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Nuclear envelope transmembrane proteins as mediators of tissue-specific diseases

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Thesis presented for the degree of Doctor of Philosophy



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Declaration

I declare that this thesis has been composed by myself and the work presented herein is my own, except where stated otherwise. This research has not been submitted for any other degree except as specified.

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September 2016

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Lay abstract

Nuclear envelope (NE) linked diseases that range from neuropathy to muscular dystrophies to fat and bone disorders are a conundrum because the mutated proteins are ubiquitously expressed yet pathology is focused in particular tissues. The recent discovery of many tissue-specific NE proteins has led to the hypothesis that the tissue-specific pathologies in NE-linked diseases is due in part to tissue-specific NE proteins interacting aberrantly with the mutated widely expressed proteins to mediate disease pathologies. Furthermore, NE linked muscle disorders often affect different distinct muscle groups. How specific muscle groups are affected is still unknown. It is postulated that if a NE protein were to contribute to muscle disease pathology, it will be specific for the muscle set affected. Such tissue-specific NE proteins could also be candidates for additional alleles in unlinked patients with similar presentations and indeed our preliminary sequencing data on unlinked Emery-Dreifuss muscular dystrophy patients suggests this to be the case. This study generated antibodies to several muscle-specific NE proteins and propose to test these on both control and muscular dystrophy patient cells to determine if they are aberrantly distributed in patient cells. Previous studies on NE-linked muscular dystrophies have revealed that several NE proteins involved in NE-cytoskeletal connections are aberrantly targeted in patient cells. If these tissue-specific NE proteins are similarly mislocalised, this could prove to be a valuable diagnostic tool for muscle diseases that often have overlapping presentations and could pave the way for further mechanistic studies that could result in therapeutic interventions. This study also looks at the interaction between NETs and lamin A using a binding assay.

Abstract

Many tissue-restricted diseases are linked to mutations in lamins and nuclear envelope transmembrane proteins (NETs). How these mutations in ubiquitously expressed proteins cause such defined diseases is still unknown. It is hypothesized that tissue restricted NETs that are partners of the nuclear lamins/existing linked proteins mediate tissue-specific disease pathologies. Proteomic studies have identified many tissue restricted NETs with effects on the cytoskeleton, gene positioning and regulation. This study investigates potential roles of candidate NETs in mediating tissue restricted disease pathology and their interactions with known factors such as emerin and lamins, mutations in which have been linked to a variety of tissue-specific dystrophies. This study looks into candidate tissue-specific NETs distribution in human tissues and in vitro using a solid phase binding assay to study candidate NETs interactions. I confirmed the tissue-specificity of the candidate NETs in human and mouse tissue sections but did not find clear reproducible distribution of these NETs in patient tissue biopsy. One postulate is that NETs bind WT lamin for localisation and/or function and disruption of this interaction leads to disease. Using a solid phase binding assay approach to study NETs/lamin interactions, we demonstrate that Tmem120a, an adipocyte-specific NET binds WT lamin but has a reduced Bmax when tested for binding against a lipodystrophy causing lamin mutant (R482Q and G465D). This is consistent with the hypothesis that tissue-specific NET partners might mediate tissue-specific disease pathology in lamin-linked nuclearenvelopathies.

Table of contents

Declaration	
Acknowledgements	
Lay abstract	
Abstract.....	i
Table of contents	2
List of figures	7
List of tables	10
List of abbreviations.....	11
Chapter 1.....	13
Introduction	13
1.1. Diseases of the nuclear envelope	16
1.2. Lamins and diseases	18
1.3. Protein diversity at the nuclear membrane	22
1.4. Mechanisms underlying pathology	24
1.5. Mechanical stress and NETs.....	26
1.6. Summary	29
Chapter 2.....	32
Materials and Methods	32
2.1. Materials	32

2.1.1.	Bacterial strains and genotypes	32
2.1.2.	Vectors.....	33
2.1.3.	Buffers and solutions.....	33
2.1.4.	Primary antibodies	37
2.1.5.	Secondary antibodies.....	39
2.1.6.	Affinity matrixes.....	40
2.1.7.	Mammalian cells	40
2.1.8.	Tissue sections	43
2.1.9.	Kits.....	44
2.2.	Mammalian cell culture.....	45
2.2.1.	Cell maintenance	45
2.2.2.	Primary human myoblast differentiation	45
2.2.3.	Production of monoclonal antibody from hybridoma cells.....	45
2.3.	Nucleic acid methods	46
2.3.1.	Sequencing of plasmid DNA	46
2.3.2.	Site-directed mutagenesis.....	46
2.4.	Microscopy methods	47
2.4.1.	Immunofluorescence on cultured cells	47
2.4.2.	Microscopy and analysis	49
2.5.	Protein methods.....	49
2.5.1.	Total protein extraction from mammalian cells	49

2.5.2.	Western blotting	50
2.5.3.	Lamin purification from inclusion bodies.....	51
2.5.4.	MBP-tagged protein purification	51
2.5.5.	GST-tagged protein purification	52
2.5.6.	HIS-tagged protein purification.....	52
2.5.7.	Affinity purification of antibodies.....	53
2.5.8.	Size exclusion chromatography	54
2.6.	Protein interaction methods.....	54
2.6.1.	Solid phase binding assay.....	54
2.6.2.	Surface Plasmon Resonance (SPR)	55
Chapter 3.....		56
Introduction		56
3.1.	Confirming expression of NETs in muscle nuclei and comparison between human muscle groups	59
3.2.	Comparison between mouse muscle groups.....	64
3.3.	Results summary	70
Chapter 4.....		71
Introduction		71
4.1.	EDMD variants.....	75
4.2.	EDMD mechanisms	79
4.3.	Distribution of EDMD-linked proteins in cultured patient muscle cells.....	83

4.4.	Distribution of muscle-specific NETs in cultured EDMD patient myotubes..	90
4.5.	Distribution of muscle-specific NETs in EDMD patient skeletal muscle sections	95
4.6.	Results summary	100
Chapter 5.....		104
Tissue-specific NETs interactions with lamin A.....		104
Introduction		104
5.1.	Lamin functions.....	105
5.2.	Lamin A, diseases and tissue-specific interaction postulate	106
5.3.	Known lamin-associated proteins.....	109
5.4.	Designing a solid phase binding assay to measure interactions between lamins and nucleoplasmic regions of NETs	111
5.5.	GST tag shows high unspecific background binding to WT lamin A	116
5.6.	Transfer of NET fragments into MBP tag vector and background testing.	117
5.7.	Tmem120A has reduced binding to lipodystrophic mutants.....	120
5.8.	Surface Plasmon Resonance revealed two step binding of NETs to lamin A	122
5.9.	Size exclusion chromatography revealed large protein complexes.....	127
5.10.	Qualitative analysis of the solid phase binding assay results.	134
5.11.	Results summary.....	138
Chapter 6.....		140

Discussion.....	140
6.1. Tissue-specificity of NETs	140
6.2. Problems of working with NETs fragments.....	143
6.3. Interactions between NETs and lamins	144
6.4. Future directions	145
6.5. Final remarks	148

List of figures

Figure 1. Schematic of the nuclear envelope.....	15
Figure 2. Networks within the cell.....	21
Figure 3. Distribution of NETs identified in different tissues.....	22
Figure 4. Affected muscle groups in different disorders can be caused by mutations in the same gene.	56
Figure 5. Muscle anatomy of the human body. Diagram showing the muscle groups present in the human body.	58
Figure 6. Staining of NETs identified in the proteomic studies in Bicep Brachii.....	61
Figure 7. Staining of NETs identified in the proteomic studies in Vastus Lateralis.	62
Figure 8. Staining of NETs identified in the proteomic studies in Gastrocnemius.	63
Figure 9. Staining of NETs identified in the proteomic studies in mouse Gastrocnemius.	65
Figure 10. Staining of NETs identified in the proteomic studies in mouse Tibialis Anteria.	66
Figure 11. Staining of NETs identified in the proteomic studies in mouse Diaphragm.	67
Figure 12. Staining of NETs identified in the proteomic studies in mouse Extensor Digitorum Longus.	68
Figure 13. Staining of NETs identified in the proteomic studies in mouse Musculus Soleus.....	69
Figure 14. Staining of NETs identified in the proteomic studies in a muscular dystrophic mouse Gastrocnemius.	70
Figure 15. The current known network of the cell.....	82
Figure 16. Staining of control (A) and patient (B) myoblasts with antibodies to EDMD-linked proteins.	86
Figure 17. Staining of myoblast/fibroblast cultures and differentiated myotubes with nesprin antibodies.....	89
Figure 18. Testing of antibodies for muscle NETs. C2C12 cells were transduced with GFP fusions to the NETs or GFP alone and these cells were divided into two populations.	92

Figure 19. Staining of muscle NETs in patient myoblast/fibroblast cultures where myoblasts were induced to differentiate into myotubes.	94
Figure 20. Muscle NET antibody staining in patient skeletal muscle sections.	98
Figure 21. Schematic of the binding assay used in this study.....	112
Figure 22. Purification of lamins and GST-tagged Tmem120A.	116
Figure 23. Purification of MBP tagged NET fragments and testing of background binding to WT lamin.	119
Figure 24. Tmem120A has a lower Bmax against lipodystrophic mutants of lamin A.	121
Figure 25. Response curve of emerin-MBP binding to immobilised WT HIS-lamin A.	123
Figure 26. Response curve of emerin-MBP binding to immobilised HIS-lamin A delta coil.	124
Figure 27. Response curve produced by Tmem120A-MBP binding to immobilised WT HIS-lamin A and lipodystrophic mutants (HIS-G465D and HIS-R482Q).	125
Figure 28. Response curve produced by Tmem38A-MBP binding to immobilised WT HIS-lamin A and muscular dystrophic mutants (HIS-R377H and HIS-R527H).	126
Figure 29. Size exclusion chromatography of emerin-MBP over a superdex 200 column in TBS.	129
Figure 30. Size exclusion chromatography of Tmem120A-MBP over a superdex 200 column in TBS. ...	130
Figure 31. Size exclusion chromatography of emerin-MBP (*) over a superpose6 column in low salt buffer (20mM Tris, 30mM NaCl).	131
Figure 32. Size exclusion chromatography of emerin-MBP (*) over a superpose6 column in detergent containing buffer (20mM Tris, 200mM NaCl, 0.03% TX-100).	132
Figure 33. Size exclusion chromatography of emerin-MBP (*) over a superpose6 column in low pH buffer (10mM NaHPO ₄ , pH6.3).....	133
Figure 34. Qualitative analysis of multiple experiments of Tmem120A-MBP binding to immobilised HIS-lamin A WT and lipodystrophic mutants (G465D and R482Q).	135

Figure 35. Qualitative analysis of multiple experiments of Tmem120A-MBP binding to immobilised HIS-lamin A WT and other non-lipodystrophic mutants revealed no significant differences.136

Figure 36. One-way ANOVA analysis of Tmem38A-MBP binding to HIS-lamin A WT and disease mutants revealed no significant differences.137

List of tables

Table 1. Functional screen of NETs carried out by the Schirmer lab. Muscle NETs are highlighted in the red boxes.....	31
Table 2. Bacterial strains and genotypes	32
Table 3. Vectors used in the study.....	33
Table 4. Buffer used and composition	33
Table 5. Primary antibodies used in this study (IF – Immunofluorescence, WB – Western Blot).....	37
Table 6. Secondary antibodies used in this study	39
Table 7. Affinity matrixes used in this study.....	40
Table 8. Cell lines used in this study	40
Table 9. Tissue sections used in this study.....	43
Table 10. Kits used in this study	44
Table 11. List of mutagenesis primers used in this study	46
Table 12. Patient and control myoblast/fibroblast cultures used in this study	84
Table 13. Tissue sections used in this study.....	96
Table 14. Primary antibodies used in this study	99
Table 15. Mutations in lamin A causes a wide range of disorders	108
Table 16. List of disease mutations generated in this study	114
Table 17. Protein used and their expected molecular weight	118

List of abbreviations

°C	Degrees Celsius	Min	Minutes
AD-EDMD	Autosomal Dominant EDMD	MBP	Maltose Binding Protein
AR-EDMD	Autosomal Recessive EDMD	MTOC	Microtubule Organising Centre
BAF	Barrier to Autointergration Factor	NE	Nuclear Envelope
cDNA	Complementary DNA	NET	Nuclear Envelope Transmembrane protein
DAPI	4,6-diamidino-2-phenylindole, dihydrochloride	NPC	Nuclear Pore Complex
ECM	Extracellular Matrix	NaAz	Sodium Azide
EDMD	Emery-Dreifuss Muscular Dystrophy	Nesprin	Nuclear Envelope Spectrin Repeat Proteins
EMD	Emerin	ONM	Outer Nuclear Membrane
EPPF	Edinburgh Protein Purification Facility	PBS	Phosphate Buffered Saline
FBS	Foetal Bovine Serum	pRb	Retinoblastoma protein
FHL1	Four and Half LIM 1	RPM	Rotations Per Minutes
FXR1P	Fragile X related Protein 1	RT	Room Temperature
FPLD	Familial Partial Lipodystrophy	S	Seconds
GST	Glutathione S-transferase	SAMP1	Spindle-Associated Membrane Protein 1
HRP	Horseradish Peroxidase	siRNA	Short inhibitory RNA
H	Hours	SPR	Surface Plasmon Resonance
INM	Inner Nuclear Membrane	SREBP1	Sterol Regulatory Element-Binding Protein 1
IPTG	Isopropyl β -D-1-thiogalactopyranoside	SUN2	Sad1 And UNC84 Domain Containing 2
ITC	Isothermal Titration Calorimetry	TAN	Transmembrane Cctin-associated Nuclear
KASH	Klarsicht, ANC-1, Syne Homology	TBS	Tris Buffered Saline
LAP	Lamin Associated Polypeptide	Tmem	Transmembrane protein
LBR	Lamin B Receptor	TRIC-A	Trimeric Cation channel protein A
LINC	Linker of Nucleoskeleton and Cytoskeleton	TX-100	Triton X-100
LGMD	Limb Girdle Muscular Dystrophy	WT	Wild Type
LMNA	Lamin A	XMPMA	X-linked Myopathy with Postural Muscle Atrophy
LMNB	Lamin B		

LMNC
MAD

Lamin C
Mandibuloacral Dysplasia

Chapter 1

Introduction

The nuclear envelope (NE) (Fig. 1) is a double layer membrane, which separates the nucleoplasmic and cytoplasmic contents. It consists of the inner and outer nuclear membranes (INM/ONM), each with their own unique set of proteins and studded with nuclear pore complexes (NPC) (Hetzer 2010). Functionally, the NE has been linked to DNA damage repair, cell cycle regulation, gene regulation, cytoskeleton connections and cellular stress responses (Bermejo, Kumar, and Foiani 2012, Malhas and Vaux 2011, Srsen, Korfali, and Schirmer 2011, Tapley and Starr 2013, Van de Vosse et al. 2011). The protein diversity at the NE has long been thought to be uniform and ubiquitous across all tissues; as such it has presented a dilemma for the study of tissue-specific or tissue-restricted diseases commonly termed laminopathies or nuclear envelopopathies such as muscular dystrophies, lipodystrophies and progeria. These diseases have been linked to certain Nuclear Envelope Transmembrane proteins (NETs), and the nuclear lamina, both of which are widely expressed in most tissues (Worman, Ostlund, and Wang 2010). Further compounding the issue, mutations in one protein can cause multiple disorders (Worman, Ostlund, and Wang 2010). Another caveat of laminopathies or nuclear envelopopathies is that mutations in multiple interacting proteins can cause variations of the same disorders. An example of this is cardiomyopathy with conduction defects, which was first linked to mutations in lamin A/C, but is also linked to mutations in lamin associated polypeptide 2 α (LAP2- α) (Taylor et al. 2005), a known interacting partner of lamin A (Dechat et al. 2000). Another disorder with multiple variants is Emery-Dreifuss muscular dystrophy (EDMD), for which genetic variants have been linked to emerin,

lamins, four and half limb 1 (FHL1), LUMA and the Linker of the Nucleoskeleton and Cytoskeleton (LINC) complex members (Mejat and Misteli 2010, Meinke, Nguyen, and Wehnert 2011, Meinke et al. 2014). How these mutated proteins cause such a wide range of tissue-restricted disorders is still largely unknown. Mechanisms that attempt to explain the specific nature conundrum include mechanical stress, gene regulation and failure of stem cell maintenance. Currently, no one mechanism can fully explain the different pathologies and the fact that many of these disorders are linked to protein complexes suggests the hypothesis that:

Tissue-restricted partner proteins may mediate the tissue-restricted pathologies of nuclear envelopathies.

In this chapter, I will describe what is known about existing NETs and their functions and the pathology of laminopathies or nuclear envelopathies. I will present the data currently available about novel, tissue restricted NETs and their potential to mediate tissue restricted disorders. Finally, I will discuss the intricacy of NET interactions with each other and proteins associated with the NE and how they fit in with current models of disease pathologies.

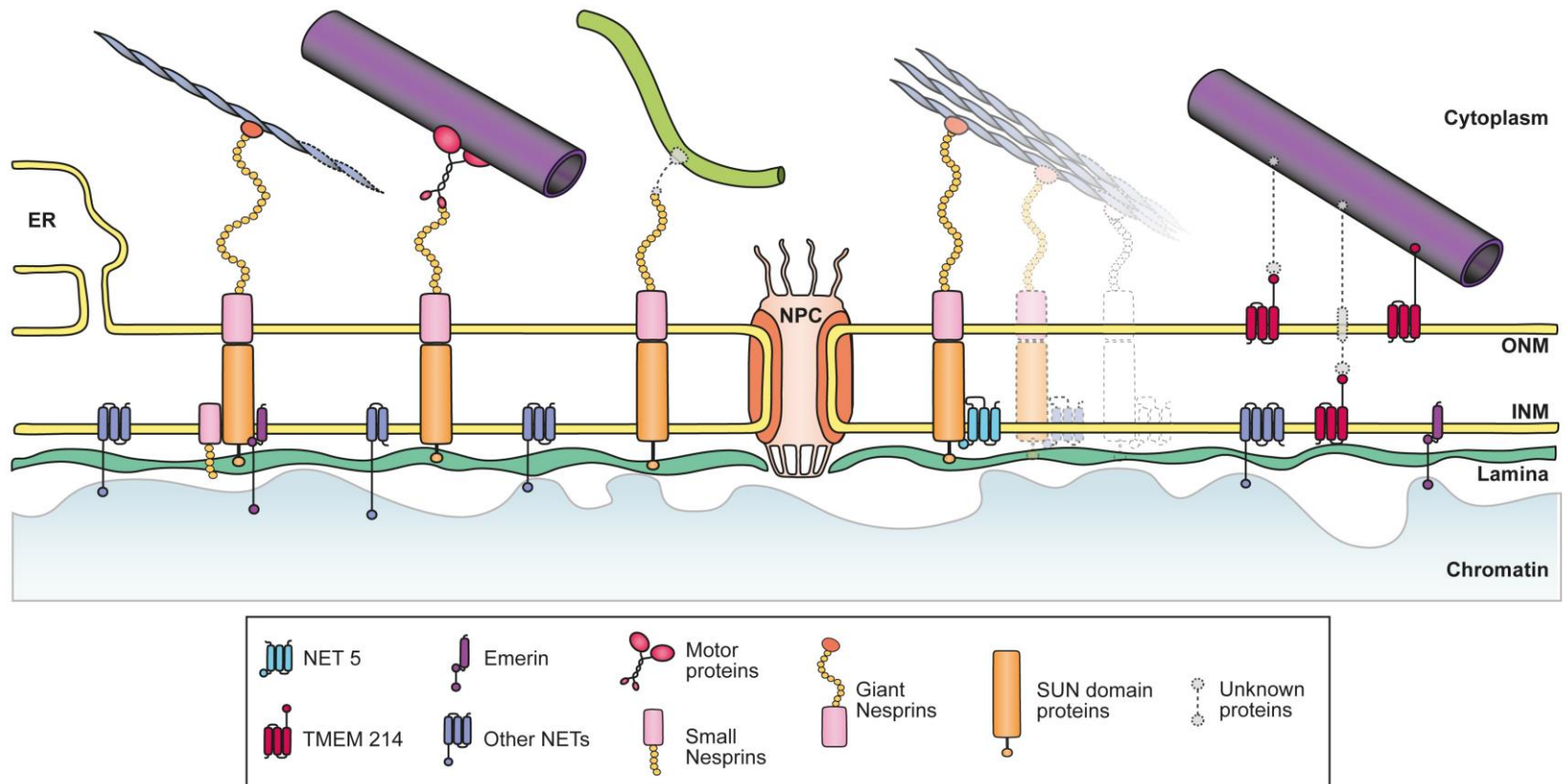


Figure 1. Schematic of the nuclear envelope. The nuclear envelope is studded with NETs which have specific localization at the inner or outer NE. Some NETs such as nesprins and SUNs form the LINC complex which link the nucleoskeleton to the cytoskeleton. Nesprin proteins in the outer nuclear membrane (ONM) connect directly or indirectly to a variety of cytoplasmic filament systems (from left to right) mainly, actin, tubulin, and intermediate filaments (IFs). They also connect to more specialized structures such as on the right side of the NPC, TAN lines, and other nuclear envelope transmembrane (NET) proteins also appear to have interactions with cytoskeletal filaments (furthest right depiction) (Peter Meinke 2015).

1.1. Diseases of the nuclear envelope

The largest organelle in a cell is the nucleus. The nucleus contains the DNA content of a cell, separated from the surrounding cytoplasm by a double layer lipid membrane, the nuclear envelope (NE). This is supported by a meshwork of protein termed the nuclear lamina. This meshwork provides the structural support for the NE. Surrounding the NE is the cytoplasm containing cellular organelles, positioning of which is determined by the microtubule network. The microtubule network, along with the actin and intermediate filaments make up most what is called the cytoskeleton. This group of protein filaments are responsible for cellular integrity. The intermediate filaments provide the mechanical strength while the other two filament types provide the dynamic functions. The actin filaments work in conjunction with another protein fibre called myosin to facilitate cell contraction, which is important for functions such as cytokinesis and cell motility. The cytoskeleton also provides a mean to which the nucleus is connected to the plasma membrane and the extracellular matrix (Figure 2) (Crisp et al. 2006, Kaminski, Fedorchak, and Lammerding 2014). This study will focus on the nuclear envelope and its protein complements.

The NE and associated proteins have been linked to many diseases (termed “nuclear envelopopathies/laminopathies), with a wide range of tissue-specific phenotypes linked to ubiquitously expressed proteins. Many of the proteins thus far linked to NE diseases are ubiquitously expressed. It has been previously thought that mutations in one gene cause one disease. However, this is not true, and perhaps the best example of this is lamin A (*LMNA*). Also, known as type V intermediate filament proteins (Burke and Stewart 2013), nuclear lamins play an important role in maintaining NE stability. There are two types of lamins, type A and type B. The majority of A-type lamins consists of lamin A and lamin

C, both encoded by the *LMNA* gene. B-type lamins consist mainly of lamin B1 and B2, encoded by two separate genes *LMNB1* and *LMNB2*. Lamin C2 and lamin B3 belong to A-type and B-type lamins respectively, but are testis specific and will not be discussed in this report. Lamins have been shown to interact with NE associated proteins (Wilson and Foisner 2010) with potential roles in NE assembly and disassembly (Goldman et al. 2002). The first two reports of a human disease caused by *LMNA* mutations were an autosomal dominant and a recessively inherited form of Emery-Dreifuss muscular dystrophy (EDMD) (Bonne et al. 1999, Raffaele Di Barletta et al. 2000). EDMD pathologies include early contracture of the elbows, Achilles tendons, rigidity of the spine, progressive muscle degradation in the upper arms and lower legs, and dilated cardiomyopathy with conduction defects (Emery 2000). Soon after, *LMNA* mutations were identified as the cause of two other diseases, dilated cardiomyopathy 1A and limb-girdle muscular dystrophy (LGMD) (Fatkin et al. 1999, Muchir et al. 2000). Unlike EDMD however, LGMD patients lack the early joint contractures that are characteristic of EDMD, and in dilated cardiomyopathy 1A patients, skeletal muscle is either minimally affected or unaffected. Subsequently, *LMNA* mutations have been linked to Dunnigan-type familial partial lipodystrophy affecting fatty tissues (Cao and Hegele 2000, Shackleton et al. 2000, Speckman et al. 2000). The picture was further complicated by the discovery of *LMNA* mutations in an autosomal recessive form of Charcot-Marie-Tooth disease type 2, a hereditary motor and sensory neuropathy (De Sandre-Giovannoli et al. 2002). Mutations in *LMNA* have also been identified in autosomal recessive mandibuloacral dysplasia (MAD), a developmental disease that affects specific bones, but also has aspects of dermopathy and lipodystrophy (Novelli et al. 2002). Eriksson et al. 2003 also identified 11 mutations in *LMNA* that cause Hutchinson-Gilford progeria syndrome, a premature

aging disease. These represent some of the major diseases that are caused by mutations in *LMNA* nuclear envelopathies (Eriksson et al. 2003).

Mutations in other genes encoding NE proteins have also been linked to nuclear envelopathies. One of the most well-known NE proteins linked to envelopathies is emerin, which was first reported in X-linked EDMD (Bione et al. 1994, Bione et al. 1995). Emerin principally localizes at the INM (Manilal et al. 1996, Nagano et al. 1996) and interacts with lamin A. This interaction was shown to be important for localization of emerin at the NE (Clements et al. 2000, Fairley, Kendrick-Jones, and Ellis 1999, Sullivan et al. 1999). The range of diseases caused by mutations in the gene encoding emerin (*EMD*), has been reported to be much wider than the original reported classical EDMD phenotype, which includes variants of EDMD and LGMD, along with dilated cardiomyopathy (Astejada et al. 2007). Genetic variants of EDMD have been linked to emerin, lamins, FHL1, LUMA and the LINC complex members (Mejat and Misteli 2010, Meinke, Nguyen, and Wehnert 2011, Meinke et al. 2014). The LINC complex acts as an important bridge between the cytoskeleton and nucleoskeleton. This connection is required for mechanosensing and signal transduction between the extracellular matrix and the nucleus. Disorders linked to the NE are varied and further complicated by genetic variants caused by interacting proteins.

1.2. Lamins and diseases

Lamins are type V-intermediate filaments (Steinert and Roop 1988). In mammals, there are four major forms of lamins; lamin A, C, B1 and B2, encoded by the *LMNA*, *LMNB1* and *LMNB2* genes with lamin C being a splice variant encoded by *LMNA*. Lamins are expressed in most mammalian cells in various combinations, however, lamin A is mainly

expressed in differentiated cells (Lehner et al. 1987, Rober, Weber, and Osborn 1989, Stewart and Burke 1987). Lamins have different properties during mitosis, the A-type lamins remain soluble, while the B-types remain associated with membranes (Gerace, Blum, and Blobel 1978, Moir et al. 2000). Lamin A and C have been shown *in vitro* to be able to form heterodimers, but have only been found as homodimers *in vivo* (Schirmer, Guan, and Gerace 2001, Kolb et al. 2011).

Among nuclear envelopathies, *LMNA* is the gene mutated most frequently with hundreds of reported mutations (<http://www.umd.be/LMNA>). Mutations in *LMNA* have been linked to many diseases, including muscular dystrophies, lipodystrophies, neuropathies and one of the most notable disorders, Hutchinson Gilford Progeria (Worman and Bonne 2007). Mutations in *LMNA* have been shown to interfere with folding, stability and assembly of the protein (Bank et al. 2011, Bank et al. 2012, Wiesel et al. 2008, Ben-Harush et al. 2009, Bollati et al. 2012). These disruptions may contribute to the pathological mechanism(s) from which tissue-specific diseases arise.

Lamins have many roles. Firstly, they form the structural components of the nucleoskeleton. Loss of or mutation of lamins have been shown to cause nuclear deformation (Lammerding et al. 2005). Indeed, change in nuclear shape is one of the effects of lamin mutations that leads to disease (Davidson and Lammerding 2014). The ratio of lamins is important for cellular properties and functions. Lamin A overexpression has been shown to increase nuclear stiffness and impair migration (Rowat et al. 2013, Harada et al. 2014). Conversely, nuclear fragility increases when lamin A is lost or reduced leading to increase in an nuclear rupture (De Vos et al. 2011, Hatch et al. 2013, Vargas et al. 2012).

Lamins also play a crucial role in mechanosignal transduction between the extracellular matrix (ECM) and the nucleus, via the LINC complex (Lombardi et al. 2011). It has been shown that interactions between the ECM and the nucleus affect levels of lamins and the structural properties of the cell and nucleus. Lamin regulation is important as the levels of the different isoforms are linked to cell fate and differentiation (Swift et al. 2013). Lamins have been found to interact with IMN NETs such as emerin (Clements et al. 2000). These interactions are important as mutations in lamin A, emerin, nesprins and SUN proteins have all been linked to EDMD. This suggests that NET interactions may mediate disease pathology. We propose the hypothesis that:

Tissue-specific NETs also bind lamins and that disease mutants of lamins and/or NETs would disrupt this interaction.

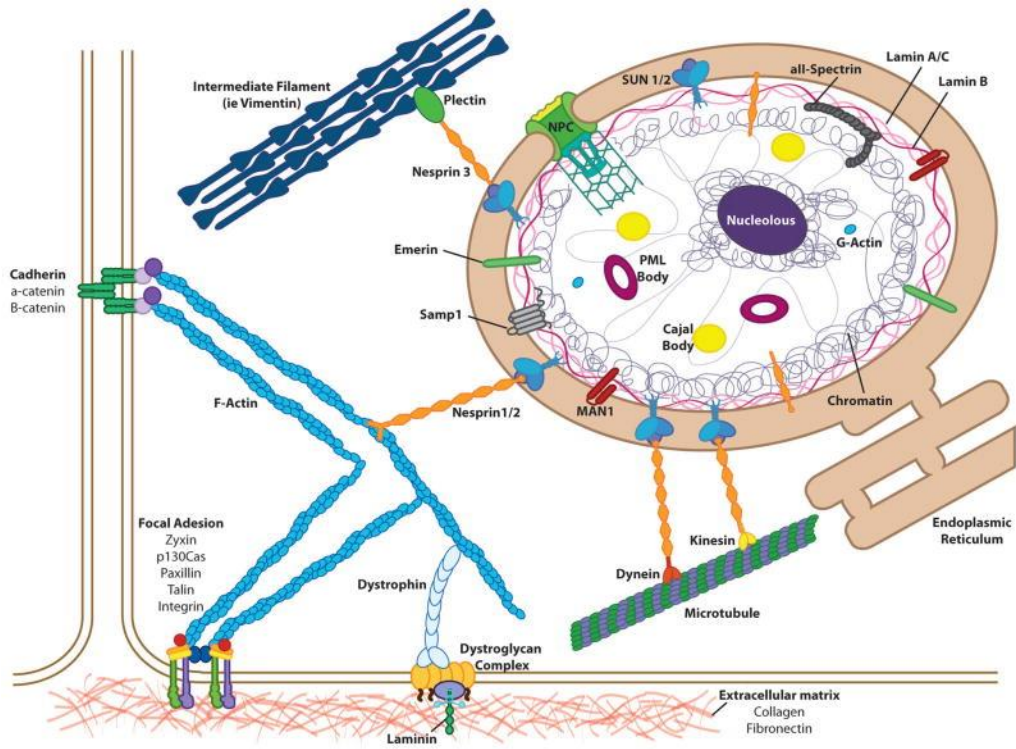


Figure 2. Networks within the cell. The nucleus is connected to the cytoskeleton via the LINC complex which intern connects to the plasma membrane and the extracellular matrix via focal adhesion points, dystroglycan complex and cadherin. (Figure taken from (Kaminski, Fedorchak, and Lammerding 2014))

1.3. Protein diversity at the nuclear membrane

NETs have been shown to be important in many processes, such as myogenesis (Brosig et al. 2010), nuclear migration (Folker et al. 2011, Razafsky et al. 2012) and cell cycle regulation (Korfali et al. 2011). The dilemma of how mutations in widely expressed proteins as well as how mutations in the same proteins selectively cause different restricted pathologies in laminopathies gave rise to the postulate that other, tissue-specific factors (i.e. other NETs) must be involved.

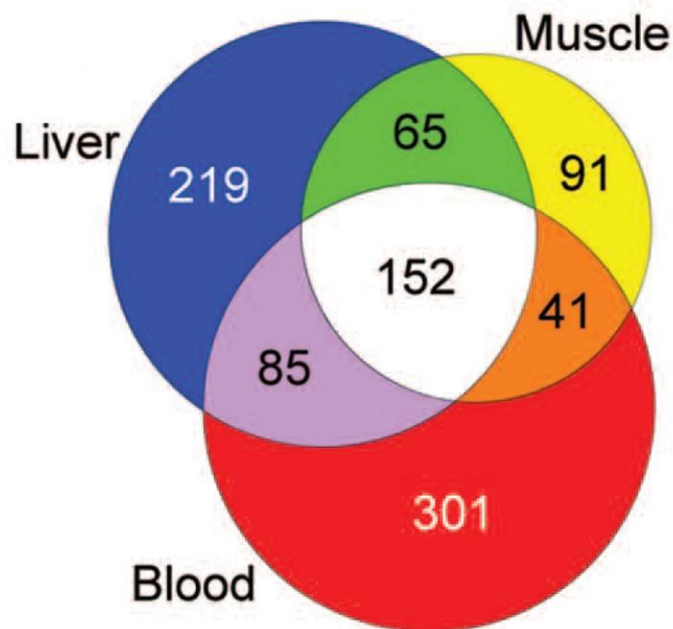


Figure 3. Distribution of NETs identified in different tissues. Only a third is shared between muscle, liver and blood tissue suggesting a high number of tissue-specific NETs.

The number of known proteins at the NE has increased in the last few years, many of which have been found to be tissue-restricted. Three studies by the Schirmer lab revealed that only a third of putative NETs are shared between muscle, blood and liver tissues (figure 3) (Korfali et al. 2010, Wilkie et al.

2011, Korfali et al. 2012). One hypothesis for the variation in the NE proteome in different

tissues is evolutionary complexity. It has been reported that highly conserved proteins such as lamin B receptor (LBR), Lamin A and B have homologs in yeast (Georgatos, Maroulakou, and Blobel 1989). It has been hypothesized that evolutionarily conserved proteins are more likely to contribute to central functions, and so are expressed in all

tissues, while more recently evolved proteins are more likely to have evolved to fill specific functions, driving divergence of different tissues in higher organisms and so could be tissue-specific (Worman and Schirmer 2015). Many NETs have specific localizations, such as the LEM-domain proteins that exclusively reside at the INM, while others such as the nesprin proteins resides at the ONM. Emerin has been reported to have non-NE localisation such as intercalated discs (Cartegni et al. 1997) potentially explaining heart involvement in EDMD. However, a later study was unable to reproduce this finding (Manilal et al. 1999). The three proteomic studies mentioned earlier identified hundreds of NETs from blood leukocytes, muscle and liver nuclei. So far 93 have been experimentally confirmed to target to the NE, and that only approximately 15% were shared between the different tissues (reviewed in (Worman and Schirmer 2015)). Some NETs are present in different tissues, while others show very specific expression. The discovery of novel tissue-restricted NETs poses the question, could any of these mediate disorders with tissue-restricted defects? It is postulated that mutations in the known linked, ubiquitously expressed proteins, disrupt interactions with tissue-specific component(s) resulting in pathology. NET interactions in tissue-specific disorders have been characterized in cases such as EDMD, where several linked proteins are known to be binding partners. It has been shown that nesprin 1 and 2 associate with EDMD linked proteins lamin A and emerin (Mislow, Holaska, et al. 2002) (Zhang et al. 2005). Along with interacting with already linked proteins, mutations in the nesprin binding, LINC complex member, SUN proteins have been linked to EDMD (Zhang et al. 2007) (Meinke et al. 2014). A clear example of tissue-specific NET linked to pathology is nesprin4, which has limited expression including the cochlea. Mutations in the gene encoding nesprin4 correlate to a high frequency of hearing loss (Roux et al. 2009, Horn et al. 2013).

However, the tissue-specific components of most nuclear envelopathies/laminopathies are still elusive.

So far, several tissue-specific NETs identified have been linked to diseases such as neonatal encephalocardiomyopathy (Tmem70)(Cizkova et al. 2008), Wolfram syndrome (WFS1) (Strom et al. 1998, Domenech, Gomez-Zaera, and Nunes 2002, Bernalova et al. 2001) and a variant of EDMD, linked to the NET designated as LUMA, with more promising candidates as mediators of disease, which will be discussed in later chapters. The evidence suggests that as we understand more about tissue-specific NETs, more will be revealed about tissue-specific disorders.

1.4. Mechanisms underlying pathology

With disorders, such as EDMD which so far had eight linked NE proteins, it is widely accepted that the mechanism(s) underlying tissue-specific disorders lie with or have links to the NE. Linked proteins have been found to be ubiquitously expressed. It is still unclear how seemingly widely expressed proteins cause tissue-specific diseases such as EDMD. Current mechanisms proposed to try and explain the pathology of these diseases includes gene regulation, mechanical stress and failure of stem cell maintenance (Gruenbaum and Foisner 2015).

The nuclear envelope and its associated proteins have been documented to interact with chromatin, transcriptional regulators and to regulate gene expression (Zuleger, Robson, and Schirmer 2011, Gruenbaum and Foisner 2015). Lamin A has been shown to bind the retinoblastoma susceptibility gene product (pRb) (Ozaki et al. 1994) and the LEM-domain family proteins, which includes emerin, binds chromatin via interaction with the Barrier to Autointegration Factor (BAF) (Brachner and Foisner 2011). Loss of

heterochromatin at the NE periphery has been reported in autosomal dominant EDMD patients (Sabatelli et al. 2001) as well as loss of emerin altering heterochromatin distribution (Maraldi et al. 2006). Extensive roles of NETs with genome organisation make it a reasonable assumption that disruption of any regulatory interactions may lead to impairment of differentiation pathways, leading to muscle loss after damage.

Failure of stem cell maintenance and differentiation could also help explain muscle loss, as if the population of muscle satellite cells are depleted, then muscles would lose their ability to replace damaged fibers. Impaired differentiation of several cell types such as muscle satellite cells, adipocytes and osteoblasts have been linked to *LMNA* mutations or dysregulation of their expression levels (reviewed in (Gruenbaum and Foisner 2015)). Complete *LMNA* knockout results in severe postnatal myogenic and adipogenic development defects (Kubben et al. 2011).

Nuclear envelope stability is very important and NE deformation is often a hallmark of disease (Davidson and Lammerding 2014). Studies have found NE deformation associated with *LMNA* and *EMD*(emerin) mutations (Lammerding et al. 2004) (Lammerding and Lee 2005) (Lammerding et al. 2005). Cultured primary cells from LINC complex deficient patients often show defects in nuclear morphology and impaired association with binding partners. Mutations in nesprins have all been found in the domain that binds to lamin or emerin. Defects in how cells deal with mechanical stress could lead to inability of muscle to repair damage caused by use. I will discuss the mechanical stress hypothesis in greater detail in the next section.

There is evidence supporting all the mechanisms mentioned above, with advocates for each. However, no single mechanism sufficiently explains how tissue-specific diseases

arise. Most likely, depending on the disease, a combination of mechanisms is responsible for tissue-specific pathology. Recent studies by the Schirmer lab identified several tissue restricted NETs with roles in chromatin organization (Zuleger et al. 2013, Korfali et al. 2010, Malik et al. 2014, Robson et al. 2016), association with the cytoskeleton (Wilkie et al. 2011) and differentiation (Batrakou et al. 2015). These proteins were identified in the proteomic studies mentioned in section 1.3 and their identification suggests that tissue-specific NETs may have roles related to the disease mechanisms above and could potentially be involved in mediating tissue-specific pathology.

1.5. Mechanical stress and NETs

One main focus in this thesis will be testing the possibility of tissue-specific NETs contributing to defects according to the mechanical stress hypothesis. Several NETs were already implicated in mechanical stress, particularly ones from the LINC complex and mutations in all of these cause EDMD. In the case of nuclear envelopathies connected to mutations lamins and emerin, there are links to both gene regulation and mechanical stress. The discovery of mutations in nesprins and SUN proteins strengthened the idea of mechanical stress as a mechanism. The LINC complex connects the nucleoskeleton with the cytoskeleton (Figure 1). The cytoskeleton consists of filamentous proteins divided in to three main groups; microtubules, actin filaments and intermediate filaments. The largest of the three are microtubules with a typical diameter of 25 nm and are made up of the protein subunit tubulin. Microtubules radiates out from a microtubule organising centre (MTOC) with the main MTOC being the centrosome. The microtubule network provides a structure which organelles used to define their positioning. Actin filaments are the smallest out of the three groups. They are important in cytokinesis and cell movement as they work alongside other filaments such as myosin

to allow for cell contraction. Intermediate filaments are strong but less dynamic than microtubules and actin filaments. They work alongside the other types to provide the mechanical support in a cell (O'Connor 2010). The core components of the LINC complex consist of the SUN INM protein family and the nesprins ONM (principally) protein family (Crisp et al. 2006). In mammals, several proteins have been grouped into the SUN family, as they all share a highly-conserved SUN domain that binds lamins; these include SUN1, SUN2, SUN3, SPAG4 and SPAG4L. The main members of the nesprin family are nesprin 1 and nesprin 2, each of which has a diverse range of splice variants and some are more tissue-specific (Rajgor et al. 2012). Nesprins bind directly to SUN domain proteins through their Klarsicht/ANC-1/Syne homologue (KASH) domain (Zhou et al. 2012). There has been many reported combinations of SUN-domain and KASH-domain proteins that make up the LINC complex, the most common comprised of SUN2 and nesprin 1 (Meinke and Schirmer 2015). The crystal structure of this SUN2/nesprin 1 complex revealed a trimer of SUN-domain proteins interacting with a corresponding trimer of KASH peptide via a covalent disulphide bond (Sosa et al. 2012). Nesprins also bind the actin cytoskeleton via their N-terminal actin-binding (calponin homology, CH) domain (Taranum et al. 2012). Furthermore, the SUN2/nesprin2G LINC complex is required for transmembrane actin-associated nuclear (TAN) lines formation and nuclear movement (Luxton et al. 2011, Luxton et al. 2010).

The involvement of the LINC complex in disease has been shown in several studies. SUN2 has been shown to be mislocalized in mouse models with absence or mutant A-type lamins (Mejat et al. 2009). Also in mice, disruption of the KASH domain of nesprin-1 led to having a phenotype that resembles EDMD (Puckelwartz et al. 2009). In humans, mutations in nesprin-1 and nesprin-2 have been linked to the pathology of EDMD (Zhang

et al. 2007). The nesprin isoforms nesprin-1alpha and nesprin-2beta binds to emerin; in X-linked EDMD, this interaction is disrupted (Wheeler et al. 2007). This implies that mutations in interacting partners of the LINC complex such as emerin can lead to defects in the complex, and that this disruption can lead to disease manifestation. This further emphasizes the importance of protein complexes in disease pathology, with strong evidence of the involvement of the LINC complex and associated NETs in disease. Mutations in nesprin-1 have also been reported to cause non-muscle related disease such as autosomal recessive cerebella ataxia (Gros-Louis et al. 2007).

The evidence outlined above indicates an important role of NETs in linking the nucleoskeleton and cytoskeleton; they also show that this link is very important as disruption leads to disease manifestation. After the identification of tissue-specific proteins in the muscle dataset from the proteomic study performed the Schirmer lab, screens were carried out on these NETs (Table 1), to see whether any have cytoskeleton effects, as muscle-specific NETs with cytoskeletal connections could potentially mediate or lead to disease when disrupted. Over 60 NETs were exogenously expressed in U2OS cells, followed by fixation and immunofluorescence staining for cytoskeletal components. Microscopy was then used to identify any abnormalities in cytoskeleton component levels or organisation. For some NETs, siRNA knockdowns were also carried out. The observations of this screen include NETs colocalizing with microtubules at the nuclear surface, microtubule accumulation at multiple foci proximal to the nucleus without a distinctive microtubule organizing center (MTOC), vimentin disorganization, reduced actin filaments and altered SUN2 levels (Table 1). Out of the positive hits, the most promising candidates with functions that may mediate muscular dystrophy pathology were NET5, Tmem70, Tmem214 and WFS1. Research is on-going with these NETs with

little known about them. Mutations in Tmem70 has been linked to neonatal encephalocardiomyopathy and mutations in WFS1 has been linked to Wolfram syndrome.

NET5, WFS1 and Tmem214 showed accumulation at the spindle base during mitosis, and Tmem214 appears to associate with microtubules at the nuclear surface (Wilkie et al. 2011). More strikingly, WFS1 and Tmem70 showed reduced SUN2 levels in knockdown experiments (unpublished data).

In addition to the results from the cytoskeleton screen, NET5, also known as SAMP1, has been shown to be a component of transmembrane actin-associated nuclear (TAN) lines (Borrego-Pinto et al. 2012) which are required for the movement of the nucleus. TAN lines are formed when the LINC complex components nesprin-2G and SUN2 interact with actin. The study above showed SAMP1 to be a component of TAN lines and that it interacts with SUN2 and lamin A/C. They also show that the NE localization of SAMP1 is dependent on lamin A/C. This study supports the idea that SAMP1 could be an interacting partner with the LINC complex, and also supports the possibility that other proteins can take part in the same or similar interactions.

Based on the phenotypes observed in the cytoskeletal screen, I planned on testing these NETs for changes in patient's muscle tissues.

1.6. Summary

This thesis sets out to investigate the hypothesis that novel tissue restricted NETs are involved in mediating pathology in laminopathies/nuclear envelopathies. In chapter 3, I investigated the expression and the distribution pattern of several muscle specific NETs

in different muscle groups, to identify potential differences as different muscular dystrophies can have distinct phenotypes. I postulate that tissue-specific NETs could yield differences between muscle groups which may contribute to disease pathology. Chapter 4 compares differences between patients and control muscle tissues. I tested NETs expression and distribution patterns as a form of diagnostic marker. In chapter 5, I test the hypothesis that NETs may mediate disease pathology through interactions between linked, ubiquitously expressed proteins such as lamin A, and that disease-causing mutations abolish or reduce these interactions leading to disease pathology.

Table 1. Functional screen of NETs carried out by the Schirmer lab. Muscle NETs are highlighted in the red boxes.

NET	Microtubules		Actin		Vimentin	SUN2		Nuclear Shape
	OE	KD	OE	KD	OE	OE	KD	KD
NET4	Variable effects	Variable effects	Mildly disrupted	NE	Mildly disrupted	Reduced	NE	Lobules
NET5	I – NE M- Spindle pole accumulation	ND	NE	NE	NE	NE	NE	NE
NET30	No MTOC	Disrupted	NE	NE	NE	Cytoplasmic accumulation	NE	NE
NET31	ND	NE	ND	NE	ND	Reduced	Strongly reduced	NE
NET50	I - Disorganised	ND	ND	NE	NE	Cytoplasmic accumulation	NE	Micronuclei
NKP9	Perinuclear accumulation	Disrupted MTOC	NE	NE	NE	Cytoplasmic accumulation	reduced	NE
TAPBPL	NE	NE	ND	NE	ND	ND	NE	Lobules Micronuclei
WFS1	I – Disorganised M – Spindle pole accumulation	NE	Perinuclear accumulation	Mildly disrupted	Partly tracking	NE	Reduced	NE
mNET8	I - Tracking	ND	NE	ND	Less organised	NE	ND	ND
Tmem70	I – Disorganised, No MTOC	Less MTOC	ND	Few filaments	ND	ND	Strongly reduced	Micronuclei
mNET20	I – NE M – Spindle pole accumulation	ND	NE	ND	Mildly disrupted	Cytoplasmic accumulation	ND	ND
Tmem214	I – Tracking M – Spindle pole accumulation	ND	ND	NE	Collapsed	NE	NE	NE

I – Interphase, M – Mitosis, ND – Not Done, NE – No Effect, OE – Overexpression, KD - Knockdown

Chapter 2

Materials and Methods

2.1. Materials

2.1.1. Bacterial strains and genotypes

Table 2. Bacterial strains and genotypes

DH5-α	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 hsdR17(rK- mK+) λ -
BL21	F- ompT gal [dcm] [lon] hsdSB (mb-; rb-)
BL21(DE3)-pLysS	F- ompT hsdSB (rB-mB-) gal dcm(DE3) pLysS (camR)
BL21(DE3)-pLysE	F- ompT hsdSB(rB-mB-) gal dcm (DE3) pLysE (camR)
BL21-CodonPlus(DE3)-RIPL	E. coli B F- ompT hsdS(rB- mB-) dcm+ Tetr gal ? (DE3) endA Hte [argU proL Camr]
StrataClone Solopack	F- endA1 glnV44 thi-1 recA1 relA1 gyrA96 deoR nupG Φ 80dlacZ Δ M15 Δ (lacZYA-argF)U169 hsdR17(rK- mK+) λ -. (Stratagene, 240207)

2.1.2. Vectors

Table 3. Vectors used in the study

pSC-B-Amp/Kan	Intermediate vector used for blunt end cloning.
pET28b	HIS-tag protein expression vector.
pMAL-CRI	MBP-tag protein expression vector
pGEX-4T1	GST-tag protein purification vector

2.1.3. Buffers and solutions

Table 4. Buffer used and composition

Buffer	Composition
LB	1% (w/v) tryptone 0.5% (w/v) yeast extract 10 mM NaCl pH 7.4
PBS (pH 7.2)	55 mM Na ₂ HPO ₄ 154 mM NaCl
PBS (pH 7.3)	55 mM Na ₂ HPO ₄ 154 mM NaCl

	pH adjusted with NaOH
PBS (pH 7.4)	65 mM Na ₂ PO ₄ 8.8 mM KH ₂ PO ₄ 137 mM NaCl 2.7 mM KCl
TBS (pH 7.4)	25 mM Tris-Base 137 mM NaCl 2.7 mM KCl
TAE	40 mM Tris-acetate 1 mM EDTA
DMEM	Dulbecco's Modified Eagle's Medium (Lonza, 12-604F)
RPMI 1640	Roswell Park Memorial Institute medium 1640 (Gibco, 11875-093)
Opti-MEM	Opti-MEM® I Reduced Serum Medium (Gibco, 31985062)
SDS-PAGE buffer	25 mM Tris pH 8.3 192 mM glycine

	0.1% (w/v) SDS
Immunofluorescence blocking buffer (in PBS pH 7.2)	1% (v/v) Horse Serum 1% (v/v) Foetal Calf Serum 0.1% (w/v) Bovine Serum Albumin
MBP lysis buffer (pH 7)	10 mM Phosphate (pH 7.2) 30 mM NaCl 0.25% (v/v) Tween-20 10 mM EDTA (pH 8) 10 mM EGTA (pH 7)
MBP elution buffer (pH 7)	10 mM Phosphate (pH 7.2) 0.5 M NaCl 1 mM NaAz 1 mM EGTA (pH 7) 10 mM Maltose
MBP column buffer	20 mM Tris-HCl 200 mM NaCl 1 mM EDTA

Lamin/GST lysis buffer	25 mM HEPES (pH 8) 0.1 M MgCl ₂ 3 mM 2-Mercaptoethanol
Lamin solubilisation buffer	20 mM HEPES (pH 8) 8 M Urea 3 mM 2-Mercaptoethanol
Skeletal muscle cell growth medium	Promocell skeletal muscle cell growth medium (C-23060)
Skeletal muscle cell differentiation medium	Promocell skeletal muscle cell differentiation medium (C-23061)
Acidified Acetone/Methanol mix	50% acidified Acetone (1 mM HCL) + 50% Methanol
GST elution buffer	50 mM Tris-HCl, 10 mM reduced glutathione, pH 8.0
HIS-Select Equilibration/wash buffer	50 mM sodium phosphate, pH 8.0, 0.3 M sodium chloride 10 mM imidazole.
HIS-Select Elution buffer	50 mM sodium phosphate, pH 8.0,

	0.3 M sodium chloride
	250 mM imidazole

2.1.4. Primary antibodies

Table 5. Primary antibodies used in this study (IF – Immunofluorescence, WB – Western Blot)

Antigen	Host	IF Dilution	WB Dilutions	Band Size	Source
Myh1	Mouse	1:50	N/A	200 kDa	Sigma (M1570) clone My-32
Lamin A/C	Rabbit	1:50	1:1000	70 kDa	(Schirmer et al, 2001)
Tmem38A	Rabbit	1:50	1:200	30 kDa	Millipore (06-1005)
WFS1	Rabbit	1:50	1:200	100 kDa	Proteintech (11558-1-AP)
Tmem214	Rabbit	1:50	1:200	70 kDa	Proteintech (20125-1-AP)
NET5	Rabbit	1:20	1:100	70 kDa	
Dystrophin	Mouse	1:50	N/A	271 kDa	Glenn Morris (MANDYS1 (3B7))

Emerin	Mouse	1:50	N/A	29 kDa	Glenn Morris (MANEM1 (5D10))
Nesprin1	Mouse	1:50	N/A	N/A	Glenn Morris (MANNES1E (8C3))
Nesprin2	Mouse	1:50	N/A	N/A	Glenn Morris (MANNES1A (7A12))
Lamin A/C	Mouse	1:50	N/A	70 kDa	Glenn Morris (MANLAC1 (4A7))
SUN1	Rabbit	1:50	N/A	N/A	Atlas antibodies (HPA008346)
SUN2	Rabbit	1:50	N/A	N/A	
FHL1	Rabbit	1:50	N/A	32 kDa	Aviva Systems Biology (ARP34378_T100)
GFP	Rabbit	N/A	1:200	25 kDa	Generated by Dzmitry Batrakou
GFP	Mouse	N/A	1:1000	25 kDa	Clontech (632381)
H3	Mouse	N/A	1:200	17 kDa	Abcam (10799)
MBP	Mouse	N/A	1:1000	42 kDa	NEB (E8032L)

2.1.5. Secondary antibodies

The table below lists all the secondary antibodies used in the study. The Licor IRDyes® listed were used for fluorescence based Licor Western blotting. The Alexa antibodies were used for immunofluorescence (IF). Anti-mouse Alexa 488 was also used to detect binding in the solid phase binding assay presented later in this thesis.

Table 6. Secondary antibodies used in this study

Antibody	Host	Dye	Dilution	Source
Anti-mouse	Donkey	Alexa 488	1:500	Invitrogen (A21202)
Anti-rabbit	Donkey	Alexa 488	1:500	Invitrogen (A21206)
Anti-mouse	Donkey	Alexa 568	1:500	Invitrogen (A10037)
Anti-rabbit	Donkey	Alexa 568	1:500	Invitrogen (A10042)
Anti-mouse	Donkey	IRDye® 800CW	1:1000	Licor (926-32212)
Anti-rabbit	Donkey	IRDye® 800CW	1:1000	Licor (926-32213)

Anti-mouse	Donkey	IRDye® 680CW	1:1000	Licor (926-68073)
Anti-rabbit	Donkey	IRDye® 680CW	1:1000	Licor (926-68073)

2.1.6. Affinity matrixes

Table 7. Affinity matrixes used in this study

Matrix	Source
Amylose resin	NEB (E8021L)
Glutathione Sepharose 4B	GE Healthcare (17-0756-01)
HIS-Select® Nickle Affinity Gel	Sigma-Aldrich (P6611)
Affi-Gel® 10	Biorad (1536099)
Pierce™ Nickel Coated Plates, Black, 96-Well	ThermoFisher Scientific (15342)

2.1.7. Mammalian cells

All primary human cells were acquired in compliance with ethical approval procedures and patient consent.

Table 8. Cell lines used in this study

Cell line	Type	Source
P2F1-g	Hybridoma	Developmental Studies Hybridoma Bank
155/09	Primary human myoblast	Muscle Tissue Culture Collection at the Friedrich-Baur-Institut (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany)
464/04	Primary human myoblast	Muscle Tissue Culture Collection at the Friedrich-Baur-Institut (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany)
103/05	Primary human myoblast	Muscle Tissue Culture Collection at the Friedrich-Baur-Institut (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany)
175/04	Primary human myoblast	Muscle Tissue Culture Collection at the Friedrich-Baur-Institut (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany)
226/05	Primary human myoblast	Muscle Tissue Culture Collection at the Friedrich-Baur-Institut (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany)
245/01	Primary human myoblast	Muscle Tissue Culture Collection at the Friedrich-Baur-Institut (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany)

402/03	Primary human myoblast	Muscle Tissue Culture Collection at the Friedrich-Baur-Institut (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany)
GB2912	Primary human myoblast	Muscle Tissue Culture Collection at the Friedrich-Baur-Institut (Department of Neurology, Ludwig-Maximilians-University, Munich, Germany)
NH11-567A	Primary human myoblast	MRC Centre for Neuromuscular Disorders Biobank London
NH13-1586A	Primary human myoblast	MRC Centre for Neuromuscular Disorders Biobank London
6467	Primary human myoblast	MRC Centre for Neuromuscular Disorders Biobank London
NH11-567B	Primary human fibroblast	MRC Centre for Neuromuscular Disorders Biobank London
NH13-1586B	Primary human fibroblast	MRC Centre for Neuromuscular Disorders Biobank London
NH11-069B	Primary human fibroblast	MRC Centre for Neuromuscular Disorders Biobank London
6468	Primary human fibroblast	MRC Centre for Neuromuscular Disorders Biobank London

6217	Primary human fibroblast	MRC Centre for Neuromuscular Disorders Biobank London
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2.1.8. Tissue sections

Listed below are all the tissue sections used in this study. All human patient sections were acquired in compliance with ethical approval procedures and patient consent.

Table 9. Tissue sections used in this study

Section ID	Type	Source
780	Human patient muscle sections	Caroline Sewry, CIND, Oswestry
NH10-648	Human patient muscle sections	MRC Centre for Neuromuscular Disorders Biobank London
NH11-069	Human patient muscle sections	MRC Centre for Neuromuscular Disorders Biobank London
NH13-1586	Human patient muscle sections	MRC Centre for Neuromuscular Disorders Biobank London
6680	Human patient muscle sections	MRC Centre for Neuromuscular Disorders Biobank London
6262	Human patient muscle sections	MRC Centre for Neuromuscular Disorders Biobank London

5808	Human patient muscle sections	MRC Centre for Neuromuscular Disorders Biobank London
6424	Human patient muscle sections	MRC Centre for Neuromuscular Disorders Biobank London
227/02	Human patient muscle sections	Benedikt Schoser, Friedrich-Baur Institute, Department of Neurology
228/02	Human patient muscle sections	Benedikt Schoser, Friedrich-Baur Institute, Department of Neurology
233/02	Human patient muscle sections	Benedikt Schoser, Friedrich-Baur Institute, Department of Neurology
234/02	Human patient muscle sections	Benedikt Schoser, Friedrich-Baur Institute, Department of Neurology
271/02	Human patient muscle sections	Benedikt Schoser, Friedrich-Baur Institute, Department of Neurology
320/02	Human patient muscle sections	Benedikt Schoser, Friedrich-Baur Institute, Department of Neurology

2.1.9. Kits

Table 10. Kits used in this study

Kits	Source
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QuikChange II XL Site-Directed Mutagenesis Kit	Agilent Technologies (200521)
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2.2. Mammalian cell culture

2.2.1. Cell maintenance

Hybridoma cell lines were maintained in RPMI 1640 medium supplemented with L-Glutamine and sodium pyruvate with 10% (v/v) FBS.

Primary human myoblasts were maintained in skeletal muscle cell growth medium (PromoCell). Cells were kept from reaching confluency to avoid differentiation.

Primary human fibroblasts were maintained in DMEM medium supplemented with 10% FBS (v/v).

All cells were grown at 37°C in a 5% CO₂ incubator.

2.2.2. Primary human myoblast differentiation

Primary human myoblasts were grown to confluency and changed into low serum differentiation medium (Skeletal muscle cell differentiation medium, Promocell).

2.2.3. Production of monoclonal antibody from hybridoma cells

Hybridoma cells were grown in suspension and withdrawn into serum free medium by 50% every 2 days in 4 steps. The cultures were left to grow for up to a week to allow the production and secretion of the antibodies. Samples of medium supernatant were taken at regular intervals to test the level of antibody production and activity by Western blot.

The supernatant containing antibodies was cleared of debris by centrifugation at 1000 RPM for 10 min and kept.

2.3. Nucleic acid methods

2.3.1. Sequencing of plasmid DNA

The entire sequence of the cDNAs cloned into expression vectors was verified by sequencing. Sequencing was performed by the GenePool sequencing facility (University of Edinburgh). Provided chromatograms were analysed using the freeware program GENTle.

2.3.2. Site-directed mutagenesis

Lamin mutants were generated by mutating the cDNA sequence situated in the expression vectors. Mutagenesis was carried out using primers designed to be compatible with the protocol described in the QuikChange II XL Site-Directed Mutagenesis Kit. The mutagenesis procedure was carried out according to the kit's protocol.

Table 11. List of mutagenesis primers used in this study

Lamin mutation	Primers used
E203K	Forward: AACTGGACTTCCAGAAGAACA Reverse: TCTCCTTCATGGTCTGCAGC
R298C	Forward: AACTGGACTTCCAGAAGAACA

	Reverse: TCTCCTTCATGGTCTGCAGC
R377H	Forward: AACTGGACTTCCAGAAGAACA Reverse: TCTCCTTCATGGTCTGCAGC
R435C	Forward: AACTGGACTTCCAGAAGAACA Reverse: TCTCCTTCATGGTCTGCAGC
R453W	Forward: AACTGGACTTCCAGAAGAACA Reverse: TCTCCTTCATGGTCTGCAGC
G465D	Forward: AACTGGACTTCCAGAAGAACA Reverse: TCTCCTTCATGGTCTGCAGC
R482Q	Forward: AACTGGACTTCCAGAAGAACA Reverse: TCTCCTTCATGGTCTGCAGC
R527H	Forward: AACTGGACTTCCAGAAGAACA Reverse: TCTCCTTCATGGTCTGCAGC

2.4. Microscopy methods

2.4.1. Immunofluorescence on cultured cells

Adherent cells were grown on coverslips and washed in PBS to remove cellular debris and remaining serum prior to fixation with ice cold (-20°C) 100% methanol and immediately placed on dry ice or stored at -20°C. Following, the cells were washed for 10

min in PBS or TBS-0.1% Tween-20. Coverslips were blocked in 1X immunofluorescence blocking buffer (Table 1) for 20 min at room temperature (RT) and subsequently incubated with the appropriate primary antibody (dilutions listed in Table 2). Following 3 washes in PBS or TBS-0.1% Tween-20, coverslips were incubated with goat secondary antibodies conjugated with Alexa Fluor® dyes (summarised in Table 3) and 4,6-diamidino-2 phenylindole, dihydrochloride (DAPI) at a final concentration of 4 µg/ml (1:1000). All antibodies and dyes were prepared in blocking solution. Coverslips were then extensively washed in PBS or TBS-0.1% Tween-20 multiple times over the course of 30 minutes and then mounted on cover slips with VectaShield (Vector Labs). The coverslips were sealed in place using nail varnish.

2.4.2. Human tissue section immunohistochemistry

Tissue blocks were snap frozen using liquid nitrogen in OCT mounting medium and mounted on cryostat chucks using OCT mounting medium (ThermoFisher). Sections were cut using a cryostat (Leica CM1900). The sections were cut to a thickness of 10µm and mounted by layering on SuperFrost Plus (VWR) slides and immediately placed on dry ice. Sections are kept at -20°C to -80°C.

The sections were allowed to equilibrate to RT before staining. Using a PAP hydrophobic marker pen (Daido Sangyo), a working area was drawn around each section. The OCT mounting medium (ThermoFisher) was then washed off using TBS. The sections were incubated in immunofluorescence blocking buffer (Table 1) for 30 min after which the solution was removed gently by blotting with tissue paper and the primary antibodies were applied. The sections were left incubating in primary antibodies overnight at 4°C in a damp chamber. After primary antibody incubation, the sections were washed 3 x 5 min

using TBS + 0.1% Tween-20. Secondary antibody was then applied and the sections were left incubating for 1 h. The secondary antibodies were then removed gently by blotting with tissue paper and DAPI applied for 10-15 min. Sections were then washed 3x 10 min in TBS + 0.1% Tween-20 and 1x 10 min in TBS. Excess liquid was blotted off carefully using tissue paper and a drop of Vectashield (Vector Laboratories) was applied on top of the sections and a coverslip applied. The coverslip was sealed in place using nail varnish. All antibodies and dyes were prepared in blocking solution.

2.4.3. Microscopy and analysis

Images were acquired on a Nikon TE-200 microscope using a 1.45 NA 100x objective, Sedat quad filter set, PIFOC Z-axis focus drive (Physik Instruments) and a CoolSnapHQ High Speed Monochrome CCD camera (Photometrics) run by Metamorph image acquisition software. For deconvolution analysis, Z-stacks were acquired at intervals of 0.2 μm from the 1 μm above to 1 μm below the imaged nucleus using the software AutoQuant X3.

2.5. Protein methods

2.5.1. Total protein extraction from mammalian cells

Protein samples were prepared from cells after directly lysing in TRIzol™. Cells were first washed with PBS. TRIzol reagent was then added and the cells scraped from the dish using cell scrapers. Cell suspensions were collected in 1.5 ml eppendorf tubes and mixed. 0.2 ml of chloroform was then added and mixed by shaking vigorously for 15-20 s. The top phase was discarded after centrifugation at 12,000 x *g* for 15 min at 4°C. Acidified methanol acetone was then added, mixed and the solution was left at -20°C

overnight for the protein to precipitate. Protein pellets were collected by centrifugation at 1,000 x g and the supernatant discarded. The protein pellets were then suspended in acidified methanol acetone and the centrifugation was then repeated and the pellets were allowed to air dry. Once dry, the pellets were resuspended in 20 mM Tris, pH 8.0, 1% SDS. Acidified methanol acetone was added and the proteins were once again precipitated at -20°C overnight. The precipitates were then collected by centrifugation at 1,000 x g. The air dried pellets were then resuspended in protein sample buffer (20 mM Tris, pH 8.0, 1% SDS with coomassie brilliant blue dye and glycerol).

2.5.2. Western blotting

Protein samples were separated on 8-12% Tris-glycine-SDS or Bis-Tris gels. Subsequently the gels were transferred onto nitrocellulose membranes (Odyssey 926-31092) by means of semidry transfer (BIO-RAD). After transfer the membrane was blocked in Western blot blocking buffer (5% milk powder (w/v) in PBS/TBS with 0.05% Tween-20) for 30 min. Subsequently, the membrane was incubated with the primary antibody diluted in western blot blocking buffer at the dilutions indicated in Table 3 for 60 min at room temperature or overnight at 4°C. Six washes in PBS/TBS, 0.05% Tween-20 were then followed by incubation with the secondary antibody conjugated to horseradish peroxidase (HRP) or an IRDye[®] for 60 min at room temperature. After 6 washes in PBS/TBS, 0.05% Tween-20, the membrane was incubated with the ECL reagent (Amersham) to allow the HRP to react with hydrogen peroxide and luminol to generate a fluorescent signal that was measured on an X-Ray film (CP-BU NEW, Agfa). Alternatively, for Li-Cor visualisation of blots, membranes IRdye[®]-conjugated antibodies were detected on a Li-Cor Odyssey Quantitative Fluorescence Imager.

2.5.3. Lamin purification from inclusion bodies

pET28-b vector containing pre-processed WT lamin A or point mutant variants were transformed into BL21 protein expressing bacteria. A preculture was grown overnight at 37°C. This culture was diluted the next day and allowed to grow for 3-4 h until the culture reached OD 0.6 at 600 nm. Induction was carried out with 0.3 mM IPTG for 4 h at 37°C. Bacterial cells were then harvested via centrifugation and the pellet resuspended in lamin lysis buffer (Table 1). Lysis was done through sonication and DNase was used to digest residual DNA. Inclusion bodies were collected by centrifugation and washed with 1% Triton X-100. This centrifugation and wash step was repeated three times. After removing all Triton X-100 wash buffer, the inclusion body pellet was solubilised overnight in urea containing buffer (Table 1).

2.5.4. MBP-tagged protein purification

Plasmids carrying maltose binding protein (MBP) fusions to NET nucleoplasmic fragments in the pMAL-CRI vector were transformed into BL21 bacterial cells. A preculture was grown overnight at 37°C. This culture was diluted the next day and allowed to grow for 3-4 h until the culture reached OD 0.6 at 600 nm. Induction was carried out with 0.3 mM IPTG for 4 hours at 37°C or overnight at 18°C (for long fragments, it is recommended to induced for longer at lower temperatures). Bacterial cells were then harvested by centrifugation and the pellet resuspended in MBP lysis buffer (Table 1). Lysis was done through sonication and DNase was used to digest residual DNA.

Protein fragments were then affinity purified using standard protocols set out by the affinity matrix manufacturer, using stated buffers or buffers listed in Table 1. Protein lysates were diluted in MBP column buffer (1/10) before loading onto the amylose resin.

Bound proteins were washed with at least 50x bed volume using the same column buffer before eluting using MBP elution buffer. The eluted proteins were dialyzed into TBS and concentrated using spin concentrator columns simultaneously in an attempt to remove free maltose.

2.5.5. GST-tagged protein purification

pGEX-4T1 vector containing nucleoplasmic fragments of NETs fused to a glutathione S-transferase (GST) tag were transformed into BL21 protein expressing bacteria. A preculture was grown overnight at 37°C. This culture was diluted the next day and allowed to grow for 3-4 hours until the culture reached OD 0.6 at 600 nm. Induction was carried out with 0.3 mM IPTG for 4 hours at 37°C or overnight at 18°C (for long fragments, it is recommended to induce for longer at lower temperatures). Bacterial cells were then harvested via centrifugation and the pellet resuspended in GST lysis buffer (Table 1). Lysis was done through sonication and DNase was used to digest residual DNA.

Protein fragments were then affinity purified using standard protocols set out by the affinity matrix manufacturer using stated buffers or buffers listed in Table 1. Glutathione Sepharose 4B beads were packed and washed with at least 20x bed volume of PBS (pH 7.3). Cell lysate was applied and incubated with the beads for 30 min at room temperature. After the lysate had been removed, the beads were washed with at least 50x bed volume of PBS (pH 7.3). The proteins were eluted using GST elution buffer.

2.5.6. HIS-tagged protein purification

pET28-b vector containing nucleoplasmic fragments of NETs were transformed into BL21 protein expressing bacteria. A preculture was grown overnight at 37°C. This culture was

diluted the next day and allow to grow for 3-4 hours until the culture reach OD 0.6 at 600nm. Induction was carried out with 0.3 mM IPTG for 4 hours at 37°C. Bacterial cells were then harvested via centrifugation and the pellet resuspended in lysis buffer (Table 3). Lysis was done through sonication and DNase was used to digest residual DNA.

Protein fragments were then affinity purified using standard protocols set out by the affinity matrix manufacturer using stated buffers or buffers listed in Table 1. Nickel matrix was packed and washed with at least 20x bed volume of equilibration buffer. Cell lysate was applied and allowed to bind for at least 30 min. Bound proteins were washed with at least 50x bed volume of equilibration buffer before eluting using elution buffer.

Matrix was regenerated before subsequent use using the manufacturer's protocol (His-Select Nickel Affinity Gel).

2.5.7. Affinity purification of antibodies

Antibodies were affinity purified against the protein fragments/peptide used in their generation.

Affi-gel matrix was activated with ice cold water. The protein antibody baits were dialysed out of Tris containing buffer into 0.01 M NaHCO₃, 0.1 M to 0.5 M NaCl before incubation overnight with the matrix at 4°C. Supernatant was then removed and the matrix washed three time with 0.01 M NaHCO₃, 0.1 M to 0.5 M NaCl. The matrix was then incubated with 0.1 M Tris, pH 8 or with 1 M ethanolamine, pH 8 overnight at 4°C. 3 alternating washes were carried out with 0.1 M NaAc, 0.5 M NaCl pH 4 and 0.1 M Tris NaCl pH8. The matrix was then either used or stored in 0.1 M Tris, 0.5 M NaCl, pH 8, 0.02% NaN₃.

For use, all supernatant was drained and the matrix equilibrated using PBS. Serum was passed over the matrix via a gravity column. PBS washes were carried out before elution. Elution of antibodies was done using 0.2 M glycine pH 2.8 or 4 M MgCl₂. Glycine elution was collected on a bed of 1 M Tris pH 8.8 (50-100 µl per 500 µl elution). MgCl₂ elution was dialyzed into PBS using spin concentrator columns. Elution was monitored using a Nanodrop 2000c measuring absorbance at 210 nm.

Glycerol was added to a final concentration of 10-25% to the eluted antibodies to help with stability. Antibody solutions were concentrated using standard spin concentrators with the appropriate molecular cut off.

2.5.8. Size exclusion chromatography

Size-exclusion chromatography coupled with multi-angle light scattering (SEC-MALS) was performed using an ÄKTA micro HPLC with a Superdex 200 10/300 GL column coupled with an on-line UV detector, Viscotek MALS-20 scattering detector (Malvern) and refractive index detector Viscotek VE3580 (Malvern). The Superdex column was equilibrated with buffer not containing any protein overnight (0.5 ml/min flow rate). 250 µl of protein samples were injected for analysis. Analysis of the mass was performed using the OmniSEC software package (Malvern). Sample runs and analysis were performed with Dr Martin Wear.

2.6. Protein interaction methods

2.6.1. Solid phase binding assay

Excess HIS-Tagged lamins (>9 pM) were bound to the nickel-plated wells in 100 µl urea buffer for 1 h. Excess lamins were washed off using 200 µl of TBS-0.05% Tween 20 three

times 10 min washes. MBP-Tagged NET fragments in TBS were then added on top of the lamins in multiples of 9 pM (0, 20, 50, 100) and left to incubate at room temperature for 1 hour. The wash steps were then repeated as previous before anti-MBP antibodies were added onto each well and left for 1 h. Excess antibodies were then washed off. Alexa-488 antibodies were diluted 1/400 and 45 μ l was added to each well and incubated for 1 h. Before reading, the wells were washed 3 times with TBS-0.05% Tween-20 and 1 time with TBS. The fluorescence signal at was read using a Modulus™ II Microplate Multimode Reader (Turner BioSystems) using their blue optical kit with a max excitation wavelength of 490 nm and emission wavelength of 510-570 nm. An absolute read out was taken and blanked using control empty wells on each plate. The plates used were 96 wells nickel coated plates (Pierce 15342)

2.6.2. Surface Plasmon Resonance (SPR)

SPR single cycle kinetic experiments (5 injections) were performed, at 25°C. A 2-fold concentration series of NETs fragments ranging from 0.3125 μ M – 5 μ M, in TBS, 0.05 mM EDTA, was injected over the sensor surface bound with 70 response unit (RU) of immobilised His-Lamin A, at 30 μ l.min⁻¹ with 90 sec contact and dissociation times. The equilibrium dissociation constant was calculated from the sensorgrams by global fitting of a 1:1 binding model, with mass transport considerations, using analysis software (v2.02) provided with the Biacore T200 instrument. The experiments were carried out on Sensor Chip NTA (BR100034) from GE Healthcare life sciences. Lamins were bound to the surface in 6 M urea, 25 mM HEPES. The same urea buffer was used to dissociate binding between the solid phase and the soluble components. The sensor chip was regenerated using TBS containing 350 mM EDTA.

Chapter 3

Introduction

All tissue-specific diseases require a mechanism to explain their pathology. In the case of EDMD, where all currently linked proteins are widely expressed, one postulate is that there are tissue-specific factors involved. Indeed, it seems improbable that mutations in the same gene can cause multiple diseases with unique aspects to their phenotype unless other proteins mediate the tissue-specific aspects of phenotype. *LMNA* is an example of such a gene (figure 4).

Muscular dystrophies such as EDMD and LGMD can affect different muscles. The work presented in this chapter, attempts to identify possible differences between muscle sub-groups, to elucidate any tissue-specific components which may contribute to pathology. Antibodies to some NETs, identified in the Schirmer lab proteomic studies (Tmem38A, NET5, WFS1, Tmem214) were acquired and used in this study to see if they yielded expression and/or distribution differences amongst muscle subtype.

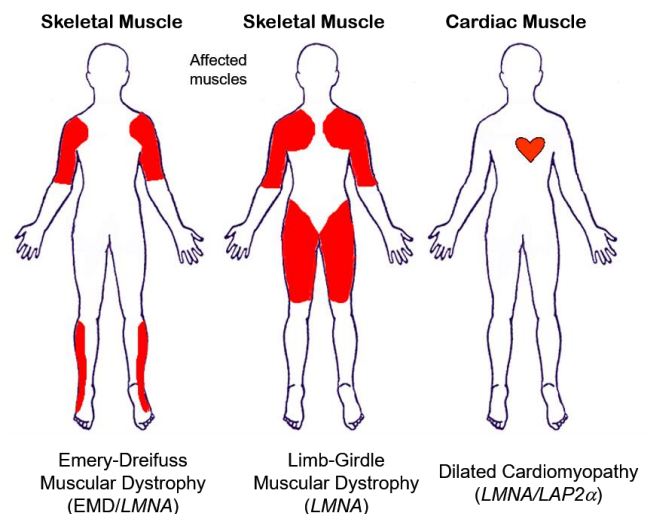


Figure 4. Affected muscle groups in different disorders can be caused by mutations in the same gene. Lamin A has been linked to diseases affecting different muscle groups, sometimes with overlapping phenotype such as EDMD and LGMD while other times with specific pathology such as DCM. In brackets are some of the genes linked to each disease.

As well as antibody availability, these proteins were chosen for their relevancy to the existing proposed disease mechanisms. NET5 (SAMP1), Wolfram syndrome 1 (WFS1) and Tmem214 (FLJ20254) all exhibit association with cytoskeleton components during functional testing (Wilkie et al. 2011). As mentioned previously, NET5 was also shown to associate with TAN lines and some LINC complex members and is reported to be required for nuclear migration (Borrego-Pinto et al. 2012). Tmem38a (TRIC-A) is a muscle specific NET that is involved in Ca²⁺ ion regulation (Yazawa et al. 2007). Tmem38A is reported to form a trimeric cation channel, which is responsible for maintaining intracellular stores and is important for regulation of hypertension in smooth vascular muscles and maintaining sarcoplasmic and endoplasmic reticulum calcium homeostasis (Yamazaki et al. 2011, Zhou et al. 2014). NET5, Tmem38A, WFS1 and Tmem214 have also been shown to be involved in recruiting chromatin to the nuclear periphery (Robson et al. 2016, Zuleger et al. 2013). I hypothesize that these NETs may contribute to tissue-specific muscle disease pathology through either mechanical instability or genome regulation and that it may be possible to identify differences between tissue sub-types, by looking at normal human muscle samples as well as matched mouse muscles (figure 5).

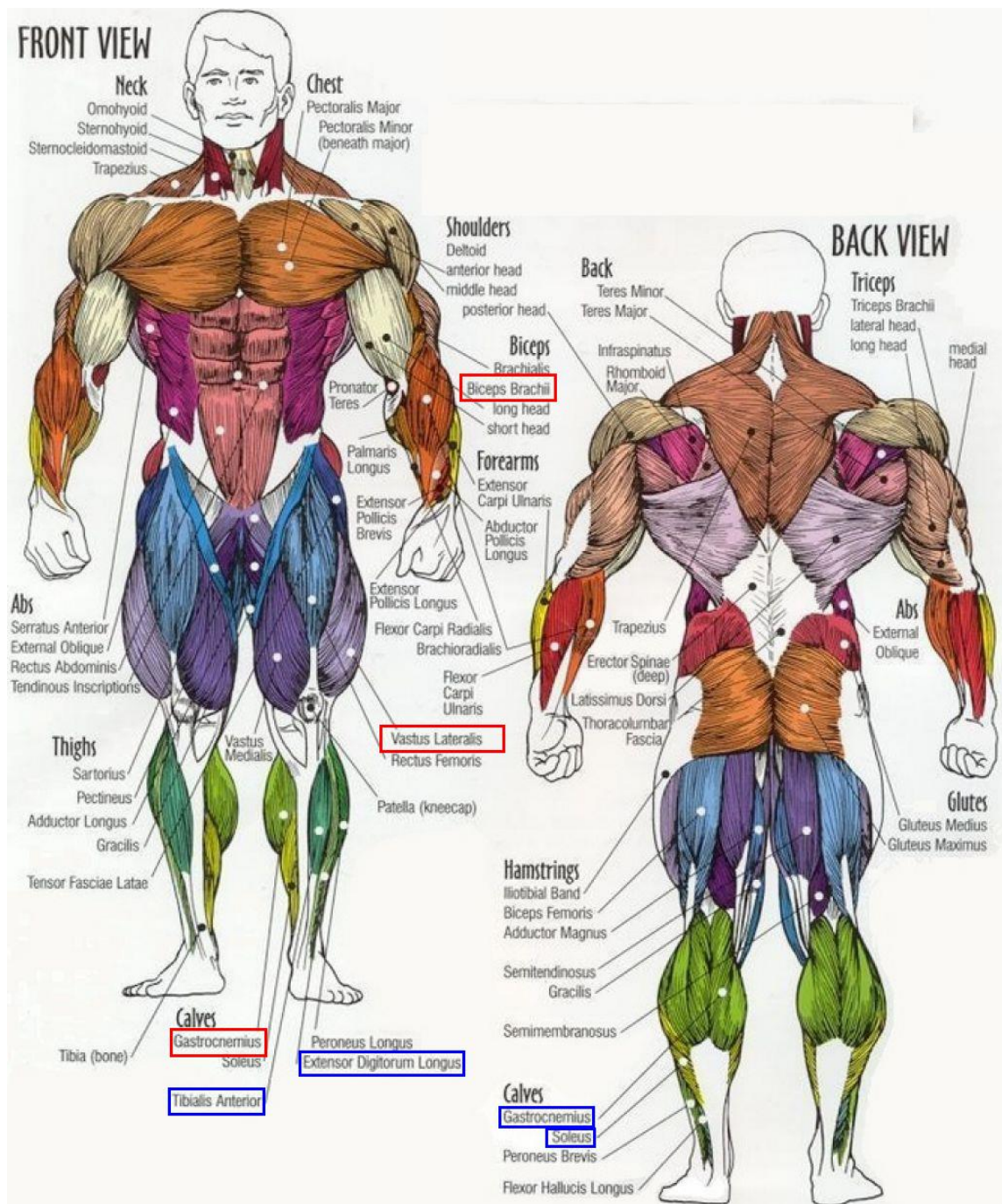


Figure 5. Muscle anatomy of the human body. Diagram showing the muscle groups present in the human body. Highlighted in RED are the human muscle groups obtained in this study, while in blue are the matched muscles obtained from mice. The diaphragm is not shown (adapted from <https://www.anatomylibrary99.com/wp-content/uploads/2016/08/anatomical-picture-human-body-anatomy-of-the-human-body-pictures-gethumananatomy.jpeg>)

3.1. Confirming expression of NETs in muscle nuclei and comparison between human muscle groups

Through collaboration with clinicians, I was able to obtain several muscle groups from control individuals (muscular dystrophy excluded). All muscle samples were obtained from several distinct sources with full ethical approval. I proceeded to stain these different muscle groups from both male and female samples. Dystrophin, a sarcoplasmic membrane marker, was used to distinguish between the nuclei of the inside and outside of muscle fibers. The tissues examined were *biceps brachii* (figure 6), vastus lateralis (figure 7) and gastrocnemius (figure 8). Pathology in *biceps brachii* is shared between EDMD and LGMD, with the latter affecting a broader upper arm area, while *gastrocnemius* pathology is exclusive to EDMD and *vastus lateralis* pathology is exclusive to LGMD (figure 4).

Muscle sections were stained using antibodies raised against selected NETs. Muscle fibers were defined by co-staining with either dystrophin or laminin2a. Nuclei were stained using DAPI. Tmem38A was found to have the most specific expression pattern, residing exclusively in muscle fiber nuclei. Tmem38A antibodies yielded staining exclusively in muscle nuclei as affirmed by co-staining with dystrophin and DAPI. In figure 6, 7 and 8 the nuclei indicated by the asterisks have Tmem38A staining as well as being inside the dystrophin-marked cells, while the DAPI-stained nuclei outside these cells and do not stain with the Tmem38A antibodies. Staining with NET5, Tmem214 and WFS1 antibodies yielded ubiquitous muscle staining with positive antibody staining in nuclei inside and outside muscle fibers. In all three muscle groups, tested NETs were all found to stain nuclei inside muscle fibers. However, only Tmem38A showed exclusive fiber staining with NET5, Tmem214 and WFS1 present in nuclei in the surrounding connective tissues. No

observable differences were found to exist between muscle groups. Quantification could not be carried out due to the different sources and storage conditions of the samples which led to cutting and freezing artifacts.

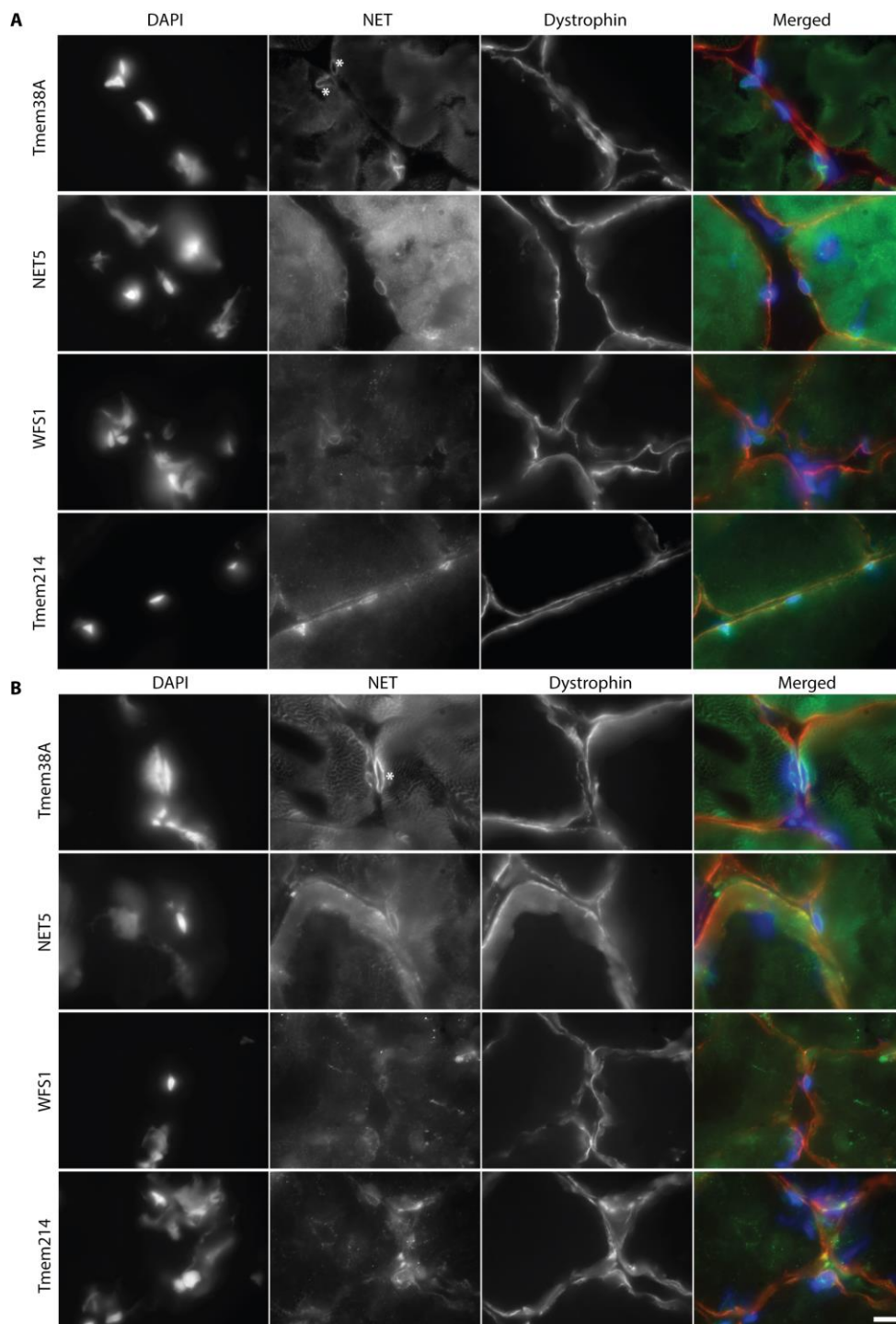


Figure 6. Staining of NETs identified in the proteomic studies in Bicep Brachii. (A) Male control (B) Female control. (*) Tmem38A positive nuclei inside muscle fibers. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Scale bar = 10 μ m

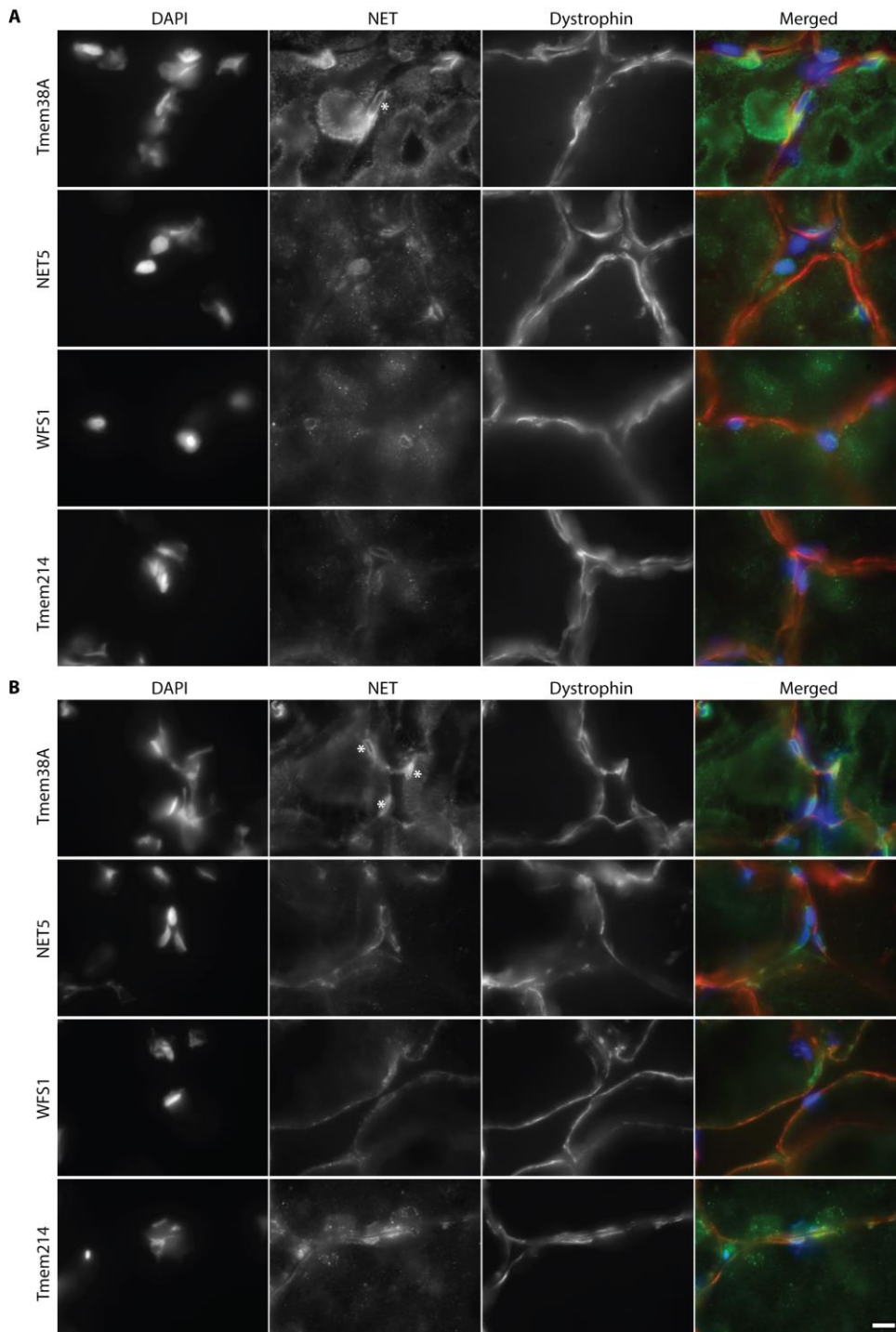


Figure 7. Staining of NETs identified in the proteomic studies in Vastus Lateralis. (A) Male control (B) Female control. (*) Tmem38A positive nuclei inside muscle fibers. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Scale bar = 10 μ m

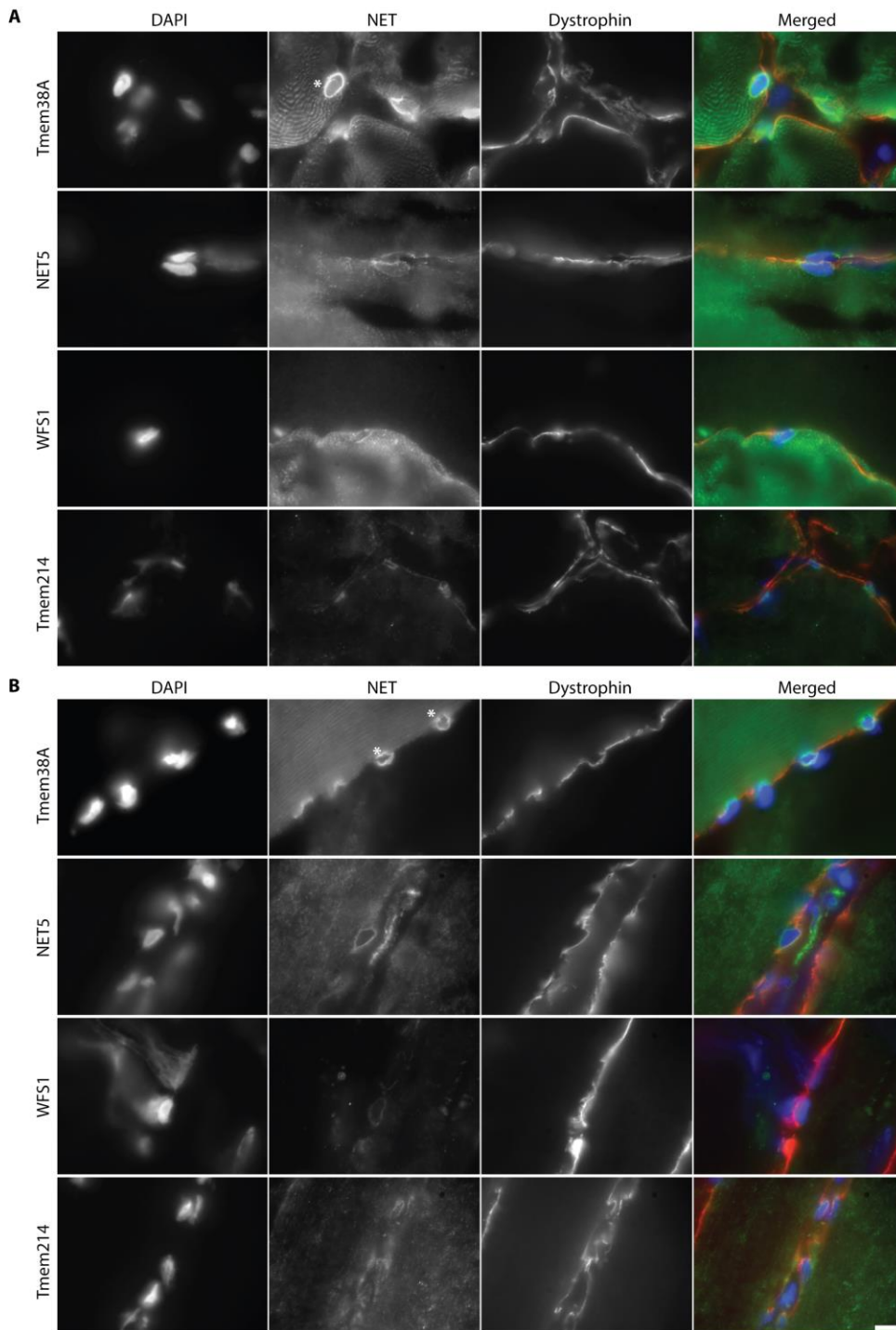


Figure 8. Staining of NETs identified in the proteomic studies in Gastrocnemius. (A) Male control (B) Female control. (*) Tmem38A positive nuclei inside muscle fibers. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Scale bar = 10 μ m

3.2. Comparison between mouse muscle groups

With the difficulties in obtaining control human sections, only three human muscle groups were examined. To further study the postulate that tissue-specific factors are involved in disease pathology, the study was expanded to include mouse muscles. Five different muscle groups were obtained: *gastrocnemius* (figure 9), *tibialis anterior* (figure 10), *diaphragm* (figure 11), *extensor digitorum longus* (figure 12) and *musculus soleus* (figure 13). These samples were obtained through collaboration with Heinrich Brinkmeier. The muscle groups obtained were matched with those present in human (figure 5). After sectioning, immunofluorescent staining was carried out under the conditions and using the same antibodies used on the human samples. Due to poor sectioning, we could not confirm WFS1 expression in the *musculus soleus* samples.

As with the human sections, the mouse samples were stained with dystrophin (red) to mark the plasma membrane of muscle fibers to distinguish between the inside and outside. NETs staining are in green and nuclei were stained with DAPI. Tested NETs were present in muscle fiber nuclei in all tested muscle groups (figure 9-13). Tmem38A showed the same specificity as was found in the human tissues with staining absent in connective tissue nuclei. No observable differences were seen between the different muscle groups and freezing/cutting artifacts preventing quantification.

Samples of the *gastrocnemius* of a muscular dystrophic (MDX) mouse were also acquired. This mouse line has a single substitution in exon 23 in the dystrophin gene, resulting in a premature stop codon (Sicinski et al. 1989, Carberry et al. 2013). To see whether there is any abnormality in dystrophic muscle, the same staining of the NETs was carried out on this MDX sample. Staining of the muscle section revealed the presence of all NETs with no observable difference when compared with controls (figure

14). Poor sectioning and freezing artifacts present meant it was not possible to confirm WFS1 expression.

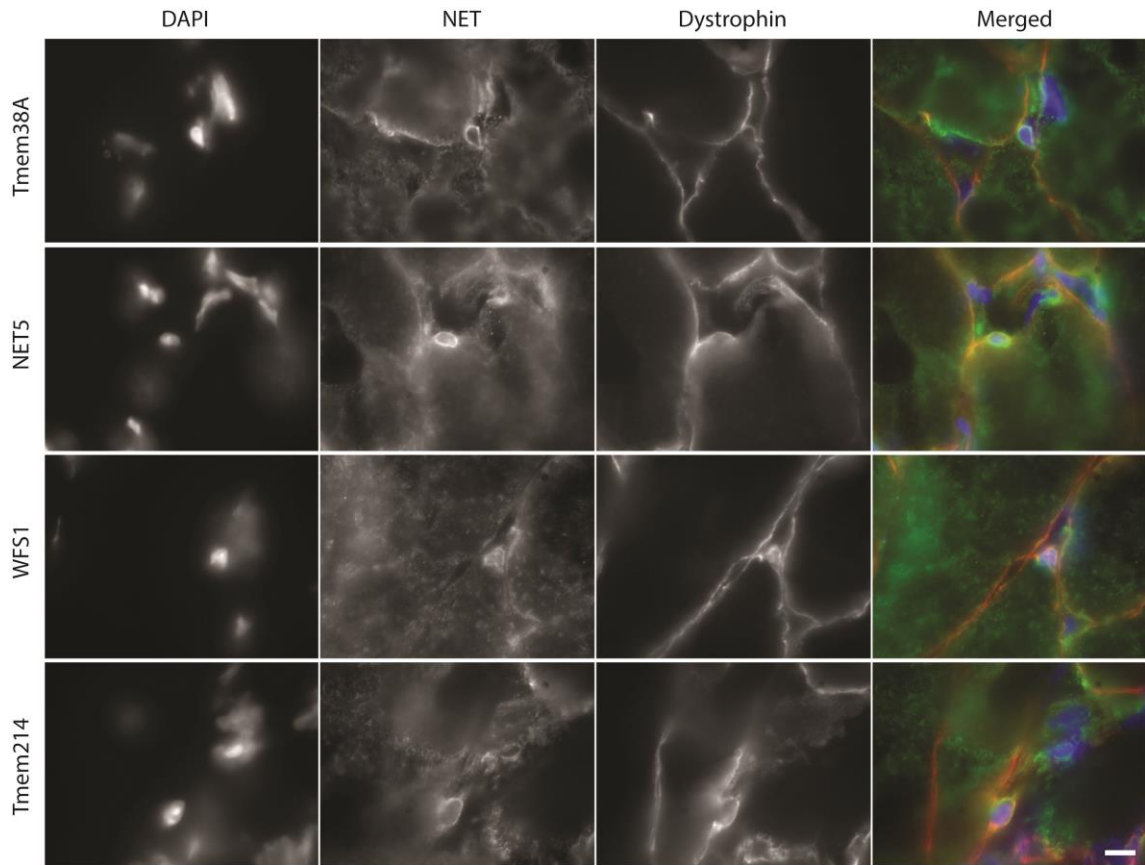


Figure 9. Staining of NETs identified in the proteomic studies in mouse Gastrocnemius. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Tested NETs stained positively in muscle nuclei. Scale bar = 10 μ m

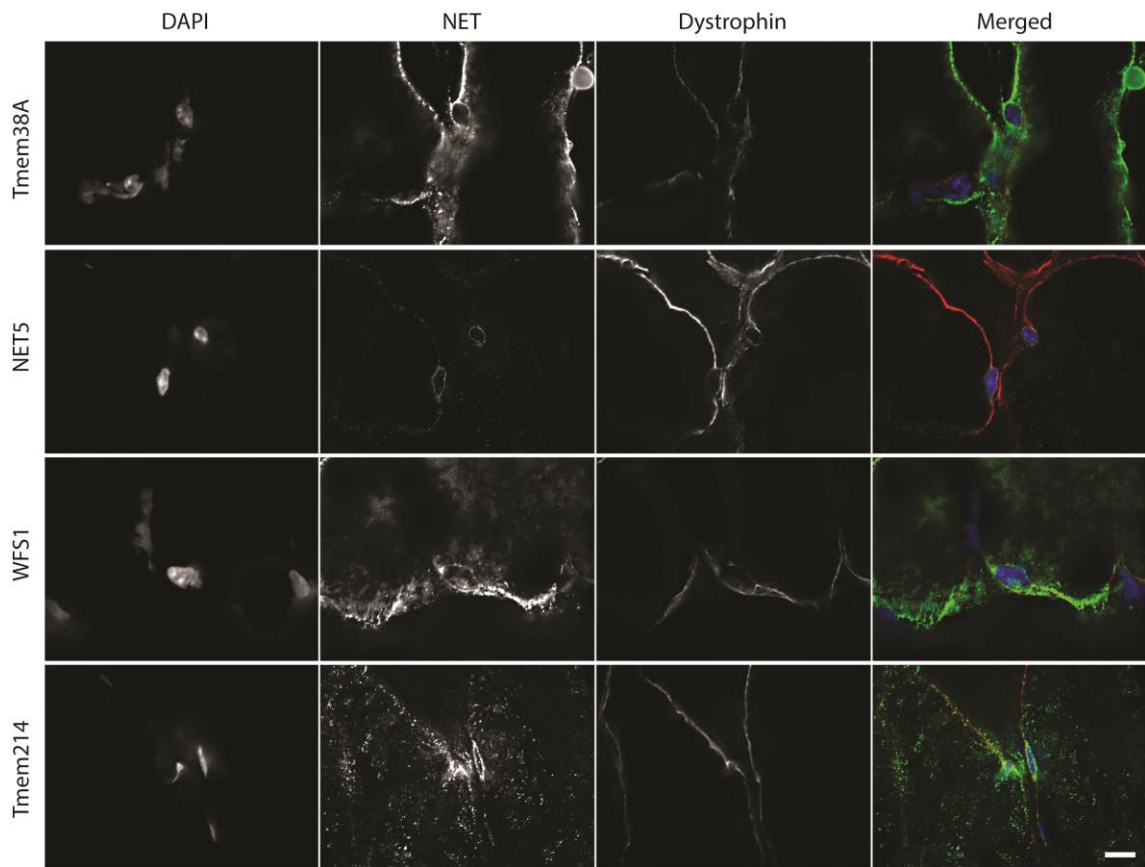


Figure 10. Staining of NETs identified in the proteomic studies in mouse Tibialis Anteria. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Tested NETs stained positively in muscle nuclei. Scale bar = 10 μ m

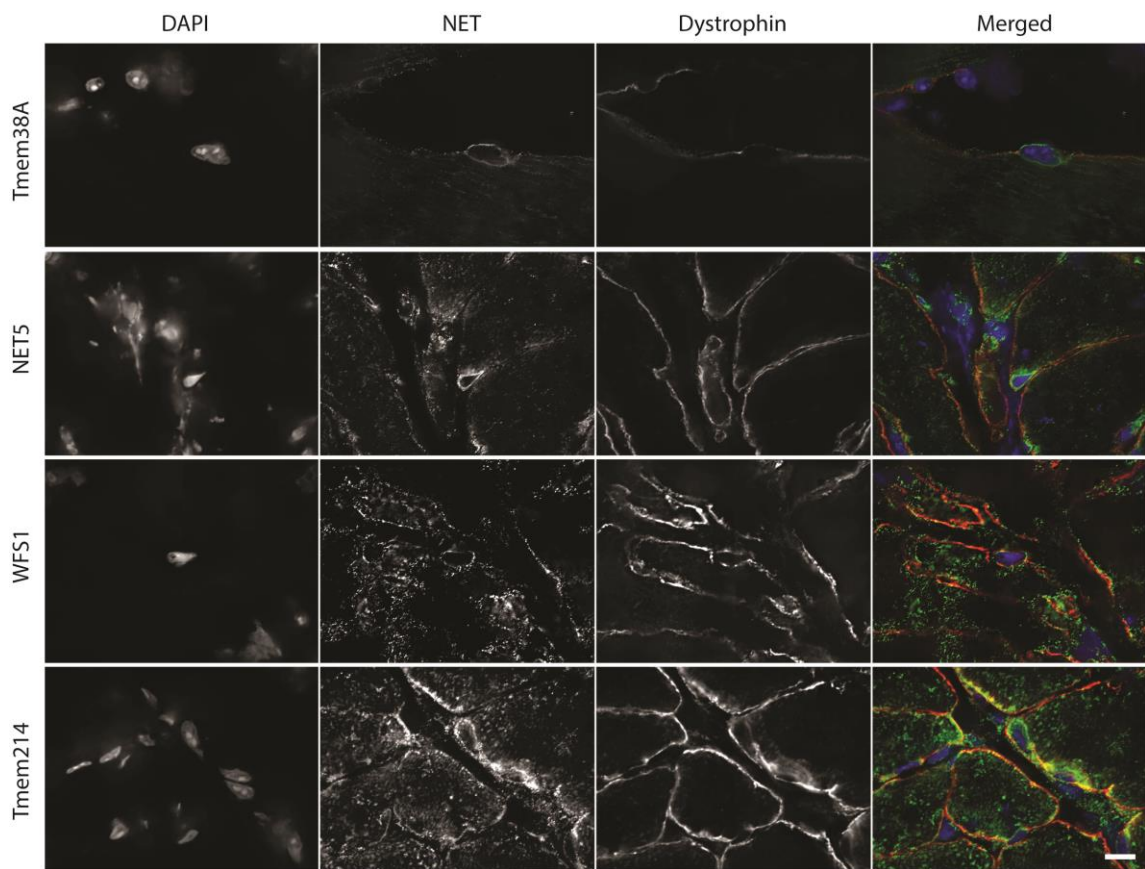


Figure 11. Staining of NETs identified in the proteomic studies in mouse Diaphragm. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Tested NETs stained positively in muscle nuclei. Scale bar = 10 μ m

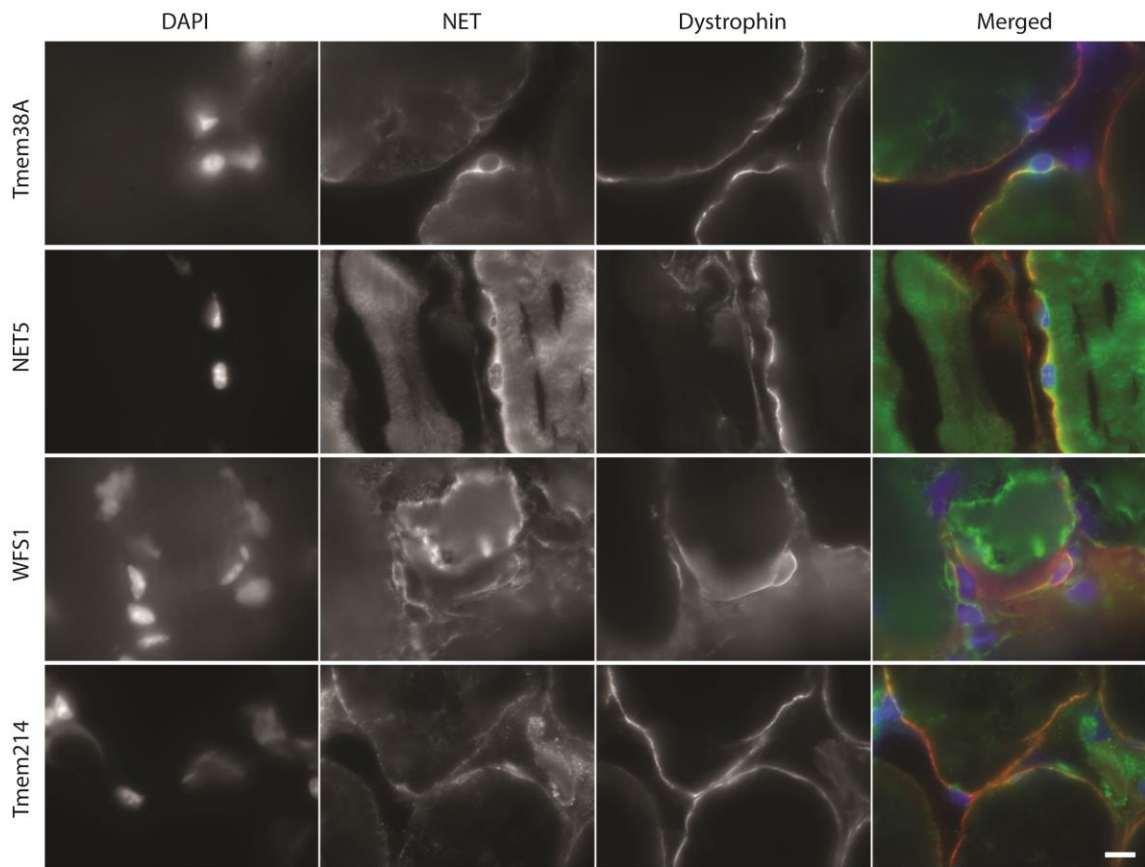


Figure 12. Staining of NETs identified in the proteomic studies in mouse Extensor Digitorum Longus. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Tested NETs stained positively in muscle nuclei. Scale bar = 10 μ m

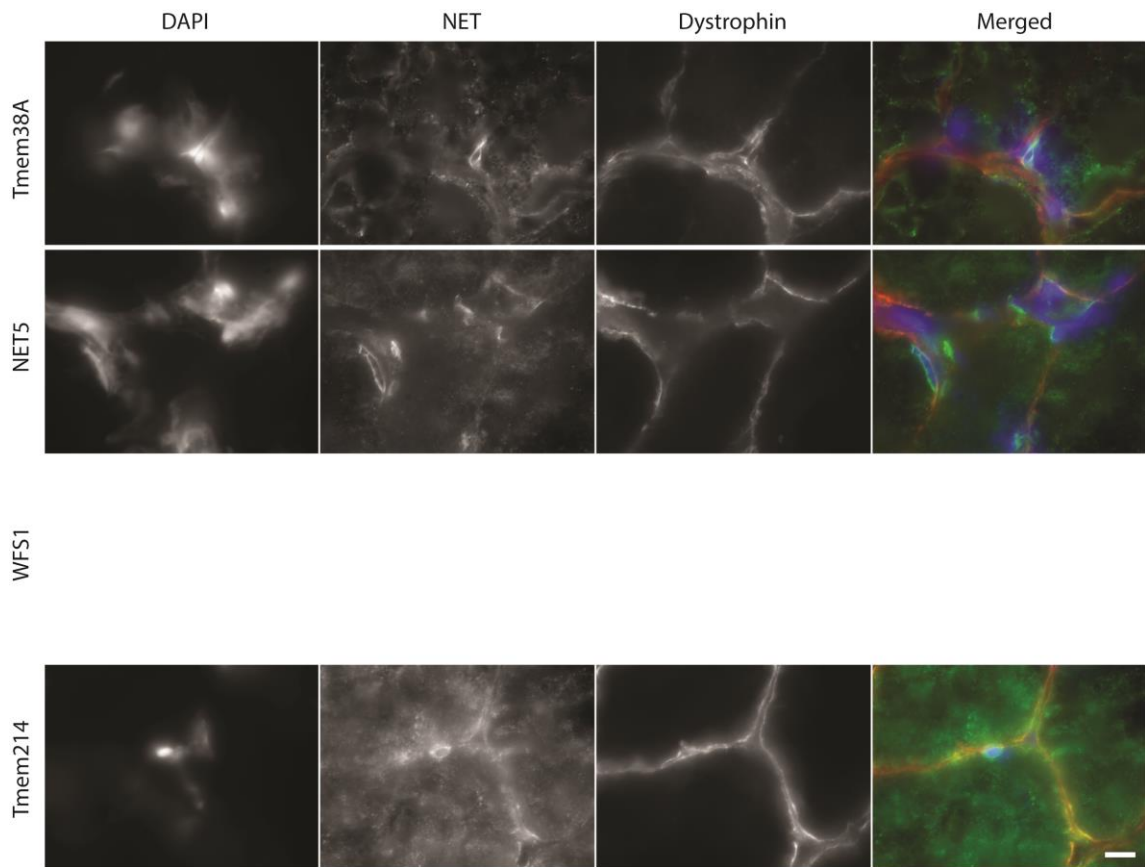


Figure 13. Staining of NETs identified in the proteomic studies in mouse *Musculus Soleus*. Due to poor staining, expression and distribution of WFS1 was not confirmed. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Tested NETs stained positively in muscle nuclei. Scale bar = 10 μ m

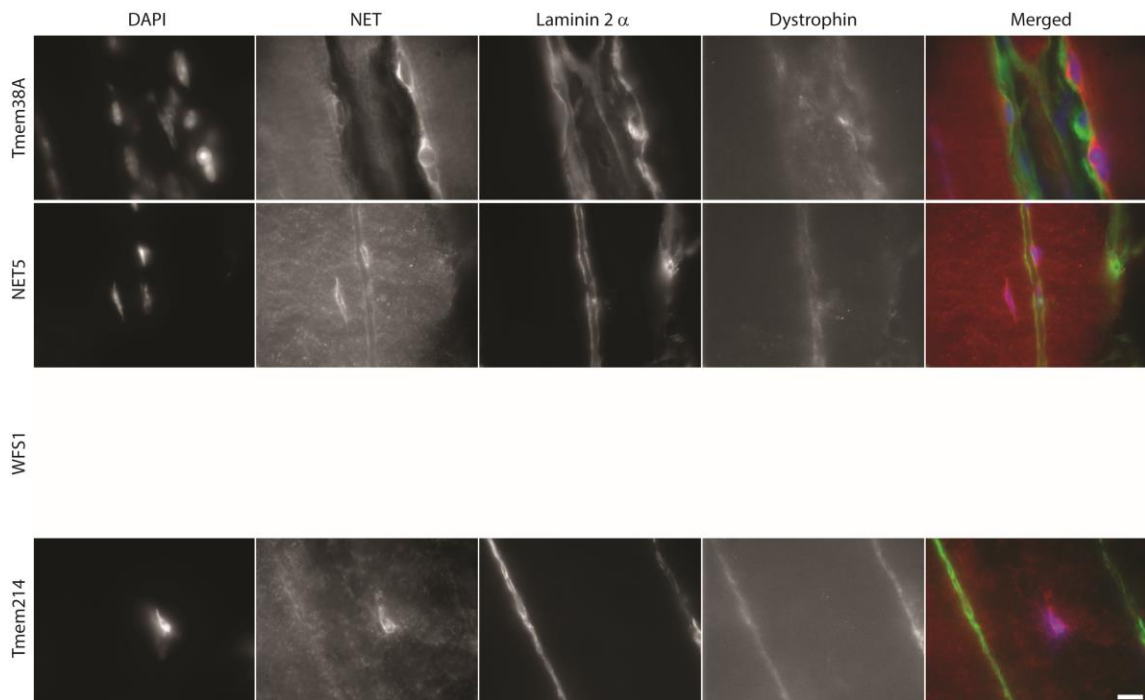


Figure 14. Staining of NETs identified in the proteomic studies in a muscular dystrophic mouse *Gastrocnemius*. Due to poor staining, expression and distribution of WFS1 was not confirmed. (Red) Dystrophin staining marking plasma membrane of muscle fibers. (Green) NET staining. (Blue) DAPI staining marking nuclei. Tested NETs stained positively in muscle nuclei. Scale bar = 10 μ m

3.3. Results summary

In this chapter, I investigated the expression and distribution of Tmem38A, Tmem214, WFS1 and NET5 using immunofluorescence staining on tissue sections obtained from both human and mouse (human matched muscles). The presence of all four NETs was confirmed in muscle fiber nuclei in both species. The staining of the muscle sections did not reveal differences between muscle groups, and three NETs (Tmem214, WFS1 and NET5) were found at the NE of nuclei in the surrounding connective tissues. Tmem38A was found exclusively to reside at the NE of nuclei inside muscle fibers. Normal mouse muscles were compared to samples of dystrophic mouse gastrocnemius and found no observable changes.

Chapter 4

Introduction

Emery-Dreifuss muscular dystrophy (EDMD) typically presents in early childhood with slow progression, though adult onset also occurs (Bonne and Quijano-Roy 2013, Bonne et al. 2000). Three defining features of this disorder include early contractures of the elbows and Achilles' tendons in the absence of major muscular defects, progressive wasting of the lower leg and upper arm muscles and cardiac conduction defects (Buckley, Dean, and Mahy 1999). All these features are variable in clinical presentation; while typical patients remain ambulatory, severe cases require wheelchairs. Likewise, cardiac defects do not always present, but complete heart block can occur in the most severe cases. Conduction defects can also present in the absence of prior muscular involvement (Emery 2000) and female carriers of the X-linked form can develop cardiac problems (Merchut, Zdonczyk, and Gujrati 1990). Even within the same family, the same mutation can yield highly variable clinical presentation among family members (Bonne et al. 2000, Mercuri et al. 2004, Rankin et al. 2008).

With this clinical variability, it was not surprising to find that EDMD is also genetically variable. Mutations in eight nuclear envelope proteins account for ~47% of patients. The vast majority of mutations are X-linked in *EMD* (encoding emerin) (Bione et al. 1994) and autosomal dominant in *LMNA* (encoding lamin A and C) (Bonne et al. 1999) though more rare autosomal recessive *LMNA* mutations also occur (Raffaele Di Barletta et al. 2000). Lamin A is a nuclear intermediate filament protein that lines the inner surface of the nuclear envelope, while emerin is a nuclear envelope transmembrane protein (NET). Roughly 3% of patients are linked to mutations in five other NETs: TMEM43, SYNE1,

SYNE2, SUN1 and SUN2 (Liang et al. 2011, Meinke et al. 2014, Zhang et al. 2007). The remaining 3% of known mutations are linked to FHL1 (encoding Four and a half LIM domain 1) (Gueneau et al. 2009). FHL1 has many splice variants that have multiple cellular localizations including muscle z-bands and the nucleus, but FHL1B also targets to the nuclear envelope (Ziat et al. 2016). FHL1 is also linked to other myopathies such as X-linked myopathy with postural muscle atrophy (XMPMA) (Windpassinger et al. 2008) and deletion in mice yields to muscle hypertrophy (Sheikh et al. 2008). The strong nuclear envelope links for nearly half of all cases raises the possibility of a common pathway at the nuclear envelope affected in EDMD.

The principal mechanisms proposed to explain how nuclear envelope disruption can yield pathology are genome misregulation, mechanical instability and failure of stem cell maintenance, all potentially leading to impaired differentiation. However, it is unclear how mutations in these widely-expressed proteins can cause this muscle-specific disease. One proposed model is that muscle-specific partners that function in complexes with these widely expressed nuclear envelope proteins might mediate the muscle-specific pathologies. Several candidates were identified by proteomics of muscle nuclear envelopes (Wilkie et al. 2011). WFS1, Tmem214 and Tmem38A/TRIC-A were identified only in muscle, out of several tissues separately analyzed by proteomics for nuclear envelopes (Korfali et al. 2012). NET5/SAMP1 was found in nuclear envelopes from other tissues, but has a muscle-specific splice variant (Zuleger et al. 2013). Several of these are candidates for mechanical functions due to implied connections to the cytoskeleton: NET5/SAMP1, WFS1 and Tmem214 localize to the mitotic spindle (Buch et al. 2009, Wilkie et al. 2011) and NET5/SAMP1 knockdown dissociates centrosomes from the NE (Buch et al. 2009). Tmem214 tracked with microtubules on the nuclear surface (Wilkie et

al. 2011). WFS1 also has a separate function shared by Tmem38A/TRIC-A in genome organization and regulation of gene expression during myogenesis (Robson et al. 2016). Tmem38A/TRIC-A separately contributes to the regulation of calcium ion transport (Tao et al. 2013, Venturi et al. 2013, Yamazaki et al. 2011, Yazawa et al. 2007). That some of these muscle-specific NETs had overlap in their functions further supports the possibility of their working in a common pathway towards EDMD pathophysiology.

I postulate that if a central mechanism at the NE underlies EDMD disease pathology through disruption of a functional complex then components of that complex might redistribute away from the NE. Previous studies reported that emerin depends on lamin A for its localization to the nuclear envelope (Sullivan et al. 1999, Vaughan et al. 2001) and that lamin EDMD mutations similarly yield a loss of emerin at the nuclear envelope (Charniot et al. 2003, Raharjo et al. 2001). Additionally, the loss of nesprin localization with emerin EDMD mutations and corresponding loss of emerin localization with nesprin (*SYNE*) EDMD mutations was reported in muscle sections (Zhang et al. 2007). However, no study has comprehensively tested for the mislocalization of the wider range of EDMD-linked proteins in a panel of patients covering the genetic spectrum of EDMD. Here a wide panel of EDMD muscle biopsy samples and cultured myoblasts was stained with a range of antibodies to the EDMD-linked proteins. To investigate potential muscle-specific NET involvement in disease mechanisms, the samples were also stained with antibodies against the muscle-specific NETs NET5/Samp1, WFS1, Tmem214 and Tmem38A. It was found that neither emerin nor lamin A nor any of the other NETs are uniformly altered in all patient samples. However, nesprin 1, SUN2, and several muscle-specific NETs exhibited unusual distribution patterns in a subset of samples. These findings indicate

that there are likely to be multiple pathways leading to EDMD pathology and suggest the possible involvement also of these muscle-specific NETs in the disease.

4.1. EDMD variants

Emerin

Like most nuclear envelope transmembrane proteins (NETs), most of the functions of emerin remain a mystery. Emerin belongs to the LEM-domain family of proteins (Lin et al. 2000). Members of this family directly bind barrier-to-autointegration factor (BAF) (Lee et al. 2001). It has been shown that this emerin-BAF interaction is important for the reintegration of emerin into reforming NE (Haraguchi et al. 2001). BAF has been shown to associate with histones and chromatin-regulatory partners (Montes de Oca, Lee, and Wilson 2005) (Montes de Oca et al. 2009), suggesting that this protein plays an important role in chromatin regulation (Segura-Totten et al. 2002) and is important in regulating cell fusion, an important process in myogenesis (Margalit et al. 2007). Emerin has been shown to bind to and regulate the activity of histone deacetylase 3 (HDAC3) a protein involved in chromatin silencing (Demmerle, Koch, and Holaska 2012). Interactions of emerin with chromatin modifying factors suggests that emerin may have important roles in genome organisation and regulation. Emerin has also been implicated in regulating β -catenin activity (Markiewicz et al. 2006), suggesting a role in signalling control. Emerin and BAF have also been found to bind lamin A (Clements et al. 2000) (Simon and Wilson 2013), an intermediate filament protein and core component of the nuclear lamina. A-type lamin has been shown to be involved in many important processes such as chromatin organisation, gene regulation, mechanotransduction and nuclear structural integrity amongst others (Dechat et al. 2008). Emerin and *LMNA* null cultured cells showed increases in NE defects and mechanotransduction (Lammerding et al. 2005) (Lammerding et al. 2004), suggesting important roles for both lamin A and emerin in NE structural integrity and also mechanotransduction. Mutations in the lamin A gene have

been linked to many tissuespecific diseases including EDMD. The evidence presented so far suggests an important role of the emerin and its interaction network in EDMD parthenogenesis.

FHL1

Another X-linked gene, Four and a half LIM domain 1 (*FHL1*) has also been linked to EDMD (Gueneau et al. 2009). There are three known isoforms of the FHL1 protein and so far, isoform A shows high expression in skeletal and heart muscle (Lee et al. 1998). A high frequency of mutations are clustered around FHL1 p.C224W, which was found to be present in unrelated EDMD patients along with a X-linked myopathy with postural muscle atrophy (XMPMA) (Windpassinger et al. 2008). All mutations that are linked to EDMD are found in the distal region of the gene and have been found to disrupt the LIM domains or nuclear localisation sequence (NLS) and FHL1 shows reduced expression in affected individuals (Windpassinger et al. 2008). One aspect of FHL1 associated X-linked EDMD is that affected individuals can show postural muscle atrophy while other muscle groups show hypertrophy, leading to the appearance of an athletic constitution (Morris, Sewry, and Wehnert 2001) (Windpassinger et al. 2008). Affected individuals can also show hypertrophy of the heart, which could lead to hypertrophic cardiomyopathy (Gueneau et al. 2009). Localisation of FHL1 has recently been confirmed at the nuclear envelope and it has been shown to interact with emerin and lamin A (Ziat et al. 2016). However, the other LIM domain proteins have been extensively studied and shown to be involved in processes such as cytoskeletal organisation among numerous other biological pathways (Kadmas and Beckerle 2004).

Lamin A

Autosomal dominant EDMD (AD-EDMD) also exists, the first gene linked to this form of EDMD was lamin A (Bonne et al. 1999). There are four main types of lamins, A, B1, B2 and C, that all localise to the INM with compositional differences between cell types. The differences in lamin sub-type composition contribute to the relative stability of the nuclear lamina for each cell type (Schirmer and Gerace 2004). Apart from structural roles of lamins as part of the underlying nucleoskeleton, lamins have been linked to many functions and mutations in lamin A in particular have been linked to many diseases (Worman and Bonne 2007). Lamin A linked AD-EDMD is more clinically variable than its X-linked cousins with variations in severity and pathology within families (Bonne et al. 2000) (Morris, Sewry, and Wehnert 2001). Many tested NETs have been shown to interact with lamins either directly or indirectly. Given its many functions and interaction partners, it is likely that lamins and NETs form a large network at the NE and disruptions to this network can lead to pathology and the variations seen in EDMD. A rare lamin A associated autosomal recessive case of EDMD (AR-EDMD) has also been reported (Raffaele Di Barletta et al. 2000).

LINC complex (nesprins and SUN proteins)

Another important set of mutated proteins linked to EDMD are proteins of the Linker of nucleoskeleton complex (LINC). This complex consists of the core proteins nesprin 1, nesprin 2 SUN1 and SUN2. Components of the LINC complex have been proven to reside at the NE, nesprins to the outer nuclear membrane (ONM) and SUNs to the INM (Zhang et al. 2001) (Crisp et al. 2006) (Hodzic et al. 2004). The role of the LINC complex in connecting the nucleoskeleton network with the cytoskeleton is well characterised

(Meinke and Schirmer 2015). It has been shown that nesprin 1 and 2 associate with EDMD linked proteins lamin A and emerin (Mislow, Holaska, et al. 2002) (Zhang et al. 2005). Along with interacting with already linked proteins, mutations in both the nesprin and SUN proteins have been linked to EDMD (Zhang et al. 2007) (Meinke et al. 2014). Cultured primary cells from LINC complex deficient patients often show defects in NE morphology and impaired association with binding partners. Mutations in nesprins have all been found in the lamin/emerin binding domain. The data suggests that structural integrity of the NE plays an important role in disease pathology and that perturbation of the interactions between the LINC complex, proteins of the NE, nuclear lamina emerin and lamins may cause EDMD.

Tmem43

Recent patient analysis revealed Tmem43/LUMA, a NET identified to reside at the INM (Dreger et al. 2001) is linked to a EDMD-related myopathy (Liang et al. 2011). The index patient (p.Glu85Lys) was diagnosed with EDMD, muscle pathology revealed fiber size variations as well as internal nuclei. Patient 2 was reported to have slow progressive proximal muscle weakness requiring a pacemaker transplant at 64 years, due to atrial fibrillation with bradycardia. Immunohistochemistry revealed normal positive staining for emerin among other markers. It is reported that LUMA can form homo-oligomers and the reported mutation (p.Glu85Lys) disrupts this oligomerization. Co-immunoprecipitation assay carried out by the same group showed interactions with emerin and SUN2, but not SUN1. They also reported no marked differences between binding to WT and mutant LUMA (Liang et al. 2011). The same report also found that over-expression of mutant LUMA disrupts NE morphology and forms extranuclear aggregates (Liang et al. 2011). Additionally, it was found that emerin and SUN2 showed decreased staining at the NE

with emerin mislocalisation to the ER (Liang et al. 2011). Electroporation of mouse Tibialis Anterior muscle with mutant LUMA showed the same decrease in emerin and SUN2. LUMA disruption of linked NE protein in patients suggests pathology may arise due to disruption of protein complexes.

Other

More recently, mutation resulting in the loss of expression of another NE protein LAP1 β in skeletal muscle nuclei, was reported in the case of a patient with muscle weakness and atrophy with contractures, rigid spine and cardiac defects (Kayman-Kurekci et al. 2014). LAP1 β is an INM protein which has been shown to bind lamins directly (Senior and Gerace 1988) (Foisner and Gerace 1993) and it also interacts with emerin (Shin, Mendez-Lopez, et al. 2013). LAP1 appears to have higher expression in mouse striated muscle than in human striated muscle, a pattern that is opposite to emerin. Also unlike emerin, a conditional LAP1 β knockout mice showed a muscular dystrophy phenotype (Shin, Mendez-Lopez, et al. 2013). Expression patterns in mice and the loss of expression in muscular dystrophic patients suggests that LAP1 β potentially has overlapping functions with emerin and may cause disease phenotypes in much the same way. Although not currently classified as EDMD due to the lack of elbow contractures, we should keep an open mind as the case of LAP1 β may be a new variant of EDMD not yet seen before.

4.2. EDMD mechanisms

With potentially 8 NE/nuclear lamina proteins linked to the disease, there is no doubt that EDMD is a disorder of the NE. It is still unclear how seemingly widely expressed protein cause tissue-specific diseases such as EDMD, but separately from this question is the

more fundamental question of how NE defects can yield pathology. Current mechanisms proposed to try and explain the pathology of these diseases include altered gene regulation, mechanical stress and failure of stem cell maintenance. Indeed, there is much evidence supporting all of these mechanisms. Extensive roles of NETs in genome organisation and the binding and regulation of transcriptional regulators would be consistent with the assumption that disruption of such regulatory interactions could lead to impairment of differentiation pathways, ultimately leading to muscle loss after damage. Studies have found NE deformation associated with *LMNA* and emerin mutations (Lammerding et al. 2004) (Lammerding and Lee 2005) (Lammerding et al. 2005, Lee et al. 2007, Broers et al. 2005, De Vos et al. 2010, De Vos et al. 2011, Houben et al. 2009). Defects in how cells deal with mechanical stress could lead to inability of muscle to repair damage caused by use. Failure of stem cell maintenance could also help explain muscle loss, as if the population of muscle satellite cells are depleted then muscles would lose their ability to replace damaged fibres. While these mechanisms may explain some of the pathways to pathology, none of the mechanisms proposed so far can fully explain the tissue-specificity of NE linked diseases and it is likely that a combination of mechanisms are required for a disease phenotype.

Although we've begun to understand the interconnectivity of the nuclear envelope and its protein complement (figure 15), there is much still unknown to us. It is postulated that tissue-specificity is conferred by interactions between the known linked NE proteins and as yet unidentified tissue-specific proteins that reside at or interact with the NE. Proteomic studies have found a subset of tissue-specific NETs (Schirmer et al. 2005) (Wilkie et al. 2011) (Korfali et al. 2012) which could potentially add to our understanding of the specificity of phenotypes in diseases such as EDMD. Indeed, studies on these new NETs

have found them to have functions in genome organisation, cell cycle control and differentiation (Zuleger et al. 2013) (Malik et al. 2014) (Batrakou et al. 2015). Functional screens carried out on these NETs also revealed other NETs that have associations with structural components of the cell. In particular, NET5/SAMP1 has been shown to be an INM protein which localises to the mitotic spindle poles, and knockdown of NET5/SAMP1 was shown to increase the distance between the centrosomes and the NE (Buch et al. 2009) (Figueroa et al. 2010). NET5/SAMP1 has also been shown to interact with some of the LINC complex components along with emerin and lamin A (Gudise et al. 2011) (Jafferli et al. 2014). On top of these findings, NET5/SAMP1 has also been found to associate with TAN lines, playing an important role in nuclear migration (Borrego-Pinto et al. 2012). These associations with linked components of EDMD suggest NET5/SAMP1 could play a role in disease pathology through the mechanical stability mechanism. Also, identified in the functional screens were three other proteins with apparent cytoskeleton associations, namely Tmem70, Tmem214 and WFS1. Apart from cytoskeletal associations and genome organisation functions, tissue-specific NETs have also been found to be involved in signalling. Tmem38A/TRIC-A, a muscle specific NET is reported to form cation channels transporting calcium (Yazawa et al. 2007) (Yamazaki et al. 2011) (Venturi et al. 2013) (Tao et al. 2013) (Zhou et al. 2014). Signalling is important in muscle function and defective muscle function may lead to disease pathology.

Finding new or similar functions of these NETs to existing linked NE proteins suggests that as a tissue-specific component of the network, they could potentially contribute to pathology via the mechanisms mentioned above. As all proteins linked to EDMD have been shown to interact with each other, albeit separately, a postulate is that linked proteins and tissue-specific NETs interact in a large network with the tissue-specificity

explained by interaction with new novel NETs. About 50% of EDMD cases are linked to 8 NE proteins described in section 4.1. This leaves a large number of patients with EDMD and no linked cause that could be due to mutations in these muscle-specific proteins with functions linked to the postulated mechanisms for EDMD disease pathology. To further study the involvement of existing and new NETs (NET5/SAMP1, Tmem38A, Tmem70, Tmem214, WFS1), presented here is panel screen of nine EDMD patients, looking at linked (already found to cause disease) and unlinked (no diseases associated) NETs and their expression pattern in different stages of muscle development as well as different cell type and also potential interactions between novel NETs and existing NE proteins. I postulate that if other NETs mediate tissue-specific pathology, they might be disrupted in their distribution in patients.

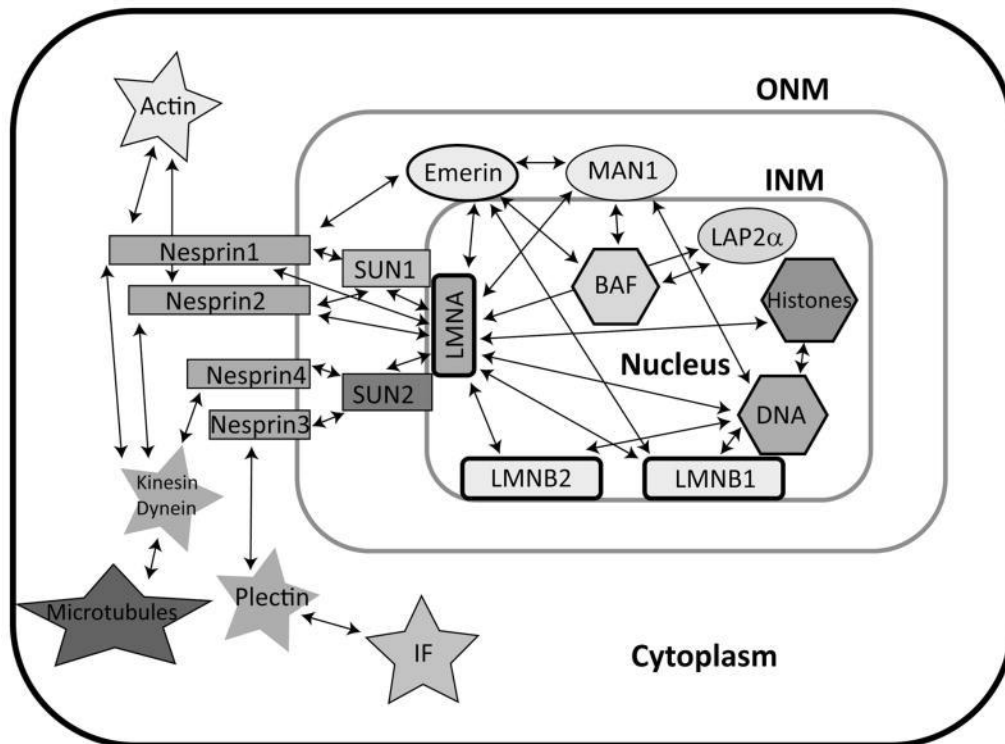


Figure 15. The current known network of the cell. (Figure taken from (Kaminski, Fedorchak, and Lammerding 2014))

4.3. Distribution of EDMD-linked proteins in cultured patient muscle cells

Several earlier reports presented data showing that emerin, nesprins and lamin A/C staining, normally concentrated at the nuclear envelope, was aberrant in cells expressing EDMD mutations (Raharjo et al. 2001, Sullivan et al. 1999, Vaughan et al. 2001, Zhang et al. 2007). However, typically only a single patient mutation was tested and only lamin A/C and a few NETs were tested for any given sample, though EDMD has now been linked to eight different nuclear envelope proteins. To determine if any particular one of these proteins is recurrently defective in its intracellular distribution, a panel of three control and eight EDMD patient myoblasts (Table 12) were stained for emerin, lamin A/C, nesprin 1, nesprin 2, SUN1, SUN2, and FHL1. All stainings were done in parallel and all images were taken with the same exposure times and microscope software settings. The samples in table 12 were obtained from CNDB (Centre for Neuromuscular Disorders Biobank) and MTCC (Muscle Tissue Culture Collection).

Table 12. Patient and control myoblast/fibroblast cultures used in this study

	Type	Gender	Age at biopsy	Source
C1	Control	Female	36	MTCC
C2	Control	Male	35	MTCC
C3	Control	Male	5yrs	CNDB
P1	FHL1	Male	51	MTCC
P2	LMNA p.R453W	Female	12	MTCC
P3	EMD p.Y59*	Female	17	MTCC
P4	LMNA p.R545C	Male	18	MTCC
P5	Unknown	Male	In teens	MTCC
P6	LMNA p.T582K	Male	2yrs	CNDB
P7	LMNA p.E358K	Female	2yrs	CNDB

This panel included patients with lamin A/C-linked (P2, 4, 6 and 7), emerin-linked (P3), and FHL1-linked (P1) disease (table 12). Surprisingly, emerin, despite previous reports of its aberrant distribution, exhibited strong nuclear envelope staining, with a crisp rim of fluorescence at the nuclear perimeter (nuclear rim) in all patient cells and was indistinguishable from the control cells (Fig. 16). Patient P3 was a female with a heterozygous truncation mutation (p.Y59*) in the X chromosomal gene encoding emerin. This patient atypically had a muscle phenotype, but expressed full-length emerin in a subset of cells, likely indicating an additional unknown mutation (Meinke et al. 2015). Here this subset of emerin-positive cells exhibited a moderately weaker staining compared to other patients. While some emerin accumulation in the ER appeared in patients P2 and P5, it was not more than for control C2, and this control had more ER accumulation than other EDMD patient cells. Thus, any minor differences in emerin distribution were within the same breadth of such differences exhibited by the control group.

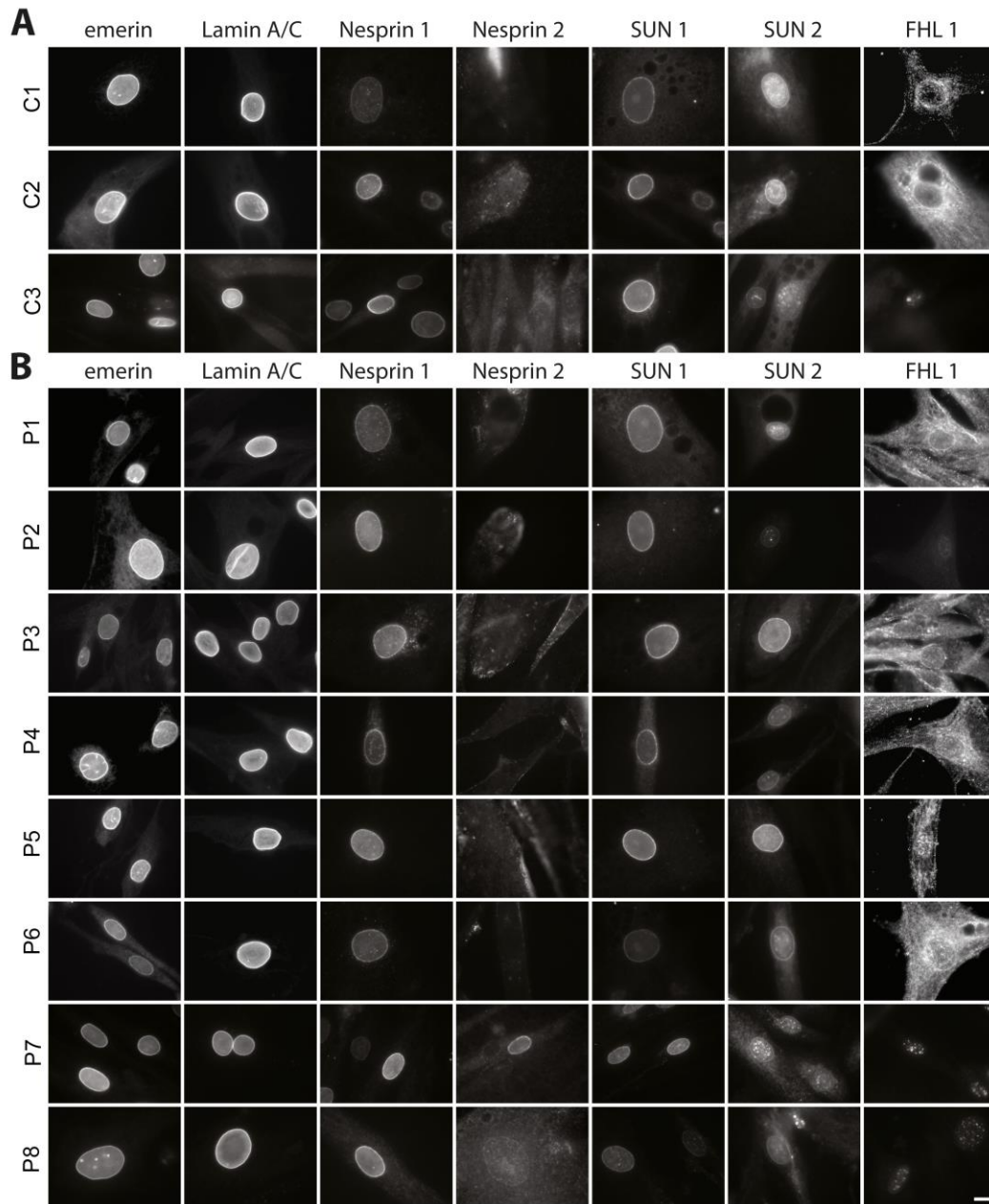


Figure 16. Staining of control (A) and patient (B) myoblasts with antibodies to EDMD-linked proteins. The patient and control descriptions are given in Table 13 and the antibodies used are described in Table 14. Most nuclear envelope proteins gave strong crisp nuclear rim staining in both control and patient groups. Though some EDMD-linked patient cells exhibited partial mislocalisation, no linked proteins exhibited uniform mislocalisations in the wide range of patient mutations investigated. The nesprin 2 antibodies do not stain well in the myoblast/fibroblast cultures; so, these were retested on differentiated cells in Figure 15. Widefield images are shown. Scale bar, 10 μ m.

No visible differences were observed for lamin A/C staining between the patient and control cells and even within each set, unlike emerin where both some control and some patient cells exhibited minimal ER accumulation (Fig. 16). The image selected for control C3 was chosen because the cell was smaller, likely due to being at an earlier cell cycle stage, and characteristically a larger nucleoplasmic lamin pool is present before the lamina is fully reassembled and nuclear volume increases after mitosis. None of the larger or smaller cells from the patients had more nucleoplasmic lamin accumulation than this control, further underscoring the fact that any minor visible differences can be discounted.

For the other NETs and FHL1 there was greater variation amongst samples, but in nearly all cases a similar range of variation was observed for the controls (Fig. 16). For example, in multiple controls nesprin 1 staining was variable in intensity at the nuclear membrane compared to the nucleoplasm (presumably representing out of focus light from the top and bottom of the nucleus). Roughly half of the control cells also exhibited some punctate staining in the nucleoplasm, most likely due to invaginations, but possibly also soluble splice variants (the antibody used, MANES1E(8C3), was generated to full-length nesprin1- α). Within the patient population similar variation was observed in overall intensity, relative rim intensity and punctate areas. However, patients P3 and P4 exhibited minor staining in the ER that was not observed for either the controls or the other patients. Although this is a different specific mutation, the P3 staining is consistent with the previous report of nesprin mislocalization with an emerin EDMD mutation (Zhang et al. 2007). This is a new observation for the P4 LMNA p.R545C mutation, but notably other lamin and the FHL1 mutant myoblasts did not exhibit similar ER accumulations; thus, this difference is not a general characteristic of EDMD. SUN2 also exhibited some ER

accumulation in myoblasts from two patients, but these were different patients with lamin mutations (LMNA p.T528K and LMNA p.E358K) and some ER accumulation was also observed in the control myoblasts. In general, SUN2 and FHL1 exhibited the most variable staining patterns, but as variability was also observed in the controls this may reflect effects of the cell cycle or differentiation state.

This latter issue of differentiation state is likely the reason for the poor staining of nesprin 2, which is stained well by this antibody in differentiated myofibers (Duong et al. 2014). Notably, the one patient with clear rim staining, P6 (LMNA p.T528K), had the appearance of multiple nuclei lined up in a myotube, while the weak rim staining for P7 (LMNA p.E358K) appears to reflect a senescent cell by its extremely large nucleus and spread cytoplasm. Therefore, nesprin 2 was also stained for after induction of differentiation in reduced serum differentiation medium (Fig. 17). Not all patient cells differentiated efficiently into fused myotubes, perhaps due to myoblast passage number in culture or different amounts of contaminating fibroblasts. Nonetheless, a distinct rim-staining pattern could be observed in both the C1 control and all EDMD patient cells tested.

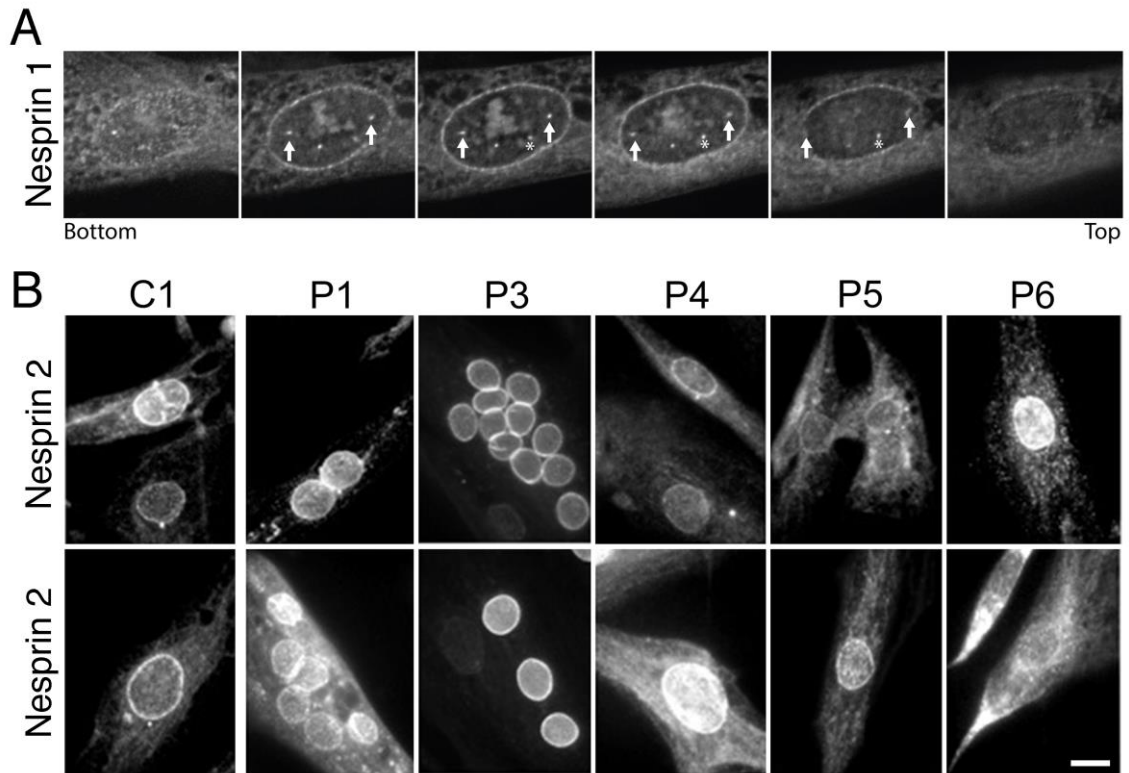


Figure 17. Staining of myoblast/fibroblast cultures and differentiated myotubes with nesprin antibodies. A: To determine whether intranuclear spots staining with nesprin antibodies reflected invaginations of the nuclear envelope as opposed to possible degradation/cleavage products, z-series images of cells stained with nesprin 1 antibodies were taken every 0.2 μm . In the images shown the arrows and asterisk point to different intranuclear spots that can be traced through the different focal planes to invaginations from the nuclear membrane. **B:** Staining after induction of differentiation into myotubes for nesprin 2. Large multinucleated myotubes were not obtained from all patients; however, changes in morphology such as elongating of the cell body or much larger cells indicative of cell cycle withdrawal were generally evident and rim staining could be readily observed compared to the very poor rim staining in the myoblast/fibroblast cultures stained in Figure 14. Multiple images for each patient are shown to show the variability between cells. Widefield images are shown. Scale bar, 10 μm .

4.4. Distribution of muscle-specific NETs in cultured EDMD patient myotubes

As the EDMD-linked NETs are all widely expressed and known to have many binding partners, their failure to exhibit aberrant distribution patterns uniformly through the set of patient samples might reflect redundancy in the partners to retain them at the nuclear membrane. As mutations in widely expressed nuclear envelope proteins cause a much wider range of tissue-specific diseases including also lipodystrophy, dermatopathy, neuropathies and bone disorders, it has been proposed that tissue-specific binding partners might mediate the tissue-specific pathologies (Worman and Schirmer 2015). Therefore, muscle-specific partners might contribute to disease pathology, have fewer binding sites and be more likely to be disrupted in their distribution in patients.

Antibodies were obtained for Tmem38A, NET5/Samp1, Tmem214 and WFS1 and tested for their specificity. C2C12 cells were transduced with lentiviruses encoding GFP fusions to these NETs, fixed, and stained with the NET antibodies. In all cases the GFP-signal co-localized with the NET antibody signal (Fig. 18A). Notably, for NET5/Samp1 and WFS1 the endogenous rim staining was sufficiently stronger than the GFP-fusions that an even more pronounced rim was observed in the antibody stained sample than for the GFP signal. The antibodies were also tested by Western blot from lysates generated from additional cells from the same transfections (Fig. 18B). In all cases the band recognized by GFP antibodies for the muscle NET-GFP fusion was also recognized by the muscle NET antibody. Hence, all antibodies are specific and function for immunohistochemistry.

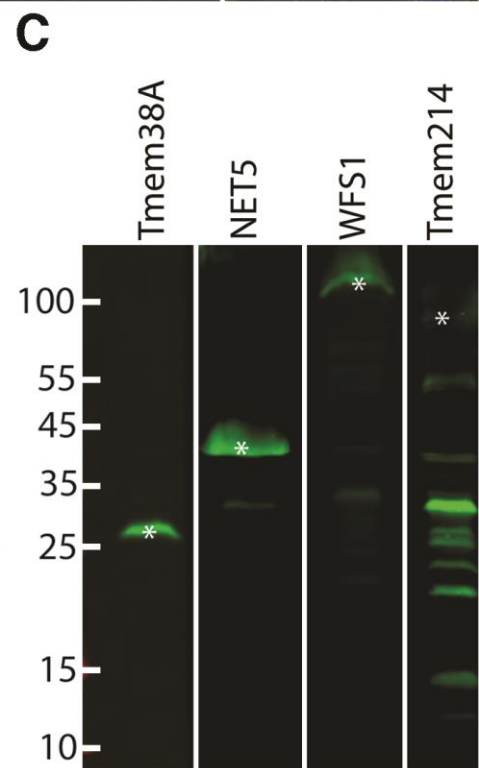
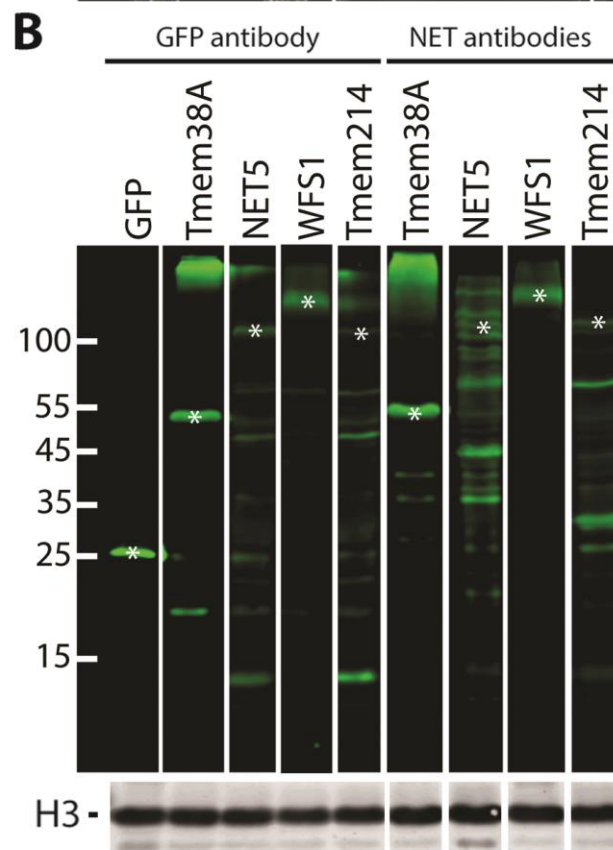
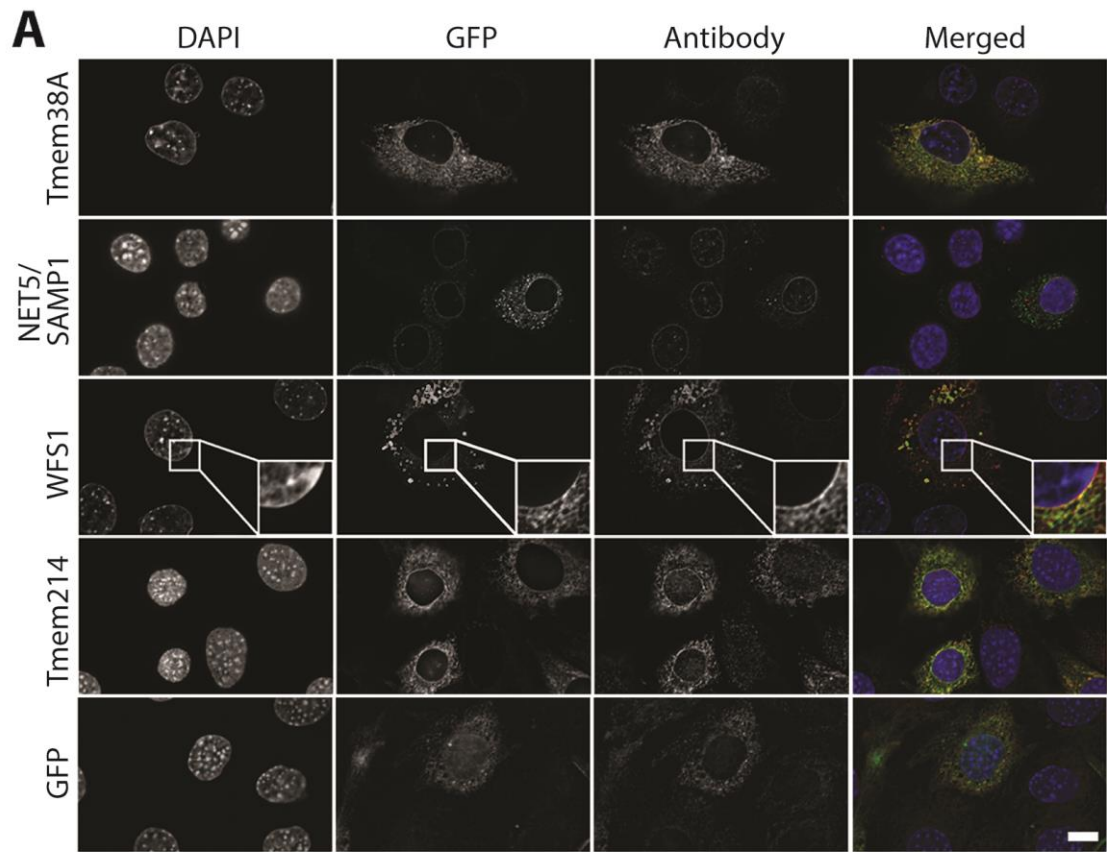


Figure 18. Testing of antibodies for muscle NETs. C2C12 cells were transduced with GFP fusions to the NETs or GFP alone and these cells were divided into two populations. A: The first was used to check for co-localisation between the antibody and GFP signal for the expressed protein. The GFP signal and NET and GFP antibody signals overlapped in all cases. The antibodies used in the antibody column match the GFP fusion proteins being expressed that are labeled on the left hand side. In the case of WFS1 as the GFP signal around the nuclear rim was weaker evidently than the endogenous protein staining with the antibody a box at the rim is enlarged so that the clear co-localisation can be seen. Widefield images shown. Scale bar, 10 μ m. B: The second population was used to generate lysates to test by Western blot. The expected size for GFP-NET fusion bands is marked by asterisks and it can be observed that the same expressed protein fused to GFP is recognised by both the GFP and muscle NET antibodies. Histone H3 (lower panel) was used as a loading control. C: Because there is often species cross-reactivity and extra bands from degradation products of overexpressed proteins, the antibodies were also used to stain a lysate from a control human muscle biopsy. This indicated that the Tmem38A, NET5 and WFS1 antibodies are quite specific so that any protein redistribution observed in immunofluorescence experiments should be specific to the NET. Tmem214 stained additional lower molecular weight bands that might be degradation products, but could also indicate cross-reactivity with other proteins. Therefore, immunofluorescence experiments with this last antibody should be interpreted with caution.

Tmem38A and WFS1 are induced during muscle differentiation (Robson et al. 2016) and a muscle-specific isoform of NET5/Samp1 has been reported (Zuleger et al. 2013). Therefore, patient myoblast cultures were induced to differentiate into myotubes for staining. These cells were co-stained with myosin (type 2) (Fig. 19) or PCM1 (data not shown) as markers for differentiation to distinguish cells that may have poorly differentiated due to the EDMD mutation and contaminating fibroblasts (Fig. 19). The necessity of performing this analysis in differentiated cells was highlighted in all cases by the lack of rim staining in cells lacking the red myosin (type 2) signal. A clear rim with some punctate areas inside the nucleus was observed in the C3 control for the Tmem38A antibody. Similar staining was observed for P5, but a significant loss of rim staining and strong increase in the punctate areas was visually clear for the other lamin and emerin mutations (Fig. 19, upper left panels).

NET5/SAMP1 exhibited clear nuclear rim staining in all differentiated cells for both the control and EDMD patient myotubes; however, a visible relative increase in ER staining was observed for the P5 and P3 patient samples (Fig. 19, upper right panels). For Tmem214 a weak rim could be discerned in all samples except for EDMD patient sample P4, while no WFS1 rim could be discerned in EDMD patient sample P5, though much stronger ER staining was observed for patients P6 and P4. Thus, none of the muscle-specific NETs yielded a uniform redistribution phenotype in all patient samples; however, each yielded different aberrant distribution patterns in cells from distinct subsets of patients.

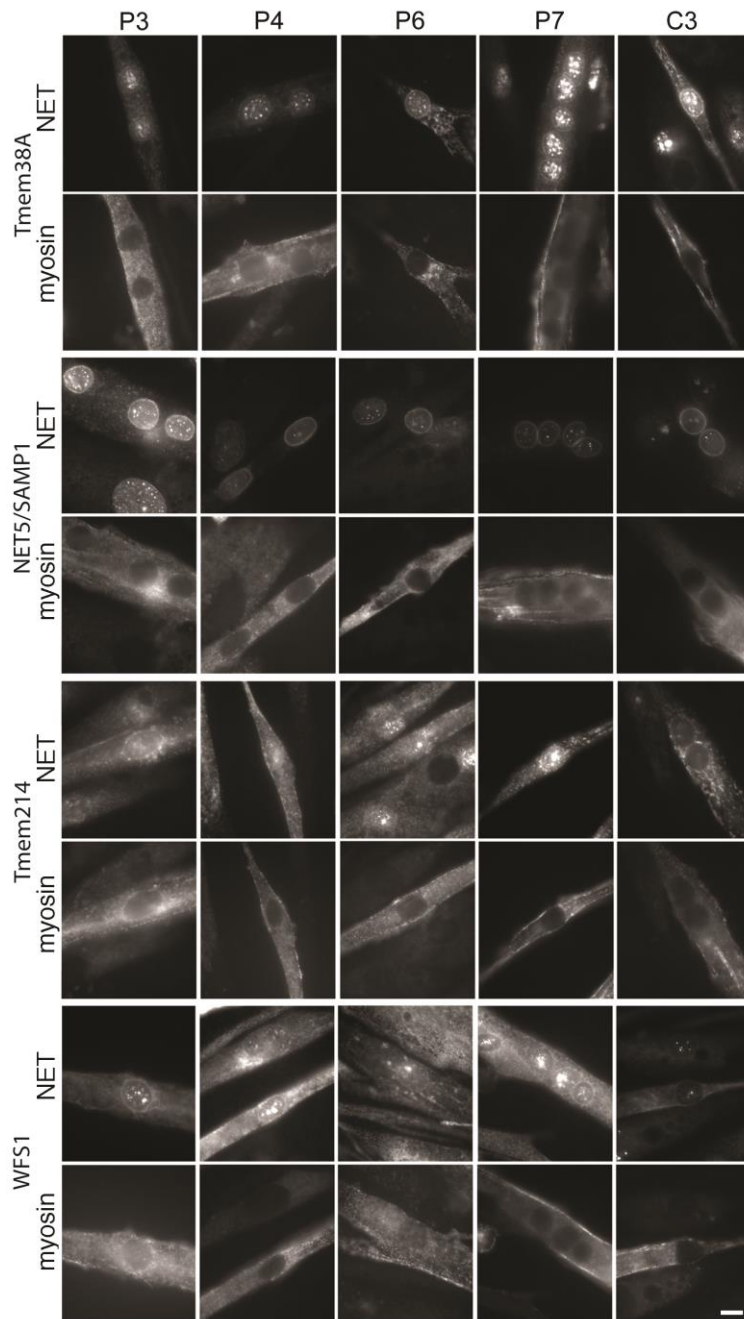


Figure 19. Staining of muscle NETs in patient myoblast/fibroblast cultures where myoblasts were induced to differentiate into myotubes. Antibodies to the muscle NETs listed were used to stain the control and patient cells listed. The cells were co-stained with myosin (type 2), a later differentiation marker, to identify cells that had differentiated within the population. Several EDMD patient cells exhibited more ER signal for the NETs compared with the control cells. Widefield images are shown. Scale bar, 10 μ m.

4.5. Distribution of muscle-specific NETs in EDMD patient skeletal muscle sections

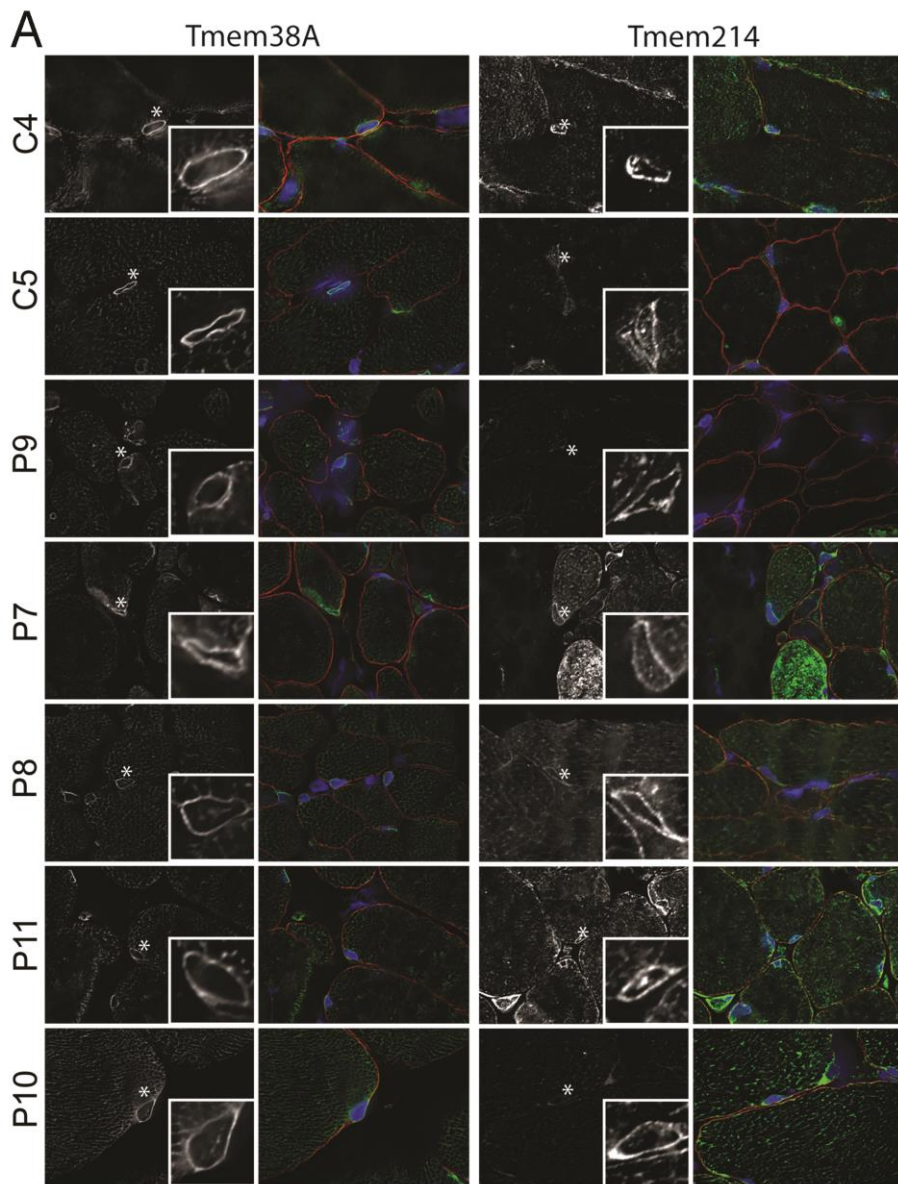
As there are many random aspects of cultured cell growth including differing passage numbers from patient myoblasts and thus progress towards senescence, EDMD patients skeletal muscle biopsies from CIND (Centre for Inherited Neuromuscular Diseases) and CNDB (Centre for Neuromuscular Disorder Biobank) were used to confirm these results (Table 13). As muscle sections contain other cell types, these were co-stained for dystrophin to delineate the plasma membrane of muscle cells. All images were taken at the same microscope settings. For Tmem38A the controls C4 and C5 exhibited crisp nuclear rim staining with weaker distribution through the sarcoplasmic reticulum (Fig. 20, left top two panels). Crisp nuclear rim staining could be observed in all patient sections (Fig. 20, left lower panels). However, the relative intensity of nuclear rim to sarcoplasmic reticulum staining was notably diminished compared to the controls. Unlike differences in the cultured cells that were patient-mutation specific, this difference was observed generally.

For Tmem214 a nuclear rim stain could be observed in all samples, both control and patient; however, this time differences in the relative and absolute intensities varied between patient samples so that no generalized difference could be observed. Notably, the nuclear rim staining for this NET was much more crisp and clear than in the cultured myotubes. In patients P6 and P9, a nucleus for a cell in the space between the myofibers as delineated by dystrophin staining, possibly a capillary nucleus (Fig. 20, red), had a much stronger nuclear rim staining than the nuclei in the muscle fibers. This was in contrast with Tmem38A staining where nuclei outside the muscle fibers were completely negative.

NET5/SAMP1 stained the control nuclei very strongly against a weak background in the sarcoplasmic reticulum and this was the same for most patients. Moreover, some staining could be observed at the plasma membrane co-localized with the dystrophin membrane marker in the controls and most patients, but this was not present in patients P6 and P7. Finally, WFS1 exhibited weak staining at both the nuclear rim and sarcoplasmic reticulum in all fibres. Taking all images using the same settings the intensity of staining varied much more than for other muscle NETs, but this could reflect accessibility in the different sections, as when the intensity of staining was equalized in the enlarged region boxes the character of staining was quite similar between patients. Thus in summary, Tmem38A generally appeared to have more accumulation in the sarcoplasmic reticulum in all the patients and both Tmem214 and NET5/SAMP1 appeared to exhibit some differences from the controls in different subsets of patients.

Table 13. Tissue sections used in this study

	Type	Gender	Age at biopsy	Source
C4	Control	Female	14 yrs	CIND, Oswestry
C5	Control	Male	3yrs	CNDB
P6	LMNA p.T582K	Male	10yrs	CNDB
P7	LMNA p.E358K	Female	2yrs	CNDB
P8	LMNA p.E31del	Female	2yrs	CNDB
P9	LMNA de novo in exon 3	Female	5yrs	CNDB



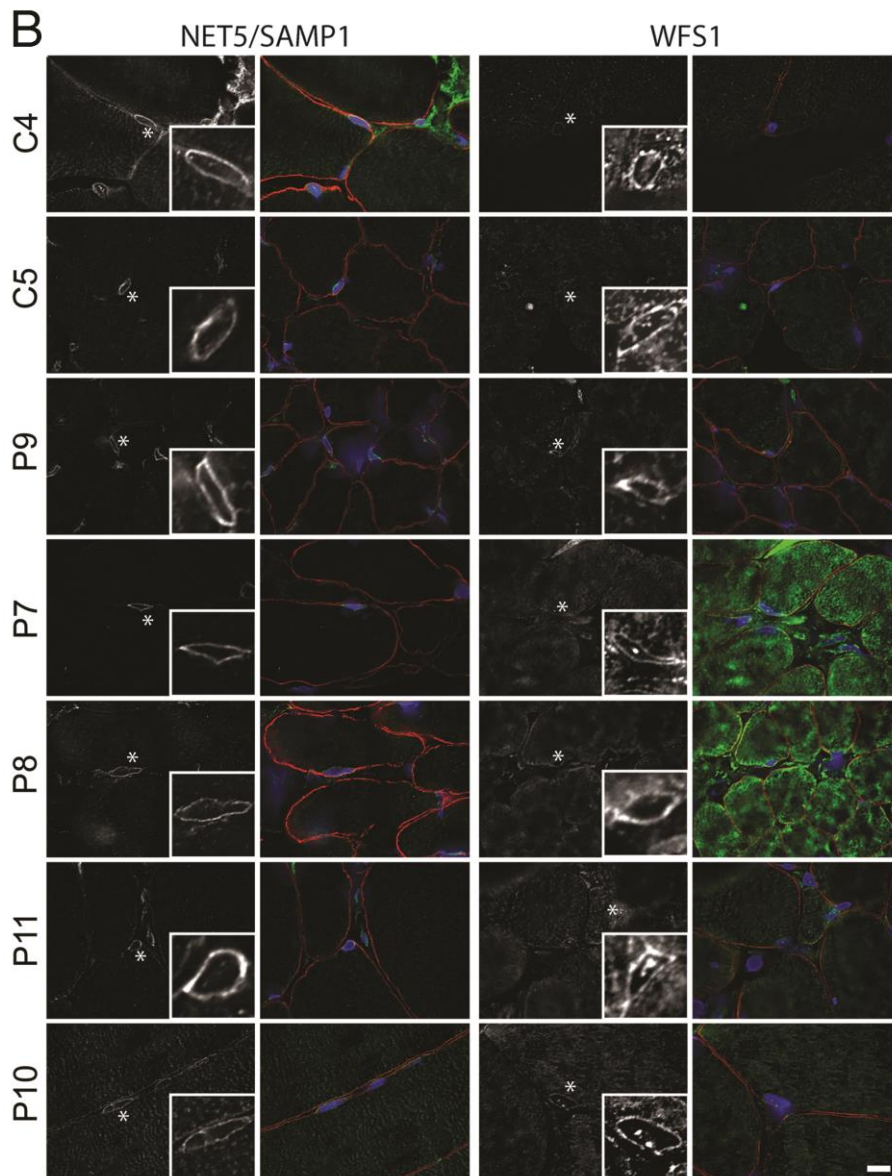


Figure 20. Muscle NET antibody staining in patient skeletal muscle sections. C4 and C5 are controls. The patient mutations are listed in Table 13. All sections were stained with muscle NET antibodies in parallel and images were taken using identical settings. DNA staining for nuclei is shown in blue, the muscle NET in green, and Dystrophin staining is shown in red to delineate the sarcolemmal nuclei of myofibers from those of nerves and the vasculature. Asterisks mark the nuclei that are enlarged in the lower right boxes. Note that the levels are adjusted on these nuclei so the intensities are similar and they can be compared for general characteristics. A: Tmem38A and Tmem214 antibodies. B: NET5/Samp1 and WFS1 antibodies. Tmem38A and NET5/Samp1 distribution and intensity were much more uniform across the samples than were Tmem214 and WFS1. Deconvolved images are shown. Scale bar, 10 μ m.

Table 14. Primary antibodies used in this study

Antigen	Host	IF Dilution	WB Dilutions	Band Size	Source
Skeletal myosin (type 2)	Mouse	1:50	N/A	200 kDa	Sigma (M1570) clone My-32
Lamin A/C	Rabbit	1:50	1:1000	70 kDa	Schirmer et al, 2001 (3262)
Tmem38A	Rabbit	1:50	1:200	30 kDa	Millipore (06-1005)
WFS1	Rabbit	1:50	1:200	100 kDa	Proteintech (11558-1-AP)
Tmem214	Rabbit	1:50	1:200	70 kDa	Proteintech (20125-1-AP)
NET5	Rabbit	1:20	1:100	70 kDa	Millipore (06-1013)
Dystrophin	Mouse	1:50	N/A	271 kDa	Glenn Morris (MANDYS1 (3B7))
Emerin	Mouse	1:50	N/A	29 kDa	Glenn Morris (MANEM1 (5D10))
Nesprin1	Mouse	1:50	N/A	N/A	Glenn Morris (MANNES1E (8C3))
Nesprin2	Mouse	1:50	N/A	N/A	Glenn Morris (MANNES2A (11A3))
Lamin A/C	Mouse	1:50	N/A	70 kDa	Glenn Morris (MANLAC1 (4A7))
SUN1	Rabbit	1:50	N/A	N/A	Atlas antibodies (HPA008346)
SUN2	Rabbit	1:50	N/A	N/A	Millipore (06-1038)
FHL1	Rabbit	1:50	N/A	32 kDa	Aviva Systems Biology (ARP34378_T100)
GFP	Rabbit	N/A	1:200	25 kDa	Generated in Schirmer Lab to whole protein
GFP	Mouse	N/A	1:1000	25 kDa	Clontech (632381)
H3	Mouse	N/A	1:200	17 kDa	Abcam (10799)

4.6. Results summary

These results indicate that the previous finding of emerin redistributing away from the nuclear envelope with lamin A mutations (Charniot et al. 2003, Raharjo et al. 2001, Sullivan et al. 1999, Vaughan et al. 2001) is not a general characteristic of AD-EDMD. Only a few patients had been tested for this before, but by comparing a wider panel of EDMD mutations it is now clear that the emerin redistribution effects are only characteristic of those few mutations. Notably, the use of 3 separate controls revealed that to some extent emerin redistribution can occur even in the absence of disease. Thus, the relevance of this redistribution to EDMD pathology is unclear even in the patients where it was observed. One recent study suggested a link between emerin cytoplasmic accumulation and pathology in that emerin-p.P183T assembles into oligomers that perhaps cannot pass through the peripheral channels of the nuclear pore complexes (Herrada et al. 2015). Nonetheless, emerin mutations result in a loss of protein.

While the specific mutations analysed in this study and earlier studies differed, another aspect that may have contributed to redistribution phenotypes previously reported is the use of complete knockout or mutant over-expression and the use of rapidly dividing cancer cell lines. Two of the earlier studies focused on lamin knockout or loss (Sullivan et al. 1999, Vaughan et al. 2001), but most lamin EDMD mutations are dominant, total lamin levels generally appear normal where tested, and the point mutations by prediction should not block targeting and integration into the lamin polymer. The lamin mutations analysed here included mutations in the N-terminus (p.N31del), the rod (exon 3, p.E358K), the Ig fold (p.R453W), the edge of the Ig fold (p.R545C) and the unstructured region after the Ig fold (p.T582K). These should all yield different effects on the protein. Lacking the rod domain, the N-terminal deletion should act like a null, though it might

dominant-negatively interfere with head-to-tail assembly. The rod p.E358K mutation has yielded conflicting results in assembly studies with one reporting no disruption of filaments and the other reporting deficient assembly in vitro, more soluble protein in the nucleoplasm and reduced mechanical stability (Wiesel et al. 2008, Zwerger et al. 2013). In contrast the Ig fold mutation is on the surface, but with the backbone buried so that it should still enable the beta sheet, that it is a part of, to form, but push it out relative to the adjacent beta sheet. p.R545C is in a basic patch and so might change charged interactions and p.T582K is hard to predict as it is in an unstructured region.

Other studies showing redistribution used mutant over-expression in tissue culture cells (Charniot et al. 2003, Raharjo et al. 2001), which may have influenced results. In these cases, the cells used were MEFs, lymphoblastoid cell lines and standard cancer cell lines as opposed to the myoblasts, myotubes and patient muscle tissue sections used here. In the study where emerin and nesprin were found to affect the localization of each other's (Zhang et al. 2007) patient cells and muscle sections were used. However, this study only compared nesprin and emerin and analysed a patient with a strong combination of nesprin 1 and 2 (*SYNE1* and *SYNE2*) mutations. Nonetheless, in keeping with their results, more intense relative nesprin 1 staining in the ER in the patient with an emerin mutation was found. However, for the EDMD-linked proteins, none exhibited a consistent redistribution phenotype throughout the wider collection of patient mutations analysed here and only nesprin 1 and SUN2 yielded phenotypes in subsets of patients that were stronger than the range of phenotypes observed when also considering a wider panel of three controls.

It is also noteworthy that many of the reports using over-expressed mutant proteins in cancer cell lines or dermal fibroblasts in culture highlighted defects in nuclear morphology

and blebbing. In contrast, here using patient myoblasts and myotubes at relatively early passage number and skeletal muscle sections, very little nuclear morphology defects or blebbing were observed. This argues that aspects of 2-dimensional tissue culture, rapidly dividing cancer cell lines and senescence of dermal fibroblasts probably underlie these phenotypes. Such changes, particularly senescence, could also have influenced previous reports of aberrant distribution of EDMD-linked proteins.

While notable shared differences for any of the EDMD-linked proteins were not observed, many differences for the muscle-specific NETs in myotubes were found. In tissue culture these tended, like the nesprin 1 and SUN2 effects, to be observed only in distinct subsets of patient cells. The redistribution of Tmem38A to the sarcoplasmic reticulum was observed in all but one of the patient in vitro differentiated myotubes and was observed in all patient skeletal muscle tissue sections, though it was not sufficiently striking to be used effectively diagnostically. Differences were also observed in both in vitro differentiated myotubes and muscle tissue sections for Tmem214 and NET5/SAMP1, though as for nesprin 1 and SUN2 these were only observed in subsets of patients.

NET5/SAMP1 is particularly interesting because it also interacts with lamin B1 and SUN1 (Jafferli, Figueroa, and Hallberg 2016) and its mutation affects the distribution of SUN1, emerin and lamin A/C (Gudise et al. 2011). SAMP1 also associates with TAN-lines that are important for nuclear migration (Borrego-Pinto et al. 2012). This provides it with a function that could underlie disease pathology and a molecular network that parallels that of the nesprins (Mislow, Kim, et al. 2002, Zhang et al. 2005). WFS1 and Tmem38A are also interesting because they are important for proper muscle gene expression and for muscle differentiation (Robson et al. 2016). In fact, disruption of three muscle-specific NETs participating in this function together almost completely blocked myogenesis,

though knockdown of each alone had little effect (Robson et al. 2016). Thus, these NETs are prime candidates to mediate EDMD pathology, because muscles appear to develop normally and then exhibit defects when they begin to be more heavily used i.e. gene expression defects that prevent the muscle from fully functioning make for a reasonable explanation of pathophysiology. Tmem38A could also influence Ca^{2+} regulation (Tao et al. 2013, Venturi et al. 2013, Yamazaki et al. 2011, Yazawa et al. 2007), especially considering its relative increase in the sarcoplasmic reticulum. Though much still needs to be done to prove their participation in EDMD pathophysiology, the finding of stronger redistribution effects for these muscle-specific NETs across a panel of EDMD patient mutations than for the already linked proteins raises the strong possibility of their involvement as new players in EDMD.

Taken together this shows that the clinical variability of EDMD is also mirrored on a cellular level. Several different proteins at the NE can be affected to varying degrees, yet many of them exhibit interactions that suggest their co-functioning in a larger network. In addition to WFS1 and Tmem38A co-functioning in myogenic genome regulation and the NET5/SAMP1 partners, redundancy of functions is observed for emerin and MAN1 and for SUN1 and SUN2 (Crisp et al. 2006, Liu et al. 2003). This study shows that several different NETs can be affected to varying degrees in EDMD muscle and further clarifies EDMD as a NE disease, indicating that many different pathways to disrupt NE organization yield a similar muscle phenotype.

Chapter 5

Tissue-specific NETs interactions with lamin A

Introduction

Lamin A has been linked to many distinct diseases (Worman and Bonne 2007), while the underlying cause(s) are still unknown. Lamins form the structural meshwork of the nuclear lamina, which is present in all cell types, except for erythrocytes that become enucleated during differentiation. How then does this ubiquitously expressed protein lead to tissue-specific pathology? One hypothesis is that a complex of proteins work together to define lamin functions and therefore disruption of these complexes is the real cause of tissue-specific disorders. This is an attractive hypothesis as many NETs have also been linked to diseases (reviewed in (Worman and Bonne 2007, Worman, Ostlund, and Wang 2010)). However, these NETs are also widely expressed and therefore cannot explain tissue-specificity.

Previous work carried out by the Schirmer lab identified a host of tissue-specific NETs (Korfali et al. 2012, Korfali et al. 2010, Schirmer et al. 2005, Wilkie et al. 2011). The discovery of differences in the proteome of nuclear envelope subsets gave rise to the postulate that one of these tissue-specific NETs interacts with lamin A to give pathology.

Until now, only a small subset of principally known, ubiquitous NETs have been tested to interact with lamins (reviewed in (Gruenbaum and Foisner 2015)). In this chapter, I test tissue-specific NETs for their ability to bind lamins and whether this binding is reduced for a variety of lamin mutants linked to disease. I show that the fat-specific NET

Tmem120A has a statistically significant reduced binding activity to lipodystrophic mutants of lamin A when compared to wild type (WT) while the muscle-specific NET Tmem38A has reduced (not statistically strong, but consistent) binding to muscular dystrophy mutants of lamin A compared to WT. This chapter will also highlight some of the difficulties in working with full length lamins and transmembrane proteins.

5.1. Lamin functions

Lamins form the structural components of the nuclear lamina, the underlying meshwork of the nucleus, which contributes to its flexibility and stiffness. This is very important in cell migration as nuclear stiffness is the rate-limiting factor. Too much lamin and the nuclei will be too stiff and present a blocking factor when navigating small pores (Rowat et al. 2013). Too little lamins and the nucleus will be too fragile and prone to disruption (Davidson and Lammerding 2014, Lammerding and Lee 2005, Lammerding et al. 2004). As mentioned previously, nuclear shape is frequently used as a hallmark of disease. The lamina has important roles in mechanotransduction via the LINC complex proteins, which has been indicated to affect lamins expression (Swift et al. 2013). The amount of different lamin subtypes present is also directly linked to differentiation. The correct levels of lamins are required for specific lineage; stiffer tissues generally require more lamins while softer tissues require less. High levels of lamin A have been shown to inhibit adipocyte and megakaryocyte differentiation, (Swift et al. 2013, Shin, Spinler, et al. 2013) while promoting erythrocyte differentiation. Unpublished data from Dr Schirmer showed that lamins also take part in chromatin organization and regulation. NETs have been shown to recruit chromosomes to the nuclear periphery for silencing, and loss of NETs resulted in abolishment of this recruitment and regulation (Robson et al. 2016, Zuleger et al. 2013).

5.2. Lamin A, diseases and tissue-specific interaction postulate

Lamin A has the most number of distinct mutated genes linked to diseases in humans. As mentioned earlier in chapter 1, there are over 500 mutations currently reported in *LMNA* linked to multiple diseases. Some of these diseases have overlapping clinical phenotypes. However most preferentially affect distinct tissue types (table 15). The tissue-specific mechanism(s) of how mutations in lamin A cause disease still eludes us. Mutations in lamin A are spread out over the entire protein with at least 1 reported case for almost every amino acid (figure 22E).

The current disease mechanisms proposed are gene regulation, stem cell maintenance and mechanical stress. Lamins and other NE proteins have been shown to interact with chromatin and chromatin associated factors (Burke and Stewart 2013). This interaction may have an important function in regulating gene expression by silencing specific genes at the nuclear periphery (Towbin, Meister, and Gasser 2009). Disruption in NE/lamina interaction with the silenced genes at the nuclear periphery may mediate pathologies and disruption in this interaction has been reported in several NE-linked diseases (Wilkie and Schirmer 2006). This suggests that gene regulation is an important mechanism in mediating pathologies. Mechanical instability is a good model to explain muscular dystrophies, as the nuclear lamina is responsible for maintaining nuclear stability, so mutation in lamins can increase fragility of the NE. Indeed, it has been reported that loss of NE structural integrity could lead to muscular dystrophies (Sullivan et al. 1999). Studies simulating the forces exerted on cells in contracting organs on lamin-deficient models have shown that lamin-deficient cells are more susceptible to mechanical forces (Lammerding et al. 2005, Lammerding et al. 2004, Broers et al. 2005). In addition,

different lamin subtypes have different mechanical strengths and tissues differ in lamin composition, which may provide a means to mediate tissue-specificity of diseases (Schirmer and Gerace 2004, Guilly et al. 1987, Broers et al. 1997).

The dilemma of these pathologies is not only, how do mutations in the same gene cause such a wide range of diseases that can affect vastly different tissue types such as muscle and fat to nerve tissues (Table 12), but also how do these supposedly widely expressed proteins cause such specific tissue restricted pathologies? This dilemma has confounded understanding of these diseases and is yet to be clarified. This led to the hypothesis that other factors must play important roles in mediating disease pathology, more importantly, these other factors must be tissue-specific. Further support for this hypothesis comes from the fact that multiple genetic variants of cardiomyopathy with conduction defects are also caused by mutations in LAP2- α (Taylor et al. 2005) a known interacting partner of lamin A/C (Dechat et al. 2000). Also genetic variants of EDMD caused by proteins such as emerin, lamins and LINC complex members have been identified (Mejat and Misteli 2010, Meinke, Nguyen, and Wehnert 2011). This suggests that disease pathologies are mediated by disruption of interactions in protein complexes and not the individual proteins themselves. Since these complexes consist of widely expressed proteins, other yet identified interacting partners may be involved.

Table 15. Mutations in lamin A causes a wide range of disorders

Laminopathies
Autosomal Emery–Dreifuss muscular dystrophy
Cardiomyopathy dilated 1A
Limb girdle muscular dystrophy type 1B
Congenital muscular dystrophy
‘Heart–hand’ syndrome
Dunnigan-type familial partial lipodystrophy
Lipoatrophy with diabetes and other features of insulin resistance
Mandibuloacral dysplasia
Charcot–Marie–Tooth disorder type 2B1
Hutchinson–Gilford progeria syndrome
Atypical Werner syndrome
Restrictive dermopathy
Variant progeroid disorders

5.3. Known lamin A-associated proteins

One of the groups of proteins that have been found to bind lamins is the LEM domain family. This evolutionarily conserved group of proteins is characterized by a bihelical LEM motif. This motif is responsible for mediating binding to the protein BAF. The LEM domain family has been shown numerous times to depend upon members of the lamin family for localization and their functions (reviewed in (Brachner and Foisner 2011, Wilson and Foisner 2010)). In mammals, 5 members have been identified, emerin, LAP2, LEMD1, MAN1 and LEM2 (Barton, Soshnev, and Geyer 2015). Some of these proteins have been shown to partly overlap in their functions (Lin et al. 2000, Mansharamani and Wilson 2005). LEM domain residence of the INM works with lamins to recruit heterochromatin to the nuclear periphery. Interactions with chromatin have been shown to be via LEM-BAF interaction, however, direct interactions have also been reported for LAP2 (Cai et al. 2001). One of the most studied LEM domain members is emerin, which has been shown to be the cause of X-linked Emery-Dreifuss muscular dystrophy.

Another lamin partner is lamin associated polypeptide 1 (LAP1), the first protein identified to interact with lamins (Foisner and Gerace 1993, Martin, Crimando, and Gerace 1995). LAP1 interacts with torsin A, a mutant of which has been linked to DYT1 dystonia (Goodchild and Dauer 2005), a disease affecting the nervous system. LAP1 also forms a complex with emerin, this has been shown to be important in the maintenance of striated muscle and that in emerin-null mice, LAP1 knock out causes muscular dystrophy (Shin, Mendez-Lopez, et al. 2013). Recently the isoform LAP1B has been linked directly with muscular dystrophy in humans (Kayman-Kurekci et al. 2014).

The LINC complex proteins are also important lamin interactors. They form the bridge between the ECM and the nucleoskeleton. Lamin interaction is achieved via the SUN

proteins with the KASH proteins connecting with the cytoskeletal components (Crisp et al. 2006, Tapley and Starr 2013). The SUN-KASH family of proteins have a wide range of functions, which include mechanotransduction, nuclear positioning (Brosig et al. 2010, Lombardi et al. 2011, Tapley and Starr 2013). Recently, the LINC complex proteins have been found to cause muscular dystrophy (Meinke et al. 2014, Puckelwartz et al. 2009, Zhang et al. 2007). What is more interesting is that many isoforms of the LINC complex members are preferentially expressed in different tissues (reviewed in (Mejat and Misteli 2010, Rothballer, Schwartz, and Kutay 2013, Tapley and Starr 2013)) and this fits with the hypothesis that tissue-specific components must be required for disease pathology.

Emerin, an INM protein, and BAF have also been found to bind lamin A (Clements et al. 2000) (Simon and Wilson 2013). Emerin and *LMNA* null cultured cells showed increases in NE defects and mechanotransduction (Lammerding et al. 2005) (Lammerding et al. 2004), suggesting important roles for both lamin A and emerin in NE structural integrity and also mechanotransduction. Mutations in the *LMNA* gene have been linked to many tissue-specific diseases including EDMD.

One other partner is the protein Lamin B receptor (LBR). LBR has 8 transmembrane domains and mutations in the gene have been linked to diseases (Hoffmann et al. 2002, Waterham et al. 2003). Interaction studies have also identified hundreds of potential nuclear lamins interacting partners (reviewed in (Simon and Wilson 2013, Wilson and Foisner 2010)). Furthermore, Tmem120A an adipocyte-specific NET identified in the Schirmer proteomic studies has also come up as a hit in one of these studies (Dittmer et al. 2014). Tmem120A has been reported to be upregulated in adipogenesis and also has chromosomal repositioning effects. Knockdown of Tmem120A also inhibits adipogenesis

in mouse 3T3-L1 cell line (Batrakou et al. 2015). With many potential lamin binding partners, I wanted to test the following hypotheses:

- 1. Tissue-specific diseases arise through interactions between ubiquitously expressed proteins such as lamin A and tissue-specific NET components.*
- 2. The interactions between the NETs and lamin A will be disrupted in the presence of disease causing mutations.*

5.4. Designing a solid phase binding assay to measure interactions between lamins and nucleoplasmic regions of NETs

There are many ways of measuring interactions between proteins currently available. The most straightforward being pulldowns, while other technologies include surface plasmon resonance (SPR) and isothermal titration calorimetry (ITC). I set out to design and test a solid phase style binding assay which would be able to be quantitative while, remaining affordable and quick. The assay was based on previous solid phase work carried out by Taniura et al 1995 (Taniura, Glass, and Gerace 1995) where a similar set up was used, however, in that set up, the detection was via radioactivity. With the advent of fluorescent probes, this solid phase technique was adapted for the purpose of measuring interactions between NETs and lamins. I wanted to design an assay which could produce results interpretable by Michaelis-Menten kinetics, which would allow for binding constants. If the results were corroborated using techniques such as SPR and are reproducible, testing would be expanded to include more NETs and chromatin.

The experimental design consists of binding full length lamin A to a nickel coated surface to form the solid phase (figure 21A). This set up is advantageous because full length lamin A is known to be insoluble, also mutations are present throughout the protein.

A nucleoplasmic NET fragment with a tag was added on top of the solid phase (figure 21B). We chose largest nucleoplasmic fragment which was determined by prediction software TMHMM. Binding was then detected using antibody against the tag and a further antibody coupled to a fluorophore against the species to which the tag antibody was raised figure 21C). Fluorescence signals from the fluorophore Alexa 488 was read using plate readers at 490 nm wavelength. Having tagged NET fragments not only allows for easy purification, but also for consistency of detection. Antibodies raised directly against a target can have huge variation between different

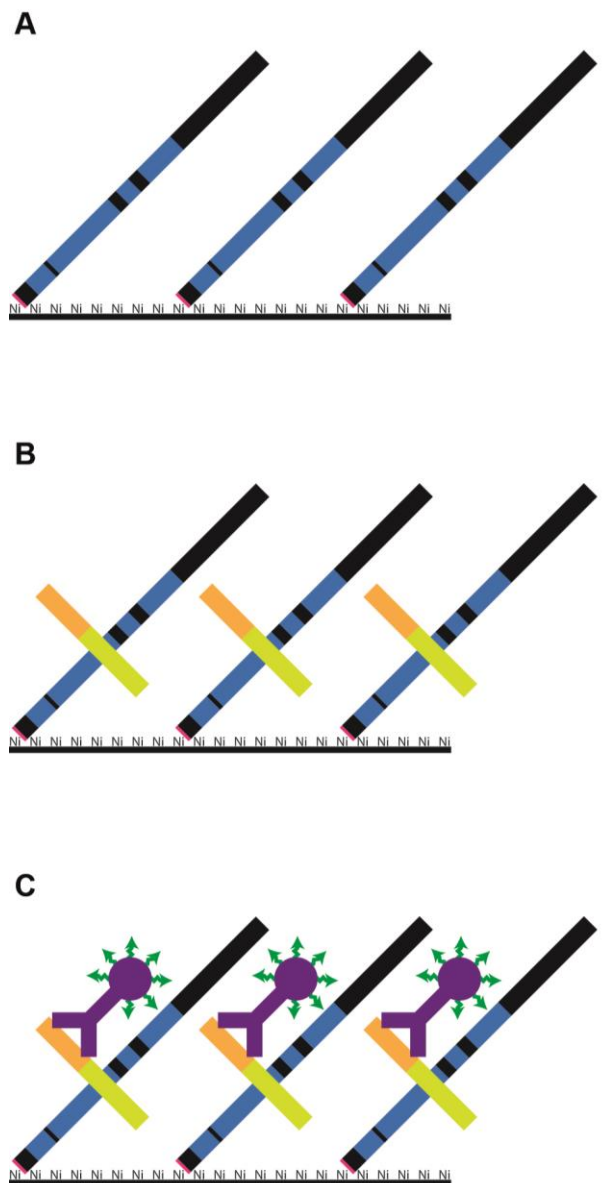


Figure 21. Schematic of the binding assay used in this study. (A) Binding of lamin (black/blue) to the nickel coated surface. (B) NET fragments (orange/yellow) are added on top of the lamin solid phase. (C) Detection of binding using fluorescently tagged antibody (purple/green).

batches, as well as between antibodies. By using only one antibody against a tag, this variation is greatly reduced.

For this study, a range of lamin A mutants were chosen (Table 16) that have been linked to very distinct diseases. The mutations were also chosen to test a wide range of position along the protein (figure 22D). It is postulated that NETs interact with WT lamins, however, tissue-specific NETs will lose or have greatly reduced binding to mutants affecting the tissue in which they are expressed.

The mutants were generated using a site-directed mutagenesis kit (chapter 2) and expressed from pET28b vector in BL21 cells. Purification of the lamins was carried out from inclusion bodies and then solubilised in urea. The purity of the lamins were checked using protein gel electrophoresis on laemmli gels (figure 22A).

Table 16. List of disease mutations generated in this study

Lamin mutation	Disease linked
E203K	Dilated Cardio Myopathy
R298C	Charcot-Marie Tooth 2B1
R377H	Emery Dreifuss Muscular dystrophy
R435C	Restricted Dermopathy-like proregia
R453W	Emery Dreifuss Muscular dystrophy
G465D	Familial Partial lipodystrophy (FPLD)
R482Q	Familial Partial lipodystrophy
R527P	EDMD with FPLD

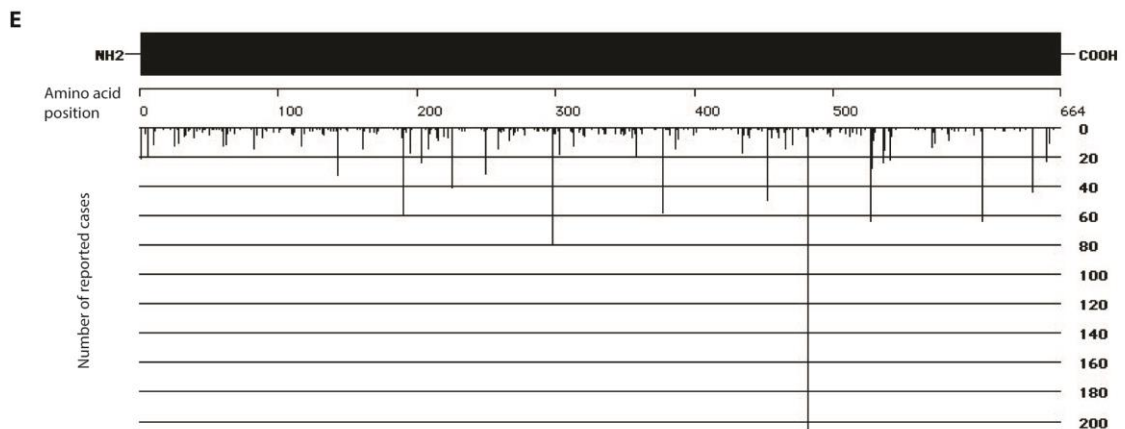
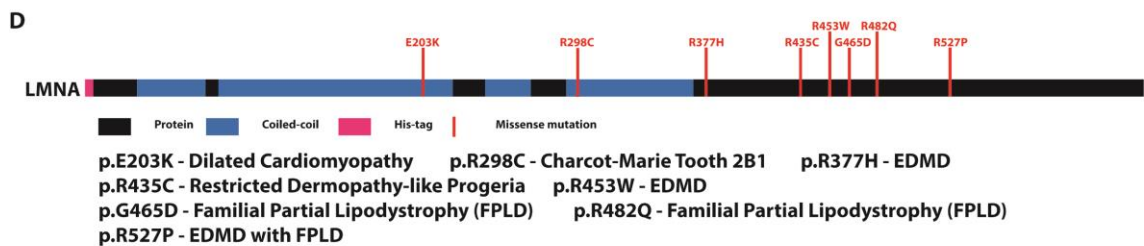
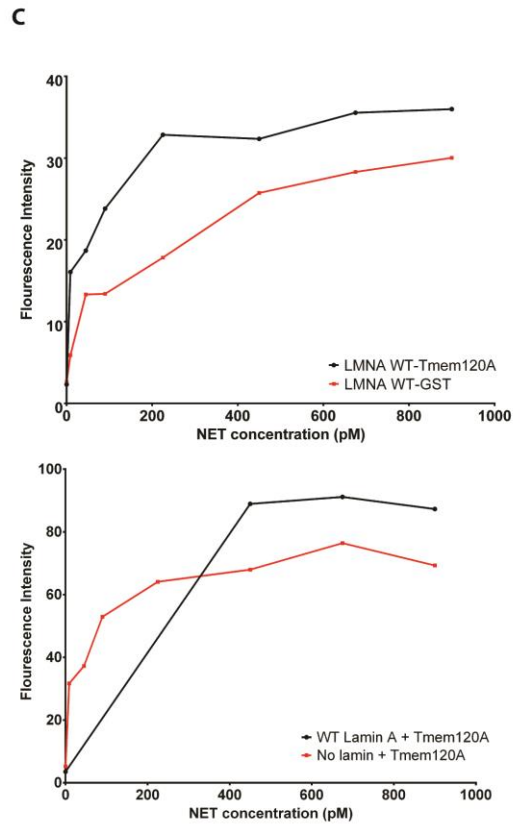
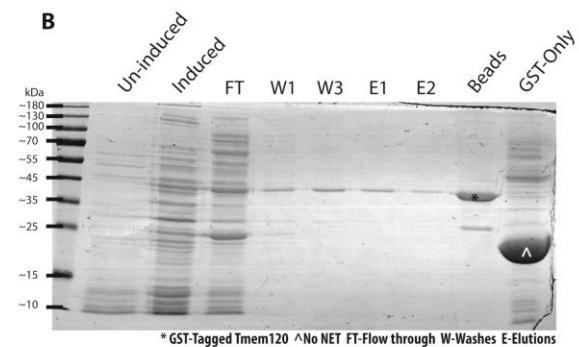
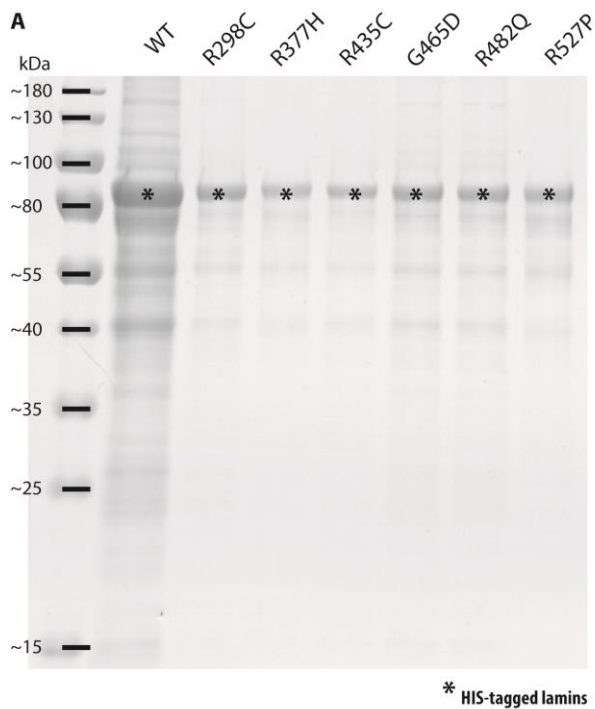


Figure 22. Purification of lamins and GST-tagged Tmem120A. (A) Lamin A WT and disease mutant purification from inclusion bodies. (B) Purification of GST tagged Tmem120A on glutathione matrix. (C) Test binding of GST tag to lamin WT shows high background unspecific binding (top graph). Tmem120A-GST also showed high unspecific binding to the wells where no lamin was present (bottom graph) (D) Location of the lamin mutants chosen in this study. The majority of mutations are clustered around the C-terminal tail segment. (E) Distribution map of mutations with their associated number of reported cases. Mutations can be found throughout the protein. Distribution map was generated using tools from <http://www.umd.be/LMNA/>.

5.5. GST tag shows high unspecific background binding to WT lamin A

NETs have been notorious for being insoluble, with many previous studies into protein-protein interactions requiring harsh refolding steps in order to produce usable proteins (Holaska et al. 2003). Additionally, proteins expressed in bacteria may not have post-translational modifications, harsh treatment can potentially alter the protein chemistry and skew any interaction results. I decided to clone nucleoplasmic domains of NETs coupled to tags that have been cited to improve solubility (reviewed in (Costa et al. 2014)). As the assay is reliant on detecting a tag to produce the readout signal, the tag itself should not bind to the lamins.

The NET fragments were first cloned into pGEX-4T1 vector expressing a C-terminal GST. This enabled the expression and purifications of the NET fragments (figure 22B) with high purity. However, when testing for background binding towards WT lamin A (figure 22 top graph), Tmem120a-GST also showed high background binding where no lamin was present (figure 22C, bottom graph), the tag alone gave very high background. This was not alleviated with more stringent buffer conditions (data not shown). Maltose binding protein (MBP) tag was then looked at as a candidate for the assay.

5.6. Transfer of NET fragments into MBP tag vector and background testing.

The MBP tag was tested for background binding to WT lamin A using several concentrations (0, 9, 45, 90, 180, 450, 900, 1800 pM). Using emerin as a positive control, a protein which has been extensively shown to interact with lamin A (Clements et al. 2000, Holaska et al. 2003), the response of emerin tagged with MBP when exposed to lamin A in the solid phase binding assay was significantly different to the response given by the MBP tag alone (figure 23C). The MBP tag alone showed increases in unspecific lamin A binding at the higher concentration (>500 pM), however, this interaction appears to be greatly reduced in the presence of a NET fragment such as emerin. Testing showed that emerin gave a much higher response than the tag alone, more significantly and much earlier, at low concentrations. At higher concentrations, there will inevitably be unspecific binding, however this interaction between the tag and the solid phase will be insignificant and most of the signal will be of the NET binding. After discussion, this background binding of the MBP tag was deemed to be acceptable, testing was expanded to other NETs. MBP-tag binding to wells in the absence of lamin A was not significantly different than a blank well with only TBS (figure 23D)

The nucleoplasmic NET fragments were subsequently cloned into pMAL-CRI vector expressing an MBP tag. It was possible to express and purify the fragments with high purity (figure 23A). One of the NET fragments, Tmem38A is very small and highly charged, and when run on SDS-PAGE gel is indistinguishable from the MBP tag alone, but its expression was confirmed by western blotting using affinity-purified antibodies produced in chapter 3 that specifically recognize this region of the protein. Although

indistinguishable via laemmli gel, Tmem38A was indeed expressed and purified (figure 23B).

Table 17. Protein used and their expected molecular weight

Protein	Expected molecular weight (kDa)
His-Tagged Lamin A inc mutants (aa1-646)	~72 kDa
MBP-Tagged Tmem38A (aa233-299)	~49.2 kDa
MBP-Tagged Tmem120A (aa1-137)	~58.3 kDa
MBP-Tagged Emerin (aa1-224)	~67.7 kDa
MBP-Tagged Lap2β (aa1-398)	~86.5 kDa
MBP-Tagged LBR (aa1-211)	~65 kDa
MBP (no protein fragment)	~42 kDa

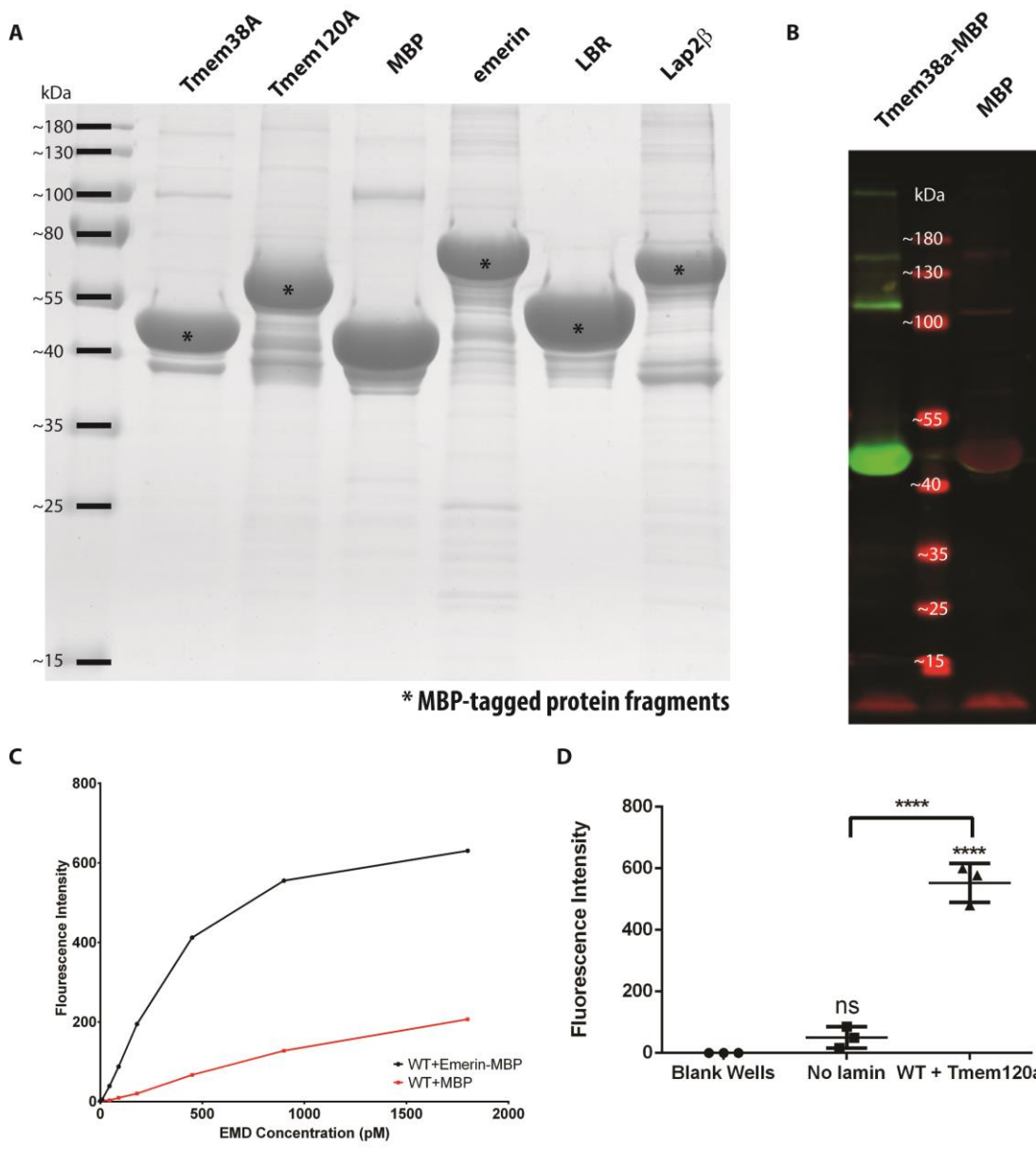


Figure 23. (A) Purification of MBP tagged NET fragments and testing of background binding to WT lamin. Laemmli gel of the purified NET fragments showing a main band of expressed protein and the background impurities present in each preparation. (B) Western blot using both anti-Tmem38A (GREEN) and anti-MBP (RED) antibodies. Although they run at the same apparent molecular weight Tmem38A is not detected in the purification of the tag alone. (C) Response curves of emerin-MBP (black) and MBP tag (red) binding to WT lamin A. (D) MBP binding to wells without lamin A present was not significantly different than wells with nothing in them.

5.7. Tmem120A has reduced binding to lipodystrophic mutants

While testing the NETs, it was found that from experiment to experiment the baseline readout can be highly variable (fluorescence intensity could be +/- 50% between experiments). This made it impossible to calculate an accurate absolute Kd. Additionally, some of the binding curves obtained do not fit a classic Michaelis-Menton curve (data not shown). However, a trend in Tmem120A was found. Although the numbers obtained from each experiment were different, in all experiments the Bmax of Tmem120A was always lower than WT lamin when tested against G465D and R483Q, two lipodystrophic mutants (figure 24 and 34). In contrast, the Bmax of Tmem120A binding to the myopathy, neuropathy and progeria lamin A mutants did not differ when compared to WT (figure 35).

The bulky nature of this sandwich assay system may hide any differences at low concentrations. The differences in Bmax persisted with repeats, however, the differences between repeats varies in magnitude.

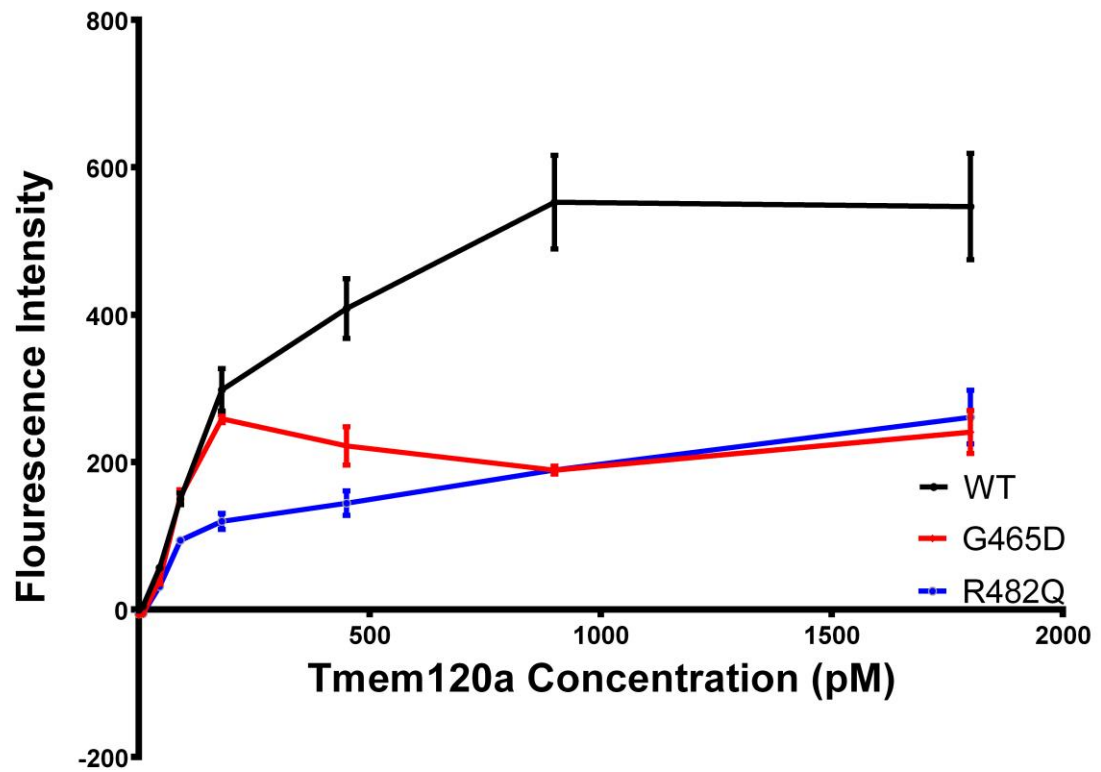


Figure 24. *Tmem120A* has a lower B_{max} against lipodystrophic mutants of lamin A. *Tmem120A* showed a higher binding saturation plateau to WT lamin A than the lipodystrophic mutants G465D and R482Q. Graph shows representative experiment with error bar calculated using 3 repeats at each concentration.

5.8. Surface Plasmon Resonance revealed two step binding of NETs to lamin A

To try and get a clearer picture of the results obtained in the solid phase binding assay, SPR experiments were carried out to try and replicate the results using a different approach. SPR is a sensitive assay which measures the differences in refractive changes when analytes bind to a predetermined surface in real time. Much in the same way as the solid phase assay, His-tagged lamin A was bound to a NTA-chip (70 Response unit (RU)) in urea as a solid phase. After binding, buffer tests (running buffer, Section 2.6.2) were ran and found no significant detachment of lamin A from the basal matrix.

Attempts to validate the interactions found with the solid phase assay were carried out, beginning with emerin (figure 25) as it was previously tested using SPR (Clements et al. 2000). The experiment was composed of 90 s injections followed by 90 s washing step. After 5 sequential injections (2-fold concentration series of NETs fragments ranging from 0.3125 μ M – 5 μ M), a plateau was not found. Tmem120A (adipocyte specific) and Tmem38A (muscle specific) were also tested, both against WT lamin A, lipodystrophic mutants and muscular dystrophic mutants respectively (figure 27 and 28). No differences were found using SPR in the interactions between WT lamin A and these NETs when compared with the disease mutants. The response curves showed a two-step reaction with a quick initial binding followed by a slower interaction.

I hypothesized that this could be due to either 1) the unstructured coiled-coil domain of lamin A may be promiscuous in tested conditions and/or 2) the protein sample is not homogenous and the steps are due to different species having different interactions. To try and eliminate this unspecific binding, emerin's binding to lamin A without its

unstructured coil domain (figure 26) was first tested. Removing the coiled-coil domain did not eliminate the two-step interaction.

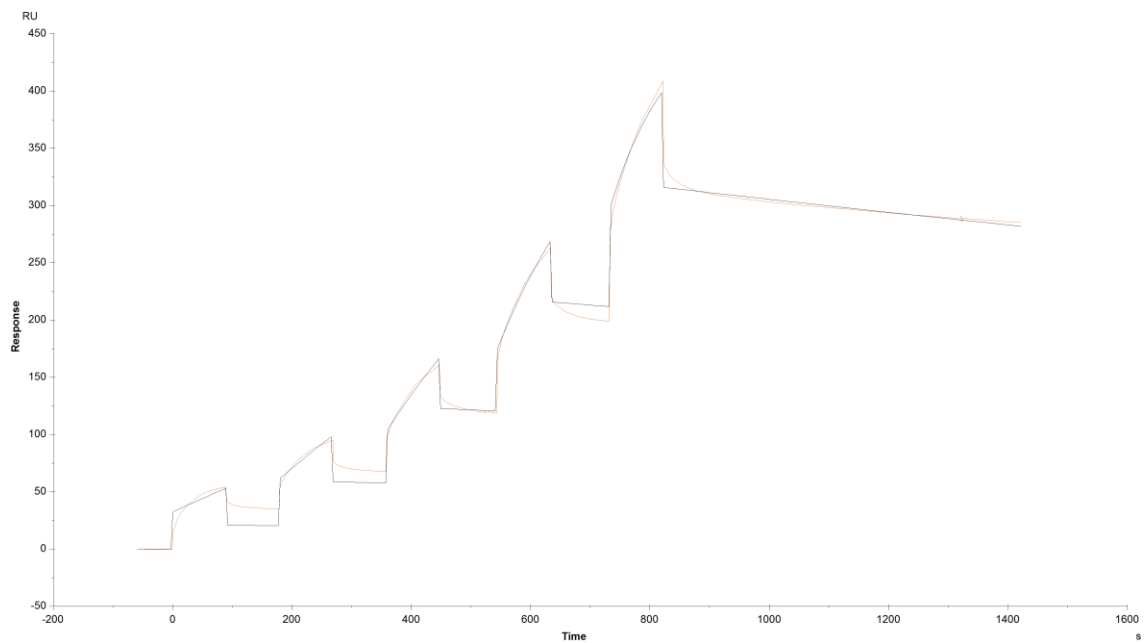


Figure 25. Response curve of emerlin-MBP binding to immobilised WT HIS-lamin A. 5 sequential injection of emerlin tagged with MBP in a two-fold dilution series from $5\mu\text{M}$ - $0.365\mu\text{M}$ (lowest concentration injected first). No binding saturation was seen. All steps showed a two-stage binding.

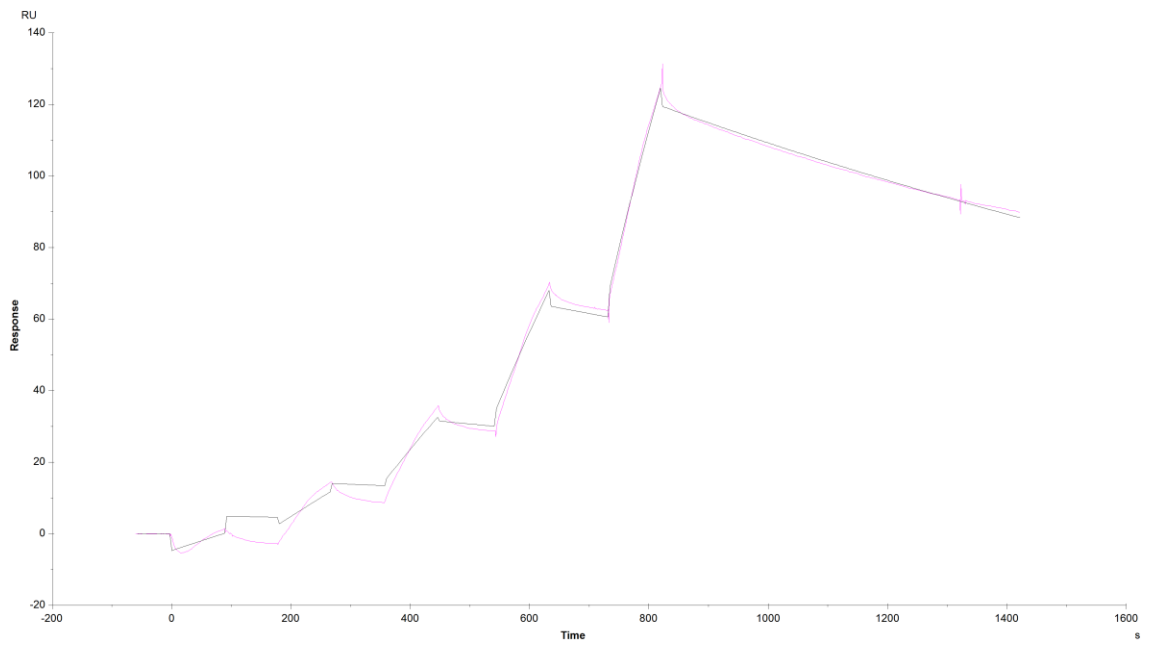


Figure 26. Response curve of emerlin-MBP binding to immobilised HIS-lamin A delta coil. 5 sequential injections of emerlin tagged with MBP in a two-fold dilution series from 5µm-0.365µm (lowest concentration injected first). No binding saturation was seen. All steps showed a two-stage binding.

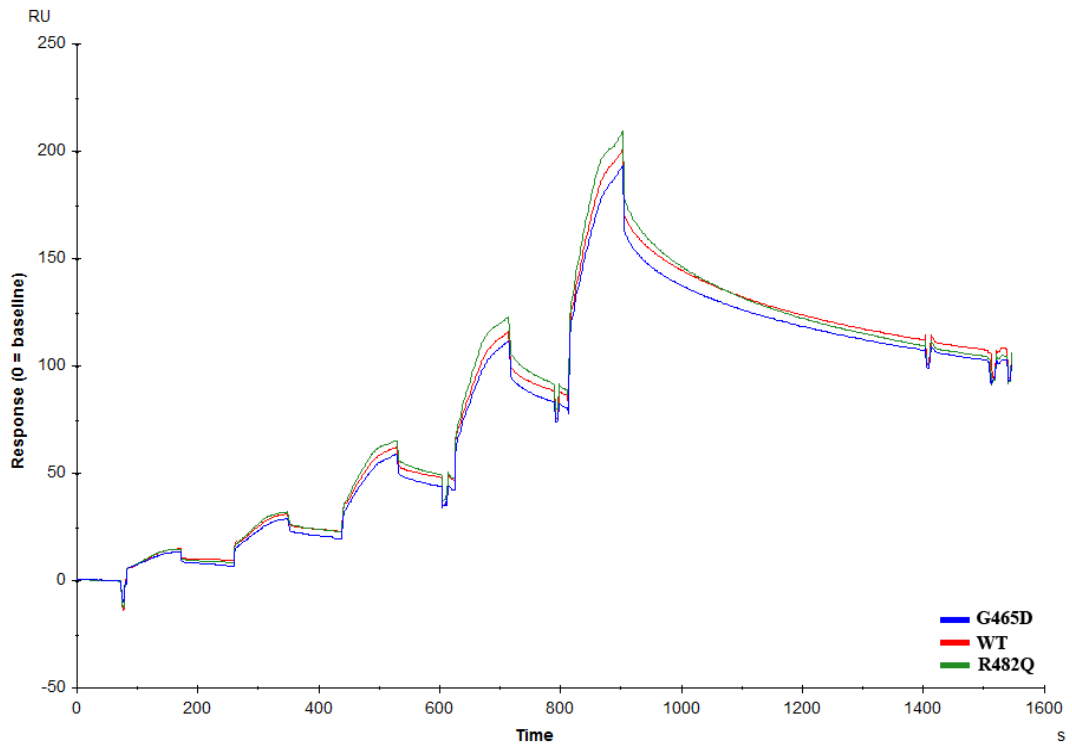


Figure 27. Response curve produced by Tmem120A-MBP binding to immobilised WT HIS-lamin A and lipodystrophic mutants (HIS-G465D and HIS-R482Q). 5 sequential injections of Tmem120A tagged with MBP in a two-fold dilution series from $5\mu\text{m}$ - $0.365\mu\text{m}$ (lowest concentration injected first). No binding saturation was seen. All steps showed a two-stage binding.

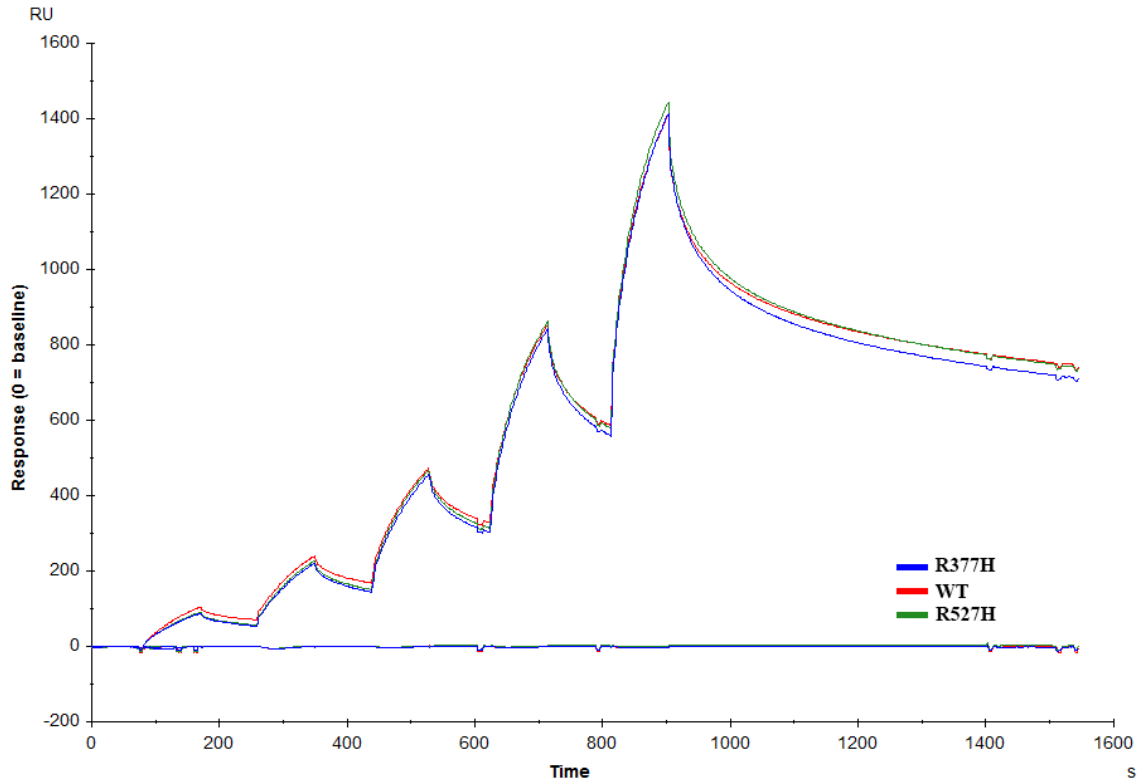


Figure 28. Response curve produced by *Tmem38A*-MBP binding to immobilised WT HIS-lamin A and muscular dystrophic mutants (HIS-R377H and HIS-R527H). 5 sequential injections of *Tmem38A* tagged with MBP in a two-fold dilution series from 5 μ M-0.365 μ M (lowest concentration injected first). No binding saturation was seen. All steps showed a two-stage binding.

5.9. Size exclusion chromatography revealed large protein complexes.

As the SPR experiments yielded ambiguous results, size exclusion chromatography to find out the oligomerization state of the protein samples was carried out. Samples of MBP tagged emerin and Tmem120A were run through superdex 200 (10 kDa- 600 kDa) column in the same buffer used for the SPR experiments. The elutions were then analysed by protein electrophoresis.

For both emerin-MBP and Tmem120A-MBP (predicted monomeric mass can be found in table 17), it was found that all the full-length NET fragments were found in the void volume (7.8 ml for the superdex 200 column) (Figure 29 and 30). This meant that the protein complexes ran at an apparent Mw of over 600 kDa. The majority of proteins that made up the elution peaks were comprised of lower Mw species. The results raised questions on the oligomeric status of the protein fragments.

Previous attempts at purifying emerin-MBP which reported monomeric forms utilized either low pH or low salt buffer (Holaska et al. 2003, Berk et al. 2014). I also postulate that as the plate based binding assay used has a higher tolerance for detergent compared with SPR, the presence of detergent could help maintain monomeric protein fragments and so could explain why differences in the assay that were not recapitulated in the SPR. Attempts to purify emerin-MBP into different buffers was carried out. The buffers tested include; low salt (20mM Tris, 30mM NaCl), detergent containing buffer (20mM Tris, 200mM NaCl, 0.02% TX-100) and low pH phosphate buffer (pH 6.3). Purification of emerin into these buffers yielded lower amounts of full length fragments (data not shown). Assuming solubility, attempts to separate the full-length fragments from the rest of the material present in the samples were carried out. A superose6 column was utilized to

give higher resolution in case of large soluble complexes. It was observed from the elution profile of the low salt buffer (figure 31), detergent containing buffer (figure 32) and low pH phosphate buffer (figure 33) that more proteins were running at the expected MW for monomers. No void peak (~6.7 ml for the superpose 6 column) was present in the detergent buffer and the low pH buffer. The low salt buffer showed a much-reduced void peak. Analysis of the elution peaks by protein gel electrophoresis however revealed that the full-length protein fragment was not separated from the rest of the contaminants.

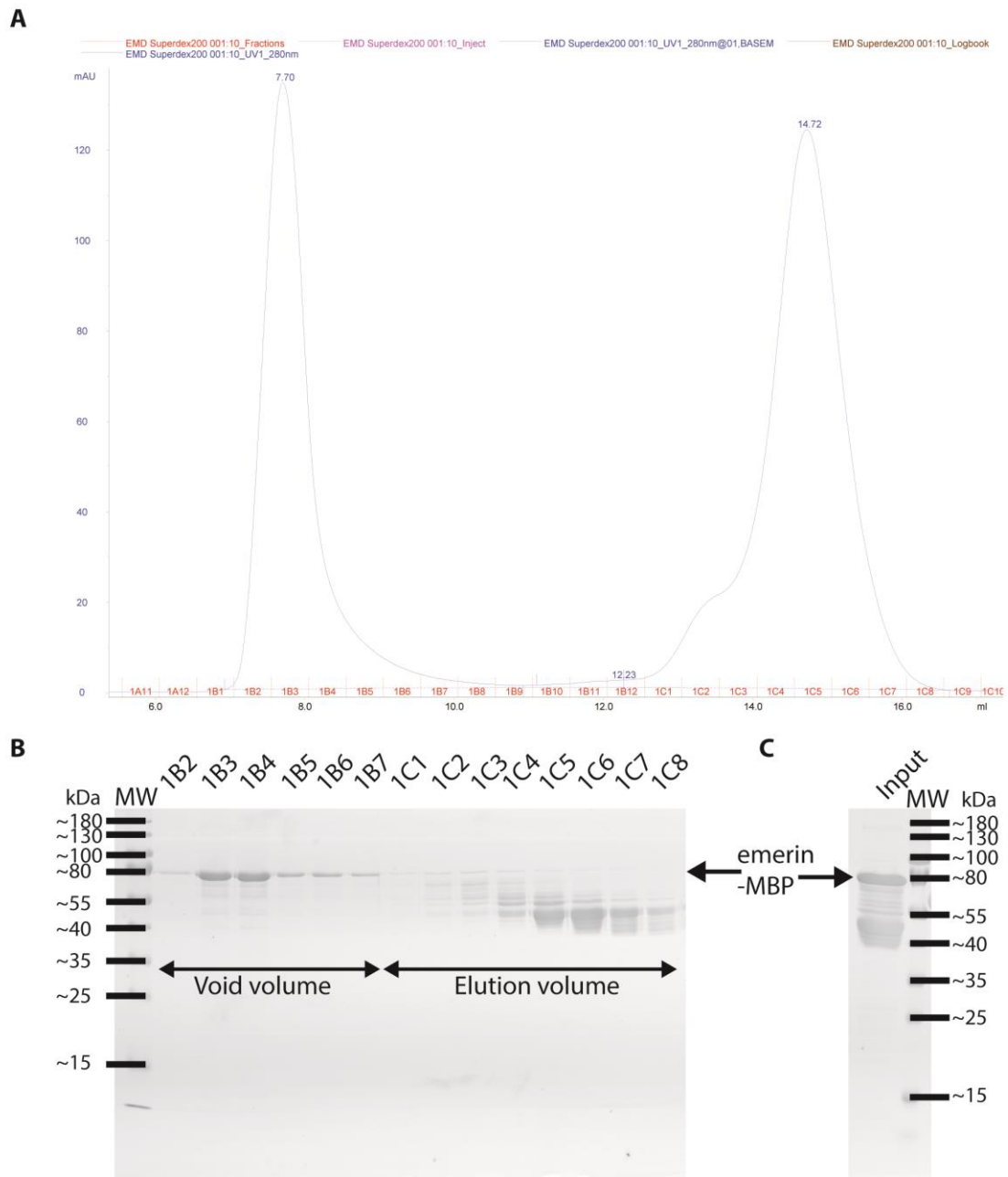


Figure 29. Size exclusion chromatography of emerin-MBP over a superdex 200 column in TBS. (A) The elution profile showed two main peaks, 1B2-1B7 being the void volume and 1C1-1C8 being the elution peak. The elution peak showed a slight shoulder where some of the full-length protein remained (1C1-1C3). (B) Protein electrophoresis analysis of the peaks. (C) Initial input material.

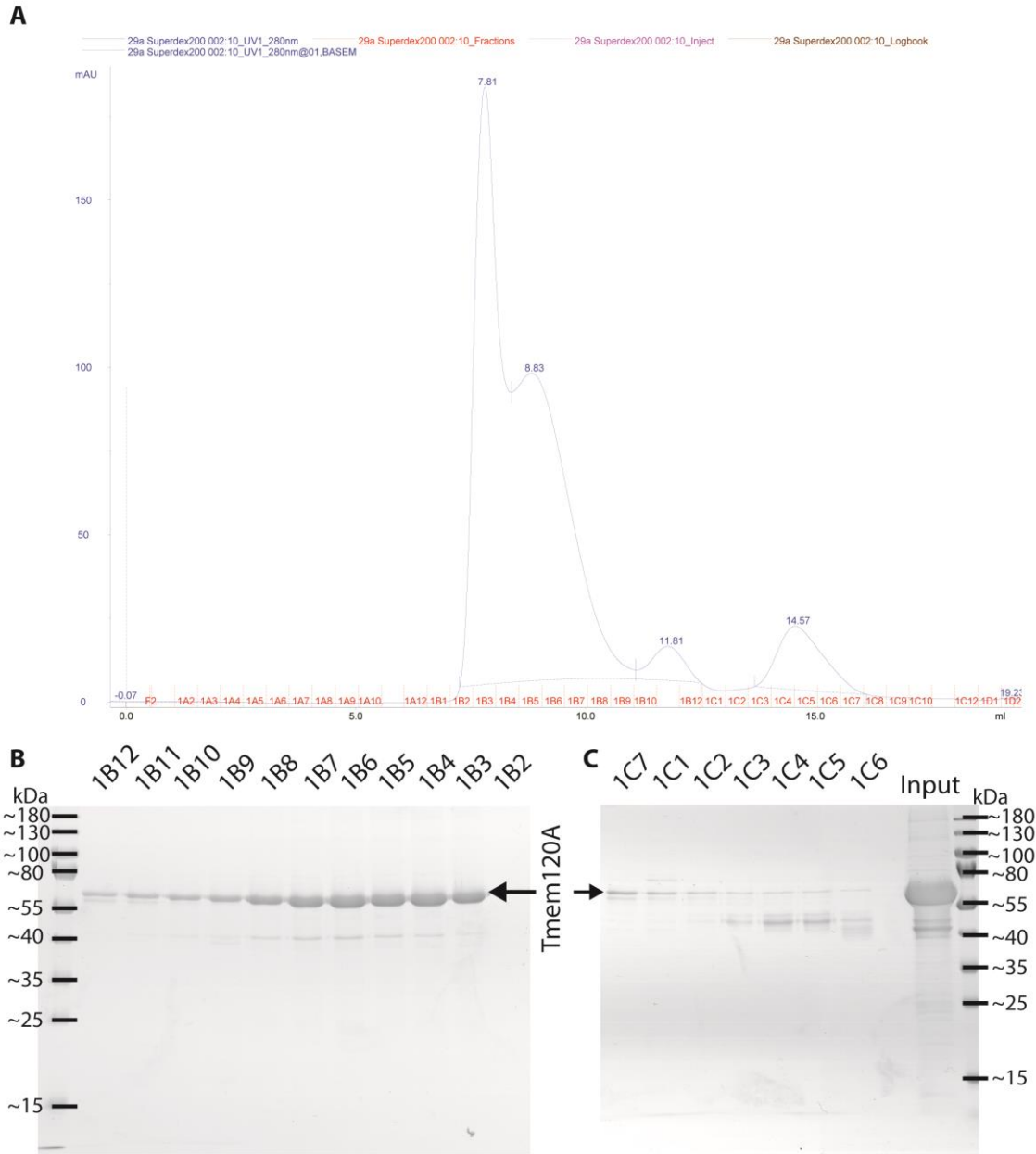
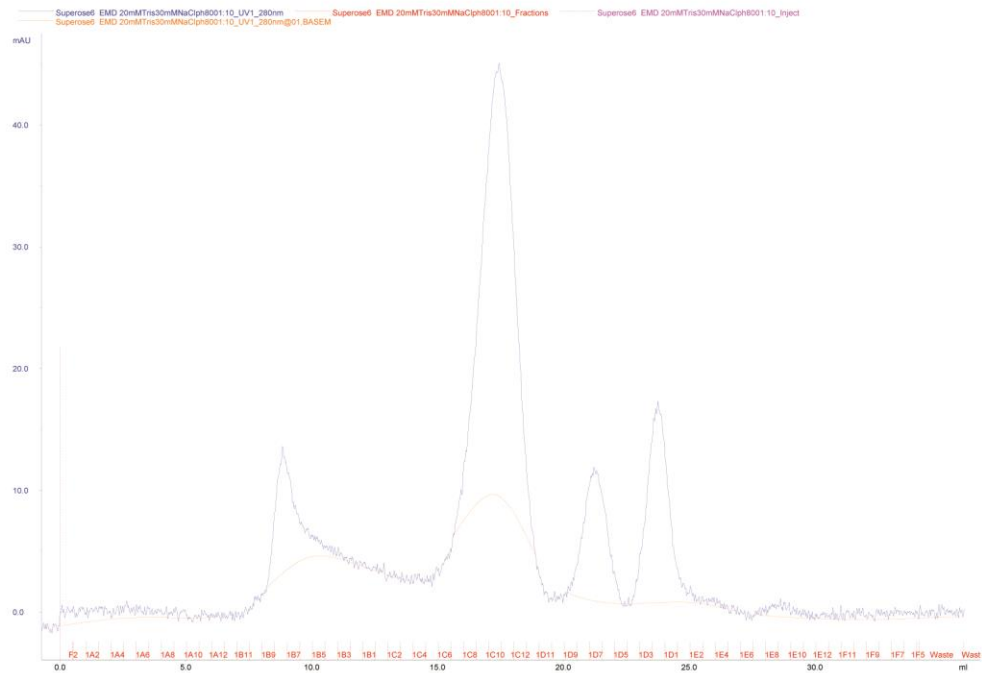
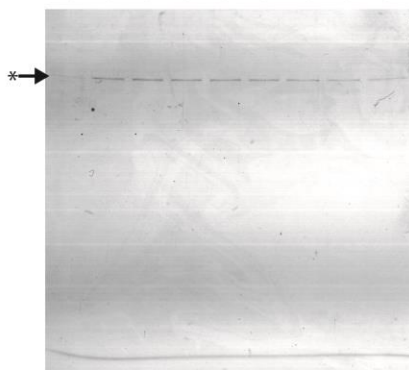


Figure 30. Size exclusion chromatography of Tmem120A-MBP over a superdex 200 column in TBS. (A) The elution profile of Tmem120A showed the majority of the protein was present in the void volume with a small elution peak 1C1-1C7. (B) Protein electrophoresis analysis of the void volume peaks. (C) Protein electrophoresis analysis of the elution peak.

A**B**

B1 B2 B3 B4 B5 B6 B7 B8 B9

**C**

C1 C2 C3 C4 C5 C6 C7 C8 C9 C10 C11 C12 D12 D11

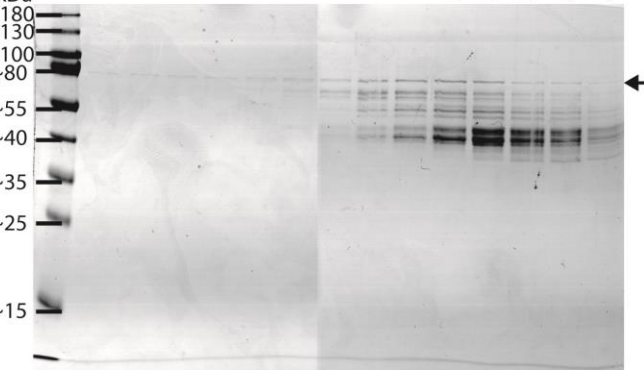


Figure 31. Size exclusion chromatography of emerlin-MBP (*) over a superpose6 column in low salt buffer (20mM Tris, 30mM NaCl). (A) Chromatography profile of emerlin showed an improved profile with a smaller void volume B9-B1 with the majority of the protein in the elution peak C1-C12, D12-D11. Analysis of the void volume (B) and the elution peak (C) showed that most of the full-length protein still remained in the void volume.

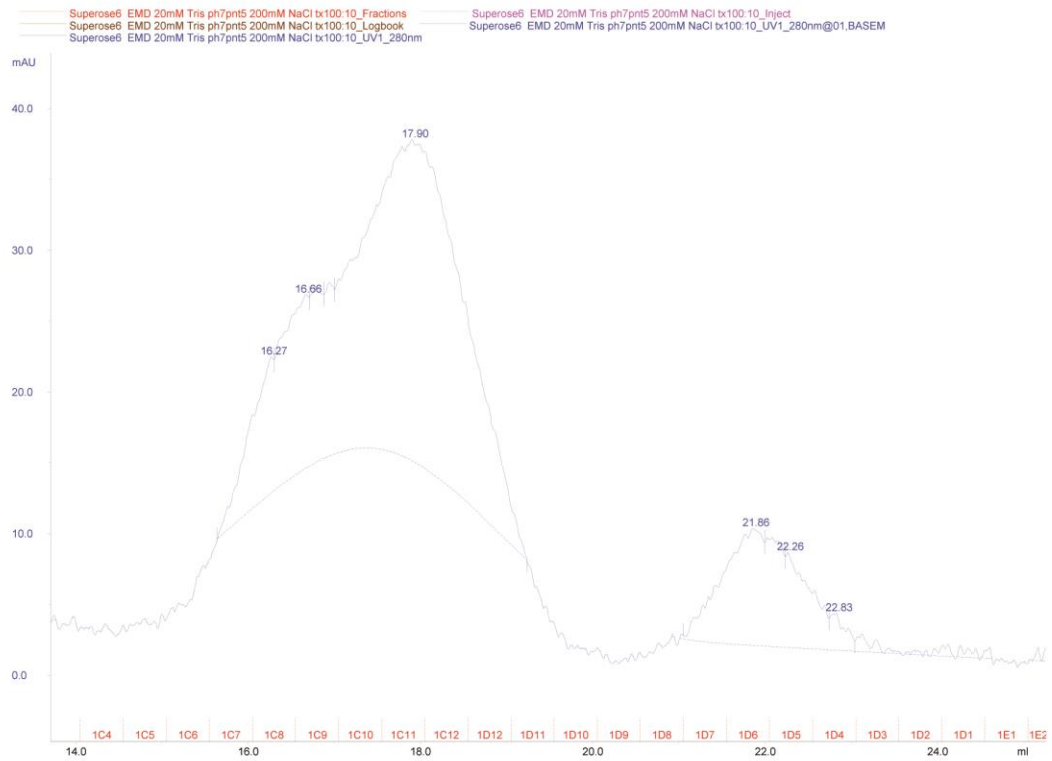
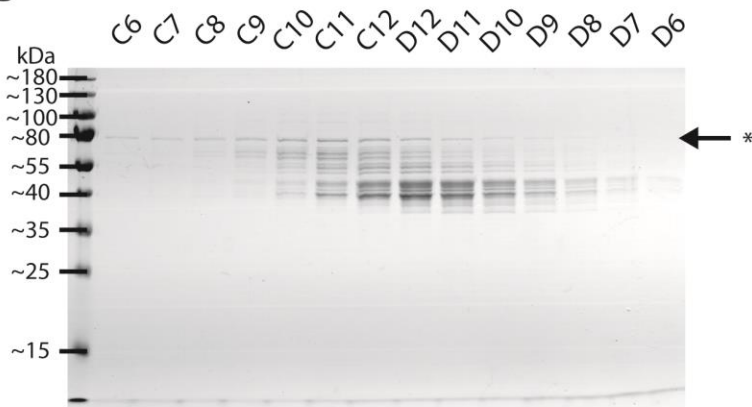
A**B**

Figure 32. Size exclusion chromatography of emerin-MBP (*) over a superpose6 column in detergent containing buffer (20mM Tris, 200mM NaCl, 0.03% TX-100). (A) The elution profile showed 1 main elution peak with a shoulder. Protein analysis of the elution peaks showed the cleanest full length fractions was present in the shoulder C6-C9. (B) Protein electrophoresis analysis of the elution peak.

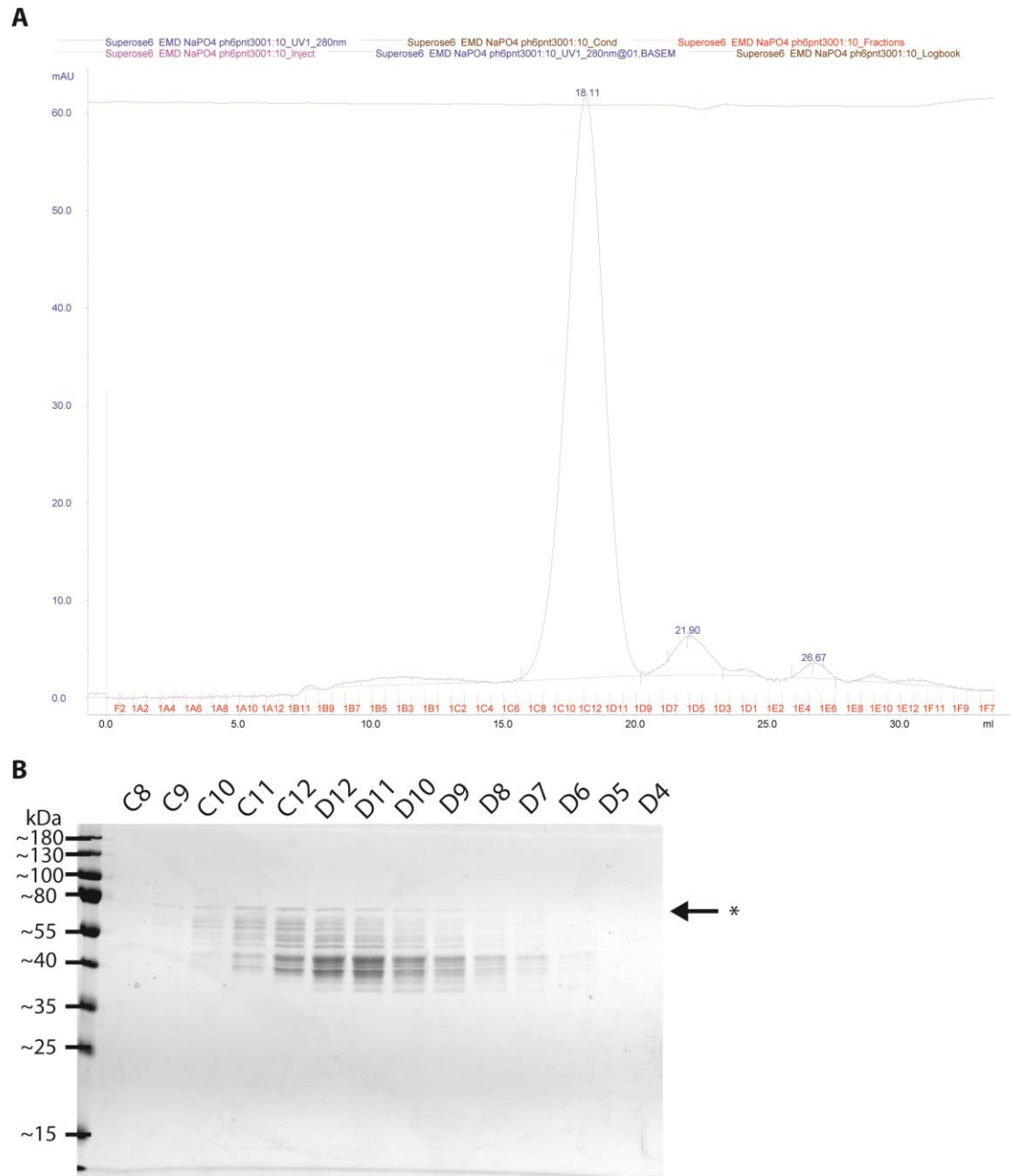


Figure 33. Size exclusion chromatography of emerlin-MBP (*) over a superpose6 column in low pH buffer (10mM NaHPO₄, pH6.3). (A) Elution profile of emerlin in low pH buffer showed a main elution peak C8-12, D12-D4. (B) Protein electrophoresis analysis of the elution peak.

5.10. Qualitative analysis of the solid phase binding assay results.

With the limitations of the protein fragments, it was not possible to get a clear interaction picture from the SPR, or perform quantitative analysis with the solid phase binding data. It was hypothesized that the differences in results from the assay compared with the SPR, could be due to detergent tolerance. The solid phase assay contained extensive washing steps which used above CMC level of detergent, as it does not interfere with the read out. This is not possible with the SPR method. I postulate that the washing step may allow time for the non-specific binding to be removed and/or maintain monomeric form of the full-length protein fragments. Another barrier against quantitative analysis was the differences in read out between experiments.

Using the solid phase approach, I believe patterns in binding between NETs and lamin A can still be analysed qualitatively. By normalizing binding of NETs to WT lamin A at B_{max} to 100% for each experiment and comparing the binding to that of lamin A mutants, I wanted to see if the differences observed with Tmem120A binding to lipodystrophic mutants was significant. ANOVA analysis of the B_{max} obtained using the plate based assay revealed significant differences between the binding of Tmem120A-MBP to WT HIS-lamin A and that to the G465D (***) and R482Q (**) mutants (figure 34). No significant differences were seen when comparing between the binding of the mutants. The binding of Tmem120A to other non-lipodystrophic mutants was also analysed. It was found that there were no significant differences when comparing against WT binding (Figure 35). The muscle specific NET Tmem38A did not reveal any differences in binding to disease mutants (figure 36).

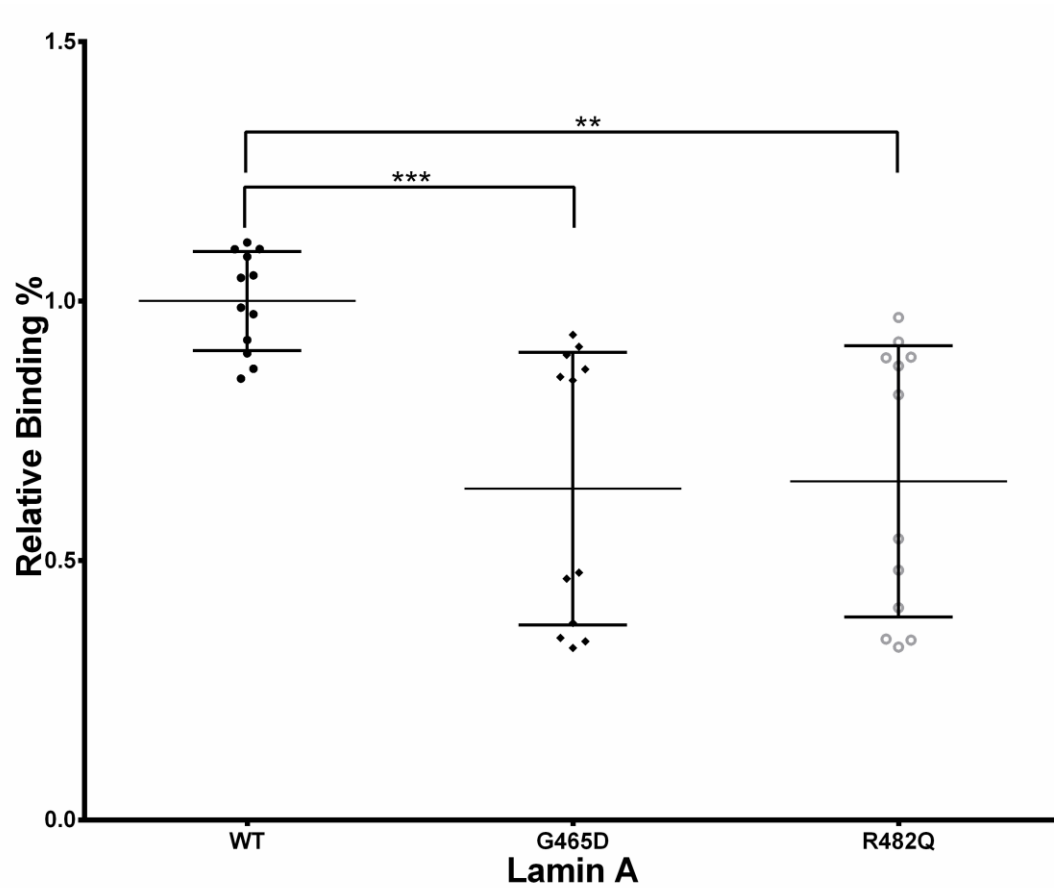


Figure 34. Qualitative analysis of multiple experiments of Tmem120A-MBP binding to immobilised HIS-lamin A WT and lipodystrophic mutants (G465D and R482Q). One-way ANOVA analysis revealed significant differences between binding of Tmem120A to WT lamin A when compared to that of its binding to lipodystrophic mutants. No significant differences were found between the binding of the mutants. Saturation concentration was decided at 900 pM using the binding curve in figure 24.

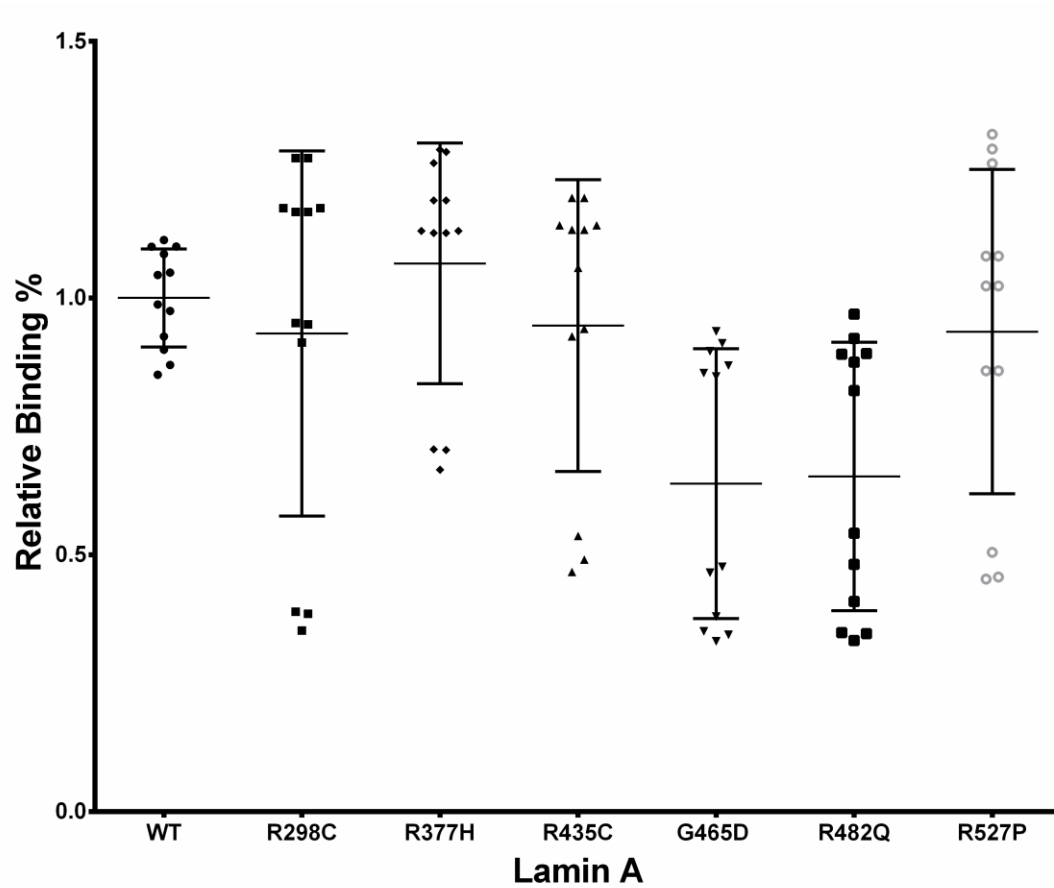


Figure 35. Qualitative analysis of multiple experiments of Tmem120A-MBP binding to immobilised HIS-lamin A WT and other non-lipodystrophic mutants revealed no significant differences. One-way ANOVA analysis revealed significant differences between binding of Tmem120A to WT lamin A when compared to that of its binding to non-lipodystrophic mutants. Saturation concentration was decided at 900 pM using the binding curve in figure 24.

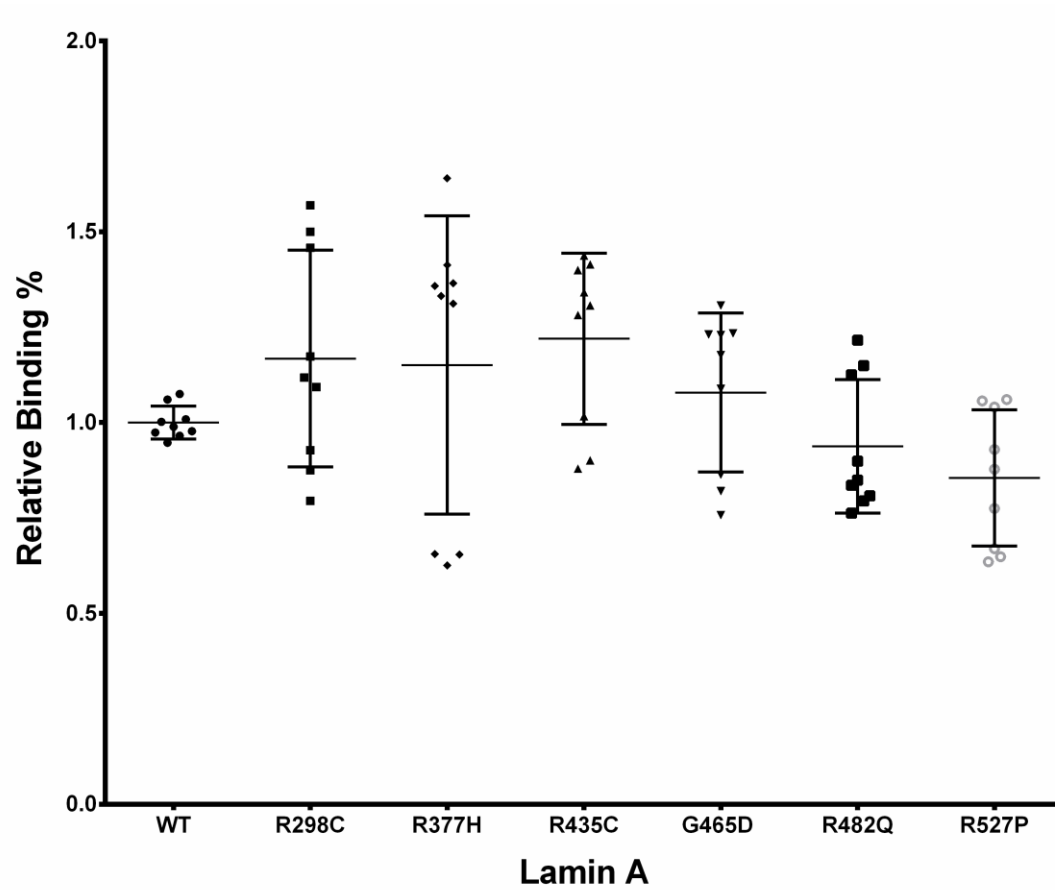


Figure 36. One-way ANOVA analysis of *Tmem38A*-MBP binding to HIS-lamin A WT and disease mutants revealed no significant differences.

5.11. Results summary

In this chapter, I showed the viability of the solid phase binding assay as a method of measuring interactions between NETs and lamin A. Using emerin as a positive control, I was able to show that the MBP tag used had minimal background binding compared to emerin/WT lamin A interaction. However, the measurements obtained from the binding assay were found to be very variable making a quantitative analysis difficult.

I postulated that the variability in the binding assay measurements could be due to differential washing steps between experiments. To test this, I carried out SPR experiments which measures interactions in real-time, eliminating washing steps. The SPR results showed a concentration dependant two stage binding for all 3 tested proteins (emerin, Tmem120A and Tmem38A).

Suspecting heterogeneity, size exclusion analyses were carried out. The data showed the fragments of emerin and Tmem120A used were forming large, soluble complexes. This improved when using detergent or low pH buffers to purify the protein fragments, but not in low salt buffers. These buffers, however, lowered the yield of the protein purification.

Qualitative analysis of the binding data revealed a reproducible pattern of reduction in Tmem120A binding to the lipodystrophic mutants G465D and R482Q, but not to lamin A WT and other non lipodystrophic mutants. The reduction in binding of Tmem120A to G465D and R482Q could be important, as it has been previously shown that knockdown of Tmem120A inhibits adipogenesis. Tmem38A testing did not reveal significant differences in binding between WT lamin A and mutants.

Time constraints resulted in several flaws in the experimental set up of both the solid phase binding assay and the SPR experiments. The lack of a comprehensive set of controls for both set up mean that interpretations of any differences observed may not be as large or significant as initially seem. Testing for background binding of Tmem120A-GST did not include a GST tag only control binding to a blank well and as such, some background binding to an empty well could be due to the Tmem120A NET fragment as well as the tag. However, later experiment with Tmem120A-MBP, with MBP tag only control versus empty well showed minimal binding of both to a blank well without lamin present. This points to the GST tag being the main source of the background binding. With the time constraint, arguments can be said about running the SPR experiment first to see if it is worth it to carry on with the project. In this case, we did not find any significant differences in binding of NETs to lamin A. However, in the presence of observable changes, this approach would take more time as proper controls would be required to properly interpret any results.

Chapter 6

Discussion

6.1. Tissue-specificity of NETs

Extensive research carried out by the Schirmer lab has previously revealed that only about a third of NE proteins are shared among the liver, muscles and blood leukocytes. Further studies have shed insights into the roles and functions of a minor portion of these proteins. NET5/SAMP1 is important in centrosome anchoring and associated with TAN lines, with implications in nuclear migration (Borrego-Pinto et al. 2012). Others, such as Tmem120A, have effects on transcription and cell fate (Batrakou et al. 2015). Though the functions of tissue-specific NETs are becoming an increasingly important field, as of yet many have not been directly tested and NE residence has only been determined by proteomic approaches.

I set out to test the hypothesis that tissue-specific disease pathology could arise from properties of tissue-specific NETs, which differ between tissue sub-types. I confirmed through immunofluorescent staining using specific antibodies on muscle tissue sections and myoblasts/myotube cell cultures, the NE localisation of Tmem38A, NET5, WFS1 and Tmem214. Although expressed at the NE in muscles nuclei for NET5, Tmem214 and WF1, they were also found at the NE of nuclei in the surrounding connective tissues and in undifferentiated myoblasts.

Tmem38A was found to be exclusively expressed in differentiated muscle nuclei with other work done in the lab showing Tmem38A upregulation during myogenesis (Robson et al. 2016), suggesting that it plays an important role in muscle function and regulation.

Although found at the NE of nuclei in the surrounding connective tissues, knockdown of WFS1 in combination with Tmem38A was shown to almost completely block myogenesis (Robson et al. 2016). Potentially, it may require subtle changes in a combination of several tissue-specific NETs to cause a disease. Unpublished data from the Schirmer lab has also identified several mutations in Tmem38A, WFS1 and NET5 through genome analysis of families with unlinked causes, suggesting these NETs may still be involved.

It is yet to be seen if there is another layer to the complexity of the NE proteome. Previous proteomic research and this study could not answer whether there might be differences in the levels of the more widely expressed NETs in different tissues and cell types. Potentially, a NET may be expressed in more than one type of tissue, but its expression levels regulate its roles and functions. Indeed, a prime example is lamins. As mention in chapter 5, different expression levels of lamins can have differing effects on cell fate and function (Swift et al. 2013, Shin, Spinler, et al. 2013). The lack of differences seen in the samples tested may be an indicator of small sample size. This study may be more suited for long term time scale to allow for a large sample library to be collected and more extensive testing.

6.2. Relevancy of biopsy in modern diagnostic medicine

Diagnosis of disease has been increasingly reliant on sequencing. New technology has made it possible to sequence faster and more cheaply than ever before. It could be argued that invasive techniques such as biopsy are no longer required. This is definitely the case for diseases with a clear and documented single source such as Duchenne/Becker's muscular dystrophy, where there is only one known linked gene (*Dystrophin*). Other diseases however, are not so clear cut. A heterogeneous disease

such as EDMD for example currently has eight linked genes responsible for approximately 50% of all cases, with half of EDMD cases still unlinked. With many potential pathways which could lead to variable manifestation of phenotypes, the relevancy of what a simple biopsy staining can tell us is questionable.

While genetic approaches have been taken to identify causal genes in many cases, biopsies are still required to confirm localisation and expression of proteins in research. It is still important to study proteins in their native, mature environments. While tissue culture techniques have improved, they still cannot replicate the conditions that are happening in mature tissues. Comparisons of affected muscle groups of EDMD and LGMD revealed no clear distinguishing features. This suggests that no overt mechanical mechanism is involved in these diseases and maybe there is a genome organization aspect to them.

Another problem with biopsies is that, for many muscular disease studies, the accepted normal control samples consist mostly of muscle excluded sections. This makes it questionable whether they are truly normal and have no effects on any observations made.

In conclusion, biopsies should be used, albeit sparingly, in conjunction with less invasive techniques to fully understand the pathology of diseases. To overcome the sample size dilemma for non-clinician researchers, all biopsies should be made available through biobank and this should be made clear to the patient when obtaining permission.

6.3. Problems of working with NETs fragments

During the course of this study, attempts at purifying NETs has brought up many obstacles and questions; the first being the solubility of NETs. Purification techniques used in this study failed to conclusively purify mono-disperse NET samples. Although NETs tagged with MBP appear to be soluble, further analysis by size exclusion chromatography showed the protein fragments forming large soluble complexes which prevented quantitative analysis.

Previous research using NET fragments generated data which on inspection, showed similar results to those obtained using SPR in this study (Clements et al. 2000). The response profile of their data also showed a two-stage response, suggesting the protein samples used were not mono-disperse. Other studies into binding of emerlin to lamins which reported soluble emerlin used various lengths of the nucleoplasmic region, all of which are shorter than the fragment used in this study (Holaska et al. 2003, Wilkinson et al. 2003, Haraguchi et al. 2004, Demmerle, Koch, and Holaska 2012, Berk et al. 2014). Apart from being bacterially expressed, which means proteins could lack post-translational modifications, these previous studies into emerlin interactions utilized shorter fragments of the protein, often just the LEM domain. While molecularly dissecting protein down to monodispersic fragments may be able to shed light on small areas of interactions, it is questionable whether these interactions are the native state of the proteins within the cell. Lamin A and NETs are by nature insoluble proteins, unpublished data from the lab shows that lamin A unstructured regions have a tendency to fold over and interact with other regions and so binding data may not represent normal interactions.

6.4. Interactions between NETs and lamins

I demonstrated in this study the interaction between NETs and lamin A using two methods, SPR and a solid phase binding assay. Attempts at obtaining quantitative binding affinity using both methods were made more complicated with the discovery that MBP tagged NETs samples have a tendency to form large, soluble aggregates. Experiments are in motion to further dissect the binding sites of NETs and lamin A. Preliminary data (not shown) from the Schirmer lab appears to indicate that for some NETs, the C-terminal domain of lamin A is sufficient for interaction. Other studies have also shown that not all of the nucleoplasmic domains of NETs are required for binding to lamin, such as emerin which only requires amino acid 1-188 (Clements et al. 2000).

Using a qualitative approach to analyse the binding data, it was found that Tmem120A had reduced binding to lipodystrophic mutants of lamin A. This supports the hypothesis that disruption of interactions between widely expressed, disease linked proteins, with more tissue-specific NETs, may be a factor in manifestation of tissue-specific pathology.

It would be interesting to narrow down the binding sites of NETs on lamin A and compare them to the mutations present. Whether disease causing mutations disrupts specific binding sites or the overall structure of lamin A, which in turn could disrupt NET binding, is still to be seen. The solid phase binding assay did not find reduced binding of Tmem120A and Tmem38A to WT and other non-lipodystrophic mutants, suggesting that these interactions require other specific binding sites and which are not disrupted by the mutations G465D and R482Q. The lipodystrophic lamin A mutant R482Q has also been shown to disrupt interaction with an adipocyte differentiation factor sterol response element binding protein 1 (SREBP1) (Lloyd, Trembath, and Shackleton 2002, Vadrot et al. 2015), as well as deregulating Fragile X-related protein 1 (FXR1P), resulting in a

myogenic gene expression response in preadipocytes (Oldenburg et al. 2014). When combined with published data showing Tmem120A is important in adipocyte differentiation (Batrakou et al. 2015), it suggests that Tmem120A may be part of the regulatory complex which, when disrupted, results in lipodystrophy.

Mutational analysis of unlinked families carried out by the Schirmer lab revealed potential causal mutations in several NETs (WFS1, Tmem38A and NET5). It would be interesting in the future to carry out studies to see whether mutations in the NETs alter binding to WT lamin A. Altered binding in the presence of NET mutations in unlinked patients could indicate causality. Further experimentation is required to see if the reduced binding has any contribution to disease pathology. However, this data represents a potentially exciting step forward in the understanding of tissue-specific pathology.

6.5. Future directions

The question of establishing normal expression and distributions of NETs in tissue, and whether changes in patient samples can be used reliably to indicate specific disease, still require further study. In hindsight, even though the sample size used in this study is greater than many previous reports, it is still not enough to establish a baseline pattern for the tested NETs. Further expansion to this study should take steps to increase the number of collaborations with clinicians to build up a bigger sample size. Establishing ethical approval with muscle banks such as the one in London and Munich would significantly help in this matter. As these orphan diseases are often rare and patients may not be readily available, it could also be argued that without a significant quantifiable difference, it may not be worth carrying out invasive procedures such as biopsies.

A study of this scope would also be best carried out long term; the requirements of generating specific antibodies against NETs are often complicated and time consuming, building up a portfolio of antibodies may take much longer than that allowed in a PhD program in the UK. Epitope masking has also been reported in certain cases and so great care needs to be taken when reporting negative signals (Tunnah et al. 2005). It is possible that certain NET distribution would only be seen with particular antibodies due to epitope masking. Generating such a panel of monoclonal antibodies would certainly take time.

Identification and purification of NET fragments can be very problematic, as shown by this study. Nucleoplasmic fragments of NETs have shown tendency to be insoluble and my attempts have found that they form soluble aggregates. Generating antibodies against soluble aggregate could generate unspecific antibodies. On the other hand, the unstructured nature of NETs could mean that they may form large complexes natively and present native epitopes. For a study which hopes to decipher differences between samples, clean antibodies to native epitopes are required to make sure no false signals are detected, which is why large nucleoplasmic fragments were used in this study.

The tendency of NETs to be insoluble or form large aggregates means that careful planning is required when purifying them. Although this study set out to design and build a system which would allow faster analysis of interactions, perhaps a more prudent approach would be to take time to molecularly dissect each individual protein separately to achieve monodisperse, soluble samples. However, the usefulness of this approach could also be called into question. In this study, I cloned and expressed the entire nucleoplasmic domain of NETs i.e. emerin aa1-224, which was shown to form soluble aggregates, despite the fact that it has been reported that emerin aa1-222 (almost all of the nucleoplasmic domain) could be purified as soluble monomers (Holaska et al. 2003).

The absence of 2 amino acids before the transmembrane domain may help with solubility and aggregation. Whether this is a universal rule which applies to all NETs needs further investigation.

Future investigation into interactions of NETs and lamin A could also molecularly dissect the protein, to narrow down the binding site. However, the relevancy of interactions between much reduced protein, as mentioned previously, could be questionable. No conclusive structure of lamin A has been found so far, and as mentioned earlier in the chapter, it is not yet known whether mutations disrupt the whole protein structure or specific binding sites, with my results suggesting the latter. It would also be interesting to find out the effects of stacking multiple mutations. Tissue-specific diseases can have a wide range of clinical variations and multiple mutations present may cause more severe phenotypes, potentially in more than one protein. A genetic study had been carried out by the Schirmer lab which identified several mutations in NETs from unlinked EDMD patients.

Future studies should also attempt to establish *in vivo* experiments to confirm the effects of loss/reduced NET/lamin interactions. Rescue experiments such as overexpressing WT NETs in a lamin mutant cell line, in the absence of WT, could give further indication of involvement in pathology.

6.6. Final remarks

While further studies are needed to understand the mechanisms behind tissue-specific diseases, the work presented in this thesis suggests changes to protein expression or distributions resulting in disease may be very subtle. No differences were found in the muscle sub-types which could explain why EDMD affects the gastrocnemius, while LGMD affects the upper leg muscles. This study also showed that care is needed when observing changes in the distribution of NETs, as they may not be indicative markers of disease. Finally, I showed that Tmem120A has a reduced binding to lipodystrophic mutants of lamin A (G465D and R482Q) suggesting it could be involved in mediating lipodystrophy.

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