower than C53:54A and C53:54:56A. When comparing the synthetic mutants that remove predicted C56 palmitoylation to the endogenous SNP, there is no significant difference between H55Q, G57A and C53:54:56A in 25 or 5mM glucose.

4.2.6 Assessment of the effect of oleate on the cellular distribution of the SNP mimetic BK channels in HEK293 cells

In a final series of experiments the influence of oleate on BK channel distribution was investigated. Oleate has been shown to compensate for the perturbing effects of excess palmitate and its effect on BK channel distribution has not previously been described.

Exemplar confocal field of view images of HEK293 cells cultured in 25mM glucose with and without addition of 0.4mM oleate are shown in Figure 4.12A. In 25mM glucose with 0.4mM oleate, there was no significant difference between the cell membrane expression of ZERO and the SNP-mimetic mutants. The membrane expression of the H55Q channel construct was significantly increased by 18% in comparison to H55Q:G57A. As H55Q was once again significantly different from a mutant with a higher predicted palmitoylation score than ZERO, it indicates that the cellular distribution of BK channels may still be influenced by palmitoylation in the presence of oleate (Figure 4.12B).

Furthermore, there was no significant difference in the cell membrane expression of ZERO as a result of oleate addition (Figure 4.12C). Unlike palmitate, which increased the proportion of all channels in the membrane oleate only increased the proportion of the H55Q mutant, which was 48% higher than the control equivalent. H55Q was the only SNP-mimetic mutant to have a mean raw ratio in the oleate group that was significantly different from that of the equivalent in the control group.

To determine if the effects of oleate addition were conditional on the baseline glucose concentration of the cell culture medium, the experiments were repeated in 5mM glucose where the H55Q mean raw ratio was again significantly higher than H55Q:G57A, with a difference of 15% between the groups (Figure 4.13A). There was also a significant difference in the cell membrane expression of H55Q and H55Q:G57A of 18%.

In 5mM glucose, there was no significant difference between the cell membrane expression of ZERO in control or oleate treated groups (Figure 4.13B). In these low glucose conditions, the G57A mutant alone was the only SNP-mimetic mutant to have a significantly different raw ratio as a result of oleate addition, with a mean change of 17% in cell membrane expression between oleate and control groups.

4.2.7 Assessment of the effect of oleate on the cellular distribution of the cysteine mutants BK channels in HEK293 cells

The C53:54A and C53:54:56A mutants exposed to 25mM glucose and 0.4mM oleate showed no significant difference in the cell membrane expression at 57% and 54% respectively. There was also no significant difference in the cellular distribution of ZERO and the cysteine mutant channels under these conditions nor was there any significant difference between control and oleate treated groups in 25mM, with differences in cell membrane expression of 9% and 6% respectively. Finally, the mean raw ratio of the C53:54A and C53:54:56A mutants exposed to 5mM glucose and 0.4mM oleate were analysed and there was no significant difference in the cell membrane expression between the groups (56% and 60% respectively).

To get a better understanding of the relationship between the SNP-mimetic and the cysteine mutant channels in conditions of raised oleate, the cell membrane expression values of the two groups were compared. There was a significant 22% difference in the mean raw ratio of H55Q in comparison to C53:54:56A in 25mM glucose but there was no significant difference in the mean raw ratio values of the SNP-mimetic and the cysteine mutants in 5mM glucose and 0.4mM oleate.

4.2.8 Assessment of the differential effect of fatty acids on the membrane expression of ZERO and the BK channel mutants.

The addition of 0.4mM extracellular oleate resulted in an inverted SNP-mimetic palmitoylation pattern in comparison to cells treated with 0.4mM palmitate, therefore it was of interest to compare the effect of oleate and palmitate addition on the cellular distribution of BK channel constructs individually. In 25mM glucose, palmitate addition resulted in a significantly increased cell membrane expression in ZERO and H55Q:G57A (increases of 18% and 22% respectively) in comparison to oleate; whilst on the other hand the cell membrane expression of H55Q was significantly reduced by 16% (Figure 4.14A). The cellular distribution of the G57A mutant was not affected. In low glucose, the pattern was similar; although the type of fatty acid added did not significantly affect the cell membrane expression of ZERO with a difference of 5%, palmitate significantly increased that of H55Q:G57A and G57A by 16% and 11% respectively, however reduced H55Q membrane expression by 36% in comparison to the oleate groups (Figure 4.14B). As was the case in 5mM glucose plus palmitate in comparison to the same 25mM group, inter-SNP-mimetic differences are most clear in low glucose; here there is a clear pattern that reduced C56 palmitoylation status makes the channel more likely to be affected by oleate concentration, whilst increased likelihood of C56 palmitoylation results in channels that are more likely to go to the membrane in the presence of palmitate.

There was no significant difference between the cellular distribution of the cysteine mutant channels in 25mM glucose; for C53:54A and C53:54:56A there was an 11% and 8% difference between palmitate and oleate groups respectively. However, under low glucose conditions, there was a significant difference in C53:54A, where exposure to palmitate increased cell membrane expression by 15% in comparison to the oleate group. Once again, there was no difference in C53:54:56A membrane expression in palmitate in comparison to oleate.

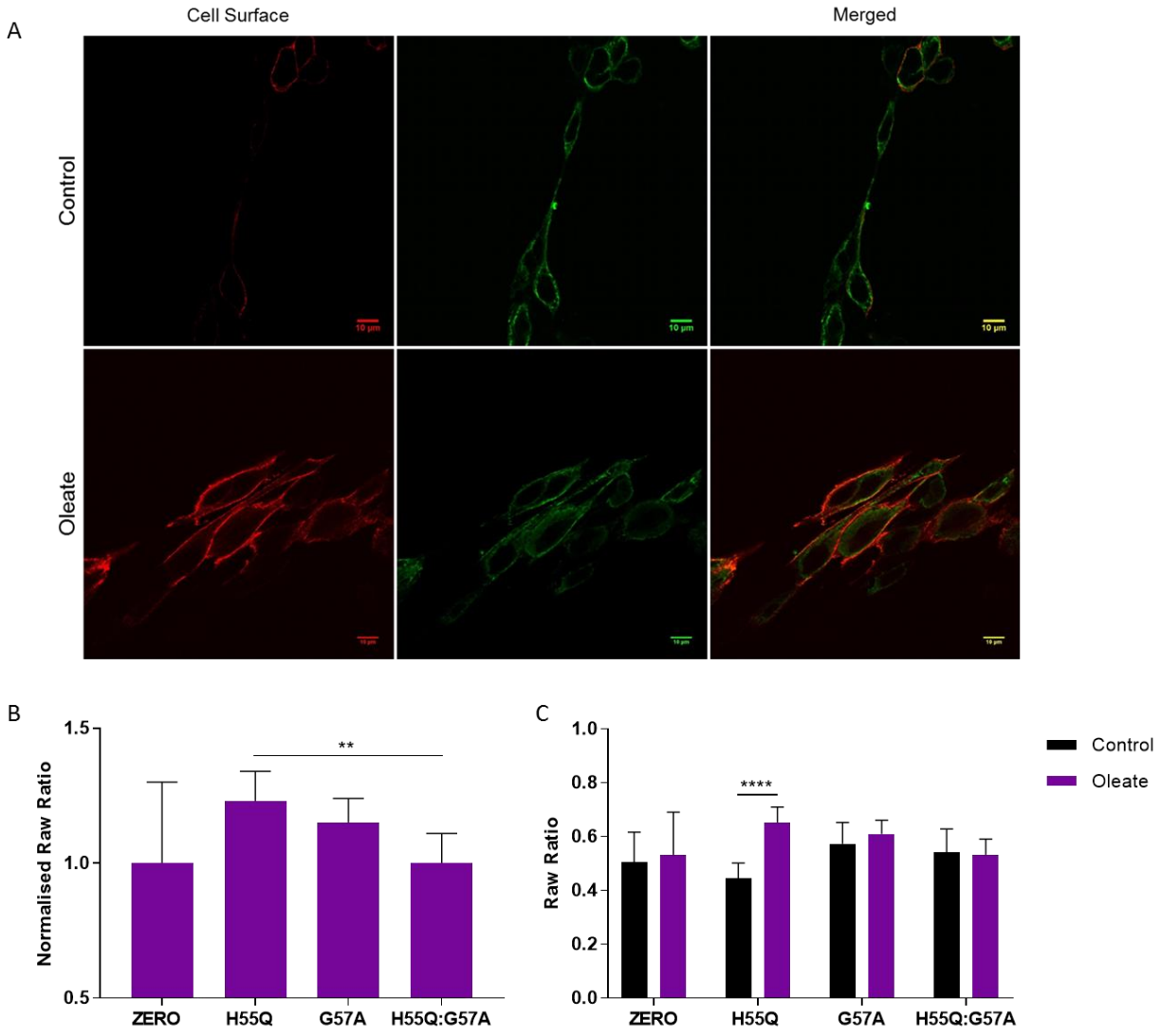


Figure 4.12- ZERO and BK channel SNP mimetic mutants channel distribution in HEK293 cells cultured in 25mM glucose with and without 0.4mM oleate. A) Exemplar FOV image of H55Q transfected cells in 25mM glucose with non-permeabilized and permeabilized BK channel fluorescent tagging. Red (Alexa® 546) labelling indicates BK channels located at the cell membrane; the middle column contains Green (Alexa® 488) labelled intracellular BK channels and red and green channel images were then merged in the right-hand column. Image intensity settings were adjusted to allow channel labelling to be clear for illustrative purposes; intensity settings were not altered in images used for quantitative analysis. FOV area 146µm2. B) Bar graph showing significantly different membrane expression between H55Q and H55Q:G57A. Raw ratio: 25mM glucose and 0.4mM oleate ZERO 0.54 ±0.16, H55Q 0.65 ±0.06, G57A 0.61 ± 0.05, H55Q:G57A 0.53 ±0.06. **C)** Comparison of BK membrane expression of cells cultured in 25mM glucose with and without oleate. Oleate significantly increased the membrane expression of H55Q. Data expressed as mean ± SD. N=3 n=13-18, **=p<0.01, ****=p<0.0001. One-way ANOVA with post hoc Tukey test.

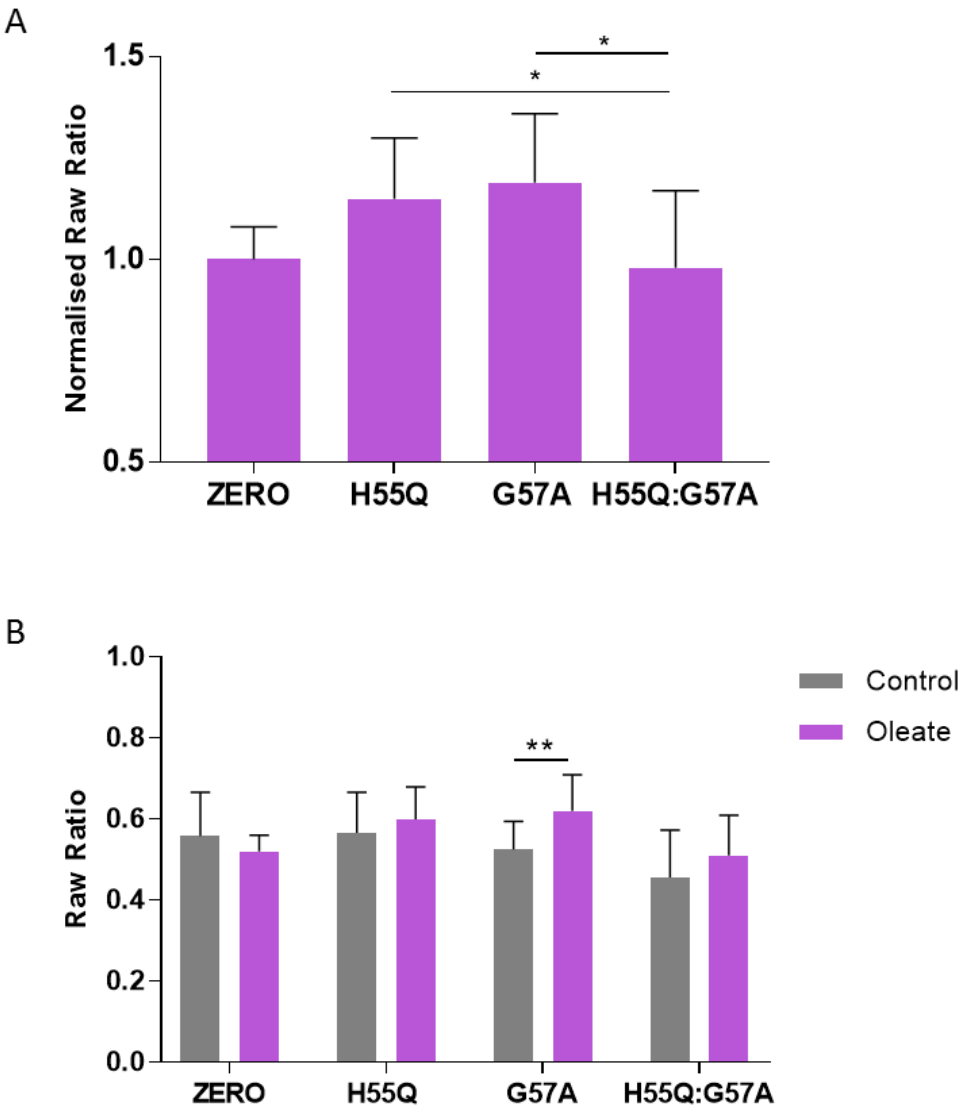


Figure 4.13- ZERO and BK channel SNP mimetic mutants channel distribution in HEK293 cells cultured in 5mM glucose with and without 0.4mM oleate. A) There was a significant difference in the raw ratio values between H55Q and H55Q:G57A in HEK293 cells cultured in 5mM glucose and 0.4mM oleate. 5mM glucose Zero 0.52 ±0.04, H55Q 0.60 ± 0.08, G57A 0.62 ±0.09, H55Q:G57A 0.51 ± 0.10. N=3 n=14-18. B) Comparison of BK membrane expression of cells cultured in 5mM glucose with and without oleate. Oleate significantly increased the membrane expression of G57A.Data expressed as mean ± SD. N=3 n=18. *=p<0.05, **=p<0.01, one-way ANOVA with *post hoc* Tukey test.

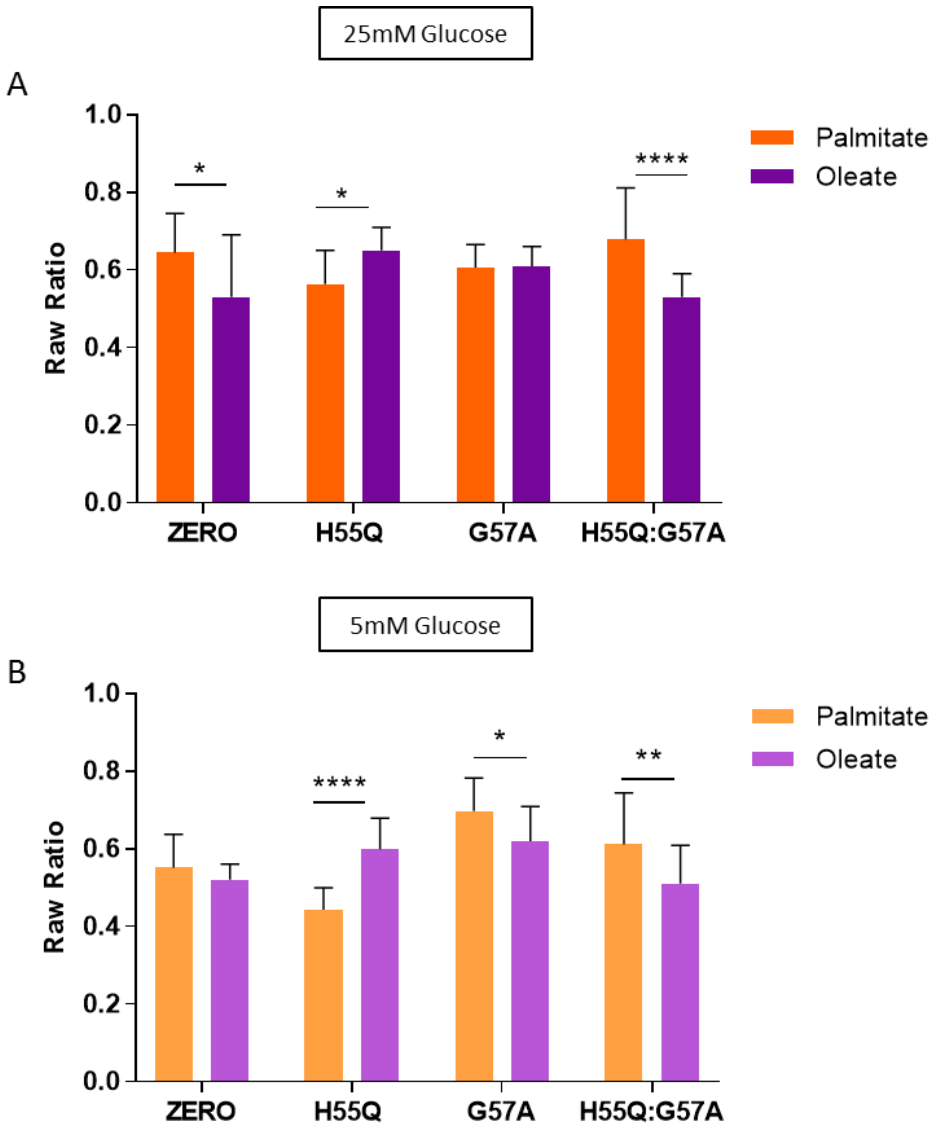


Figure 4.14- The effect of fatty acid on the cell membrane expression of ZERO and SNP-mimetic channels cultured in 25mM or 5mM glucose. The raw ratio of ZERO and the SNP-mimetic mutants in HEK293 cells cultured in either **A)** 25mM glucose or **B)** 5mM glucose and treated with either 0.4mM palmitate or 0.4mM oleate for 24 hours. In 25mM glucose, there were significant differences in fatty acid treatments for ZERO, H55Q and H55Q:G57A constructs, whilst in 5mM glucose H55Q, G57A and H55Q:G57A had fatty acid mediated differences in cell membrane expression Data expressed as mean ± SD. N=3 n=13-18, *=p<0.05, **=p<0.01, ****=p<0.0001. One-way ANOVA with post hoc Tukey test.

4.3 Discussion

This is the first study to investigate the relationship between the cellular distribution of the SNP-mimetic BK channels (H55Q, G57A and H55Q:G57A), the cysteine mutant channels (C53:54A and C53:54:56A), and the predicted palmitoylation score of cysteine residues within the S0-S1 linker region of the channel. In addition, there is novel data on the effects of differing concentrations of glucose, with the addition of palmitate or oleate, on the membrane-bound ratio of BK channel SNP-mimetics.

4.3.1 The cell membrane expression of the BK 'wild-type' ZERO channel

The first step of the investigation focused on assessing the cellular distribution of the ZERO isoform of the BK channel in 25mM glucose as utilised in previous BK channel studies with the HEK293 cell line (Shipston *et al.*, 1999; Shipston, 2001; McCartney *et al.*, 2005; Tong *et al.*, 2006; Tian *et al.*, 2008; Saleem, Rowe and Shipston, 2009; Chang *et al.*, 2011).

The aim was to quantify, via ratio, the distribution of the ZERO channel variant in the cell membrane using double-FLAG antigen tag labelling of the BK channel in stacked sections across a field of view that contained several cells rather than the single cell or single stack approach that had been utilised in a previous study (Chen *et al.*, 2005). Many studies do not state the confocal imaging protocol in detail (Barden *et al.*, 2003; Ishiwata *et al.*, 2004; Sheng and Acquaah-Mensah, 2011; Wu *et al.*, 2013) and thus it can be difficult to make direct comparisons.

Nevertheless, the FLAG-FLAG immunocytochemistry method provided data from which the percentage of BK channels in the membrane could be calculated and indicating that between 31% and 68% of total BK channels were located at the cell membrane at the time of staining, with a mean value of 51%. The FLAG-FLAG method had been used in one other study of BK channel membrane distribution. Unfortunately, the results do not include a breakdown of the relative proportion of total BK channels located in the cell membrane and thus results could not be compared (Chen *et al.*, 2013). In other research investigating the surface expression of BK channels, it was not possible to extract the membrane-bound BK channel percentage as the method of calculation did not allow for a comparison of membrane-bound fraction against the total number of BK channels (Kim, Alvarez-Baron and Dryer, 2009; Jeffries *et al.*, 2010; Kim and Dryer, 2011; Föller *et al.*, 2012; Tian *et al.*, 2012). These studies only allowed for the assessment of treatment or mutant channel deviation from the control group. This deviation is expressed as an arbitrary ratio or normalised value without reference to the total amount of selected protein in isolation.

One study that used biotinylation to label surface BK channels, in combination with western blotting using an anti-BK antibody for an assessment of the total number of channels, calculated the BK channel cell membrane-bound proportion to be approximately 80% of total in 25mM glucose (Kim *et al.*, 2014). The difference in cell membrane expression of the BK channel between the studies may arise for several reasons. For example, the species of BK channel used differs; the current study used the murine ZERO (mSlo), whilst Kim *et al* (2014) was rat Slo1 (rSlo). When directly compared, there is a small difference (89% identity) between the amino acid sequences of the two channels, though the S0-S1 loop domain was completely conserved and neither channel possessed the STREX insert. In addition, this current study was conducted using the HEK293 cell line, whilst Kim *et al* used COS-7 cells. Despite being derived from different species (human for HEK293, monkey for COS), both cell types are mammalian and renal in origin with no documented evidence that the cell types would have differing impacts on ion channel overexpression, and neither cell type express the BK channel endogenously.

Both the fluorescent tagging of the channel in this study, and biotinylation and western blotting used by Kim *et al* require the use of antibodies for protein identification, however with that exception, the two methodologies vary in many aspects (confocal microscopy vs gel separation) and are thus difficult to directly compare.

In other research investigating the surface expression of BK channels, it was not possible to extract the membrane-bound BK channel percentage as the method of calculation did not allow for a comparison of membrane-bound fraction against the total number of BK channels (Kim, Alvarez-Baron and Dryer, 2009; Jeffries *et al.*, 2010; Kim and Dryer, 2011; Föller *et al.*, 2012; Tian *et al.*, 2012). These studies only allowed for the assessment of treatment or mutant channel deviation from the control group. This deviation is expressed as an arbitrary ratio or normalised value without reference to the total amount of selected protein in isolation.

A publication addressed the mean membrane-bound ratio of another ion channel; in a study of Kir2.1 channels expressed in Opossum kidney (OK) cells, the cell membrane-bound percentage was 15% of total, a value obtained by using HA antigen tagging for surface and immunoblot for total (Stockklausner and Klocker, 2003). Additional studies investigated the effect of endogenous and exogenous factors on the surface/cell membrane expression of a number of ion channels, but normalised the results of the control and details of the ratio of cell membrane-bound channels to total are not provided (Zhang *et al.*, 2005; Frindt and Palmer, 2010). In conclusion, the difference in absolute values obtained for percentage of membrane bound BK channel is probably a product of the different techniques used- for example, the specificity of the primary and secondary antibodies. The absolute value of channel expressed in an overexpression assay may also depend on the size of the protein product and the

efficiency of the vector, but the main finding is that the percentage and/or ratio was consistent for BK channels in HEK293 cells under normal culture conditions (25mM) with a normal distribution and standard deviation of ~10%- in line with the variance seen in previous studies (Jeffries, 2010; Jeffries *et al.*, 2010; Bi, 2014; Kim *et al.*, 2014). Therefore the double FLAG approach can be used to investigate BK channel membrane distribution.

4.3.2 The cellular distribution of BK 'SNP-mimetic channels' and the predicted palmitoylation status of the C56 cysteine residue of the S0-S1 linker.

Key aims were to investigate whether the BK channel SNP-mimetic mutants H55Q, G57A and H55Q:G57A would alter the cellular distribution of the channels and whether there was a relationship with the CSS-Palm 4.0 determined pattern of predicted C56 palmitoylation. The confocal imaging screen revealed no significant difference in the distribution of the SNP-mimetics and the ZERO wild-type channel. Although there were statistically significant differences in distribution between the SNP-mimetics channels themselves, and a trend emerged where the proportion of channel in the membrane rose from 44% for H55Q to 51% for ZERO onto 54% for H55Q:G57A to peak at 57% for G57A.

This pattern of membrane expression of the SNP-mimetic mutants appeared to correlate with the CSS-Palm 4.0 generated predicted palmitoylation scores. SNP-mimetic mutants that decreased the predicted palmitoylation score for C56 in comparison to ZERO had a lower membrane expression (H55Q membrane expression was 88% that of ZERO), and those that increased C56 palmitoylation score lead to a greater membrane expression of BK (G57A 113% of ZERO and H55Q:G57A 107% of ZERO). The strong positive correlation between predicted C56 palmitoylation score and SNP-mimetic cell membrane expression (PCC of +0.95) suggests that palmitoylation plays an important role in the targeting of BK channels to the cell membrane.

The current theory is that the BK channel is: a) palmitoylated (Tian *et al.*, 2008); and b) that this palmitoylation modulates cell membrane translocation (Jeffries, 2010; Jeffries *et al.*, 2010). This relationship has been based on investigation of those palmitoylated cysteine residues in the amino acid sequence alone. The implication of the current study is that naturally occurring mutations to amino acid residues flanking the cysteine residues, but not just mutations to the cysteine residues themselves, 1) have an impact on the palmitoylation status of that residue and, 2) results in a predictable effect on the cellular distribution of that construct.

Despite evidence linking C56 palmitoylation to the inter-mutant differences, other factors may also determine the cellular distribution of the channel. In the case of the H55Q mutant, the histidine to

glutamine substitution itself is significant because mutations of this type have been shown to reduce zinc-binding affinity in proteins such as calbindin D28k, a calcium-binding protein (Bauer *et al.*, 2008). Zinc binding in proteins can participate directly in chemical catalysis and help to maintain protein structure, which stabilises and may induce folding of protein subdomains (Coleman, 1992). A study investigating the effects of histidine to glutamine mutation concluded that the structure of the surrounding hydrogen bond network is crucial for metal ion affinity of the zinc-binding site (McCall, Huang and Fierke, 2000). Alteration of this network caused by the increased hydrogen-bonding capability of glutamine may be the basis of the reduced zinc affinity and possible local loss of proper folding structure. Mutation of histidine residues in the C-terminus of EEA1, an auto-antigen, reduced zinc binding and importantly, as a result, reduced protein membrane association (Stenmark *et al.*, 1996). Although zinc binding has been shown to increase BK channel activity, and histidine residues were found to be crucial to this modulation (Hou *et al.*, 2010), there is no current evidence that zinc affects BK channel membrane expression. Further investigation to determine if this impacts channel membrane translocation, in addition to C56 palmitoylation, may be worthwhile.

Similarly, the glycine to alanine mutation (as seen with G57A) could affect BK cellular distribution as it affects the stability of proteins depending on the location of the amino acid position in the α-helix structure of the protein (Serrano *et al.*, 1992). Alanine is the most powerful α-helix stabiliser with glycine associated with α-helix destabilization (López-Llano, Campos and Sancho, 2006; Scott *et al.*, 2007). The mechanics of this are complex; however, it is possible that increased stability of the protein structure enables more efficient palmitoylation enzyme binding to the C56 site, thus greater ease of palmitoylation and increased cell membrane expression. This information may shed some light as to why the predicted palmitoylation of C56 is raised when the DNA includes the G57A mutation.

The location of the amino acid substitutions in the SNP-mimetic channels, therefore have the potential for causing a conformational change to the region where the protein is palmitoylated. As stated, this could be due to a change in zinc association near a PAT-binding site, a change to the local environment of a binding site, or possibly as a direct result of the different mass, shape and binding potential associated with the substituted amino acid residue, as was discussed in the bioinformatics chapter.

4.3.3 The cellular distribution of the cysteine mutant channels C53:54A and C53:54:56A

In contrast with the SNP-mimetic mutants that had not been previously described, the double and triple cysteine mutants have been investigated before in BK channels; these results form the basis of the C56 model as the C53:54:56A mutant and the C53:54A mutants significantly reduce membrane expression (Jeffries, 2010; Jeffries *et al.*, 2010; Bi, 2014; Kim *et al.*, 2014). The data presented here is

not identical to that produced in previous studies as C53:54A was associated with an increase in membrane insertion in comparison with ZERO and though C53:54:56A has reduced membrane expression from the double mutant it was not significantly lower than ZERO. There are a number of possible explanations, based on the difference in tagging, that may contribute to the disparity.

Whilst there is information available about each of the antigen tags (Zhao, Li and Liang, 2013), no specific measure of sensitivity/selectivity is given, and there is no publication with comparison of the sensitivity and specificity of FLAG and HA antibodies and further information could not be sourced from the manufacturers. The dilution of primary antibody used in permeabilised tagging was the same for non-permeabilised tagging at 1:100, which was considerably higher than the 1:500 dilution used by Jeffries *et al* 2010 and Chen *et al* 2013. The 1:100 dilution was used to reduce the number of variables in the protocol and to ensure equity in the measurement of total channel by adding red and green fluorescence and using a higher concentration of primary antibody is a consistent change and should not affect the magnitude of change between groups. Antibody controls, conducted on non-transfected HEK293 cells, did not show any significant non-specific fluorescence, indicating this was unlikely to be the cause of the disparity.

For FLAG-FLAG and FLAG-HA tagging methods to be comparable, both antigens must be equally accessible. As FLAG and HA antigens are positioned on opposite terminus of the channel (N- and C- respectively), the orientation of the channel in the organelle membranes or transport vesicles may be important as this may render sites more or less accessible. If all membranes are fully permeabilised to allow for antibody access there should be no restriction of access. At the concentration used in this study, Triton-X is predicted to permeabilise all cell membranes and thus incomplete permeabilisation is an unlikely explanation for the difference in cell membrane expression (Willingham, 2010). Whilst surface expression of C53:54:56A channels was reduced by 2-fold in COS-7 cells when compared with a ZERO control (Kim *et al.*, 2014), the final membrane-bound ratio of approximately 35% for the triple mutant is in agreement with the results obtained in this study.

4.3.4 The cellular distribution of the cysteine mutant channels C53:54A and C53:54:56A and the predicted palmitoylation status of C56

The data obtained also raised issues with the potential relationship between the cellular distribution of the cysteine mutant channels and the SNP-mimetic channels, based upon the palmitoylation status of the C56 cysteine residue. Firstly, because the triple mutant does not contain C56 and secondly because the predictive score for the double cysteine mutant was 0, even though the C56 residue in the double mutant has been shown to be significantly palmitoylated in a previous study utilising

radiolabelled palmitate (Jeffries, 2010). This highlights a limitation in the predictive software program CSS-Palm 4.0, as the palmitoylation results extracted to compile training data for the software is only taken from results published in peer reviewed journal articles, rather than PhD theses. In this case, an important piece of information for software accuracy at an amino acid has been omitted.

One interpretation of the cysteine mutant data produced in this current study is that a higher predicted palmitoylation score at C56 is indeed associated with raised membrane insertion of the BK channel, but this is counterbalanced by the weaker negative correlations between C53 and C54 palmitoylation and membrane insertion (Figure 4.6). Thus the removal of C53 and C54 would result in loss of the "negative" drive- so the removal of these residues that would normally reduce insertion would in real terms enhance BK membrane expression. Thus, providing an explanation as to why C53:54A had a higher raw ratio than ZERO. Equally, in the triple mutant the loss of the stronger positive influence of C56 is balanced by the loss of the two weaker negative drivers at C53 and C54, resulting in an almost neutral impact.

4.3.5 Low glucose concentration affected BK channel membrane expression in a manner related to C56 palmitoylation status

Changes in extracellular glucose concentration have previously been shown to have variable effects on ion channel expression and membrane translocation; raised glucose resulted in a 2-fold increase in TRPM7 and a significant increase in TRPC6 protein expression (Sun *et al.*, 2013).(Daoyan Liu *et al.*, 2008). While a reduction in glucose concentration resulted in increased KATP ion channel expression by 50% in 30 minutes and by 270% within 10 hours (Smith *et al.*, 2006). Liu *et al* 2008 also showed that glucose concentration may have no effect; the expression of TRPC1, TRPC3, TRPC4, or TRPC5 were unchanged when cells expressing the channels were exposed to high glucose.

An aim of this investigation was to uncover the effects of reducing the glucose concentration from 25mM to 5mM. The data could be split into two patterns with ZERO, H55Q and C53:C54:C56A cell membrane ratio increased in 5mM glucose (by 10%, 30% and 10% respectively), whilst G57A, H55Q:G57A and C53:54A membrane-bound ratio decreased in 5mM glucose (by 8%, 17% and 10% respectively) relative to levels seen in the control conditions of 25mM glucose. Within the context of the current model, the channel constructs with potentially lower C56 palmitoylation – the H55Q and the triple cysteine mutant plus ZERO, have an increased membrane insertion in lowered (5mM) glucose. While those channels with higher predicted C56 palmitoylation scores: G57A; H55Q:G57A; and the double cysteine mutant whose predicted score appears inaccurate; all show reduced membrane insertion with lowered glucose.

Thus, the channels in 5mM glucose display a reversed 'C56 palmitoylation status pattern' in comparison to the 25mM results; with the H55Q mean raw ratio was now significantly raised in comparison to H55Q:G57A.

As far as other BK channel data is concerned, experiments by Kim & Dryer (2011) with endogenously BK channel expressing mouse podocytes found that cell exposure to high glucose led to a 70% reduction in the proportion of BK channels at the cell membrane. As the BK channels used by Kim & Dryer (2011) did not contain any of the SNP-mimetic mutations, the most appropriate comparator channel was ZERO. In this case, whilst a cell membrane expression reduction of that magnitude was not seen, there was nonetheless a reduction in ZERO in 25mM glucose.

In mouse podocytes, the high glucose effect was proposed to be mediated by a reduction in cell membrane expression of the adhesion and scaffolding protein nephrin; a protein that binds almost exclusively to BK channels containing the extreme C-terminal amino acid sequence VEDEC, which increases its cell surface expression by 800% (Kim, Choi & Dryer 2008). The nephrin theory is not directly applicable to this current study as HEK293 cells do not endogenously express nephrin despite their distant renal cell lineage (Khoshnoodi *et al.*, 2003), and the protein was not co-transfected alongside the BK channel. However, as the BK channel constructs used in this study do contain the VEDEC amino acid sequence in the C-terminus, it is possible that other proteins affect BK channel surface expression in a glucose dependent manner and could be targets for future investigation.

The novel findings in 5mM glucose indicate that a) BK channel cellular distribution can indeed be affected by glucose concentration and suggests that b) the extent of the channel distribution change is influenced by the nature of the amino acid mutation in the S0-S1 linker region.

4.3.6 The addition of extracellular palmitate, but not oleate, increases the cell membrane expression of BK channels in HEK293 cells

Raising extracellular palmitate by 0.4mM significantly increased the mean cell membrane expression of the constructs, with ZERO rising from 51% to 64% in 25mM glucose with 0.4mM palmitate and echo data published in the literature, where the addition of palmitate can increase cell membrane expression of proteins by 60% and 100% (Cazanave *et al.*, 2011; Pararasa, 2013). The magnitude of cell membrane expression change observed in the current experiments is lower, but with a common trend with glucose and palmitate concentrations having an additive effect to increase protein expression (Sathanoori *et al.*, 2015; Hong *et al.*, 2016).

Oleate, an unsaturated fatty acid, has been shown to have a variable effect on protein membrane expression (Mastrangelo, Jeitner and Eaton, 1998; Hannah *et al.*, 2001; Benoit *et al.*, 2009), and in this study the addition of 0.4mM oleate did not result in a change in ZERO cellular distribution nor the cysteine mutants but significantly increased the cell membrane expression of the H55Q mutant by 48% in 25mM glucose.

4.3.7 The effect of palmitate and oleate, in combination with changes in glucose concentration, on BK channel distribution

The data acquired from experiments where alterations in glucose concentration (5mM vs 25mM) were combined with the addition of palmitate or oleate produced a range of changes in the membrane expression of BK channels. One key observation was that the inversion of C56-dependent distribution pattern produced by exposure of ZERO and the SNP-mimetic channels to 5mM glucose was reversed by 0.4mM palmitate but not by 0.4mM oleate. Furthermore, the specific order and any significant difference in BK channel membrane distribution associated with the change in metabolic substrates was dependent on the specific constructs. Thus, the pattern displayed by H55Q was different from G57A and H55Q:G57A and in most occasions showed reversed trends. Thus where 0.4mM palmitate would be associated with raised levels of membrane expression over 0.4mM oleate for the G57A and H55Q:G57A constructs, the opposite was observed for H55Q in both 25mM and 5mM glucose.

In the literature, there is evidence to suggest that palmitate and oleate have either a) opposing actions, or that b) oleate can compensate for the perturbing effects of excess palmitate. For example oleate, in comparison to palmitate, did not cause mitochondrial DNA damage in skeletal muscle cells (Yuzefovych, Wilson and Rachek, 2010). Potentially, the differences in the effect of oleate and palmitate are due to a shift in metabolic status of the HEK293 cells. In a 2014 study, oleate increased cellular triglyceride (TG) levels and thus mitochondrial β-oxidation, whilst palmitate increased the protein kinase C activator, diacylglycerol (DAG) levels associated with PKC/NF-κB activations (Coll *et al.*, 2008; Kwon, Lee and Querfurth, 2014). Thus it may be that there is competition between palmitate and oleate to bind the cysteines in the BK channel S0-S1 loop, or that the binding and resulting efficacy of modulation is dependent on the palmitoylation profile of the cysteines and thus the different predicted score for the separate constructs informs the likely outcomes (Greaves *et al.*, 2017).

4.3.8 The metabolic substrates glucose, palmitate and oleate may be moderating BK channel membrane expression in HEK293 cells via multiple mechanisms

Are the effects of glucose and fatty acids on BK channel membrane expression mediated by AMPK?

Glucose, as the main metabolic substrate in humans, plays a role in many physiological processes; therefore, there are a number of routes by which a reduction in glucose concentration may alter the cellular distribution of the BK channel in HEK293 cells. As introduced earlier, many of the downstream effects of glucose (such as fatty acid synthesis, glyconeogenesis and protein synthesis) are regulated by a powerful kinase enzyme called AMPK (Figure 4.15). AMPK is strongly activated by the low energy status of a cell where the supply of ATP cannot match demand, increasing the ratio of AMP/ADP to ATP; this occurs in times of limited glucose, lipid or oxygen availability (Kudo *et al.*, 1995; Salt *et al.*, 1998; Marsin *et al.*, 2000; da Silva Xavier *et al.*, 2003; Minokoshi *et al.*, 2004; Evans *et al.*, 2005), and during exercise (Hutber, Hardie and Winder, 1997; Wojtaszewski *et al.*, 2000; Chen *et al.*, 2003).

As part of the cell treatment protocol, the glucose concentration of the HEK293 cell growth medium was reduced from 25mM to 5mM; this may have been sensed by the cells as a relative hypoglycaemia, activating AMPK and inhibiting protein synthesis. This has been shown to occur via a) inhibition of eukaryotic elongation factor 2 (EF2) required for protein elongation by the ribosomes (Horman *et al.*, 2002; Yan *et al.*, 2003) and b) via inhibition of the mammalian target of rapamycin (mTOR), a serine/threonine kinase and a key regulator of protein translation/synthesis (Bolster *et al.*, 2002; Kimura *et al.*, 2003; Cheng *et al.*, 2004). Based upon the results, a reduction in glucose concentration did not result in a reduction in BK channel synthesis, as there was no significant reduction in raw ratio in the 5mM glucose groups. However, it is important to note that the aim of the immunofluorescent tagging method in combination with confocal microscopy was to assess differences in the *proportion* of total BK channel situated in the cell membrane, rather than the *absolute* quantity of BK channel protein translated. Therefore, in future experiments to more accurately quantify changes in protein synthesis, a protein quantification technique such as Western blotting, could be undertaken. Western blotting could also support investigation into the production of other proteins involved in BK channel translocation such as the PAT and APT/PPT enzymes involved in palmitoylation and de-palmitoylation.

AMPK activation also results in the inhibition of fatty acid synthesis and an increase in fatty acid oxidation via inhibition of acetyl-CoA carboxylase (ACC) isoforms 1 and 2, which are responsible for the formation of malonyl-CoA, an essential component in the pathway for fatty acid synthesis (Saddiksj *et al.*, 1993; O'Neill *et al.*, 2014). This may have implications for the amount of palmitate available, as AMPK activation would reduce palmitate production and increase its breakdown. Reduced palmitate availability may contribute to the differential effects of glucose on the cell-membrane expression of the SNP-mimetic mutants, as expression is closely linked to C56 palmitoylation status.

It has been hypothesised that regulation of AMPK by glucose may normally be restricted to specialised energy sensitive cells, such as pancreatic beta cells, skeletal muscle cells, adipocytes and hepatocytes (Hardie, Hawley and Scott, 2006); however significant glucose mediated changes were observed in this study. Interestingly, a publication investigating AMPK mediated changes in BK channel protein abundance in Xenopus oocytes using confocal immuno-microscopy (Föller *et al.*, 2012) showed that AMPK increased BK channel abundance in the cell membrane. This result ties in with the increased BK channel cell membrane expression seen with the H55Q construct. However, the effect of AMPK on BK channels may be splice variant specific (Ross *et al.*, 2011). In this chapter, results indicate that glucose concentration can affect the cellular distribution of BK channels expressing SNP-mimetic mutations, however, the precise mechanisms behind this have not been determined and further investigation is required.

As fatty acids are metabolic substrates it would be logical that the addition of excess palmitate or oleate to the cell environment would inhibit AMPK activation in the same manner as glucose; however, results in the literature do not consistently support this logic. For example, when cultured bovine aortic endothelial cells (BAECs) were exposed to 0.4mM palmitate for 40 hours, there was a 72% reduction in AMPK activation (Wu *et al.*, 2007), which supports the hypothesis. Similar effects on AMPK inhibition have been shown *in vitro*, and *in vivo* in rodents fed a diet high in fatty acids (Yi Liu *et al.*, 2006; Fujii *et al.*, 2008; Kwon and Querfurth, 2015).

On the other hand, L6 rat skeletal muscle cells were exposed to a range of palmitate concentrations from 0.01mM to 0.8mM for 1 hour, which resulted in up to a 4-fold increase in AMPK activation (Fediuc, Gaidhu and Ceddia, 2006). This effect has been reported in other publications (Clark, Carling and Saggerson, 2004; Watt *et al.*, 2006).

Results of the experiments conducted in this chapter highlight that fatty acids of different length and saturation may not have the same downstream effects; addition of palmitate and not oleate resulted in an increase in BK wild-type channel cell membrane expression. Differences have been shown in the literature; oleate increased cellular triglyceride (TG) levels and thus mitochondrial β-oxidation, whilst palmitate increased the protein kinase C activator, diacylglycerol (DAG) levels associated with PKC/NF-κB activations (Coll *et al.*, 2008; Kwon, Lee and Querfurth, 2014). In addition, fatty acids can have opposing effects on AMPK activation; monounsaturated oleate has been shown to activate AMPK whilst saturated palmitate inhibits (Hickson-Bick, Buja and McMillin, 2000; Wu *et al.*, 2007; Yuzefovych, Wilson and Rachek, 2010; Kwon and Querfurth, 2015).

Are the BK channel membrane expression changes mediated by lipid raft formation and composition?

The increased cell membrane expression of proteins associated with palmitate addition extracellularly has been attributed to an increase in protein association with lipid rafts (Cazanave *et al.*, 2011). Lipid rafts are a membrane sub-compartmentalisation, composed of sphingolipid, cholesterol and proteins, that play a role in lipid–lipid, protein–lipid, and protein–protein interactions (as reviewed by Calder and Yaqoob, 2007). The percentage of the cell membrane occupied by lipid rafts appears to be variable depending on the cell type (Pike, 2003), with estimates ranging from approximately 50% (Mayor and Maxfield, 1995; Hao, Mukherjee and Maxfield, 2001), to below 15% (Koutny, 2013).

However, there are challenges with accurately determining lipid raft composition and quantity due to limitations in the methods available to isolate and to study rafts (Lai, 2003; Calder and Yaqoob, 2007).

BK channels have been shown to localise in lipid rafts (Bravo-Zehnder *et al.*, 2000; Lam, Shaw and Duszyk, 2004; Weaver *et al.*, 2007), and as palmitate is a component of lipid rafts (Laethem *et al.*, 2003; Schumann *et al.*, 2011), it is possible that the increased palmitate concentration in the cell culture medium resulted in an increase in the number and or size of lipid rafts in the cell membrane, allowing for increased BK channel – cell membrane association.

Association of proteins to lipid rafts has been shown to increase when the protein has been palmitoylated; palmitoylation of DR4 is essential for the redistribution of this receptor into lipid rafts (Rossin *et al.*, 2009). This brings into question whether an increase in the availability of palmitate not only affects the cellular structure of the cell allowing for increased BK channels in the cell membrane, or if the effects can be attributed to an increase in the extent of protein palmitoylation. Association of BK channels with cell membrane lipid rafts has been linked with palmitoylation; the SNP-mimetic mutants increase or decrease the palmitoylation status of C56, this may be the link between the amino acid mutations and their differing cell membrane expression. Further research is required to uncover the specific mechanisms involved in palmitate mediated cell membrane expression regulation.

If the palmitate and lipid raft theory is correct, then it is possible that the extra cellular energy source, in the form of high glucose concentration, is necessary to complete the processes required to transport excess palmitate into the cell membrane (Bizzozero, Sanchez and Tetzloff, 2002) and may explain why there is an additive effect seen in cell-membrane expression in 25mM glucose and 0.4mM palmitate.

It is plausible that differences between the cell membrane expression values of BK channels in palmitate and oleate treated cells could be related to the varying properties of the lipids.

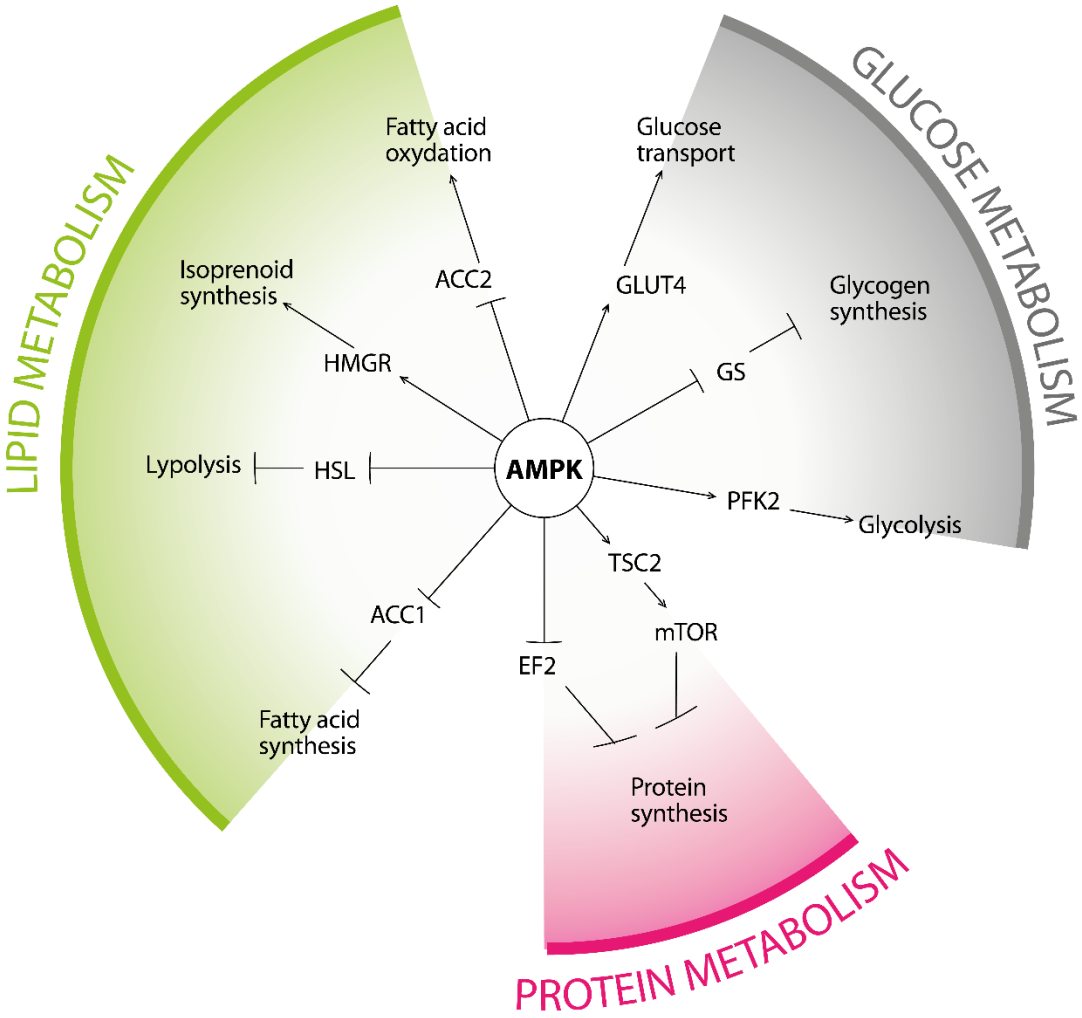


Figure 4.15- AMPK activation as a mechanism of glucose control of BK channel cell-membrane expression. Illustrated is a simplified schematic showing a small number of the potential mechanisms by which AMPK regulates lipid, protein and glucose homeostasis. Activation of AMPK promotes fatty acid oxidation and glucose utilisation, but inhibits protein, fatty acid and glycogen synthesis. Shown are pathways of activation (indicated by arrows) or pathways of inhibition (indicated by perpendicular lines) of agents by AMPK in downstream pathways. Figure adapted from (Wang, Song and Zou, 2012). (GLUT4) glucose transporter type 4; (GS) glycogen synthase; (PFK2) phosphofructokinase 2; (TSC2) tuberous sclerosis complex 2; (mTOR) mammalian target of rapamycin; (EF2) eukaryotic elongation factor 2; (ACC1, ACC2) acetyl-CoA carboxylase 1 and 2; (HSL) hormone-sensitive lipase; (HMGR) HMG-CoA reductase.

In a 2005 study, extracellular oleate was found mainly as free oleic acid in phospholipids, whereas palmitate was incorporated into phospholipids and to a lesser extent found as cellular free palmitic acid (Gaster, Rustan and Beck-Nielsen, 2005). This may impact the fluidity or viscosity of the lipid rafts (with oleate being in a liquid state at cell culture temperature, whilst palmitate favours a solid state), and affect the dynamics of BK incorporation into the cell membrane.

4.4 Limitations and Further Work

Limitations

The experiments conducted in this chapter have a number of possible limitations. The investigations assess changes during a treatment period of 24 hours, a time period used in a number of other studies (Liu *et al.*, 2001; Peiró *et al.*, 2001; Hovsepyan, Sargsyan and Bergsten, 2010; Chang *et al.*, 2011; Kim and Dryer, 2011; Su *et al.*, 2013). However, for a better understanding of the changes, it would be of value to determine responses over a range of timepoints, as has been conducted in other studies (Samikkannu *et al.*, 2006; Cho *et al.*, 2013). The results obtained in the 24-hour period in this study are still valid and show significant responses over a moderate timeframe. Confocal microscopy was utilised as a method to assess trends in the proportion of total BK channels that were at the cell membrane at the time of fixing, a method that has been utilised to assess surface expression in many other studies (Barden *et al.*, 2003; Jeffries, 2010; Jeffries *et al.*, 2010; Sun *et al.*, 2010; Bi, 2014). However, as this study did not use live cell imaging, it is not possible to determine the real-time change in BK channel cell membrane distribution.

Future Work

To gain further clarity of the mechanisms behind the C56 palmitoylation pattern and the variations in pattern depending on cell culture medium composition, further investigation is required in several areas. Firstly, to more accurately quantify the effect of SNP-mimetic mutation on C56 palmitoylation status, it would be of great interest to determine the level of C56 palmitoylation of each of the mutants via ^{3}H-palmitate incorporation. This method has been used in many studies to establish the extent of amino acid palmitoylation (Pouliot and Béliveau, 1995; Luiken *et al.*, 1997; Ducker *et al.*, 2004; Liang *et al.*, 2004; Jeffries, 2010; Dasgupta *et al.*, 2011).

Another aim of future interest would be to test adherence to the C56 palmitoylation pattern using different amino acid mutations; as for example, H55G mutation, in place of H55Q, leads to increased predicted palmitoylation of C56. This was not the case for C53 or C54, as mutation to C53:54G still

resulted in removed C56 predicted palmitoylation. This may indicate a complexity to the pattern that would be of interest to investigate in the future. Another complicating factor is that amino acid substitutions can have effects other than the augmentation of palmitoylation, such as potential changes in zinc binding and protein structure stabilisation in the case of H55Q and G57A. These may impact BK channel cell membrane expression and cause other mutated BK channels to deviate from the C56 palmitoylation pattern.

As the function of BK channels in this thesis was interpreted as the ability of the channel to insert into the cell membrane, confocal imaging was used in order to best establish the location of the BK channel as either at the cell membrane, or within the cell. However, the effect of the interplay between BK channel amino acid mutations and metabolic substrates has not yet been tested using electrophysiology; however, the effect of fatty acids on BK channel function (Denson *et al.*, 2000; Clarke *et al.*, 2002; Sun *et al.*, 2007) and S0-S1 cysteine mutations on BK channel function have been assessed (Jeffries, 2010; Jeffries *et al.*, 2010). Knowledge of the effects of SNP-mimetic mutation in the presence of excess lipids on BK channel activity would contribute considerably to the understanding of the mechanisms behind the changes observed in this thesis. Also, quantification of the amount of BK channel protein expression in each of the conditions would be of great value, as it would place results amongst those already published (Grunnet, Hay-Schmidt and Klaerke, 2005; Sakai, Harvey and Sokolowski, 2011), and for example indicate whether palmitate is associated with increased membrane expression of mutated BK channels.

Using a wider scope, an alternative way of interpreting the results of metabolic substrates on BK channel cellular distribution would be to think of the direct effects of the compounds themselves on the cell. As such, an area of future work would be to investigate the effect of fatty acid addition on the composition of the cell membrane and lipid rafts. There is evidence that BK channels localize in lipid rafts (Weaver *et al.*, 2007) and changes in their composition may either encourage or inhibit BK channel insertion. As palmitoylation is implicated in the translocation of the BK channel to the cell membrane, it would be of value to investigate the role of palmitoylation in translocation of BK channels to lipid rafts of varying compositions of palmitate and oleate. The specificity of palmitoylation enzymes for addition of particular fatty acids may also play a role in the BK channel cellular distribution differences between palmitate and oleate. As S-acylation has been associated with a number of fatty acids (such as stearate), and there is emerging evidence of differing fatty acid selectivity in the PAT family of enzymes (Greaves *et al.*, 2017). Therefore, it is of interest to investigate if the substrate of S-acylation can shift from palmitate to when oleate is in excess.

As a change in metabolic substrate concentration in the cell growth medium could result in altered protein and fatty acid synthesis via AMPK -increasing or decreasing BK channel synthesis or the availability of palmitate for palmitoylation- (Saddiksj *et al.*, 1993; Clark, Carling and Saggerson, 2004; Fediuc, Gaidhu and Ceddia, 2006; Watt *et al.*, 2006; O'Neill *et al.*, 2014), assessment of AMPK activation in low glucose, and in the presence of the fatty acids palmitate and oleate would be of value.

There is a trend towards the investigation of a combination of fatty acids (Karaskov *et al.*, 2006; Coll *et al.*, 2008; Das, Mondal and Elbein, 2010; Yuzefovych, Wilson and Rachek, 2010; Kwon, Lee and Querfurth, 2014), as well as the different effects of glucose and fructose (Meissen *et al.*, 2015; Diaz-Aguirre, Velez-Pardo and Jimenez-Del-Rio, 2016; Windemuller *et al.*, 2016; Phoomak *et al.*, 2017). Therefore, in a future expansion of this research, the effect of combinations of glucose and fatty acids on BK channel cellular distribution could be investigated.

Conclusion

The verification of BK channel nsSNP undertaken in this chapter completes step 7 of the thesis workflow (Figure 4.16). The results reinforce the link between the cell membrane expression of the BK channel and palmitoylation, and indicates that alteration of palmitoylation status by amino acid mutation need not be of the cysteine residue itself, but flanking amino acids may also have an effect. This encourages the expansion of current research practice, to investigate not only the amino acids directly involved in post-translational modification of BK channels, but also those amino acids flanking the residue of interest. This is the first study to demonstrate the effect of differing concentrations of glucose, palmitate and oleate on the membrane-bound ratio of BK channel constructs that have been mutated to mimic endogenously occurring human SNPs. The findings also suggest that this approach would be beneficial in the investigation of other ion channels and proteins.

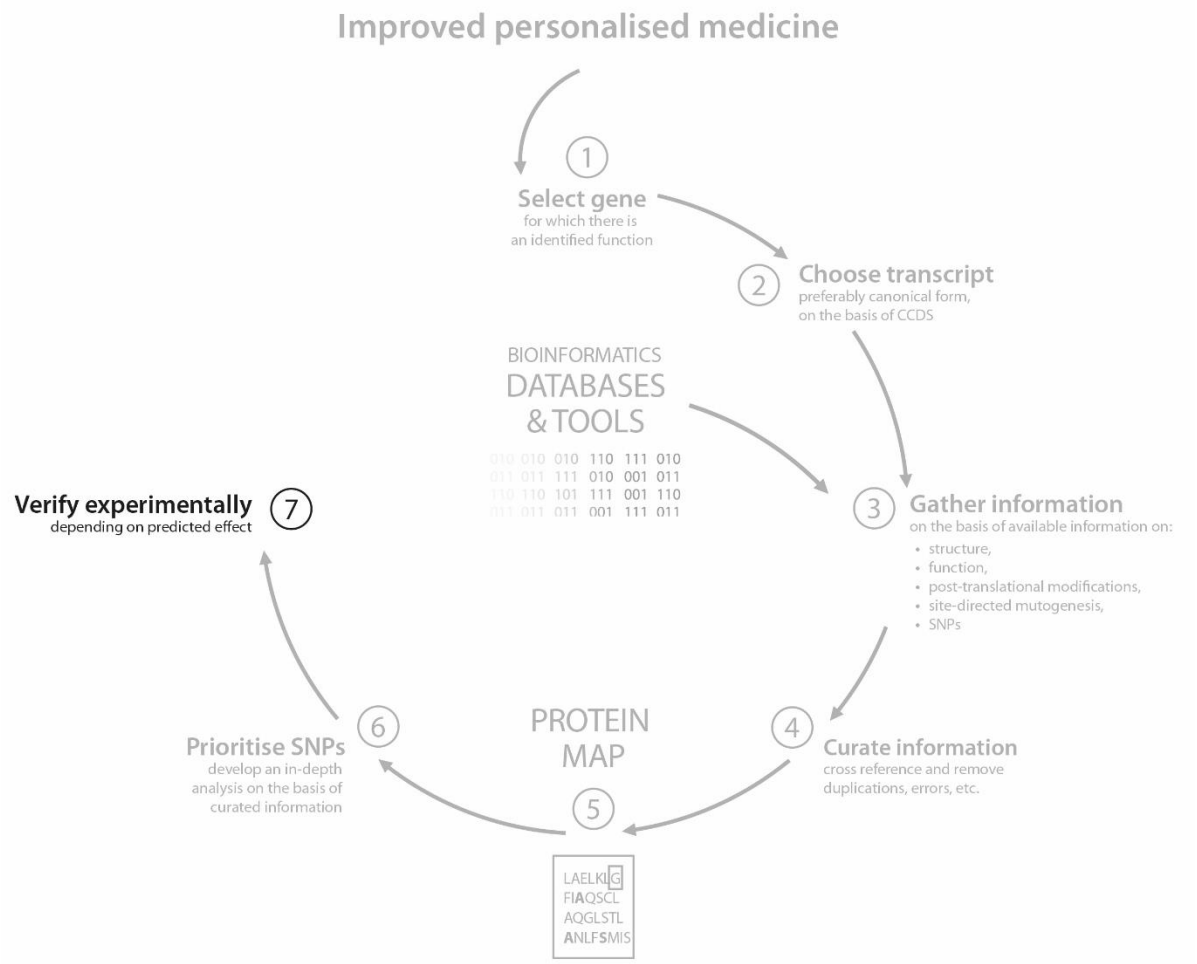


Figure 4.16- Schematic of thesis workflow: Summary of chapter 4. Described are the steps to be undertaken in this thesis work to identify new potential genetic targets for personalised medicine. Steps 1-6 have been successfully achieved in chapter 3 by the selection of the BK channel gene KCNMA1, the canonical BK channel transcript, the collation of available information, the biocuration of this work into a 'BK channel resource' and the prioritisation of nsSNP. Step 7 was achieved as a result of the experimental verification of H55Q and G57A (H120A and G122Q in human) this chapter.

5 General discussion and Further Work

5.1 Thesis aims

In this thesis, the aims were initially to; a) capture the key aspects of data relating to the human BK channel held on bioinformatics databases and b) create a novel 'at a glance' resource for the BK channel with the synthesised information, then to c) use this resource to prioritise single-nucleotide polymorphisms (SNPs) predicted to be damaging to BK channel function, and then d) forward the mutations for lab-based investigation. These aims were successfully achieved and naturally occurring mutations H120Q and G122A (H55Q and G57A in the murine form of the channel), located in the S0-S1 linker, were prioritised and selected for lab-based investigation due to their varying effect on the CSS-Palm 4.0 predicted palmitoylation status of C121.

As C121 is proven to modulate cell membrane expression of the channel, the next series of investigations in the thesis aimed to; 1) determine whether incorporation of the SNP-mimetic mutants H55Q, G57A and H55Q:G57A would alter the baseline cellular distribution of the channels in comparison to ZERO, the wild type channel and 2) ascertain if the cellular distribution pattern of the SNP-mimetic mutants is related to the CSS-Palm 4.0 determined pattern of predicted C56 palmitoylation of the SNP-mimetics mutants. This was achieved, and it was confirmed that the SNP mimetic mutants alter the cell membrane expression of BK channels in a pattern related to the predicted palmitoylation status of C56. There is evidence linking changes in the concentration of metabolic substrates to BK channel dysregulation, therefore the final aim of the thesis was to establish whether the concentration of the metabolic substrates glucose, palmitate and oleate in the cell culture medium affects the cellular distribution of the BK channel. This aim was also achieved and it was established that metabolic substrate concentration in the extracellular environment does affect the cell membrane distribution of BK channel constructs. In this chapter, the main themes that arose from the experimental chapters (chapters 3 and 4) will be discussed, in addition to suggestions of areas of future work.

5.2 Main Themes

5.2.1 Bioinformatics outputs require lab-based verification

Journal articles, online publications and opinion pieces have discussed the inter-dependency of bioinformatics and lab based experimentation (Haoudi and Bensmail, 2006; Penders, Horstman and Vos, 2008; Tachibana, 2010; Eddy, 2014). The term 'moist zone' was coined in reference to this, and is

a wordplay on the collaboration based upon lab-based experimentation being nicknamed 'wet lab' and '*in silico'* experimentation 'dry lab'. An example of 'moist zone' collaboration is the development of a comprehensive algorithm training data set for post-translational modification prediction software by those in the 'wet lab', by conducting lab-based verification experiments of potential modification sites using mass spectroscopy and site-directed mutagenesis. The interdependency of the two methodologies is illustrated as, once the algorithm has been trained in the 'dry lab', the software will produce predictions for additional sites of post-translational modification, which will then be put forward for lab based verification in the 'wet lab'. Another similar example of this interdependency is the verification of SNP damage consequences, which can be undertaken using site directed mutagenesis. The results of this experimentation can then be included in the training data of SNP damage prediction software such as PolyPhen-2, to improve the accuracy of the scores for future predictions (Adzhubei, Jordan and Sunyaev, 2013).

An exemplar advantage of this 'moist zone' collaboration is that the feedback between site prediction and validation facilitates machine learning and increases the accuracy of predictions; research can then be guided towards those sites that have the highest likelihood of affecting protein function. A potential disadvantage of this inter-dependency is the potential over reliance on bioinformatics outputs to form conclusions and to guide future lab-based investigations. This over reliance may result in errors or miscalculations in bioinformatics outputs misdirecting lab-based investigation, and lead to false results if outputs are not validated. An example of this occurred in 2001; the structures of multidrug resistance transporters EmrE and MsbA were modelled using bespoke computational biology algorithms, which resulted in 5 publications based upon the software output and further work based upon this (Chang and Roth, 2001; Chang, 2003; Ma and Chang, 2004; Pornillos *et al.*, 2005; Reyes and Chang, 2005). However, lab-based experimentation results did not agree with the findings of the publications, and a paper in 2006 was released which directly contradicted the computational biology results (Dawson and Locher, 2006). Soon after, a glitch in the software was identified which caused an inversion of parts of the protein structure; the previously published structure based upon the software output was incorrect. In this scenario, prompt verification of the protein structure by lab based experimentation may have highlighted a potential issue more quickly.

A bioinformatics methodology has been used successfully in previous studies to prioritise single nucleotide polymorphisms for further investigation (Teng *et al.*, 2012; Ghaedi *et al.*, 2015; Alipoor *et al.*, 2016), however there are no known studies in which this has been achieved for the BK channel. Therefore, the aim of the thesis was to create a BK channel resource and prioritise nsSNP using a bioinformatics methodology. This was achieved, however, the previously discussed disadvantages of a bioinformatics method manifested themselves in the thesis results in a number of ways. The Ensembl

database contained a list of nsSNP of the BK channel canonical transcript that were uploaded by a number of projects such as 1000 genomes, COSMIC and ESP; these mutations were then assigned a damaging score by damage prediction software. However, discrepancies between the validated experimental results and the predictions of bioinformatics tools for these mutants became apparent, as in the case of D434 and S722 detailed in Chapter 3. In these examples, lab-based experimentation at both sites via site-directed mutagenesis confirmed that the mutations altered channel function by increased calcium sensitivity, and decreased hypoxia sensitivity respectively. However, nsSNP damage prediction software SIFT classified both mutations as tolerated. Without knowledge of the site-directed mutagenesis results, a researcher may erroneously bypass the nsSNP occurring at these locations as they appear to be unimportant for channel function. This again highlights that lab based verification of bioinformatics outputs, and feedback of the results to the software is crucial to increase the accuracy of predictions.

Another finding from the thesis hints at the consequences of the interdependency of lab-based experimentation and bioinformatics. Site-directed mutagenesis and palmitoylation assessment of the S0-S1 linker cysteine residues C53, C54 and C56 in mSlo (C118, C118 and C121 in hSlo) (Jeffries, 2010), lead to the inclusion of the verified sites in the training dataset for CSS Palm 4.0 software (Xue, 2017). In turn, the software correctly predicted the sites as being palmitoylated on the murine BK channel and other orthologs, such as hSlo. The C56 predicted palmitoylation scores for each of the SNPmimetic mutant channels was calculated, and in chapter 4 it is shown that membrane distribution of these channels in HEK293 cells displayed a pattern similar to that of the predicted score. However, this relationship between predicted palmitoylation score and cell membrane expression results was not observed in the BK channels mutated to contain mutated cysteine residues. CSS Palm 4.0 predicted that C56 would not be palmitoylated in either cysteine mutant channel; this result was expected for the cysteine triple mutant (C53:54:56A), however as C56 is present in the cysteine double mutant, a palmitoylation score was expected. Interestingly, results from chapter 4 highlighted significant differences between the cell membrane expression of the double and triple cysteine mutant channels, indicating differences between the channels that may be related to C56. It is possible that these results were driven by the inclusion of Jeffries 2010 data into the CSS-Palm 4.0 training dataset, which may have unintentionally over weighted the lab-based validation results showing that C53 and C54 integral for palmitoylation of the cysteine rich domain. It is also plausible that when palmitoylation of the S0-S1 cysteine residues is not possible that other mechanisms modulate cell membrane expression; however, this is not predicted to be via palmitoylation as the CSS-Palm 4.0 score for the remaining cysteine residues was not altered, and no additional sites were predicted.

As discussed in chapter 3, there is a case for more extensive biocuration of BK channel data, which could potentially increase the speed of crosstalk between bioinformatics and lab based experimentation, and ensure that lab based and software outputs 'make sense' in relation to each other. In this thesis, biocuration of the data has flagged the S0-S1 cysteine residues for further investigation, and highlighted that whilst the SNP mimetic mutants were shown to affect BK channel membrane translocation, the mutations were not predicted to have an effect on channel, therefore data of this result could be added to PolyPhen-2 training to increase its accuracy. On the same theme, there is an argument for the development of a BK channel specific database or a set of BK channel specialised software tools. As discussed in chapter 3, specific databases and tools have been developed for other proteins (Elefsinioti *et al.*, 2004; Torkamani and Schork, 2007; Gallin and Boutet, 2010; Lahiry *et al.*, 2010; Ranjan *et al.*, 2011), which resulted in increased accuracy of site predictions and an increase in the density of information for that specific protein. To enable such a resource to be created for the BK channel, issues relating to intellectual property, project funding, the mechanism of granting access to data before publication, the formation of a collaboration between different research groups, and agreement of the type of resource to be developed need to be addressed. There are publications providing initial generic guidance on how to overcome a number of the aforementioned barriers (Toronto International Data Release Workshop Authors, 2009; Singh, 2014; Lin and Chen, 2015), however a comprehensive protocol for the creation of a protein specific database or tool does not yet exist.

An underlying factor that determines the accuracy of the output from bioinformatics resources is the quality and richness of the raw data included. The background information listed for the SNPs is often incomplete, likely due to the ethical issues of data disclosure (Ormond, 2008; Ross *et al.*, 2013; Clarke, 2014; Niemiec and Howard, 2016). The nsSNP included in the 'deep dive' section in chapter 3 of the thesis are examples of this as many had missing information on the gender, age and ethnicity of the subject in which the mutation was identified. Single gene and genome screening has moved from an individual basis, to screening populations (such as 100,000 genomes project); this increased the volume of SNP data included in the database and 'cast a wide net', however it may present challenges when attempting to compile detailed information on each of the subjects.

5.2.2 The S0-S1 linker cysteine residues as cell membrane expression modulators

In addition to cysteine residues located in the STREX splice insert at the C-terminus (Jeffries, 2010), other regions of the BK channel have been linked with altered cell membrane localisation. Examples include the acidic ER trafficking motif upstream of the STREX splice insert site (Chen *et al.*, 2010), BK channel splice variant mk44 in the S0-S1 linker of the channel that results in N-terminal endoproteolytic cleaving (Korovkina *et al.*, 2001). Examples also include splice variants that cause ER trapping of the channel, such as SV1 found in the S0-S1 linker (Zarei *et al.*, 2004), and Δe23 in the C-terminus (Chen *et al.*, 2005). The cysteine residues of the S0-S1 loop (C53, C56 and C56) have been linked with modulation of channel localisation in the cell membrane; this relationship has been demonstrated in work published in 2010 (Jeffries, 2010; Jeffries *et al.*, 2010), and in 2014 (Bi, 2014; Kim *et al.*, 2014). The relationship between the cysteine residues and membrane translocation is not clear cut however, as in 2016 a publication indicated that BK channels containing the S0-S1 linker cysteine residues did not reach the cell membrane (Suzuki *et al.*, 2016). One of the aims of the work undertaken in chapter 4 of this thesis was to establish if the changes in the palmitoylation status of C56 caused by mutation neighbouring amino acid residues H55 and G57 would affect cell membrane expression of the BK channel. Results indicated that this was the case, as cell membrane expression was linked to the palmitoylation status of C56, providing further evidence that the S0-S1 linker cysteine residues are implicated in cell membrane expression.

5.2.3 The potential physiological effects of H120Q and G122A mutation in humans

The impact of genetic mutation on BK channel cellular distribution was examined in chapter 4 of this thesis, where naturally occurring mutations discovered using a bioinformatics methodology were tested for differences in cell membrane expression in HEK293 cells. Results demonstrated the significant difference in the membrane expression of BK channels containing the mutations H55Q, G57A and H55Q:G57A (H120Q, G122A or H120Q:G122A in hSlo). Within the results a broad pattern emerged; high glucose concentration alone (25mM), and conditions of high palmitate concentration combined with both high or low glucose concentration (25mM or 5mM) resulted in the statistically significant reduction of H55Q membrane expression in comparison to either G57A or H55Q:G57A. This trend was reversed in conditions of low glucose concentration, and conditions of high oleate concentration combined with both high and low glucose concentration; in these scenarios H55Q cell membrane expression was significantly higher than either G57A or H55Q:G57A. These results indicate that individuals with the H55Q mutation may have an altered response to metabolic imbalances in comparison to those with a G57A or H55Q:G57A mutation. In order to pre-empt the potential

consequences of the mutations, it is important to understand the role that the BK channel plays in human physiology.

BK channels are ubiquitously expressed in the body and are implicated in a wide range of processes, such as the physiological regulation of vascular tone and blood pressure (Aldrich *et al.*, 2000; Sausbier *et al.*, 2005), neuronal cell function and neurotransmitter release (Raffaelli *et al.*, 2004; Sausbier *et al.*, 2004; Du *et al.*, 2005), bladder control (Meredith *et al.*, 2004), uterine function reviewed by (Khan *et al.*, 2001) and hearing (Rüttiger *et al.*, 2004; Föller *et al.*, 2012). The cell membrane expression of the BK channel is required for proper function, and it is plausible that individuals carrying the H55Q, or G57A / H55Q:G57A mutations may have differing 'regulation' of the aforementioned processes, as the membrane expression of the mutants is significantly different. Normal changes in BK channel membrane expression are demonstrated in pregnancy where reduced BK channel expression in myometrium smooth muscle cells facilitates progression to labour (Song *et al.*, 1999) and in aging coronary arteries (Marijic *et al.*, 2001) where a reduction in BK channel expression in the elderly increases the risk of vasospasm.

The inversion of the cell membrane expression pattern of the SNP mimetic mutants in different combinations of metabolic substrate is noteworthy. The cellular responses to excess palmitate and excess oleate differ, with palmitate commonly resulting in mitochondrial stress and cell apoptosis (Okuyama, Fujiwara and Ohsumi, 2003; Staiger *et al.*, 2004; Cnop *et al.*, 2010; Morse *et al.*, 2010; Elsner, Gehrmann and Lenzen, 2011; Cai *et al.*, 2014; Yan *et al.*, 2014; Jiaqi Liu *et al.*, 2016); whilst oleate commonly results in no change in cellular processes in comparison to control, or serves to reverse or limit the damaging effect of palmitate (Gaster, Rustan and Beck-Nielsen, 2005; Coll *et al.*, 2008; Kwon, Lee and Querfurth, 2014; Hetherington *et al.*, 2016). This raises the question of whether the cell membrane expression pattern observed in high glucose and high palmitate is indicative of 'unhealthy' metabolic substrate combinations.

Individuals who suffer from diabetes mellitus have dysregulation of glucose and lipid homeostasis, and commonly results in high glucose and palmitate concentrations. The results of this thesis may suggest that mutations of the BK channel can result in a differing response to the excess concentrations of metabolic substrates associated with diabetes, as the cell membrane expression of H55Q and G57A/H55Q:G57A change inversely to one another. In the case of BK channel control of vascular tone, the significantly reduced BK channel membrane expression observed in mSlo H55Q mutant channels may suggest that individuals with the hSlo H120Q mutation will have a higher incidence of hypertension, as reduced BK channel activity is associated with increased vessel construction (Marijic *et al.*, 2001).

5.3 Future Work

5.3.1 Further investigation of the effects of H120Q and G122A mutation on the BK channel

Investigation in chapter 4 established that mutations H55Q, G57A and H55Q:G57A affect cell membrane translocation, and results suggest this is actioned via altered C56 palmitoylation. Jeffries et al 2010 investigated the effect of cysteine mutations C56 on channel activity, and found significant differences in channel activation by calcium influx between the mutants. Therefore, to gain more information on the potential similarities between the cysteine mutants and SNP mimetic mutants, and to investigate whether the significant differences in SNP mimetic cell membrane expression translate into differences in activity, patch clamp electrophysiology of the channels could be conducted.

It would also be of interest to conduct gene sequencing in individuals suffering from diabetes mellitus to establish whether there is an increased incidence of the H120Q, G122A or H120Q:G122A mutation in that population in comparison to those without diabetes. In addition, the incidence of hypertension in those with one or both of the mutations could be assessed to determine if there is a relationship between genetic mutations and a differential response to deranged glucose and oleate conditions.

5.3.2 The effect of a combination of fatty acids on BK channel cellular distribution

Oleate is often added to cultures containing excess palmitate to assess whether an unsaturated fatty acid can reverse the perturbing effects of the saturated fatty acid. Co-incubation of skeletal muscle cells with palmitate and oleate reversed both inflammation and impairment of insulin signalling created by palmitate (Coll et al. 2008), and oleate addition to palmitate prevented palmitate-induced mitochondrial DNA damage, increased cell ATP levels and cell viability (Yuzefovych, Wilson and Rachek, 2010). As single fatty acids, such as palmitate and oleate are not present alone in normal physiology and are found in combination with other fatty acids (Havel, Naimark and Borchgrevink, 1963), it would be of interest to examine the effect of varied combinations of fatty acids on the cell membrane distribution of BK channels.

5.3.3 Additional sites for potential lab based research

An additional site for lab-based experimentation would be G694C, a nsSNP prioritised in chapter 3. This mutation was predicted to create a new cysteine rich domain at the heme binding domain in the C-terminus of the channel. Thus, assessment of the mutant channel's affinity for haeme, and potential changes in cell membrane translocation would be of interest to investigate.

Exonic SNP are commonly the target of lab based research due to their potential impact on BK channel function; however, sites of post-translational modification, which add functional diversity to the channel are also worthy of investigation. The results of the H120Q and G122A experiments in chapter 4 suggest that mutations flanking key amino acids that undergo post-translational modification can affect those processes. When comparing the locations of predicted phosphorylation and the previously identified structural and functional domains, 3 sites of interest came to light (Figure 5.1). The predicted tyrosine phosphorylation site Y355 comprises the final amino acid of the potassium selectivity domain, which spans from T352 to Y355. Predicted phosphorylation site Y359 immediately flanks the pore forming domain, which spans from A336 to V358, whilst Y1027 is located only two amino acids downstream from the calcium bowl, located in the C-terminus, spanning from T1003 to E1025.

Figure 5.1- Sites of tyrosine phosphorylation of interest for future investigation

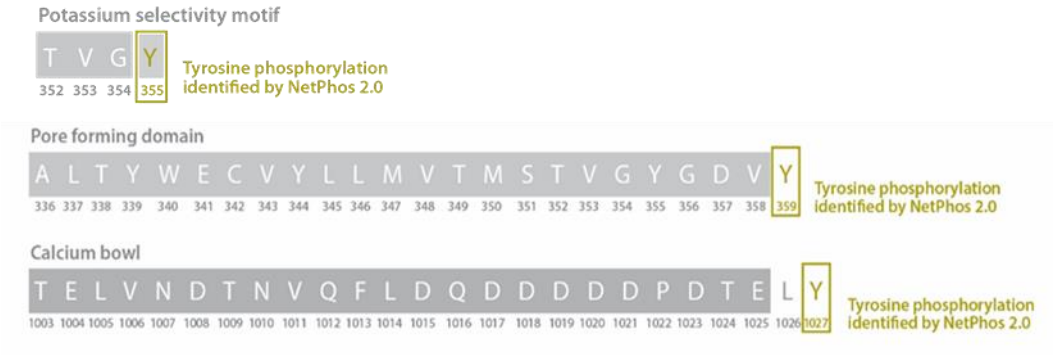


Figure 5.1- Sites of tyrosine phosphorylation of interest for future investigation. Included in the figure are predicted tyrosine phosphorylation sites Y354 within the potassium selectivity motif, Y359 adjacent to the pore forming domain and Y1027, located two amino acid residues downstream of the calcium bowl.

5.4 Conclusion

The creation of a novel BK channel resource in this thesis highlighted the limitations, and inter-dependency of bioinformatics and lab based experimentation, whilst SNP verification experiments solidified the link between S0-S1 cysteine residues and BK cellular distribution. Results also provided evidence that naturally occurring mutations and metabolic substrates affect the cell membrane expression of BK channels, and that metabolic substrates have differing effects. Figure 5.2 illustrates the steps undertaken in this thesis and summarises areas of future work. The BK channel is expressed ubiquitously in the body and plays an extensive and varied role in human physiology. Therefore, investigations that provide insight into channel regulation and the effects of naturally occurring genetic alterations will increase understanding of function and associations with disease. The potential clinical consequences of the SNPs prioritised in this thesis requires further research, but may be a target for future population stratification and personalised medicine.

Figure 5.2- Schematic of thesis workflow: Thesis summary and future work.

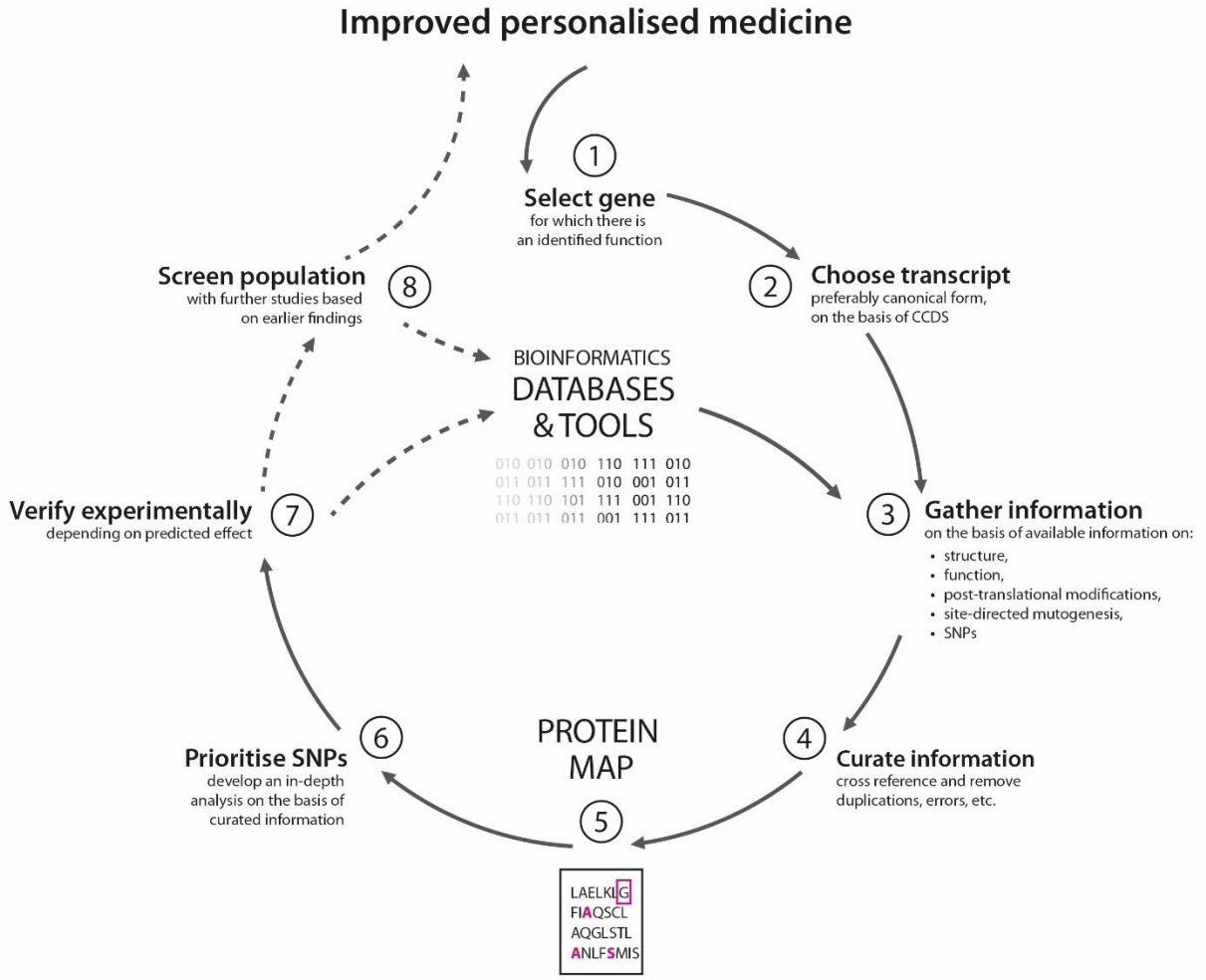


Figure 5.2- Schematic of thesis workflow: Thesis summary and future work. Described are the steps undertaken in this thesis work to identify new potential genetic targets for personalised medicine. Steps 1-6 were successfully achieved in chapter 3 by the selection of the BK channel gene KCNMA1, the canonical BK channel transcript, the collation of available information, the biocuration of this work into a 'BK channel resource' and the prioritisation of nsSNP. Step 7 was achieved as a result of the experimental verification of H55Q and G57A (H120A and G122Q in human) in chapter 4. Potential future avenues for the work would be to a) input the results into existing databases or predictive software training datasets, b) to input the data into a newly created BK channel database, c) conduct a GWAS or gene sequencing individuals to determine the incidence and linkage of H120A and G122A with disease traits.

6 References

Abdelmagid, S. A., Clarke, S. E., Nielsen, D. E., Badawi, A., El-Sohemy, A., Mutch, D. M. and Ma, D. W. L. (2015) 'Comprehensive Profiling of Plasma Fatty Acid Concentrations in Young Healthy Canadian Adults', *PLOS ONE*. Edited by P. Aspichueta. Public Library of Science, 10(2), p. e0116195. doi: 10.1371/journal.pone.0116195.

Abràmoff, M. D., Magalhães, P. J. and Ram, S. J. (2004) 'Image processing with imageJ', *Biophotonics International*, pp. 36–41. doi: 10.1117/1.3589100.

Adan, A., Alizada, G., Kiraz, Y., Baran, Y., Nalbant, A., Nel Alizada, G. and Mur Kiraz, Y. ˘ (2016) 'Flow cytometry: basic principles and applications Flow cytometry: basic principles and applications', *Yusuf Baran & Ayten Nalbant Critical Reviews in Biotechnology*. doi: 10.3109/07388551.2015.1128876.

Adelman, J., Shen, K., Kavanaugh, M., Warren, R., Wu, Y., Lagrutta, Bond, C. and North, R. (1992) 'Calcium-activated potassium channels expressed from cloned complementary DNAs.', *Neuron*, 9(2), pp. 209–16.

Adzhubei, I. A., Schmidt, S., Peshkin, L., Ramensky, V. E., Gerasimova, A., Bork, P., Kondrashov, A. S. and Sunyaev, S. R. (2010) 'A method and server for predicting damaging missense mutations.', *Nature methods*. NIH Public Access, 7(4), pp. 248–9. doi: 10.1038/nmeth0410-248.

Adzhubei, I., Jordan, D. M. and Sunyaev, S. R. (2013) 'Predicting functional effect of human missense mutations using PolyPhen-2.', *Current protocols in human genetics*. NIH Public Access, Chapter 7, p. Unit7.20. doi: 10.1002/0471142905.hg0720s76.

Aebersold, R. and Mann, M. (2003) 'Mass spectrometry-based proteomics.', *Nature*, 422(6928), pp. 198–207. doi: 10.1038/nature01511.

Aguilar-Martinez, E., Chen, X., Webber, A., Mould, A. P., Seifert, A., Hay, R. T. and Sharrocks, A. D. (2015) 'Screen for multi-SUMO-binding proteins reveals a multi-SIM-binding mechanism for recruitment of the transcriptional regulator ZMYM2 to chromatin.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 112(35), pp. E4854-63. doi: 10.1073/pnas.1509716112.

Ahern, G. (2002) 'cGMP and S-nitrosylation: two routes for modulation of neuronal excitability by NO', *Trends in Neurosciences*, 25(10), pp. 510–517. doi: 10.1016/S0166-2236(02)02254-3.

Ahn, Y.-T., Kim, Y.-M., Adams, E., Lyu, S.-C., Alvira, C. M. and Cornfield, D. N. (2012) 'Hypoxia-inducible factor-1α regulates KCNMB1 expression in human pulmonary artery smooth muscle cells.', *American journal of physiology. Lung cellular and molecular physiology*, 302(3), pp. L352-9. doi: 10.1152/ajplung.00302.2011.

Ahring, P. K., Strøbæk, D., Christophersen, P., Olesen, S.-P. and Johansen, T. E. (1997) 'Stable expression of the human large-conductance Ca2+-activated K+ channel α- and β-subunits in HEK293 cells', *FEBS Letters*, 415(1), pp. 67–70. doi: 10.1016/S0014-5793(97)01096-X.

Albert, F. W. and Kruglyak, L. (2015) 'The role of regulatory variation in complex traits and disease', *Nature Reviews Genetics*. Nature Research, 16(4), pp. 197–212. doi: 10.1038/nrg3891.

Aldrich, R. W., Brenner, R., Peréz, G. J., Bonev, A. D., Eckman, D. M., Kosek, J. C., Wiler, S. W., Patterson, A. J. and Nelson, M. T. (2000) 'Vasoregulation by the beta1 subunit of the calcium-activated potassium channel.', *Nature*, 407(6806), pp. 870–876. doi: 10.1038/35038011.

Alipoor, B., Ghaedi, H., Omrani, M. D., Bastami, M., Meshkani, R. and Golmohammadi, T. (2016) 'A

Bioinformatics Approach to Prioritize Single Nucleotide Polymorphisms in TLRs Signaling Pathway Genes', *International Journal of Molecular and Cellular Medicine*, 5(2), pp. 65–79.

Altman, R. B. (1998) 'Bioinformatics in support of molecular medicine.', *Proceedings. AMIA Symposium*. American Medical Informatics Association, pp. 53–61.

Alyass, A., Turcotte, M., Meyre, D., Hancock, W., Anderson, G. and Anderson, N. (2015) 'From big data analysis to personalized medicine for all: challenges and opportunities', *BMC Medical Genomics*. BioMed Central, 8(1), p. 33. doi: 10.1186/s12920-015-0108-y.

Antoniou, A., Pharoah, P. D. P., Narod, S., Risch, H. A., Eyfjord, J. E., Hopper, J. L., Loman, N., Olsson, H., Johannsson, O., Borg, Å., Pasini, B., Radice, P., Manoukian, S., Eccles, D. M., Tang, N., Olah, E., Anton-Culver, H., Warner, E., Lubinski, J., Gronwald, J., Gorski, B., Tulinius, H., Thorlacius, S., Eerola, H., Nevanlinna, H., Syrjäkoski, K., Kallioniemi, O.-P., Thompson, D., Evans, C., Peto, J., Lalloo, F., Evans, D. G. and Easton, D. F. (2003) 'Average Risks of Breast and Ovarian Cancer Associated with BRCA1 or BRCA2 Mutations Detected in Case Series Unselected for Family History: A Combined Analysis of 22 Studies', *The American Journal of Human Genetics*, 72(5), pp. 1117–1130. doi: 10.1086/375033.

Arias, T. D., Jorge, L. F. and Barrantes, R. (1991) 'Uses and misuses of definitions of genetic polymorphism. A perspective from population pharmacogenetics.', *British journal of clinical pharmacology*. Wiley-Blackwell, 31(1), pp. 117–9.

Auton, A., Abecasis, G. R., Altshuler, D. M., Durbin, R. M., Abecasis, G. R., Bentley, D. R., Chakravarti, A., Clark, A. G., Donnelly, P., Eichler, E. E., Flicek, P., Gabriel, S. B., Gibbs, R. A., Green, E. D., Hurles, M. E., Knoppers, B. M., Korbel, J. O., Lander, E. S., Lee, C., Lehrach, H., Mardis, E. R., Marth, G. T., McVean, G. A., Nickerson, D. A., Schmidt, J. P., Sherry, S. T., Wang, J., Wilson, R. K., Gibbs, R. A., Boerwinkle, E., Doddapaneni, H., Han, Y., Korchina, V., Kovar, C., Lee, S., Muzny, D., Reid, J. G., Zhu, Y., Wang, J., Chang, Y., Feng, Q., Fang, X., Guo, X., Jian, M., Jiang, H., Jin, X., Lan, T., Li, G., Li, J., Li, Y., Liu, S., Liu, X., Lu, Y., Ma, X., Tang, M., Wang, B., Wang, G., Wu, H., Wu, R., Xu, X., Yin, Y., Zhang, D., Zhang, W., Zhao, J., Zhao, M., Zheng, X., Lander, E. S., Altshuler, D. M., Gabriel, S. B., Gupta, N., Gharani, N., Toji, L. H., Gerry, N. P., Resch, A. M., Flicek, P., Barker, J., Clarke, L., Gil, L., Hunt, S. E., Kelman, G., Kulesha, E., Leinonen, R., McLaren, W. M., Radhakrishnan, R., Roa, A., Smirnov, D., Smith, R. E., Streeter, I., Thormann, A., Toneva, I., Vaughan, B., Zheng-Bradley, X., Bentley, D. R., Grocock, R., Humphray, S., James, T., Kingsbury, Z., Lehrach, H., Sudbrak, R., Albrecht, M. W., Amstislavskiy, V. S., Borodina, T. A., Lienhard, M., Mertes, F., Sultan, M., Timmermann, B., Yaspo, M.-L., Mardis, E. R., Wilson, R. K., Fulton, L., Fulton, R., Sherry, S. T., Ananiev, V., Belaia, Z., Beloslyudtsev, D., Bouk, N., Chen, C., Church, D., Cohen, R., Cook, C., Garner, J., Hefferon, T., Kimelman, M., Liu, C., Lopez, J., Meric, P., O'Sullivan, C., Ostapchuk, Y., Phan, L., Ponomarov, S., Schneider, V., Shekhtman, E., Sirotkin, K., Slotta, D., Zhang, H., McVean, G. A., Durbin, R. M., Balasubramaniam, S., Burton, J., Danecek, P., Keane, T. M., Kolb-Kokocinski, A., McCarthy, S., Stalker, J., Quail, M., Schmidt, J. P., Davies, C. J., Gollub, J., Webster, T., Wong, B., Zhan, Y., Auton, A., Campbell, C. L., Kong, Y., Marcketta, A., Gibbs, R. A., Yu, F., Antunes, L., Bainbridge, M., Muzny, D., Sabo, A., Huang, Z., Wang, J., Coin, L. J. M., Fang, L., Guo, X., Jin, X., Li, G., Li, Q., Li, Y., Li, Z., Lin, H., Liu, B., Luo, R., Shao, H., Xie, Y., Ye, C., Yu, C., Zhang, F., Zheng, H., Zhu, H., Alkan, C., Dal, E., Kahveci, F., Marth, G. T., Garrison, E. P., Kural, D., Lee, W.-P., Fung Leong, W., Stromberg, M., Ward, A. N., Wu, J., Zhang, M., Daly, M. J., DePristo, M. A., Handsaker, R. E., Altshuler, D. M., Banks, E., Bhatia, G., del Angel, G., Gabriel, S. B., Genovese, G., Gupta, N., Li, H., Kashin, S., Lander, E. S., McCarroll, S. A., Nemesh, J. C., Poplin, R. E., Yoon, S. C., Lihm, J., Makarov, V., Clark, A. G., Gottipati, S., Keinan, A., Rodriguez-Flores, J. L., Korbel, J. O., Rausch, T., Fritz, M. H., Stütz, A. M., Flicek, P., Beal, K., Clarke, L., Datta, A., Herrero, J., McLaren, W. M., Ritchie, G. R. S., Smith, R. E., Zerbino, D., Zheng-Bradley, X., Sabeti, P. C., Shlyakhter, I., Schaffner, S. F., Vitti, J., Cooper, D. N., Ball, E. V., Stenson, P. D., Bentley, D. R., Barnes, B., Bauer, M., Keira Cheetham, R., Cox, A., Eberle, M., Humphray, S., Kahn, S., Murray, L., Peden, J., Shaw, R., Kenny, E. E., Batzer, M. A., Konkel, M. K., Walker, J. A., MacArthur, D. G., Lek, M., Sudbrak, R., Amstislavskiy, V. S., Herwig, R., Mardis, E. R., Ding, L., Koboldt, D. C., Larson,

D., Ye, K., Gravel, S., Swaroop, A., Chew, E., Lappalainen, T., Erlich, Y., Gymrek, M., Frederick Willems, T., Simpson, J. T., Shriver, M. D., Rosenfeld, J. A., Bustamante, C. D., Montgomery, S. B., De La Vega, F. M., Byrnes, J. K., Carroll, A. W., DeGorter, M. K., Lacroute, P., Maples, B. K., Martin, A. R., Moreno-Estrada, A., Shringarpure, S. S., Zakharia, F., Halperin, E., Baran, Y., Lee, C., Cerveira, E., Hwang, J., Malhotra, A., Plewczynski, D., Radew, K., Romanovitch, M., Zhang, C., Hyland, F. C. L., Craig, D. W., Christoforides, A., Homer, N., Izatt, T., Kurdoglu, A. A., Sinari, S. A., Squire, K., Sherry, S. T., Xiao, C., Sebat, J., Antaki, D., Gujral, M., Noor, A., Ye, K., Burchard, E. G., Hernandez, R. D., Gignoux, C. R., Haussler, D., Katzman, S. J., James Kent, W., Howie, B., Ruiz-Linares, A., Dermitzakis, E. T., Devine, S. E., Abecasis, G. R., Min Kang, H., Kidd, J. M., Blackwell, T., Caron, S., Chen, W., Emery, S., Fritsche, L., Fuchsberger, C., Jun, G., Li, B., Lyons, R., Scheller, C., Sidore, C., Song, S., Sliwerska, E., Taliun, D., Tan, A., Welch, R., Kate Wing, M., Zhan, X., Awadalla, P., Hodgkinson, A., Li, Y., Shi, X., Quitadamo, A., Lunter, G., McVean, G. A., Marchini, J. L., Myers, S., Churchhouse, C., Delaneau, O., Gupta-Hinch, A., Kretzschmar, W., Iqbal, Z., Mathieson, I., Menelaou, A., Rimmer, A., Xifara, D. K., Oleksyk, T. K., Fu, Y., Liu, X., Xiong, M., Jorde, L., Witherspoon, D., Xing, J., Eichler, E. E., Browning, B. L., Browning, S. R., Hormozdiari, F., Sudmant, P. H., Khurana, E., Durbin, R. M., Hurles, M. E., Tyler-Smith, C., Albers, C. A., Ayub, Q., Balasubramaniam, S., Chen, Y., Colonna, V., Danecek, P., Jostins, L., Keane, T. M., McCarthy, S., Walter, K., Xue, Y., Gerstein, M. B., Abyzov, A., Balasubramanian, S., Chen, J., Clarke, D., Fu, Y., Harmanci, A. O., Jin, M., Lee, D., Liu, J., Jasmine Mu, X., Zhang, J., Zhang, Y., Li, Y., Luo, R., Zhu, H., Alkan, C., Dal, E., Kahveci, F., Marth, G. T., Garrison, E. P., Kural, D., Lee, W.-P., Ward, A. N., Wu, J., Zhang, M., McCarroll, S. A., Handsaker, R. E., Altshuler, D. M., Banks, E., del Angel, G., Genovese, G., Hartl, C., Li, H., Kashin, S., Nemesh, J. C., Shakir, K., Yoon, S. C., Lihm, J., Makarov, V., Degenhardt, J., Korbel, J. O., Fritz, M. H., Meiers, S., Raeder, B., Rausch, T., Stütz, A. M., Flicek, P., Paolo Casale, F., Clarke, L., Smith, R. E., Stegle, O., Zheng-Bradley, X., Bentley, D. R., Barnes, B., Keira Cheetham, R., Eberle, M., Humphray, S., Kahn, S., Murray, L., Shaw, R., Lameijer, E.-W., Batzer, M. A., Konkel, M. K., Walker, J. A., Ding, L., Hall, I., Ye, K., Lacroute, P., Lee, C., Cerveira, E., Malhotra, A., Hwang, J., Plewczynski, D., Radew, K., Romanovitch, M., Zhang, C., Craig, D. W., Homer, N., Church, D., Xiao, C., Sebat, J., Antaki, D., Bafna, V., Michaelson, J., Ye, K., Devine, S. E., Gardner, E. J., Abecasis, G. R., Kidd, J. M., Mills, R. E., Dayama, G., Emery, S., Jun, G., Shi, X., Quitadamo, A., Lunter, G., McVean, G. A., Chen, K., Fan, X., Chong, Z., Chen, T., Witherspoon, D., Xing, J., Eichler, E. E., Chaisson, M. J., Hormozdiari, F., Huddleston, J., Malig, M., Nelson, B. J., Sudmant, P. H., Parrish, N. F., Khurana, E., Hurles, M. E., Blackburne, B., Lindsay, S. J., Ning, Z., Walter, K., Zhang, Y., Gerstein, M. B., Abyzov, A., Chen, J., Clarke, D., Lam, H., Jasmine Mu, X., Sisu, C., Zhang, J., Zhang, Y., Gibbs, R. A., Yu, F., Bainbridge, M., Challis, D., Evani, U. S., Kovar, C., Lu, J., Muzny, D., Nagaswamy, U., Reid, J. G., Sabo, A., Yu, J., Guo, X., Li, W., Li, Y., Wu, R., Marth, G. T., Garrison, E. P., Fung Leong, W., Ward, A. N., del Angel, G., DePristo, M. A., Gabriel, S. B., Gupta, N., Hartl, C., Poplin, R. E., Clark, A. G., Rodriguez-Flores, J. L., Flicek, P., Clarke, L., Smith, R. E., Zheng-Bradley, X., MacArthur, D. G., Mardis, E. R., Fulton, R., Koboldt, D. C., Gravel, S., Bustamante, C. D., Craig, D. W., Christoforides, A., Homer, N., Izatt, T., Sherry, S. T., Xiao, C., Dermitzakis, E. T., Abecasis, G. R., Min Kang, H., McVean, G. A., Gerstein, M. B., Balasubramanian, S., Habegger, L., Yu, H., Flicek, P., Clarke, L., Cunningham, F., Dunham, I., Zerbino, D., Zheng-Bradley, X., Lage, K., Berg Jespersen, J., Horn, H., Montgomery, S. B., DeGorter, M. K., Khurana, E., Tyler-Smith, C., Chen, Y., Colonna, V., Xue, Y., Gerstein, M. B., Balasubramanian, S., Fu, Y., Kim, D., Auton, A., Marcketta, A., Desalle, R., Narechania, A., Wilson Sayres, M. A., Garrison, E. P., Handsaker, R. E., Kashin, S., McCarroll, S. A., Rodriguez-Flores, J. L., Flicek, P., Clarke, L., Zheng-Bradley, X., Erlich, Y., Gymrek, M., Frederick Willems, T., Bustamante, C. D., Mendez, F. L., David Poznik, G., Underhill, P. A., Lee, C., Cerveira, E., Malhotra, A., Romanovitch, M., Zhang, C., Abecasis, G. R., Coin, L., Shao, H., Mittelman, D., Tyler-Smith, C., Ayub, Q., Banerjee, R., Cerezo, M., Chen, Y., Fitzgerald, T. W., Louzada, S., Massaia, A., McCarthy, S., Ritchie, G. R., Xue, Y., Yang, F., Gibbs, R. A., Kovar, C., Kalra, D., Hale, W., Muzny, D., Reid, J. G., Wang, J., Dan, X., Guo, X., Li, G., Li, Y., Ye, C., Zheng, X., Altshuler, D. M., Flicek, P., Clarke, L., Zheng-Bradley, X., Bentley, D. R., Cox, A., Humphray, S., Kahn, S., Sudbrak, R., Albrecht, M. W., Lienhard, M., Larson, D., Craig, D. W., Izatt, T., Kurdoglu, A. A., Sherry, S. T., Xiao, C., Haussler, D., Abecasis, G. R., McVean, G. A., Durbin, R. M., Balasubramaniam, S., Keane, T. M., McCarthy, S., Stalker, J., Chakravarti, A., Knoppers, B. M.,

Abecasis, G. R., Barnes, K. C., Beiswanger, C., Burchard, E. G., Bustamante, C. D., Cai, H., Cao, H., Durbin, R. M., Gerry, N. P., Gharani, N., Gibbs, R. A., Gignoux, C. R., Gravel, S., Henn, B., Jones, D., Jorde, L., Kaye, J. S., Keinan, A., Kent, A., Kerasidou, A., Li, Y., Mathias, R., McVean, G. A., Moreno-Estrada, A., Ossorio, P. N., Parker, M., Resch, A. M., Rotimi, C. N., Royal, C. D., Sandoval, K., Su, Y., Sudbrak, R., Tian, Z., Tishkoff, S., Toji, L. H., Tyler-Smith, C., Via, M., Wang, Y., Yang, H., Yang, L., Zhu, J., Bodmer, W., Bedoya, G., Ruiz-Linares, A., Cai, Z., Gao, Y., Chu, J., Peltonen, L., Garcia-Montero, A., Orfao, A., Dutil, J., Martinez-Cruzado, J. C., Oleksyk, T. K., Barnes, K. C., Mathias, R. A., Hennis, A., Watson, H., McKenzie, C., Qadri, F., LaRocque, R., Sabeti, P. C., Zhu, J., Deng, X., Sabeti, P. C., Asogun, D., Folarin, O., Happi, C., Omoniwa, O., Stremlau, M., Tariyal, R., Jallow, M., Sisay Joof, F., Corrah, T., Rockett, K., Kwiatkowski, D., Kooner, J., Tịnh Hiê`n, T., Dunstan, S. J., Thuy Hang, N., Fonnie, R., Garry, R., Kanneh, L., Moses, L., Sabeti, P. C., Schieffelin, J., Grant, D. S., Gallo, C., Poletti, G., Saleheen, D., Rasheed, A., Brooks, L. D., Felsenfeld, A. L., McEwen, J. E., Vaydylevich, Y., Green, E. D., Duncanson, A., Dunn, M., Schloss, J. A., Wang, J., Yang, H., Auton, A., Brooks, L. D., Durbin, R. M., Garrison, E. P., Min Kang, H., Korbel, J. O., Marchini, J. L., McCarthy, S., McVean, G. A. and Abecasis, G. R. (2015) 'A global reference for human genetic variation', *Nature*. Nature Research, 526(7571), pp. 68–74. doi: 10.1038/nature15393.

Avdonin, V., Tang, X. D. and Hoshi, T. (2003) 'Stimulatory action of internal protons on Slo1 BK channels.', *Biophysical journal*, 84(5), pp. 2969–80. doi: 10.1016/S0006-3495(03)70023-X.

Baekkeskov, S. and Kanaani, J. (2009) 'Palmitoylation cycles and regulation of protein function.', *Molecular membrane biology*, 26(1), pp. 42–54. doi: 10.1080/09687680802680108.

Bagge, A., Clausen, T. R., Larsen, S., Ladefoged, M., Rosenstierne, M. W., Larsen, L., Vang, O., Nielsen, J. H. and Dalgaard, L. T. (2012) 'MicroRNA-29a is up-regulated in beta-cells by glucose and decreases glucose-stimulated insulin secretion', *Biochemical and Biophysical Research Communications*, 426(2), pp. 266–272. doi: 10.1016/j.bbrc.2012.08.082.

Bai, J.-P., Surguchev, A., Joshi, P., Gross, L. and Navaratnam, D. (2012) 'CDK5 interacts with Slo and affects its surface expression and kinetics through direct phosphorylation.', *American journal of physiology. Cell physiology*. American Physiological Society, 302(5), pp. C766-80. doi: 10.1152/ajpcell.00339.2011.

Bairoch, A. and Apweiler, R. (1996) 'The SWISS-PROT protein sequence data bank and its new supplement TREMBL.', *Nucleic acids research*. Oxford University Press, 24(1), pp. 21–5.

Bairoch, A. and Boeckmann, B. (1991) 'The SWISS-PROT protein sequence data bank.', *Nucleic acids research*. Oxford University Press, 19 Suppl(Suppl), pp. 2247–9.

Baker, W., van den Broek, A., Camon, E., Hingamp, P., Sterk, P., Stoesser, G. and Tuli, M. A. (2000) 'The EMBL nucleotide sequence database.', *Nucleic acids research*. Oxford University Press, 28(1), pp. 19–23.

Bao, L., Kaldany, C., Holmstrand, E. C. and Cox, D. H. (2004) 'Mapping the BK Ca Channel's "Ca 2+ Bowl"', *The Journal of General Physiology*, 123(5), pp. 475–489. doi: 10.1085/jgp.200409052.

Bao, L., Rapin, A. M., Holmstrand, E. C. and Cox, D. H. (2002) 'Elimination of the BKCa Channel's High-Affinity Ca 2+ Sensitivity', *The Journal of General Physiology*, 120(2), pp. 173–189. doi: 10.1085/jgp.20028627.

Bao, R., Huang, L., Andrade, J., Tan, W., Kibbe, W. A., Jiang, H. and Feng, G. (2014) 'Review of current methods, applications, and data management for the bioinformatics analysis of whole exome sequencing.', *Cancer informatics*. Libertas Academica, 13(Suppl 2), pp. 67–82. doi: 10.4137/CIN.S13779.

Barden, J. A., Sluyter, R., Gu, B. J. and Wiley, J. S. (2003) 'Specific detection of non-functional human P2X7 receptors in HEK293 cells and B-lymphocytes', *FEBS Letters*, 538(1–3), pp. 159–162. doi:

10.1016/S0014-5793(03)00172-8.

Bateman, A. (2010) 'Curators of the world unite: the International Society of Biocuration', *Bioinformatics*, 26(8), pp. 991–991. doi: 10.1093/bioinformatics/btq101.

Bauer, M. C., Nilsson, H., Thulin, E., Frohm, B., Malm, J. and Linse, S. (2008) 'Zn2+ binding to human calbindin D(28k) and the role of histidine residues.', *Protein science : a publication of the Protein Society*, 17(4), pp. 760–7. doi: 10.1110/ps.073381108.

Beck-Sickinger, A. G. and Mörl, K. (2006) 'Posttranslational Modification of Proteins. Expanding Nature's Inventory. By Christopher T. Walsh.', *Angewandte Chemie International Edition*. Roberts and Co. Publishers, 45(7), pp. 1020–1020. doi: 10.1002/anie.200585363.

Belkadi, A., Bolze, A., Itan, Y., Cobat, A., Vincent, Q. B., Antipenko, A., Shang, L., Boisson, B., Casanova, J.-L. and Abel, L. (2015) 'Whole-genome sequencing is more powerful than whole-exome sequencing for detecting exome variants.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 112(17), pp. 5473–8. doi: 10.1073/pnas.1418631112.

Benoit, S. C., Kemp, C. J., Elias, C. F., Abplanalp, W., Herman, J. P., Migrenne, S., Lefevre, A.-L., Cruciani-Guglielmacci, C., Magnan, C., Yu, F., Niswender, K., Irani, B. G., Holland, W. L. and Clegg, D. J. (2009) 'Palmitic acid mediates hypothalamic insulin resistance by altering PKC-theta subcellular localization in rodents.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 119(9), pp. 2577–89. doi: 10.1172/JCI36714.

Bentrop, D., Beyermann, M., Wissmann, R. and Fakler, B. (2001) 'NMR Structure of the "Ball-and-chain" Domain of KCNMB2, the β2-Subunit of Large Conductance Ca2+- and Voltage-activated Potassium Channels', *Journal of Biological Chemistry*, 276(45), pp. 42116–42121. doi: 10.1074/jbc.M107118200.

Bergouignan, A., Trudel, G., Simon, C., Chopard, A., Schoeller, D. A., Momken, I., Votruba, S. B., Desage, M., Burdge, G. C., Gauquelin-Koch, G., Normand, S. and Blanc, S. (2009) 'Physical inactivity differentially alters dietary oleate and palmitate trafficking', *Diabetes*, 58, pp. 367–376. doi: 10.2337/db08-0263.

Bernstein, F. C., Koetzle, T. F., Williams, G. J. B., Meyer, E. F., Brice, M. D., Rodgers, J. R., Kennard, O., Shimanouchi, T. and Tasumi, M. (1977) 'The protein data bank: A computer-based archival file for macromolecular structures', *Journal of Molecular Biology*, 112(3), pp. 535–542. doi: 10.1016/S0022-2836(77)80200-3.

Bertier, G., Joly, Y. and Hétu, M. (2016) 'Unsolved challenges of clinical whole-exome sequencing: A systematic literature review of end-users' views. Accepted 07/28/2016', *BMC medical genomics*, 9(1), pp. 1–12. doi: 10.1186/s12920-016-0213-6.

Berweck, S., Lepple-Wienhues, A., Stöss, M. and Wiederholt, M. (1994) 'Large conductance calcium-activated potassium channels in cultured retinal pericytes under normal and high-glucose conditions.', *Pflügers Archiv : European journal of physiology*, 427(1–2), pp. 9–16.

Besant, P. G., Tan, E. and Attwood, P. V (2003) 'Mammalian protein histidine kinases', *The International Journal of Biochemistry & Cell Biology*, 35(3), pp. 297–309. doi: 10.1016/S1357-2725(02)00257-1.

Bi, D. (2014) *Palmitoylation of large conductance voltage- and calcium-dependent potassium (BK) channels*. The University of Edinburgh.

Bijland, S., Mancini, S. J. and Salt, I. P. (2013) 'Role of AMP-activated protein kinase in adipose tissue metabolism and inflammation', *Clinical Science*, 124(8).

Biswas, S. and Mallikarjuna Rao, C. (2017) 'Epigenetics in cancer: Fundamentals and Beyond', *Pharmacology & Therapeutics*, 1. doi: http://dx.doi.org/10.1016/j.pharmthera.2017.02.011.

Bizzozero, O. A., Sanchez, P. and Tetzloff, S. U. (2002) 'Effect of ATP Depletion on the Palmitoylation of

Myelin Proteolipid Protein in Young and Adult Rats', *Journal of Neurochemistry*. Blackwell Science Ltd., 72(6), pp. 2610–2616. doi: 10.1046/j.1471-4159.1999.0722610.x.

Blau, N., van Spronsen, F. J., Levy, H. L., Elzaouk, L., Wolff, J. and Thony, B. (2010) 'Phenylketonuria.', *Lancet (London, England)*. Springer, Heidelberg, 376(9750), pp. 1417–27. doi: 10.1016/S0140-6736(10)60961-0.

Blom, N., Gammeltoft, S. and Brunak, S. (1999) 'Sequence and structure-based prediction of eukaryotic protein phosphorylation sites.', *Journal of molecular biology*, 294, pp. 1351–1362. doi: 10.1006/jmbi.1999.3310.

Boeckmann, B., Bairoch, A., Apweiler, R., Blatter, M.-C., Estreicher, A., Gasteiger, E., Martin, M. J., Michoud, K., O'Donovan, C., Phan, I., Pilbout, S. and Schneider, M. (2003) 'The SWISS-PROT protein knowledgebase and its supplement TrEMBL in 2003.', *Nucleic acids research*. Oxford University Press, 31(1), pp. 365–70.

Boeckmann, B., Blatter, M.-C., Famiglietti, L., Hinz, U., Lane, L., Roechert, B. and Bairoch, A. (2005) 'Protein variety and functional diversity: Swiss-Prot annotation in its biological context.', *Comptes rendus biologies*, 328(10–11), pp. 882–99. doi: 10.1016/j.crvi.2005.06.001.

Bolster, D. R., Crozier, S. J., Kimball, S. R. and Jefferson, L. S. (2002) 'AMP-activated Protein Kinase Suppresses Protein Synthesis in Rat Skeletal Muscle through Down-regulated Mammalian Target of Rapamycin (mTOR) Signaling', *Journal of Biological Chemistry*, 277(27), pp. 23977–23980. doi: 10.1074/jbc.C200171200.

Braun, A. P. and Sy, L. (2001) 'Contribution of potential EF hand motifs to the calcium-dependent gating of a mouse brain large conductance, calcium-sensitive K(+) channel.', *The Journal of physiology*. Wiley-Blackwell, 533(Pt 3), pp. 681–95. doi: 10.1111/j.1469-7793.2001.00681.x.

Bravo-Zehnder, M., Orio, P., Norambuena, A., Wallner, M., Meera, P., Toro, L., Latorre, R. and González, A. (2000) 'Apical sorting of a voltage- and Ca2+-activated K+ channel alpha -subunit in Madin-Darby canine kidney cells is independent of N-glycosylation.', *Proceedings of the National Academy of Sciences*, 97(24), pp. 13114–9. doi: 10.1073/pnas.240455697.

Brayden, J. and Nelson, M. (1992) 'Regulation of arterial tone by activation of calcium-dependent potassium channels', *Science*, 256(5056).

Brelidze, T. I. and Magleby, K. L. (2005) 'Probing the geometry of the inner vestibule of BK channels with sugars.', *The Journal of general physiology*. The Rockefeller University Press, 126(2), pp. 105–21. doi: 10.1085/jgp.200509286.

Brelidze, T. I., Niu, X. and Magleby, K. L. (2003) 'A ring of eight conserved negatively charged amino acids doubles the conductance of BK channels and prevents inward rectification.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 100(15), pp. 9017–22. doi: 10.1073/pnas.1532257100.

Brenner, R., Chen, Q. H., Vilaythong, A., Toney, G. M., Noebels, J. L. and Aldrich, R. W. (2005) 'BK channel beta4 subunit reduces dentate gyrus excitability and protects against temporal lobe seizures.', *Nature neuroscience*, 8(12), pp. 1752–1759. doi: 10.1038/nn1573.

Brenner, R., Jegla, T. J., Wickenden, A., Liu, Y. and Aldrich, R. W. (2000) 'Cloning and functional characterization of novel large conductance calcium-activated potassium channel beta subunits, hKCNMB3 and hKCNMB4', *The Journal of biological chemistry*, 275(9), pp. 6453–6461. doi: 10.1074/jbc.275.9.6453.

Broca, C., Varin, E., Armanet, M., Tourrel-Cuzin, C., Bosco, D., Dalle, S. and Wojtusciszyn, A. (2014) 'Proteasome dysfunction mediates high glucose-induced apoptosis in rodent beta cells and human

islets.', *PloS one*. Public Library of Science, 9(3), p. e92066. doi: 10.1371/journal.pone.0092066.

Bryzgalov, L. O., Antontseva, E. V., Matveeva, M. Y., Shilov, A. G., Kashina, E. V., Mordvinov, V. A. and Merkulova, T. I. (2013) 'Detection of Regulatory SNPs in Human Genome Using ChIP-seq ENCODE Data', *PLoS ONE*. Edited by L. Chen. Public Library of Science, 8(10), p. e78833. doi: 10.1371/journal.pone.0078833.

Buchanan, C. C., Torstenson, E. S., Bush, W. S. and Ritchie, M. D. (2012) 'A comparison of cataloged variation between International HapMap Consortium and 1000 Genomes Project data.', *Journal of the American Medical Informatics Association : JAMIA*. American Medical Informatics Association, 19(2), pp. 289–94. doi: 10.1136/amiajnl-2011-000652.

Buglino, J. A. and Resh, M. D. (2008) 'Hhat is a palmitoylacyltransferase with specificity for N-palmitoylation of Sonic Hedgehog.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 283(32), pp. 22076–88. doi: 10.1074/jbc.M803901200.

Buglino, J. A. and Resh, M. D. (2012) 'Palmitoylation of Hedgehog proteins.', *Vitamins and hormones*. NIH Public Access, 88, pp. 229–52. doi: 10.1016/B978-0-12-394622-5.00010-9.

Bukiya, A. N., Belani, J. D., Rychnovsky, S. and Dopico, A. M. (2011) 'Specificity of cholesterol and analogs to modulate BK channels points to direct sterol-channel protein interactions.', *The Journal of general physiology*, 137(1), pp. 93–110. doi: 10.1085/jgp.201010519.

Bunn, R. C., Cockrell, G. E., Ou, Y., Thrailkill, K. M., Lumpkin, C. K. and Fowlkes, J. L. (2010) 'Palmitate and insulin synergistically induce IL-6 expression in human monocytes.', *Cardiovascular diabetology*, 9(1), p. 73. doi: 10.1186/1475-2840-9-73.

Burge, S., Attwood, T. K., Bateman, A., Berardini, T. Z., Cherry, M., O'Donovan, C., Xenarios, L. and Gaudet, P. (2012) 'Biocurators and biocuration: surveying the 21st century challenges.', *Database : the journal of biological databases and curation*. Oxford University Press, 2012, p. bar059. doi: 10.1093/database/bar059.

Bürger, M., Zimmermann, T. J., Kondoh, Y., Stege, P., Watanabe, N., Osada, H., Waldmann, H. and Vetter, I. R. (2012) 'Crystal structure of the predicted phospholipase LYPLAL1 reveals unexpected functional plasticity despite close relationship to acyl protein thioesterases.', *Journal of lipid research*. American Society for Biochemistry and Molecular Biology, 53(1), pp. 43–50. doi: 10.1194/jlr.M019851.

Burke, W. and Psaty, B. M. (2007) 'Personalized Medicine in the Era of Genomics', *JAMA*. American Medical Association, 298(14), p. 1682. doi: 10.1001/jama.298.14.1682.

Bush, W. S. and Moore, J. H. (2012) 'Chapter 11: Genome-wide association studies.', *PLoS computational biology*. Public Library of Science, 8(12), p. e1002822. doi: 10.1371/journal.pcbi.1002822.

Butler, A., Tsunoda, S., McCobb, D., Wei, A. and Salkoff, L. (1993) 'mSlo, a complex mouse gene encoding "maxi" calcium-activated potassium channels', *Science*, 261(5118), pp. 221–224. doi: 10.1126/science.7687074.

Cai, N., Zhao, X., Jing, Y., Sun, K., Jiao, S., Chen, X., Yang, H., Zhou, Y. and Wei, L. (2014) 'Autophagy protects against palmitate-induced apoptosis in hepatocytes.', *Cell & bioscience*, 4, p. 28. doi: 10.1186/2045-3701-4-28.

Calder, P. C. and Yaqoob, P. (2007) 'Lipid Rafts — Composition , Characterization , and Controversies', 2(December 2006), pp. 545–547.

Cameron, D. (2012) *DNA tests to revolutionise fight against cancer and help 100,000 NHS patients - GOV.UK*. London.

Camp, L. A. and Hofmann, S. L. (1993) 'Purification and properties of a palmitoyl-protein thioesterase that cleaves palmitate from H-Ras.', *The Journal of biological chemistry*, 268(30), pp. 22566–74.

Cannata, N., Merelli, E., Altman, R. B., Ng, G. and Jacoby, A. (2005) 'Time to Organize the Bioinformatics Resourceome', *PLoS Computational Biology*. Public Library of Science, 1(7), p. e76. doi: 10.1371/journal.pcbi.0010076.

Castaman, G., Giacomelli, S. H., Mancuso, M. E., D'Andrea, G., Santacroce, R., Sanna, S., Santagostino, E., Mannucci, P. M., Goodeve, A. and Rodeghiero, F. (2011) 'Deep intronic variations may cause mild hemophilia A.', *Journal of thrombosis and haemostasis : JTH*, 9(8), pp. 1541–8. doi: 10.1111/j.1538-7836.2011.04408.x.

Cavalli-Sforz, L. and Bodmer, W. (1971) 'The Genetics of Human Populations', in *Segregation, Linkage Analysis, and Estimation of Gene Frequencies.* San Francisco: W. H. Freeman & Co, p. 118.

Cazanave, S. C., Mott, J. L., Bronk, S. F., Werneburg, N. W., Fingas, C. D., Meng, X. W., Finnberg, N., El-Deiry, W. S., Kaufmann, S. H. and Gores, G. J. (2011) 'Death receptor 5 signaling promotes hepatocyte lipoapoptosis.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 286(45), pp. 39336–48. doi: 10.1074/jbc.M111.280420.

Cerf, M. E. (2013) 'Beta cell dysfunction and insulin resistance.', *Frontiers in endocrinology*. Frontiers Media SA, 4, p. 37. doi: 10.3389/fendo.2013.00037.

Chaki, M., Kovacs, I., Spannagl, M. and Lindermayr, C. (2014) 'Computational Prediction of Candidate Proteins for S-Nitrosylation in Arabidopsis thaliana', *PLoS ONE*. Public Library of Science, 9(10). doi: 10.1371/JOURNAL.PONE.0110232.

Chang, G. (2003) 'Structure of MsbA from Vibrio cholera: a multidrug resistance ABC transporter homolog in a closed conformation.', *Journal of molecular biology*, 330(2), pp. 419–30.

Chang, G. and Roth, C. B. (2001) 'Structure of MsbA from E. coli: A Homolog of the Multidrug Resistance ATP Binding Cassette (ABC) Transporters', *Science*, 293(5536), pp. 1793–1800. doi: 10.1126/science.293.5536.1793.

Chang, H., Ma, Y.-G., Wang, Y.-Y., Song, Z., Li, Q., Yang, N., Zhao, H.-Z., Feng, H.-Z., Chang, Y.-M., Ma, J., Yu, Z.-B. and Xie, M.-J. (2011) 'High glucose alters apoptosis and proliferation in HEK293 cells by inhibition of cloned BK Ca channel.', *Journal of cellular physiology*, 226(6), pp. 1660–1675. doi: 10.1002/jcp.22497.

Chapman, M. L., Krovetz, H. S. and VanDongen, A. M. J. (2001) 'GYGD pore motifs in neighbouring potassium channel subunits interact to determine ion selectivity', *The Journal of Physiology*. Wiley-Blackwell, 530(1), pp. 21–33. doi: 10.1111/j.1469-7793.2001.0021m.x.

Chapman, P. B., Hauschild, A., Robert, C., Haanen, J. B., Ascierto, P., Larkin, J., Dummer, R., Garbe, C., Testori, A., Maio, M., Hogg, D., Lorigan, P., Lebbe, C., Jouary, T., Schadendorf, D., Ribas, A., O'Day, S. J., Sosman, J. A., Kirkwood, J. M., Eggermont, A. M. M., Dreno, B., Nolop, K., Li, J., Nelson, B., Hou, J., Lee, R. J., Flaherty, K. T. and McArthur, G. A. (2011) 'Improved Survival with Vemurafenib in Melanoma with BRAF V600E Mutation', *New England Journal of Medicine*. Massachusetts Medical Society , 364(26), pp. 2507–2516. doi: 10.1056/NEJMoa1103782.

Chen, L., Bi, D., Tian, L., McClafferty, H., Steeb, F., Ruth, P., Knaus, H. G. and Shipston, M. J. (2013) 'Palmitoylation of the β4-subunit regulates surface expression of large conductance calcium-activated potassium channel splice variants.', *The Journal of biological chemistry*, 288(18), pp. 13136–44. doi: 10.1074/jbc.M113.461830.

Chen, L., Jeffries, O., Rowe, I. C. M., Liang, Z., Knaus, H.-G., Ruth, P. and Shipston, M. J. (2010) 'Membrane trafficking of large conductance calcium-activated potassium channels is regulated by

alternative splicing of a transplantable, acidic trafficking motif in the RCK1-RCK2 linker.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 285(30), pp. 23265–75. doi: 10.1074/jbc.M110.139758.

Chen, L., Tian, L., MacDonald, S. H.-F., McClafferty, H., Hammond, M. S. L., Huibant, J.-M., Ruth, P., Knaus, H.-G. and Shipston, M. J. (2005) 'Functionally diverse complement of large conductance calcium- and voltage-activated potassium channel (BK) alpha-subunits generated from a single site of splicing.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 280(39), pp. 33599–609. doi: 10.1074/jbc.M505383200.

Chen, R. and Snyder, M. (2013) 'Promise of personalized omics to precision medicine.', *Wiley interdisciplinary reviews. Systems biology and medicine*. NIH Public Access, 5(1), pp. 73–82. doi: 10.1002/wsbm.1198.

Chen, S. and Parmigiani, G. (2007) 'Meta-Analysis of BRCA1 and BRCA2 Penetrance', *Journal of Clinical Oncology*, 25(11), pp. 1329–1333. doi: 10.1200/JCO.2006.09.1066.

Chen, X., Shi, S.-P., Xu, H.-D., Suo, S.-B. and Qiu, J.-D. (2016) 'A homology-based pipeline for global prediction of post-translational modification sites.', *Scientific reports*. Nature Publishing Group, 6, p. 25801. doi: 10.1038/srep25801.

Chen, Y., Tang, C.-E., Ouyang, G.-L., Ruan, L., Li, M.-Y., Zhang, P.-F., Li, C., Yi, H., Peng, F., Li, J.-L., Chen, Z.-C. and Xiao, Z.-Q. (2009) 'Identification of RKIP as a differentially tyrosine-phosphorylated protein in nasopharyngeal carcinoma and normal nasopharyngeal epithelial tissues by phosphoproteomic approach', *Medical Oncology*. Humana Press Inc, 26(4), pp. 463–470. doi: 10.1007/s12032-008-9147-y.

Chen, Z.-P., Stephens, T. J., Murthy, S., Canny, B. J., Hargreaves, M., Witters, L. A., Kemp, B. E. and McConell, G. K. (2003) 'Effect of exercise intensity on skeletal muscle AMPK signaling in humans.', *Diabetes*, 52(9), pp. 2205–12.

Cheng, K. and Rolandi, M. (2015) 'Graphic design for scientists', *Nature Nanotechnology*. Nature Research, 10(12), pp. 1084–1084. doi: 10.1038/nnano.2015.290.

Cheng, S. W. Y., Fryer, L. G. D., Carling, D. and Shepherd, P. R. (2004) 'Thr2446 is a novel mammalian target of rapamycin (mTOR) phosphorylation site regulated by nutrient status.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 279(16), pp. 15719–22. doi: 10.1074/jbc.C300534200.

Cheng, Y., Gu, X. Q., Bednarczyk, P., Wiedemann, F. R., Haddad, G. G. and Siemen, D. (2008) 'Hypoxia increases activity of the BK-channel in the inner mitochondrial membrane and reduces activity of the permeability transition pore.', *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. Karger Publishers, 22(1–4), pp. 127–36. doi: 10.1159/000149790.

Chiu, Y.-H., Alvarez-Baron, C., Kim, E. Y. and Dryer, S. E. (2010) 'Dominant-negative regulation of cell surface expression by a pentapeptide motif at the extreme COOH terminus of an Slo1 calcium-activated potassium channel splice variant.', *Molecular pharmacology*, 77(4), pp. 497–507. doi: 10.1124/mol.109.061929.

Cho, H., Wu, M., Zhang, L., Thompson, R., Nath, A. and Chan, C. (2013) 'Signaling dynamics of palmitate-induced ER stress responses mediated by ATF4 in HepG2 cells.', *BMC systems biology*, 7(1), p. 9. doi: 10.1186/1752-0509-7-9.

Christie, W. (2014) *Proteolipids*, *The American Oil Chemists' Society*. Available at: http://lipidlibrary.aocs.org/Primer/content.cfm?ItemNumber=39359 (Accessed: 30 May 2017).

Clark, H., Carling, D. and Saggerson, D. (2004) 'Covalent activation of heart AMP-activated protein kinase in response to physiological concentrations of long-chain fatty acids', *European Journal of Biochemistry*, 271(11), pp. 2215–2224. doi: 10.1111/j.1432-1033.2004.04151.x.

Clarke, A. J. (2014) 'Managing the ethical challenges of next-generation sequencing in genomic medicine', *British Medical Bulletin*. Oxford University Press, 111(1), pp. 17–30. doi: 10.1093/bmb/ldu017.

Clarke, A. L., Petrou, S., Walsh, J. V. and Singer, J. J. (2002) 'Modulation of BKCa channel activity by fatty acids: structural requirements and mechanism of action', *American Journal of Physiology - Cell Physiology*, 283(5).

Cline, M. S. and Karchin, R. (2011) 'Using bioinformatics to predict the functional impact of SNVs.', *Bioinformatics (Oxford, England)*, 27(4), pp. 441–448.

Cnop, M., Ladrière, L., Igoillo-Esteve, M., Moura, R. F. and Cunha, D. A. (2010) 'Causes and cures for endoplasmic reticulum stress in lipotoxic β-cell dysfunction.', *Diabetes, obesity & metabolism*, 12 Suppl 2, pp. 76–82. doi: 10.1111/j.1463-1326.2010.01279.x.

Cochrane, G. R. and Galperin, M. Y. (2010) 'The 2010 Nucleic Acids Research Database Issue and online Database Collection: a community of data resources.', *Nucleic acids research*. Oxford University Press, 38(Database issue), pp. D1-4. doi: 10.1093/nar/gkp1077.

Coleman, J. E. (1992) 'Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins.', *Annual review of biochemistry*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA, 61, pp. 897–946. doi: 10.1146/annurev.bi.61.070192.004341.

Coll, T., Eyre, E., Rodríguez-Calvo, R., Palomer, X., Sánchez, R. M., Merlos, M., Laguna, J. C. and Vázquez-Carrera, M. (2008) 'Oleate reverses palmitate-induced insulin resistance and inflammation in skeletal muscle cells.', *The Journal of biological chemistry*, 283(17), pp. 11107–16. doi: 10.1074/jbc.M708700200.

Collins, F. S. and Mansoura, M. K. (2001) 'The Human Genome Project. Revealing the shared inheritance of all humankind.', *Cancer*, 91(1 Suppl), pp. 221–5.

Contet, C. (2016) *Big on Bk: Current Insights into the Function of Large Conductance Voltage- and Ca2+-Activated K+ Channels at the Molecular, Cellular and Systemic Levels*. 1st edn. Academic Press.

Cooper, D., Krawczak, M. and Antonorakis, S. (1995) 'The nature and mechanisms of human gene mutation', in *The Metabolic and Molecular Bases of Inherited Disease*. McGraw-Hill, pp. 259–291.

Corvin, A., Craddock, N. and Sullivan, P. F. (2010) 'Genome-wide association studies: a primer.', *Psychological medicine*. NIH Public Access, 40(7), pp. 1063–77. doi: 10.1017/S0033291709991723.

Cox, J. J., Reimann, F., Nicholas, A. K., Thornton, G., Roberts, E., Springell, K., Karbani, G., Jafri, H., Mannan, J., Raashid, Y., Al-Gazali, L., Hamamy, H., Valente, E. M., Gorman, S., Williams, R., McHale, D. P., Wood, J. N., Gribble, F. M. and Woods, C. G. (2006) 'An SCN9A channelopathy causes congenital inability to experience pain.', *Nature*, 444(7121), pp. 894–8. doi: 10.1038/nature05413.

Cravatt, B. F., Simon, G. M. and Yates, J. R. (2007) 'The biological impact of mass-spectrometry-based proteomics.', *Nature*, 450(7172), pp. 991–1000. doi: 10.1038/nature06525.

Craven, S. E., El-Husseini, A. E. and Bredt, D. S. (1999) 'Synaptic targeting of the postsynaptic density protein PSD-95 mediated by lipid and protein motifs.', *Neuron*, 22(3), pp. 497–509.

Cui, J., Cox, D. H. and Aldrich, R. W. (1997) 'Intrinsic voltage dependence and Ca2+ regulation of mslo large conductance Ca-activated K+ channels.', *The Journal of General Physiology*, 109(5), pp. 647–673. doi: 10.1085/jgp.109.5.647.

Cunha, D. A., Hekerman, P., Ladrière, L., Bazarra-Castro, A., Ortis, F., Wakeham, M. C., Moore, F., Rasschaert, J., Cardozo, A. K., Bellomo, E., Overbergh, L., Mathieu, C., Lupi, R., Hai, T., Herchuelz, A., Marchetti, P., Rutter, G. A., Eizirik, D. L. and Cnop, M. (2008) 'Initiation and execution of lipotoxic ER stress in pancreatic beta-cells.', *Journal of cell science*, 121(Pt 14), pp. 2308–18. doi: 10.1242/jcs.026062.

Dallaporta, B., Hirsch, T., Susin, S. A., Zamzami, N., Larochette, N., Brenner, C., Marzo, I. and Kroemer, G. (1998) 'Potassium Leakage During the Apoptotic Degradation Phase', *The Journal of Immunology*, 160(11).

Das, S. K., Mondal, A. K. and Elbein, S. C. (2010) 'Distinct gene expression profiles characterize cellular responses to palmitate and oleate.', *Journal of lipid research*. American Society for Biochemistry and Molecular Biology, 51(8), pp. 2121–2131. doi: 10.1194/jlr.M004275.

Dasgupta, S., Bhattacharya, S., Maitra, S., Pal, D., Majumdar, S. S., Datta, A. and Bhattacharya, S. (2011) 'Mechanism of lipid induced insulin resistance: activated PKCε is a key regulator.', *Biochimica et biophysica acta*. Elsevier B.V., 1812(4), pp. 495–506. doi: 10.1016/j.bbadis.2011.01.001.

Dawson, R. J. P. and Locher, K. P. (2006) 'Structure of a bacterial multidrug ABC transporter', *Nature*, 443(7108), pp. 180–185. doi: 10.1038/nature05155.

Denson, D. D., Wang, X., Worrell, R. T. and Eaton, D. C. (2000) 'Effects of fatty acids on BK channels in GH3cells', *American Journal of Physiology - Cell Physiology*, 279(4).

Dephoure, N., Gould, K. L., Gygi, S. P. and Kellogg, D. R. (2013) 'Mapping and analysis of phosphorylation sites: a quick guide for cell biologists.', *Molecular biology of the cell*. American Society for Cell Biology, 24(5), pp. 535–42. doi: 10.1091/mbc.E12-09-0677.

DeWan, A., Liu, M., Hartman, S., Zhang, S. S.-M., Liu, D. T. L., Zhao, C., Tam, P. O. S., Chan, W. M., Lam, D. S. C., Snyder, M., Barnstable, C., Pang, C. P. and Hoh, J. (2006) 'HTRA1 Promoter Polymorphism in Wet Age-Related Macular Degeneration', *Science*, 314(5801), pp. 989–992. doi: 10.1126/science.1133807.

Diaz-Aguirre, V., Velez-Pardo, C. and Jimenez-Del-Rio, M. (2016) 'Fructose sensitizes Jurkat cells oxidative stress-induced apoptosis via caspase-dependent and caspase-independent mechanisms', *Cell Biology International*, 40(11), pp. 1162–1173. doi: 10.1002/cbin.10653.

Díaz, L., Meera, P., Amigo, J., Stefani, E., Alvarez, O., Toro, L. and Latorre, R. (1998) 'Role of the S4 segment in a voltage-dependent calcium-sensitive potassium (hSlo) channel.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 273(49), pp. 32430–6. doi: 10.1074/JBC.273.49.32430.

Diaz, M. R., Wadleigh, A., Hughes, B. A., Woodward, J. J. and Valenzuela, C. F. (2011) 'Bestrophin1 Channels are Insensitive to Ethanol and Do not Mediate Tonic GABAergic Currents in Cerebellar Granule Cells.', *Frontiers in neuroscience*. Frontiers Media SA, 5, p. 148. doi: 10.3389/fnins.2011.00148.

DiChiara, T. J. and Reinhart, P. H. (1997) 'Redox modulation of hslo Ca2+-activated K+ channels.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 17(13), pp. 4942–55.

Díez-Sampedro, A., Silverman, W. R., Bautista, J. F. and Richerson, G. B. (2006) 'Mechanism of increased open probability by a mutation of the BK channel.', *Journal of neurophysiology*, 96(3), pp. 1507–16. doi: 10.1152/jn.00461.2006.

Diness, J. G., Sørensen, U. S., Nissen, J. D., Al-Shahib, B., Jespersen, T., Grunnet, M. and Hansen, R. S. (2010) 'Inhibition of Small-Conductance Ca2+-Activated K+ Channels Terminates and Protects Against Atrial Fibrillation', *Circulation: Arrhythmia and Electrophysiology*, 3(4).

Doll, S. and Burlingame, A. L. (2015) 'Mass Spectrometry-Based Detection and Assignment of Protein Posttranslational Modifications', *ACS Chemical Biology*. American Chemical Society, 10(1), pp. 63–71. doi: 10.1021/cb500904b.

Dopico, A. M. and Bukiya, A. N. (2014) 'Lipid regulation of BK channel function.', *Frontiers in physiology*. Frontiers Media SA, 5, p. 312. doi: 10.3389/fphys.2014.00312.

Dopico, A. M., Lemos, J. R. and Treistman, S. N. (1996) 'Ethanol increases the activity of large conductance, Ca(2+)-activated K+ channels in isolated neurohypophysial terminals.', *Molecular Pharmacology*, 49(1).

Drenth, J. P. H., te Morsche, R. H. M., Guillet, G., Taieb, A., Kirby, R. L., Jansen, J. B. M. J., Catterall, W. A., Chan, M. K., Tucker, A. T., Madden, S., Golding, C. E., Atherton, D. J., Dillon, M. J., Cummins, T. R., Dib-Hajj, S. D., Waxman, S. G., Davis, M. D., Sandroni, P., Rooke, T. W., Low, P. A., Drenth, J. P., Finley, W. H., Breedveld, G. J., al., et, Drenth, J. P., Michiels, J. J., Drenth, J. P., Michiels, J. J., Guillet, M. H., Noach, E. Le, Milochau, P., Sassolas, B., Guillet, G., Jang, H. S., Jung, D., Kim, S., al., et, Kanadia, R. N., Johnstone, K. A., Mankodi, A., al., et, Klugbauer, N., Lacinova, L., Flockerzi, V., Hofmann, F., Kretschmer, T., Happel, L. T., England, J. D., Nguyen, D. H., Tiel, R. L., Beuerman, R. W., Kline, D. G., Kuhnert, S. M., Phillips, W. J., Davis, M. D., Lai, J., Hunter, J. C., Porreca, F., Legroux-Crespel, E., Sassolas, B., Guillet, G., Kupfer, I., Dupre, D., Misery, L., Littleford, R. C., Khan, F., Belch, J. J., Martin, J. C., Lacombe, D., Lefebvre, D., Bonafe, J. L., Taieb, A., Maleville, J., Michiels, J. J., Abels, J., Steketee, J., Vliet, H. H. van, Vuzevski, V. D., Mork, C., Asker, C. L., Salerud, E. G., Kvernebo, K., Nassar, M. A., Stirling, L. C., Forlani, G., Baker, M. D., Matthews, E. A., Dickenson, A. H., Wood, J. N., Orstavik, K., Mork, C., Kvernebo, K., Jorum, E., Orstavik, K., Weidner, C., Schmidt, R., al., et, Thompson, G. H., Hahn, G., Rang, M., Toledo-Aral, J. J., Moss, B. L., He, Z. J., al., et, Yang, Y., Wang, Y., Li, S. and al., et (2005) 'SCN9A mutations define primary erythermalgia as a neuropathic disorder of voltage gated sodium channels.', *The Journal of investigative dermatology*. Elsevier, 124(6), pp. 1333–8. doi: 10.1111/j.0022-202X.2005.23737.x.

Drenth, J. P. H. and Waxman, S. G. (2007) 'Mutations in sodium-channel gene SCN9A cause a spectrum of human genetic pain disorders.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 117(12), pp. 3603–9. doi: 10.1172/JCI33297.

Du, J.-Y., Yuan, F., Zhao, L.-Y., Zhu, J., Huang, Y.-Y., Zhang, G.-S., Wei, Y., Liu, Y., Yi, Q., Tu, Y., Zhong, X., Sun, F.-Y., Sun, H.-S., Guan, Y.-Y., Chen, W.-L. and Wang, G.-L. (2017) 'Suppression of Kv1.5 protects against endothelial apoptosis induced by palmitate and in type 2 diabetes mice', *Life Sciences*, 168, pp. 28–37. doi: 10.1016/j.lfs.2015.12.054.

Du, W., Bautista, J. F., Yang, H., Diez-Sampedro, A., You, S.-A., Wang, L., Kotagal, P., Lüders, H. O., Shi, J., Cui, J., Richerson, G. B. and Wang, Q. K. (2005) 'Calcium-sensitive potassium channelopathy in human epilepsy and paroxysmal movement disorder.', *Nature genetics*, 37(7), pp. 733–738. doi: 10.1038/ng1585.

Duck, G., Nenadic, G., Filannino, M., Brass, A., Robertson, D. L. and Stevens, R. (2016) 'A Survey of Bioinformatics Database and Software Usage through Mining the Literature', *PLOS ONE*. Edited by S. Ranganathan. Figshare, 11(6), p. e0157989. doi: 10.1371/journal.pone.0157989.

Ducker, C. E., Stettler, E. M., French, K. J., Upson, J. J. and Smith, C. D. (2004) 'Huntingtin interacting protein 14 is an oncogenic human protein: palmitoyl acyltransferase.', *Oncogene*, 23(57), pp. 9230–9237. doi: 10.1038/sj.onc.1208171.

Dukka, B. K. (2013) 'Structure-based Methods for Computational Protein Functional Site Prediction.', *Computational and structural biotechnology journal*. Research Network of Computational and Structural Biotechnology, 8, p. e201308005. doi: 10.5936/csbj.201308005.

Duncan, J. A. and Gilman, A. G. (1998) 'A cytoplasmic acyl-protein thioesterase that removes palmitate

from G protein α subunits and p21(RAS)', *Journal of Biological Chemistry*, 273(25), pp. 15830–15837. doi: 10.1074/jbc.273.25.15830.

Dunphy, J. T. and Linder, M. E. (1998) 'Signalling functions of protein palmitoylation.', *Biochimica et biophysica acta*, 1436(1–2), pp. 245–261.

Dworetzky, S. I., Boissard, C. G., Lum-Ragan, J. T., McKay, M. C., Post-Munson, D. J., Trojnacki, J. T., Chang, C.-P. and Gribkoff, V. K. (1996) 'Phenotypic Alteration of a Human BK (hSlo) Channel by hSloβ Subunit Coexpression: Changes in Blocker Sensitivity, Activation/Relaxation and Inactivation Kinetics, and Protein Kinase A Modulation', *Journal of Neuroscience*, 16(15).

Dworetzky, S. I., Trojnacki, J. T. and Gribkoff, V. K. (1994) 'Cloning and expression of a human large-conductance calcium-activated potassium channel.', *Brain research. Molecular brain research*, 27(1), pp. 189–93.

Ecker, J. R., Bickmore, W. A., Barroso, I., Pritchard, J. K., Gilad, Y. and Segal, E. (2012) 'Genomics: ENCODE explained', *Nature*. Nature Research, 489(7414), pp. 52–55. doi: 10.1038/489052a.

Eddy, S. (2014) 'High throughput sequencing for neuroscience', *Cryptogenomicon*. Cambridge, MA.

Edenberg, H. and Liu, Y. (2009) 'Laboratory Methods for High-Throughput Genotyping', *Methods for High-Throughput Genotyping*, pp. 183–193.

Edmonds, M. J. and Morgan, A. (2014) 'A systematic analysis of protein palmitoylation in Caenorhabditis elegans', *BMC Genomics*, 15(1), p. 841. doi: 10.1186/1471-2164-15-841.

Eklund, D. M. and Edqvist, J. (2003) 'Localization of nonspecific lipid transfer proteins correlate with programmed cell death responses during endosperm degradation in Euphorbia lagascae seedlings.', *Plant physiology*. American Society of Plant Biologists, 132(3), pp. 1249–59. doi: 10.1104/PP.103.020875.

El-Husseini, A. E.-D., Schnell, E., Dakoji, S., Sweeney, N., Zhou, Q., Prange, O., Gauthier-Campbell, C., Aguilera-Moreno, A., Nicoll, R. A. and Bredt, D. S. (2002) 'Synaptic strength regulated by palmitate cycling on PSD-95.', *Cell*, 108(6), pp. 849–63.

Elefsinioti, A. L., Bagos, P. G., Spyropoulos, I. C. and Hamodrakas, S. J. (2004) 'A database for G proteins and their interaction with GPCRs', *BMC Bioinformatics*. BioMed Central, 5(1), p. 208. doi: 10.1186/1471-2105-5-208.

Elias, G. M. and Nicoll, R. A. (2007) 'Synaptic trafficking of glutamate receptors by MAGUK scaffolding proteins', *Trends in Cell Biology*, 17(7), pp. 343–352. doi: 10.1016/j.tcb.2007.07.005.

Elmore, S. (2007) 'Apoptosis: a review of programmed cell death.', *Toxicologic pathology*. NIH Public Access, 35(4), pp. 495–516. doi: 10.1080/01926230701320337.

Elsner, M., Gehrmann, W. and Lenzen, S. (2011) 'Peroxisome-generated hydrogen peroxide as important mediator of lipotoxicity in insulin-producing cells.', *Diabetes*, 60(1), pp. 200–8. doi: 10.2337/db09-1401.

ENCODE Project Consortium and The ENCODE Project (2012) 'An integrated encyclopedia of DNA elements in the human genome.', *Nature*. NIH Public Access, 489(7414), pp. 57–74. doi: 10.1038/nature11247.

Erxleben, C., Everhart, A. L., Romeo, C., Florance, H., Bauer, M. B., Alcorta, D. A., Rossie, S., Shipston, M. J. and Armstrong, D. L. (2002) 'Interacting effects of N-terminal variation and strex exon splicing on slo potassium channel regulation by calcium, phosphorylation, and oxidation.', *The Journal of biological chemistry*, 277(30), pp. 27045–52. doi: 10.1074/jbc.M203087200.

Estrada, K., Styrkarsdottir, U., Evangelou, E., Hsu, Y.-H., Duncan, E. L., Ntzani, E. E., Oei, L., Albagha, O. M. E., Amin, N., Kemp, J. P., Koller, D. L., Li, G., Liu, C.-T., Minster, R. L., Moayyeri, A., Vandenput, L., Willner, D., Xiao, S.-M., Yerges-Armstrong, L. M., Zheng, H.-F., Alonso, N., Eriksson, J., Kammerer, C. M., Kaptoge, S. K., Leo, P. J., Thorleifsson, G., Wilson, S. G., Wilson, J. F., Aalto, V., Alen, M., Aragaki, A. K., Aspelund, T., Center, J. R., Dailiana, Z., Duggan, D. J., Garcia, M., Garcia-Giralt, N., Giroux, S., Hallmans, G., Hocking, L. J., Husted, L. B., Jameson, K. A., Khusainova, R., Kim, G. S., Kooperberg, C., Koromila, T., Kruk, M., Laaksonen, M., Lacroix, A. Z., Lee, S. H., Leung, P. C., Lewis, J. R., Masi, L., Mencej-Bedrac, S., Nguyen, T. V, Nogues, X., Patel, M. S., Prezelj, J., Rose, L. M., Scollen, S., Siggeirsdottir, K., Smith, A. V, Svensson, O., Trompet, S., Trummer, O., van Schoor, N. M., Woo, J., Zhu, K., Balcells, S., Brandi, M. L., Buckley, B. M., Cheng, S., Christiansen, C., Cooper, C., Dedoussis, G., Ford, I., Frost, M., Goltzman, D., González-Macías, J., Kähönen, M., Karlsson, M., Khusnutdinova, E., Koh, J.-M., Kollia, P., Langdahl, B. L., Leslie, W. D., Lips, P., Ljunggren, Ö., Lorenc, R. S., Marc, J., Mellström, D., Obermayer-Pietsch, B., Olmos, J. M., Pettersson-Kymmer, U., Reid, D. M., Riancho, J. A., Ridker, P. M., Rousseau, F., Slagboom, P. E., Tang, N. L. S., Urreizti, R., Van Hul, W., Viikari, J., Zarrabeitia, M. T., Aulchenko, Y. S., Castano-Betancourt, M., Grundberg, E., Herrera, L., Ingvarsson, T., Johannsdottir, H., Kwan, T., Li, R., Luben, R., Medina-Gómez, C., Palsson, S. T., Reppe, S., Rotter, J. I., Sigurdsson, G., van Meurs, J. B. J., Verlaan, D., Williams, F. M. K., Wood, A. R., Zhou, Y., Gautvik, K. M., Pastinen, T., Raychaudhuri, S., Cauley, J. A., Chasman, D. I., Clark, G. R., Cummings, S. R., Danoy, P., Dennison, E. M., Eastell, R., Eisman, J. A., Gudnason, V., Hofman, A., Jackson, R. D., Jones, G., Jukema, J. W., Khaw, K.-T., Lehtimäki, T., Liu, Y., Lorentzon, M., McCloskey, E., Mitchell, B. D., Nandakumar, K., Nicholson, G. C., Oostra, B. A., Peacock, M., Pols, H. A. P., Prince, R. L., Raitakari, O., Reid, I. R., Robbins, J., Sambrook, P. N., Sham, P. C., Shuldiner, A. R., Tylavsky, F. A., van Duijn, C. M., Wareham, N. J., Cupples, L. A., Econs, M. J., Evans, D. M., Harris, T. B., Kung, A. W. C., Psaty, B. M., Reeve, J., Spector, T. D., Streeten, E. A., Zillikens, M. C., Thorsteinsdottir, U., Ohlsson, C., Karasik, D., Richards, J. B., Brown, M. A., Stefansson, K., Uitterlinden, A. G., Ralston, S. H., Ioannidis, J. P. A., Kiel, D. P. and Rivadeneira, F. (2012) 'Genome-wide meta-analysis identifies 56 bone mineral density loci and reveals 14 loci associated with risk of fracture.', *Nature genetics*. NIH Public Access, 44(5), pp. 491–501. doi: 10.1038/ng.2249.

European Molecular Biology Laboratory (2014) *Protein Expression and Purification Core Facility - Cloning - Choice of Expression Systems - EMBL*. Available at: http://www.embl.de/pepcore/pepcore_services/cloning/choice_expression_systems/ (Accessed: 6 January 2015).

Evans, A. M., Mustard, K. J. W., Wyatt, C. N., Peers, C., Dipp, M., Kumar, P., Kinnear, N. P. and Hardie, D. G. (2005) 'Does AMP-activated protein kinase couple inhibition of mitochondrial oxidative phosphorylation by hypoxia to calcium signaling in O2-sensing cells?', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 280(50), pp. 41504–11. doi: 10.1074/jbc.M510040200.

Evanson, K. W., Bannister, J. P., Leo, M. D. and Jaggar, J. H. (2014) 'LRRC26 is a functional BK channel auxiliary γ subunit in arterial smooth muscle cells.', *Circulation research*. NIH Public Access, 115(4), pp. 423–31. doi: 10.1161/CIRCRESAHA.115.303407.

Farber, S. J., Berger, E. Y. and Earle, D. P. (1951) 'Effect of diabetes and insulin of the maximum capacity of the renal tubules to reabsorb glucose.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 30(2), pp. 125–9. doi: 10.1172/JCI102424.

Fediuc, S., Gaidhu, M. P. and Ceddia, R. B. (2006) 'Regulation of AMP-activated protein kinase and acetyl-CoA carboxylase phosphorylation by palmitate in skeletal muscle cells', *J. Lipid Res*, 47, pp. 412–420. doi: 10.1194/jlr.M500438-JLR200.

Feil, R. (2003) 'Cyclic GMP-Dependent Protein Kinases and the Cardiovascular System: Insights From Genetically Modified Mice', *Circulation Research*, 93(10), pp. 907–916. doi: 10.1161/01.RES.0000100390.68771.CC.

Fellermann, K., Stange, D. E., Schaeffeler, E., Schmalzl, H., Wehkamp, J., Bevins, C. L., Reinisch, W., Teml, A., Schwab, M., Lichter, P., Radlwimmer, B. and Stange, E. F. (2006) 'A Chromosome 8 Gene-Cluster Polymorphism with Low Human Beta-Defensin 2 Gene Copy Number Predisposes to Crohn Disease of the Colon', *The American Journal of Human Genetics*, 79(3), pp. 439–448. doi: 10.1086/505915.

Feng, J., Wang, C., Chen, Q., Chen, H., Ren, B., Li, X. and Zuo, J. (2013) 'S-nitrosylation of phosphotransfer proteins represses cytokinin signaling', *Nature Communications*. Nature Publishing Group, 4, p. 1529. doi: 10.1038/ncomms2541.

Fernald, G. H., Capriotti, E., Daneshjou, R., Karczewski, K. J. and Altman, R. B. (2011) 'Bioinformatics challenges for personalized medicine.', *Bioinformatics (Oxford, England)*. Oxford University Press, 27(13), pp. 1741–8. doi: 10.1093/bioinformatics/btr295.

Finn, R. D., Mistry, J., Tate, J., Coggill, P., Heger, A., Pollington, J. E., Gavin, O. L., Gunasekaran, P., Ceric, G., Forslund, K., Holm, L., Sonnhammer, E. L. L., Eddy, S. R. and Bateman, A. (2010) 'The Pfam protein families database', *Nucleic Acids Research*, 38(Database), pp. D211–D222. doi: 10.1093/nar/gkp985.

Flanagan, S. E., Patch, A.-M. and Ellard, S. (2010) 'Using SIFT and PolyPhen to Predict Loss - of - Function and Gain - of - Function Mutations', *Genetic Testing and Molecular Biomarkers*, 14(4), pp. 533–537. doi: 10.1089/gtmb.2010.0036.

Föller, M., Jaumann, M., Dettling, J., Saxena, A., Pakladok, T., Munoz, C., Ruth, P., Sopjani, M., Seebohm, G., Rüttiger, L., Knipper, M. and Lang, F. (2012) 'AMP-activated protein kinase in BK-channel regulation and protection against hearing loss following acoustic overstimulation', *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 26(10), pp. 4243–53. doi: 10.1096/fj.12-208132.

Forbes, S. A., Bindal, N., Bamford, S., Cole, C., Kok, C. Y., Beare, D., Jia, M., Shepherd, R., Leung, K., Menzies, A., Teague, J. W., Campbell, P. J., Stratton, M. R. and Futreal, P. A. (2011) 'COSMIC: mining complete cancer genomes in the Catalogue of Somatic Mutations in Cancer', *Nucleic Acids Research*, 39(Database), pp. D945–D950. doi: 10.1093/nar/gkq929.

Frindt, G. and Palmer, L. G. (2010) 'Effects of dietary K on cell-surface expression of renal ion channels and transporters', *American Journal of Physiology - Renal Physiology*, 299(4).

Frousios, K., Iliopoulos, C. S., Schlitt, T. and Simpson, M. A. (2013) 'Predicting the functional consequences of non-synonymous DNA sequence variants — evaluation of bioinformatics tools and development of a consensus strategy', *Genomics*, 102(4), pp. 223–228. doi: 10.1016/j.ygeno.2013.06.005.

Fujii, N., Ho, R. C., Manabe, Y., Jessen, N., Toyoda, T., Holland, W. L., Summers, S. A., Hirshman, M. F. and Goodyear, L. J. (2008) 'Ablation of AMP-Activated Protein Kinase 2 Activity Exacerbates Insulin Resistance Induced by High-Fat Feeding of Mice', *Diabetes*, 57(11), pp. 2958–2966. doi: 10.2337/db07-1187.

Funari, S. S., Barceló, F. and Escribá, P. V (2003) 'Effects of oleic acid and its congeners, elaidic and stearic acids, on the structural properties of phosphatidylethanolamine membranes.', *Journal of lipid research*. American Society for Biochemistry and Molecular Biology, 44(3), pp. 567–75. doi: 10.1194/jlr.M200356-JLR200.

Gallin, W. J. and Boutet, P. A. (2010) 'VKCDB: voltage-gated K + channel database updated and upgraded', *Nucleic Acids Research*, 39. doi: 10.1093/nar/gkq1000.

Gámez, A., Pérez, B., Ugarte, M. and Desviat, L. R. (2000) 'Expression analysis of phenylketonuria mutations. Effect on folding and stability of the phenylalanine hydroxylase protein.', *The Journal of*

biological chemistry. American Society for Biochemistry and Molecular Biology, 275(38), pp. 29737–42. doi: 10.1074/jbc.M003231200.

Gardos, G. (1958) 'The function of calcium in the potassium permeability of human erythrocytes.', *Biochimica et biophysica acta*, 30(3), pp. 653–4.

Gasteiger, E., Gattiker, A., Hoogland, C., Ivanyi, I., Appel, R. D. and Bairoch, A. (2003) 'ExPASy: The proteomics server for in-depth protein knowledge and analysis.', *Nucleic acids research*, 31(13), pp. 3784–8.

Gaster, M., Rustan, A. C. and Beck-Nielsen, H. (2005) 'Differential Utilization of Saturated Palmitate and Unsaturated Oleate', *Diabetes*, 54(3).

Ge, L., Hoa, N. T., Cornforth, A. N., Bota, D. A., Mai, A., Kim, D. I., Chiou, S.-K., Hickey, M. J., Kruse, C. A. and Jadus, M. R. (2012) 'Glioma Big Potassium Channel Expression in Human Cancers and Possible T Cell Epitopes for Their Immunotherapy', *The Journal of Immunology*, 189(5).

Genome Research Limited (2017) *What types of mutation are there?* Available at: http://www.yourgenome.org/facts/what-types-of-mutation-are-there (Accessed: 8 March 2017).

Geoffroy, M.-C. and Hay, R. T. (2009) 'An additional role for SUMO in ubiquitin-mediated proteolysis', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 10(8), pp. 564–568. doi: 10.1038/nrm2707.

George Priya Doss, C., Rajasekaran, R., Arjun, P. and Sethumadhavan, R. (2010) 'Prioritization of candidate SNPs in colon cancer using bioinformatics tools: An alternative approach for a cancer biologist', *Interdisciplinary Sciences: Computational Life Sciences*. International Association of Scientists in the Interdisciplinary Areas, 2(4), pp. 320–346. doi: 10.1007/s12539-010-0003-3.

Gessner, G., Schönherr, K., Soom, M., Hansel, A., Asim, M., Baniahmad, A., Derst, C., Hoshi, T. and Heinemann, S. H. (2005) 'BKCa Channels Activating at Resting Potential without Calcium in LNCaP Prostate Cancer Cells', *Journal of Membrane Biology*, 208(3), pp. 229–240. doi: 10.1007/s00232-005-0830-z.

Geurts, P., Irrthum, A., Wehenkel, L., Cai, Y., Chaga, O., Jiang, G., Beaver, W., Dobereiner, H., Freund, Y., Borisy, G., Sheetz, M., Roth, F. P., Shales, M., Zhang, X., Davey, M., Robinson, M. D., Paccanaro, A., Bray, J. E., Sheung, A., Beattie, B., Richards, D. P., Canadien, V., Lalev, A., Mena, F., Wong, P., Starostine, A., Canete, M. M., Vlasblom, J., Wu, S., Orsi, C., Collins, S. R., Chandran, S., Haw, R., Rilstone, J. J., Gandi, K., Thompson, N. J., Musso, G., Onge, P. S., Ghanny, S., Lam, M. H. Y., Butland, G., Altaf-Ul, A. M., Kanaya, S., Shilatifard, A., O'Shea, E., Weissman, J. S., Ingles, C. J., Hughes, T. R., Parkinson, J., Gerstein, M., Wodak, S. J., Emili, A. and Greenblatt, J. F. (2009) 'Supervised learning with decision tree-based methods in computational and systems biology', *Molecular BioSystems*. The Royal Society of Chemistry, 5(12), p. 1593. doi: 10.1039/b907946g.

Ghaedi, H., Bastami, M., Zare-Abdollahi, D., Alipoor, B., Movafagh, A., Mirfakhraie, R., Omrani, M. D. and Masotti, A. (2015) 'Bioinformatics prioritization of SNPs perturbing microRNA regulation of hematological malignancy-implicated genes', *Genomics*, 106(6), pp. 360–366. doi: 10.1016/j.ygeno.2015.10.004.

Ghezzi, A., Pohl, J. B., Wang, Y. and Atkinson, N. S. (2010) 'BK channels play a counter-adaptive role in drug tolerance and dependence.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 107(37), pp. 16360–5. doi: 10.1073/pnas.1005439107.

Gilissen, C., Hoischen, A., Brunner, H. G. and Veltman, J. A. (2011) 'Unlocking Mendelian disease using exome sequencing', *Genome Biology*, 12(9), p. 228. doi: 10.1186/gb-2011-12-9-228.

Glauber, H. S., Rishe, N. and Karnieli, E. (2014) 'Introduction to personalized medicine in diabetes mellitus.', *Rambam Maimonides medical journal*. Rambam Health Care Campus, 5(1), p. e0002. doi: 10.5041/RMMJ.10136.

Goldstein, D. B. (2009) 'Common Genetic Variation and Human Traits', *New England Journal of Medicine*, 36017(23), pp. 17–19. doi: 10.1056/NEJMp0806284.

Goldstein, D. and Weale, M. (2001) 'Population genomics: Linkage disequilibrium holds the key', *Current Biology*, 11, pp. R576–R579.

Goodwin, J. S., Drake, K. R., Rogers, C., Wright, L., Lippincott-Schwartz, J., Philips, M. R. and Kenworthy, A. K. (2005) 'Depalmitoylated Ras traffics to and from the Golgi complex via a nonvesicular pathway.', *The Journal of cell biology*. The Rockefeller University Press, 170(2), pp. 261–72. doi: 10.1083/jcb.200502063.

Gordon, E., Semus, S. F., Lozinskaya, I. M., Lin, Z. and Xu, X. (2010) 'Characterizing the role of Thr352 in the inhibition of the large conductance Ca2+-activated K+ channels by 1-[1-hexyl-6-(methyloxy)-1H-indazol-3-yl]-2-methyl-1-propanone.', *The Journal of pharmacology and experimental therapeutics*, 334(2), pp. 402–9. doi: 10.1124/jpet.110.166017.

Gould, N., Doulias, P.-T., Tenopoulou, M., Raju, K. and Ischiropoulos, H. (2013) 'Regulation of protein function and signaling by reversible cysteine S-nitrosylation.', *The Journal of biological chemistry*, 288(37), pp. 26473–9. doi: 10.1074/jbc.R113.460261.

Grafodatskaya, D., Chung, B., Szatmari, P. and Weksberg, R. (2010) 'Autism Spectrum Disorders and Epigenetics', *Journal of the American Academy of Child & Adolescent Psychiatry*, 49(8), pp. 794–809. doi: 10.1016/j.jaac.2010.05.005.

Greaves, J., Munro, K. R., Davidson, S. C., Riviere, M., Wojno, J., Smith, T. K., Tomkinson, N. C. O. and Chamberlain, L. H. (2017) 'Molecular basis of fatty acid selectivity in the zDHHC family of S-acyltransferases revealed by click chemistry.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 114(8), pp. E1365–E1374. doi: 10.1073/pnas.1612254114.

Greenwood, T. A. and Kelsoe, J. R. (2003) 'Promoter and intronic variants affect the transcriptional regulation of the human dopamine transporter gene', *Genomics*, 82(5), pp. 511–520. doi: 10.1016/S0888-7543(03)00142-3.

Gribkoff, V. K., Starrett, J. E. and Dworetzky, S. I. (2001) 'Maxi-K Potassium Channels: Form, Function, and Modulation of a Class of Endogenous Regulators of Intracellular Calcium', *The Neuroscientist*. Sage PublicationsSage CA: Thousand Oaks, CA, 7(2), pp. 166–177. doi: 10.1177/107385840100700211.

Grunnet, M., Hay-Schmidt, A. and Klaerke, D. A. (2005) 'Quantification and distribution of big conductance Ca2+-activated K+ channels in kidney epithelia', *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1714(2), pp. 114–124. doi: 10.1016/j.bbamem.2005.05.012.

Guglielmi, L., Servettini, I., Caramia, M., Catacuzzeno, L., Franciolini, F., D'Adamo, M. C. and Pessia, M. (2015) 'Update on the implication of potassium channels in autism: K(+) channelautism spectrum disorder.', *Frontiers in cellular neuroscience*. Frontiers Media SA, 9, p. 34. doi: 10.3389/fncel.2015.00034.

Gupta, R., Jung, E. and Brunak, S. (2002) 'Prediction of N-glycosylation sites in human proteins', *Pacific Symposium on Biocomputing*, 7, pp. 310–322.

Gutman, G. A. (2005) 'International Union of Pharmacology. LIII. Nomenclature and Molecular Relationships of Voltage-Gated Potassium Channels', *Pharmacological Reviews*, 57(4), pp. 473–508. doi: 10.1124/pr.57.4.10.

Guttmacher, A. E. and Collins, F. S. (2003) 'Welcome to the Genomic Era', *New England Journal of Medicine*. Massachusetts Medical Society , 349(10), pp. 996–998. doi: 10.1056/NEJMe038132.

Guyer, M. and Guyer, statement compiled by M. (1998) 'Statement on the rapid release of genomic DNA sequence.', *Genome research*. Cold Spring Harbor Laboratory Press, 8(5), p. 413. doi: 10.1101/GR.8.5.413.

Hagman, D. K., Hays, L. B., Parazzoli, S. D. and Poitout, V. (2005) 'Palmitate inhibits insulin gene expression by altering PDX-1 nuclear localization and reducing MafA expression in isolated rat islets of Langerhans.', *The Journal of biological chemistry*, 280(37), pp. 32413–8. doi: 10.1074/jbc.M506000200.

Haim, T. E., Wang, W., Flagg, T. P., Tones, M. A., Bahinski, A., Numann, R. E., Nichols, C. G. and Nerbonne, J. M. (2010) 'Palmitate attenuates myocardial contractility through augmentation of repolarizing Kv currents.', *Journal of molecular and cellular cardiology*. NIH Public Access, 48(2), pp. 395–405. doi: 10.1016/j.yjmcc.2009.10.004.

Hallak, H. and Rubin, R. (2004) 'Ethanol inhibits palmitoylation of G protein Gαs', *Journal of Neurochemistry*. Blackwell Science Ltd, 89(4), pp. 919–927. doi: 10.1046/j.1471-4159.2004.02364.x.

Hames, B. and Higgins, S. (1999) *Protein Expression: A Practical Approach (Practical Approach Series)*. Oxford ; Oxford University Press,.

Han, C., Rush, A. M., Dib-Hajj, S. D., Li, S., Xu, Z., Wang, Y., Tyrrell, L., Wang, X., Yang, Y. and Waxman, S. G. (2006) 'Sporadic onset of erythermalgia: A gain-of-function mutation in Nav1.7', *Annals of Neurology*. Wiley Subscription Services, Inc., A Wiley Company, 59(3), pp. 553–558. doi: 10.1002/ana.20776.

Hannah, V. C., Ou, J., Luong, A., Goldstein, J. L. and Brown, M. S. (2001) 'Unsaturated fatty acids down-regulate srebp isoforms 1a and 1c by two mechanisms in HEK-293 cells.', *The Journal of biological chemistry*, 276(6), pp. 4365–72. doi: 10.1074/jbc.M007273200.

Hao, M., Mukherjee, S. and Maxfield, F. R. (2001) 'Cholesterol depletion induces large scale domain segregation in living cell membranes.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 98(23), pp. 13072–7. doi: 10.1073/pnas.231377398.

Haoudi, A. and Bensmail, H. (2006) 'Bioinformatics and data mining in proteomics', *Expert Review of Proteomics*, 3(3), pp. 333–343. doi: 10.1586/14789450.3.3.333.

Hardie, D. G., Hawley, S. A. and Scott, J. W. (2006) 'AMP-activated protein kinase--development of the energy sensor concept.', *The Journal of physiology*. Wiley-Blackwell, 574(Pt 1), pp. 7–15. doi: 10.1113/jphysiol.2006.108944.

Hardie, D. G., Ross, F. A. and Hawley, S. A. (2012) 'AMPK: a nutrient and energy sensor that maintains energy homeostasis', *Nature Reviews Molecular Cell Biology*. Nature Publishing Group, 13(4), pp. 251–262. doi: 10.1038/nrm3311.

Hardy, S., St-Onge, G. G., Joly, E., Langelier, Y. and Prentki, M. (2005) 'Oleate Promotes the Proliferation of Breast Cancer Cells via the G Protein-coupled Receptor GPR40', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 280(14), pp. 13285–13291. doi: 10.1074/jbc.M410922200.

Harte, R. A., Farrell, C. M., Loveland, J. E., Suner, M.-M., Wilming, L., Aken, B., Barrell, D., Frankish, A., Wallin, C., Searle, S., Diekhans, M., Harrow, J. and Pruitt, K. D. (2012) 'Tracking and coordinating an international curation effort for the CCDS Project.', *Database : the journal of biological databases and curation*. Oxford University Press, 2012, p. bas008. doi: 10.1093/database/bas008.

Havel, R., Naimark, A. and Borchgrevink, C. (1963) 'Turnover rate and oxidation of free fatty acids of blood plasma in man during exercise: studies during continuous infusion of palmitate-1-C14.', 42(7), pp. 1054–63.

Heginbotham, L., Lu, Z., Abramson, T. and MacKinnon, R. (1994) 'Mutations in the K+ channel signature sequence.', *Biophysical journal*. The Biophysical Society, 66(4), pp. 1061–7. doi: 10.1016/S0006-3495(94)80887-2.

Hei, H., Gao, J., Dong, J., Tao, J., Tian, L., Pan, W., Wang, H. and Zhang, X. (2016) 'BK Knockout by TALEN-Mediated Gene Targeting in Osteoblasts: KCNMA1 Determines the Proliferation and Differentiation of Osteoblasts.', *Molecules and cells*. Korean Society for Molecular and Cellular Biology, 39(7), pp. 530–5. doi: 10.14348/molcells.2016.0033.

Heimberg, H., De Vos, A., Vandercammen, A., Van Schaftingen, E., Pipeleers, D. and Schuit, F. (1993) 'Heterogeneity in glucose sensitivity among pancreatic beta-cells is correlated to differences in glucose phosphorylation rather than glucose transport.', *The EMBO journal*. European Molecular Biology Organization, 12(7), pp. 2873–9.

Hellsten, E., Vesa, J., Olkkonen, V. M., Jalanko, A. and Peltonen, L. (1996) 'Human palmitoyl protein thioesterase: evidence for lysosomal targeting of the enzyme and disturbed cellular routing in infantile neuronal ceroid lipofuscinosis.', *The EMBO journal*. European Molecular Biology Organization, 15(19), pp. 5240–5.

Hepp, D., Gonçalves, G. L. and de Freitas, T. R. O. (2015) 'Prediction of the damage-associated non-synonymous single nucleotide polymorphisms in the human MC1R gene.', *PloS one*, 10(3), p. e0121812. doi: 10.1371/journal.pone.0121812.

Hermann, A., Sitdikova, G. and Weiger, T. (2015) 'Oxidative Stress and Maxi Calcium-Activated Potassium (BK) Channels', *Biomolecules*. Multidisciplinary Digital Publishing Institute, 5(3), pp. 1870–1911. doi: 10.3390/biom5031870.

Herrick, J. B. (1910) 'Peculiar elongated and sickle-shaped red blood corpuscles in a case of severe anemia. 1910.', *The Yale journal of biology and medicine*, 74(3), pp. 179–84.

Hetherington, A. M., Sawyez, C. G., Zilberman, E., Stoianov, A. M., Robson, D. L. and Borradaile, N. M. (2016) 'Differential Lipotoxic Effects of Palmitate and Oleate in Activated Human Hepatic Stellate Cells and Epithelial Hepatoma Cells.', *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. Karger Publishers, 39(4), pp. 1648–62. doi: 10.1159/000447866.

Hickson-Bick, D. L. ., Buja, M. L. and McMillin, J. B. (2000) 'Palmitate-mediated Alterations in the Fatty Acid Metabolism of Rat Neonatal Cardiac Myocytes', *Journal of Molecular and Cellular Cardiology*, 32(3), pp. 511–519. doi: 10.1006/jmcc.1999.1098.

Hindorff, L. A., Sethupathy, P., Junkins, H. A., Ramos, E. M., Mehta, J. P., Collins, F. S. and Manolio, T. A. (2009) 'Potential etiologic and functional implications of genome-wide association loci for human diseases and traits', *Proceedings of the National Academy of Sciences*, 106(23), pp. 9362–9367. doi: 10.1073/pnas.0903103106.

Hirschhorn, J. N. and Daly, M. J. (2005) 'Genome-wide association studies for common diseases and complex traits', *Nature Reviews Genetics*. Nature Publishing Group, 6(2), pp. 95–108. doi: 10.1038/nrg1521.

Hirschman, L., Burns, G. A. P. C., Krallinger, M., Arighi, C., Cohen, K. B., Valencia, A., Wu, C. H., Chatr-Aryamontri, A., Dowell, K. G., Huala, E., Lourenco, A., Nash, R., Veuthey, A.-L., Wiegers, T. and Winter, A. G. (2012) 'Text mining for the biocuration workflow', *Database*. Oxford University Press, 2012(0), p.

bas020-bas020. doi: 10.1093/database/bas020.

Hite, R. K., Tao, X. and MacKinnon, R. (2016) 'Structural basis for gating the high-conductance Ca2+-activated K+ channel', *Nature*. Nature Research, 541(7635), pp. 52–57. doi: 10.1038/nature20775.

Ho, F. M., Liu, S. H., Liau, C. S., Huang, P. J. and Lin-Shiau, S. Y. (2000) 'High Glucose-Induced Apoptosis in Human Endothelial Cells Is Mediated by Sequential Activations of c-Jun NH2-Terminal Kinase and Caspase-3', *Circulation*, 101(22), pp. 2618–2624. doi: 10.1161/01.CIR.101.22.2618.

Hogrefe, H. H., Cline, J., Youngblood, G. L. and Allen, R. M. (2002) *QuickChange II XL Site-Directed Mutagenesis Kit*, *BioTechniques*.

Hollox, E. J., Huffmeier, U., Zeeuwen, P. L. J. M., Palla, R., Lascorz, J., Rodijk-Olthuis, D., van de Kerkhof, P. C. M., Traupe, H., de Jongh, G., Heijer, M. den, Reis, A., Armour, J. A. L. and Schalkwijk, J. (2008) 'Psoriasis is associated with increased β-defensin genomic copy number', *Nature Genetics*, 40(1), pp. 23–25. doi: 10.1038/ng.2007.48.

Hong, O.-K., Yoo, S.-J., Son, J.-W., Kim, M.-K., Baek, K.-H., Song, K.-H., Cha, B.-Y., Jo, H. and Kwon, H.-S. (2016) 'High glucose and palmitate increases bone morphogenic protein 4 expression in human endothelial cells.', *The Korean journal of physiology & pharmacology : official journal of the Korean Physiological Society and the Korean Society of Pharmacology*. Korean Physiological Society and Korean Society of Pharmacology, 20(2), pp. 169–75. doi: 10.4196/kjpp.2016.20.2.169.

Horman, S., Browne, G., Krause, U., Patel, J., Vertommen, D., Bertrand, L., Lavoinne, A., Hue, L., Proud, C. and Rider, M. (2002) 'Activation of AMP-activated protein kinase leads to the phosphorylation of elongation factor 2 and an inhibition of protein synthesis.', *Current biology : CB*. Elsevier, 12(16), pp. 1419–23. doi: 10.1016/S0960-9822(02)01077-1.

Horrigan, F. T., Cui, J. and Aldrich, R. W. (1999) 'Allosteric voltage gating of potassium channels I. Mslo ionic currents in the absence of Ca(2+).', *The Journal of general physiology*. The Rockefeller University Press, 114(2), pp. 277–304.

Hoshi, T., Pantazis, A. and Olcese, R. (2013) 'Transduction of Voltage and Ca2+ Signals by Slo1 BK Channels', *Physiology*, 28(3).

Hou, S., Vigeland, L. E., Zhang, G., Xu, R., Li, M., Heinemann, S. H. and Hoshi, T. (2010) 'Zn2+ activates large conductance Ca2+-activated K+ channel via an intracellular domain.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 285(9), pp. 6434–42. doi: 10.1074/jbc.M109.069211.

Hou, S., Xu, R., Heinemann, S. H. and Hoshi, T. (2008) 'The RCK1 high-affinity Ca2+ sensor confers carbon monoxide sensitivity to Slo1 BK channels.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 105(10), pp. 4039–43. doi: 10.1073/pnas.0800304105.

Hovsepyan, M., Sargsyan, E. and Bergsten, P. (2010) 'Palmitate-induced changes in protein expression of insulin secreting INS-1E cells.', *Journal of proteomics*, 73(6), pp. 1148–55. doi: 10.1016/j.jprot.2010.01.012.

Howe, D. and Yon, S. (2008) 'The future of biocuration', *Nature*, 455(7209), pp. 47–50. doi: 10.1038/455047a.

Howe, K. L., Bolt, B. J., Shafie, M., Kersey, P. and Berriman, M. (2016) 'WormBase ParaSite − a comprehensive resource for helminth genomics', *Molecular and Biochemical Parasitology*. doi: 10.1016/j.molbiopara.2016.11.005.

Hu, H., He, M.-L., Tao, R., Sun, H.-Y., Hu, R., Zang, W.-J., Yuan, B.-X., Lau, C.-P., Tse, H.-F. and Li, G.-R.

(2009) ‘Characterization of ion channels in human preadipocytes’, *Journal of Cellular Physiology*. Wiley Subscription Services, Inc., A Wiley Company, 218(2), pp. 427–435. doi: 10.1002/jcp.21617.

Hu, H., Shao, L. R., Chavoshy, S., Gu, N., Trieb, M., Behrens, R., Laake, P., Pongs, O., Knaus, H. G., Ottersen, O. P. and Storm, J. F. (2001) ‘Presynaptic Ca2+-activated K+ channels in glutamatergic hippocampal terminals and their role in spike repolarization and regulation of transmitter release.’, *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 21(24), pp. 9585–97.

Huang, S., Zhou, J., Wang, Z., Ling, Q. and Shen, Y. (2017) ‘Biomedical informatics with optimization and machine learning.’, *EURASIP journal on bioinformatics & systems biology*. Springer, 2017, p. 4. doi: 10.1186/s13637-017-0058-0.

Huang, W.-T. and Ma, H.-P. (2010) ‘Glucose stimulates BK channels in human podocytes’, *FASEB J*, 24(1_MeetingAbstracts), p. 610.13-.

Hubbard, T., Barker, D., Birney, E., Cameron, G., Chen, Y., Clark, L., Cox, T., Cuff, J., Curwen, V., Down, T., Durbin, R., Eyras, E., Gilbert, J., Hammond, M., Huminiecki, L., Kasprzyk, A., Lehvaslaiho, H., Lijnzaad, P., Melsopp, C., Mongin, E., Pettett, R., Pocock, M., Potter, S., Rust, A., Schmidt, E., Searle, S., Slater, G., Smith, J., Spooner, W., Stabenau, A., Stalker, J., Stupka, E., Ureta-Vidal, A., Vastrik, I. and Clamp, M. (2002) ‘The Ensembl genome database project’, *Nucleic Acids Research*. Oxford University Press, 30(1), pp. 38–41. doi: 10.1093/nar/30.1.38.

Hull, J. (2012) ‘Cystic fibrosis transmembrane conductance regulator dysfunction and its treatment.’, *Journal of the Royal Society of Medicine*. Royal Society of Medicine Press, (Suppl 2), pp. S2-8. doi: 10.1258/jrsm.2012.12s001.

Human Genome Sequencing Consortium, I. (2004) ‘Finishing the euchromatic sequence of the human genome’, *Nature*. Nature Publishing Group, 431(7011), pp. 931–945. doi: 10.1038/nature03001.

Huntington, G. (1872) ‘On Chorea’, *Medical and Surgical Reporter*.

Hutber, C. A., Hardie, D. G. and Winder, W. W. (1997) ‘Electrical stimulation inactivates muscle acetyl-CoA carboxylase and increases AMP-activated protein kinase.’, *The American journal of physiology*, 272(2 Pt 1), pp. E262-6.

Illison, J., Tian, L., McClafferty, H., Werno, M., Chamberlain, L. H., Leiss, V., Sassmann, A., Offermanns, S., Ruth, P., Shipston, M. J. and Lukowski, R. (2016) ‘Obesogenic and Diabetogenic Effects of High-Calorie Nutrition Require Adipocyte BK Channels.’, *Diabetes*. American Diabetes Association, 65(12), pp. 3621–3635. doi: 10.2337/db16-0245.

Imlach, W. L., Finch, S. C., Miller, J. H., Meredith, A. L. and Dalziel, J. E. (2010) ‘A role for BK channels in heart rate regulation in rodents.’, *PloS one*, 5(1), p. e8698. doi: 10.1371/journal.pone.0008698.

Ingram, V. M. (1959) ‘Abnormal human haemoglobins. III. The chemical difference between normal and sickle cell haemoglobins.’, *Biochimica et biophysica acta*, 36, pp. 402–11.

International HapMap Consortium, T. (2007) ‘A second generation human haplotype map of over 3.1 million SNPs’, *Nature*, 449, pp. 851–861. doi: 10.1038/nature06258.

Ishiwata, T., Fujii, T., Ishiwata, S., Ikegawa, S. and Naito, Z. (2004) ‘Effect of morpholino antisense oligonucleotide against lumican mRNA in human embryonic kidney (HEK) 293 cells’, *Pathology International*, 54(2), pp. 77–81. doi: 10.1111/j.1440-1827.2004.01593.x.

Iwanaga, T., Tsutsumi, R., Noritake, J., Fukata, Y. and Fukata, M. (2009) ‘Dynamic protein palmitoylation in cellular signaling.’, *Progress in lipid research*. Elsevier Ltd, 48(3–4), pp. 117–27. doi: 10.1016/j.plipres.2009.02.001.

Jakab, M., Weiger, T. M. and Hermann, A. (1997) ‘Ethanol activates maxi Ca2+-activated K+ channels

of clonal pituitary (GH3) cells.', *The Journal of membrane biology*, 157(3), pp. 237–45.

Jeffries, O. (2010) *Palmitoylation of BK channels*. The University of Edinburgh.

Jeffries, O., Geiger, N., Rowe, I. C. M., Tian, L., McClafferty, H., Chen, L., Bi, D., Knaus, H. G., Ruth, P. and Shipston, M. J. (2010) 'Palmitoylation of the S0-S1 linker regulates cell surface expression of voltage- and calcium-activated potassium (BK) channels.', *The Journal of biological chemistry*, 285(43), pp. 33307–14. doi: 10.1074/jbc.M110.153940.

Jensen, B. S. (2002) 'BMS-204352: a potassium channel opener developed for the treatment of stroke.', *CNS drug reviews*, 8(4), pp. 353–60.

Jensen, B. S. (2006) 'BMS-204352: A Potassium Channel Opener Developed for the Treatment of Stroke', *CNS Drug Reviews*. Blackwell Publishing Ltd, 8(4), pp. 353–360. doi: 10.1111/j.1527-3458.2002.tb00233.x.

Jensen, M. O., Jogini, V., Borhani, D. W., Leffler, A. E., Dror, R. O. and Shaw, D. E. (2012) 'Mechanism of Voltage Gating in Potassium Channels', *Science*, 336(6078), pp. 229–233. doi: 10.1126/science.1216533.

Jiang, Y., Lee, A., Chen, J., Cadene, M., Chait, B. T. and MacKinnon, R. (2002) 'Crystal structure and mechanism of a calcium-gated potassium channel.', *Nature*, 417(6888), pp. 515–22. doi: 10.1038/417515a.

Jiang, Y., Pico, A., Cadene, M., Chait, B. T. and MacKinnon, R. (2001) 'Structure of the RCK Domain from the E. coli K+ Channel and Demonstration of Its Presence in the Human BK Channel', *Neuron*, 29(3), pp. 593–601. doi: 10.1016/S0896-6273(01)00236-7.

Jiao, H., Arner, P., Hoffstedt, J., Brodin, D., Dubern, B., Czernichow, S., van't Hooft, F., Axelsson, T., Pedersen, O., Hansen, T., Sørensen, T. I. A., Hebebrand, J., Kere, J., Dahlman-Wright, K., Hamsten, A., Clement, K. and Dahlman, I. (2011) 'Genome wide association study identifies KCNMA1 contributing to human obesity.', *BMC medical genomics*, 4(1), p. 51. doi: 10.1186/1755-8794-4-51.

Jo, S., Lee, K.-H., Song, S., Jung, Y.-K. and Park, C.-S. (2005) 'Identification and functional characterization of cereblon as a binding protein for large-conductance calcium-activated potassium channel in rat brain', *Journal of Neurochemistry*. Blackwell Science Ltd, 94(5), pp. 1212–1224. doi: 10.1111/j.1471-4159.2005.03344.x.

Kalia, N., Sharma, A., Kaur, M., Kamboj, S. S. and Singh, J. (2016) 'A comprehensive in silico analysis of non-synonymous and regulatory SNPs of human MBL2 gene.', *SpringerPlus*. Springer, 5(1), p. 811. doi: 10.1186/s40064-016-2543-4.

Kapplinger, J. D., Tester, D. J., Salisbury, B. A., Carr, J. L., Harris-Kerr, C., Pollevick, G. D., Wilde, A. A. M., Ackerman, M. J. and Ackerman, M. J. (2009) 'Spectrum and prevalence of mutations from the first 2,500 consecutive unrelated patients referred for the FAMILION long QT syndrome genetic test.', *Heart rhythm : the official journal of the Heart Rhythm Society*. NIH Public Access, 6(9), pp. 1297–303. doi: 10.1016/j.hrthm.2009.05.021.

Karas-Kuzelicki, N. and Mlinaric-Rascan, I. (2009) 'Individualization of thiopurine therapy: thiopurine *S* -methyltransferase and beyond', *Pharmacogenomics*, 10(8), pp. 1309–1322. doi: 10.2217/pgs.09.78.

Karaskov, E., Scott, C., Zhang, L., Teodoro, T., Ravazzola, M. and Volchuk, A. (2006) 'Chronic palmitate but not oleate exposure induces endoplasmic reticulum stress, which may contribute to INS-1 pancreatic beta-cell apoptosis.', *Endocrinology*. Endocrine Society, 147(7), pp. 3398–407. doi: 10.1210/en.2005-1494.

Karki, R., Pandya, D., Elston, R. C. and Ferlini, C. (2015) 'Defining "mutation" and "polymorphism" in

the era of personal genomics', *BMC medical genomics*. BioMed Central, 8, p. 37. doi: 10.1186/s12920-015-0115-z.

Kashyap, P., Sehrawat, A. and Deswal, R. (2015) 'Nitric oxide modulates Lycopersicon esculentum C-repeat binding factor 1 (LeCBF1) transcriptionally as well as post-translationally by nitrosylation', *Plant Physiology and Biochemistry*, 96, pp. 115–123. doi: 10.1016/j.plaphy.2015.07.032.

Kellis, M., Wold, B., Snyder, M. P., Bernstein, B. E., Kundaje, A., Marinov, G. K., Ward, L. D., Birney, E., Crawford, G. E., Dekker, J., Dunham, I., Elnitski, L. L., Farnham, P. J., Feingold, E. A., Gerstein, M., Giddings, M. C., Gilbert, D. M., Gingeras, T. R., Green, E. D., Guigo, R., Hubbard, T., Kent, J., Lieb, J. D., Myers, R. M., Pazin, M. J., Ren, B., Stamatoyannopoulos, J. A., Weng, Z., White, K. P. and Hardison, R. C. (2014) 'Defining functional DNA elements in the human genome.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 111(17), pp. 6131–8. doi: 10.1073/pnas.1318948111.

Khan, R. N., Matharoo-Ball, B., Arulkumaran, S. and Ashford, M. L. J. (2001) 'Potassium Channels in the Human Myometrium', *Experimental Physiology*. Blackwell Science Ltd, 86(2), pp. 255–264. doi: 10.1113/eph8602181.

Khoshnoodi, J., Sigmundsson, K., Ofverstedt, L.-G., Skoglund, U., Obrink, B., Wartiovaara, J. and Tryggvason, K. (2003) 'Nephrin promotes cell-cell adhesion through homophilic interactions.', *The American journal of pathology*, 163(6), pp. 2337–46. doi: 10.1016/S0002-9440(10)63590-0.

Kim, E. Y., Alvarez-Baron, C. P. and Dryer, S. E. (2009) 'Canonical transient receptor potential channel (TRPC)3 and TRPC6 associate with large-conductance Ca2+-activated K+ (BKCa) channels: role in BKCa trafficking to the surface of cultured podocytes.', *Molecular pharmacology*, 75(3), pp. 466–77. doi: 10.1124/mol.108.051912.

Kim, E. Y., Choi, K.-J. and Dryer, S. E. (2008) 'Nephrin binds to the COOH terminus of a large-conductance Ca2+-activated K+ channel isoform and regulates its expression on the cell surface.', *American journal of physiology. Renal physiology*. doi: 10.1152/ajprenal.00140.2008.

Kim, E. Y. and Dryer, S. E. (2011) 'Effects of insulin and high glucose on mobilization of slo1 BKCa channels in podocytes.', *Journal of cellular physiology*, 226(9), pp. 2307–15. doi: 10.1002/jcp.22567.

Kim, E. Y., Ridgway, L. D. and Dryer, S. E. (2007) 'Interactions with Filamin A Stimulate Surface Expression of Large-Conductance Ca2+-Activated K+ Channels in the Absence of Direct Actin Binding', *Molecular Pharmacology*, 72(3).

Kim, E. Y., Ridgway, L. D., Zou, S., Chiu, Y.-H. and Dryer, S. E. (2007) 'Alternatively spliced C-terminal domains regulate the surface expression of large conductance calcium-activated potassium channels', *Neuroscience*, 146(4), pp. 1652–1661. doi: 10.1016/j.neuroscience.2007.03.038.

Kim, H. and Oh, K. H. (2016) 'Protein Network Interacting with BK Channels', in, pp. 127–161. doi: 10.1016/bs.irn.2016.03.003.

Kim, M.-S., Zhong, J. and Pandey, A. (2016) 'Common errors in mass spectrometry-based analysis of post-translational modifications', *PROTEOMICS*, 16(5), pp. 700–714. doi: 10.1002/pmic.201500355.

Kim, S., Lee, B.-C., Lee, A.-R., Won, S. and Park, C.-S. (2014) 'Effects of palmitoylation on the diffusional movement of BKCa channels in live cells.', *FEBS letters*, 588(5), pp. 713–9. doi: 10.1016/j.febslet.2014.01.014.

Kimura, N., Tokunaga, C., Dalal, S., Richardson, C., Yoshino, K., Hara, K., Kemp, B. E., Witters, L. A., Mimura, O. and Yonezawa, K. (2003) 'A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway', *Genes to Cells*. Blackwell Science Ltd, 8(1), pp. 65–79. doi: 10.1046/j.1365-2443.2003.00615.x.

King, J. T., Lovell, P. V, Rishniw, M., Kotlikoff, M. I., Zeeman, M. L. and McCobb, D. P. (2006) 'Beta2 and beta4 subunits of BK channels confer differential sensitivity to acute modulation by steroid hormones.', *Journal of neurophysiology*, 95(5), pp. 2878–2888. doi: 10.1152/jn.01352.2005.

Kingsbury, K. J., Paul, S., Crossley, A. and Morgan, D. M. (1961) 'The fatty acid composition of human depot fat.', *The Biochemical journal*, 78, pp. 541–50.

Klein, R. J., Zeiss, C., Chew, E. Y., Tsai, J.-Y., Sackler, R. S., Haynes, C., Henning, A. K., SanGiovanni, J. P., Mane, S. M., Mayne, S. T., Bracken, M. B., Ferris, F. L., Ott, J., Barnstable, C. and Hoh, J. (2005) 'Complement factor H polymorphism in age-related macular degeneration.', *Science (New York, N.Y.)*. NIH Public Access, 308(5720), pp. 385–9. doi: 10.1126/science.1109557.

Knaus, H., Schwarzer, C., Koch, R., Eberhart, A., Kaczorowski, G., Glossmann, H., Wunder, F., Pongs, O., Garcia, M. and Sperk, G. (1996) 'Distribution of high-conductance Ca(2+)-activated K+ channels in rat brain: targeting to axons and nerve terminals', *Journal of Neuroscience*, 16(3).

Ko, J.-H., Ibrahim, M. A., Park, W. S., Ko, E. a, Kim, N., Warda, M., Lim, I., Bang, H. and Han, J. (2009) 'Cloning of large-conductance Ca(2+)-activated K(+) channel alpha-subunits in mouse cardiomyocytes.', *Biochemical and biophysical research communications*. Elsevier Inc., 389(1), pp. 74–79. doi: 10.1016/j.bbrc.2009.08.087.

Köhler, M., Hirschberg, B., Bond, C. T., Kinzie, J. M., Marrion, N. V, Maylie, J. and Adelman, J. P. (1996) 'Small-conductance, calcium-activated potassium channels from mammalian brain.', *Science (New York, N.Y.)*, 273(5282), pp. 1709–14.

Kordyukova, L. V., Serebryakova, M. V., Baratova, L. A. and Veit, M. (2010) 'Site-specific attachment of palmitate or stearate to cytoplasmic versus transmembrane cysteines is a common feature of viral spike proteins', *Virology*, 398(1), pp. 49–56. doi: 10.1016/j.virol.2009.11.039.

Kordyukova, L. V, Serebryakova, M. V, Baratova, L. A. and Veit, M. (2008) 'S acylation of the hemagglutinin of influenza viruses: mass spectrometry reveals site-specific attachment of stearic acid to a transmembrane cysteine.', *Journal of virology*. American Society for Microbiology (ASM), 82(18), pp. 9288–92. doi: 10.1128/JVI.00704-08.

Korovkina, V. P., Fergus, D. J., Holdiman, A. J. and England, S. K. (2001) 'Characterization of a novel 132-bp exon of the human maxi-K channel', *Am J Physiol Cell Physiol*, 281(1), pp. C361-367.

Korycka, J., Łach, A., Heger, E., Bogusławska, D. M., Wolny, M., Toporkiewicz, M., Augoff, K., Korzeniewski, J. and Sikorski, A. F. (2012) 'Human DHHC proteins: A spotlight on the hidden player of palmitoylation', *European Journal of Cell Biology*, 91(2), pp. 107–117. doi: 10.1016/j.ejcb.2011.09.013.

Koutny, T. (2013) 'Glucose predictability, blood capillary permeability, and glucose utilization rate in subcutaneous, skeletal muscle, and visceral fat tissues', *Computers in Biology and Medicine*, 43(11), pp. 1680–1686. doi: 10.1016/j.compbiomed.2013.08.008.

Koval, O. M., Fan, Y. and Rothberg, B. S. (2007) 'A role for the S0 transmembrane segment in voltage-dependent gating of BK channels.', *The Journal of general physiology*. The Rockefeller University Press, 129(3), pp. 209–20. doi: 10.1085/jgp.200609662.

Kuang, Q., Purhonen, P. and Hebert, H. (2015) 'Structure of potassium channels.', *Cellular and molecular life sciences : CMLS*. Springer, 72(19), pp. 3677–93. doi: 10.1007/s00018-015-1948-5.

Kudo, N., Barr, A. J., Barr, R. L., Desai, S. and Lopaschuk, G. D. (1995) 'High rates of fatty acid oxidation during reperfusion of ischemic hearts are associated with a decrease in malonyl-CoA levels due to an increase in 5'-AMP-activated protein kinase inhibition of acetyl-CoA carboxylase.', *The Journal of biological chemistry*, 270(29), pp. 17513–20.

Kumar, P., Henikoff, S. and Ng, P. C. (2009) 'Predicting the effects of coding non-synonymous variants on protein function using the SIFT algorithm.', *Nature protocols*, 4(7), pp. 1073–81. doi: 10.1038/nprot.2009.86.

Kwon, B., Lee, H.-K. and Querfurth, H. W. (2014) 'Oleate prevents palmitate-induced mitochondrial dysfunction, insulin resistance and inflammatory signaling in neuronal cells', *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, 1843(7), pp. 1402–1413. doi: 10.1016/j.bbamcr.2014.04.004.

Kwon, B. and Querfurth, H. W. (2015) 'Palmitate activates mTOR/p70S6K through AMPK inhibition and hypophosphorylation of raptor in skeletal muscle cells: Reversal by oleate is similar to metformin', *Biochimie*, 118, pp. 141–150. doi: 10.1016/j.biochi.2015.09.006.

Kwon, S.-H. and Guggino, W. B. (2004) 'Multiple sequences in the C terminus of MaxiK channels are involved in expression, movement to the cell surface, and apical localization.', *Proceedings of the National Academy of Sciences*, 101(42), pp. 15237–15242. doi: 10.1073/pnas.0404877101.

Kyle, B. D. and Braun, A. P. (2014) 'The regulation of BK channel activity by pre- and post-translational modifications.', *Frontiers in physiology*, 5, p. 316. doi: 10.3389/fphys.2014.00316.

Kyle, B. D., Mishra, R. C. and Braun, A. P. (2017) 'The augmentation of BK channel activity by nitric oxide signaling in rat cerebral arteries involves co-localized regulatory elements', *Journal of Cerebral Blood Flow & Metabolism*, p. 0271678X1769129. doi: 10.1177/0271678X17691291.

Laderoute, K. R., Amin, K., Calaoagan, J. M., Knapp, M., Le, T., Orduna, J., Foretz, M. and Viollet, B. (2006) '5'-AMP-activated protein kinase (AMPK) is induced by low-oxygen and glucose deprivation conditions found in solid-tumor microenvironments.', *Molecular and cellular biology*, 26(14), pp. 5336–47. doi: 10.1128/MCB.00166-06.

Laethem, F. Van, Liang, X., Andris, F., Urbain, J., Vandenbranden, M., Ruysschaert, J.-M., Resh, M. D., Stulnig, T. M. and Leo, O. (2003) 'Glucocorticoids Alter the Lipid and Protein Composition of Membrane Rafts of a Murine T Cell Hybridoma', *The Journal of Immunology*, 170(6).

Lagrutta, A., Shen, K. Z., North, R. A. and Adelman, J. P. (1994) 'Functional differences among alternatively spliced variants of Slowpoke, a Drosophila calcium-activated potassium channel', *Journal of Biological Chemistry*, 269(32), pp. 20347–20351.

Lahiry, P., Torkamani, A., Schork, N. J. and Hegele, R. A. (2010) 'Kinase mutations in human disease: interpreting genotype–phenotype relationships', *Nature Reviews Genetics*. Nature Publishing Group, 11(1), pp. 60–74. doi: 10.1038/nrg2707.

Lai, E. C. (2003) 'Lipid rafts make for slippery platforms.', *The Journal of cell biology*. The Rockefeller University Press, 162(3), pp. 365–70. doi: 10.1083/jcb.200307087.

Lam, A. C., Schouten, M., Aulchenko, Y. S., Haley, C. S., de Koning, D.-J., Babron, C., Bartlett, C. W., Beyene, J., Bickeböller, H., Culverhouse, R., Cupples, A., Warwick Daw, E., Dupuis, J., Falk, C. T., Ghosh, S., Goddard, K. A., Goode, E. L., Hauser, E. R., Martin, L. J., Martinez, M., North, K. E., Saccone, N. L., Schmidt, S., Tapper, W., Thomas, D., Tritchler, D., Vieland, V. J., Wijsman, E. M., Wilcox, M. A., Witte, J. S., Yang, Q., Ziegler, A., Almasy, L. and MacCluer, J. W. (2006) 'Rapid and robust association mapping of expression quantitative trait loci', *BMC Proceedings*, 1(1), pp. 11–15.

Lam, R. S., Shaw, A. R. and Duszyk, M. (2004) 'Membrane cholesterol content modulates activation of BK channels in colonic epithelia.', *Biochimica et biophysica acta*, 1667(2), pp. 241–248. doi: 10.1016/j.bbamem.2004.11.004.

Lander, E. S., Altshuler, D., Pollara, V. J., Cowles, C. R., Van Etten, W. J., Baldwin, J. and Linton, L. (2000) 'An SNP map of the human genome generated by reduced representation shotgun sequencing.', *Nature*, 407(6803), pp. 513–516. doi: 10.1038/35035083.

Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., Funke, R., Gage, D., Harris, K., Heaford, A., Howland, J., Kann, L., Lehoczky, J., LeVine, R., McEwan, P., McKernan, K., Meldrim, J., Mesirov, J. P., Miranda, C., Morris, W., Naylor, J., Raymond, C., Rosetti, M., Santos, R., Sheridan, A., Sougnez, C., Stange-Thomann, N., Stojanovic, N., Subramanian, A., Wyman, D., Rogers, J., Sulston, J., Ainscough, R., Beck, S., Bentley, D., Burton, J., Clee, C., Carter, N., Coulson, A., Deadman, R., Deloukas, P., Dunham, A., Dunham, I., Durbin, R., French, L., Grafham, D., Gregory, S., Hubbard, T., Humphray, S., Hunt, A., Jones, M., Lloyd, C., McMurray, A., Matthews, L., Mercer, S., Milne, S., Mullikin, J. C., Mungall, A., Plumb, R., Ross, M., Shownkeen, R., Sims, S., Waterston, R. H., Wilson, R. K., Hillier, L. W., McPherson, J. D., Marra, M. A., Mardis, E. R., Fulton, L. A., Chinwalla, A. T., Pepin, K. H., Gish, W. R., Chissoe, S. L., Wendl, M. C., Delehaunty, K. D., Miner, T. L., Delehaunty, A., Kramer, J. B., Cook, L. L., Fulton, R. S., Johnson, D. L., Minx, P. J., Clifton, S. W., Hawkins, T., Branscomb, E., Predki, P., Richardson, P., Wenning, S., Slezak, T., Doggett, N., Cheng, J.-F., Olsen, A., Lucas, S., Elkin, C., Uberbacher, E., Frazier, M., Gibbs, R. A., Muzny, D. M., Scherer, S. E., Bouck, J. B., Sodergren, E. J., Worley, K. C., Rives, C. M., Gorrell, J. H., Metzker, M. L., Naylor, S. L., Kucherlapati, R. S., Nelson, D. L., Weinstock, G. M., Sakaki, Y., Fujiyama, A., Hattori, M., Yada, T., Toyoda, A., Itoh, T., Kawagoe, C., Watanabe, H., Totoki, Y., Taylor, T., Weissenbach, J., Heilig, R., Saurin, W., Artiguenave, F., Brottier, P., Bruls, T., Pelletier, E., Robert, C., Wincker, P., Rosenthal, A., Platzer, M., Nyakatura, G., Taudien, S., Rump, A., Smith, D. R., Doucette-Stamm, L., Rubenfield, M., Weinstock, K., Lee, H. M., Dubois, J., Yang, H., Yu, J., Wang, J., Huang, G., Gu, J., Hood, L., Rowen, L., Madan, A., Qin, S., Davis, R. W., Federspiel, N. A., Abola, A. P., Proctor, M. J., Roe, B. A., Chen, F., Pan, H., Ramser, J., Lehrach, H., Reinhardt, R., McCombie, W. R., de la Bastide, M., Dedhia, N., Blöcker, H., Hornischer, K., Nordsiek, G., Agarwala, R., Aravind, L., Bailey, J. A., Bateman, A., Batzoglou, S., Birney, E., Bork, P., Brown, D. G., Burge, C. B., Cerutti, L., Chen, H.-C., Church, D., Clamp, M., Copley, R. R., Doerks, T., Eddy, S. R., Eichler, E. E., Furey, T. S., Galagan, J., Gilbert, J. G. R., Harmon, C., Hayashizaki, Y., Haussler, D., Hermjakob, H., Hokamp, K., Jang, W., Johnson, L. S., Jones, T. A., Kasif, S., Kaspryzk, A., Kennedy, S., Kent, W. J., Kitts, P., Koonin, E. V., Korf, I., Kulp, D., Lancet, D., Lowe, T. M., McLysaght, A., Mikkelsen, T., Moran, J. V., Mulder, N., Pollara, V. J., Ponting, C. P., Schuler, G., Schultz, J., Slater, G., Smit, A. F. A., Stupka, E., Szustakowki, J., Thierry-Mieg, D., Thierry-Mieg, J., Wagner, L., Wallis, J., Wheeler, R., Williams, A., Wolf, Y. I., Wolfe, K. H., Yang, S.-P., Yeh, R.-F., Collins, F., Guyer, M. S., Peterson, J., Felsenfeld, A., Wetterstrand, K. A., Myers, R. M., Schmutz, J., Dickson, M., Grimwood, J., Cox, D. R., Olson, M. V., Kaul, R., Raymond, C., Shimizu, N., Kawasaki, K., Minoshima, S., Evans, G. A., Athanasiou, M., Schultz, R., Patrinos, A. and Morgan, M. J. (2001) 'Initial sequencing and analysis of the human genome', *Nature*. Nature Publishing Group, 409(6822), pp. 860–921. doi: 10.1038/35057062.

Larsen, M. R., Trelle, M. B., Thingholm, T. E. and Jensen, O. N. (2006) 'Analysis of posttranslational modifications of proteins by tandem mass spectrometry', *BioTechniques*, pp. 790–798. doi: 10.2144/000112201.

Latorre, R., Vergara, C. and Hidalgo, C. (1982) 'Reconstitution in planar lipid bilayers of a Ca2+-dependent K+ channel from transverse tubule membranes isolated from rabbit skeletal muscle.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 79(3), pp. 805–9.

Laumonnier, F., Roger, S., Guérin, P., Molinari, F., M'Rad, R., Cahard, D., Belhadj, A., Halayem, M., Persico, A. M., Elia, M., Romano, V., Holbert, S., Andres, C., Chaabouni, H., Colleaux, L., Constant, J., Le Guennec, J.-Y. and Briault, S. (2006) 'Association of a Functional Deficit of the BKCa Channel, a Synaptic Regulator of Neuronal Excitability, With Autism and Mental Retardation', *American Journal of Psychiatry*. American Psychiatric Association, 163(9), pp. 1622–1629. doi: 10.1176/ajp.2006.163.9.1622.

Lawley, R. (2009) *A REVOLUTION IN THE MICROBIOLOGY LABORATORY*. Available at: http://www.foodsafetywatch.org/features/a-revolution-in-the-microbiology-laboratory/.

Layne, J. J., Nausch, B., Olesen, S.-P. and Nelson, M. T. (2010) 'BK channel activation by NS11021 decreases excitability and contractility of urinary bladder smooth muscle', *AJP: Regulatory, Integrative and Comparative Physiology*, 298(2), pp. R378–R384. doi: 10.1152/ajpregu.00458.2009.

Lee, H., Deignan, J. L., Dorrani, N., Strom, S. P., Kantarci, S., Quintero-Rivera, F., Das, K., Toy, T., Harry, B., Yourshaw, M., Fox, M., Fogel, B. L., Martinez-Agosto, J. A., Wong, D. A., Chang, V. Y., Shieh, P. B., Palmer, C. G. S., Dipple, K. M., Grody, W. W., Vilain, E. and Nelson, S. F. (2014) 'Clinical exome sequencing for genetic identification of rare Mendelian disorders.', *JAMA*. NIH Public Access, 312(18), pp. 1880–7. doi: 10.1001/jama.2014.14604.

Lee, P. H. (2009) *Prioritizing SNPs for Disease-Gene Association Studies: Algorithms and Systems*. Queen's University, Canada.

Lee, S.-J., Choi, S.-E., Jung, I.-R., Lee, K.-W. and Kang, Y. (2013) 'Protective effect of nicotinamide on high glucose/palmitate-induced glucolipotoxicity to INS-1 beta cells is attributed to its inhibitory activity to sirtuins.', *Archives of biochemistry and biophysics*, 535(2), pp. 187–96. doi: 10.1016/j.abb.2013.03.011.

Lee, U. S. and Cui, J. (2009) 'Beta subunit-specific modulations of BK channel function by a mutation associated with epilepsy and dyskinesia.', *The Journal of physiology*. Wiley-Blackwell, 587(Pt 7), pp. 1481–98. doi: 10.1113/jphysiol.2009.169243.

Levitan, I. B. (1994) 'Modulation of Ion Channels by Protein Phosphorylation and Dephosphorylation', *Annual Review of Physiology*, 56(1), pp. 193–212. doi: 10.1146/annurev.ph.56.030194.001205.

Levy-Lahad, E. and Friedman, E. (2007) 'Cancer risks among BRCA1 and BRCA2 mutation carriers', *British Journal of Cancer*. Nature Publishing Group, 96(1), pp. 11–15. doi: 10.1038/sj.bjc.6603535.

Levy, S., Sutton, G., Ng, P. C., Feuk, L., Halpern, A. L., Walenz, B. P., Axelrod, N., Huang, J., Kirkness, E. F., Denisov, G., Lin, Y., MacDonald, J. R., Pang, A. W. C., Shago, M., Stockwell, T. B., Tsiamouri, A., Bafna, V., Bansal, V., Kravitz, S. A., Busam, D. A., Beeson, K. Y., McIntosh, T. C., Remington, K. A., Abril, J. F., Gill, J., Borman, J., Rogers, Y. H., Frazier, M. E., Scherer, S. W., Strausberg, R. L. and Venter, J. C. (2007) 'The diploid genome sequence of an individual human', *PLoS Biology*. Edited by E. M. Rubin. Springer, 5(10), pp. 2113–2144. doi: 10.1371/journal.pbio.0050254.

Li, J., Deng, C.-L., Gao, F., Cheng, J.-H., Yu, Z.-B., Liu, L. and Xie, M.-J. (2009) 'Coexpression and characterization of the human large-conductance Ca(2+)-activated K (+) channel alpha + beta1 subunits in HEK293 cells.', *Molecular and cellular biochemistry*, 331(1–2), pp. 117–26. doi: 10.1007/s11010-009-0149-7.

Li, Q. and Yan, J. (2016) 'Modulation of BK Channel Function by Auxiliary Beta and Gamma Subunits.', *International review of neurobiology*. NIH Public Access, 128, pp. 51–90. doi: 10.1016/bs.irn.2016.03.015.

Li, W. and Aldrich, R. W. (2004) 'Unique inner pore properties of BK channels revealed by quaternary ammonium block.', *The Journal of general physiology*. The Rockefeller University Press, 124(1), pp. 43–57. doi: 10.1085/jgp.200409067.

Li, X., Uchida, M., Alpar, H. O. and Mertens, P. (2013) 'Biolistic transfection of human embryonic kidney (HEK) 293 cells.', *Methods in molecular biology (Clifton, N.J.)*, 940, pp. 119–32. doi: 10.1007/978-1-62703-110-3_10.

Liang, X., Lu, Y., Wilkes, M., Neubert, T. A. and Resh, M. D. (2004) 'The N-terminal SH4 region of the Src family kinase Fyn is modified by methylation and heterogeneous fatty acylation: role in membrane targeting, cell adhesion, and spreading.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 279(9), pp. 8133–9. doi: 10.1074/jbc.M311180200.

Liang, X., Nazarian, A., Erdjument-Bromage, H., Bornmann, W., Tempst, P. and Resh, M. D. (2001) 'Heterogeneous fatty acylation of Src family kinases with polyunsaturated fatty acids regulates raft localization and signal transduction.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 276(33), pp. 30987–94. doi: 10.1074/jbc.M104018200.

Liesa, M. and Shirihai, O. S. (2013) 'Mitochondrial Dynamics in the Regulation of Nutrient Utilization and Energy Expenditure', *Cell Metabolism*, 17(4), pp. 491–506. doi: 10.1016/j.cmet.2013.03.002.

Lin, C.-W., Yan, F., Shimamura, S., Barg, S. and Shyng, S.-L. (2005) 'Membrane phosphoinositides control insulin secretion through their effects on ATP-sensitive K+ channel activity.', *Diabetes*. NIH Public Access, 54(10), pp. 2852–8.

Lin, H. and Chen, W. (2015) 'Briefing in application of machine learning methods in ion channel prediction.', *The Scientific World Journal*. Hindawi Publishing Corporation, 2015, p. 945927. doi: 10.1155/2015/945927.

Linder, M. E. and Deschenes, R. J. (2007) 'Palmitoylation: policing protein stability and traffic', *Nature Reviews Molecular Cell Biology*, 8(1), pp. 74–84. doi: 10.1038/nrm2084.

Ling, S., Woronuk, G., Sy, L., Lev, S. and Braun, A. P. (2000) 'Enhanced Activity of a Large Conductance, Calcium-sensitive K+ Channel in the Presence of Src Tyrosine Kinase', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 275(39), pp. 30683–30689. doi: 10.1074/jbc.M004292200.

Liu, D., Maier, A., Scholze, A., Rauch, U., Boltzen, U., Zhao, Z., Zhu, Z. and Tepel, M. (2008) 'High glucose enhances transient receptor potential channel canonical type 6-dependent calcium influx in human platelets via phosphatidylinositol 3-kinase-dependent pathway.', *Arteriosclerosis, thrombosis, and vascular biology*, 28(4), pp. 746–51. doi: 10.1161/ATVBAHA.108.162222.

Liu, G., Zakharov, S. I., Yang, L., Deng, S.-X., Landry, D. W., Karlin, A. and Marx, S. O. (2008) 'Position and Role of the BK Channel α Subunit S0 Helix Inferred from Disulfide Crosslinking', *The Journal of General Physiology*, 131(6), pp. 537–548. doi: 10.1085/jgp.200809968.

Liu, G., Zakharov, S. I., Yao, Y., Marx, S. O. and Karlin, A. (2015) 'Positions of the cytoplasmic end of BK α S0 helix relative to S1–S6 and of β1 TM1 and TM2 relative to S0–S6', *The Journal of General Physiology*, 145(3).

Liu, H., Moczydlowski, E. and Haddad, G. G. (1999) 'O(2) deprivation inhibits Ca(2+)-activated K(+) channels via cytosolic factors in mice neocortical neurons.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 104(5), pp. 577–88. doi: 10.1172/JCI7291.

Liu, J., Asuncion-Chin, M., Liu, P. and Dopico, A. M. (2006) 'CaM kinase II phosphorylation of slo Thr107 regulates activity and ethanol responses of BK channels.', *Nature neuroscience*, 9(1), pp. 41–9. doi: 10.1038/nn1602.

Liu, J., Vaithianathan, T., Manivannan, K., Parrill, A. and Dopico, A. M. (2008) 'Ethanol modulates BKCa channels by acting as an adjuvant of calcium.', *Molecular pharmacology*, 74, pp. 628–640. doi: 10.1124/mol.108.048694.

Liu, J., Wu, J., Sun, A., Sun, Y., Yu, X., Liu, N., Dong, S., Yang, F., Zhang, L., Zhong, X., Xu, C., Lu, F. and Zhang, W. (2016) 'Hydrogen sulfide decreases high glucose/palmitate-induced autophagy in endothelial cells by the Nrf2-ROS-AMPK signaling pathway.', *Cell & bioscience*. BioMed Central, 6, p. 33. doi: 10.1186/s13578-016-0099-1.

Liu, J., Ye, J., Zou, X., Xu, Z., Feng, Y., Zou, X., Chen, Z., Li, Y. and Cang, Y. (2014) 'CRL4ACRBN E3 ubiquitin ligase restricts BK channel activity and prevents epileptogenesis', *Nature Communications*, 5, p. 3924. doi: 10.1038/ncomms4924.

Liu, T., Wang, Y., Luo, X., Li, J., Reed, S. A., Xiao, H., Young, T. S. and Schultz, P. G. (2016) 'Enhancing protein stability with extended disulfide bonds.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 113(21), pp. 5910–5. doi: 10.1073/pnas.1605363113.

Liu, X., Jian, X. and Boerwinkle, E. (2011) 'dbNSFP: A lightweight database of human nonsynonymous SNPs and their functional predictions', *Human Mutation*. Wiley-Blackwell, 32(8), pp. 894–899. doi: 10.1002/humu.21517.

Liu, Y., Terata, K., Rusch, N. J. and Gutterman, D. D. (2001) 'High Glucose Impairs Voltage-Gated K+ Channel Current in Rat Small Coronary Arteries', *Circulation Research*, 89(2), pp. 146–152. doi: 10.1161/hh1401.093294.

Liu, Y., Tian, X., Li, Y., Liu, D., Liu, M., Zhang, X., Zhang, Q., Yan, C. and Han, Y. (2016) 'Up-Regulation of CREG Expression by the Transcription Factor GATA1 Inhibits High Glucose- and High Palmitate-Induced Apoptosis in Human Umbilical Vein Endothelial Cells.', *PloS one*. Public Library of Science, 11(5), p. e0154861. doi: 10.1371/journal.pone.0154861.

Liu, Y., Wan, Q., Guan, Q., Gao, L. and Zhao, J. (2006) 'High-fat diet feeding impairs both the expression and activity of AMPKa in rats' skeletal muscle', *Biochemical and Biophysical Research Communications*, 339(2), pp. 701–707. doi: 10.1016/j.bbrc.2005.11.068.

Loos, R. J. F., Lindgren, C. M., Li, S., Wheeler, E., Zhao, J. H., Prokopenko, I., Inouye, M., Freathy, R. M., Attwood, A. P., Beckmann, J. S., Berndt, S. I., Jacobs, K. B., Chanock, S. J., Hayes, R. B., Bergmann, S., Bennett, A. J., Bingham, S. A., Bochud, M., Brown, M., Cauchi, S., Connell, J. M., Cooper, C., Smith, G. D., Day, I., Dina, C., De, S., Dermitzakis, E. T., Doney, A. S. F., Elliott, K. S., Elliott, P., Evans, D. M., Sadaf Farooqi, I., Froguel, P., Ghori, J., Groves, C. J., Gwilliam, R., Hadley, D., Hall, A. S., Hattersley, A. T., Hebebrand, J., Heid, I. M., Lamina, C., Gieger, C., Illig, T., Meitinger, T., Wichmann, H.-E., Herrera, B., Hinney, A., Hunt, S. E., Jarvelin, M.-R., Johnson, T., Jolley, J. D. M., Karpe, F., Keniry, A., Khaw, K.-T., Luben, R. N., Mangino, M., Marchini, J., McArdle, W. L., McGinnis, R., Meyre, D., Munroe, P. B., Morris, A. D., Ness, A. R., Neville, M. J., Nica, A. C., Ong, K. K., O'Rahilly, S., Owen, K. R., Palmer, C. N. A., Papadakis, K., Potter, S., Pouta, A., Qi, L., Kraft, P., Hankinson, S. E., Hunter, D. J., Hu, F. B., Randall, J. C., Rayner, N. W., Ring, S. M., Sandhu, M. S., Scherag, A., Sims, M. A., Song, K., Soranzo, N., Speliotes, E. K., Lyon, H. N., Voight, B. F., Ridderstrale, M., Groop, L., Syddall, H. E., Teichmann, S. A., Timpson, N. J., Tobias, J. H., Uda, M., Scheet, P., Sanna, S., Abecasis, G. R., Albai, G., Nagaraja, R., Schlessinger, D., Ganz Vogel, C. I., Wallace, C., Waterworth, D. M., Weedon, M. N., Willer, C. J., Jackson, A. U., Tuomilehto, J., Collins, F. S., Boehnke, M., Mohlke, K. L., Wraight, V. L., Yuan, X., Zeggini, E., Hirschhorn, J. N., Strachan, D. P., Ouwehand, W. H., Caulfield, M. J., Samani, N. J., Frayling, T. M., Vollenweider, P., Waeber, G., Mooser, V., Deloukas, P., McCarthy, M. I., Wareham, N. J. and Barroso, I. (2008) 'Common variants near MC4R are associated with fat mass, weight and risk of obesity', *Nature Genetics*. Nature Publishing Group, 40(6), pp. 768–775. doi: 10.1038/ng.140.

López-Llano, J., Campos, L. A. and Sancho, J. (2006) 'Alpha-helix stabilization by alanine relative to glycine: roles of polar and apolar solvent exposures and of backbone entropy.', *Proteins*, 64(3), pp. 769–78. doi: 10.1002/prot.21041.

Lu, T. and Lee, H. (2010) 'Impaired Vascular BK Channel Function in Type 2 Diabetes Mellitus', in Croniger, C. (ed.) *Medical Complications of Type 2 Diabetes*, pp. 53–71.

Luiken, J. J., van Nieuwenhoven, F. A., America, G., van der Vusse, G. J. and Glatz, J. F. (1997) 'Uptake and metabolism of palmitate by isolated cardiac myocytes from adult rats: involvement of sarcolemmal proteins.', *Journal of lipid research*, 38(4), pp. 745–58.

Luscombe, N. M., Greenbaum, D. and Gerstein, M. (2001) 'What is bioinformatics ? An introduction and overview', *Yearbook of Medical Informatics*, pp. 83–100. doi: 10.1053/j.ro.2009.03.010.

Lynch, S. J., Snitkin, H., Gumper, I., Philips, M. R., Sabatini, D. and Pellicer, A. (2015) 'The differential palmitoylation states of N-Ras and H-Ras determine their distinct Golgi subcompartment localizations.', *Journal of cellular physiology*. NIH Public Access, 230(3), pp. 610–9. doi: 10.1002/jcp.24779.

Ma, B., Tsai, C.-J., Haliloğlu, T. and Nussinov, R. (2011) 'Dynamic Allostery: Linkers Are Not Merely Flexible', *Structure*, 19(7), pp. 907–917. doi: 10.1016/j.str.2011.06.002.

Ma, C. and Chang, G. (2004) 'Structure of the multidrug resistance efflux transporter EmrE from Escherichia coli', *Proceedings of the National Academy of Sciences*, 101(9), pp. 2852–2857. doi: 10.1073/pnas.0400137101.

Ma, Z., Lou, X. J. and Horrigan, F. T. (2006) 'Role of charged residues in the S1-S4 voltage sensor of BK channels.', *The Journal of general physiology*. The Rockefeller University Press, 127(3), pp. 309–28. doi: 10.1085/jgp.200509421.

MacKinnon, R. (2003) 'Potassium channels', *FEBS Letters*, 555(1), pp. 62–65. doi: 10.1016/S0014-5793(03)01104-9.

Maedler, K., Oberholzer, J., Bucher, P., Spinas, G. A. and Donath, M. Y. (2003) 'Monounsaturated Fatty Acids Prevent the Deleterious Effects of Palmitate and High Glucose on Human Pancreatic -Cell Turnover and Function', *Diabetes*, 52(3), pp. 726–733. doi: 10.2337/diabetes.52.3.726.

Majewski, J., Schwartzentruber, J., Lalonde, E., Montpetit, A. and Jabado, N. (2011) 'What can exome sequencing do for you?', *Journal of Medical Genetics*, 48(9), pp. 580–589. doi: 10.1136/jmedgenet-2011-100223.

Manolio, T. A. and Collins, F. S. (2009) 'The HapMap and Genome-Wide Association Studies in Diagnosis and Therapy', *Annual Review of Medicine*, 60(1), pp. 443–456. doi: 10.1146/annurev.med.60.061907.093117.

Marijic, J., Li, Q., Song, M., Nishimaru, K., Stefani, E. and Toro, L. (2001) 'Decreased Expression of Voltage- and Ca2+-Activated K+ Channels in Coronary Smooth Muscle During Aging', *Circulation Research*, 88(2), pp. 210–216. doi: 10.1161/01.RES.88.2.210.

Maris, M., Robert, S., Waelkens, E., Derua, R., Hernangomez, M. H., D'Hertog, W., Cnop, M., Mathieu, C. and Overbergh, L. (2013) 'Role of the saturated nonesterified fatty acid palmitate in beta cell dysfunction.', *Journal of proteome research*. American Chemical Society, 12(1), pp. 347–62. doi: 10.1021/pr300596g.

Marsin, A. S., Bertrand, L., Rider, M. H., Deprez, J., Beauloye, C., Vincent, M. F., Van den Berghe, G., Carling, D. and Hue, L. (2000) 'Phosphorylation and activation of heart PFK-2 by AMPK has a role in the stimulation of glycolysis during ischaemia.', *Current biology : CB*. Elsevier, 10(20), pp. 1247–55. doi: 10.1016/S0960-9822(00)00742-9.

Martin-Moreno, J. M., Willett, W. C., Gorgojo, L., Banegas, J. R., Rodriguez-Artalejo, F., Fernandez-Rodriguez, J. C., Maisonneuve, P. and Boyle, P. (1994) 'Dietary fat, olive oil intake and breast cancer risk.', *International journal of cancer*, 58(6), pp. 774–80.

Martin, A. (2016) *A Summary of Missense Prediction Methods*, *www.bioinf.org.uk*. Available at: http://www.bioinf.org.uk/saap/methods/#ref68.

Martino, L., Masini, M., Novelli, M., Beffy, P., Bugliani, M., Marselli, L., Masiello, P., Marchetti, P. and De Tata, V. (2012) 'Palmitate activates autophagy in INS-1E β-cells and in isolated rat and human pancreatic islets.', *PloS one*. Public Library of Science, 7(5), p. e36188. doi: 10.1371/journal.pone.0036188.

Marty, A. (1981) ‘Ca-dependent K channels with large unitary conductance in chromaffin cell membranes.’, *Nature*, 291(5815), pp. 497–500.

Marx, V. (2013) ‘Biology: The big challenges of big data’, *Nature*. Nature Research, 498(7453), pp. 255–260. doi: 10.1038/498255a.

Mastrangelo, A. M., Jeitner, T. M. and Eaton, J. W. (1998) ‘Oleic Acid Increases Cell Surface Expression and Activity of CD11b on Human Neutrophils’, *The Journal of Immunology*, 161(8).

Mattanovich, D. and Borth, N. (2006) ‘Applications of cell sorting in biotechnology.’, *Microbial cell factories*. BioMed Central, 5, p. 12. doi: 10.1186/1475-2859-5-12.

Matthews, H. R. (1995) ‘Protein kinases and phosphatases that act on histidine, lysine, or arginine residues in eukaryotic proteins: A possible regulator of the mitogen-activated protein kinase cascade’, *Pharmacology & Therapeutics*, 67(3), pp. 323–350. doi: 10.1016/0163-7258(95)00020-8.

Mayor, S. and Maxfield, F. R. (1995) ‘Insolubility and redistribution of GPI-anchored proteins at the cell surface after detergent treatment.’, *Molecular biology of the cell*. American Society for Cell Biology, 6(7), pp. 929–44. doi: 10.1091/MBC.6.7.929.

Mazzio, E. A. and Soliman, K. F. A. (2012) ‘Basic concepts of epigenetics: impact of environmental signals on gene expression.’, *Epigenetics*. Taylor & Francis, 7(2), pp. 119–30. doi: 10.4161/epi.7.2.18764.

McCall, K. A., Huang, C. and Fierke, C. A. (2000) ‘Function and mechanism of zinc metalloenzymes.’, *The Journal of nutrition*, 130(5), p. 1437S–46S.

McCarroll, S. A., Huett, A., Kuballa, P., Chilewski, S. D., Landry, A., Goyette, P., Zody, M. C., Hall, J. L., Brant, S. R., Cho, J. H., Duerr, R. H., Silverberg, M. S., Taylor, K. D., Rioux, J. D., Altshuler, D., Daly, M. J. and Xavier, R. J. (2008) ‘Deletion polymorphism upstream of IRGM associated with altered IRGM expression and Crohn’s disease’, *Nature Genetics*, 40(9), pp. 1107–1112. doi: 10.1038/ng.215.

McCartney, C. E., McClafferty, H., Huibant, J.-M., Rowan, E. G., Shipston, M. J. and Rowe, I. C. M. (2005) ‘A cysteine-rich motif confers hypoxia sensitivity to mammalian large conductance voltage- and Ca-activated K (BK) channel alpha-subunits.’, *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 102(49), pp. 17870–6. doi: 10.1073/pnas.0505270102.

McCobb, D. P., Fowler, N. L., Featherstone, T., Lingle, C. J., Saito, M., Krause, J. E. and Salkoff, L. (1995) ‘A human calcium-activated potassium channel gene expressed in vascular smooth muscle’, *American Journal of Physiology - Heart and Circulatory Physiology*, 269(3).

McGahon, M. K., Dash, D. P., Arora, A., Wall, N., Dawicki, J., Simpson, D. a, Scholfield, C. N., McGeown, J. G. and Curtis, T. M. (2007) ‘Diabetes downregulates large-conductance Ca2+-activated potassium beta 1 channel subunit in retinal arteriolar smooth muscle.’, *Circulation research*, 100(5), pp. 703–11. doi: 10.1161/01.RES.0000260182.36481.c9.

McLeod, J. F., Leempoels, J. M., Peng, S. X., Dax, S. L., Myers, L. J. and Golder, F. J. (2014) ‘GAL-021, a new intravenous BKCa-channel blocker, is well tolerated and stimulates ventilation in healthy volunteers’, *British Journal of Anaesthesia*. Oxford University Press, 113(5), pp. 875–883. doi: 10.1093/bja/aeu182.

McManus, O. B. and Magleby, K. L. (1991) ‘Accounting for the Ca(2+)-dependent kinetics of single large-conductance Ca(2+)-activated K+ channels in rat skeletal muscle.’, *The Journal of physiology*. Wiley-Blackwell, 443, pp. 739–77.

Meech, R. W. (1972) ‘Intracellular calcium injection causes increased potassium conductance in Aplysia

nerve cells', *Comparative Biochemistry and Physiology -- Part A: Physiology*, 42(2), pp. 493–499. doi: 10.1016/0300-9629(72)90128-4.

Meera, P., Wallner, M., Song, M. and Toro, L. (1997) 'Large conductance voltage- and calcium-dependent K+ channel, a distinct member of voltage-dependent ion channels with seven N-terminal transmembrane segments (S0-S6), an extracellular N terminus, and an intracellular (S9-S10) C terminus.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 94(25), pp. 14066–71.

Meienberg, J., Bruggmann, R., Oexle, K. and Matyas, G. (2016) 'Clinical sequencing: is WGS the better WES?', *Human genetics*. Springer, 135(3), pp. 359–62. doi: 10.1007/s00439-015-1631-9.

Meissen, J. K., Hirahatake, K. M., Adams, S. H. and Fiehn, O. (2015) 'Temporal metabolomic responses of cultured HepG2 liver cells to high fructose and high glucose exposures', *Metabolomics*, 11(3), pp. 707–721. doi: 10.1007/s11306-014-0729-8.

Meredith, A. L., Thorneloe, K. S., Werner, M. E., Nelson, M. T. and Aldrich, R. W. (2004) 'Overactive bladder and incontinence in the absence of the BK large conductance Ca2+-activated K+ channel.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 279(35), pp. 36746–52. doi: 10.1074/jbc.M405621200.

Miles, J. M., Wooldridge, D., Grellner, W. J., Windsor, S., Isley, W. L., Klein, S. and Harris, W. S. (2003) 'Nocturnal and Postprandial Free Fatty Acid Kinetics in Normal and Type 2 Diabetic Subjects: Effects of Insulin Sensitization Therapy', *Diabetes*, 52(3), pp. 675–681. doi: 10.2337/diabetes.52.3.675.

Millburn, G. H., Crosby, M. A., Gramates, L. S., Tweedie, S. and FlyBase Consortium, the F. (2016) 'FlyBase portals to human disease research using Drosophila models.', *Disease models & mechanisms*. Company of Biologists, 9(3), pp. 245–52. doi: 10.1242/dmm.023317.

Miller, C. (2000) 'An overview of the potassium channel family.', *Genome Biology*, 1(4), p. reviews0004.1. doi: 10.1186/gb-2000-1-4-reviews0004.

Minokoshi, Y., Alquier, T., Furukawa, N., Kim, Y.-B., Lee, A., Xue, B., Mu, J., Foufelle, F., Ferré, P., Birnbaum, M. J., Stuck, B. J. and Kahn, B. B. (2004) 'AMP-kinase regulates food intake by responding to hormonal and nutrient signals in the hypothalamus', *Nature*, 428(6982), pp. 569–574. doi: 10.1038/nature02440.

Mishra, P. and Chan, D. C. (2016) 'Metabolic regulation of mitochondrial dynamics', *The Journal of Cell Biology*, 212(4).

Mitchell, C. S., Cates, A., Kim, R. B. and Hollinger, S. K. (2015) 'Undergraduate Biocuration: Developing Tomorrow's Researchers While Mining Today's Data.', *Journal of undergraduate neuroscience education : JUNE : a publication of FUN, Faculty for Undergraduate Neuroscience*. Faculty for Undergraduate Neuroscience, 14(1), pp. A56-65.

Mitchell, D. A., Vasudevan, A., Linder, M. E. and Deschenes, R. J. (2006) 'Protein palmitoylation by a family of DHHC protein S-acyltransferases', *Journal of Lipid Research*, 47(6), pp. 1118–1127. doi: 10.1194/jlr.R600007-JLR200.

Miteva, M., Keusekotten, K., Hofmann, K., Praefcke, G. J. K. and Dohmen, R. J. (2010) 'Sumoylation as a signal for polyubiquitylation and proteasomal degradation', *Sub-cellular biochemistry*. Landes Bioscience, 54, pp. 195–214. doi: 10.1007/978-1-4419-6676-6_16.

Moharir, A., Peck, S. H., Budden, T., Lee, S. Y., Zeman, W., Dyken, P., Mole, S., Williams, R., Goebel, H., Bennett, M., Hofmann, S., Seehafer, S., Pearce, D., Palmer, D., Fearnley, I., Walker, J., Hall, N., Lake, B., Tyynela, J., Palmer, D., Baumann, M., Haltia, M., Haltia, M., Savukoski, M., Klockars, T., Holmberg, V., Santavuori, P., Lander, E., Xin, W., Mullen, T., Kiely, R., Min, J., Feng, X., Cannelli, N., Nardocci, N.,

Cassandrini, D., Morbin, M., Aiello, C., Isosomppi, J., Vesa, J., Jalanko, A., Peltonen, L., Schmiedt, M., Bessa, C., Heine, C., Ribeiro, M., Jalanko, A., Mamo, A., Jules, F., Dumaresq-Doiron, K., Costantino, S., Lefrancois, S., Schulz, A., Mousallem, T., Venkataramani, M., Persaud-Sawin, D., Zucker, A., Haddad, S., Khoury, M., Daoud, M., Kantar, R., Harati, H., Wujek, P., Kida, E., Walus, M., Wisniewski, K., Golabek, A., Steinfeld, R., Heim, P., Gregory, H. von, Meyer, K., Ullrich, K., Steinfeld, R., Steinke, H., Isbrandt, D., Kohlschutter, A., Gartner, J., Pohl, S., Marschner, K., Storch, S., Braulke, T., Sleat, D., Zheng, H., Qian, M., Lobel, P., Sun, Q., Westphal, W., Wong, K., Tan, I., Zhong, Q., Ohtsubo, K., Marth, J., Helenius, A., Aebi, M., Brodsky, J., Bard, F., Malhotra, V., Woodman, P., Stenmark, H., Parton, R., Steele-Mortimer, O., Lutcke, A., Gruenberg, J., Petersen, T., Brunak, S., Heijne, G. von, Nielsen, H., Qian, M., Sleat, D., Zheng, H., Moore, D., Lobel, P., Kollmann, K., Mutenda, K., Balleininger, M., Eckermann, E., Figura, K. von, Tretter, V., Altmann, F., Marz, L., Lebrun, A., Storch, S., Ruschendorf, F., Schmiedt, M., Kyttala, A., Vesa, J., Chin, M., Oelgeschlager, K., Isosomppi, J. and DellAngelica, E. (2013) 'The Role of N-Glycosylation in Folding, Trafficking, and Functionality of Lysosomal Protein CLN5', *PLoS ONE*. Edited by C. Beh. Public Library of Science, 8(9), p. e74299. doi: 10.1371/journal.pone.0074299.

Moon, J. S. and Won, K. C. (2015) 'Pancreatic α-Cell Dysfunction in Type 2 Diabetes: Old Kids on the Block.', *Diabetes & metabolism journal*. Korean Diabetes Association, 39(1), pp. 1–9. doi: 10.4093/dmj.2015.39.1.1.

Moore, M. C., Coate, K. C., Winnick, J. J., An, Z. and Cherrington, A. D. (2012) 'Regulation of hepatic glucose uptake and storage in vivo.', *Advances in nutrition (Bethesda, Md.)*. American Society for Nutrition, 3(3), pp. 286–94. doi: 10.3945/an.112.002089.

Moritz, W., Leech, C. A., Ferrer, J. and Habener, J. F. (2001) 'Regulated expression of adenosine triphosphate-sensitive potassium channel subunits in pancreatic beta-cells.', *Endocrinology*. Endocrine Society, 142(1), pp. 129–38. doi: 10.1210/endo.142.1.7885.

Morrison, A. C., Felix, J. F., Cupples, L. A., Glazer, N. L., Loehr, L. R., Dehghan, A., Demissie, S., Bis, J. C., Rosamond, W. D., Aulchenko, Y. S., Wang, Y. A., Haritunians, T., Folsom, A. R., Rivadeneira, F., Benjamin, E. J., Lumley, T., Couper, D., Stricker, B. H., O'Donnell, C. J., Rice, K. M., Chang, P. P., Hofman, A., Levy, D., Rotter, J. I., Fox, E. R., Uitterlinden, A. G., Wang, T. J., Psaty, B. M., Willerson, J. T., van Duijn, C. M., Boerwinkle, E., Witteman, J. C. M., Vasan, R. S. and Smith, N. L. (2010) 'Genomic variation associated with mortality among adults of European and African ancestry with heart failure: the cohorts for heart and aging research in genomic epidemiology consortium.', *Circulation. Cardiovascular genetics*. NIH Public Access, 3(3), pp. 248–55. doi: 10.1161/CIRCGENETICS.109.895995.

Morrow, J. P., Zakharov, S. I., Liu, G., Yang, L., Sok, A. J. and Marx, S. O. (2006) 'Defining the BK channel domains required for beta1-subunit modulation.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 103(13), pp. 5096–101. doi: 10.1073/pnas.0600907103.

Morse, E., Schroth, J., You, Y.-H., Pizzo, D. P., Okada, S., RamachandraRao, S., Vallon, V., Sharma, K. and Cunard, R. (2010) 'TRB3 is stimulated in diabetic kidneys, regulated by the ER stress marker CHOP, and is a suppressor of podocyte MCP-1', *American Journal of Physiology - Renal Physiology*, 299(5).

Moskowitz, S. M., Chmiel, J. F., Sternen, D. L., Cheng, E., Gibson, R. L., Marshall, S. G. and Cutting, G. R. (2008) 'Clinical practice and genetic counseling for cystic fibrosis and CFTR-related disorders.', *Genetics in medicine : official journal of the American College of Medical Genetics*. NIH Public Access, 10(12), pp. 851–68. doi: 10.1097/GIM.0b013e31818e55a2.

Muka, T., Koromani, F., Portilla, E., O'Connor, A., Bramer, W. M., Troup, J., Chowdhury, R., Dehghan, A. and Franco, O. H. (2016) 'The role of epigenetic modifications in cardiovascular disease: A systematic review', *International Journal of Cardiology*, 212, pp. 174–183. doi: 10.1016/j.ijcard.2016.03.062.

Munday, A. D. and López, J. a (2007) 'Posttranslational protein palmitoylation: promoting platelet

purpose.', *Arteriosclerosis, thrombosis, and vascular biology*, 27(7), pp. 1496–9. doi: 10.1161/ATVBAHA.106.136226.

Muszbek, L., Haramura, G., Cluette-Brown, J. E., Van Cott, E. M. and Laposata, M. (1999) 'The pool of fatty acids covalently bound to platelet proteins by thioester linkages can be altered by exogenously supplied fatty acids.', *Lipids*, 34 Suppl, pp. S331-7.

N'Gouemo, P. (2014) 'BKCa channel dysfunction in neurological diseases.', *Frontiers in physiology*. Frontiers Media SA, 5, p. 373. doi: 10.3389/fphys.2014.00373.

Nageshwaran, S. and Festenstein, R. (2015) 'Epigenetics and Triplet-Repeat Neurological Diseases', *Frontiers in Neurology*, 6, p. 262. doi: 10.3389/fneur.2015.00262.

Naruse, K., Tang, Q.-Y. and Sokabe, M. (2009) 'Stress-Axis Regulated Exon (STREX) in the C terminus of BK(Ca) channels is responsible for the stretch sensitivity.', *Biochemical and biophysical research communications*. Elsevier Inc., 385(4), pp. 634–639. doi: 10.1016/j.bbrc.2009.05.105.

Nascimbeni, A. C., Fanin, M., Tasca, E. and Angelini, C. (2010) 'Transcriptional and translational effects of intronic CAPN3 gene mutations.', *Human mutation*. Wiley-Blackwell, 31(9), pp. E1658-69. doi: 10.1002/humu.21320.

National Center for Biotechnology Information (2016) *NCBI retiring HapMap Resource*, *National Center for Biotechnology Information Website*. Available at: https://www.ncbi.nlm.nih.gov/variation/news/NCBI_retiring_HapMap/ (Accessed: 9 March 2017).

Nausch, B., Rode, F., Jørgensen, S., Nardi, A., Korsgaard, M. P. G., Hougaard, C., Bonev, A. D., Brown, W. D., Dyhring, T., Strøbæk, D., Olesen, S.-P., Christophersen, P., Grunnet, M., Nelson, M. T. and Rønn, L. C. B. (2014) 'NS19504: a novel BK channel activator with relaxing effect on bladder smooth muscle spontaneous phasic contractions.', *The Journal of pharmacology and experimental therapeutics*. American Society for Pharmacology and Experimental Therapeutics, 350(3), pp. 520–30. doi: 10.1124/jpet.113.212662.

Nelson, M. T., Cheng, H., Rubart, M., Santana, L. F., Bonev, A. D., Knot, H. J. and Lederer, W. J. (1995) 'Relaxation of Arterial Smooth Muscle by Calcium Sparks', *Science*, 270(5236).

Nelson, M. T. and Quayle, J. M. (1995) 'Physiological roles and properties of potassium channels in arterial smooth muscle.', *The American journal of physiology*, 268(4 Pt 1), pp. C799-822.

Nepveu-Traversy, M.-É., Demogines, A., Fricke, T., Plourde, M. B., Riopel, K., Veillette, M., Diaz-Griffero, F., Sawyer, S. L. and Berthoux, L. (2016) 'A putative SUMO interacting motif in the B30.2/SPRY domain of rhesus macaque TRIM5α important for NF-κB/AP-1 signaling and HIV-1 restriction', *Heliyon*, 2(1), p. e00056. doi: 10.1016/j.heliyon.2015.e00056.

Ng, P. C. and Henikoff, S. (2001) 'Predicting deleterious amino acid substitutions.', *Genome research*. Cold Spring Harbor Laboratory Press, 11(5), pp. 863–74. doi: 10.1101/gr.176601.

Niemiec, E. and Howard, H. C. (2016) 'Ethical issues in consumer genome sequencing: Use of consumers' samples and data', *Applied & Translational Genomics*, 8, pp. 23–30. doi: 10.1016/j.atg.2016.01.005.

Nishi, H., Hashimoto, K. and Panchenko, A. R. (2011) 'Phosphorylation in Protein-Protein Binding: Effect on Stability and Function', *Structure*, 19(12), pp. 1807–1815. doi: 10.1016/j.str.2011.09.021.

Nutt, L. K. and O'Neil, R. G. (2000) 'Effect of elevated glucose on endothelin-induced store-operated and non-store-operated calcium influx in renal mesangial cells.', *Journal of the American Society of Nephrology : JASN*, 11(7), pp. 1225–35.

O'Brien, P. J. and Zatz, M. (1984) 'Acylation of bovine rhodopsin by [3H]palmitic acid', *Journal of*

Biological Chemistry, 259(8), pp. 5054–5057.

O'Neill, H. M., Lally, J. S., Galic, S., Thomas, M., Azizi, P. D., Fullerton, M. D., Smith, B. K., Pulinilkunnil, T., Chen, Z., Samaan, M. C., Jorgensen, S. B., Dyck, J. R. B., Holloway, G. P., Hawke, T. J., van Denderen, B. J., Kemp, B. E. and Steinberg, G. R. (2014) 'AMPK phosphorylation of ACC2 is required for skeletal muscle fatty acid oxidation and insulin sensitivity in mice', *Diabetologia*, 57(8), pp. 1693–1702. doi: 10.1007/s00125-014-3273-1.

Oberbach, A., Schlichting, N., Heinrich, M., Till, H., Stolzenburg, J.-U. and Neuhaus, J. (2012) 'Free fatty acid palmitate impairs the vitality and function of cultured human bladder smooth muscle cells.', *PloS one*. Edited by H. Koul. Public Library of Science, 7(7), p. e41026. doi: 10.1371/journal.pone.0041026.

Ohno, Y., Kihara, A., Sano, T. and Igarashi, Y. (2006) 'Intracellular localization and tissue-specific distribution of human and yeast DHHC cysteine-rich domain-containing proteins', *Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids*, 1761(4), pp. 474–483. doi: 10.1016/j.bbalip.2006.03.010.

Oku, S., Takahashi, N., Fukata, Y. and Fukata, M. (2013) 'In silico screening for palmitoyl substrates reveals a role for DHHC1/3/10 (zDHHC1/3/11)-mediated neurochondrin palmitoylation in its targeting to Rab5-positive endosomes.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 288(27), pp. 19816–29. doi: 10.1074/jbc.M112.431676.

Okuyama, R., Fujiwara, T. and Ohsumi, J. (2003) 'High glucose potentiates palmitate-induced NO-mediated cytotoxicity through generation of superoxide in clonal β-cell HIT-T15', *FEBS Letters*, 545(2–3), pp. 219–223. doi: 10.1016/S0014-5793(03)00534-9.

Orio, P., Rojas, P., Ferreira, G. and Latorre, R. (2002) 'New disguises for an old channel: MaxiK channel beta-subunits', *Physiology*, 17(4), pp. 156–161. doi: 10.1152/nips.01387.2002.

Ormond, K. E. (2008) 'Medical ethics for the genome world: a paper from the 2007 William Beaumont hospital symposium on molecular pathology.', *The Journal of molecular diagnostics : JMD*. American Society for Investigative Pathology, 10(5), pp. 377–82. doi: 10.2353/jmoldx.2008.070162.

Overby, C. L. and Tarczy-Hornoch, P. (2013) 'Personalized medicine: challenges and opportunities for translational bioinformatics.', *Personalized medicine*, 10(5), pp. 453–462. doi: 10.1016/j.biotechadv.2011.08.021.Secreted.

Pabinger, S., Dander, A., Fischer, M., Snajder, R., Sperk, M., Efremova, M., Krabichler, B., Speicher, M. R., Zschocke, J. and Trajanoski, Z. (2014) 'A survey of tools for variant analysis of next-generation genome sequencing data.', *Briefings in bioinformatics*. Oxford University Press, 15(2), pp. 256–78. doi: 10.1093/bib/bbs086.

Pal, T., Permuth-Wey, J., Betts, J. A., Krischer, J. P., Fiorica, J., Arango, H., LaPolla, J., Hoffman, M., Martino, M. A., Wakeley, K., Wilbanks, G., Nicosia, S., Cantor, A. and Sutphen, R. (2005) 'BRCA1 and BRCA2 mutations account for a large proportion of ovarian carcinoma cases', *Cancer*, 104(12), pp. 2807–2816. doi: 10.1002/cncr.21536.

Pallanck, L. and Ganetzky, B. (1994) 'Cloning and characterization of human and mouse homologs of the Drosophila calcium-activated potassium channel gene, slowpoke.', *Human molecular genetics*, 3(8), pp. 1239–43.

Pallotta, B. S. (1985) 'Calcium-activated potassium channels in rat muscle inactivate from a short-duration open state.', *J. Physiol.*, 363(1), pp. 501–516. doi: 10.1113/jphysiol.1985.sp015724.

Pallotta, B. S., Magleby, K. L. and Barrett, J. N. (1981) 'Single channel recordings of Ca2+-activated K+ currents in rat muscle cell culture', *Nature*, 293(October), pp. 471–474. doi: 10.1017/CBO9781107415324.004.

Pararasa, C. (2013) *Fatty acids, monocytes and ageing*. Aston University.

Park, M. K., Lee, S. H., Lee, S. J., Ho, W. K. and Earm, Y. E. (1995) 'Different modulation of Ca-activated K channels by the intracellular redox potential in pulmonary and ear arterial smooth muscle cells of the rabbit.', *Pflugers Archiv : European journal of physiology*, 430(3), pp. 308–14.

Park, S. M., Liu, G., Kubal, A., Fury, M., Cao, L. and Marx, S. O. (2004) 'Direct interaction between BKCa potassium channel and microtubule-associated protein 1A', *FEBS Letters*, 570(1–3), pp. 143–148. doi: 10.1016/j.febslet.2004.06.037.

Parker, C. E., Mocanu, V., Mocanu, M., Dicheva, N. and Warren, M. R. (2010) 'Mass Spectrometry for Post-Translational Modifications', in *Neuroproteomics*. CRC Press/Taylor & Francis.

Parker, S. M. M., Moore, P. C. C., Johnson, L. M. M. and Poitout, V. (2003) 'Palmitate potentiation of glucose-induced insulin release: a study using 2-bromopalmitate', *Metabolism*, 52(10), pp. 1367–1371. doi: 10.1016/S0026-0495(03)00279-8.

Paumen, M. B., Ishida, Y., Muramatsu, M., Yamamoto, M. and Honjo, T. (1997) 'Inhibition of Carnitine Palmitoyltransferase I Augments Sphingolipid Synthesis and Palmitate-induced Apoptosis', *Journal of Biological Chemistry*, 272(6), pp. 3324–3329. doi: 10.1074/jbc.272.6.3324.

Pearson, T. A. and Manolio, T. A. (2008) 'How to Interpret a Genome-wide Association Study', *JAMA*. American Medical Association, 299(11), p. 1335. doi: 10.1001/jama.299.11.1335.

Peiró, C., Lafuente, N., Matesanz, N., Cercas, E., Llergo, J. L., Vallejo, S., Rodríguez-Mañas, L. and Sánchez-Ferrer, C. F. (2001) 'High glucose induces cell death of cultured human aortic smooth muscle cells through the formation of hydrogen peroxide.', *British journal of pharmacology*, 133(7), pp. 967–74. doi: 10.1038/sj.bjp.0704184.

Peiró, C., Romacho, T., Azcutia, V., Villalobos, L., Fernández, E., Bolaños, J. P., Moncada, S. and Sánchez-Ferrer, C. F. (2016) 'Inflammation, glucose, and vascular cell damage: the role of the pentose phosphate pathway.', *Cardiovascular diabetology*. BioMed Central, 15, p. 82. doi: 10.1186/s12933-016-0397-2.

Pekkurnaz, G., Trinidad, J. C., Wang, X., Kong, D. and Schwarz, T. L. (2014) 'Glucose regulates mitochondrial motility via Milton modification by O-GlcNAc transferase.', *Cell*. NIH Public Access, 158(1), pp. 54–68. doi: 10.1016/j.cell.2014.06.007.

dela Peña, I. and Cheong, J. H. (2011) 'On benzofuroindole analogues as smooth muscle relaxants.', *Journal of biomedicine & biotechnology*. Hindawi Publishing Corporation, 2011, p. 389056. doi: 10.1155/2011/389056.

Peña, T. L. and Rane, S. G. (1999) 'The fibroblast intermediate conductance K(Ca) channel, FIK, as a prototype for the cell growth regulatory function of the IK channel family.', *The Journal of membrane biology*, 172(3), pp. 249–57.

Penders, B., Horstman, K. and Vos, R. (2008) 'Walking the Line between Lab and Computation: The "Moist" Zone', *BioScience*. Oxford University Press, 58(8), p. 747. doi: 10.1641/B580811.

Pepinsky, R. B., Zeng, C., Wen, D., Rayhorn, P., Baker, D. P., Williams, K. P., Bixler, S. A., Ambrose, C. M., Garber, E. A., Miatkowski, K., Taylor, F. R., Wang, E. A. and Galdes, A. (1998) 'Identification of a Palmitic Acid-modified Form of Human Sonic hedgehog', *Journal of Biological Chemistry*. American Society for Biochemistry and Molecular Biology, 273(22), pp. 14037–14045. doi: 10.1074/jbc.273.22.14037.

Perona, J. S., Cañizares, J., Montero, E., Sánchez-Domínguez, J. M., Catalá, A. and Ruiz-Gutiérrez, V. (2004) 'Virgin olive oil reduces blood pressure in hypertensive elderly subjects', *Clinical Nutrition*, 23(5), pp. 1113–1121. doi: 10.1016/j.clnu.2004.02.004.

Petersen, B.-S., Fredrich, B., Hoeppner, M. P., Ellinghaus, D. and Franke, A. (2017) 'Opportunities and challenges of whole-genome and -exome sequencing.', *BMC genetics*. BioMed Central, 18(1), p. 14. doi: 10.1186/s12863-017-0479-5.

Petersén A, Larsen, K. E., Behr, G. G., Romero, N., Przedborski, S., Brundin, P. and Sulzer, D. (2001) 'Expanded CAG repeats in exon 1 of the Huntington's disease gene stimulate dopamine-mediated striatal neuron autophagy and degeneration.', *Human molecular genetics*, 10(12), pp. 1243–54.

Petty, L. (2014) *Characterization of variation in transmembrane domain coding regions*, *Texas Medical Center Dissertations (via ProQuest)*.

Phoomak, C., Vaeteewoottacharn, K., Silsirivanit, A., Saengboonmee, C., Seubwai, W., Sawanyawisuth, K., Wongkham, C. and Wongkham, S. (2017) 'High glucose levels boost the aggressiveness of highly metastatic cholangiocarcinoma cells via O-GlcNAcylation', *Scientific Reports*, 7, p. 43842. doi: 10.1038/srep43842.

Pike, L. J. (2003) 'Lipid rafts: bringing order to chaos.', *Journal of lipid research*, 44(4), pp. 655–667.

Pipili, A. (2012) *Does metabolic stress affect palmitoylation of calcium dependent BK channels?* University of Edinburgh.

Plante, A. E. (2016) 'Effects of Single Nucleotide Polymorphisms (SNPs) on BK KD Channel Properties', in *SEVENTIETH ANNUAL MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS ANNUAL MEETING OF THE SOCIETY OF GENERAL PHYSIOLOGISTS*, p. 1.

Poitout, V. and Robertson, R. P. (2008) 'Glucolipotoxicity: fuel excess and beta-cell dysfunction.', *Endocrine reviews*. Endocrine Society, 29(3), pp. 351–66. doi: 10.1210/er.2007-0023.

Pornillos, O., Chen, Y.-J., Chen, A. P. and Chang, G. (2005) 'X-ray Structure of the EmrE Multidrug Transporter in Complex with a Substrate', *Science*, 310(5756), pp. 1950–1953. doi: 10.1126/science.1119776.

Pouliot, J. F. and Béliveau, R. (1995) 'Palmitoylation of the glucose transporter in blood-brain barrier capillaries.', *Biochimica et biophysica acta*, 1234(2), pp. 191–196.

Prades, J., Funari, S. S., Escribá, P. V and Barceló, F. (2003) 'Effects of unsaturated fatty acids and triacylglycerols on phosphatidylethanolamine membrane structure.', *Journal of lipid research*. American Society for Biochemistry and Molecular Biology, 44(9), pp. 1720–7. doi: 10.1194/jlr.M300092-JLR200.

Pruitt, K. D., Harrow, J., Harte, R. A., Wallin, C., Diekhans, M., Maglott, D. R., Searle, S., Farrell, C. M., Loveland, J. E., Ruef, B. J., Hart, E., Suner, M.-M., Landrum, M. J., Aken, B., Ayling, S., Baertsch, R., Fernandez-Banet, J., Cherry, J. L., Curwen, V., DiCuccio, M., Kellis, M., Lee, J., Lin, M. F., Schuster, M., Shkeda, A., Amid, C., Brown, G., Dukhanina, O., Frankish, A., Hart, J., Maidak, B. L., Mudge, J., Murphy, M. R., Murphy, T., Rajan, J., Rajput, B., Riddick, L. D., Snow, C., Steward, C., Webb, D., Weber, J. A., Wilming, L., Wu, W., Birney, E., Haussler, D., Hubbard, T., Ostell, J., Durbin, R. and Lipman, D. (2009) 'The consensus coding sequence (CCDS) project: Identifying a common protein-coding gene set for the human and mouse genomes', *Genome Research*, 19(7), pp. 1316–1323. doi: 10.1101/gr.080531.108.

Psaltopoulou, T., Naska, A., Orfanos, P., Trichopoulos, D., Mountokalakis, T. and Trichopoulou, A. (2004) 'Olive oil, the Mediterranean diet, and arterial blood pressure: the Greek European Prospective Investigation into Cancer and Nutrition (EPIC) study.', *The American journal of clinical nutrition*, 80(4), pp. 1012–8.

Pyott, S. J., Meredith, A. L., Fodor, A. A., Vázquez, A. E., Yamoah, E. N. and Aldrich, R. W. (2007) 'Cochlear function in mice lacking the BK channel alpha, beta1, or beta4 subunits.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 282(5), pp. 3312–24.

doi: 10.1074/jbc.M608726200.

Qiu, H., Honey, D. M., Kingsbury, J. S., Park, A., Boudanova, E., Wei, R. R., Pan, C. Q. and Edmunds, T. (2015) 'Impact of cysteine variants on the structure, activity, and stability of recombinant human α-galactosidase A', *Protein Science*, 24(9), pp. 1401–1411. doi: 10.1002/pro.2719.

Quirk, J. C. and Reinhart, P. H. (2001) 'Identification of a Novel Tetramerization Domain in Large Conductance KCa Channels', *Neuron*, 32(1), pp. 13–23. doi: 10.1016/S0896-6273(01)00444-5.

Raffaelli, G., Saviane, C., Mohajerani, M. H., Pedarzani, P. and Cherubini, E. (2004) 'BK potassium channels control transmitter release at CA3-CA3 synapses in the rat hippocampus.', *The Journal of physiology*. Wiley-Blackwell, 557(Pt 1), pp. 147–57. doi: 10.1113/jphysiol.2004.062661.

Rafiee, A., Fatemi, S., Jamili, S., Ajdari, S., Riazi-Rad, F., Memarnejadian, A. and Alimohammadian, M. (2012) 'Cloning, Expression and Characterization of Zebra Fish Ferroportin in Hek 293T Cell Line.', *Iranian journal of public health*, 41(1), pp. 79–86.

Ranjan, R., Khazen, G., Gambazzi, L., Ramaswamy, S., Hill, S. L., Schürmann, F. and Markram, H. (2011) 'Channelpedia: an integrative and interactive database for ion channels.', *Frontiers in neuroinformatics*. Frontiers Media SA, 5, p. 36. doi: 10.3389/fninf.2011.00036.

Rasband, W. S. (2012) *ImageJ*, *U. S. National Institutes of Health, Bethesda, Maryland, USA. http://imagej.nih.gov/ij/, 1997-2012*. Available at: http://imagej.nih.gov/ij/%5Cnhttp://ci.nii.ac.jp/naid/10024044120/.

Reiser, L., Berardini, T. Z., Li, D., Muller, R., Strait, E. M., Li, Q., Mezheritsky, Y., Vetushko, A. and Huala, E. (2016) 'Sustainable funding for biocuration: The Arabidopsis Information Resource (TAIR) as a case study of a subscription-based funding model', *Database*. Oxford University Press, p. baw018. doi: 10.1093/database/baw018.

Remizov, O., Jakubov, R., Düfer, M., Krippeit Drews, P., Drews, G., Waring, M., Brabant, G., Wienbergen, A., Rustenbeck, I. and Schöfl, C. (2003) 'Palmitate-induced Ca2+-signaling in pancreatic beta-cells', *Molecular and Cellular Endocrinology*, 212(1–2), pp. 1–9. doi: 10.1016/j.mce.2003.09.026.

Ren, J., Gao, X., Jin, C., Zhu, M., Wang, X., Shaw, A., Wen, L., Yao, X. and Xue, Y. (2009) 'Systematic study of protein sumoylation: Development of a site-specific predictor of SUMOsp 2.0', *PROTEOMICS*. WILEY-VCH Verlag, 9(12), pp. 3409–3412. doi: 10.1002/pmic.200800646.

Ren, J., Wen, L., Gao, X., Jin, C., Xue, Y. and Yao, X. (2008) 'CSS-Palm 2.0: An updated software for palmitoylation sites prediction', *Protein Engineering, Design and Selection*, 21, pp. 639–644. doi: 10.1093/protein/gzn039.

Resh, M. D. (2006) 'Trafficking and signaling by fatty-acylated and prenylated proteins', *Nature Chemical Biology*, 2(11), pp. 584–590. doi: 10.1038/nchembio834.

Resnik, E., Herron, J., Fu, R., Ivy, D. D. and Cornfield, D. N. (2006) 'Oxygen tension modulates the expression of pulmonary vascular BKCa channel alpha- and beta-subunits.', *American journal of physiology. Lung cellular and molecular physiology*, 290(4), pp. L761–L768. doi: 10.1152/ajplung.00283.2005.

Retterer, K., Juusola, J., Cho, M. T., Vitazka, P., Millan, F., Gibellini, F., Vertino-Bell, A., Smaoui, N., Neidich, J., Monaghan, K. G., McKnight, D., Bai, R., Suchy, S., Friedman, B., Tahiliani, J., Pineda-Alvarez, D., Richard, G., Brandt, T., Haverfield, E., Chung, W. K. and Bale, S. (2016) 'Clinical application of whole-exome sequencing across clinical indications', *Genetics in Medicine*. Springer Nature, 18(7), pp. 696–704. doi: 10.1038/gim.2015.148.

Reva, B., Antipin, Y. and Sander, C. (2007) 'Determinants of protein function revealed by combinatorial

entropy optimization.', *Genome biology*. BioMed Central, 8(11), p. R232. doi: 10.1186/gb-2007-8-11-r232.

Reyes, C. L. and Chang, G. (2005) 'Structure of the ABC Transporter MsbA in Complex with ADP{middle dot}Vanadate and Lipopolysaccharide', *Science*, 308(5724), pp. 1028–1031. doi: 10.1126/science.1107733.

Riccomini, D. R. (2013) *Visual Strategies: A Practical Guide to Graphics for Scientists & Engineers*, *Technical Communication*. Yale University Press. doi: 10.1063/PT.3.2119.

Ridgway, L. D., Kim, E. Y. and Dryer, S. E. (2009) 'MAGI-1 interacts with Slo1 channel proteins and suppresses Slo1 expression on the cell surface.', *American journal of physiology. Cell physiology*. American Physiological Society, 297(1), pp. C55-65. doi: 10.1152/ajpcell.00073.2009.

Rigden, D., Fernández-Suárez, X. and Galperin, M. (2016) 'The 2016 database issue of Nucleic Acids Research and an updated molecular biology database collection', *Nucleic Acids Research*. Oxford University Press, 44(D1), pp. D1–D6. doi: 10.1093/nar/gkv1356.

Rimington, C. (1927) 'Phosphorylation of Proteins.', *The Biochemical journal*. Portland Press Ltd, 21(2), pp. 272–81.

Risch, N. and Merikangas, K. (1996) 'The future of genetic studies of complex human diseases.', *Science (New York, N.Y.)*, 273(5281), pp. 1516–7.

Robitaille, R. and Charlton, M. P. (1992) 'Presynaptic calcium signals and transmitter release are modulated by calcium-activated potassium channels.', *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 12(1), pp. 297–305.

Rocks, O., Peyker, A., Kahms, M., Verveer, P. J., Koerner, C., Lumbierres, M., Kuhlmann, J., Waldmann, H., Wittinghofer, A. and Bastiaens, P. I. H. (2005) 'An acylation cycle regulates localization and activity of palmitoylated Ras isoforms.', *Science (New York, N.Y.)*, 307(5716), pp. 1746–1752. doi: 10.1126/science.1105654.

Rodríguez Estrada, F. C. and Davis, L. S. (2015) 'Improving Visual Communication of Science Through the Incorporation of Graphic Design Theories and Practices Into Science Communication', *Science Communication*. SAGE PublicationsSage CA: Los Angeles, CA, 37(1), pp. 140–148. doi: 10.1177/1075547014562914.

Rolandi, M., Cheng, K. and Pérez-Kriz, S. (2011) 'A Brief Guide to Designing Effective Figures for the Scientific Paper', *Advanced Materials*. WILEY-VCH Verlag, 23(38), pp. 4343–4346. doi: 10.1002/adma.201102518.

Ross, F. A., Rafferty, J. N., Dallas, M. L., Ogunbayo, O., Ikematsu, N., McClafferty, H., Tian, L., Widmer, H., Rowe, I. C. M., Wyatt, C. N., Shipston, M. J., Peers, C., Hardie, D. G. and Evans, A. M. (2011) 'Selective expression in carotid body type I cells of a single splice variant of the large conductance calcium- and voltage-activated potassium channel confers regulation by AMP-activated protein kinase.', *The Journal of biological chemistry*, 286(14), pp. 11929–36. doi: 10.1074/jbc.M110.189779.

Ross, L. F., Saal, H. M., David, K. L., Anderson, R. R. and Genomics, and the A. A. of P. A. C. of M. G. and (2013) 'Technical report: ethical and policy issues in genetic testing and screening of children', *Genetics in Medicine*. Springer Nature, 15(3), pp. 234–245. doi: 10.1038/gim.2012.176.

Rossin, A., Derouet, M., Abdel-Sater, F. and Hueber, A.-O. (2009) 'Palmitoylation of the TRAIL receptor DR4 confers an efficient TRAIL-induced cell death signalling', *Biochemical Journal*, 419(1).

Rovelet-Lecrux, A., Hannequin, D., Raux, G., Meur, N. Le, Laquerrière, A., Vital, A., Dumanchin, C., Feuillette, S., Brice, A., Vercelletto, M., Dubas, F., Frebourg, T. and Campion, D. (2006) 'APP locus

duplication causes autosomal dominant early-onset Alzheimer disease with cerebral amyloid angiopathy', *Nature Genetics*, 38(1), pp. 24–26. doi: 10.1038/ng1718.

Ruchat, S.-M., Rankinen, T., Weisnagel, S. J., Rice, T., Rao, D. C., Bergman, R. N., Bouchard, C. and Pérusse, L. (2010) 'Improvements in glucose homeostasis in response to regular exercise are influenced by the PPARG Pro12Ala variant: results from the HERITAGE Family Study.', *Diabetologia*. NIH Public Access, 53(4), pp. 679–89. doi: 10.1007/s00125-009-1630-2.

Rusiecka, I. and Kocic, I. (2012) *Potassium Channels as a Target for Clinical Therapeutics*, *Potassium Channels as a Target for Clinical Therapeutics*. doi: 10.2174/97816080523561120101.

Rüttiger, L., Sausbier, M., Zimmermann, U., Winter, H., Braig, C., Engel, J., Knirsch, M., Arntz, C., Langer, P., Hirt, B., Müller, M., Köpschall, I., Pfister, M., Münkner, S., Rohbock, K., Pfaff, I., Rüsch, A., Ruth, P. and Knipper, M. (2004) 'Deletion of the Ca2+-activated potassium (BK) alpha-subunit but not the BKbeta1-subunit leads to progressive hearing loss.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 101(35), pp. 12922–7. doi: 10.1073/pnas.0402660101.

Ryan, N. M., Morris, S. W., Porteous, D. J., Taylor, M. S. and Evans, K. L. (2014) 'SuRFing the genomics wave: an R package for prioritising SNPs by functionality.', *Genome medicine*. BioMed Central, 6(10), p. 79. doi: 10.1186/s13073-014-0079-1.

Sabin, M. A., De Hora, M., Holly, J. M. P., Hunt, L. P., Ford, A. L., Williams, S. R., Baker, J. S., Retallick, C. J., Crowne, E. C. and Shield, J. P. H. (2007) 'Fasting nonesterified fatty acid profiles in childhood and their relationship with adiposity, insulin sensitivity, and lipid levels.', *Pediatrics*, 120(6), pp. e1426-33. doi: 10.1542/peds.2007-0189.

Sachidanandam, R., Weissman, D., Schmidt, S. C., Kakol, J. M., Stein, L. D., Marth, G., Sherry, S., Mullikin, J. C., Mortimore, B. J., Willey, D. L., Hunt, S. E., Cole, C. G., Coggill, P. C., Rice, C. M., Ning, Z., Rogers, J., Bentley, D. R., Kwok, P.-Y., Mardis, E. R., Yeh, R. T., Schultz, B., Cook, L., Davenport, R., Dante, M., Fulton, L., Hillier, L., Waterston, R. H., McPherson, J. D., Gilman, B., Schaffner, S., Van Etten, W. J., Reich, D., Higgins, J., Daly, M. J., Blumenstiel, B., Baldwin, J., Stange-Thomann, N., Zody, M. C., Linton, L., Lander, E. S. and Altshuler, D. (2001) 'A map of human genome sequence variation containing 1.42 million single nucleotide polymorphisms', *Nature*. Nature Publishing Group, 409(6822), pp. 928–933. doi: 10.1038/35057149.

Saddiksj, M., Gamblesll, J., Wittersll, L. A. and Lopaschuks, G. D. (1993) 'Acetyl-coA Carboxylase Regulation of Fatty Acid Oxidation in the Heart*', *The Journal of Biological Chemistry*, 268(34).

Sah, P. (1996) 'Ca(2+)-activated K+ currents in neurones: types, physiological roles and modulation.', *Trends in neurosciences*, 19(4), pp. 150–4.

Sah, P. and Louise Faber, E. S. (2002) 'Channels underlying neuronal calcium-activated potassium currents', *Progress in Neurobiology*, 66(5), pp. 345–353. doi: 10.1016/S0301-0082(02)00004-7.

Saito, M., Nelson, C., Salkoff, L. and Lingle, C. J. (1997) 'A cysteine-rich domain defined by a novel exon in a slo variant in rat adrenal chromaffin cells and PC12 cells.', *The Journal of biological chemistry*, 272(18), pp. 11710–11717.

Sakagami, H., Makino, Y., Mizumoto, K., Isoe, T., Takeda, Y., Watanabe, J., Fujita, Y., Takiyama, Y., Abiko, A. and Haneda, M. (2014) 'Loss of HIF-1α impairs GLUT4 translocation and glucose uptake by the skeletal muscle cells', *American Journal of Physiology - Endocrinology and Metabolism*, 306(9).

Sakai, Y., Harvey, M. and Sokolowski, B. (2011) 'Identification and quantification of full-length BK channel variants in the developing mouse cochlea.', *Journal of neuroscience research*. NIH Public Access, 89(11), pp. 1747–60. doi: 10.1002/jnr.22713.

Salaun, C., Greaves, J. and Chamberlain, L. H. (2010) 'The intracellular dynamic of protein palmitoylation.', *The Journal of cell biology*, 191(7), pp. 1229–1238. doi: 10.1083/jcb.201008160.

Saleem, F., Rowe, I. C. M. and Shipston, M. J. (2009) 'Characterization of BK channel splice variants using membrane potential dyes.', *British journal of pharmacology*, 156(1), pp. 143–152. doi: 10.1111/j.1476-5381.2008.00011.x.

Salkoff, L., Butler, A., Ferreira, G., Santi, C. and Wei, A. (2006) 'High-conductance potassium channels of the SLO family', *Nature Reviews Neuroscience*, 7(12), pp. 921–931. doi: 10.1038/nrn1992.

Salt, I. P., Johnson, G., Ashcroft, S. J. and Hardie, D. G. (1998) 'AMP-activated protein kinase is activated by low glucose in cell lines derived from pancreatic beta cells, and may regulate insulin release.', *The Biochemical journal*. Portland Press Ltd, 335 (Pt 3)(Pt 3), pp. 533–9.

Samaranayake, H., Saunders, J. C., Greene, M. I. and Navaratnam, D. S. (2004) 'Ca(2+) and K(+) (BK) channels in chick hair cells are clustered and colocalized with apical-basal and tonotopic gradients.', *The Journal of physiology*, 560(Pt 1), pp. 13–20. doi: 10.1113/jphysiol.2004.069856.

Samikkannu, T., Thomas, J. J., Bhat, G. J., Wittman, V. and Thekkumkara, T. J. (2006) 'Acute effect of high glucose on long-term cell growth: a role for transient glucose increase in proximal tubule cell injury.', *American journal of physiology. Renal physiology*, 291(1), pp. F162-75. doi: 10.1152/ajprenal.00189.2005.

Dos Santos, H. G., Nunez-Castilla, J. and Siltberg-Liberles, J. (2016) 'Functional Diversification after Gene Duplication: Paralog Specific Regions of Structural Disorder and Phosphorylation in p53, p63, and p73.', *PloS one*. Public Library of Science, 11(3), p. e0151961. doi: 10.1371/journal.pone.0151961.

Sastre, L. (2014) 'Exome sequencing: what clinicians need to know', *Advances in Genomics and Genetics*. Dove Press, Volume 4, p. 15. doi: 10.2147/AGG.S39108.

Sathanoori, R., Swärd, K., Olde, B. and Erlinge, D. (2015) 'The ATP Receptors P2X7 and P2X4 Modulate High Glucose and Palmitate-Induced Inflammatory Responses in Endothelial Cells.', *PloS one*. Public Library of Science, 10(5), p. e0125111. doi: 10.1371/journal.pone.0125111.

Sausbier, M., Arntz, C., Bucurenciu, I., Zhao, H., Zhou, X.-B., Sausbier, U., Feil, S., Kamm, S., Essin, K., Sailer, C. a, Abdullah, U., Krippeit-Drews, P., Feil, R., Hofmann, F., Knaus, H.-G., Kenyon, C., Shipston, M. J., Storm, J. F., Neuhuber, W., Korth, M., Schubert, R., Gollasch, M. and Ruth, P. (2005) 'Elevated blood pressure linked to primary hyperaldosteronism and impaired vasodilation in BK channel-deficient mice.', *Circulation*, 112(1), pp. 60–68. doi: 10.1161/01.CIR.0000156448.74296.FE.

Sausbier, M., Hu, H., Arntz, C., Feil, S., Kamm, S., Adelsberger, H., Sausbier, U., Sailer, C. A., Feil, R., Hofmann, F., Korth, M., Shipston, M. J., Knaus, H.-G., Wolfer, D. P., Pedroarena, C. M., Storm, J. F. and Ruth, P. (2004) 'Cerebellar ataxia and Purkinje cell dysfunction caused by Ca2+-activated K+ channel deficiency.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 101(25), pp. 9474–8. doi: 10.1073/pnas.0401702101.

Savas, S. and Ozcelik, H. (2005) 'Phosphorylation states of cell cycle and DNA repair proteins can be altered by the nsSNPs.', *BMC cancer*. BioMed Central, 5, p. 107. doi: 10.1186/1471-2407-5-107.

Schilling, J. D., Machkovech, H. M., He, L., Diwan, A. and Schaffer, J. E. (2013) 'TLR4 activation under lipotoxic conditions leads to synergistic macrophage cell death through a TRIF-dependent pathway.', *Journal of immunology (Baltimore, Md. : 1950)*. American Association of Immunologists, 190(3), pp. 1285–96. doi: 10.4049/jimmunol.1202208.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., Tinevez, J.-Y., White, D. J., Hartenstein, V., Eliceiri, K., Tomancak, P. and Cardona, A. (2012) 'Fiji: an open-source platform for biological-image analysis.', *Nature methods*.

Nature Publishing Group, a division of Macmillan Publishers Limited. All Rights Reserved., 9(7), pp. 676–82. doi: 10.1038/nmeth.2019.

Scholl, U. I., Stölting, G., Nelson-Williams, C., Vichot, A. A., Choi, M., Loring, E., Prasad, M. L., Goh, G., Carling, T., Juhlin, C. C., Quack, I., Rump, L. C., Thiel, A., Lande, M., Frazier, B. G., Rasoulpour, M., Bowlin, D. L., Sethna, C. B., Trachtman, H., Fahlke, C., Lifton, R. P., Adeyemo, A., Gerry, N., Chen, G., Herbert, A., Doumatey, A., Huang, H., Zhou, J., Lashley, K., Chen, Y., Christman, M., Rotimi, C., Azizan, E., Poulsen, H., Tuluc, P., Zhou, J., Clausen, M., Lieb, A., Maniero, C., Garg, S., Bochukova, E., Zhao, W., Shaikh, L., Brighton, C., Teo, A., Davenport, A., Dekkers, T., Tops, B., Küsters, B., Ceral, J., Yeo, G., Neogi, S., McFarlane, I., Rosenfeld, N., Marass, F., Hadfield, J., Margas, W., Chaggar, K., Solar, M., Deinum, J., Dolphin, A., Farooqi, I., Striessnig, J., Nissen, P., Brown, M., Berridge, M., Lipp, P., Bootman, M., Beuschlein, F., Boulkroun, S., Osswald, A., Wieland, T., Nielsen, H., Lichtenauer, U., Penton, D., Schack, V., Amar, L., Fischer, E., Walther, A., Tauber, P., Schwarzmayr, T., Diener, S., Graf, E., Allolio, B., Samson-Couterie, B., Benecke, A., Quinkler, M., Fallo, F., Plouin, P., Mantero, F., Meitinger, T., Mulatero, P., Jeunemaitre, X., Warth, R., Vilsen, B., Zennaro, M., Strom, T., Reincke, M., Bock, G., Gebhart, M., Scharinger, A., Jangsangthong, W., Busquet, P., Poggiani, C., Sartori, S., Mangoni, M., Sinnegger-Brauns, M., Herzig, S., Striessnig, J., Koschak, A., Browning, S., Browning, B., Cannon, S., Jr, R. B., Corey, D., Chen, Y., Lu, J., Pan, H., Zhang, Y., Wu, H., Xu, K., Liu, X., Jiang, Y., Bao, X., Yao, Z., Ding, K., Lo, W., Qiang, B., Chan, P., Shen, Y., Wu, X., Chiang, C., Huang, C., Chieng, H., Chang, Y., Chang, D., Chen, J., Chen, Y., Chen, Y., Shin, H., Campbell, K., Chen, C., Chioza, B., Everett, K., Aschauer, H., Brouwer, O., Callenbach, P., Covanis, A., Dulac, O., Durner, M., Eeg-Olofsson, O., Feucht, M., Friis, M., Heils, A., Kjeldsen, M., Larsson, K., Lehesjoki, A., Nabbout, R., Olsson, I., Sander, T., Sirén, A., Robinson, R., Rees, M., Gardiner, R., Choi, M., Scholl, U., Yue, P., Bjorklund, P., Zhao, B., Nelson-Williams, C., Ji, W., Cho, Y., Patel, A., Men, C., Lolis, E., Wisgerhof, M., Geller, D., Mane, S., Hellman, P., Westin, G., Åkerström, G., Wang, W., Carling, T., Lifton, R., Conn, J., Coulter, D., Huguenard, J., Prince, D., Cribbs, L., Lee, J., Yang, J., Satin, J., Zhang, Y., Daud, A., Barclay, J., Williamson, M., Fox, M., Rees, M., Perez-Reyes, E., Consortium, E. A., Felizola, S., Maekawa, T., Nakamura, Y., Satoh, F., Ono, Y., Kikuchi, K., Aritomi, S., Ikeda, K., Yoshimura, M., Tojo, K., Sasano, H., Funder, J., Carey, R., Fardella, C., Gomez-Sanchez, C., Mantero, F., Stowasser, M., Jr, W. Y., Montori, V., Society, E., Geller, D., Rodriguez-Soriano, J., Boado, A. V., Schifter, S., Bayer, M., Chang, S., Lifton, R., Genin, E., Tullio-Pelet, A., Begeot, F., Lyonnet, S., Abel, L., Hebeisen, S., Fahlke, C., Hering, S., Aczel, S., Kraus, R., Berjukow, S., Striessnig, J., Timin, E., Hering, S., Berjukow, S., Aczel, S., Timin, E., Heron, S., Khosravani, H., Varela, D., Bladen, C., Williams, T., Newman, M., Scheffer, I., Berkovic, S., Mulley, J., Zamponi, G., Heron, S., Phillips, H., Mulley, J., Mazarib, A., Neufeld, M., Berkovic, S., Scheffer, I., Hu, C., Rusin, C., Tan, Z., Guagliardo, N., Barrett, P., Studies, I. C. for B. P. G.-W. A., Ehret, G., Munroe, P., Rice, K., Bochud, M., Johnson, A., Chasman, D., Smith, A., Tobin, M., Verwoert, G., Hwang, S., Pihur, V., Vollenweider, P., O'Reilly, P., Amin, N., Bragg-Gresham, J., Teumer, A., Glazer, N., Launer, L., Zhao, J., Aulchenko, Y., Heath, S., Sõber, S., Parsa, A., Luan, J., Arora, P., Dehghan, A., Zhang, F., Lucas, G., Hicks, A., Jackson, A., Peden, J., Tanaka, T., Wild, S., Rudan, I., Igl, W., Milaneschi, Y., Parker, A., Fava, C., Chambers, J., Fox, E., Kumari, M., Go, M., Harst, P. van der, Kao, W., Sjögren, M., Vinay, D., Alexander, M., Tabara, Y., Shaw-Hawkins, S., Whincup, P., Liu, Y., Shi, G., Kuusisto, J., Tayo, B., Seielstad, M., Sim, X., Nguyen, K., Lehtimäki, T., Matullo, G., Wu, Y., Gaunt, T., Onland-Moret, N., Cooper, M., Platou, C., Org, E., Hardy, R., Dahgam, S., Palmen, J., Vitart, V., Braund, P., Kuznetsova, T., Uiterwaal, C., Adeyemo, A., Palmas, W., Campbell, H., Ludwig, B., Tomaszewski, M., Tzoulaki, I., Palmer, N., consortium, Cardi., Consortium, Ckdg., Consortium, K., consortium, E., consortium, C.-H., Aspelund, T., Garcia, M., Chang, Y., O'Connell, J., Steinle, N., Grobbee, D., Arking, D., Kardia, S., Morrison, A., Hernandez, D., Najjar, S., McArdle, W., Hadley, D., Brown, M., Connell, J., Hingorani, A., Day, I., Lawlor, D., Beilby, J., Lawrence, R., Clarke, R., Hopewell, J., Ongen, H., Dreisbach, A., Li, Y., Young, J., Bis, J., Kähönen, M., Viikari, J., Adair, L., Lee, N., Chen, M., Olden, M., Pattaro, C., Bolton, J., Köttgen, A., Bergmann, S., Mooser, V., Chaturvedi, N., Frayling, T., Islam, M., Jafar, T., Erdmann, J., Kulkarni, S., Bornstein, S., Grässler, J., Groop, L., Voight, B., Kettunen, J., Howard, P., Taylor, A., Guarrera, S., Ricceri, F., Emilsson, V., Plump, A., Barroso, I., Khaw, K., Weder, A., Hunt, S., Sun, Y., Bergman, R., Collins, F., Bonnycastle, L., Scott, L., Stringham, H., Peltonen, L.,

Perola, M., Vartiainen, E., Brand, S., Staessen, J., Wang, T., Burton, P., Artigas, M. S., Dong, Y., Snieder, H., Wang, X., Zhu, H., Lohman, K., Rudock, M., Heckbert, S., Smith, N., Wiggins, K., Doumatey, A., Shriner, D., Veldre, G., Viigimaa, M., Kinra, S., Prabhakaran, D., Tripathy, V., Langefeld, C., Rosengren, A., Thelle, D., Corsi, A., Singleton, A., Forrester, T., Hilton, G., McKenzie, C., Salako, T., Iwai, N., Kita, Y., Ogihara, T., Ohkubo, T., Okamura, T., Ueshima, H., Umemura, S., Eyheramendy, S., Meitinger, T., Wichmann, H., Cho, Y., Kim, H., Lee, J., Scott, J., Sehmi, J., Zhang, W., Hedblad, B., Nilsson, P., Smith, G., Wong, A., Narisu, N., Stančáková, A., Raffel, L., Yao, J., Kathiresan, S., O'Donnell, C., Schwartz, S., Ikram, M., Jr, W. L., Mosley, T., Seshadri, S., Shrine, N., Wain, L., Morken, M., Swift, A., Laitinen, J., Prokopenko, I., Zitting, P., Cooper, J., Humphries, S., Danesh, J., Rasheed, A., Goel, A., Hamsten, A., Watkins, H., Bakker, S., Gilst, W. van, Janipalli, C., Mani, K., Yajnik, C., Hofman, A., Mattace-Raso, F., Oostra, B., Demirkan, A., Isaacs, A., Rivadeneira, F., Lakatta, E., Orru, M., Scuteri, A., Ala-Korpela, M., Kangas, A., Lyytikäinen, L., Soininen, P., Tukiainen, T., Würtz, P., Ong, R., Dörr, M., Kroemer, H., Völker, U., Völzke, H., Galan, P., Hercberg, S., Lathrop, M., Zelenika, D., Deloukas, P., Mangino, M., Spector, T., Zhai, G., Meschia, J., Nalls, M., Sharma, P., Terzic, J., Kumar, M., Denniff, M., Zukowska-Szczechowska, E., Wagenknecht, L., Fowkes, F., Charchar, F., Schwarz, P., Hayward, C., Guo, X., Rotimi, C., Bots, M., Brand, E., Samani, N., Polasek, O., Talmud, P., Nyberg, F., Kuh, D., Laan, M., Hveem, K., Palmer, L., Schouw, Y. van der, Casas, J., Mohlke, K., Vineis, P., Raitakari, O., Ganesh, S., Wong, T., Tai, E., Cooper, R., Laakso, M., Rao, D., Harris, T., Morris, R., Dominiczak, A., Kivimaki, M., Marmot, M., Miki, T., Saleheen, D., Chandak, G., Coresh, J., Navis, G., Salomaa, V., Han, B., Zhu, X., Kooner, J., Melander, O., Ridker, P., Bandinelli, S., Gyllensten, U., Wright, A., Wilson, J., Ferrucci, L., Farrall, M., Tuomilehto, J., Pramstaller, P., Elosua, R., Soranzo, N., Sijbrands, E., Altshuler, D., Loos, R., Shuldiner, A., Gieger, C., Meneton, P., Uitterlinden, A., Wareham, N., Gudnason, V., Rotter, J., Rettig, R., Uda, M., Strachan, D., Witteman, J., Hartikainen, A., Beckmann, J., Boerwinkle, E., Vasan, R., Boehnke, M., Larson, M., Järvelin, M., Psaty, B., Abecasis, G., Chakravarti, A., Elliott, P., Duijn, C. van, Newton-Cheh, C., Levy, D., Caulfield, M., Johnson, T., Kidambi, S., Ghosh, S., Kotchen, J., Grim, C., Krishnaswami, S., Kaldunski, M., Jr, A. C., Patel, S., Kotchen, T., Lafferty, A., Torpy, D., Stowasser, M., Taymans, S., Lin, J., Huggard, P., Gordon, R., Stratakis, C., Lemaire, M., Frémeaux-Bacchi, V., Schaefer, F., Choi, M., Tang, W., Quintrec, M. Le, Fakhouri, F., Taque, S., Nobili, F., Martinez, F., Ji, W., Overton, J., Mane, S., Nürnberg, G., Altmüller, J., Thiele, H., Morin, D., Deschenes, G., Baudouin, V., Llanas, B., Collard, L., Majid, M., Simkova, E., Nürnberg, P., Rioux-Leclerc, N., Moeckel, G., Gubler, M., Hwa, J., Loirat, C., Lifton, R., Lerche, H., Heine, R., Pika, U., Jr, A. G., Mitrovic, N., Browatzki, M., Weiss, T., Rivet-Bastide, M., Franke, C., Lomonaco, M., Liang, J., Zhang, Y., Wang, J., Pan, H., Wu, H., Xu, K., Liu, X., Jiang, Y., Shen, Y., Wu, X., Lifton, R., Dluhy, R., Powers, M., Rich, G., Cook, S., Ulick, S., Lalouel, J., Manichaikul, A., Mychaleckyj, J., Rich, S., Daly, K., Sale, M., Chen, W., Marcantoni, A., Vandael, D., Mahapatra, S., Carabelli, V., Sinnegger-Brauns, M., Striessnig, J., Carbone, E., Marksteiner, R., Schurr, P., Berjukow, S., Margreiter, E., Perez-Reyes, E., Hering, S., Michels, G., Matthes, J., Handrock, R., Kuchinke, U., Groner, F., Cribbs, L., Pereverzev, A., Schneider, T., Perez-Reyes, E., Herzig, S., Mukhopadhyay, N., Almasy, L., Schroeder, M., Mulvihill, W., Weeks, D., Mulatero, P., Cella, S. di, Williams, T., Milan, A., Mengozzi, G., Chiandussi, L., Gomez-Sanchez, C., Veglio, F., Mulatero, P., Tauber, P., Zennaro, M., Monticone, S., Lang, K., Beuschlein, F., Fischer, E., Tizzani, D., Pallauf, A., Viola, A., Amar, L., Williams, T., Strom, T., Graf, E., Bandulik, S., Penton, D., Plouin, P., Warth, R., Allolio, B., Jeunemaitre, X., Veglio, F., Reincke, M., Neville, A., O'Hare, M., Perez-Reyes, E., Perez-Reyes, E., Deusen, A. Van, Vitko, I., Purcell, S., Neale, B., Todd-Brown, K., Thomas, L., Ferreira, M., Bender, D., Maller, J., Sklar, P., Bakker, P. de, Daly, M., Sham, P., Ragueneau, I., Sao, A., Démolis, J., Darne, B., Funck-Brentano, C., Jaillon, P., Rossi, G., Bernini, G., Caliumi, C., Desideri, G., Fabris, B., Ferri, C., Ganzaroli, C., Giacchetti, G., Letizia, C., Maccario, M., Mallamaci, F., Mannelli, M., Mattarello, M., Moretti, A., Palumbo, G., Parenti, G., Porteri, E., Semplicini, A., Rizzoni, D., Rossi, E., Boscaro, M., Pessina, A., Mantero, F., Investigators, P. S., Rossier, M., Ertel, E., Vallotton, M., Capponi, A., Schmitt, R., Kleinbloesem, C., Belz, G., Schroeter, V., Feifel, U., Pozenel, H., Kirch, W., Halabi, A., Woittiez, A., Welker, H., Brummelen, P. van, Scholl, U., Goh, G., Stolting, G., Oliveira, R. de, Choi, M., Overton, J., Fonseca, A., Korah, R., Starker, L., Kunstman, J., Prasad, M., Hartung, E., Mauras, N., Benson, M., Brady, T., Shapiro, J., Loring, E., Nelson-Williams, C., Libutti, S.,

Mane, S., Hellman, P., Westin, G., Åkerström, G., Björklund, P., Carling, T., Fahlke, C., Hidalgo, P., Lifton, R., Scholl, U., Lifton, R., Scholl, U., Nelson-Williams, C., Yue, P., Grekin, R., Wyatt, R., Dillon, M., Couch, R., Hammer, L., Harley, F., Farhi, A., Wang, W., Lifton, R., Schrier, A., Wang, H., Talley, E., Perez-Reyes, E., Barrett, P., Spät, A., Hunyady, L., Stowasser, M., Bachmann, A., Jonsson, J., Tunny, T., Klemm, S., Gordon, R., Stowasser, M., Gordon, R., Tunny, T., Klemm, S., Finn, W., Krek, A., Torpy, D., Gordon, R., Lin, J., Huggard, P., Taymans, S., Stowasser, M., Chrousos, G., Stratakis, C., Youssoufian, H., Pyeritz, R., Zaidi, S., Choi, M., Wakimoto, H., Ma, L., Jiang, J., Overton, J., Romano-Adesman, A., Bjornson, R., Breitbart, R., Brown, K., Carriero, N., Cheung, Y., Deanfield, J., DePalma, S., Fakhro, K., Glessner, J., Hakonarson, H., Italia, M., Kaltman, J., Kaski, J., Kim, R., Kline, J., Lee, T., Leipzig, J., Lopez, A., Mane, S., Mitchell, L., Newburger, J., Parfenov, M., Pe'er, I., Porter, G., Roberts, A., Sachidanandam, R., Sanders, S., Seiden, H., State, M., Subramanian, S., Tikhonova, I., Wang, W., Warburton, D., White, P., Williams, I., Zhao, H., Seidman, J., Brueckner, M., Chung, W., Gelb, B., Goldmuntz, E., Seidman, C. and Lifton, R. (2015) 'Recurrent gain of function mutation in calcium channel CACNA1H causes early-onset hypertension with primary aldosteronism.', *eLife*. eLife Sciences Publications Limited, 4, p. e06315. doi: 10.7554/eLife.06315.

Schreiber, M. and Salkoff, L. (1997) 'A novel calcium-sensing domain in the BK channel.', *Biophysical journal*. The Biophysical Society, 73(3), pp. 1355–63. doi: 10.1016/S0006-3495(97)78168-2.

Schrijver, I., Aziz, N., Farkas, D. H., Furtado, M., Gonzalez, A. F., Greiner, T. C., Grody, W. W., Hambuch, T., Kalman, L., Kant, J. A., Klein, R. D., Leonard, D. G. B., Lubin, I. M., Mao, R., Nagan, N., Pratt, V. M., Sobel, M. E., Voelkerding, K. V. and Gibson, J. S. (2012) 'Opportunities and Challenges Associated with Clinical Diagnostic Genome Sequencing', *The Journal of Molecular Diagnostics*, 14(6), pp. 525–540. doi: 10.1016/j.jmoldx.2012.04.006.

Schubert, R. and Nelson, M. T. (2001) 'Protein kinases: tuners of the BKCa channel in smooth muscle', *Trends in Pharmacological Sciences*, 22(10), pp. 505–512. doi: 10.1016/S0165-6147(00)01775-2.

Schuit, F., De Vos, A., Farfari, S., Moens, K., Pipeleers, D., Brun, T. and Prentki, M. (1997) 'Metabolic fate of glucose in purified islet cells. Glucose-regulated anaplerosis in beta cells.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 272(30), pp. 18572–9. doi: 10.1074/JBC.272.30.18572.

Schumann, J., Leichtle, A., Thiery, J., Fuhrmann, H., Singer, S., Nicolson, G., Simons, K., Ikonen, E., Pike, L., Pike, L., Ye, S., Tan, L., Ma, J., Shi, Q., Li, J., Zhang, T., Zhang, X., Sun, Z., Simons, K., Toomre, D., Gaus, K., Rodriguez, M., Ruberu, K., Gelissen, I., Sloane, T., Zhang, N., Shaw, A., Li, N., Chen, R., Mak, A., Hartlova, A., Cerveny, L., Hubalek, M., Krocova, Z., Stulik, J., Van, L., Leo, O., Dhungana, S., Merrick, B., Tomer, K., Fessler, M., Benatti, P., Peluso, G., Nicolai, R., Calvani, M., Ma, D., Seo, J., Switzer, K., Fan, Y., McMurray, D., Calder, P., Wassall, S., Stillwell, W., Ikonen, E., Gomez-Mouton, C., Abad, J., Mira, E., Lacalle, R., Gallardo, E., Smith, A., Helenius, A., Harder, T., Siddiqui, R., Harvey, K., Zaloga, G., Stillwell, W., Schmitz, G., Grandl, M., Michel, V., Bakovic, M., Biondo, P., Brindley, D., Sawyer, M., Field, C., Kelley, E., Buettner, G., Burns, C., Stulnig, T., Huber, J., Leitinger, N., Imre, E., Angelisova, P., Kim, W., Fan, Y., Barhoumi, R., Smith, R., McMurray, D., Li, Q., Tan, L., Wang, C., Li, N., Li, Y., Chen, W., Jump, D., Esselman, W., Busik, J., Wassall, S., Brzustowicz, M., Shaikh, S., Cherezov, V., Caffrey, M., Huster, D., Jin, A., Arnold, K., Gawrisch, K., Koenig, B., Strey, H., Gawrisch, K., Smaby, J., Momsen, M., Brockman, H., Brown, R., Epand, R., Kafrawy, O., Zerouga, M., Stillwell, W., Jenski, L., Williams, E., Jenski, L., Stillwell, W., Stillwell, W., Wassall, S., Armstrong, V., Brzustowicz, M., Wassall, S., Jenski, L., Stillwell, W., Stulnig, T., Berger, M., Sigmund, T., Raederstorff, D., Stockinger, H., Zeyda, M., Staffler, G., Horejsi, V., Waldhausl, W., Stulnig, T., Webb, Y., Hermida-Matsumoto, L., Resh, M., Wong, S., Kwon, M., Choi, A., Kim, H., Nakahira, K., Li, Q., Wang, M., Tan, L., Wang, C., Ma, J., Anderson, R., Jacobson, K., Yanagida, M., Iwabuchi, K., Fiedler, G., Leichtle, A., Kase, J., Baumann, S., Ceglarek, U., Leaf, D., Connor, W., Barstad, L., Sexton, G., Macdonald, J., Pike, L., J, Leichtle, A., Fuhrmann, H., Kim, M., Kim, B., Min, Y., Kim, S., Yi, T., Lee, H., Cha, J., Ko, S., Kim, H., Kanayama, M., Yamaguchi, S., Shibata, T., Shibata, N.,

Kobayashi, M., Seidel, A., Gueck, T. and Fuhrmann, H. (2011) 'Fatty Acid and Peptide Profiles in Plasma Membrane and Membrane Rafts of PUFA Supplemented RAW264.7 Macrophages', *PLoS ONE*. Edited by D. Holowka. Public Library of Science, 6(8), p. e24066. doi: 10.1371/journal.pone.0024066.

Schwarz, F. and Aebi, M. (2011) 'Mechanisms and principles of N-linked protein glycosylation', *Current Opinion in Structural Biology*, 21, pp. 576–582. doi: 10.1016/j.sbi.2011.08.005.

Schwarz, J. M., Rödelsperger, C., Schuelke, M. and Seelow, D. (2010) 'MutationTaster evaluates disease-causing potential of sequence alterations', *Nature Methods*. Nature Research, 7(8), pp. 575–576. doi: 10.1038/nmeth0810-575.

Scott, K. A., Alonso, D. O. V, Sato, S., Fersht, A. R. and Daggett, V. (2007) 'Conformational entropy of alanine versus glycine in protein denatured states.', *Proceedings of the National Academy of Sciences of the United States of America*, 104(8), pp. 2661–6. doi: 10.1073/pnas.0611182104.

Seabright, M. (1971) 'A RAPID BANDING TECHNIQUE FOR HUMAN CHROMOSOMES', *The Lancet*, pp. 971–972. doi: 10.1016/S0140-6736(71)90287-X.

Serrano, L., Neira, J. L., Sancho, J. and Fersht, A. R. (1992) 'Effect of alanine versus glycine in alpha-helices on protein stability.', *Nature*, 356(6368), pp. 453–5. doi: 10.1038/356453a0.

Shastry, B. S. (2009) 'SNPs: Impact on Gene Function and Phenotype', in, pp. 3–22. doi: 10.1007/978-1-60327-411-1_1.

Sheng, J. J. and Acquaah-Mensah, G. K. (2011) 'Subcellular location and molecular mobility of human cytosolic sulfotransferase 1C1 in living human embryonic kidney 293 cells.', *Drug metabolism and disposition: the biological fate of chemicals*, 39(8), pp. 1334–7. doi: 10.1124/dmd.111.039537.

Sherry, S. T., Ward, M. H., Kholodov, M., Baker, J., Phan, L., Smigielski, E. M. and Sirotkin, K. (2001) 'dbSNP: the NCBI database of genetic variation.', *Nucleic acids research*, 29(1), pp. 308–11.

Sheta, R., Roux-Dalvai, F., Woo, C. M., Fournier, F., Bourassa, S., Bertozzi, C. R., Droit, A. and Bachvarov, D. (2016) 'Proteomic dataset for altered glycoprotein expression upon GALNT3 knockdown in ovarian cancer cells.', *Data in brief*. Elsevier, 8, pp. 342–9. doi: 10.1016/j.dib.2016.05.060.

Shihab, H. A., Gough, J., Cooper, D. N., Stenson, P. D., Barker, G. L. A., Edwards, K. J., Day, I. N. M. and Gaunt, T. R. (2013) 'Predicting the Functional, Molecular, and Phenotypic Consequences of Amino Acid Substitutions using Hidden Markov Models', *Human Mutation*, 34(1), pp. 57–65. doi: 10.1002/humu.22225.

Shimabukuro, M., Zhou, Y.-T., Levi, M. and Unger, R. H. (1998) 'Fatty acid-induced cell apoptosis: A link between obesity and diabetes', *Proceedings of the National Academy of Sciences*, 95(5), pp. 2498–2502. doi: 10.1073/pnas.95.5.2498.

Shipston, M. J. (2001) 'Alternative splicing of potassium channels: a dynamic switch of cellular excitability.', *Trends in cell biology*, 11(9), pp. 353–358.

Shipston, M. J. (2011) 'Ion channel regulation by protein palmitoylation.', *The Journal of biological chemistry*, 286(11), pp. 8709–8716. doi: 10.1074/jbc.R110.210005.

Shipston, M. J. (2014) 'S-acylation dependent post-translational cross-talk regulates large conductance calcium- and voltage- activated potassium (BK) channels.', *Frontiers in physiology*, 5, p. 281. doi: 10.3389/fphys.2014.00281.

Shipston, M. J., Duncan, R. R., Clark, A. G., Antoni, F. A. and Tian, L. (1999) 'Molecular components of large conductance calcium-activated potassium (BK) channels in mouse pituitary corticotropes.', *Molecular endocrinology (Baltimore, Md.)*, 13, pp. 1728–1737. doi: 10.1210/me.13.10.1728.

Shipston, M. J. and Tian, L. (2016) 'Chapter Three – Posttranscriptional and Posttranslational Regulation of BK Channels', in *International Review of Neurobiology*, pp. 91–126. doi: 10.1016/bs.irn.2016.02.012.

Shruti, S., Urban-Ciecko, J., Fitzpatrick, J. A., Brenner, R., Bruchez, M. P. and Barth, A. L. (2012) 'The brain-specific Beta4 subunit downregulates BK channel cell surface expression.', *PloS one*, 7(3), p. e33429. doi: 10.1371/journal.pone.0033429.

Siemen, D., Loupatatzis, C., Borecky, J., Gulbins, E., Lang, F., Sievert, H., Kohler, K. P., Kaltenbach, M. and Kober, G. (1999) 'Ca2+-activated K channel of the BK-type in the inner mitochondrial membrane of a human glioma cell line', *Biochemical and Biophysical Research Communications*, 257(2), pp. 549–554.

da Silva Xavier, G., Leclerc, I., Varadi, A., Tsuboi, T., Moule, S. K. and Rutter, G. A. (2003) 'Role for AMP-activated protein kinase in glucose-stimulated insulin secretion and preproinsulin gene expression.', *The Biochemical journal*, 371(Pt 3), pp. 761–74. doi: 10.1042/BJ20021812.

Sim, N.-L., Kumar, P., Hu, J., Henikoff, S., Schneider, G. and Ng, P. C. (2012) 'SIFT web server: predicting effects of amino acid substitutions on proteins', *Nucleic Acids Research*. Oxford University Press, 40(W1), pp. W452–W457. doi: 10.1093/nar/gks539.

Singh, K. K. (2014) 'Intellectual Property Protection in Bioinformatics and Open Bio Development', *Asian Biotechnology and Development Review*, 16(3), pp. 25–45.

Smith, A. J., Partridge, C. J., Asipu, A., Mair, L. A., Hunter, M. and Sivaprasadarao, A. (2006) 'Increased ATP-sensitive K+ channel expression during acute glucose deprivation.', *Biochemical and biophysical research communications*, 348(3), pp. 1123–31. doi: 10.1016/j.bbrc.2006.07.170.

Sommese, L., Zullo, A., Mancini, F. P., Fabbricini, R., Soricelli, A. and Napoli, C. (2017) 'Clinical relevance of epigenetics in the onset and management of type 2 diabetes mellitus', *Epigenetics*, pp. 1–15. doi: 10.1080/15592294.2016.1278097.

Song, M., Zhu, N., Olcese, R., Barila, B., Toro, L. and Stefani, E. (1999) 'Hormonal control of protein expression and mRNA levels of the MaxiK channel α subunit in myometrium', *FEBS Letters*, 460(3), pp. 427–432. doi: 10.1016/S0014-5793(99)01394-0.

Staiger, H., Staiger, K., Stefan, N., Wahl, H. G., Machicao, F., Kellerer, M. and Haring, H.-U. (2004) 'Palmitate-Induced Interleukin-6 Expression in Human Coronary Artery Endothelial Cells', *Diabetes*, 53(12), pp. 3209–3216. doi: 10.2337/diabetes.53.12.3209.

Stanford, K. I., Middelbeek, R. J. W., Townsend, K. L., An, D., Nygaard, E. B., Hitchcox, K. M., Markan, K. R., Nakano, K., Hirshman, M. F., Tseng, Y.-H. and Goodyear, L. J. (2013) 'Brown adipose tissue regulates glucose homeostasis and insulin sensitivity.', *The Journal of clinical investigation*. American Society for Clinical Investigation, 123(1), pp. 215–23. doi: 10.1172/JCI62308.

Stead, L. F., Wood, I. C., Westhead, D. R. and Bateman, A. (2011) 'KvSNP: accurately predicting the effect of genetic variants in voltage-gated potassium channels', *BIOINFORMATICS*, 27(16), pp. 2181–218610. doi: 10.1093/bioinformatics/btr365.

Stenmark, H., Aasland, R., Toh, B. H. and D'Arrigo, A. (1996) 'Endosomal Localization of the Autoantigen EEA1 Is Mediated by a Zinc-binding FYVE Finger', *Journal of Biological Chemistry*, 271(39), pp. 24048–24054. doi: 10.1074/jbc.271.39.24048.

Stenson, P. D., Mort, M., Ball, E. V, Shaw, K., Phillips, A. and Cooper, D. N. (2014) 'The Human Gene Mutation Database: building a comprehensive mutation repository for clinical and molecular genetics, diagnostic testing and personalized genomic medicine.', *Human genetics*. Springer, 133(1), pp. 1–9. doi: 10.1007/s00439-013-1358-4.

Stockklausner, C. and Klocker, N. (2003) 'Surface expression of inward rectifier potassium channels is controlled by selective Golgi export.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 278(19), pp. 17000–5. doi: 10.1074/jbc.M212243200.

Su, X., Theberge, A. B., January, C. T. and Beebe, D. J. (2013) 'Effect of microculture on cell metabolism and biochemistry: do cells get stressed in microchannels?', *Analytical chemistry*, 85(3), pp. 1562–70. doi: 10.1021/ac3027228.

Sudmant, P. H., Rausch, T., Gardner, E. J., Handsaker, R. E., Abyzov, A., Huddleston, J., Zhang, Y., Ye, K., Jun, G., Hsi-Yang Fritz, M., Konkel, M. K., Malhotra, A., Stütz, A. M., Shi, X., Paolo Casale, F., Chen, J., Hormozdiari, F., Dayama, G., Chen, K., Malig, M., Chaisson, M. J. P., Walter, K., Meiers, S., Kashin, S., Garrison, E., Auton, A., Lam, H. Y. K., Jasmine Mu, X., Alkan, C., Antaki, D., Bae, T., Cerveira, E., Chines, P., Chong, Z., Clarke, L., Dal, E., Ding, L., Emery, S., Fan, X., Gujral, M., Kahveci, F., Kidd, J. M., Kong, Y., Lameijer, E.-W., McCarthy, S., Flicek, P., Gibbs, R. A., Marth, G., Mason, C. E., Menelaou, A., Muzny, D. M., Nelson, B. J., Noor, A., Parrish, N. F., Pendleton, M., Quitadamo, A., Raeder, B., Schadt, E. E., Romanovitch, M., Schlattl, A., Sebra, R., Shabalin, A. A., Untergasser, A., Walker, J. A., Wang, M., Yu, F., Zhang, C., Zhang, J., Zheng-Bradley, X., Zhou, W., Zichner, T., Sebat, J., Batzer, M. A., McCarroll, S. A., Mills, R. E., Gerstein, M. B., Bashir, A., Stegle, O., Devine, S. E., Lee, C., Eichler, E. E., Korbel, J. O. and Korbel, J. O. (2015) 'An integrated map of structural variation in 2,504 human genomes', *Nature*. Nature Research, 526(7571), pp. 75–81. doi: 10.1038/nature15394.

Sun, H., Leng, T., Zeng, Z., Gao, X., Inoue, K. and Xiong, Z.-G. (2013) 'Role of TRPM7 channels in hyperglycemia-mediated injury of vascular endothelial cells.', *PloS one*, 8(11), p. e79540. doi: 10.1371/journal.pone.0079540.

Sun, L., Shen, X., Liu, Y., Zhang, G., Wei, J., Zhang, H., Zhang, E. and Ma, F. (2010) 'The location of endogenous wild-type p53 protein in 293T and HEK293 cells expressing low-risk HPV-6E6 fusion protein with GFP', *Acta Biochimica et Biophysica Sinica*, 42(3), pp. 230–235. doi: 10.1093/abbs/gmq009.

Sun, X., Zhou, D., Zhang, P., Moczydlowski, E. G. and Haddad, G. G. (2007) 'β-Subunit–Dependent Modulation of hSlo BK Current by Arachidonic Acid', *Journal of Neurophysiology*, 97(1).

Suzuki, Y., Ohya, S., Yamamura, H., Giles, W. R. and Imaizumi, Y. (2016) 'A New Splice Variant of Large Conductance Ca2+-activated K+ (BK) Channel α Subunit Alters Human Chondrocyte Function.', *Journal of Biological Chemistry*, 291(46), pp. 24247–24260. doi: 10.1074/jbc.M116.743302.

Swope, S. L., Moss, S. J., Blackstone, C. D. and Huganir, R. L. (1992) 'Phosphorylation of ligand-gated ion channels: a possible mode of synaptic plasticity.', *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 6, pp. 2514–2523.

Sylow, L., Møller, L. L. V, Kleinert, M., Richter, E. A. and Jensen, T. E. (2015) 'Stretch-stimulated glucose transport in skeletal muscle is regulated by Rac1.', *The Journal of physiology*. Wiley-Blackwell, 593(3), pp. 645–56. doi: 10.1113/jphysiol.2014.284281.

Szabo, I. and Zoratti, M. (2014) 'Mitochondrial Channels: Ion Fluxes and More', *Physiological Reviews*, 94(2), pp. 519–608. doi: 10.1152/physrev.00021.2013.

Tabangin, M. E., Woo, J. G. and Martin, L. J. (2009) 'The effect of minor allele frequency on the likelihood of obtaining false positives.', *BMC proceedings*. BioMed Central, 3 Suppl 7(Suppl 7), p. S41. doi: 10.1186/1753-6561-3-S7-S41.

Tachibana, C. (2010) 'Systems Biology and Bioinformatics: Something for Everyone', *Science*, pp. 255–257. doi: 10.1126/science.opms.r1000087.

Tajima, N., Itokazu, Y., Korpi, E. R., Somerharju, P. and Käkelä, R. (2011) 'Activity of BK(Ca) channel is

modulated by membrane cholesterol content and association with Na+/K+-ATPase in human melanoma IGR39 cells.', *The Journal of biological chemistry*, 286(7), pp. 5624–5638. doi: 10.1074/jbc.M110.149898.

Taliaferro, W. H. and Huck, J. G. (1923) 'The Inheritance of Sickle-Cell Anaemia in Man.', *Genetics*. American Association for the Advancement of Science, 8(6), pp. 594–8. doi: 10.1001/ARCHINTE.1944.00210210032005.

Tanabe (2007) *Research and Development Pipeline*, *Tanabe Website*. Available at: http://www.kalbemed.com/Portals/6/komelib/musculo-skeletal system/Rheumatologi/Mediflex/r&d pipeline.pdf.

Tanaka, M., Sasaki, Y., Kimura, Y., Fukui, T., Hamada, K. and Ukai, Y. (2003) 'A novel pyrrole derivative, NS-8, suppresses the rat micturition reflex by inhibiting afferent pelvic nerve activity', *BJU International*. Blackwell Publishing Ltd., 92(9), pp. 1031–1036. doi: 10.1111/j.1464-410X.2003.04512.x.

Tang, X. D., Xu, R., Reynolds, M. F., Garcia, M. L., Heinemann, S. H. and Hoshi, T. (2003) 'Haem can bind to and inhibit mammalian calcium-dependent Slo1 BK channels.', *Nature*, 425(6957), pp. 531–5. doi: 10.1038/nature02003.

Tao, X., Hite, R. K. and MacKinnon, R. (2016) 'Cryo-EM structure of the open high-conductance Ca2+-activated K+ channel', *Nature*. Nature Research, 541(7635), pp. 46–51. doi: 10.1038/nature20608.

Tao, X., Lin, M. T., Thorington, G. U., Wilson, S. M., Longo, L. D. and Hessinger, D. A. (2015) 'Long-term hypoxia increases calcium affinity of BK channels in ovine fetal and adult cerebral artery smooth muscle.', *American journal of physiology. Heart and circulatory physiology*. American Physiological Society, 308(7), pp. H707-22. doi: 10.1152/ajpheart.00564.2014.

Tatara, Y., Yoshida, T. and Ichishima, E. (2005) 'A Single Free Cysteine Residue and Disulfide Bond Contribute to the Thermostability of Aspergillus saitoi 1,2-α-Mannosidase', *Bioscience, Biotechnology, and Biochemistry*, 69(11), pp. 2101–2108. doi: 10.1271/bbb.69.2101.

Tavtigian, S. V., Greenblatt, M. S., Lesueur, F. and Byrnes, G. B. (2008) 'In silico analysis of missense substitutions using sequence-alignment based methods', *Human Mutation*. Wiley Subscription Services, Inc., A Wiley Company, 29(11), pp. 1327–1336. doi: 10.1002/humu.20892.

Taylor, R. (2012) 'Insulin Resistance and Type 2 Diabetes', *Diabetes*, 61(4), pp. 778–779. doi: 10.2337/db12-0073.

Teng, M., Ichikawa, S., Padgett, L. R., Wang, Y., Mort, M., Cooper, D. N., Koller, D. L., Foroud, T., Edenberg, H. J., Econs, M. J. and Liu, Y. (2012) 'regSNPs: a strategy for prioritizing regulatory single nucleotide substitutions', 28(14), pp. 1879–188610. doi: 10.1093/bioinformatics/bts275.

Terés, S., Barceló-Coblijn, G., Benet, M., Alvarez, R., Bressani, R., Halver, J. E. and Escribá, P. V (2008) 'Oleic acid content is responsible for the reduction in blood pressure induced by olive oil.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 105(37), pp. 13811–6. doi: 10.1073/pnas.0807500105.

The ENCODE Project Consortium (2004) 'The ENCODE (ENCyclopedia Of DNA Elements) Project', *Science*, 306(5696), pp. 636–640. doi: 10.1126/science.1105136.

The ENCODE Project Consortium (2007) 'Identification and analysis of functional elements in 1% of the human genome by the ENCODE pilot project The ENCODE Project Consortium*', *Nature*, 447. doi: 10.1038/nature05874.

The European Bioinformatics Institute (2000) *Ensembl Genome Server*. Available at:

http://web.archive.org/web/20000815203739/http://www.ensembl.org/ (Accessed: 15 March 2017).

The European Bioinformatics Institute (2017) *Primary and secondary databases | EMBL-EBI Train online*. Available at: https://www.ebi.ac.uk/training/online/course/bioinformatics-terrified/what-database/relational-databases/primary-and-secondary-databases (Accessed: 10 March 2017).

The Huntington's Disease Collaborative Research Group (1993) 'A novel gene containing a trinucleotide repeat that is expanded and unstable on Huntington's disease chromosomes. The Huntington's Disease Collaborative Research Group.', *Cell*, 72(6), pp. 971–83.

The International HapMap Consortium, Gibbs, R. A., Belmont, J. W., Hardenbol, P., Willis, T. D., Yu, F., Yang, H., Ch'ang, L.-Y., Huang, W., Liu, B., Shen, Y., Tam, P. K.-H., Tsui, L.-C., Waye, M. M. Y., Wong, J. T.-F., Zeng, C., Zhang, Q., Chee, M. S., Galver, L. M., Kruglyak, S., Murray, S. S., Oliphant, A. R., Montpetit, A., Hudson, T. J., Chagnon, F., Ferretti, V., Leboeuf, M., Phillips, M. S., Verner, A., Kwok, P.-Y., Duan, S., Lind, D. L., Miller, R. D., Rice, J. P., Saccone, N. L., Taillon-Miller, P., Xiao, M., Nakamura, Y., Sekine, A., Sorimachi, K., Tanaka, T., Tanaka, Y., Tsunoda, T., Yoshino, E., Bentley, D. R., Deloukas, P., Hunt, S., Powell, D., Altshuler, D., Gabriel, S. B., Zhang, H., Zeng, C., Matsuda, I., Fukushima, Y., Macer, D. R., Suda, E., Rotimi, C. N., Adebamowo, C. A., Aniagwu, T., Marshall, P. A., Matthew, O., Nkwodimmah, C., Royal, C. D. M., Leppert, M. F., Dixon, M., Stein, L. D., Cunningham, F., Kanani, A., Thorisson, G. A., Chakravarti, A., Chen, P. E., Cutler, D. J., Kashuk, C. S., Donnelly, P., Marchini, J., McVean, G. A. T., Myers, S. R., Cardon, L. R., Abecasis, G. R., Morris, A., Weir, B. S., Mullikin, J. C., Sherry, S. T., Feolo, M., Altshuler, D., Daly, M. J., Schaffner, S. F., Qiu, R., Kent, A., Dunston, G. M., Kato, K., Niikawa, N., Knoppers, B. M., Foster, M. W., Clayton, E. W., Wang, V. O., Watkin, J., Gibbs, R. A., Belmont, J. W., Sodergren, E., Weinstock, G. M., Wilson, R. K., Fulton, L. L., Rogers, J., Birren, B. W., Han, H., Wang, H., Godbout, M., Wallenburg, J. C., L'Archevêque, P., Bellemare, G., Todani, K., Fujita, T., Tanaka, S., Holden, A. L., Lai, E. H., Collins, F. S., Brooks, L. D., McEwen, J. E., Guyer, M. S., Jordan, E., Peterson, J. L., Spiegel, J., Sung, L. M., Zacharia, L. F., Kennedy, K., Dunn, M. G., Seabrook, R., Shillito, M., Skene, B., Stewart, J. G., Valle (chair), D. L., Clayton (co-chair), E. W., Jorde (co-chair), L. B., Belmont, J. W., Chakravarti, A., Cho, M. K., Duster, T., Foster, M. W., Jasperse, M., Knoppers, B. M., Kwok, P.-Y., Licinio, J., Long, J. C., Marshall, P. A., Ossorio, P. N., Wang, V. O., Rotimi, C. N., Royal, C. D. M., Spallone, P., Terry, S. F., Lander (chair), E. S., Lai (co-chair), E. H., Nickerson (co-chair), D. A., Abecasis, G. R., Altshuler, D., Bentley, D. R., Boehnke, M., Cardon, L. R., Daly, M. J., Deloukas, P., Douglas, J. A., Gabriel, S. B., Hudson, R. R., Hudson, T. J., Kruglyak, L., Kwok, P.-Y., Nakamura, Y., Nussbaum, R. L., Royal, C. D. M., Schaffner, S. F., Sherry, S. T., Stein, L. D., Tanaka, T. and -, - (2003) 'The International HapMap Project', *Nature*. Nature Publishing Group, 426(6968), pp. 789–796. doi: 10.1038/nature02168.

The Wellcome Trust (2003) *Sharing Data from Large-scale Biological Research Projects: A System of Tripartite Responsibility Sharing Data from Large-scale Biological Research Projects*. Fort Lauderdale.

Thermo Scientific (2014) *Overview of Protein Expression Systems*. Available at: http://www.piercenet.com/method/overview-protein-expression-systems#methods (Accessed: 6 January 2015).

Thomas, P. D., Campbell, M. J., Kejariwal, A., Mi, H., Karlak, B., Daverman, R., Diemer, K., Muruganujan, A. and Narechania, A. (2003) 'PANTHER: a library of protein families and subfamilies indexed by function.', *Genome research*. Cold Spring Harbor Laboratory Press, 13(9), pp. 2129–41. doi: 10.1101/gr.772403.

Thomas, P. and Smart, T. G. (2005) 'HEK293 cell line: a vehicle for the expression of recombinant proteins.', *Journal of pharmacological and toxicological methods*, 51(3), pp. 187–200. doi: 10.1016/j.vascn.2004.08.014.

Thompson, E. A. (2013) 'Identity by Descent: Variation in Meiosis, Across Genomes, and in Populations', *Genetics*, 194(2), pp. 301–326. doi: 10.1534/genetics.112.148825.

Thorisson, G. A., Smith, A. V, Krishnan, L. and Stein, L. D. (2005) 'The International HapMap Project Web site', *Genome Research*, 15(11), pp. 1592–1593. doi: 10.1101/gr.4413105.

Thorn, K. (2010) *Palmitate-induced Apoptosis in Insulin-producing Beta Cells*.

Tian, L., Chen, L., McClafferty, H., Sailer, C. a, Ruth, P., Knaus, H.-G. and Shipston, M. J. (2006) 'A noncanonical SH3 domain binding motif links BK channels to the actin cytoskeleton via the SH3 adapter cortactin.', *The FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 20(14), pp. 2588–2590. doi: 10.1096/fj.06-6152fje.

Tian, L., Coghill, L. S., McClafferty, H., MacDonald, S. H.-F., Antoni, F. A., Ruth, P., Knaus, H.-G. and Shipston, M. J. (2004) 'Distinct stoichiometry of BKCa channel tetramer phosphorylation specifies channel activation and inhibition by cAMP-dependent protein kinase.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 101(32), pp. 11897–902. doi: 10.1073/pnas.0402590101.

Tian, L., Duncan, R., Hammond, M., Coghill, L., Wen, H., Rusinova, R., Clark, A., Levitan, I. and Shipston, M. (2001) 'Alternative splicing switches potassium channel sensitivity to protein phosphorylation.', *The Journal of biological chemistry*, 276(11), pp. 7717–20. doi: 10.1074/jbc.C000741200.

Tian, L., Hammond, M. S., Florance, H., Antoni, F. A. and Shipston, M. J. (2001) 'Alternative splicing determines sensitivity of murine calcium-activated potassium channels to glucocorticoids.', *The Journal of physiology*. Wiley-Blackwell, 537(Pt 1), pp. 57–68. doi: 10.1111/J.1469-7793.2001.0057K.X.

Tian, L., Jeffries, O., McClafferty, H., Molyvdas, A., Rowe, I. C. M., Saleem, F., Chen, L., Greaves, J., Chamberlain, L. H., Knaus, H.-G., Ruth, P. and Shipston, M. J. (2008) 'Palmitoylation gates phosphorylation-dependent regulation of BK potassium channels.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 105(52), pp. 21006–11. doi: 10.1073/pnas.0806700106.

Tian, L., McClafferty, H., Jeffries, O. and Shipston, M. J. (2010) 'Multiple palmitoyltransferases are required for palmitoylation-dependent regulation of large conductance calcium- and voltage-activated potassium channels.', *The Journal of biological chemistry*, 285(31), pp. 23954–23962. doi: 10.1074/jbc.M110.137802.

Tian, L., McClafferty, H., Knaus, H.-G., Ruth, P. and Shipston, M. J. (2012) 'Distinct acyl protein transferases and thioesterases control surface expression of calcium-activated potassium channels.', *The Journal of biological chemistry*, 287(18), pp. 14718–25. doi: 10.1074/jbc.M111.335547.

Tjong, Y.-W., Li, M., Hung, M.-W., Wang, K. and Fung, M.-L. (2008) 'Nitric oxide deficit in chronic intermittent hypoxia impairs large conductance calcium-activated potassium channel activity in rat hippocampal neurons.', *Free radical biology & medicine*, 44(4), pp. 547–57. doi: 10.1016/j.freeradbiomed.2007.10.033.

Tomás, M., Vázquez, E., Fernández-Fernández, J. M., Subirana, I., Plata, C., Heras, M., Vila, J., Marrugat, J., Valverde, M. A. and Sentí, M. (2008) 'Genetic variation in the KCNMA1 potassium channel α subunit as risk factor for severe essential hypertension and myocardial infarction', *Journal of Hypertension*, 26(11), pp. 2147–2153. doi: 10.1097/HJH.0b013e32831103d8.

Tong, L., He, T., Katusic, Z. S., Lee, H.-C. C., Lu, T., He, T., Katusic, Z. S. and Lee, H.-C. C. (2006) 'Molecular mechanisms mediating inhibition of human large conductance Ca2+-activated K+ channels by high glucose', *Circ Res*, 99(6), pp. 607–616. doi: 10.1161/01.RES.0000243147.41792.93.

Torjesen, I. (2013) 'Genomes of 100,000 people will be sequenced to create an open access research resource.', *BMJ (Clinical research ed.)*, 347, p. f6690.

Torkamani, A. and Schork, N. J. (2007) 'Accurate prediction of deleterious protein kinase

polymorphisms', *Bioinformatics*. Oxford University Press, 23(21), pp. 2918–2925. doi: 10.1093/bioinformatics/btm437.

Toronto International Data Release Workshop Authors (2009) 'Prepublication data sharing', *Nature*. NIH Public Access, 461(7261), pp. 168–170. doi: 10.1038/461168a.

Torres, Y. P., Granados, S. T. and Latorre, R. (2014) 'Pharmacological consequences of the coexpression of BK channel α and auxiliary β subunits.', *Frontiers in physiology*. Frontiers Media SA, 5, p. 383. doi: 10.3389/fphys.2014.00383.

Trusheim, M. R., Berndt, E. R. and Douglas, F. L. (2007) 'Stratified medicine: strategic and economic implications of combining drugs and clinical biomarkers', *Nature Reviews Drug Discovery*. Nature Publishing Group, 6(4), pp. 287–293. doi: 10.1038/nrd2251.

Tsai, J., Lee, J. T., Wang, W., Zhang, J., Cho, H., Mamo, S., Bremer, R., Gillette, S., Kong, J., Haass, N. K., Sproesser, K., Li, L., Smalley, K. S. M., Fong, D., Zhu, Y.-L., Marimuthu, A., Nguyen, H., Lam, B., Liu, J., Cheung, I., Rice, J., Suzuki, Y., Luu, C., Settachatgul, C., Shellooe, R., Cantwell, J., Kim, S.-H., Schlessinger, J., Zhang, K. Y. J., West, B. L., Powell, B., Habets, G., Zhang, C., Ibrahim, P. N., Hirth, P., Artis, D. R., Herlyn, M. and Bollag, G. (2008) 'Discovery of a selective inhibitor of oncogenic B-Raf kinase with potent antimelanoma activity.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 105(8), pp. 3041–6. doi: 10.1073/pnas.0711741105.

Tseng-Crank, J., Foster, C. D., Krause, J. D., Mertz, R., Godinot, N., DiChiara, T. J. and Reinhart, P. H. (1994) 'Cloning, expression, and distribution of functionally distinct Ca(2+)-activated K+ channel isoforms from human brain.', *Neuron*, 13(6), pp. 1315–30.

Tsutsumi, R., Fukata, Y. and Fukata, M. (2008) 'Discovery of protein-palmitoylating enzymes.', *Pflügers Archiv : European journal of physiology*, 456(6), pp. 1199–206. doi: 10.1007/s00424-008-0465-x.

Tunduguru, R., Chiu, T. T., Ramalingam, L., Elmendorf, J. S., Klip, A. and Thurmond, D. C. (2014) 'Signaling of the p21-activated kinase (PAK1) coordinates insulin-stimulated actin remodeling and glucose uptake in skeletal muscle cells.', *Biochemical pharmacology*. NIH Public Access, 92(2), pp. 380–8. doi: 10.1016/j.bcp.2014.08.033.

Venter, J. C., Adams, M. D., Myers, E. W., Li, P. W., Mural, R. J., Sutton, G. G., Smith, H. O., Yandell, M., Evans, C. A., Holt, R. A., Gocayne, J. D., Amanatides, P., Ballew, R. M., Huson, D. H., Wortman, J. R., Zhang, Q., Kodira, C. D., Zheng, X. H., Chen, L., Skupski, M., Subramanian, G., Thomas, P. D., Zhang, J., Gabor Miklos, G. L., Nelson, C., Broder, S., Clark, A. G., Nadeau, J., McKusick, V. A., Zinder, N., Levine, A. J., Roberts, R. J., Simon, M., Slayman, C., Hunkapiller, M., Bolanos, R., Delcher, A., Dew, I., Fasulo, D., Flanigan, M., Florea, L., Halpern, A., Hannenhalli, S., Kravitz, S., Levy, S., Mobarry, C., Reinert, K., Remington, K., Abu-Threideh, J., Beasley, E., Biddick, K., Bonazzi, V., Brandon, R., Cargill, M., Chandramouliswaran, I., Charlab, R., Chaturvedi, K., Deng, Z., Di Francesco, V., Dunn, P., Eilbeck, K., Evangelista, C., Gabrielian, A. E., Gan, W., Ge, W., Gong, F., Gu, Z., Guan, P., Heiman, T. J., Higgins, M. E., Ji, R. R., Ke, Z., Ketchum, K. A., Lai, Z., Lei, Y., Li, Z., Li, J., Liang, Y., Lin, X., Lu, F., Merkulov, G. V, Milshina, N., Moore, H. M., Naik, A. K., Narayan, V. A., Neelam, B., Nusskern, D., Rusch, D. B., Salzberg, S., Shao, W., Shue, B., Sun, J., Wang, Z., Wang, A., Wang, X., Wang, J., Wei, M., Wides, R., Xiao, C., Yan, C., Yao, A., Ye, J., Zhan, M., Zhang, W., Zhang, H., Zhao, Q., Zheng, L., Zhong, F., Zhong, W., Zhu, S., Zhao, S., Gilbert, D., Baumhueter, S., Spier, G., Carter, C., Cravchik, A., Woodage, T., Ali, F., An, H., Awe, A., Baldwin, D., Baden, H., Barnstead, M., Barrow, I., Beeson, K., Busam, D., Carver, A., Center, A., Cheng, M. L., Curry, L., Danaher, S., Davenport, L., Desilets, R., Dietz, S., Dodson, K., Doup, L., Ferriera, S., Garg, N., Gluecksmann, A., Hart, B., Haynes, J., Haynes, C., Heiner, C., Hladun, S., Hostin, D., Houck, J., Howland, T., Ibegwam, C., Johnson, J., Kalush, F., Kline, L., Koduru, S., Love, A., Mann, F., May, D., McCawley, S., McIntosh, T., McMullen, I., Moy, M., Moy, L., Murphy, B., Nelson, K., Pfannkoch, C., Pratts, E., Puri, V., Qureshi, H., Reardon, M., Rodriguez, R., Rogers, Y. H., Romblad, D., Ruhfel, B., Scott, R., Sitter, C., Smallwood, M., Stewart, E., Strong, R., Suh, E., Thomas, R., Tint, N. N., Tse, S., Vech, C.,

Wang, G., Wetter, J., Williams, S., Williams, M., Windsor, S., Winn-Deen, E., Wolfe, K., Zaveri, J., Zaveri, K., Abril, J. F., Guigó, R., Campbell, M. J., Sjolander, K. V, Karlak, B., Kejariwal, A., Mi, H., Lazareva, B., Hatton, T., Narechania, A., Diemer, K., Muruganujan, A., Guo, N., Sato, S., Bafna, V., Istrail, S., Lippert, R., Schwartz, R., Walenz, B., Yooseph, S., Allen, D., Basu, A., Baxendale, J., Blick, L., Caminha, M., Carnes-Stine, J., Caulk, P., Chiang, Y. H., Coyne, M., Dahlke, C., Mays, A., Dombroski, M., Donnelly, M., Ely, D., Esparham, S., Fosler, C., Gire, H., Glanowski, S., Glasser, K., Glodek, A., Gorokhov, M., Graham, K., Gropman, B., Harris, M., Heil, J., Henderson, S., Hoover, J., Jennings, D., Jordan, C., Jordan, J., Kasha, J., Kagan, L., Kraft, C., Levitsky, A., Lewis, M., Liu, X., Lopez, J., Ma, D., Majoros, W., McDaniel, J., Murphy, S., Newman, M., Nguyen, T., Nguyen, N., Nodell, M., Pan, S., Peck, J., Peterson, M., Rowe, W., Sanders, R., Scott, J., Simpson, M., Smith, T., Sprague, A., Stockwell, T., Turner, R., Venter, E., Wang, M., Wen, M., Wu, D., Wu, M., Xia, A., Zandieh, A. and Zhu, X. (2001) 'The Sequence of the Human Genome', *Science*, 291(5507), pp. 1304–1351. doi: 10.1126/science.1058040.

Vergara, C., Latorre, R., Marrion, N. V and Adelman, J. P. (1998) 'Calcium-activated potassium channels', *Current Opinion in Neurobiology*, 8(3), pp. 321–329. doi: 10.1016/S0959-4388(98)80056-1.

Viskupicova, J., Blaskovic, D., Galiniak, S., Soszyński, M., Bartosz, G., Horakova, L. and Sadowska-Bartosz, I. (2015) 'Effect of high glucose concentrations on human erythrocytes in vitro', *Redox Biology*, 5, pp. 381–387. doi: 10.1016/j.redox.2015.06.011.

Visscher, P. M., Brown, M. A., McCarthy, M. I. and Yang, J. (2012) 'Five years of GWAS discovery.', *American journal of human genetics*. Elsevier, 90(1), pp. 7–24. doi: 10.1016/j.ajhg.2011.11.029.

Voolstra, O. and Huber, A. (2014) 'Post-Translational Modifications of TRP Channels.', *Cells*. Multidisciplinary Digital Publishing Institute (MDPI), 3(2), pp. 258–87. doi: 10.3390/cells3020258.

Wallner, M., Meera, P. and Toro, L. (1996) 'Determinant for beta-subunit regulation in high-conductance voltage-activated and Ca(2+)-sensitive K+ channels: an additional transmembrane region at the N terminus.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 93(25), pp. 14922–7.

Walsh, G. and Jefferis, R. (2006) 'Post-translational modifications in the context of therapeutic proteins', *Nature Biotechnology*, 24(10), pp. 1241–1252. doi: 10.1038/nbt1252.

Wang, B., Rothberg, B. S. and Brenner, R. (2009) 'Mechanism of increased BK channel activation from a channel mutation that causes epilepsy.', *The Journal of general physiology*. The Rockefeller University Press, 133(3), pp. 283–94. doi: 10.1085/jgp.200810141.

Wang, J., Alexanian, A., Ying, R., Kizhakekuttu, T. J., Dharmashankar, K., Vasquez-Vivar, J., Gutterman, D. D. and Widlansky, M. E. (2012) 'Acute exposure to low glucose rapidly induces endothelial dysfunction and mitochondrial oxidative stress: role for AMP kinase.', *Arteriosclerosis, thrombosis, and vascular biology*. NIH Public Access, 32(3), pp. 712–20. doi: 10.1161/ATVBAHA.111.227389.

Wang, K., Li, W.-D., Zhang, C. K., Wang, Z., Glessner, J. T., Grant, S. F. A., Zhao, H., Hakonarson, H. and Price, R. A. (2011) 'A Genome-Wide Association Study on Obesity and Obesity-Related Traits', *PLoS ONE*. Edited by Z. Zhao. Public Library of Science, 6(4), p. e18939. doi: 10.1371/journal.pone.0018939.

Wang, L. and Sigworth, F. J. (2009) 'Structure of the BK potassium channel in a lipid membrane from electron cryomicroscopy.', *Nature*. NIH Public Access, 461(7261), pp. 292–5. doi: 10.1038/nature08291.

Wang, S., Song, P. and Zou, M.-H. (2012) 'AMP-activated protein kinase, stress responses and cardiovascular diseases', *Clinical Science*, 122(12).

Wang, T.-H. and Wang, H.-S. (2009) 'A Genome-Wide Association Study Primer for Clinicians', *Taiwanese Journal of Obstetrics and Gynecology*, 48(2), pp. 89–95. doi: 10.1016/S1028-

4559(09)60265-5.

Wang, Z.-W. (2008) 'Regulation of Synaptic Transmission by Presynaptic CaMKII and BK Channels', *Molecular Neurobiology*, 38(2), pp. 153–166. doi: 10.1007/s12035-008-8039-7.

Wang, Z. and Moult, J. (2001) 'SNPs, protein structure, and disease.', *Human mutation*, 17(4), pp. 263–70. doi: 10.1002/humu.22.

Wang, Z. and Prelich, G. (2009) 'Quality control of a transcriptional regulator by SUMO-targeted degradation.', *Molecular and cellular biology*. American Society for Microbiology, 29(7), pp. 1694–706. doi: 10.1128/MCB.01470-08.

Warsi, J., Singh, Y., Elvira, B., Hosseinzadeh, Z. and Lang, F. (2015) 'Regulation of Large Conductance Voltage-and Ca2+-Activated K+ Channels by the Janus Kinase JAK3.', *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. Karger Publishers, 37(1), pp. 297–305. doi: 10.1159/000430354.

Watson, M. L., Macrae, K., Marley, A. E. and Hundal, H. S. (2011) 'Chronic effects of palmitate overload on nutrient-induced insulin secretion and autocrine signalling in pancreatic MIN6 beta cells.', *PloS one*. Edited by I. Agoulnik. Public Library of Science, 6(10), p. e25975. doi: 10.1371/journal.pone.0025975.

Watt, M. J., Steinberg, G. R., Chen, Z.-P., Kemp, B. E. and Febbraio, M. A. (2006) 'Fatty acids stimulate AMP-activated protein kinase and enhance fatty acid oxidation in L6 myotubes.', *The Journal of physiology*. Wiley-Blackwell, 574(Pt 1), pp. 139–47. doi: 10.1113/jphysiol.2006.107318.

Weaver, A. K., Olsen, M. L., McFerrin, M. B. and Sontheimer, H. (2007) 'BK channels are linked to inositol 1,4,5-triphosphate receptors via lipid rafts: a novel mechanism for coupling [Ca(2+)](i) to ion channel activation.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 282(43), pp. 31558–68. doi: 10.1074/jbc.M702866200.

Wei, A. D. (2005) 'International Union of Pharmacology. LII. Nomenclature and Molecular Relationships of Calcium-Activated Potassium Channels', *Pharmacological Reviews*, 57(4), pp. 463–472. doi: 10.1124/pr.57.4.9.

Wei, A., Jegla, T. and Salkoff, L. (1996) 'Eight Potassium Channel Families Revealed by the C. elegans Genome Project', *Neuropharmacology*, 35(7), pp. 805–829. doi: 10.1016/0028-3908(96)00126-8.

Weigert, C., Brodbeck, K., Staiger, H., Kausch, C., Machicao, F., Häring, H. U. and Schleicher, E. D. (2004) 'Palmitate, but not unsaturated fatty acids, induces the expression of interleukin-6 in human myotubes through proteasome-dependent activation of nuclear factor-kappaB.', *The Journal of biological chemistry*, 279(23), pp. 23942–23952. doi: 10.1074/jbc.M312692200.

Weikel, K. A., Cacicedo, J., Ruderman, N. B. and Ido, Y. (2014) 'Glucose and Palmitate Uncouple AMPK from Autophagy in Human Aortic Endothelial Cells.', *American journal of physiology. Cell physiology*, p. ajpcell.00265.2014. doi: 10.1152/ajpcell.00265.2014.

Wellcome Trust Case Control Consortium, T. W. T. C. C. (2007) 'Genome-wide association study of 14,000 cases of seven common diseases and 3,000 shared controls.', *Nature*. Europe PMC Funders, 447(7145), pp. 661–78. doi: 10.1038/nature05911.

Werner, M. E., Zvara, P., Meredith, A. L., Aldrich, R. W. and Nelson, M. T. (2005) 'Erectile dysfunction in mice lacking the large-conductance calcium-activated potassium (BK) channel.', *The Journal of physiology*. Wiley-Blackwell, 567(Pt 2), pp. 545–56. doi: 10.1113/jphysiol.2005.093823.

White, R. E., Kryman, J. P., El-Mowafy, A. M., Han, G. and Carrier, G. O. (2000) 'cAMP-Dependent Vasodilators Cross-Activate the cGMP-Dependent Protein Kinase to Stimulate BKCa Channel Activity in Coronary Artery Smooth Muscle Cells', *Circulation Research*, 86(8), pp. 897–905. doi:

10.1161/01.RES.86.8.897.

Widmer, H. a, Rowe, I. C. and Shipston, M. J. (2003) 'Conditional protein phosphorylation regulates BK channel activity in rat cerebellar Purkinje neurones', *J Physiol*, 552(Pt 2), pp. 379–91. doi: 10.1113/jphysiol.2003.046441.

Wieland, T. and Attwood, P. V. (2015) 'Alterations in reversible protein histidine phosphorylation as intracellular signals in cardiovascular disease', *Frontiers in Pharmacology*. Frontiers, 6, p. 173. doi: 10.3389/fphar.2015.00173.

Wieland, T., Hippe, H.-J., Ludwig, K., Zhou, X.-B., Korth, M. and Klumpp, S. (2010) 'Reversible Histidine Phosphorylation in Mammalian Cells', in *Methods in Enzymology*, pp. 379–402. doi: 10.1016/S0076-6879(10)71020-X.

Williams, S. E. J., Wootton, P., Mason, H. S., Bould, J., Iles, D. E., Riccardi, D., Peers, C. and Kemp, P. J. (2004) 'Hemoxygenase-2 is an oxygen sensor for a calcium-sensitive potassium channel.', *Science (New York, N.Y.)*, 306(5704), pp. 2093–7. doi: 10.1126/science.1105010.

Willingham, M. C. (2010) 'Fluorescence labeling of intracellular antigens of attached or suspended tissue-culture cells.', *Methods in molecular biology (Clifton, N.J.)*, 588, pp. 153–164. doi: 10.1007/978-1-59745-324-0_17.

Windemuller, F., Xu, J., Rabinowitz, S. S., Hussain, M. M. and Schwarz, S. M. (2016) 'Lipogenesis in Huh7 cells is promoted by increasing the fructose: Glucose molar ratio', *World Journal of Hepatology*, 8(20), p. 838. doi: 10.4254/wjh.v8.i20.838.

Wojtaszewski, J. F., Nielsen, P., Hansen, B. F., Richter, E. A. and Kiens, B. (2000) 'Isoform-specific and exercise intensity-dependent activation of 5'-AMP-activated protein kinase in human skeletal muscle.', *The Journal of physiology*, 528 Pt 1, pp. 221–6.

Won, S., Kim, H. D., Kim, J. Y., Lee, B. C., Chang, S. and Park, C. S. (2010) 'Movements of individual BKCa channels in live cell membrane monitored by site-specific labeling using quantum dots', *Biophysical Journal*. doi: 10.1016/j.bpj.2010.08.049.

Won, S., Lee, B.-C. and Park, C.-S. (2011) 'Functional effects of cytoskeletal components on the lateral movement of individual BK $_{Ca}$ channels expressed in live COS-7 cell membrane', *FEBS Letters*, 585(14), pp. 2323–2330. doi: 10.1016/j.febslet.2011.05.069.

Wong, G. K.-S., Yang, Z., Passey, D. A., Kibukawa, M., Paddock, M., Liu, C.-R., Bolund, L. and Yu, J. (2003) 'A Population Threshold for Functional Polymorphisms', *Genome Research*. Cold Spring Harbor Laboratory Press, 13(8), pp. 1873–1879. doi: 10.1101/GR.1324303.

Woodsmall, R. M. and Benson, D. A. (1993) 'Information resources at the National Center for Biotechnology Information.', *Bulletin of the Medical Library Association*. Medical Library Association, 81(3), pp. 282–4.

Wren, J. D. and Bateman, A. (2008) 'Databases, data tombs and dust in the wind', *Bioinformatics*. Oxford University Press, 24(19), pp. 2127–2128. doi: 10.1093/bioinformatics/btn464.

Wu, J. and Jiang, R. (2013) 'Prediction of deleterious nonsynonymous single-nucleotide polymorphism for human diseases.', *TheScientificWorldJournal*. Hindawi Publishing Corporation, 2013, p. 675851. doi: 10.1155/2013/675851.

Wu, Y., Liu, Y., Hou, P., Yan, Z., Kong, W., Liu, B., Li, X., Yao, J., Zhang, Y., Qin, F., Ding, J., Caterina, M., Schumacher, M., Tominaga, M., Rosen, T., Levine, J., Liu, B., Zhang, C., Qin, F., Schwartz, E., Christianson, J., Chen, X., La, J., Davis, B., Staaf, S., Oerther, S., Lucas, G., Mattsson, J., Ernfors, P., Negri, L., Lattanzi, R., Giannini, E., Colucci, M., Margheriti, F., Alsalem, M., Wong, A., Millns, P., Arya, P., Chan,

M., Nakanishi, M., Hata, K., Nagayama, T., Sakurai, T., Nishisho, T., Gorman, A., Thomas, M., Heyer, C., Lux, H., Li, W., Gao, S., Lv, C., Wu, Y., Guo, Z., Yao, J., Qin, F., Earley, S., Heppner, T., Nelson, M., Brayden, J., Kwan, H., Shen, B., Ma, X., Kwok, Y., Huang, Y., Zimmermann, M., Zhang, X., Aman, K., Hökfelt, T., Yao, J., Liu, B., Qin, F., Wu, Y., Xiong, Y., Wang, S., Yi, H., Li, H., Xia, X., Zeng, X., Lingle, C., Berkefeld, H., Sailer, C., Bildl, W., Rohde, V., Thumfart, J., Prakriya, M., Lingle, C., Jahnel, R., Dreger, M., Gillen, C., Bender, O., Kurreck, J., Mergler, S., Skrzypski, M., Sassek, M., Pietrzak, P., Pucci, C., Tóth, A., Wang, Y., Kedei, N., Tran, R., Pearce, L., Zong, X., Hofmann, F., Fakler, B., Adelman, J., Kostyuk, P., Pronchuk, N., Savchenko, A. and Verkhratsky, A. (2013) 'TRPV1 Channels Are Functionally Coupled with BK(mSlo1) Channels in Rat Dorsal Root Ganglion (DRG) Neurons', *PLoS ONE*. Edited by A. G. Obukhov. Public Library of Science, 8(10), p. e78203. doi: 10.1371/journal.pone.0078203.

Wu, Y., Song, P., Xu, J., Zhang, M. and Zou, M.-H. (2007) 'Activation of protein phosphatase 2A by palmitate inhibits AMP-activated protein kinase.', *The Journal of biological chemistry*, 282(13), pp. 9777–88. doi: 10.1074/jbc.M608310200.

Wulff, H. and Zhorov, B. S. (2008) 'K+ channel modulators for the treatment of neurological disorders and autoimmune diseases.', *Chemical reviews*. NIH Public Access, 108(5), pp. 1744–73. doi: 10.1021/cr078234p.

Xia, X., Ding, J. P. and Lingle, C. J. (1999) 'Molecular Basis for the Inactivation of Ca2+- and Voltage-Dependent BK Channels in Adrenal Chromaffin Cells and Rat Insulinoma Tumor Cells', *J. Neurosci.*, 19(13), pp. 5255–5264.

Xia, X. M., Ding, J. P., Zeng, X. H., Duan, K. L. and Lingle, C. J. (2000) 'Rectification and rapid activation at low Ca2+ of Ca2+-activated, voltage-dependent BK currents: Consequences of rapid inactivation by a novel beta subunit', *Journal of Neuroscience*, 20(13), pp. 4890–4903.

Xie, J. and McCobb, D. P. (1998) 'Control of alternative splicing of potassium channels by stress hormones.', *Science (New York, N.Y.)*, 280(5362), pp. 443–446.

Xue, Y. (2017) 'CSS-Palm 4.0 Cysteine Residue Training Dataset'.

Xue, Y., Liu, Z., Cao, J. and Ren, J. (2010) 'Computational Prediction of Post-Translational Modification Sites in Proteins', *Systems and Computational Biology - Molecular and Cellular Experimental Systems*. InTech, pp. 105–124. doi: 10.5772/18559.

Xue, Y., Liu, Z., Gao, X., Jin, C., Wen, L., Yao, X. and Ren, J. (2010) 'GPS-SNO: Computational Prediction of Protein S-Nitrosylation Sites with a Modified GPS Algorithm', *PLoS ONE*. Edited by V. B. Bajic. Public Library of Science, 5(6), p. e11290. doi: 10.1371/journal.pone.0011290.

Xue, Y. and Ren, J. (2013) 'CSS-Palm Manual: Prediction of Palmitoylation sites', pp. 1–26.

Yan, J. and Aldrich, R. W. (2010) 'LRRC26 auxiliary protein allows BK channel activation at resting voltage without calcium', *Nature*, 466(7305), pp. 513–516. doi: 10.1038/nature09162.

Yan, J. and Aldrich, R. W. (2012) 'BK potassium channel modulation by leucine-rich repeat-containing proteins.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 109(20), pp. 7917–22. doi: 10.1073/pnas.1205435109.

Yan, J., Olsen, J. V, Park, K.-S., Li, W., Bildl, W., Schulte, U., Aldrich, R. W., Fakler, B. and Trimmer, J. S. (2008) 'Profiling the phospho-status of the BKCa channel alpha subunit in rat brain reveals unexpected patterns and complexity.', *Molecular & cellular proteomics : MCP*. American Society for Biochemistry and Molecular Biology, 7(11), pp. 2188–98. doi: 10.1074/mcp.M800063-MCP200.

Yan, L., Nairn, A. C., Palfrey, H. C. and Brady, M. J. (2003) 'Glucose Regulates EF-2 Phosphorylation and Protein Translation by a Protein Phosphatase-2A-Dependent Mechanism in INS-1 Derived 832/13 Cells', *JBC Papers in Press. Published on*.

Yan, W., Wang, Y., Xiao, Y., Wen, J., Wu, J., Du, L. and Cai, W. (2014) 'Palmitate induces TRB3 expression and promotes apoptosis in human liver cells.', *Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology*. Karger Publishers, 33(3), pp. 823–34. doi: 10.1159/000358655.

Yang, C., Zeng, X.-H., Zhou, Y., Xia, X.-M. and Lingle, C. J. (2011) 'LRRC52 (leucine-rich-repeat-containing protein 52), a testis-specific auxiliary subunit of the alkalization-activated Slo3 channel.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 108(48), pp. 19419–24. doi: 10.1073/pnas.1111104108.

Yang, J. (2014) 'Enhanced Skeletal Muscle for Effective Glucose Homeostasis', in *Progress in molecular biology and translational science*, pp. 133–163. doi: 10.1016/B978-0-12-800101-1.00005-3.

Yang, J., Krishnamoorthy, G., Saxena, A., Zhang, G., Shi, J., Yang, H., Delaloye, K., Sept, D. and Cui, J. (2010) 'An epilepsy/dyskinesia-associated mutation enhances BK channel activation by potentiating Ca2+ sensing.', *Neuron*. NIH Public Access, 66(6), pp. 871–83. doi: 10.1016/j.neuron.2010.05.009.

Yang, J., Robert, C. E., Burkhardt, B. R., Young, R. A., Wu, J., Gao, Z. and Wolf, B. A. (2005) 'Mechanisms of Glucose-Induced Secretion of Pancreatic-Derived Factor (PANDER or FAM3B) in Pancreatic β-Cells', *Diabetes*, 54(11).

Yang, Q., Alemany, R., Casas, J., Kitajka, K., Lanier, S. M. and Escribá, P. V. (2005) 'Influence of the Membrane Lipid Structure on Signal Processing via G Protein-Coupled Receptors', *Molecular Pharmacology*, 68(1).

Yang, W., Di Vizio, D., Kirchner, M., Steen, H. and Freeman, M. R. (2009) 'Proteome-scale characterization of human s-acylated proteins in lipid raft-enriched and non-raft membranes.', *Molecular & cellular proteomics : MCP*. American Society for Biochemistry and Molecular Biology, 9(1), pp. 1–53. doi: 10.1074/mcp.M800448-MCP200.

Yang, Y., Wang, Y., Li, S., Xu, Z., Li, H., Ma, L., Fan, J., Bu, D., Liu, B., Fan, Z., Wu, G., Jin, J., Ding, B., Zhu, X. and Shen, Y. (2004) 'Mutations in SCN9A, encoding a sodium channel alpha subunit, in patients with primary erythermalgia.', *Journal of medical genetics*, 41(3), pp. 171–4.

Yasuda, K., Miyake, K., Horikawa, Y., Hara, K., Osawa, H., Furuta, H., Hirota, Y., Mori, H., Jonsson, A., Sato, Y., Yamagata, K., Hinokio, Y., Wang, H.-Y., Tanahashi, T., Nakamura, N., Oka, Y., Iwasaki, N., Iwamoto, Y., Yamada, Y., Seino, Y., Maegawa, H., Kashiwagi, A., Takeda, J., Maeda, E., Shin, H. D., Cho, Y. M., Park, K. S., Lee, H. K., Ng, M. C. Y., Ma, R. C. W., So, W.-Y., Chan, J. C. N., Lyssenko, V., Tuomi, T., Nilsson, P., Groop, L., Kamatani, N., Sekine, A., Nakamura, Y., Yamamoto, K., Yoshida, T., Tokunaga, K., Itakura, M., Makino, H., Nanjo, K., Kadowaki, T. and Kasuga, M. (2008) 'Variants in KCNQ1 are associated with susceptibility to type 2 diabetes mellitus', *Nature Genetics*. Nature Publishing Group, 40(9), pp. 1092–1097. doi: 10.1038/ng.207.

Yen, C.-J., Beamer, B. A., Negri, C., Silver, K., Brown, K. A., Yarnall, D. P., Burns, D. K., Roth, J. and Shuldiner, A. R. (1997) 'Molecular Scanning of the Human Peroxisome Proliferator Activated Receptor γ (hPPARγ) Gene in Diabetic Caucasians: Identification of a Pro12Ala PPARγ2 Missense Mutation', *Biochemical and Biophysical Research Communications*, 241(2), pp. 270–274. doi: 10.1006/bbrc.1997.7798.

Yi, L., Morgan, J. T. and Ragsdale, S. W. (2010) 'Identification of a thiol/disulfide redox switch in the human BK channel that controls its affinity for heme and CO.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 285(26), pp. 20117–27. doi: 10.1074/jbc.M110.116483.

Yoo, Y. J., Sun, L. and Bull, S. B. (2013) 'Gene-based multiple regression association testing for combined examination of common and low frequency variants in quantitative trait analysis', *Frontiers in Genetics*.

Frontiers, 4, p. 233. doi: 10.3389/fgene.2013.00233.

Yool, A. J. and Schwarz, T. L. (1991) 'Alteration of ionic selectivity of a K+ channel by mutation of the H5 region', *Nature*, 349(6311), pp. 700–704. doi: 10.1038/349700a0.

Yu, F. H., Yarov-Yarovoy, V., Gutman, G. A. and Catterall, W. A. (2005) 'Overview of Molecular Relationships in the Voltage-Gated Ion Channel Superfamily', *Pharmacological Reviews*, 57(4).

Yuan, P., Leonetti, M. D., Pico, A. R., Hsiung, Y. and MacKinnon, R. (2010) 'Structure of the Human BK Channel Ca2+-Activation Apparatus at 3.0 Å Resolution', *Science*, 329(5988).

Yuzefovych, L., Wilson, G. and Rachek, L. (2010) 'Different effects of oleate vs. palmitate on mitochondrial function, apoptosis, and insulin signaling in L6 skeletal muscle cells: role of oxidative stress.', *American journal of physiology. Endocrinology and metabolism*. American Physiological Society, 299(6), pp. E1096-105. doi: 10.1152/ajpendo.00238.2010.

Zahid, S., Khan, R., Oellerich, M., Ahmed, N. and Asif, A. R. (2014) 'Differential S-nitrosylation of proteins in Alzheimer's disease', *Neuroscience*, 256, pp. 126–136. doi: 10.1016/j.neuroscience.2013.10.026.

Zarei, M. M., Eghbali, M., Alioua, A., Song, M., Knaus, H.-G., Stefani, E. and Toro, L. (2004) 'An endoplasmic reticulum trafficking signal prevents surface expression of a voltage- and Ca2+-activated K+ channel splice variant.', *Proceedings of the National Academy of Sciences of the United States of America*, 101(27), pp. 10072–7. doi: 10.1073/pnas.0302919101.

Zarei, M. M., Zhu, N., Alioua, A., Eghbali, M., Stefani, E. and Toro, L. (2001) 'A Novel MaxiK Splice Variant Exhibits Dominant-negative Properties for Surface Expression', *Journal of Biological Chemistry*, 276(19), pp. 16232–16239. doi: 10.1074/jbc.M008852200.

Zarrei, M., MacDonald, J. R., Merico, D. and Scherer, S. W. (2015) 'A copy number variation map of the human genome', *Nature Reviews Genetics*. Nature Research, 16(3), pp. 172–183. doi: 10.1038/nrg3871.

Zhang, D.-M., He, T., Katusic, Z. S., Lee, H.-C. and Lu, T. (2010) 'Muscle-specific f-box only proteins facilitate bk channel β(1) subunit downregulation in vascular smooth muscle cells of diabetes mellitus.', *Circulation research*, 107(12), pp. 1454–9. doi: 10.1161/CIRCRESAHA.110.228361.

Zhang, F., Gu, W., Hurles, M. E. and Lupski, J. R. (2009) 'Copy number variation in human health, disease, and evolution.', *Annual review of genomics and human genetics*. NIH Public Access, 10, pp. 451–81. doi: 10.1146/annurev.genom.9.081307.164217.

Zhang, F. and Lupski, J. R. (2015) 'Non-coding genetic variants in human disease.', *Human molecular genetics*. Oxford University Press, 24(R1), pp. R102-10. doi: 10.1093/hmg/ddv259.

Zhang, G. and Horrigan, F. T. (2005) 'Cysteine modification alters voltage- and Ca(2+)-dependent gating of large conductance (BK) potassium channels.', *The Journal of general physiology*. The Rockefeller University Press, 125(2), pp. 213–36. doi: 10.1085/jgp.200409149.

Zhang, X., Huang, J., McNaughton, P. A., Barik, S., Galinski, M., Besson, J., Chaouch, A., Bessou, P., Perl, E., Bonnington, J., McNaughton, P., Caterina, M., Leffler, A., Malmberg, A., Martin, W., Trafton, J., Petersen-Zeitz, K., Koltzenburg, M., Basbaum, A., Julius, D., Caterina, M., Rosen, T., Tominaga, M., Brake, A., Julius, D., Caterina, M., Schumacher, M., Tominaga, M., Rosen, T., Levine, J., Julius, D., Cesare, P., Dekker, L., Sardini, A., Parker, P., McNaughton, P., Cesare, P., McNaughton, P., Cho, C., Song, W., Leitzell, K., Teo, E., Meleth, A., Quick, M., Lester, R., Chuang, H., Prescott, E., Kong, H., Shields, S., Jordt, S., Basbaum, A., Chao, M., Julius, D., Davis, J., Gray, J., Gunthorpe, M., Hatcher, J., Davey, P., Overend, P., Harries, M., Latcham, J., Clapham, C., Atkinson, K., Hughes, S., Rance, K., Grau, E., Harper, A., Pugh, P., Rogers, D., Bingham, S., Randall, A., Sheardown, S., Frank, C., Burkhardt, C., Imhof, D., Ringel, J.,

Zschornig, O., Wieligmann, K., Zacharias, M., Bohmer, F., Galoyan, S., Petruska, J., Mendell, L., Gamper, N., Stockand, J., Shapiro, M., Hayashi, T., Huganir, R., Jahnel, R., Dreger, M., Gillen, C., Bender, O., Kurreck, J., Hucho, F., Jin, X., Morsy, N., Winston, J., Pasricha, P., Garrett, K., Akbarali, H., Jordt, S., Tominaga, M., Julius, D., Kaplan, D., Miller, F., Martin, K., Steinberg, T., Cooley, L., Gee, K., Beechem, J., Patton, W., Morenilla-Palao, C., Planells-Cases, R., Garcia-Sanz, N., Ferrer-Montiel, A., Numazaki, M., Tominaga, T., Toyooka, H., Tominaga, M., Obermeier, A., Lammers, R., Wiesmuller, K., Jung, G., Schlessinger, J., Ullrich, A., Pawson, T., Gish, G., Nash, P., Premkumar, L., Ahern, G., Prescott, E., Julius, D., Shah, B., Catt, K., Shiratori, T., Miyatake, S., Ohno, H., Nakaseko, C., Isono, K., Bonifacino, J., Saito, T., Shu, X., Mendell, L., Shu, X., Mendell, L., Tominaga, M., Wada, M., Masu, M., Buren, J. Van, Bhat, S., Rotello, R., Pauza, M., Premkumar, L., Vellani, V., Mapplebeck, S., Moriondo, A., Davis, J., McNaughton, P., Vriens, J., Watanabe, H., Janssens, A., Droogmans, G., Voets, T., Nilius, B., Wang, W., Lin, D., Sterling, H., Wennstrom, S., Downward, J., Woodbury, C., Zwick, M., Wang, S., Lawson, J., Caterina, M., Koltzenburg, M., Albers, K., Koerber, H., Davis, B., Xu, H., Zhao, H., Tian, W., Yoshida, K., Roullet, J., Cohen, D., Zhao, Z., Tan, Z., Diltz, C., You, M., Fischer, E., Zhuang, Z., Xu, H., Clapham, D. and Ji, R. (2005) 'NGF rapidly increases membrane expression of TRPV1 heat-gated ion channels.', *The EMBO journal*. EMBO Press, 24(24), pp. 4211–23. doi: 10.1038/sj.emboj.7600893.

Zhang, X., Solaro, C. R. and Lingle, C. J. (2001) 'Allosteric regulation of BK channel gating by Ca(2+) and Mg(2+) through a nonselective, low affinity divalent cation site.', *The Journal of general physiology*, 118(5), pp. 607–36.

Zhao, N., Han, J. G., Shyu, C.-R. and Korkin, D. (2014) 'Determining effects of non-synonymous SNPs on protein-protein interactions using supervised and semi-supervised learning.', *PLoS computational biology*. Public Library of Science, 10(5), p. e1003592. doi: 10.1371/journal.pcbi.1003592.

Zhao, Q., Xie, Y., Zheng, Y., Jiang, S., Liu, W., Mu, W., Liu, Z., Zhao, Y., Xue, Y. and Ren, J. (2014) 'GPS-SUMO: A tool for the prediction of sumoylation sites and SUMO-interaction motifs', *Nucleic Acids Research*. Oxford University Press, 42(W1), pp. W325-30. doi: 10.1093/nar/gku383.

Zhao, X., Li, G. and Liang, S. (2013) 'Several affinity tags commonly used in chromatographic purification.', *Journal of analytical methods in chemistry*. Hindawi Publishing Corporation, 2013, p. 581093. doi: 10.1155/2013/581093.

Zhao, Y., Wieman, H. L., Jacobs, S. R. and Rathmell, J. C. (2008) 'Mechanisms and methods in glucose metabolism and cell death.', *Methods in enzymology*. NIH Public Access, 442, pp. 439–57. doi: 10.1016/S0076-6879(08)01422-5.

Zheng, Z. and Diamond, M. I. (2012) 'Huntington Disease and the Huntingtin Protein', in *Progress in molecular biology and translational science*, pp. 189–214. doi: 10.1016/B978-0-12-385883-2.00010-2.

Zhong, Y., Zhong, Y., Wang, J., Wang, J., Liu, W., Liu, W., Zhu, Y. and Zhu, Y. (2013) *Molecular medicine reports.*, *Molecular Medicine Reports*. D.A. Spandidos.

Zhou, F.-F., Xue, Y., Chen, G.-L. and Yao, X. (2004) 'GPS: a novel group-based phosphorylation predicting and scoring method', *Biochemical and Biophysical Research Communications*, 325(4), pp. 1443–1448. doi: 10.1016/j.bbrc.2004.11.001.

Zhou, F., Xue, Y., Yao, X. and Xu, Y. (2006) 'CSS-Palm: palmitoylation site prediction with a clustering and scoring strategy (CSS).', *Bioinformatics (Oxford, England)*, 22(7), pp. 894–6. doi: 10.1093/bioinformatics/btl013.

Zhou, X.-B., Wulfsen, I., Utku, E., Sausbier, U., Sausbier, M., Wieland, T., Ruth, P. and Korth, M. (2010) 'Dual role of protein kinase C on BK channel regulation.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 107(17), pp. 8005–10. doi: 10.1073/pnas.0912029107.

Zhou, X., Wulfsen, I., Korth, M., McClafferty, H., Lukowski, R., Shipston, M. J., Ruth, P., Dobrev, D. and Wieland, T. (2012) 'Palmitoylation and membrane association of the stress axis regulated insert (STREX) controls BK channel regulation by protein kinase C.', *The Journal of biological chemistry*. American Society for Biochemistry and Molecular Biology, 287(38), pp. 32161–71. doi: 10.1074/jbc.M112.386359.

Zoltewicz, S. J., Lee, S., Chittoor, V. G., Freeland, S. M., Rangaraju, S., Zacharias, D. A. and Notterpek, L. (2012) 'The palmitoylation state of PMP22 modulates epithelial cell morphology and migration.', *ASN neuro*. SAGE Publications, 4(6), pp. 409–21. doi: 10.1042/AN20120045.

Zou, S., Jha, S., Kim, E. Y. and Dryer, S. E. (2008) 'A Novel Actin-Binding Domain on Slo1 Calcium-Activated Potassium Channels Is Necessary for Their Expression in the Plasma Membrane', *Molecular Pharmacology*, 73(2).

Zu, X.-L., Besant, P. G. and Attwood, P. V. (2008) 'Chapter 14 Protein Histidine Phosphorylation', *Comprehensive Analytical Chemistry*, 52, pp. 315–352. doi: 10.1016/S0166-526X(08)00214-6.