2-3-2016

IMPLICATIONS OF MITOTIC ONSET REGULATION IN TUBEROUS SCLEROSIS

Jessica Morgan Dare-Shih
University of Windsor

IMPLICATIONS OF MITOTIC ONSET REGULATION IN TUBEROUS SCLEROSIS

by
Jessica M. Dare-Shih

A Thesis
Submitted to the Faculty of Graduate Studies
Through Biological Sciences
In Partial Fulfillment of the Requirements for
The Degree of Master of Science at the
University of Windsor

Windsor, Ontario, Canada
2016

IMPLICATIONS OF MITOTIC ONSET REGULATIONS IN TUBEROUS SCLEROSIS

by

Jessica M. Dare-Shih

APPROVED BY:

_____

M. Boffa
Department of Chemistry and Biochemistry

_____

A. Swan
Department of Biological Sciences

_____

E. Fidalgo Da Silva, Co-Advisor
Department of Biological Sciences

_____

L. Porter, Advisor
Department of Biological Sciences

Tuesday December 15th 2015

AUTHOR'S DECLARATION OF ORIGINALITY

I hereby certify that I am the sole author of this thesis and that no part of this thesis has been published or submitted for publication.

I certify that, to the best of my knowledge, my thesis does not infringe upon anyone's copyright nor violate any proprietary rights and that any ideas, techniques, quotations, or any other material from the work of other people included in my thesis, published or otherwise, are fully acknowledged in accordance with the standard referencing practices. Furthermore, to the extent that I have included copyrighted material that surpasses the bounds of fair dealing within the meaning of the Canada Copyright Act, I certify that I have obtained a written permission from the copyright owner(s) to include such material(s) in my thesis and have included copies of such copyright clearances to my appendix.

I declare that this is a true copy of my thesis, including any final revisions, as approved by my thesis committee and the Graduate Studies office, and that this thesis has not been submitted for a higher degree to any other University or Institution.

ABSTRACT

The cell cycle is a highly dynamic and phasic process controlling cell growth, DNA duplication, and successful division into two daughter cells. Regulation of this process is key to avoiding errors and activation of cell death. Tumour suppressor proteins, such as Tuberin, and cell cycle proteins, such as Cyclin B1 are highly important in co-ordinating adequate cell growth and properly timed cell division, respectively. It is known that mutation, truncation, and misregulation of the Tuberin protein can result in diseases like Tuberous Sclerosis and cancer. This study demonstrated that a clinically relevant Tuberin truncation, S664Δ, increases cellular proliferation and exhibits aberrant localization compared to wild-type Tuberin. Additionally, our study showed that S664Δ is able to bind Cyclin B1, which may derange proper co-ordination of the G2/M transition. Findings here may have clinical implications towards better understanding the progression of disease involving misregulated Tuberin.

DEDICATION

This thesis is dedicated to my husband and family. Your constant love and support were the fuel I harnessed during the completion of this effort. Your encouragement is incorporated into every page of this work. May this serve as a source of inspiration for many years to come.

ACKNOWLEDGEMENTS

I would like to extend my sincerest gratitude to my two supervisors, Dr. Lisa A. Porter and Dr. Elizabeth Fidalgo da Silva.

To Dr. Lisa Porter, I am so appreciative for the opportunity you gave me during my graduate studies. Your enthusiasm and understanding allowed me to flourish within your dynamic and lively lab. I would like to personally thank you for this invaluable experience as it has shaped me for the future. Every day without fail, you have been a remarkable example of hard work, dedication, and passion to all of us graduate students. These special qualities are sought after in both a supervisor and scientist. I want you to know that they never went unnoticed. Thank you again for this amazing opportunity. I look forward to what the future holds.

To Dr. Elizabeth Fidalgo da Silva, I want to take the time to whole heartedly thank you for every time you picked me up when I was down and every time you single handily saved the day. You have been extremely influential to me; in both the way I develop a scientific question and how I handle everyday tasks. I could not have asked for a more knowledgeable and technically gifted scientist to work closely with during the duration of this degree. Your immense patience, acceptance, and support came through in everything you did and continue to do for me I will treasure that forever. Thank you for your never ending guidance and kindness. It was such a privilege to be one of your Tuberin "girls".

Thank you to my committee members Dr. Andrew Swan and Dr. Michael Boffa for your time, energy and valuable input.

Thank you to my colleagues and fellow lab rats, both past and present. Ingrid, Rosa, Nicole, Janice, Dorota, Bre-Anne, Ellen, Bashaer, Frank, Kaitlyn, and Sabrina; it has been a true pleasure to grow alongside you as scientists. I want to thank all of you for your technical assistance and most importantly for your acceptance and kindness. I will never forget the comfort, encouragement, and never ending

laughs we went through together. Jiamila Miamati, MSc. thank you for your technical assistance and being the first person to welcome me into the lab. I will never forget the many lessons you taught me.

Last, but certainly not least, thank you to my family and husband, for your never failing love and support. Thank you so much for bailing me out when I was in over my head and thank you for understanding when I was pre-occupied with the lab. David, you have been my rock and never fail to endure whatever lie may throw at us. I couldn't have done it without you. Thank you.

TABLE OF CONTENTS

Author's Declaration of Originality iii

Abstract iv

Dedication v

Acknowledgements vi

List of Figures & Appendices x

List of Abbreviations xi

Introduction 1

I. The Cell Cycle and Control Mechanisms 1

II. Tuberin facilitates the G1/S Transition 6

III. Tuberin Activity is regulated through Nutrient and Growth Factor Sensing Proteins 9

IV. Tuberin Regulates Downstream Cell Growth Proteins 10

V. Tuberin and Cyclin B1 regulate Mitotic Onset 13

VI. Tuberin is implicated in Tumour Formation 14

Materials and Methods 21

I. DNA Plasmids 21

II. Mammalian Cell Culture 21

III. Over-expression and Protein Concentration Determination 21

IV. SDS-PAGE, Western Blotting, and Chemiluminescence Detection Methods 22

V. Antibodies 23

VI. Co-Immunoprecipitation (Co-IP) 24

VII. Immunofluorescence (IF) 24

VIII. Quantification of Immunofluorescent Protein Localization 25

IX. Flow Cytometry and Cell Cycle Profile Analysis 26

Results 27

I. Characterizing the effects of Tuberin truncation S664Δ over-expression 27

II. Effects of the clinically relevant truncation S664Δ on the mitotic transition 37

Discussion 45
Appendices 55
References 66
VITA AUCTORIS 74

LIST OF FIGURES & APPENDICES

Figure 1.	An illustration of the Cyclin B1 protein and its key domains	5
Figure 2.	An illustration of the Tuberin protein and its key domains	8
Figure 3.	A schematic depicting the most influential players upstream and downstream of Tuberin protein signaling	12
Figure 4.	Mutations occur across the entire *TSC2* gene, but the CyclinB1 binding domain exons are an area of frequent mutation	18
Figure 5.	Over-expression of the Tuberin truncation, S664Δ significantly increases cell proliferation compared to control	28
Figure 6.	Over-expression of S664Δ significantly increases mitotic activity compared to Tuberin WT	29
Figure 7.	Mutant Tuberin S664Δ exhibits perinuclear localization significantly more than WT Tuberin protein	32
Figure 8.	Over-expression of S664Δ does not significantly alter average cell size compared to Tuberin WT	35
Figure 9.	S664Δ over-expression does not result in significantly increased autophagy protein markers compared to WT Tuberin or control	36
Figure 10.	Tuberin WT and S664Δ regulate the sub-cellular localization of Cyclin B1 WT	38
Figure 11.	S664Δ binds Cyclin B1 WT protein less than Tuberin WT *in vivo*	42
Figure 12.	Tuberin WT and S664Δ both bind all three Cyclin B1 NES variants to significantly different degrees *in vivo*	43
Appendix 1.	Site-Directed Mutagenesis and cloning of the novel, clinically relevant Tuberin truncation vector, pCMV-FLAG-TSC2-S664Δ	56
Appendix 2.	Mutant Tuberin S664Δ exhibits perinuclear localization significantly more than WT Tuberin protein	58
Appendix 3.	Tuberin S664Δ protein is significantly more clustered compared to WT Tuberin protein	61
Appendix 4.	Site-Directed Mutagenesis and cloning of novel Cyclin B1 NES vector, pCMX Cyc B1 4A (A126E)	65

LIST OF ABBREVIATIONS

4A	Cyclin B1 4A (A126E) protein variant
4E-BP1	4E binding protein 1
5A	Cyclin B1 5A protein variant
5E	Cyclin B1 5E protein variant
Akt	protein kinase B
AMP	adenosine monophosphate
AMPK	AMP- activated protein kinase
Atg	autophagy associated gene
ATP	adenosine triphosphate
BIFC	Bimolecular Fluorescence Complementation
BPB	bromophenol blue
CAK	Cdk activating kinase
CDK	cyclin dependent kinase
CKI	cyclin dependent kinase inhibitor
Co-IP	co-immunoprecipitation
CRM1	chromosome region maintenance 1/exportin 1
CRS	cytoplasmic retention sequence
DMEM	Dulbecco's modified eagle's medium
DNA	deoxyribose nucleic acid
ECL	enhanced chemiluminescent reagent
EGFR	epidermal growth factor receptor
eIF4E	eukaryotic initiation factor 4E
Erk / MAPK	extracellular signal regulated kinase
FBS	fetal bovine serum
FKBP38	FK506-binding protein 8
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GFP	green fluorescent protein
GTP	guanosine triphosphate

HERC1	HECT and RLD Domain Containing E3 Ubiquitin Protein Ligase Family Member 1
IGF	insulin like growth factor
IGFR	insulin like growth factor receptor
IRS	insulin receptor substrate
IF	immunofluorescence
IP	immunoprecipitation
LB	lysis buffer
LC3	light chain associated protein 3
MAPK (ERK)	mitogen activated protein kinase
MB	medulloblastoma
MEK	mitogen activated protein kinase kinase
MPF	maturation promoting factor
mTOR	mammalian target of rapamycin
mTORC1	mammalian target of rapamycin complex 1
mTORC2	mammalian target of rapamycin complex 2
Myt1	Myelin Transcription Factor 1
NEBD	nuclear envelope breakdown
NES	nuclear export signal
p70S6K	Ribosomal protein S6 Kinase 1
PAGE	polyacrylamide gel electrophoresis
PDK1	phosphoinositide dependent kinase-1
PEI	polyethylenimine
PI	propidium iodide
PI3K	phosphatidylinositide 3-kinases
PLK1	polo-like kinase 1
PMSF	phenylmethanesulfonyl fluoride
P/S	penicillin/streptomycin antiiotics
PtdIns-3	phosphatidylinostitol 3-phosphate
PVDF	Polyvinylidene fluoride
Raptor	regulatory-associated protein of mTOR complex 1

RCC	Renal Cell Carcinoma
Rheb	Ras homolog enriched in brain
Rictor	rapamycin insensitive companion of mTOR complex 2
SDS	sodium dodecyl sulfate
SDM	site-directed mutagenesis
SEGA	subependymal giant cell astrocytomas
SENs	subependymal nodules
TBST	tris buffered saline and tween 20
TS	tuberous sclerosis disease
TSC	tuberous sclerosis complex
TSC1	tuberous sclerosis 1 gene
TSC2	tuberous sclerosis 2 gene
Wee1	Mitosis Inhibitor Protein Kinase
Y2H	yeast 2 hybrid

INTRODUCTION

I. The Cell Cycle and Control Mechanisms

The cell cycle is a tightly regulated process through which cell growth and cell division occur. The mammalian cell cycle can be divided into two main stages known as *interphase* and *mitosis*. Interphase is further subdivided into phases of growth (Gap-phases) and DNA synthesis (S-phase). Immediately following division, a cell enters its first growth phase (G1) and upon commitment to the cell cycle prepares for S-Phase. Following successful DNA replication, the cell begins its second phase of growth (G2) in preparation for mitosis (Norbury and Nurse, 1992; Nurse, 2002). Mitosis is a very dynamic process which consists of four distinct phases; prophase, metaphase, anaphase and telophase. Chromosome condensation occurs at prophase and is followed by organization at the metaphase plate. Anaphase is observed as chromosomes separate to opposite ends of the cell. Subsequently, telophase begins and allows for the re-assembly of a nuclear membrane around the chromosomes (Vermeulen et al., 2003). Beginning at anaphase and occurring concurrently to telophase is an extremely important event known as cytokinesis, which divides the cell into separate daughter cells by pinching of the cellular membrane. Together, mitosis and cytokinesis are referred to as M-phase (Glotzer, 2001). Notably, if a cell is unable to commit to the cell cycle during G1, it may enter a phase known as G0. A cell may remain in this quiescent phase indefinitely until reaching satisfactory conditions for growth and proliferation (Vermeulen et al., 2003).

The cell cycle is a vigorous process and many cellular proteins, markedly the cyclins and their cyclin dependent kinase (CDK) partners, tightly regulate "checkpoints" as a cell progresses from one phase to the next (Morgan, 1995; Pines, 1995). CDKs fall into a family of serine/threonine protein kinases, whose activity ultimately leads to cell cycle advancement. CDKs are expressed at relatively constant levels throughout the cell cycle and are regulated largely through post-translational modifications. The kinase activity of a CDK is dependent upon cyclin binding. In the absence of cyclin binding, a CDK is inactive due to blockage of the CDK active site by the T-loop (Jeffrey et al., 1995). CDKs are also regulated by phosphorylation and dephosphorylation on conserved tyrosine (Y) and threonine (T) residues. Phosphorylation on T14 and Y15 by Myt1 and Wee1 kinases, respectively, render a CDK inactive.

Phosphorylation on these residues ultimately inhibits ATP binding and full CDK activation. Phosphatase activity by Cdc25 on these two sites is essential for CDK1 activation and cell cycle progression overall (Lew and Kornbluth, 1996). Following cyclin binding, CDK-activating enzymes (CAKs) phosphorylate a conserved threonine residue within the T-loop. For example, CDK1 requires phosphorylation on residue T161 by CAK for its full activation (Jeffrey et al., 1995; Paulovich and Hartwell, 1995). Phosphorylation by CAK induces a conformational change in the CDK resulting in enhanced cyclin binding and complete activation. Upon complete activation, CDKs are able to phosphorylate specific protein substrates.

Similarly, cyclins are a family of temporally regulated cell cycle proteins that can be categorized by type (Vermeulen et al., 2003). Cyclin proteins are able to interact with CDKs through a conserved protein motif, known as the cyclin box. Classically, there are D-type, E-type, A-type, and B-type cyclins (Vermeulen et al., 2003). Unlike their CDK partners, many cyclin proteins are expressed in a cyclical fashion, with levels rising and falling through progression of the cell cycle (Norbury and Nurse, 1992; Nurse, 2002). At the onset of G1, levels of D-type cyclins rise and are able to bind CDK4 and CDK6 (Sherr, 1994). Following this interaction, Cyclin E-CDK2 interaction allows for the transition from G1 to the onset of S-phase (Ohtsubo et al., 1995). Co-currently, through S-phase levels of Cyclin A rise and association with CDK2 is observed into G2 (Girard et al., 1991; Walker and Maller, 1991). Subsequently, in late G2, the Cyclin A-CDK1 interaction promotes M phase entry (Ford et al., 1994). Continued regulation and progression of mitosis is controlled by CDK1 interactions with Cyclin B1 and B2 (Arellano and Moreno, 1997). Cyclin proteins, with the exception of D-type cyclins (Assoian and Zhu, 1997), are heavily regulated first by their synthesis and second through ubiquitin-mediated degradation (Glotzer et al., 1991). Degradation of Cyclins A and B is mediated through a domain known as the destruction box (D-box), while Cyclin D and E degradation is mediated through a segment rich in proline, glutamic acid, serine, and threonine residues; commonly referred to as the "PEST" sequence (Glotzer et al., 1991; Rechsteiner and Rogers, 1996).

There are additional mechanisms that govern CDK activity, furthering cell cycle control. Binding of CDK inhibitor (CKI) proteins negatively regulates Cyclin-CDK complexes (Sherr and Roberts, 1995; Vermeulen et al., 2003). CKIs are classified into two main families; the CIP/KIP (CDK inhibiting protein) family and the INK4 tumor suppressor family. The INK4 branch of CKIs consists of $p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$, and $p19^{INK4d}$. The INK4 proteins act to inhibit CDK4 and CDK6 at G1. This inhibition is accomplished by CKIs forming a stable complex with the CDK; ultimately preventing Cyclin D binding (Carnero and Hannon, 1998). Within the branch of the CIP/KIP family are $p21^{Cip1}$, $p27^{Kip1}$, and $p57^{Kip2}$ proteins; which ultimately inhibit CDK1 and CDK2 activity (Harper et al., 1995; Hengst et al., 1998; Lee et al., 1995; Polyak et al., 1994). The CIP/KIP proteins bind at the interface of a Cyclin-CDK complex essentially blocking the Adenosine Triphosphate (ATP) region of the CDK. This blockage restricts proper folding of the CDK at its catalytic cleft, preventing CDK activation (Sherr and Roberts, 1999). Additionally, Cyclin-CDK complexes are inhibited through chaperone proteins and upstream inhibition. Phosphorylated Cdc25 is inactive and displays increased binding to the chaperone protein, 14-3-3σ within the cytoplasm (Dunphy and Kumagai, 1991). This interaction has direct consequences on mitotic entry, as the Cdc25-14-3-3σ interaction prevents Cdc25 from activating CDK1 and overall Cyclin B1-CDK1 complex formation (Hengst and Reed, 1998). Moreover, binding of Cyclin B1 with 14-3-3σ actively enhances nuclear export (Peng et al., 1997; Yang et al., 1998).

Cell cycle progression is also regulated spatially, via intracellular Cyclin-CDK localization. For example, Cyclin B1 contains a variety of domains and protein modifications, which contribute to its fluctuating localization (Figure 1) (Porter and Donoghue, 2003). Cyclin B1 forms a complex with CDK1 to control mitotic onset. The Cyclin B1 gene, *CCNB1*, is located on chromosome 5q13.2 and results in a 62kDa protein (Sartor et al., 1992). The mammalian Cyclin B1 protein, as depicted in Figure 1, contains the classical cyclin box domain (aa210-348), which allows for its direct CDK1 interaction. In 1998 Yang et al. observed that during interphase, Cyclin B1 is actively shuttled from the nucleus to the cytoplasm until prophase begins. From here, Cyclin B1 protein accumulates in the nucleus only when the rate of Cyclin B1

import/export is altered (Yang et al., 1998). Surprisingly, Cyclin B1 lacks a classical nuclear localization signal (NLS) (Pines and Hunter, 1994), but contains a nuclear export signal (NES) within a region classically coined the cytoplasmic retention sequence (CRS). This CRS domain is of key importance since it contains five conserved serine residues, which are phosphorylation targets. It was originally thought that these sites, when phosphorylated, facilitate nuclear accumulation (Hagting et al., 1999; Hagting et al., 1998; Li et al., 1997a). However, later data revealed that phosphorylation prevented the binding of nuclear export proteins within the CRS, resulting in nuclear accumulation (Pines and Hunter, 1994). The exportin protein chromosome region maintenance 1 (CRM1) is one export protein responsible for the active nuclear export of Cyclin B1 (Yang et al., 1998). CRM1 mediated export is an extremely important regulator of Cyclin B1 localization. Shuttling of Cyclin B1 from the nucleus to the cytoplasm is in a constant equilibrium (Hagting et al., 1998; Yang et al., 1998).

The aforementioned NES is located within the CRS region (aa88-154) of Cyclin B1 (Porter and Donoghue, 2003). The Cyclin B1-CDK1 complex is an extremely important cell cycle activator at the G2/M transition because it is responsible for chromosome condensation, nuclear envelope breakdown (NEBD), and mitotic spindle assembly (Ciemerych and Sicinski, 2005; Draviam et al., 2001; Ianzini and Mackey, 1997; Jackman et al., 2003). During S-phase, Cyclin B1 protein accumulation begins, peaking at prophase and metaphase, and significantly declining at the start of anaphase via degradation by the cyclosome/anaphase-promoting complex (APC) (Chang et al., 2003; Hershko, 1999). However, hyper-phosphorylation of the mentioned serine residues results in hindering of NES activity and promotes nuclear accumulation (Hagting et al., 1999; Hagting et al., 1998; Li et al., 1997b). Cyclin B1 residues S126 and S128 are known CDK1 targets and are the first residues responsible for promoting B1 nuclear translocation (Borgne et al., 1999; Hagting et al., 1999; Izumi and Maller, 1991). S147 is phosphorylated by polo-like kinase 1 (Plk1), a kinetochore localizing protein that is a known activator of the APC (Toyoshima-Morimoto et al., 2001). These events; however, are not fully sufficient to prevent Cyclin B1 export, indicating additional mechanisms are involved in this complex process.

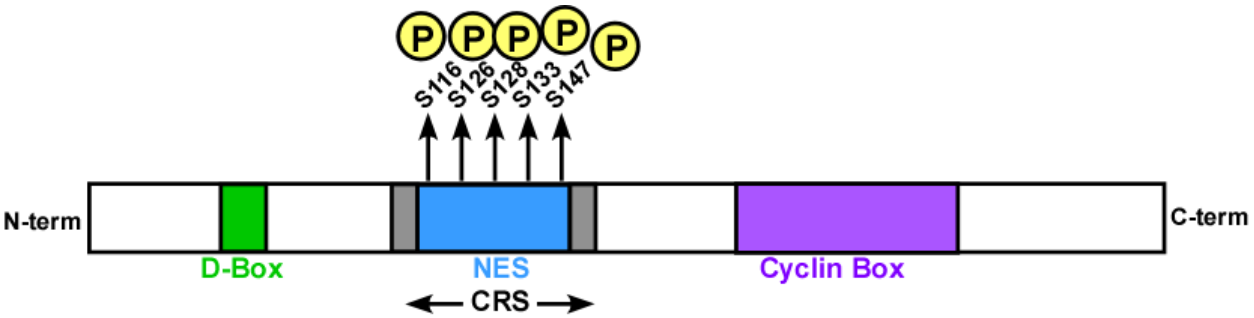


Figure 1: An illustration of the Cyclin B1 protein and its key domains

From N to C-terminus, the Destruction Box (residues 42-50), the Nuclear Export Sequence (NES) residing in the Cytoplasmic Retention Sequence (CRS) from residues 88-154, and the Cyclin Box (residues 210-348), which is responsible for correct CDK1 association. The NES also contains 5 serine residues, which regulate Cyclin B1 trans-localization.

II. Tuberin facilitates the G1/S Transition

Cell cycle control is regulated through a variety of complex mechanisms regulated by a plethora of other proteins not mentioned here. One protein of particular importance to the cell cycle is the tumor suppressor protein, Tuberin. The Tuberin protein is encoded by the *TSC2* gene located on chromosome 16p13.3 consisting of 41 exons. The Tuberin protein is comprised of 1804 amino acids and results in a 200 kDa protein (European Tuberous Sclerosis Consortium, 1993) (1993). This large protein contains a variety of conserved domains responsible for its function reviewed by Rosner et al. in 2008. Tuberin specifically contains two coiled-coil domains (aa346-371), a leucine zipper (aa75-107), a GTPase activating protein (GAP) homology domain (aa1517-1674) and a calmodulin domain (aa1740-1758) (Figure 2) (Rosner et al., 2008). It is well established that the Tuberin protein plays cell cycle regulatory roles at both the G1/S and G2/M transition.

The most widely known role for Tuberin is to act as a tumor suppressor at the G1/S transition by responding to nutrient and growth factor stimuli. Tuberin in a complex with a protein Hamartin form the Tuberous Sclerosis Complex (TSC), which negatively regulates cell growth and proliferation (Green et al., 1994a; Green et al., 1994b; Tee et al., 2003a; van Slegtenhorst et al., 1998). Tuberin, independent of Hamartin, is known to interact with the important CKI, p27, which markedly inhibits the G1/S transition. Tuberin accomplishes this action by binding to the CDK2 inhibitor, p27, protecting it from ubiquitin-mediated degradation. Tuberin also allows for nuclear accumulation of p27 by interfering with the 14-3-3σ dependent cytoplasmic retention of p27. These results, taken together, demonstrate the ability of Tuberin to negatively regulate the cell cycle at the G1/S transition (Rosner et al., 2007a; Rosner et al., 2006).

The Hamartin protein arises from the *TSC1* gene located on chromosome 9q34. The *TSC1* gene consists of 23 exons and once translated produces a 130kDa protein consisting of 1164 amino acids (van Slegtenhorst et al., 1997). Together Tuberin and Hamartin act as a tumor suppressor complex. Hamartin is known to bind the N-terminus (aa1-418) of the Tuberin protein (Rosner et al., 2008). Hamartin has a large role in the stabilization of Tuberin primarily by inhibiting its interaction with the ubiquitin ligase, HECT

and RLD Domain Containing E3 Ubiquitin Protein Ligase Family Member 1, HERC1 (Benvenuto et al., 2000; Chong-Kopera et al., 2006).

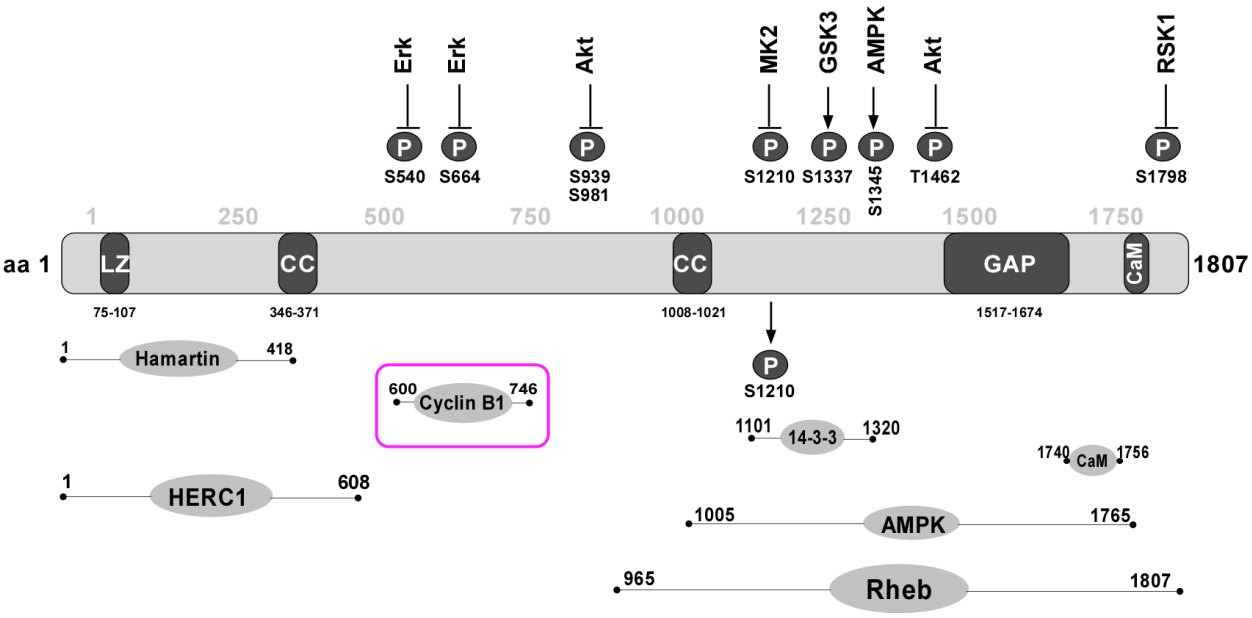


FIGURE 2: An illustration of the Tuberin protein and its key domains.

Structurally, the Tuberin protein contains a leucine zipper region (LZ) from residues 75-107, two coiled-coil regions (CC) from residues 346-371 and 1008-1021 and a GTPase activating protein domain (GAP) from residues 1517-1674. Tuberin also has a variety of functionally important binding domains. From N to C terminus, he Hamartin binding domain (residues 1-418), the HERC1 binding domain (residues 1-608), CyclinB1 binding domain (pink) (residues 600-746), the Rheb binding domain (residues 965-1807), and CaM, Calcium/Calmodulin binding domain (residues 1740-1756). Phosphorylation at an array of residues also spans the entire protein, and of particular interest are Erk and Akt phosphorylation residues. (J.Dare 2015; Adapted from Rosner et al., 2008)

III. Tuberin Activity is regulated through Nutrient and Growth Factor Sensing Proteins

Together, Tuberin and Hamartin integrate levels of nutrients, growth factors, and energy stores to regulate cell growth and proliferation (Figure 2 and Figure 3) (Huang and Manning, 2008; Orlova and Crino, 2010). This integration is important since it results in the inhibition of the activity of downstream Tuberin targets (Gao and Pan, 2001; Inoki et al., 2003; Tee et al., 2002). They accomplish this by responding to a variety of upstream proteins that regulate their protein-protein interactions as well as the phosphorylation, and subcellular localization of Tuberin. Specifically, energy is sensed in the cell via ATP levels. In the event of high energy levels, the cell will be in an ATP high flux. Conversely, if energy levels fall low, an adenosine monophosphate (AMP) high flux will result. If high levels of AMP are present within the cell, AMP will bind to the AMP-activated protein kinase (AMPK). Activating phosphorylation of Tuberin occurs on residues T1227 and S1345 by AMPK, resulting in cell growth inhibition (Corradetti et al., 2004; Huang and Manning, 2008; Shaw, 2009; Shaw et al., 2004a; Shaw et al., 2004b).

Mitogen stimulation in the form of growth factors, cytokines, or hormones is also communicated through Tuberin and the TSC. Growth factor levels of insulin and insulin-like growth factor (IGF) are transmitted through IGF transmembrane receptors (IGFR) binding. Transmembrane activation results in insulin receptor substrate (IRS) recruitment and subsequent phosphoinositide 3-kinase (PI3K) activation (reviewed by (Orlova and Crino, 2010)). PI3K exerts its affects through lipids, specifically through phosphorylation of membrane associated phosphatidylinostitol 3-phosphate (PtdIns 3). These events recruit phosphoinositide dependent kinase-1 (PDK1) to active membrane associated lipids. Once bound, PDK1 becomes active and is allowed to activate Protein Kinase B (Akt) (Manning and Cantley, 2003; Manning et al., 2002). The serine threonine kinase, Akt, has the ability to bind and negatively regulate the Tuberin protein. Inhibitory phosphorylation by Akt occurs on S939 and T1462 of the Tuberin protein in the event of high nutrient and insulin signalling (Dan et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et

al., 2002). This inhibition results in decreased binding between Hamartin and Tuberin (Manning et al., 2002).

Conversely, cell growth regulation by Tuberin is not only controlled through Akt, but also through mitogen activated protein kinase (MAPK)/extracellular signaling related kinase (Erk) signaling (Ma et al., 2005). Epidermal growth factor (EGF) is known to bind the transmembrane associated epidermal growth factor receptor (EGFR). This binding induces intrinsic membrane activation, through tyrosine phosphorylation, resulting in signal transduction events involving the GTPase, Ras. Ras, becomes activated in its GTP bound form and is now able activate the kinase activity of Raf. Raf, a serine/threonine protein kinase is known to phosphorylate and activate Mek1/2 and ultimately activate Erk via phosphorylation (Ma et al., 2005; Payne et al., 1991; Pearson et al., 2001). In 2007, data from Ma et al. identified Tuberin as a substrate for Erk on sites S540 and S664, with S664 being the primary and functionally important regulatory site on Tuberin. Dual phosphorylation on Tuberin through Erk decreases the affinity of Tuberin for Hamartin, resulting in enhanced S6K activity through mTOR and, strikingly, Tuberin modification via Erk has significantly increased effects on cell proliferation and oncogenic transformation (Ma et al., 2005; Ma et al., 2007).

IV. Tuberin Regulates Downstream Cell Growth Proteins

Tuberin is a fundamental player in the regulation of cell growth and protein synthesis through its downstream inhibition of mTOR during the G1/S transition (Figure 4) (Tee et al., 2002). Tuberin contains a C-terminal GAP domain allowing its GTPase activating functions. Previous research has shown Tuberin binds to Ras homolog enriched in brain (Rheb) (Inoki et al., 2003; Tee et al., 2003b). Rheb, a member of the Ras super family, functions as small GTPase protein, which shuttles between its active (GTP bound) and inactive (GDP bound) form.. GTPases can weakly and slowly hydrolyze their bound GTP to GDP. Tuberin acts as a GAP towards Rheb through direct binding. The GAP function of Tuberin accelerates the G-proteins built in hydrolysis timer. Specifically, Tuberin binds the G-protein, Rheb, directing a conformational change, which allows for nucleophilic attack and quick hydrolysis, rendering Rheb in the

inactive GDP form. GDP bound Rheb is unable to bind and activate mTOR, thereby halting an increase in cell growth. In this manner, Tuberin GAP activity is prevented by Akt phosphorylation on sites previously mentioned (Li et al., 2006; Maheshwar et al., 1997; Tee et al., 2003b; Wienecke et al., 1995).

When active, Rheb acts as a mediator, inhibiting mTORs repression, by abrogating the association of mTOR with the endogenous inhibitor FKBP38 (Bai et al., 2007). The mTOR protein is a complex protein involved in many more cellular events than described here. Briefly, mTOR retains hierarchical control over protein synthesis, cell growth and autophagy, by responding to upstream nutrient, growth factor, and amino acid stimuli (Beugnet et al., 2003; Tee et al., 2002; Tee et al., 2003b). mTOR is a large serine/threonine protein kinase that is composed of two distinct complexes: mTORC1 and mTORC2 (Wullschleger et al., 2006). Both complexes are known to localize to distinct subcellular components, contributing to their differential roles within the cell (Betz and Hall, 2013). The mTORC2 subunit, with its rapamycin insensitive partner, rictor, is known to regulate the actin cytoskeleton and provide input to cellular metabolism through feedback to Akt (Sarbassov et al., 2004).

The more described subunit, mTORC1, is known to associate with its rapamycin sensitive partner, raptor. Interactions between mTORC1 and raptor are of particular interest because together these proteins serve as an interface in the regulation of protein synthesis (Kim et al., 2002). mTORC1 accomplishes the activation of protein synthesis by upstream regulation of p70S6K (Ribosomal protein S6 Kinase 1) and 4E binding protein 1 (4E-BP1) (Wullschleger et al., 2006). mTORC1 phosphorylates S6K resulting in activation of the ribosomal S6 protein subunit leading to increase translation and cellular growth (Garami et al., 2003). Concomitantly, mTORC1 phosphorylates and inhibits 4E-BP1, thereby allowing the eukaryotic initiation factor 4E (eIF4E) to be free, participate in mRNA stabilization by cap binding and initiate 5’ cap dependent translation. These events also lead to an increase in protein synthesis, cell size, and cell cycle advancement (Dann et al., 2007; Kim et al., 2002; Rosner et al., 2009; Wullschleger et al., 2006).

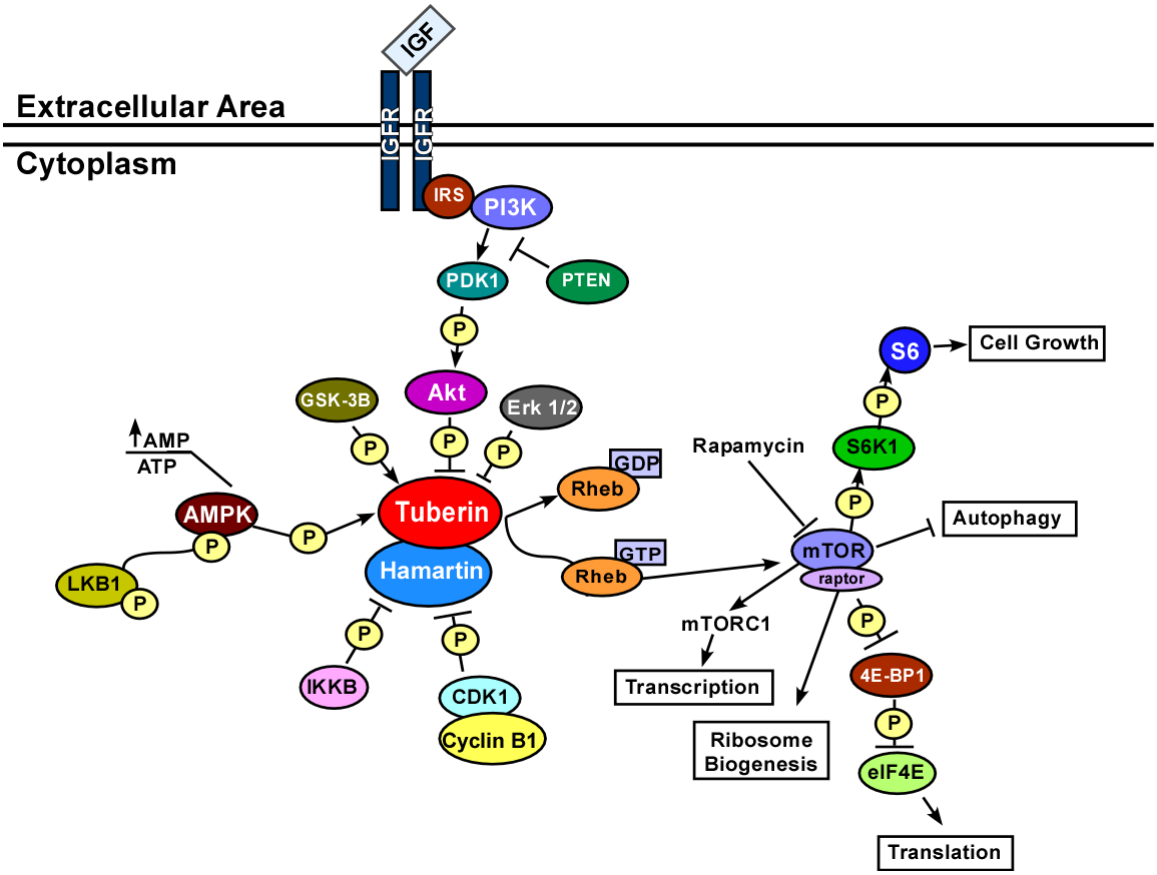


FIGURE 3: A schematic depicting the most influential players upstream and downstream of Tuberin protein signaling. (J. Dare 2015, Adapted from (Orlova and Crino, 2010))

mTOR involvement in cell growth is heavily dependent on available amino acids and growth factor levels; however, when the cell is found in a nutrient poor state, the initiation of events such as autophagy may occur. Autophagy is described as a process in which cells undergo rearrangement and sequester a portion of cytoplasm, organelles, and intracellular proteins for lysosomal degradation in order to maintain cellular homeostasis and to produce energy in times of stress (Parkhitko et al., 2011). Classically, active mTOR negatively regulates the onset of autophagy through mammalian autophagy kinase (ULK1/2) inhibitory phosphorylation. Inhibitory release of ULK allows downstream activation of Atg13, FIP200, and ULK itself; ultimately resulting in a cascade of autophagy associated gene (Atg) activation and biosynthesis of the autophagosome (Klionsky et al., 2008). Autophagy can be initiated by AMPK indirectly through Tuberin phosphorylation and mTORC1-mediated signals (Kim et al., 2011). Moreover, autophagy is also known to be activated downstream and independently of Tuberin, by AMPK dependent raptor phosphorylation (Kim et al., 2011). It has been found recently that the autophagy substrate p62/sequestosome 1 (SQSTM1) are critical components of TSC/mTORC1 driven tumor development (Parkhitko et al., 2011). In particular, the survival of *TSC2*-Null cells is dependent upon autophagy in an mTORC1-dependent fashion (Parkhitko et al., 2011). Nonetheless, it is clear that mTORC1 is a convergent mechanism through which autophagy is regulated. It is noteworthy to think about the possibilities that Tuberin and the TSC may have on autophagy initiation independently of mTOR.

V. Tuberin and Cyclin B1 regulate Mitotic Onset

In addition to inhibitory roles with cell cycle proteins and growth inhibition at the G1/S transition, our lab has elucidated a novel and important role for Tuberin at the G2/M transition. Since mitosis is directly responsible for cell division, cells must grow to double their original size and have appropriately segregated DNA. The exact mechanisms regulating the transition of cell growth to cell division are not fully understood.

In 2011, Fidalgo da Silva et al. observed, through a Yeast 2 Hybrid (Y2H) screen, that Tuberin forms a transient complex with Cyclin B1 at the G2/M transition, providing an intriguing mechanism

through which Cyclin B1 localization is regulated. Using small fragments of Tuberin (aa 600-746), positive interactions were seen with the CRS region of Cyclin B1 protein (Fidalgo da Silva et al., 2011). Fishing with a small fragment of Cyclin B1 containing the CRS region yielded a small fragment of Tuberin spanning from amino acids 610-746 (Fidalgo da Silva et al., 2011). It is now established that this region of Tuberin is responsible for both Cyclin B1 binding and cytoplasmic localization of Cyclin B1. This data presents the possibility that Tuberin can regulate premature mitotic onset. The Tuberin-Cyclin B1 complex is known to associate with both Hamartin and CDK1, presenting the possibility that feedback can be provided to regulate G1/S. Utilizing Cyclin B1 NES variants, it was seen that nearly 100% of the cyclin variant, Cyclin B1 5E (which mimics fully phosphorylated Cyclin B1), localizes to the nucleus after 24 hours. Fidalgo da Silva et al. (2011) has shown that in the presence of Tuberin, approximately 90% of the Cyclin B1 5E variant remains cytoplasmic. This further verifies the interaction and localization of Cyclin B1 and its dependence on Tuberin. Moreover, very recent findings from our lab indicate that both low nutrient levels and Akt non-phosphorylatable Tuberin mutants, diminishes the Tuberin-CyclinB1 interaction (Fidalgo da Silva et al. manuscript in preparation). The factors contributing to the regulation of this novel interaction are not fully understood. Further dissection of both upstream regulators and protein complex modifications are needed to better understand Tuberin involvement.

VI. Tuberin is implicated in Tumour Formation

Tuberous Sclerosis (TS) is known to arise from mutations in both the *TSC1* and *TSC2* genes; however, mutations in *TSC2* result in more severe disease related phenotypes compared to that of *TSC1 (*Dabora et al., 2001; Zeng et al., 2011). TS is characterized by the formation of benign tumours, referred to as hamartomas, that occur in a variety of organ types such as the brain, heart, skin, kidney, and lung (Maheshwar et al., 1997; Orlova and Crino, 2010; Rosner et al., 2008). TS most frequently occurs during development and displays itself in early childhood (Webb et al., 1991). Traditionally, TS is considered an autosomal-dominate disorder acquired through inheritance; however, approximately two thirds of TS cases arise from sporadic germ-line mutations. Disease prevalence affects approximately 1 in 6000 live births

annually and is estimated to affect roughly 1.5 million individuals worldwide (Curatolo et al., 2008; Jones et al., 1999; Maheshwar et al., 1997). The Tuberin protein has proven itself a fundamental and highly important protein. Deletion, truncation, or mutation can result in very severe cellular outcomes. This protein is known to be essential for neural tube closure and *TSC2* gene deletion results in embryonic lethality at day 10.5 in Eker rat models (Rennebeck et al., 1998).

The severity and prognosis of the disease is widely variable (Wataya-Kaneda et al., 2013). Over 95% of patients have dermalogical manifestations, such as facial angiofibroma, which often causes no detriment to life (Jones et al., 1999; Jozwiak et al., 2005; Kwiatkowski et al., 2015). However, manifestations affecting the brain, such as cortical tubers, subependymal nodules (SENs), or subependymal giant cell astrocytomas (SEGA) can cause blood vessel obstruction and cerebrospinal fluid accumulation, which can be lethal (Han and Sahin, 2011; Henske et al., 1997; Kohrman, 2012; Orlova and Crino, 2010). The central nervous system is affected in nearly 50% of TS sufferers and these mentioned brain abnormalities can result in forms of autism, epilepsy (in 60-90% of cases), mild to severe learning difficulties, and behavioural disorders, such as Attention Deficit Hyperactive Disorder (de Vries and Howe, 2007; Ehninger et al., 2009; Numis et al., 2011). Conversely, TS outcomes such as angiomyolipomas (AMLs) developing within the kidney may lead to kidney failure and impaired function, haemorrhaging and possible development of carcinomas (Orlova and Crino, 2010; Rosner et al., 2008). In addition to the role of Tuberin in the benign tumours of TS patients, Tuberin mutations have also been found to cooperate with oncogenic mutations aiding in the initiation and progression of a number of malignant cancers including medulloblastoma (MB), renal cell carcinomas (RCC), and lung, kidney, and breast cancers (Bhatia et al., 2009; Dabora et al., 2001; Franz et al., 2010; O'Callaghan et al., 2004; Orlova and Crino, 2010).

The tremendous variability in the severity of the tumour formed in TS are ascribed to the fact that mutations in *TSC2* are known to span the entire length of the gene, resulting in a plethora of possible Tuberin

protein mutations. It is notable however that mutational hotspots have been identified, and that one of these is located within the Cyclin B1 binding domain of Tuberin (Figure 4) (Fidalgo da Silva et al., 2011).

There is data to support that clinically relevant missense mutations within the *TSC2* gene do abrogate the function of the protein. In 2001, Nellist et al. found that the mutations to Tuberin R611Q, R611W, A614D, C696Y, F615S, and V796E all disrupted the chaperone function of Tuberin with respect to Hamartin. Specifically, Tuberin functions as a chaperone protein by maintaining the complex in the soluble form and by preventing the self-aggregation of Hamartin (Nellist et al., 2001). In 2005, Nellist et al. also found that phosphorylation of known Akt target sites was inhibited in the mentioned Tuberin mutants. Data here suggests that clinically relevant Tuberin mutations likely render the Tuberin protein in conformations that are unfavourable for important regulatory modifications, indicative of impaired Tuberin function (Nellist et al., 2008; Nellist et al., 2005b). Mutations resulting in changes of Tuberin residues R611, A614, F615, C696, V769 and Y1571 all exhibit decreased binding to Hamartin (Aicher et al., 2001; Astrinidis and Henske, 2005; Nellist et al., 2001). In addition, mutations such as these have been associated with increased S6K phosphorylation and increased Rheb-GTPase activity compared to that of wild type Tuberin (Nellist et al., 2005a). However, mutations residing outside of the Cyclin B1 binding domain, such as N525S, K599M, R905Q, G1556S along with others, do not elicit the same effects and disease severity (Nellist et al., 2005a). Moreover, immunofluorescence studies and protein binding densitometry analysis confirm that C696Y significantly exhibits more than a 50% decrease in binding affinity to Cyclin B1 compared to that of wild type Tuberin (Fidalgo da Silva et al., 2011). This finding may indicate increased cell cycle progression and may explain the increase in nuclear localization of Cyclin B1. In a recent medical study, Yu et al. (2014) performed mutational analysis on a family affected with Tuberous Sclerosis. Sequencing analysis revealed a novel Tuberin truncation mutant that arises from a deletion at exon 24 in the *TSC2* gene. This mutation results in a 946 amino acid protein, which is expected to have intact Hamartin binding (Yu et al., 2014). A mutation in the *TSC2* gene such as this lacks the C-terminal GAP domain, transcriptional activation domains, and many critical phosphorylation sites, including the important

regulatory Erk and Akt phosphorylation sites. Naturally, it can be expected that loss of these critical domains can impair proper Tuberin function and lead to uncontrolled growth resulting in tumor initiation.

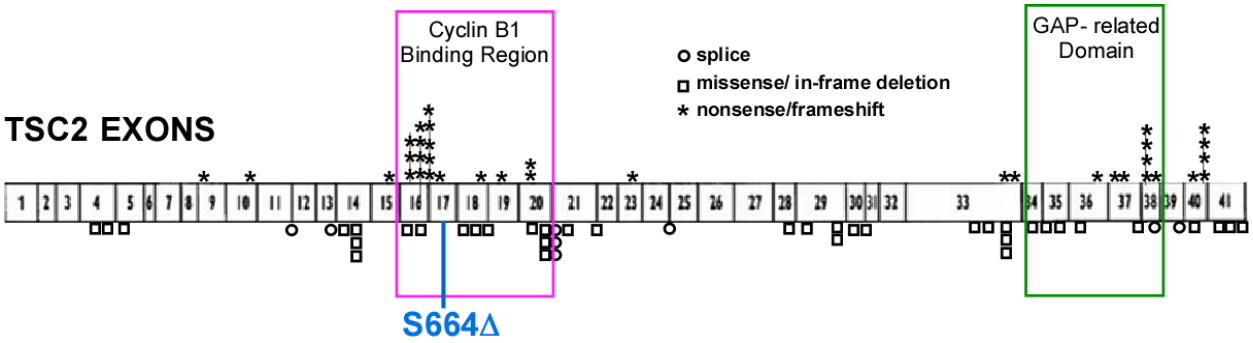


FIGURE 4: Mutations occur across the entire *TSC2* gene, but the CyclinB1 binding domain exons are an area of frequent mutation.

Exons 1 to 41 of Tuberin are depicted in this schematic. Mutations are indicated by the legend above and we can see from this figure that the Cyclin B1 binding domain of Tuberin is found to be a mutational hotspot, outlined in pink above. This area is littered with a variety of different classes of mutations. Exon 17 of the *TSC2* gene is an area of interest since a mutation resulting in a stop codon occurs here, resulting in a truncated Tuberin protein, S664Δ (blue). In addition, the GAP-related domain is also frequently mutated, which may be a reason for downstream misregulation and disease pathogenesis. (J. Dare 2015; Adapted from Jones et al. 1999)

Unfortunately, TS patients have a minimal number of accessible treatment options. The growth of these benign tumours must be monitored closely, and, if possible, surgically removed. One of the most prevalent treatment options for life threatening manifestations is treatment with mTOR inhibitors such as rapamycin, sirolimus, and everolimus (Franz, 2013; Kohrman, 2012; Orlova and Crino, 2010; Sengupta et al., 2010; Zeng et al., 2008). Many clinical trials are underway to increase efficacy of these treatments and determine additional mTOR inhibitors. As explained, there is a vast amount of research in this field targeted towards understanding Tuberin's role with respect to protein synthesis and regulation of the G1/S transition, but it is imperative that we better understand the Tuberin's cell division regulation at the G2/M transition, as traditional measures of treating TS may not be applicable to this transient interaction.

The involvement of Tuberin at the onset of mitosis is still poorly understood. Given the diversity in the phenotypes found with different mutations, this work will focus on one of the most severe mutations found within Tuberin, specifically a truncation occurring at serine residue 664 in the Tuberin protein. This truncation in Tuberin will be referred to as S664Δ. We will also dissect aspects of the Tuberin-Cyclin B1 interaction further using this truncated Tuberin protein mutant. e hypothesize that **protein modifications to both Tuberin and Cyclin B1 will have effects with respect to mitotic onset, binding, and localization.**

Objective 1: Elucidate where clinically relevant Tuberin truncation proteins localize and reveal possible phenotypic outcomes. To accomplish this, site directed mutagenesis was utilized to create the truncated Tuberin protein,S664Δ. Using mammalian cell culture, determination of truncation mutant S664Δ activity was assessed through proliferation assays, autophagy marker activation, cell size determination through flow cytometry, and pH3 immunofluorescent staining.

Objective 2: Determine if Tuberin truncation proteins can co-localize and directly bind Cyclin B1 *in vitro.* Using Tuberin and Cyclin B1 in wild-type, truncated and variant forms, we measured protein binding using co-immunoprecipitation. Additionally, a Cyclin B1 NES variant was made to determine importance

of different serine sites to the Tuberin-Cyclin B1 interaction. We assessed localization through immunofluorescent measures.

MATERIALS & METHODS

I. DNA Plasmids

The following mammalian DNA plasmids were constructed by Dr. Elizabeth Fidalgo da Silva and were utilized during transfections:

pCMV Tag2C Mlu^- FLAG (empty control vector)
pCMV Mlu^- FLAG-TSC2 WT

The following mammalian DNA plasmids were constructed by Jessica M. Dare-Shih and were utilized during transfections:

pCMV Tag2C Mlu^- FLAG-TSC2-S664Δ (Appendix 1)
pCMX Cyclin B1-4A-(A126E)-GFP (Appendix 2)

pCMX Cyclin B1-WT-GFP, pCMX Cyclin B1-5A-GFP, pCMX Cyclin B1-5E-GFP vectors were given as a kind gift from Dr. A. Hagting.

II. Mammalian Cell Culture of HeLa and Human Embryonic Kidney 293 cells (HEK 293)

HEK 293 cells were maintained in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS; Thermo-Fisher, Lot#906532) and 1% Penicillin-Streptomycin (Thermo-Fisher; Cat# 15140-1322). Cells were cultured in a 10 cm tissue culture dish in 7mL of complete fresh media at 37°C in 5% CO_2.

III. Overexpression and Protein Concentration Determination

Transfection protocols varied slightly over the course of several months. DNA of either 5 or 10 μg was incubated with 500 μL of supplemented DMEM and 20-30 μg of branched

polyethylenimine (PEI) reagent (Sigma; Cat# 408719), immediately vortexed for 10 seconds and followed by 10 minutes of incubation at room temperature. After incubation, the DNA mixtures were added drop wise onto 10 cm tissue culture plates of cells grown to approximately 80% confluency.

Transfected cells were incubated at 37°C in 5% CO_2 for 14-20 hours, followed by collection by cell scraping. Cells were pelleted by centrifugation at 800 xg for 5 minutes at 4°C. Based on pellet size cells were resuspended with 200-600 μL of 1X LB (0.1% NP-40, 20 mM Tris pH 7.5, 100 mM NaCl, 5 mM EDTA and 1 mM PMSF, 5μg/mL aprotinin, and 5μg/mL leupeptin). Cells were lysed on ice for 30 minutes followed by centrifugation for 10 minutes at 10000 rpm at 4°C. Cell debris pellets were removed using a sterile toothpick.

A standard Bradford Assay was used to determine protein concentrations of collected samples. In short, 995 μL of Bradford reagent was added to 5 μL of protein sample in a 1.5mL Eppendorf Tube and vortexed for 10 seconds. Protein samples were analyzed in a cuvette and and absorbance readings at 595nm were detected by a Thermo Electron Concentration Spectrophotometer. Following protein concentration determination samples were calculated based on loading of either 100, 150 or 200 μg of protein and added with 4X Sample Buffer with 20% B-mercaptoethanol. Samples were then boiled to 95°C for 5 to 7 minutes in a standard heat block.

4X Sample Buffer (LAP recipe):

0.8g SDS powder, 2.5 mL 1 M Tris pH 6.8, 4mL glycerol, 80 μL of bromophenolblue dye (BPB; Sigma Aldrich; Cat #B0126-25G), diH2O up to 8mL.

IV. SDS-PAGE, Western Blotting, and Chemiluminescence Detection Methods

After denaturation, samples were subjected to SDS-PAGE and western blotting. Samples were loaded on either a 7.5%, 10% or 12.5% SDS-PAGE (sodium dodecyl sulfate- polyacrylamide gel) for 3 to 4 hours at 100-110V and then transferred onto 0.45 μm Polyvinyidene fluoride (PVDF) membrane (Millipore; Cat# IPVH00010) for 2 hours and 45 minutes at 30V at room temperature. Following transferring, the PVDF membrane was subjected to blocking by shaking for 30 minutes to 1 hour at room temperature in 1% milk powder diluted in TSBT (Tris-buffered saline- with 10% Tween-20). Blotted PVDF membrane was incubated with specific primary antibodies at a ratio of 1:1000 in 1% milk overnight at 4^{o}C on a rotator or at room temperature rotating for 2 hours. Membranes were then washed in TBST (3X for 10 minutes) and incubated with the appropriate species-specific secondary antisera for 45 minutes to 1 hour at room temperature. Following a brief washing (3X 5minutes), Enhanced Chemiluminescent Substrate (ECL) and 0.01% H_2O_2 reagents (Thermo-Fisher; Cat# 32106) were applied to PVDF membranes in equal volumes and chemiluminescence was detected by Alphainnotech equipment. Densitometric analysis of protein was also conducted with AlphaEase FC software.

V. Antibodies

The following antibodies were utilized during Western Blotting, co-immunoprecipitation and indirect immunofluorescent analysis:

Mouse α-CycB1 (monoclonal; Santa Cruz; Cat# H1809), rabbit α -Tuberin (C-4; polyclonal; Santa Cruz; Cat# 2210), mouse α-actin (monoclonal; Millipore; Cat# MAB1501), mouse α-FLAG (Sigma; Cat# F1365), goat α- mouse IgG (Sigma; Cat# A4416), goat α-rabbit IgG (Sigma; Cat#A6154), Alexa 488- conjugated α- mouse IgG (Invitrogen; Cat# 488735), Alexa 568-conjugated α- mouse IgG (Invitrogen; Cat# A-11004), rabbit α - phospho-H3 (AbCam Technologies; Cat# ab32107), rabbit α-LC3-II (Novus Biologicals; Cat# NB100-2220), rabbit α-

p62/SQSTM1-(Sigma; Cat# P0067) Agarose conjugated α-FLAG primary antibody affinity gel beads (Biotool; Cat# B23101)

VI. Co-Immunoprecipitation (Co-IP)

Transfected HEK 293 cells were collected and lysed with 1X LB as described previously. 5μL of α-CyclinB1 mouse was added to 1000μg to 1500μg of protein and the volume was completed to 500μL total with 1X LB complete with protease inhibitors and rotated overnight at 4^{o}C. 5μL of Protein G Sepharose beads (Sigma) were added to each sample and were immediately rotated for 3.5 hours at 4^{o}C. Alternatively, protein samples were incubated with 2μL of 2X diluted Agarose conjugated α-FLAG primary antibody affinity gel beads (Biotool) for 3.5 hours at 4^{o}C. Following incubation, samples were centrifuged at 4800 xg for 6 minutes at 4^{o}C, the supernatant was aspirated and samples were manually inverted for washing with 1mL of 1X LB supplemented with protease inhibitors as described above. Samples were centrifuged at 4^{o}C for 3 minutes at 2400 xg to immunoprecipitate protein and washed by inverting and gentle pipeting. Three cycles of washing were undergone and after the final spin, all but 75μL of supernatant was aspirated. To the remaining protein, 4X Sample Buffer was added to a final volume of 100μL. Samples were then stored at -20^{o}C until being subjected to SDS-PAGE and western blotting as described above.

VII. Immunofluorescent Microscopy (IF)

HEK 293 cells were seeded onto sterile baked coverslips in 10 cm cell culture dishes containing 7 mL of warmed media. Cells were grown to approximately 80% confluency and transfected with 5 or 10μg of DNA as previously described. After 16-20 hours of incubation at 37^{o}C in 5% CO_2, cell lysates and coverslips were collected. Coverslips were collected in 12 well plates and fixed with 100 μL of 4% paraformaldehyde (PFA) for 15 minutes at room temperature.

Slips were then washed (3x for 5 minutes) in 100μL of 1X PBS (Phosphate buffered saline) and permeabilized with 100μL of 0.2% Triton-X-100 for 5-7 minutes. Following washing, slips were blocked with 100μL of LAP blocker (500μL of 100% Triton X, 0.7 5g glycine, 1.25 mL calf serum, and 5mL of 10X PBS) for 30 minutes at room temperature and then immediately incubated with 100μL of primary antibody diluted in LAP blocker for 1 hour. Slips were then washed (3X for 5 minutes) with 1X PBS and incubated with fluorophore conjugated species-specific secondary antibody for 30 minutes at room temperature. Slips were aspirated and stained with 100μL of Hoescht (1:1300) in LAP blocker for 10 minutes to complete nuclear staining. Slips were again washed (3X for 5 minutes) and mounted on glass slides with mounting solution. Treated coverslips were additionally adhered to slides via clear nail polish and allowed to dry for 24 hours in the dark before imaging. Slides were then analyzed by fluorescence microscopy using Leica Microsystems Microscopy (Leica CTR 6500 microscope / FX360 digital colour camera) and Leica Application Suite (LAS) Autofluorescence (AF) accompanying software.

Alternatively, coverslips were treated and stained as described above and subjected to immunofluorescence microscopy using a laser scanning confocal microscope (Olympus FluoView FV1000 Confocal Microscope; IX81) laser (Olympus Ix2-UCB and FV10-MARAD) and analyzed by accompanying software (Olympus View Ver.2.1c). Images were taken at a 60x objective with oil and non-artificially zoomed to scale.

VIII. Quantification of Immunofluorescent Protein Localization

Slides with fixed and appropriately stained protein were analyzed by fluorescence microscopy under equal exposure times and saved as numbered .tiff files. Number labelled images were analyzed by an unbiased researcher. Images of protein localization were counted as

cytoplasmic if protein was observed as very spread out and elongated within the cell, whether it was free in the cytosol (diffuse), or organelle bound (punctate). Perinuclear localization was classified as protein being very concentrated around, but not within the nucleus and this form of localization was mainly observed in mutated versions of Tuberin protein. Nuclear localization was classified as protein that was found within the nucleus (overlapping with DAPI filters) and appeared very condensed. Quantification in this manner was used for Figures 7 and 10 and Appendices 2 and 3.

IX. Flow Cytometry and Cell Cycle Profile Analysis

HEK 293 cells were cultured, treated and collected as described previously. Following initial centrifugation, cell pellets were fixed with ice cold 70% ethanol while vortexing on medium speed until completely re-suspended. Fixed cells were then stored at -20^{o}C or immediately prepared for immune-intracellular flow cytometry. Fixed HEK 293 cells were washed once with 2 mL of 1X PBS and pelleted by centrifugation for 5 minutes at 1000rpm at 4^{o}C. Cell pellets were then subjected to a staining protocol and treated with fixation/permeabilization/washing procedures using the BD Biosciences (Pharmingen) Flow Cytometry Transcription Factor Buffer Set following manufacturer's instructions (Cat# 562574). Cells were dual immuno-labelled with 100 μL of diluted antibody (1:100) mouse anti-FLAG primary antibody (1:300) and species specific Alexa 488 anti-mouse fluorescent secondary antibody (1:800) for 1 hour at 4^{o}C in the dark). Using BD Biosciences LSR Fortessa X-20 Cell Analyzer, cells were gated based on presence of Alexa 488 (green) fluorescence above a set threshold to control. Cell cycle profiles were collected based on DNA content assessed by propidium iodide (PI) staining. Overall mean cell area was acquired from a G2/M subpopulation using forward/side scatter ratios.

RESULTS

I. *Characterizing the effects of Tuberin truncation S664Δ over-expression.*

To analyze the effects of a clinically relevant Tuberin truncation S664Δ *in vitro*, over-expression of this protein was performed using HEK 293 cells under normal culture conditions (Figure 5). To verify over-expression levels of FLAG tagged wild-type and FLAG tagged S664Δ Tuberin protein, western blotting analysis was used (Figure 5A). Cells transfected for 24 hours were counted via trypan blue exclusion and graphed as total cell numbers of alive vs. dead cells (Figure 5B). This proliferation assay demonstrates that S664Δ over-expression results in significantly increased alive cell numbers compared to Tuberin WT or pCMV control. Over-expression of proteins was confirmed (Figure 5A) and protein levels were analyzed by densitometry (Figure 5C). Analysis of protein band intensity confirms that Tuberin truncation S664Δ is expressed 75% less compared to that of Tuberin WT.

To validate proliferation results (Figure 5B), immunofluorescent techniques analyzing phospho-Histone 3 (pH3) protein, were used to assess mitotic index (Figure 6). Mitotic index of cells over-expressing Tuberin WT or S664Δ in regular nutrient conditions, containing 10% FBS (Figure 6A) or starvation conditions, containing 0.5% FBS (Figure 6B) was of particular interest. Over-expression of protein was confirmed by western blot analysis (Figure 6C). pH3 positive FLAG over-expressing cells were counted and graphed as a percent of mitotic cells over total FLAG positive cells counted (Figure 6D). In FLAG over-expressing cells, results indicate that S664Δ significantly increases mitotic index compared to Tuberin WT in normal serum conditions. Additionally, in starvation conditions, this increased mitotic effect is significantly exaggerated by approximately 40%. These data confirm increased proliferation results from the presence of the S664Δ mutant.

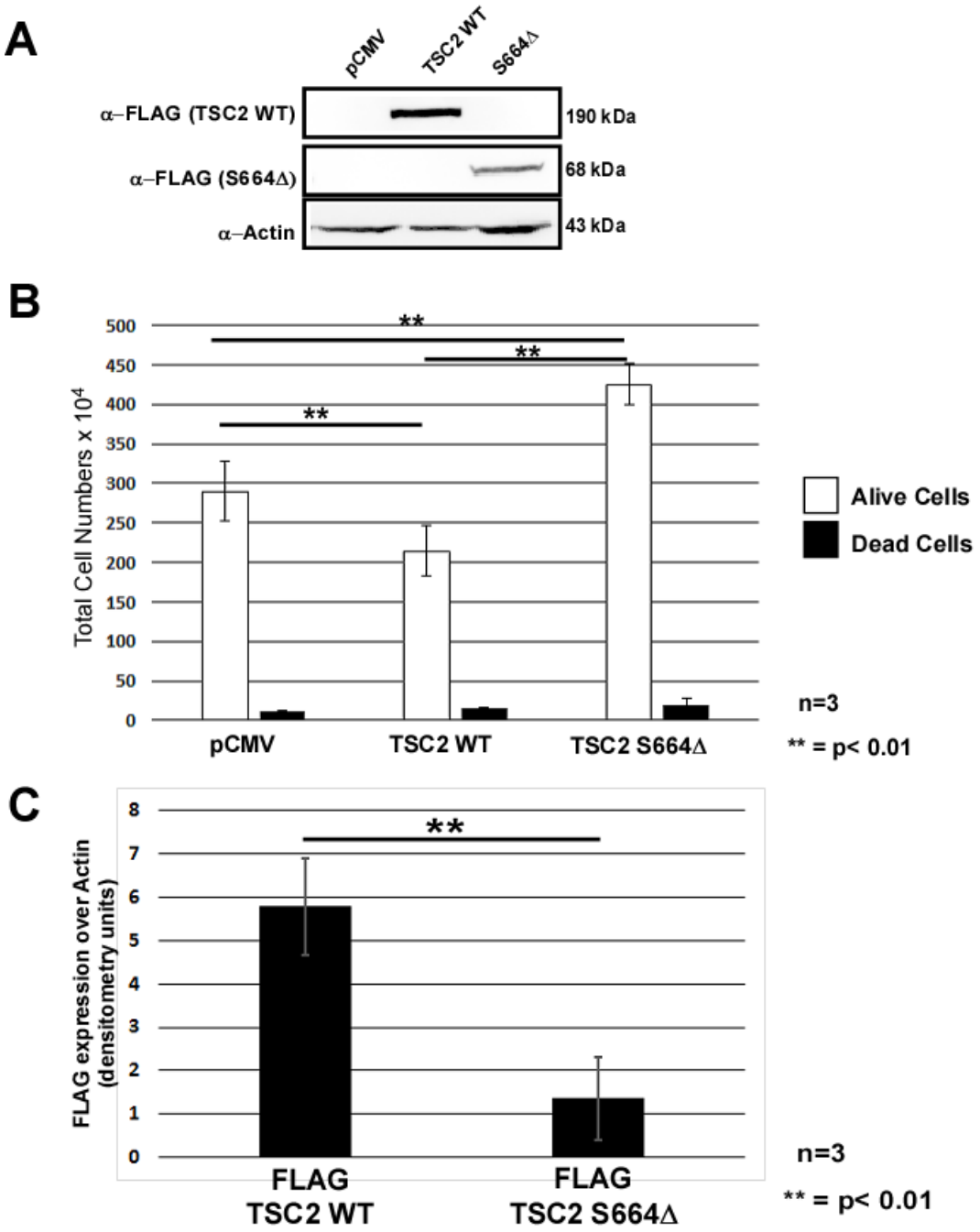
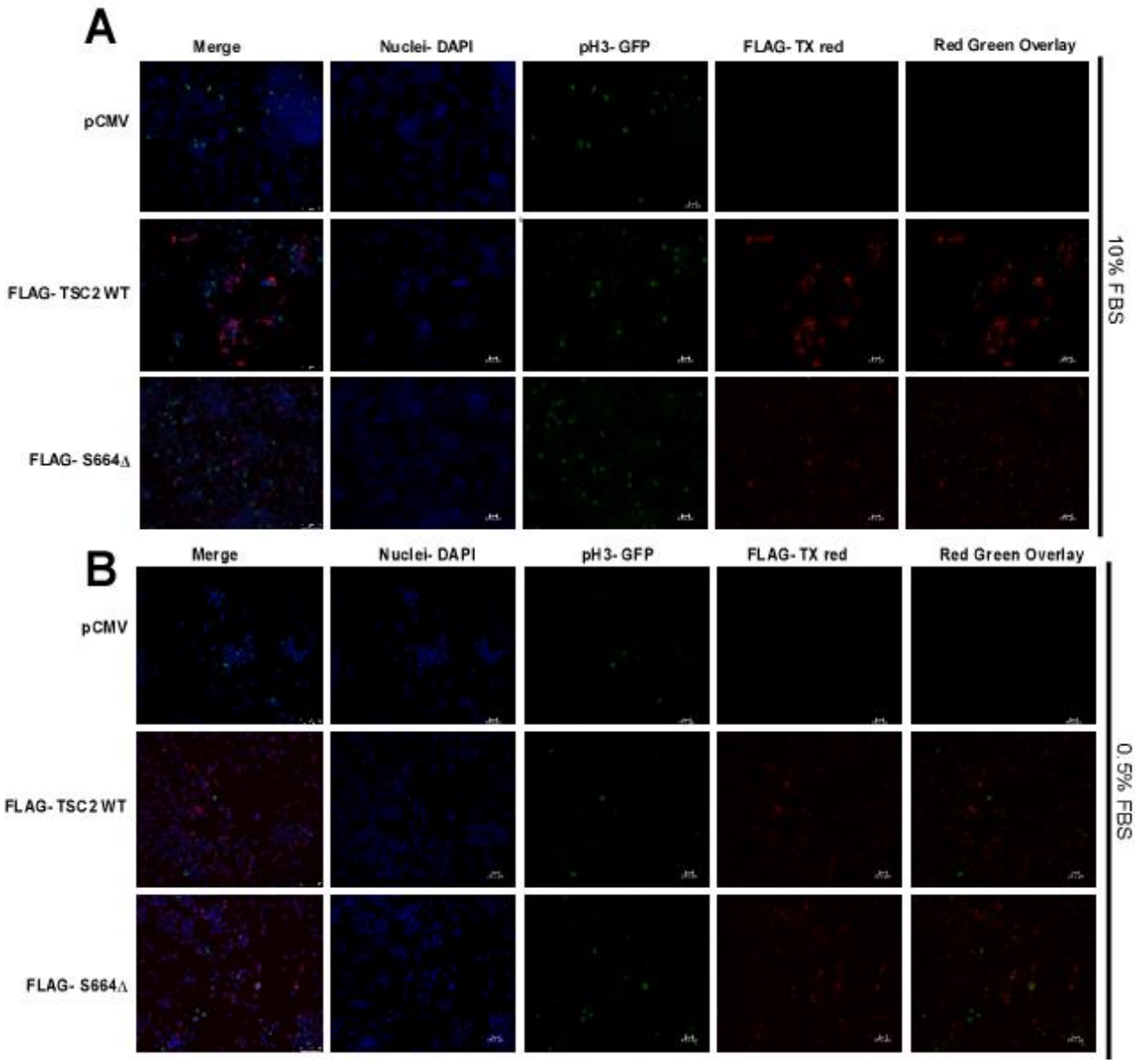


Figure 5. Over-expression of the Tuberin truncation, S664Δ significantly increases cell numbers compared to Tuberin WT and empty control. HEK 293 cells were seeded in equal numbers and transiently transfected for 24 hours with mammalian expression vectors containing pCMV control, Tuberin WT, or truncation S664Δ. (A) Tuberin WT and S664Δ over-expression was confirmed by FLAG tag presence via western blot analysis. Actin served as a loading control. (B) 24 hours post-transfection cells were collected and were subsequently counted via trypan blue exclusion. Total cell numbers of alive cells (white bars) and dead cells (black bars) are depicted. (C) Over-expression intensity (protein levels) was measured via western blot and densitometry analysis to determine transfection efficiency and consistency among treatments. **p<0.01. Statistical significance was assessed using student's unpaired t-test.


A
Merge
Nuclei- DAPI
pH3- GFP
FLAG- TX red
Red Green Overlay
pCMV
FLAG- TSC2 WT
FLAG- S664Δ
10% FBS
B
Merge
Nuclei- DAPI
pH3- GFP
FLAG- TX red
Red Green Overlay
pCMV
FLAG- TSC2 WT
FLAG- S664Δ
0.5% FBS

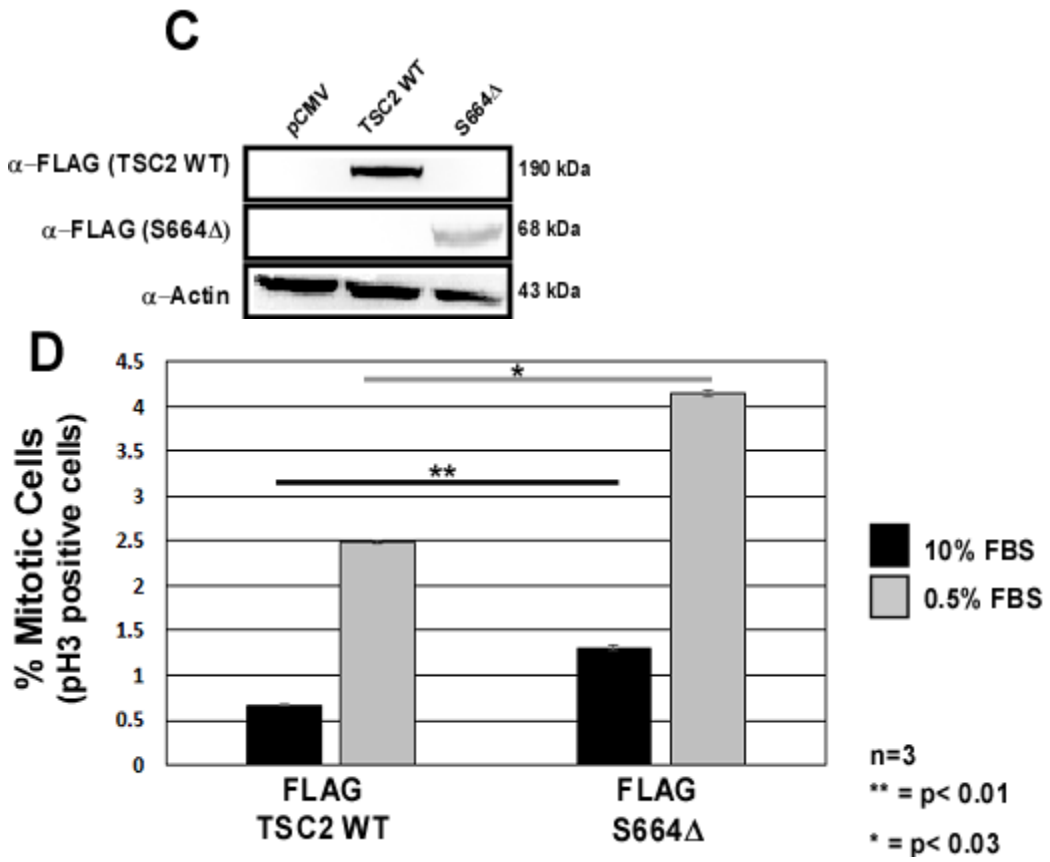
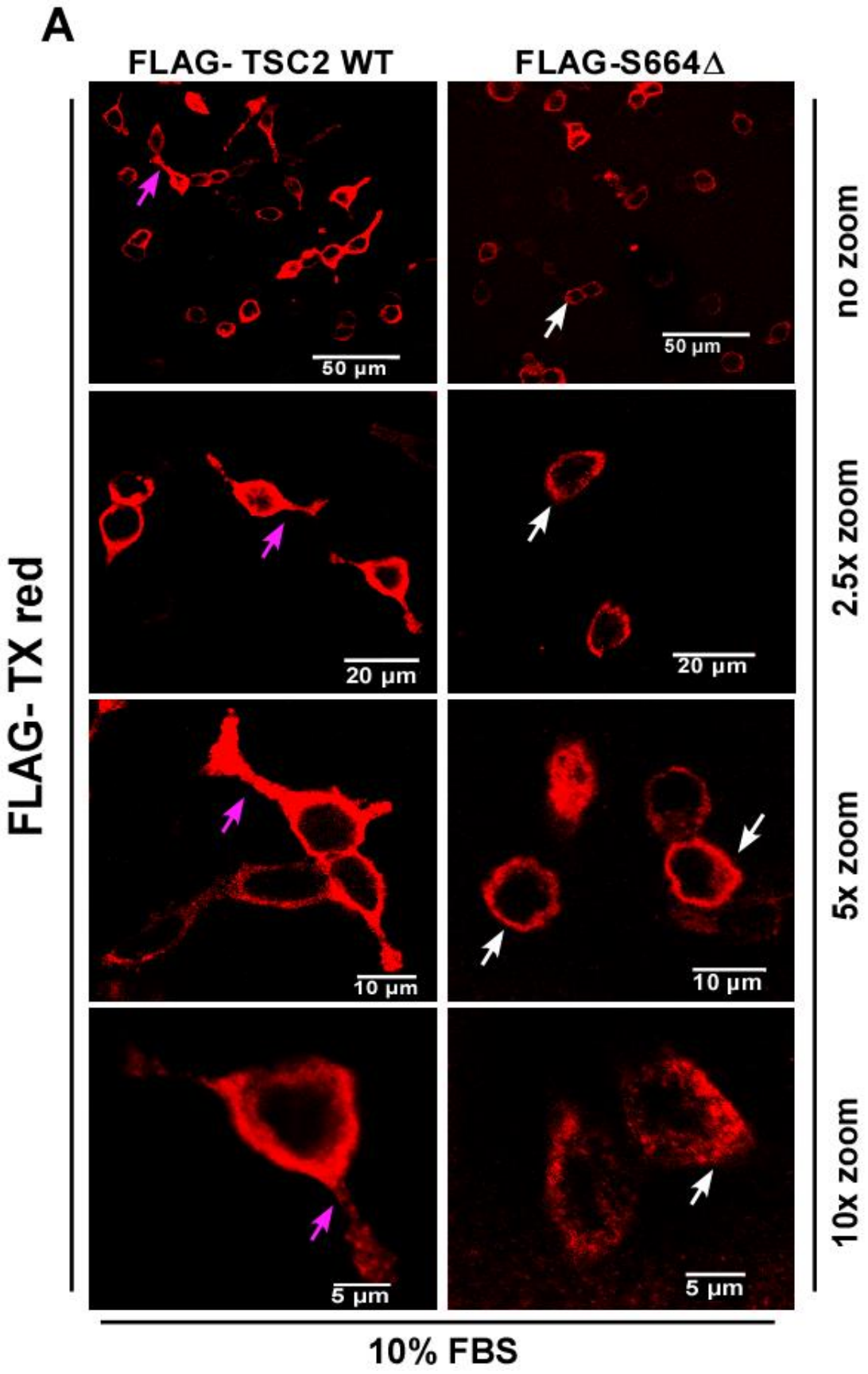


Figure 6. Over-expression of S664Δ significantly increases mitotic activity compared to Tuberin WT. HEK 293 cells were seeded onto cover slips and transiently transfected with indicated vectors, treated with 10% or 0.5% serum 24 hours later and collected 48 hours post transfection. Cell pellets and coverslips were immediately collected. Coverslips were subjected to immunofluorescence and treated with fluorescent antibodies. (A) Immunofluorescence images depicting nuclei (blue), FLAG protein over-expression (red), and phospho-Histone3 (pH3) protein (green) of 10% FBS treated cells at 20x objective. (B) Immunofluorescence images depicting nuclei (blue), FLAG protein over-expression (red), and phospho-Histone 3 (pH3) protein (green) of 0.5% FBS treated cells at 20x objective. (C) Representative western blot depicting immunoblot for FLAG over-expression (top two panels) and actin control (bottom panel). (D) Mitotic activity depicted in the graph was measured as a function of pH3 positive-FLAG over-expressing cells. **p<0.01,*p<0.03. Statistical significance was assessed using student's unpaired t-test.

Protein localization of the clinically relevant mutant S664Δ was determined using immunofluorescent studies (Figure 7). FLAG tagged Tuberin WT or S664Δ were over-expressed in regular nutrient conditions (Figure 7A) or starvation conditions (Appendix 2). Over-expression of protein was confirmed by western blot analysis (Figure 7B). FLAG over-expressing cells were counted based on cellular localization (cytoplasmic vs. perinuclear) (Figure 7C and 7D) and graphed as a percentage. Wild-type Tuberin protein is known to localize primarily to the cytoplasm (Figure 7A; pink arrows), but can also be observed in the nucleus under certain conditions (York et al. 2006, Rosner et al. 2007). In normal culture conditions, S664Δ significantly increases perinuclear localization (white arrows) compared to its WT counterpart (Figure 7A; right panels). This perinuclear localization was significantly attenuated under starvation conditions in S664 over-expressing cells (Appendix 2A; lower panels). Moreover, after immunofluorescent examination, it was observed that cells over-expressing the Tuberin truncation display a unique "clustered", condensed or aggregated phenotype, mainly located around the nucleus (Appendix 3).


A
FLAG- TSC2 WT
FLAG-S664Δ
FLAG- TX red
no zoom
2.5x zoom
5x zoom
10x zoom
50 µm
50 µm
20 µm
20 µm
10 µm
10 µm
5 µm
5 µm
10% FBS

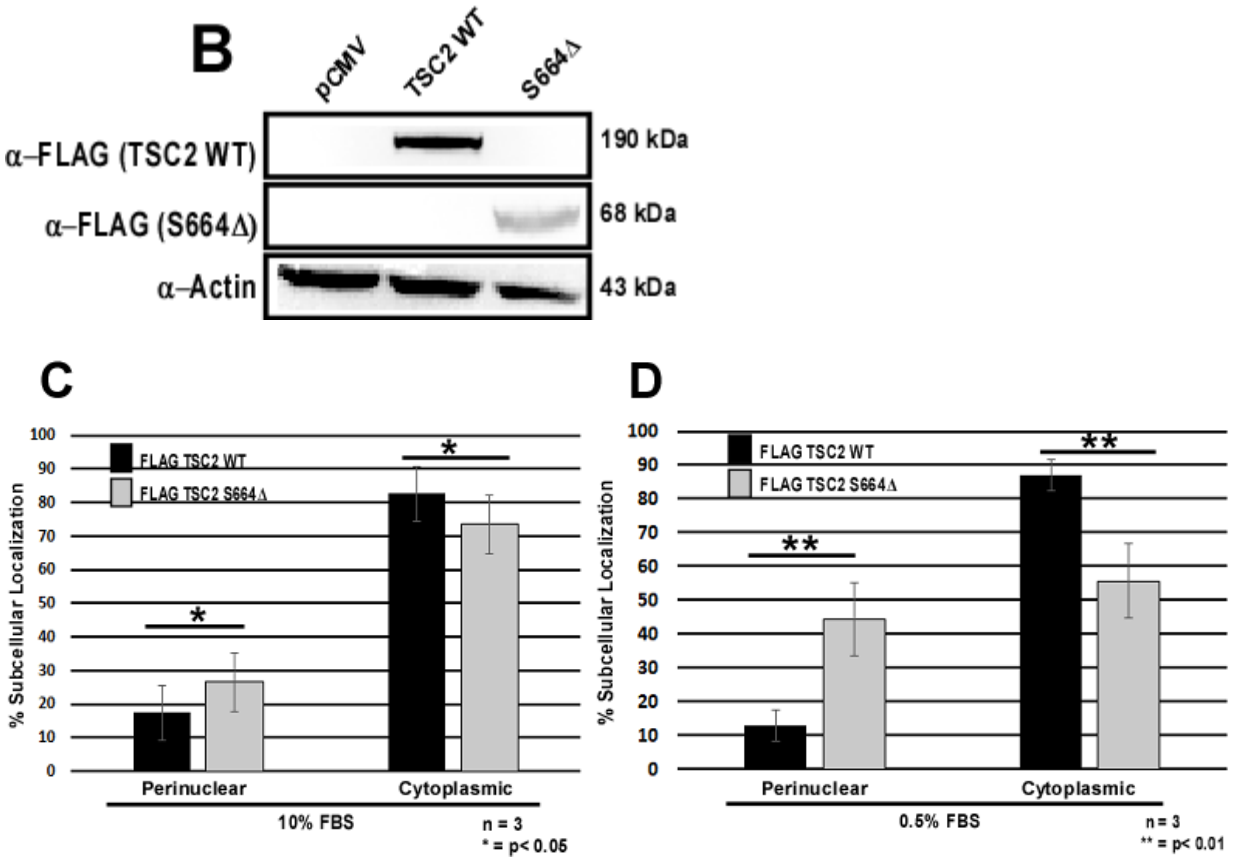


Figure 7. Mutant Tuberin S664Δ exhibits perinulcear localization significantly more than WT Tuberin protein. HEK 293 cells were seeded onto glass cover slips and transiently transfected with indicated vectors, treated with 10% serum 24 hours later and collected 48 hours post-transfection. Cell pellets and coverslips were immediately collected and treated with fluorescent antibodies. (A) Immunofluorescence images depicting FLAG protein over-expression (red) of 10% FBS treated cells at 60x oil objective. Images were zoomed in on this same objective and fully representative of protein expression (non-artificial zoom). (B) Representative western blot depicting immunoblot for FLAG over-expression (top two panels) and actin control (bottom panel). Sub-cellular localization of FLAG WT and FLAG S664Δ protein in normal culture conditions (C) or starvation conditions (D; See Appendix 2) depicted as a percentage of cells in a perinuclear (left bars) or cytoplasmic (right bars) phenotype. Example of Tuberin cytoplasmic localization identified by pink arrows and example of perinuclear localization of Tuberin protein identified by white arrows. *p<0.05, **p<0.01, ***p<0.001. Statistical significance was assessed using a student's unpaired t-test.

Increased proliferation and alterations in subcellular localization as a result of S664Δ overexpression may result in changes in cellular characteristics and phenotypes. Our lab has found that Tuberin has the ability to regulate cell size independently of mTOR (Fidalgo da Silva, manuscript in preparation), which led us to question the ability of a clinically relevant truncation to maintain cell size. To assess this, flow cytometry (Figure 8) and physiological assays (Figure 9) were utilized. Cells over-expressing WT or S664Δ Tuberin were grown in normal serum or under starvation conditions and were analyzed by flow cytometry. Following adequate gating based on singlet cell population expressing FLAG tag, forward vs side scatter information generated an average cell size. Cell size is plotted as a percent of cell area increase (Figure 8). There are no significant differences in cell area between WT and truncated protein over-expression ($p=0.14$) (Figure 8). This lack of difference holds true for serum condition changes as well.

Since over-expression of S664Δ increases proliferation without changes in cell size, it was hypothesized that cells may be continually dividing even in the face of starvation. Moreover, truncation S664Δ lacks regions responsible for downstream mTOR regulation, known to inhibit autophagy, leading us to determine if autophagy regulation differs in cells over-expressing this mutant. To assess this, we analyzed levels of autophagy associated proteins as a possible physiological outcome of increased cell proliferation (Figure 9). Over-expression of WT or truncated protein was confirmed by western blotting (Figure 9A, FLAG blot). Levels of p62 protein (Figure 9B) and LC3 protein conversion (Figure 9C) were compared using densitometry. When comparing control, Tuberin WT and S664Δ, levels of p62 and LC3 conversion differ slightly but are not significantly different from one another ($p>0.05$).

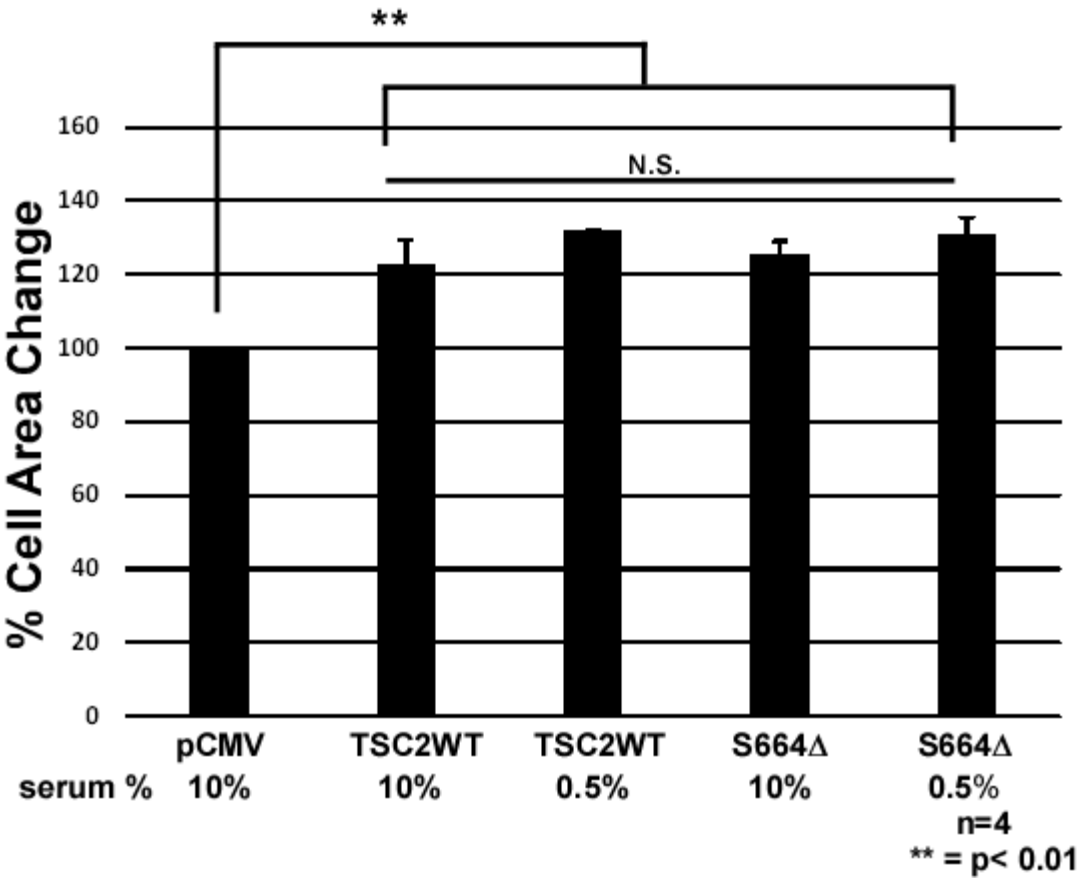


Figure 8. Over-expression of S664Δ does not significantly alter average cell size compared to Tuberin wild-type. HEK 293 cells were transiently transfected with pCMV empty control, FLAG TSC2 WT, or FLAG S664Δ and grown in 10% or 0.5% serum. 48 hours post-transfection cells were harvested and fixed for flow cytometry analysis. Cells were intracellularly labelled with anti-FLAG/Alexa-488 green and subjected to flow cytometry. Cells were also stained with PI, isolated for singlets and gated for green fluorescence. This cell population was then analyzed for mean cell area by forward vs. side scatter between all treatments. **$p<0.01$, NS= $p>0.05$. Statistical significance was assessed using student's unpaired t-test.

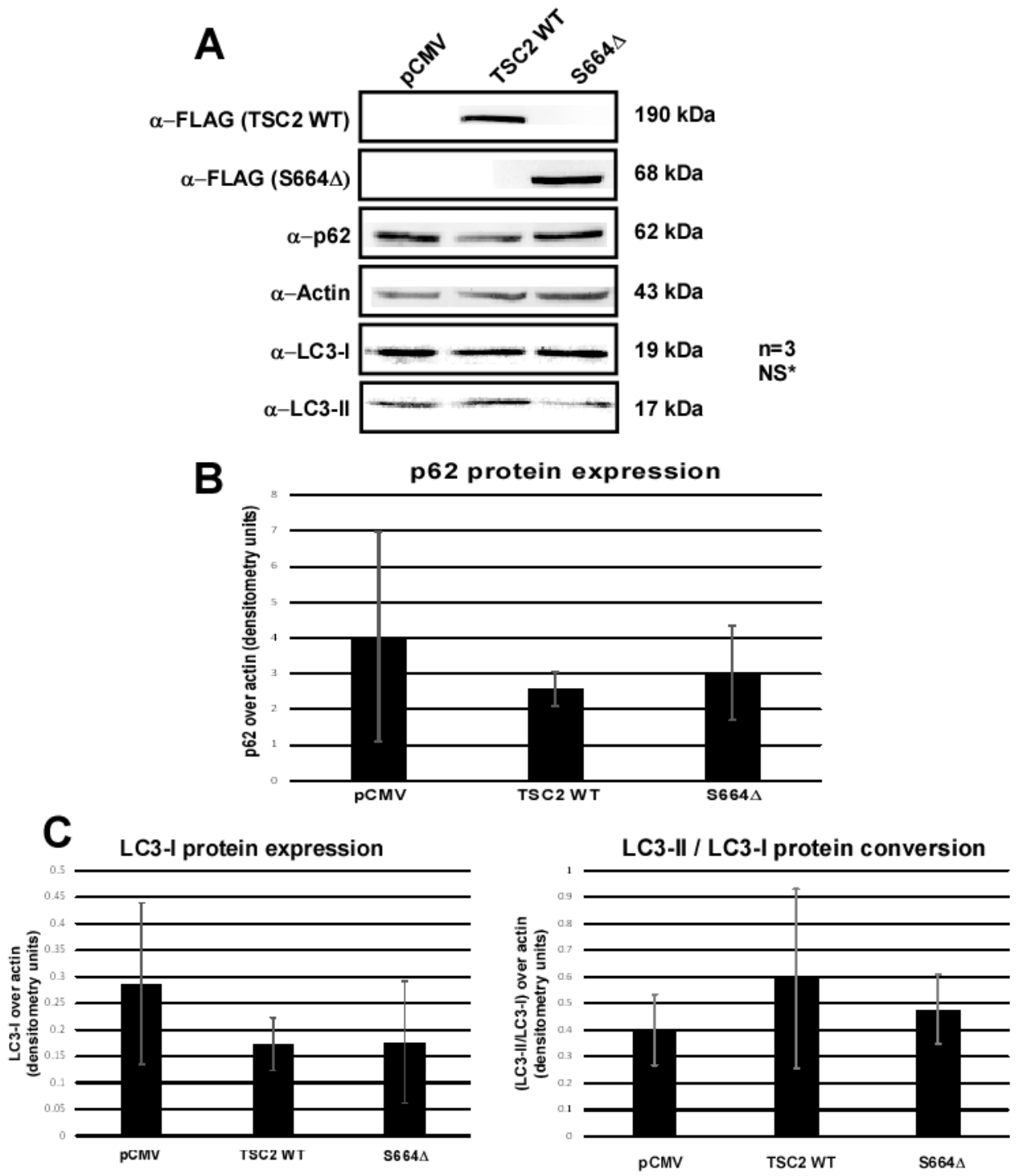
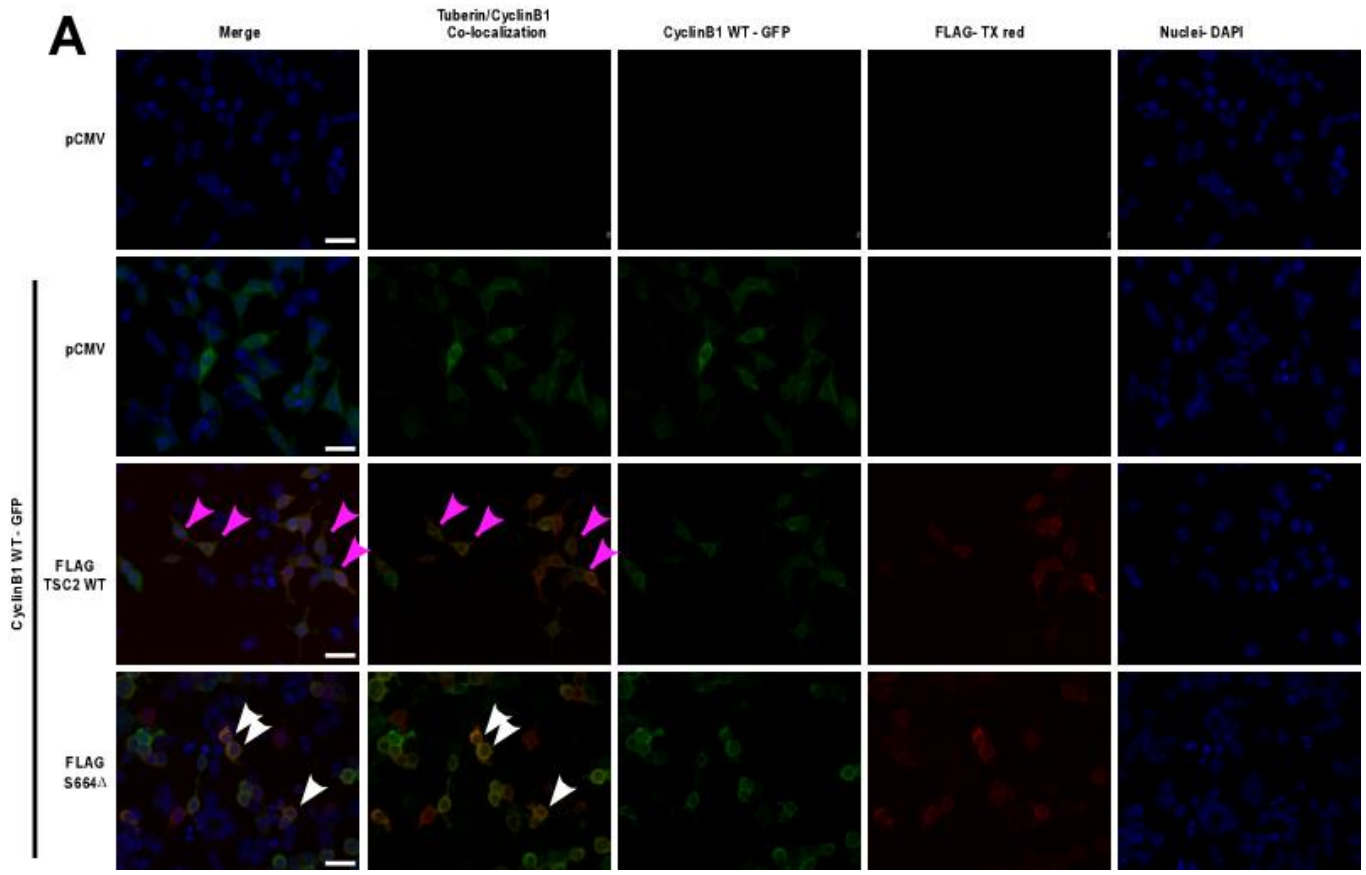
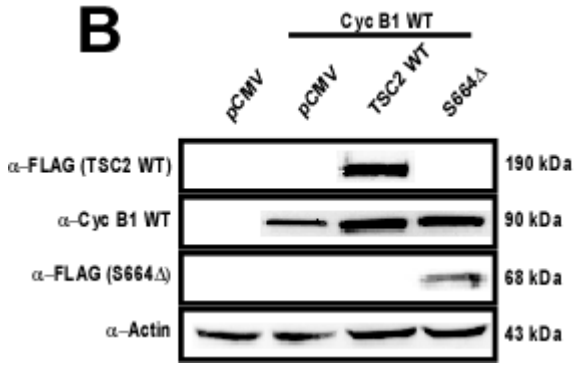


Figure 9. S664Δ over-expression does not result in significantly increased autophagy protein markers compared to WT Tuberin or pCMV control. HEK 293 cells were transiently transfected with indicated vectors above and cultured in normal serum conditions. (A) Western blotting confirming over-expression of indicated vectors and autophagy associated protein markers. Actin was used as a loading control. (B) Densitometry analysis of p62 protein expression in cells over-expressing indicated vectors. (C) Densitometry analysis of autophagosome associated protein LC3 and corresponding conversion of this protein. $p>0.05$. Statistical significance was assessed using student's unpaired t-test.

II. *Effects of the clinically relevant truncation S664Δ on the mitotic transition.*

Since localization of Tuberin WT and S664Δ protein were seen to be significantly different, alterations to Cyclin B1 co-localization is a possibility. Cyclin B1 localizes to both the nucleus and the cytoplasm. (Li et al. 1997, Yang et al. 1998, Fidalgo Da Silva et al. 2011). Based on studies from Fidalgo Da Silva, Tuberin WT protein retains Cyclin B1 in the cytoplasm and holds back mitosis. To compare possible G2/M effects between Tuberin WT and S664Δ, immunofluorescent studies utilizing Cyclin B1 WT were performed (Figure 10). pCMV control, FLAG tagged (red) WT or S664Δ Tuberin were over-expressed with Cyclin B1 WT (green) and analyzed using immunofluorescent microscopy (Figure 10A). Typical cytoplasmic phenotype identified by pink arrows, and traditional perinuclear phenotype identified by white arrows (Figure 10A). Protein over-expression was confirmed by western blot analysis (Figure 10B). Both Tuberin WT and S664Δ were seen to co-localize with Cyclin B1 WT (yellow in left merge panel; Figure 10A). Cyclin B1 WT protein localization (green) was counted as perinuclear/nuclear vs. cytoplasmic in FLAG over-expressing cells (red) and graphed as a percentage (Figure 10C). Consistent with previous literature, nearly 90% of Cyclin B1 WT protein was found to be retained in the cytoplasm (Figure 10C; grey bars) (Fidalgo da Silva et al., 2011). Interestingly, when Cyclin B1 WT is over-expressed with S664Δ, Cyclin B1 localization is altered and is significantly more perinuclear than compared to WT Tuberin or control cells (Figure 10C; black bars).


A
Merge
Tuberin/CyclinB1 Co-localization
CyclinB1 WT - GFP
FLAG- TX red
Nuclei- DAPI
pCMV
pCMV
FLAG TSC2 WT
FLAG S664Δ
CyclinB1 WT - GFP


B
Cyc B1 WT
pCMV
pCMV
TSC2 WT
S664Δ
α–FLAG (TSC2 WT)
α–Cyc B1 WT
α–FLAG (S664Δ)
α–Actin
190 kDa
90 kDa
68 kDa
43 kDa

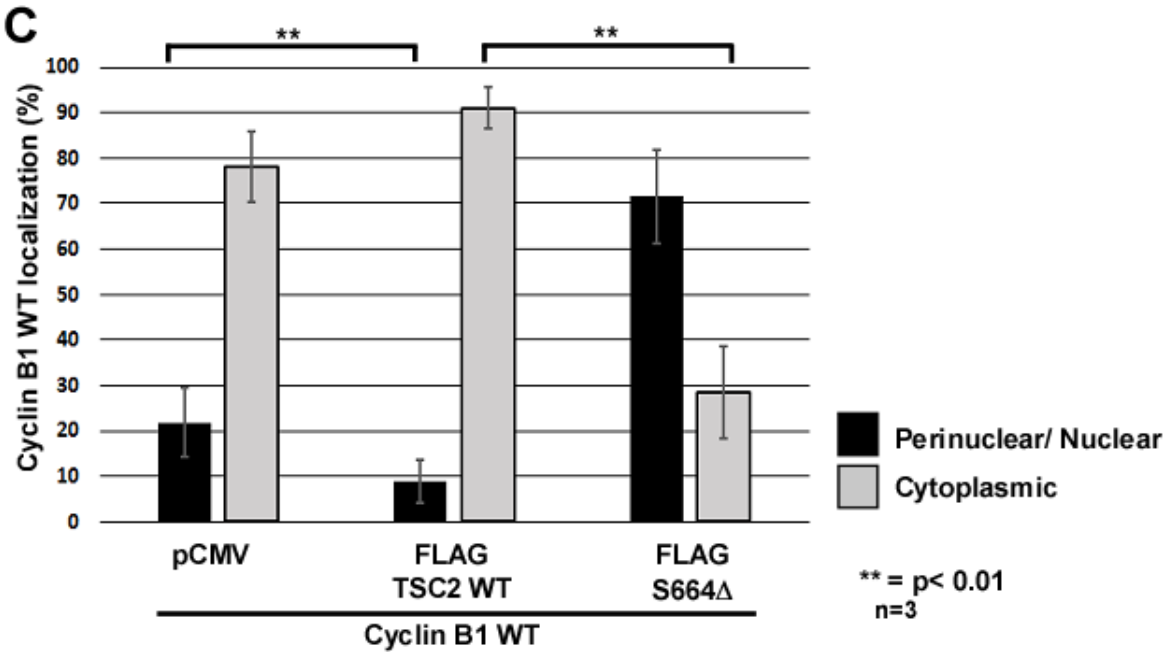


Figure 10. Tuberin WT and S664Δ regulate the sub-cellular localization of Cyclin B1 WT. HEK 293 cells were seeded onto cover slips and transiently transfected with indicated vector and collected 24 hours post transfection. Cell pellets and coverslips were immediately collected and treated with fluorescent antibodies. (A) Immunofluorescence images depicting nuclei (blue), FLAG protein over-expression (red), and Cyclin B1 WT protein (green) at 40x objective. (B) Representative blot of successful protein over-expression. (C) Graph depicting perinuclear/nuclear localization (black bars) vs. cytoplasmic localization (grey bars) of Cyclin B1 WT protein (when co-expressed with WT Tuberin or S664Δ) as a percentage. Cytoplasmic co-localization phenotype identified with pink arrows and perinuclear/nuclear co-localization phenotype identified with white arrows in panel A. **p<0.01. Statistical significance was assessed using student's unpaired t-test.

Co-localization was positively verified between Cyclin B1 WT and the Tuberin proteins used above, so direct protein binding studies were assessed by co-immunoprecipitation (co-IP) (Figure 11). pCMV control, Tuberin WT, or S664Δ were over-expressed with Cyclin B1 WT in normal culture conditions and confirmed by western blot (Figure 11A). Using FLAG conjugated protein G beads, *in vivo* co-IP experiments yielded successful pulldown of FLAG tagged Tuberin WT and S664Δ (Figure 11B). Interestingly, Cyclin B1 WT protein was seen to successfully pulldown with both Tuberin WT and S664Δ (Figure 11B). To determine the strength of binding, levels of over-expression in immunoblot were normalized to actin (from Figure 11A). Levels of FLAG and Cyclin B1 precipitate (from Figure 11B) were normalized to the level of immunoblot expression. Subsequently, these ratios were divided as Cyclin B1 WT binding over FLAG and graphed in Figure 11C. Surprisingly, there were no significant differences found between the strength of Cyclin B1 to that of FLAG over-expressing Tuberin WT or S664Δ.

Since Cyclin B1 WT was found to bind both Tuberin WT and S664Δ (Figure 11), we were interested in the possibility of Cyclin B1 NES variant binding with both Tuberin WT and S664Δ. To assess this, we performed co-IP experiments using these same Tuberin proteins and the Cyclin B1 NES variants (Figure 12). It has been established that Cyclin B1 5A tends to have a cytoplasmic localization and bind significantly more to Tuberin WT, while Cyclin B1 5E tends to have nuclear accumulation and bind significantly less to Tuberin WT (Fidalgo Da Silva et al. 2011). Therefore, we sought to determine which phosphorylation site is more influential in binding to Tuberin WT. A novel Cyclin B1 NES variant mammalian expression vector, pCMX Cyc B1 4A (A126E) was constructed to assess this (Appendix 3). The Cyclin B1 NES variant 4A is indicative of a Cyclin B1 protein which is only phosphorylated on S126. Cells were over-expressed with pCMV control or FLAG Tuberin WT in combination with Cyclin B1 5A, 5E, or 4A (A126E) and protein over-expression was confirmed by western blot analysis (Figure 12A). Using FLAG conjugated protein G beads, *in vivo* co-immunoprecipitation experiments yielded successful pulldown of FLAG tagged Tuberin WT (Figure 12B). Cyclin B1 WT protein was seen to successfully precipitate with Tuberin WT and not with control cells (Figure 12B). To determine protein affinity, levels

of over-expression in immunoblot were normalized to actin (from Figure 12A). Levels of FLAG and Cyclin B1 variant precipitate (from Figure 12B) were normalized to the level of immunoblot expression. Subsequently, these ratios were divided as Cyclin B1 variant binding over FLAG and graphed in Figure 12E. As expected, there were statistically significant differences found between the affinity of Cyclin B1 5A with Tuberin WT (left bars) compared to that of 5E with Tuberin WT (right bars) (Figure 12E). Interestingly, it was found that the Cyclin B1 4A (A126E) variant binds significantly less than 5A, but significantly more than 5E (middle bars) (Figure 12E).

Additionally, since S664Δ was seen to bind Cyclin B1 WT *in vivo (*Figure 11), we sought to evaluate if Cyclin B1 variants had the ability bind to S664Δ, since this truncated protein is known to lack a portion of the Cyclin B1 protein binding domain. Cells were over-expressed with pCMV control or FLAG S664Δ in combination with Cyclin B1 5A, 5E, and 4A (A126E) and confirmed by western blot analysis (Figure 12C). Using FLAG conjugated protein G beads, *in vivo* co-IP experiments yielded successful pulldown of FLAG tagged S664Δ Tuberin (Figure 12D). Strikingly, all three Cyclin B1 variant proteins were seen to successfully precipitate with S664Δ, but not with control cells (Figure 12D). To determine the strength of protein binding, levels of over-expression in immunoblot were normalized to actin (from Figure 12C). Levels of FLAG and Cyclin B1 variant precipitate (from Figure 12D) were normalized to the level of immunoblot expression. Subsequently these ratios were divided as Cyclin B1 variant binding over FLAG and graphed in Figure 12E. Interestingly, when comparing degrees of binding, there were no statistically significant differences between all three Cyclin B1 variants (Figure 12E).

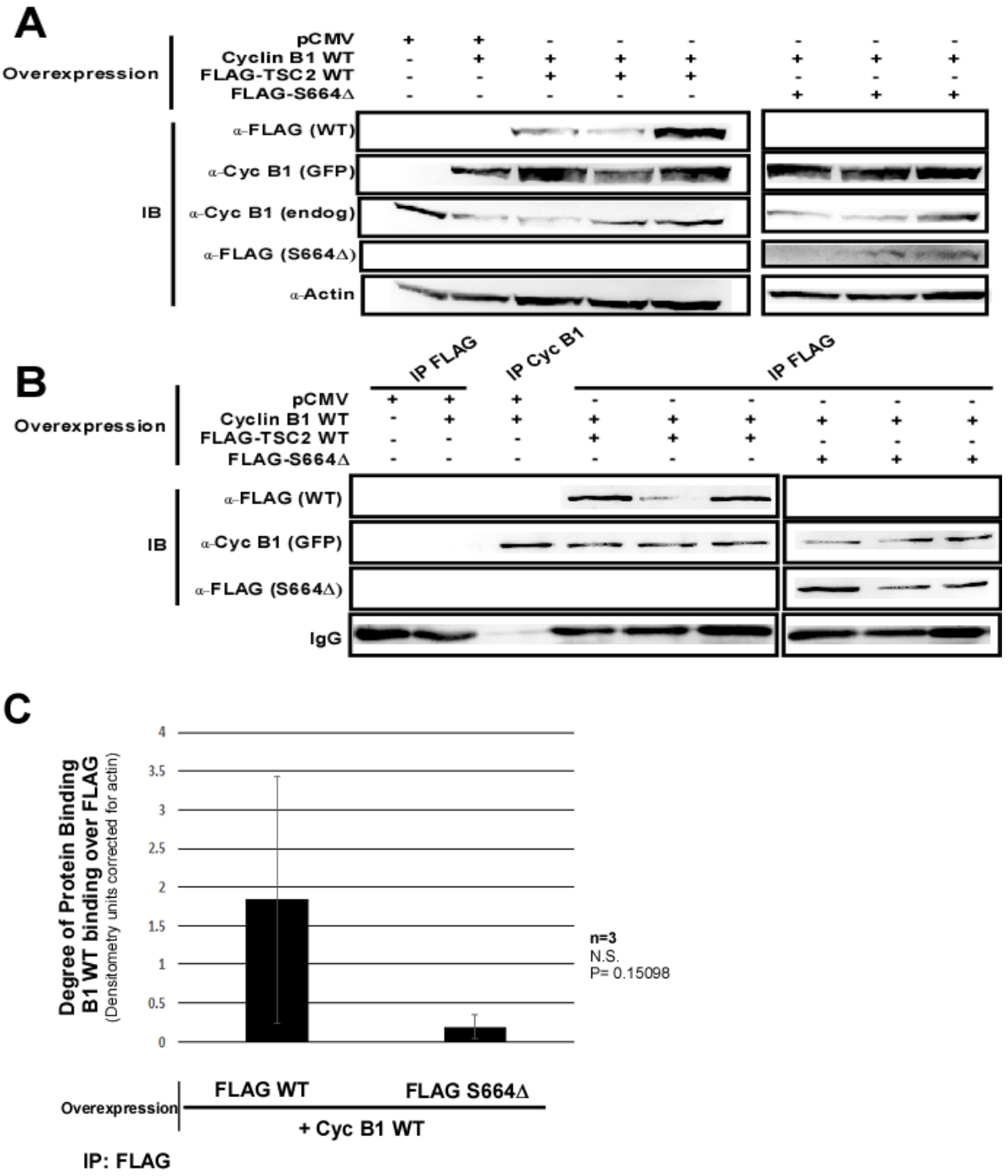


Figure 11. S664Δ binds Cyclin B1 WT protein less than Tuberin WT *in vivo*. HEK 293 cells transfected with Cyclin B1 WT-GFP were co-transfected with empty control vector, FLAG-tagged Tuberin WT, or FLAG-tagged S664Δ. (A) Western blots confirming successful over-expression of vectors. Actin was used as a loading control. (B) Co-immunoprecipitation blots of FLAG precipitated protein and co-immunoprecipitated Cyclin B1 protein. (C) Graph depicting the degree of Cyclin B1 binding to that of Tuberin WT or S664Δ. $p>0.05$. Statistical significance was assessed using student's unpaired t-test.

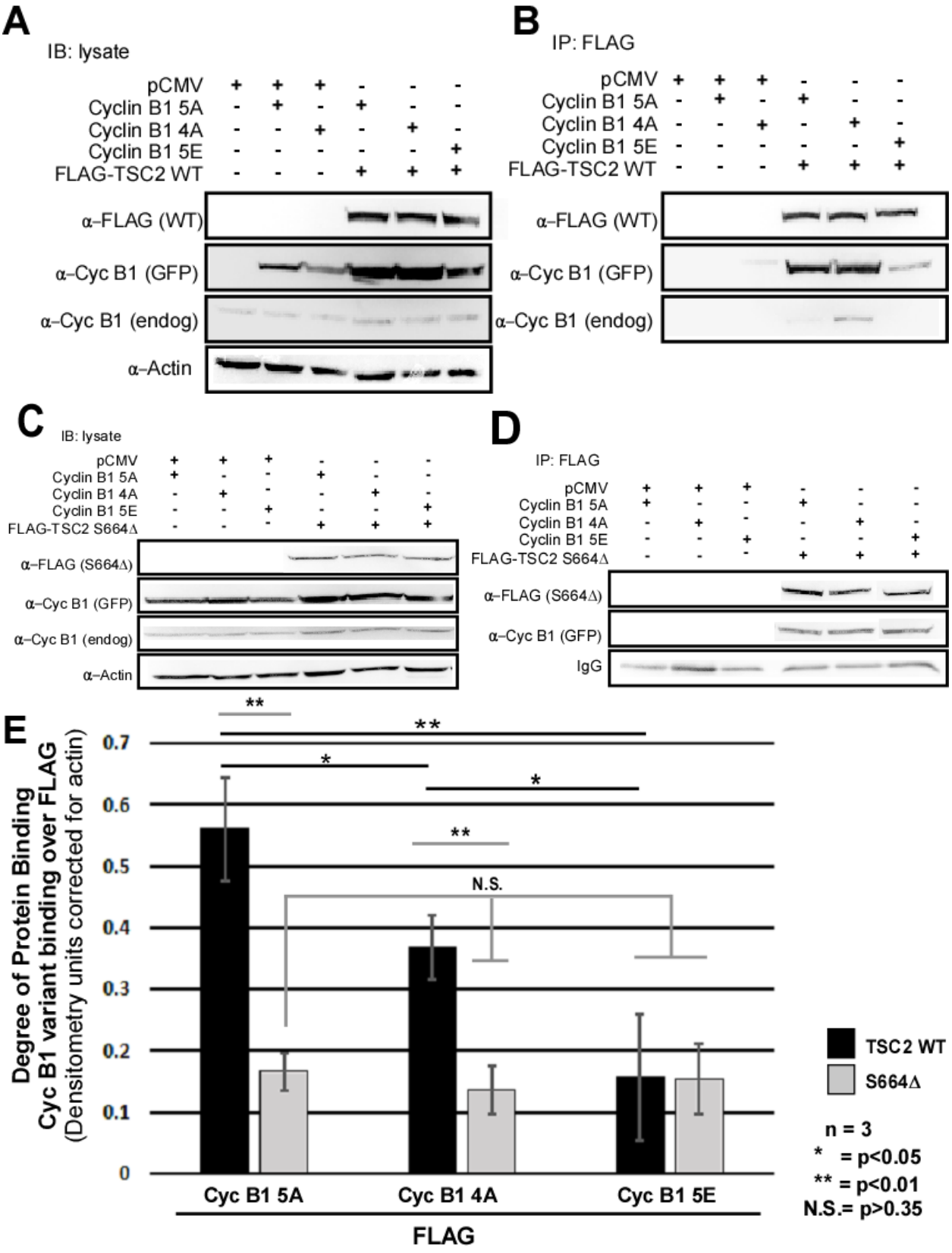

A
IB: lysate
pCMV
Cyclin B1 5A
Cyclin B1 4A
Cyclin B1 5E
FLAG-TSC2 WT
α–FLAG (WT)
α–Cyc B1 (GFP)
α–Cyc B1 (endog)
α–Actin
B
IP: FLAG
pCMV
Cyclin B1 5A
Cyclin B1 4A
Cyclin B1 5E
FLAG-TSC2 WT
α–FLAG (WT)
α–Cyc B1 (GFP)
α–Cyc B1 (endog)
C
IB: lysate
pCMV
Cyclin B1 5A
Cyclin B1 4A
Cyclin B1 5E
FLAG-TSC2 S664Δ
α–FLAG (S664Δ)
α–Cyc B1 (GFP)
α–Cyc B1 (endog)
α–Actin
D
IP: FLAG
pCMV
Cyclin B1 5A
Cyclin B1 4A
Cyclin B1 5E
FLAG-TSC2 S664Δ
α–FLAG (S664Δ)
α–Cyc B1 (GFP)
IgG
E
Degree of Protein Binding
Cyc B1 variant binding over FLAG
(Densitometry units corrected for actin)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
**
**
*
*
**
N.S.
Cyc B1 5A
Cyc B1 4A
Cyc B1 5E
FLAG
TSC2 WT
S664Δ
n = 3
* = p<0.05
** = p<0.01
N.S.= p>0.35

Figure 12. Tuberin WT and S664Δ both bind all three Cyclin B1 NES variants to significantly different degrees *in vivo*. HEK 293 cells transfected with wild-type FLAG-Tuberin (black bars) or S664Δ (grey bars) were co-transfected with empty control vector, or indicated Cyclin B1 variant vectors. (A) Western blots confirming over-expression of indicated vectors with Tuberin WT. Actin was used as a loading control. (B) Co-immunoprecipitation blots of FLAG precipitated protein and co-immunoprecipitated Cyclin B1 variant protein. (C) Western blots confirming over-expression of indicated vectors with S664Δ. Actin was used as a loading control. (D) Co-immunoprecipitation blots of FLAG precipitated protein and co-immunoprecipitated Cyclin B1 variant protein. (E) Graph depicting the degree of Cyclin B1 5A, 4A, 5E variant binding to that of Tuberin WT (black bars) or to that of S664Δ (grey bars). *$p<0.05$, **$p<0.01$. Statistical significance was assessed using a student's unpaired t-test.

DISCUSSION

A wide array of diseases and cancers have misregulated cell growth signalling networks and a variety of protein mutations. There is still very little molecular understanding of how clinically relevant Tuberin mutations and truncations lead to the onset of Tuberous Sclerosis and cancer. Our investigation of a clinically relevant Tuberin truncation, S664Δ, may be able to shed some light onto the mechanistic changes involved in the pathways regulated by the tumor suppressor Tuberin. Additionally, work here has gained insight on how Tuberin and modified Cyclin B1 proteins may influence the onset of mitosis.

Tuberin S664Δ lacks the Rheb binding domain at the C-terminus that is responsible for mTOR inhibition thereby allowing for increased cell growth or proliferation. Mammalian cells over expressing techniques were utilized *in vitro* to investigate the effects of Tuberin truncation in the cell cycle regulation. As expected, Tuberin WT over-expression exhibits significantly less cell proliferation compared to control cells. Furthermore, over-expression of S664Δ showed significantly increased proliferation (total cell number) compared to Tuberin WT and control over-expression (Figure 4). However, densitometry analysis revealed that over-expression between Tuberin WT and S664Δ was not equal (Figure 5). Since Tuberin S664Δ protein is 75% less abundant compared to Tuberin WT, differences here may contribute to the drastic increase in proliferation. Although endogenous Tuberin protein is present, we observed significant changes in total cell numbers, between S664Δ and control cells, leading us to believe that S664Δ does not have the ability to act as a tumor suppressor and may have gained additional functions contributing to proliferation, perhaps through misregulated binding or mislocalization. Nellist et al. 2001 used a Tuberin protein truncation (aa 1-604) to assess Hamartin binding and found that this small N-terminal fragment was able to bind Hamartin. From this, we can hypothesize that truncation protein S664Δ may still be able to bind Hamartin *in vitro*, but may not be able to contribute to Tuberin's known chaperone function towards Hamartin. Additionally, since the S664Δ truncation lacks domains known to be important for Hamartin binding, it may not be able to undergo proper protein folding to prevent Hamartin's degradation (Nellist et al., 2001). In 2014, Munkley et al. discovered a novel Tuberin isoform which increases cell proliferation in

response to androgen stimulation. This isoform is quite different however, it lacks the N-terminal domain responsible for Hamartin interactions and only contains a partial Rheb inactivation domain (Munkley et al., 2014). To date, there are no *in vitro* Tuberin truncation proliferation studies utilizing only the N-terminal domain seen here.

The rate of S664Δ protein expression may be equal to Tuberin WT; however, since it lacks many functional domains, its stability is questionable (Figure 5). Degradation of S664Δ protein compared to Tuberin WT protein through ubiquitin mediated processes is still under investigation. It is well established that Tuberin is ubiquitinated by HERC1 through N-terminal interactions, and that Hamartin binding protects Tuberin from HERC1 mediated degradation (Chong-Kopera et al., 2006).We can hypothesize that S664Δ is still target for degradation by HERC1 through the truncations N-terminus and we hypothesize that lack of regulation may be leading to accelerated protein degradation.

To more accurately assess the effect of this clinically relevant truncation on cell proliferation, we evaluated cell mitotic index using immunofluorescence and analysis of pH3 (Figure 6). Findings indicate that over-expressing Tuberin WT results in less mitotic cells compared to pCMV control cells, as observed by Fidalgo et al. 2011. Conversely, S664Δ over-expressing cells have significantly more mitotic cells than Tuberin WT and control cells. Moreover, data indicates that S664Δ regulation of mitotic onset is dependent on serum, since we see significantly exaggerated effects with serum starvation. This agrees with and further validates the increased proliferation observed with the S664Δ mutant (Figure 4). These data indicate that clinically relevant Tuberin S664Δ may have lost its ability to act as a tumor suppressor controlling cell division; however, this requires further investigation. In quiescent fibroblasts, it is known that silencing of endogenous Tuberin results in cell cycle re-entry (Soucek et al., 1997). Moreover, a shortened G1 phase results in Tuberin null cell types (Miloloza et al., 2000). We can hypothesize that mTOR activity will not be hindered in cells over-expressing S664Δ. This clinically relevant truncation lacks its C-terminal GAP domain, which is responsible for Rheb binding and downstream mTOR inhibition (Figure 2 and Figure 3)

(Dan et al., 2002; Goncharova et al., 2002; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002; Tee et al., 2002). Moreover, it is currently unknown where Tuberin WT and the CKI, p27, bind one another. The possibility exists that truncation S664Δ may not be able to sequester p27 and prevent its degradation; thereby, leading to enhanced cell cycle progression at the G1/S transition (Dan et al., 2002; Rosner et al., 2007a; Rosner et al., 2006; Short et al., 2010). It is well established that Tuberin mutations are a large contributing factor to misregulated cell growth pathways, like mTOR hyperactivation and development of SEGA cells mentioned previously (Henske et al., 1997; Ichikawa et al., 2005; Miloloza et al., 2000; Sancak et al., 2005; van Eeghen et al., 2012a; van Eeghen et al., 2012b; van Eeghen et al., 2013). Proliferation data found here may be relevant and important with respect to disease pathogenesis, as it is the first of its kind to assess a clinically relevant truncation *in vitro*.

Additionally, since S664Δ exhibits unique cellular effects, we were interested in determining differences in cellular localization between Tuberin WT and S664Δ . As expected, Tuberin WT was found to localize primarily to the cytoplasm in both normal and starvation conditions (Figure 7 and Appendix 2) (Cai et al., 2006; Rosner et al., 2007b; York et al., 2006) (see Materials and Methods for explanation of protein localization quantification). Literature shows us that mutated and truncated Tuberin protein has been observed to be mislocalized in the cell. Tuberin mutant C696Y was observed to be primarily nuclear compared to its wild-type counterpart (Fidalgo da Silva et al., 2011). Similarly, we discovered that truncation S664Δ localizes significantly more to a perinuclear region within the cell compared to Tuberin WT, but the significance of this altered localization is still under investigation. We know that cellular nutrient levels and Tuberin regulation affect Tuberin-Cyclin B1 interactions, localization, and the onset of mitosis (Fidalgo da Silva et al., 2011; Rosner et al., 2007b). Rosner et al. 2007 showed that phosphorylation by Akt leads to cytoplasmic Tuberin localization. Additionally, it is known that Tuberin nuclear localization occurs during times of serum starvation in LAM transfected cells (York et al., 2006) We also wanted to investigate if serum alterations could lead to Tuberin WT and truncation protein mislocalization. Interestingly, mislocalization was exaggerated to significantly increased perinuclear accumulation in

starvation conditions in S664Δ over-expressing cells compared to Tuberin WT. Although classical C-terminal domains leading to nuclear accumulation are not present in this truncation (York et al., 2006), other mechanisms, such as nutrient conditions may exist leading to mislocalization (Rosner et al., 2007b). Gaining knowledge of S664Δ mislocalization may be important in dissecting disease propagation and tumorigenesis.

Although, Tuberin WT and S664Δ localize primarily to different subcellular regions, similar unique phenotypes were also observed. It was noted that S664Δ protein tends to appear in a dense "clustered" or aggregated appearance significantly more than Tuberin WT protein (Appendix 3). White arrows in images from Appendix 3A and 3B display examples of cells exhibiting clustered wild-type or clustered truncated protein. This clustered appearance appears to be mainly perinuclear in high magnification (Appendix 3C). This clustering of protein is observed in both Tuberin WT and S664Δ over-expressing cells. However, clustering is observed significantly more in cells over-expressing S664Δ. The percent of cells exhibiting this phenotype, albeit low, may have implications in protein localization and binding interactions (Barnes et al., 2010; Liu et al., 1997; Wienecke et al., 1996). Tuberin is known to localize to both the lysosome and Golgi apparatus (Demetriades et al., 2014; Jones et al., 2004; Menon et al., 2014; Wienecke et al., 1996). These known localizations display themselves in a manner similar to the clustered phenotype observed here. We plan to perform additional work involving co-staining of Tuberin WT and S664Δ with classical organelle associated proteins. From this we may be able to further elucidate if this protein clustering has physiological importance and will help to determine if clustering of WT or truncated Tuberin protein is localizing to a specific cellular region *in vitro*.

Since Tuberin classically acts to inhibit protein synthesis and proliferation, preventing excess cellular growth when conditions are not adequate, we were interested in determining any effects over-expression of S664Δ had on cell size. To accomplish this, HEK-293 cells were over-expressed with FLAG-Tuberin WT or FLAG-S664Δ and subjected to flow cytometry (Figure 8). It is important to note that control

cells did not have a fluorescent label, so their size changes, although significantly smaller, cannot be compared equally to Tuberin WT or S664 cells. Samples were subjected to doublet discrimination, only the singlet population was analyzed and cells were gated for FLAG over-expression by green fluorescence. Surprisingly, data here shows that there are no significant changes in cell size between cells overexpressing wild-type or truncated protein. We postulated that S664Δ over-expression would yield increased cell size, due to our suspected lack of mTOR inhibition (Fingar et al., 2002; Rosner et al., 2009; Tee et al., 2002). However, it can be noted that cells were only cultured for 24 hours after transfection, and cell size changes may take a longer amount (up to 72 hours) of incubation time to develop significant changes (Fingar et al., 2002). Moreover, there may be additional cell growth regulation contributing to this finding. Assessing individual cell cycle phases and altering timing of analysis may help to gather insight regarding if and when any cell size changes occur.

Our interest in the clinically relevant truncation protein also extends to elucidate which other pathways may be regulated by Tuberin. To assess this, studies to assess autophagy activation were undergone (Figure 9). Since S664Δ over-expressing cells have increased proliferation, we hypothesized that these cells may be continually dividing even in the face of low nutrients and growth factors. Under normal serum conditions, cells overexpressing pCMV control, Tuberin WT, or S664Δ were investigated for the protein markers of autophagy, such as the protein expression of p62 and protein conversion ofLC3. Protein levels indicate there are no significant differences between Tuberin WT or S664Δ treated cells. Since S664Δ lacks a C-terminal Rheb binding domain, this protein likely does not inhibit mTOR to the same degree as wild-type. mTOR is known to inhibit the initiators of autophagy signalling (ULK1, Atg13, and FIP200) (Alers et al., 2012). We can extrapolate that cells over-expressing S664Δ cannot inhibit mTOR, allowing for constitutively active mTOR even in the face of starvation, leading to differences from wild-type. This was somewhat surprising to us; however, cells were over-expressed for 24 hours and immediately collected. Studies revealed that basal autophagy in HEK 293 cells mechanistically mimics events which occur with mTOR inhibition (Musiwaro et al. 2013). Autophagy markers are known to be elevated and

fluctuating as soon as 2-6 hours after starvation or with the addition of autophagy initiating drugs, such as Bafilomycin A1 (Klionsky et al., 2008; Mizushima, 2010; Mizushima and Levine, 2010; Tanida, 2011; Tanida et al., 2008; Tanida and Waguri, 2010; Zhang et al., 2013). Perhaps if cultured in normal serum and collected over a short and long-time course, we would have observed a more pronounced effect on autophagy protein marker variations (Tanida and Waguri, 2010).

It is well established that Tuberin WT protein has the ability to bind Cyclin B1 *in vivo*. We wanted to assess if clinically relevant truncation S664Δ can also bind to or interact with Cyclin B1 *in vivo*. To assess this we used a variety of immunofluorescence and immunoprecipitation techniques (Figures 10 and 11). As expected, Cyclin B1 WT protein was found to be approximately 90% cytoplasmic when over-expressed with Tuberin WT protein. It is known that Tuberin is able to retain Cyclin B1 in the cytoplasm, in both its wild-type and hyper-phosphomimic form (Cyclin B1 5E) (Fidalgo da Silva et al., 2011). The consequences of this enhanced binding interaction at G2 ultimately allows for slower progression and an increase in cell size (Fidalgo et al. 2015 manuscript in preparation).

Conversely, we observed a significant change in Cyclin B1 WT to a perinuclear/nuclear localization when co-expressed with S664Δ protein. Moreover, recent data from our lab indicates that Cyclin B1 localization is altered when co-expressed with mutant forms of Tuberin (C696Y, ΔGAP) and in changing nutrient conditions (Fidalgo da Silva et al., 2011), so it was not surprising to us that Cyclin B1 protein localization was altered with S664Δ (Figure 10). These data indicate that S664Δ protein may not be able to bind and interact with Cyclin B1 WT and retain it in the cytoplasm in a manner similar to Tuberin WT. Furthermore, we learned earlier that S664Δ protein is itself localized primarily to a perinuclear region, so perinuclear co-localization between S664Δ and Cyclin B1 proteins is a tangible possibility (Figure 10A; yellow in merged images).

To accurately determine if Cyclin B1 WT and S664Δ protein can bind *in vivo*, co-immunoprecipitation experiments were performed (Figure 11). Y2H screens previously showed that a

147aa fragment (aa 600-746) of Tuberin was sufficient for Tuberin-Cyclin B1 binding (Fidalgo da Silva et al., 2011). Presence of Cyclin B1 co-immunoprecipitation was confirmed by immunoblotting back for Cyclin B1 (Figure 11B). Surprisingly, we observed Cyclin B1 pulldown in both Tuberin WT and S664Δ sample lanes. S664Δ protein does contain some overlapping residues with the Tuberin fragment, and since we see successful binding, data here may indicate that residues 610-664 of Tuberin have a significantly important role in binding and securing Cyclin B1 protein. We were interested in comparing the affinity of Tuberin WT and S664Δ to bind Cyclin B1 WT protein (Figure 11C). Although differences in degrees of binding are not significant, it does suggest that S664Δ likely has lower affinity to Cyclin B1 than does Tuberin WT. These results are rather preliminary and this experiment likely has an outlier from Tuberin WT IP. In the future, repeating co-immunoprecipitation experiments to have larger sample sizes will truly help in validating results, especially since this is such a sensitive set of experiments. Moreover, it is important to comment on this surprising result. We have shown that S664Δ over-expression leads to increased cell proliferation, but it is known that Tuberin binding to Cyclin B1 ultimately hold back mitosis and prevents premature division (Fidalgo da Silva et al., 2011). It is possible that S664Δ and Cyclin B1 protein binding occurs because of decreased protein size and lack of regulation due to the truncation within Tuberin. Although, for Cyclin B1 WT did not significantly differ between Tuberin WT and truncation, the interaction of S664Δ may be very low and not have substantial effects on cell division. Physiological and mechanistically important roles for the observed S664Δ-Cyclin B1 WT complex within the cell is still under investigation.

Our lab is highly interested in Cyclin B1 NES variant binding with Tuberin WT and variant proteins (Fidalgo da Silva et al., 2011). As previously noted, Cyclin B1 5A (5A) binds to Tuberin WT significantly more than it does to Cyclin B1 5E (5E). Our lab is currently designing and cloning additional Cyclin B1 variants, in which each individual phosphorylation site will be mutated a glutamic acid (E) to mimic the charge of phosphorylation. Since, serine reside S126 is one of the first sites phosphorylated by Cdk1 on Cyclin B1, we decided to use site-directed mutagenesis and create a new Cyclin B1 binding variant, Cyclin

B1 4A (A126E) (termed 4A) (Appendix 3). There is currently very little known about which phosphorylation site on Cyclin B1 in the NES region is most important with respect to Tuberin binding. We want to fully elucidate this using this new variant (4A) and other variants currently in progress (Figure 12). Our results suggest that Tuberin WT binds Cyclin B1 5A significantly more than 5E, which is in agreeance with work demonstrated by Fidalgo da Silva et al. 2011. To further validate this, we plan to assess protein affinity using increasing gradients of protein over-expression (of Cyclin B1) and determining maximum protein binding values in a manner similar to Fidalgo da Silva (Fidalgo da Silva et al., 2011). Interestingly, we discovered that 4A binds to Tuberin WT protein significantly more than 5E but significantly less the 5A. These exciting results may indicate to us that S126 on Cyclin B1 is a site of critical importance for Tuberin WT binding. Based on these data, we can extrapolate that when Cyclin B1 becomes phosphorylated by Cdk1 on S126, alterations between Tuberin-CyclinB1 binding dynamics occurs. Specifically, we can expect to observe a decrease in complex binding affinity and perhaps advanced mitotic onset. These hypotheses need to be confirmed through future protein binding work. We can use a the novel G2/M report vector, pCCNB1-ECFP, developed in our lab to accurately measure the rate of mitosis when 4A and other Cyclin B1 variants still in progress are present within the cell (Fidalgo da Silva et al., 2014).

Concomitantly, we were interested in determining if S664Δ protein is able to bind Cyclin B1 variants in the same manner as Tuberin WT (Figure 13). Experiments here were performed in a similar manner as Figure 12. We observed that the clinically relevant truncation S664Δ is able to successfully bind all three Cyclin B1 variants. To no surprise, no significant differences were found between Cyclin B1 treatments. Computational proteomic data from our lab indicates that Tuberin protein folding is likely very important in Cyclin B1 binding (Market 2015, undergraduate thesis). Since this truncation lacks the C-terminal domain that is predicted to fold over when Tuberin binds to Cyclin B1, we can hypothesize that post-translational modifications such as Cyclin B1 phosphorylation cannot be monitored by this truncation protein. We can assume that the lack of predicted conformational changes may account for the lack of difference noted between the three Cyclin B1 NES variants used here.

It is a recurring theme that longer transfection incubation periods may be necessary to better assess disease pathology related to clinically relevant truncations. Moreover, transfection is not an optimal choice for most assays utilizing mutant over-expression, since endogenous protein is still present in the cell and may be able to offset or hinder mutant affects. It is also well established that mammalian DNA transfection is transient, unstable and can be uneven. To overcome this, we hope to use new genetic manipulation techniques including the very current crispr/cas9 system in future experiments to gain reliable protein expression and be able to consistently carry out long course experiments (Cong et al., 2013; Mali et al., 2013; Sander and Joung, 2014; Wiles et al., 2015). We also have developed a set of viral knock-down/over-expression vectors, which have been designed to silence endogenous Tuberin and over-express the desired mutant cloned into this vector. Our lab has recently developed a Bimolecular Fluorescence Complementation system (BIFC), utilizing both Tuberin and Cyclin B1, that can be used with confocal and live cell microscopy to assess binding *in vivo*. We plan to experiment with the timing of complex formation and can also alter nutrient conditions to assess binding affinity. Moreover, Tuberin and Cyclin B1 mutants can be inserted into these vectors allowing for a plethora of studies to be performed.

Changing of nutrient conditions in cells over-expressing Tuberin WT and Cyclin B1 NES variants is also of interest since we know that levels of nutrients and Tuberin protein modifications by Akt phosphorylation have significant effects on Cyclin B1 binding (Fidalgo et al. 2015 manuscript in preparation). We plan to culture cells under high and low nutrient conditions and assess binding of Cyclin B1 4A and other Cyclin B1 NES mutant currently under construction. Furthermore, binding studies using Tuberin protein with modifications to Erk phosphorylation sites (S540 and S664) in conjunction with Cyclin B1 WT and Cyclin B1 NES protein variants may shed light on how growth factor signalling effects G2/M progression.

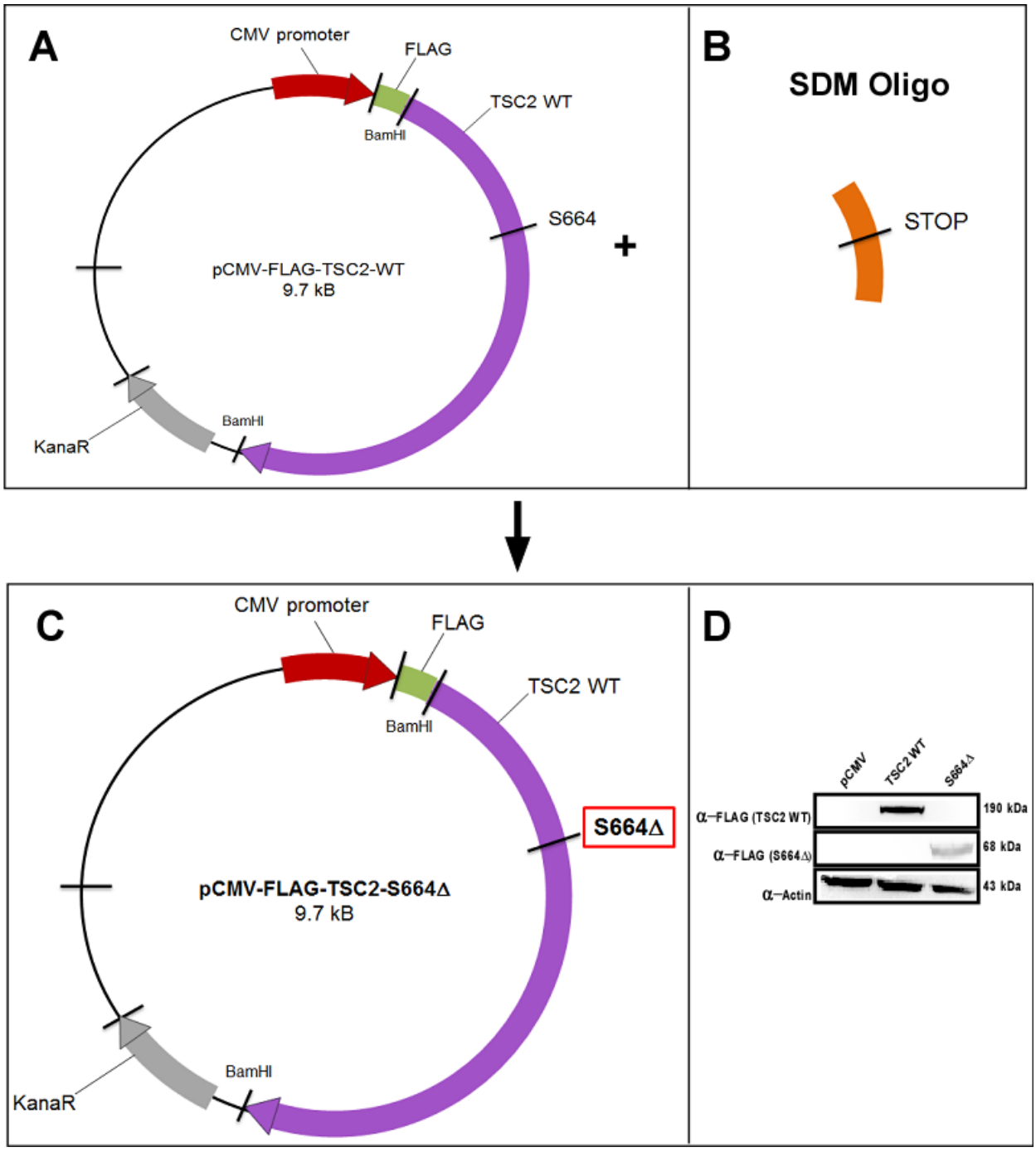
This is the first study of its kind to assess how clinically relevant truncations contribute to disease manifestation *in vitro*. In summary, S664Δ over-expression leads to significantly increased cell proliferation compared to Tuberin WT, significant changes to protein localization compared to Tuberin WT

and we learned that this truncation can successfully bind Cyclin B1 WT and Cyclin B1 variants *in vivo*. Together, these findings provide insight into how Tuberin related diseases may develop and the signalling networks that may be involved in this process.

APPENDIX 1

Construction of a novel, clinically relevant Tuberin truncation vector, pCMV-FLAG-TSC2-S664Δ by Site-Directed Mutagenesis

In order to assess the mechanistic role of a clinically relevant truncation protein of Tuberin, we utilized site directed mutagenesis (SDM) and molecular cloning techniques to produce a new mammalian expression vector. Our aim was to positively clone a premature stop codon into the *TSC2* gene of the parent vector, to ultimately produce a truncated Tuberin protein, at serine residue 664 (S664Δ) upon successful mammalian over-expression using transient transfection. We began with the pCMV Tag2C FLAG-TSC2-WT-Mlu$^-$ mammalian expression vector (the parent vector) (Stratagene & E. Fidalgo Da Silva) (Appendix 1A). A SDM oligo was designed to anneal to the *TSC2* WT gene and create a stop codon at serine 664 (Appendix 1B). Upon successful PCR and parent strand destruction by Dpn1 restriction enzyme digest, PCR product was transformed into Stbl3 *E.coli* bacteria, colonies were selected, and screened. Successfully cloned vector was analyzed by DNA sequencing with the T7 promoter (Eurofins). The resulting vector we cloned, pCMV-Tag2C-FLAG-TSC2-S664 Δ contains the full length TSC2 gene with a successfully inserted stop codon at S664. Additionally, we evaluated if cloning was successful by transfection of newly synthesized DNA and western blotting to ensure successful truncated protein over-expression (right-most lane, 68kDa protein) (Appendix 1C).

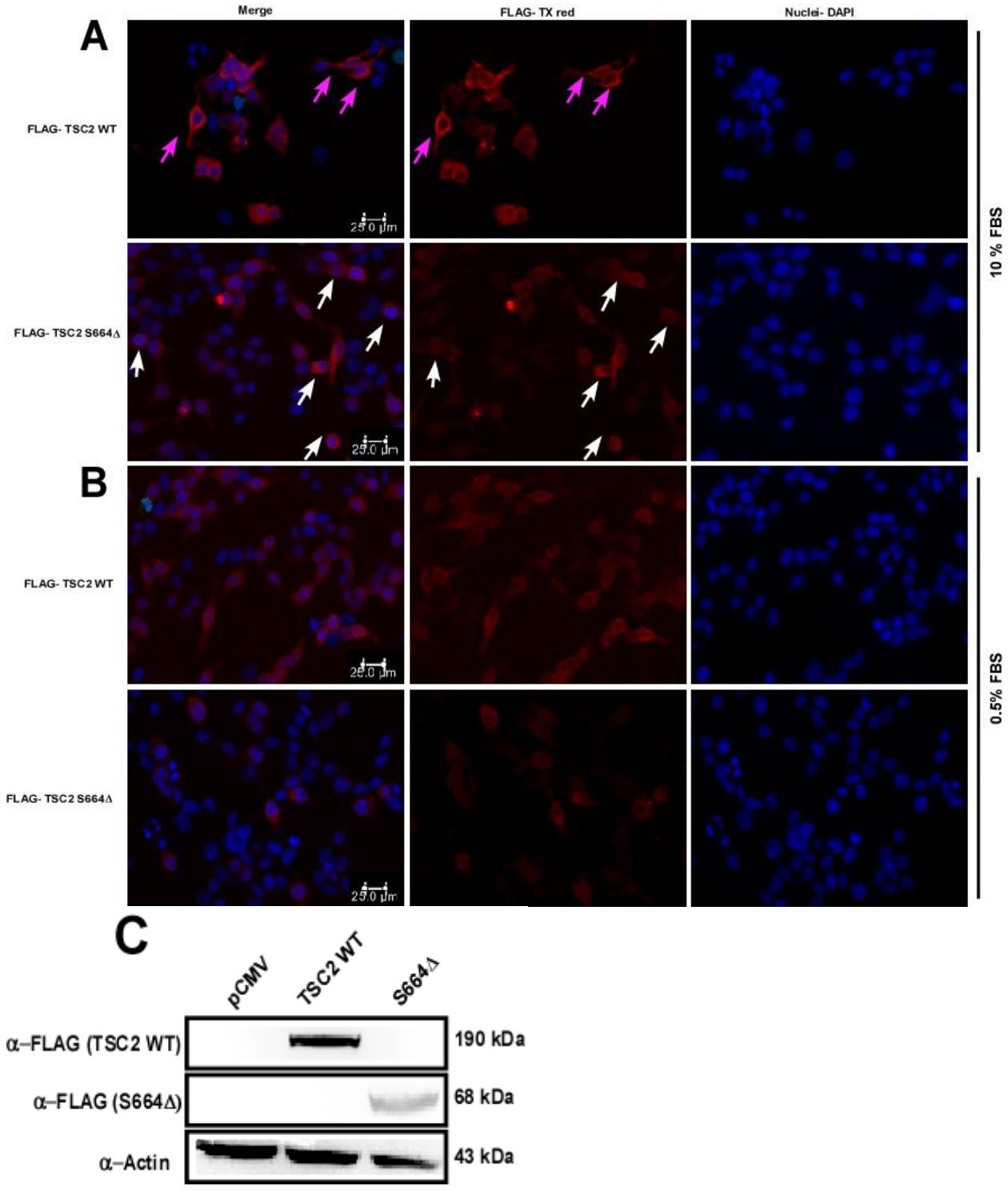


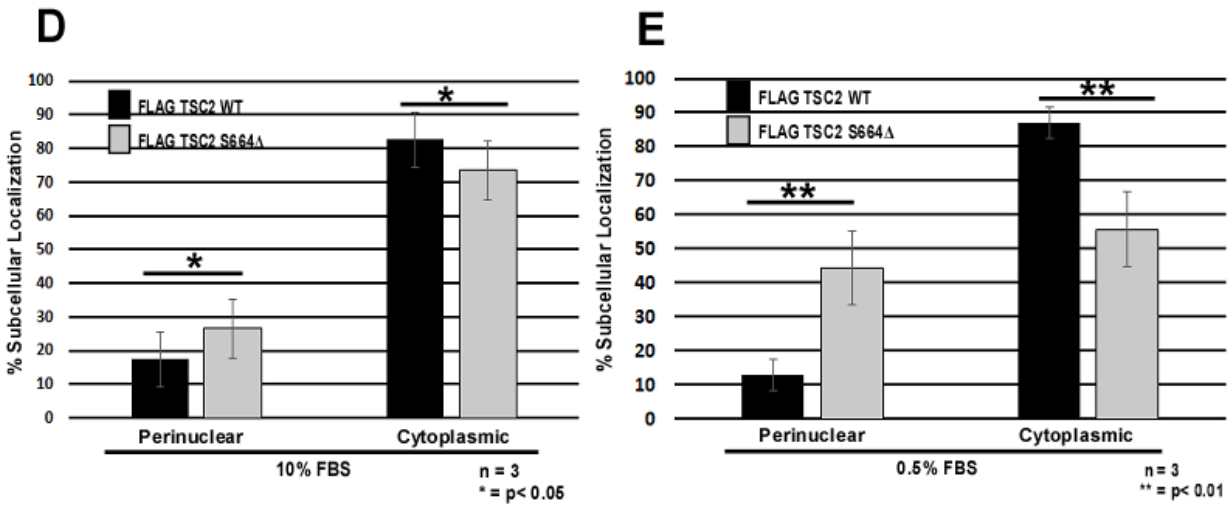
Appendix 1. Site-Directed Mutagenesis and cloning of the novel, clinically relevant Tuberin truncation vector, pCMV-FLAG-TSC2-S664Δ. (A) pCMV FLAG TSC2 WT DNA was utilized as the parent vector. (B) The parent vector was subjected to SDM PCR with the stop codon mutation designed oligo at residue S664 (orange DNA fragment). PCR product was screened by DNA sequencing for the acquired stop codon and truncation mutation. (C) A depiction of resulting mammalian expression vector product, pCMV-FLAG-TSC2-S664Δ. (D) Protein over-expression analysis by western blot to confirmation stop codon insertion and truncated protein product. S664Δ protein over-expression results in a 68kDa protein.

APPENDIX 2

Phenotypic evaluation of the clinically relevant Tuberin truncation, S664Δ.

Protein localization of the clinically relevant mutant S664Δ was determined using immunofluorescent studies (Figure 7 and Appendix 2). FLAG tagged WT or S664Δ Tuberin were over-expressed in regular nutrient conditions (Appendix 2A) or starvation conditions (Appendix 2B). Over-expression of protein was confirmed by western blot analysis (Appendix 2B). FLAG over-expressing cells were counted based on cellular localization (cytoplasmic vs. perinuclear) (Appendix 2D and 2E) and graphed as a percentage. Wild-type Tuberin protein is known to localize primarily to the cytoplasm, but can also be observed in the nucleus under certain conditions (York et al. 2006, Rosner et al. 2007). In normal culture conditions, cells over-expressing S664Δ exhibit perinuclear localization significantly more than the WT counterpart (Appendix 2D). Cells treated with the same over-expression and faced with starvation conditions exhibit the same trend, but have a significantly exaggerated effect on perinuclear localization (Appendix 2E). Moreover, after immunofluorescent examination, it was observed that cells over-expressing the Tuberin truncation display a unique phenotype that appears “clustered”, condensed or aggregated, mainly located near the nucleus or perinuclear (Appendix 3).


Merge
FLAG- TX red
Nuclei- DAPI
A
FLAG- TSC2 WT
FLAG- TSC2 S664Δ
10 % FBS
B
FLAG- TSC2 WT
FLAG- TSC2 S664Δ
0.5% FBS
C
pCMV
TSC2 WT
S664Δ
α–FLAG (TSC2 WT)
α–FLAG (S664Δ)
α–Actin
190 kDa
68 kDa
43 kDa

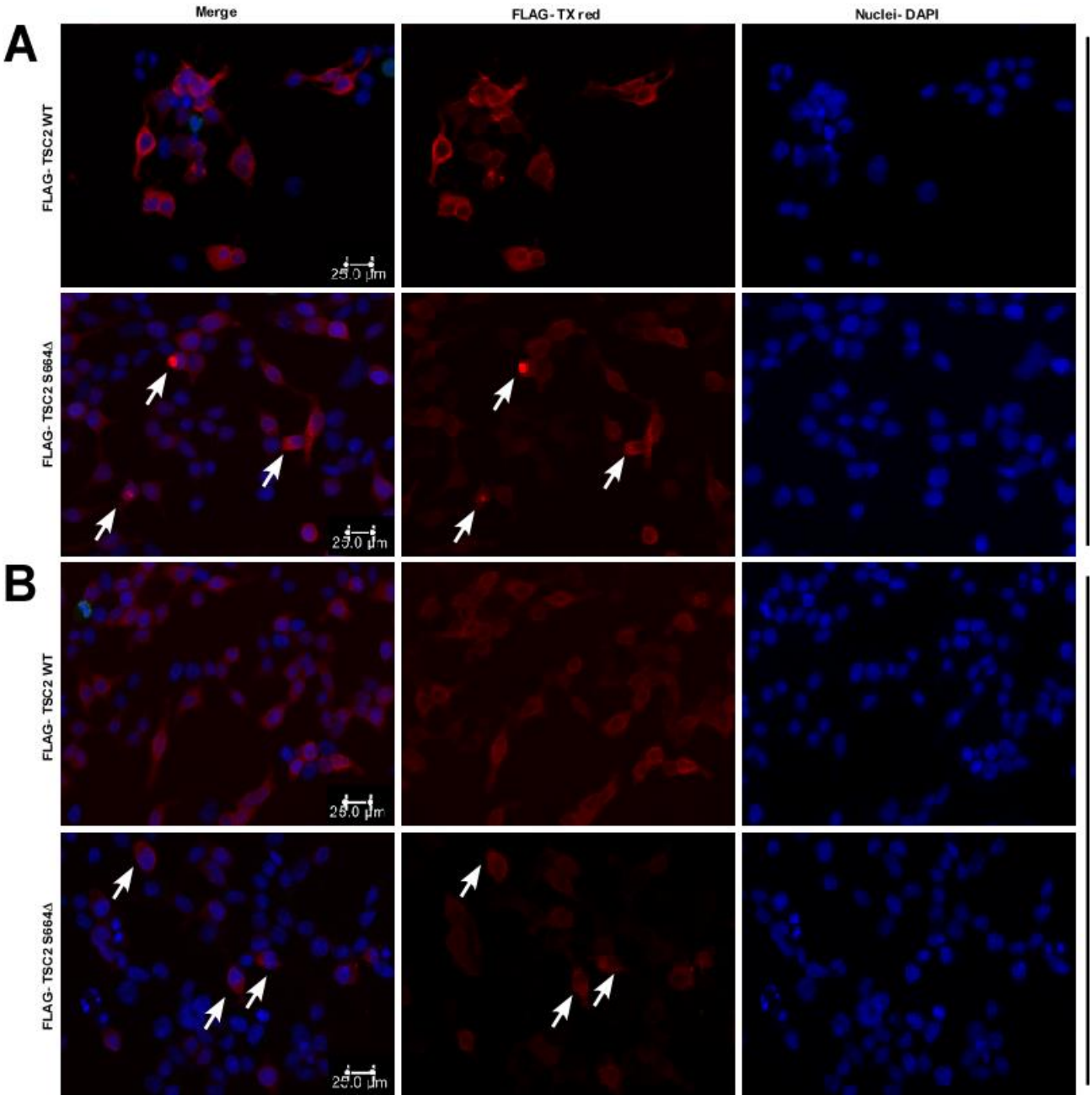


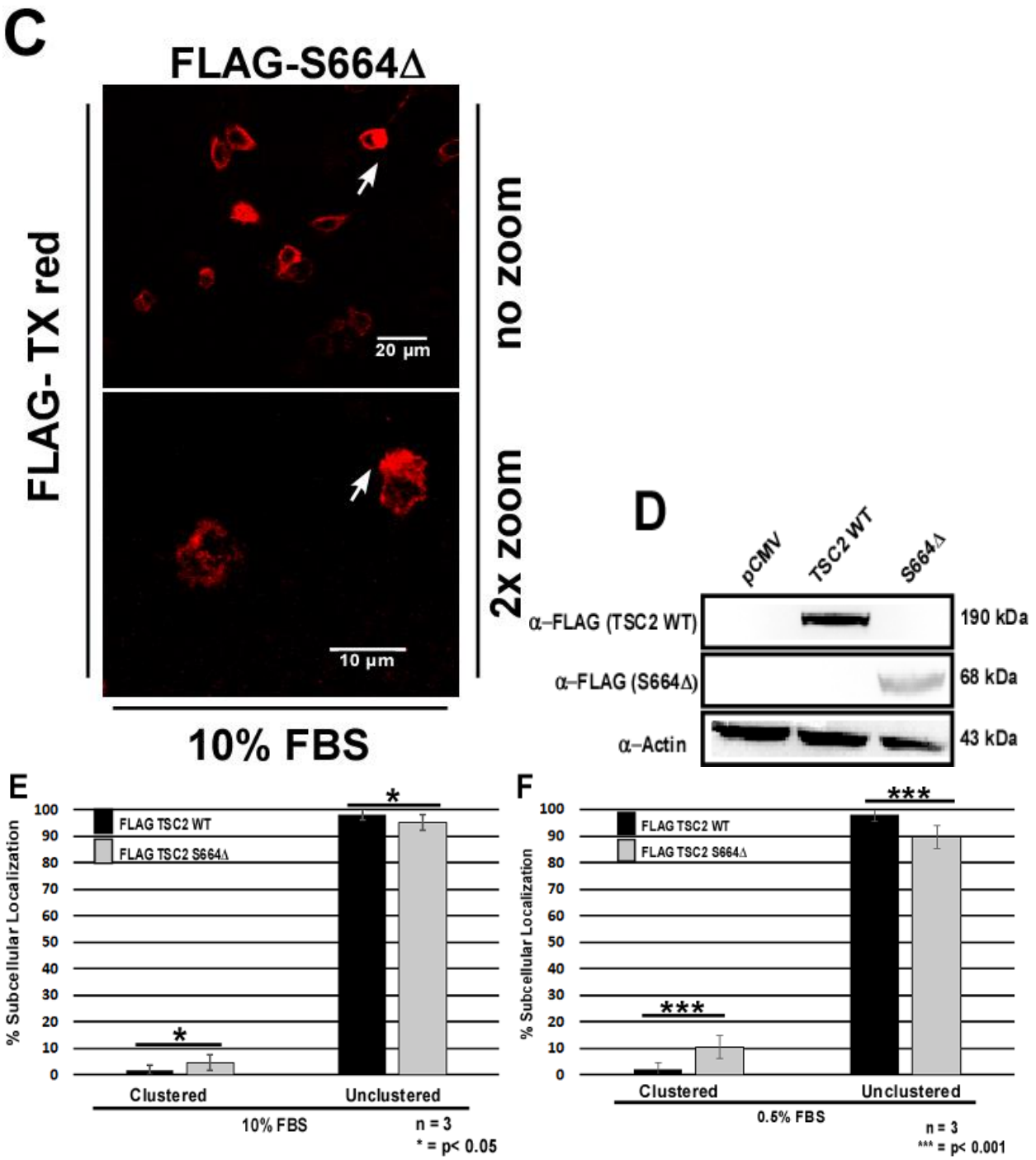
Appendix 2. Mutant Tuberin S664Δ exhibits perinuclear localization significantly more than WT Tuberin protein. HEK 293 cells were seeded onto cover slips and transiently transfected with indicated vectors, treated with 10% or 0.5% serum 24 hours later and collected 48 hours post-transfection. Cell pellets and coverslips were immediately collected and treated with fluorescent antibodies. (A) Immunofluorescence images depicting nuclei (blue) and FLAG protein over-expression (red) of 10% FBS treated cells at 20x objective. (B) Immunofluorescence images depicting nuclei (blue) and FLAG tagged protein over-expression (red) of 0.5% FBS treated cells at 20x objective. (C) Representative blot of successful protein over-expression. Subcellular localization of FLAG WT and FLAG S664Δ protein in normal culture conditions (D) or starvation conditions (E) depicted as a percentage of cells in a perinuclear (left bars) or cytoplasmic (right bars) phenotype. Example of Tuberin cytoplasmic localization identified by pink arrows and example of perinuclear/nuclear localization of Tuberin identified by white arrows. *p<0.05, **p<0.01, ***p<0.001. Statistical significance was assessed using student's unpaired t-test.

APPENDIX 3

Phenotypic evaluation of the clinically relevant Tuberin truncation, S664Δ.

To determine protein localization of the clinically relevant mutant S664Δ, immunofluorescent studies were undergone (Figure 7). During this localization study, S664Δ protein presented itself in a unique or "clustered" form (Appendix 3A and 3B; white arrows). FLAG tagged WT or S664Δ Tuberin were over-expressed in regular nutrient conditions (10% FBS) (Appendix 3A) or starvation conditions (0.5% FBS) (Appendix 3B). Clustered phenotype example of FLAG tagged S664Δ over-expressed protein in 10% nutrient conditions (Appendix 3C; white arrows). Over-expression of protein was confirmed by western blot analysis (Appendix 3D). FLAG over-expressing cells were counted based on protein localization (clustered vs. unclustered) (Appendix 3E and 3F) and graphed as a percentage. Wild-type Tuberin protein is known to localize primarily to the cytoplasm, but can also be observed in the nucleus under certain conditions (Rosner et al., 2007b; Rosner and Hengstschlager, 2007; York et al., 2006). In normal culture conditions, over-expressed S664Δ protein (grey bars) exists in a clustered and perinuclear region significantly more than its WT counterpart (black bars) (Appendix 3E). Cells treated with the same over-expression and faced with starvation conditions exhibit the same trend, but have a significantly exaggerated effect on clustered localization (Appendix 3F).


A
B
Merge
FLAG-TX red
Nuclei-DAPI
FLAG-TSC2 WT
FLAG-TSC2 S664Δ
FLAG-TSC2 WT
FLAG-TSC2 S664Δ
25.0 µm



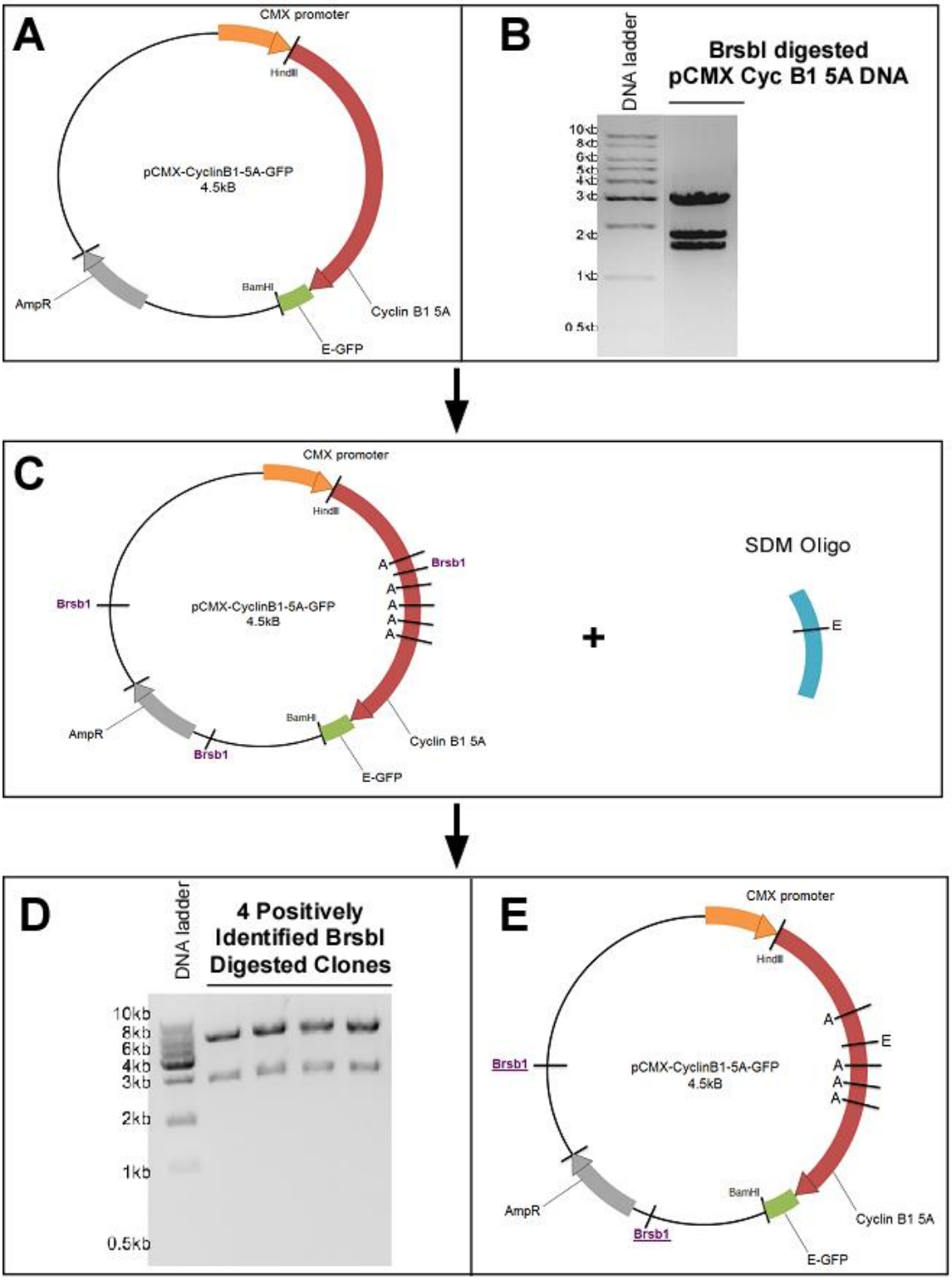
Appendix 3. Tuberin S664Δ protein is significantly more clustered compared to WT Tuberin protein. HEK 293 cells were seeded onto cover slips and transiently transfected with indicated vectors, treated with 10% or 0.5% serum 24 hours later and collected 48 hours post-transfection. Cell pellets and coverslips were immediately collected and treated with fluorescent antibodies. (A) Immunofluorescence images depicting nuclei (blue) and FLAG protein over-expression (red) of 10% FBS treated cells at 20x objective. (B) Immunofluorescence images depicting nuclei (blue) and FLAG tagged protein over-expression (red) of 0.5% FBS treated cells at 20x objective. (C) Immunofluorescence images depicting FLAG tagged protein over-expression (red) of 10% FBS treated cells at 60X oil objective (white arrows depicting clustered phenotype observation). (D) Representative blot of successful protein over-expression. Subcellular

localization of FLAG WT and FLAG S664Δprotein in normal culture conditions (E) or starvation conditions (F) depicted as a percentage of cells in a clustered (left bars) or unclustered (right bars) phenotype. Example of clustered Tuberin WT or truncated protein identified by white arrows. *$p<0.05$, **$p<0.01$, ***$p<0.001$. Statistical significance was assessed using a student's unpaired t-test.

APPENDIX 4

Construction of a novel Cyclin B1 NES variant vector, pCMX-Cyclin B1-4A (A126E) by site-directed mutagenesis.

In order to assess the essentiality of the conserved serine residues found within the NES domain of Cyclin B1, we utilized SDM and molecular cloning techniques to produce a new mammalian expression vector. Our aim was to assess which phosphorylation site is most detrimental in regulating Tuberin-Cyclin B1 protein interactions. We began with the pCMX-Cyclin B1-5A mammalian expression vector (the parent vector) (a gift from A. Hagting) (Appendix 4A). The gene and backbone of the parent vector were screened for Brsb1 restriction endonuclease cut sites; three sites were positively identified, resulting in three DNA fragments after Brsb1 restriction enzyme digestion. Two sites were found in the backbone, and one Brsb1 site was found near the SDM cloning site, in which the oligo was designed to anneal (Appendix 4B). A designed cloning oligo and the parent vector were subjected to PCR (Appendix 4C). The SDM oligo was designed to mutate the alanine 126 residue to a glutamic acid 126 residue and to destroy the Brsb1 site upstream. The Brsb1 site was destroyed as a tool to positively identify successfully mutated clones. The Brsb1 site was destroyed without alteration to the protein coding sequence; commonly referred to as a silent site screening mutation. Successful clones that have the SDM sequence should also only contain two Brsb1 sites and result in two DNA fragments when subjected to Brsb1 digestion (Appendix 4D). Upon successful PCR and parent strand destruction by Dpn1 restriction enzyme digest, PCR product was transformed into Stbl3 *E.coli* bacteria, colonies were selected, and screened. Successfully cloned vector was first screened by Brsb1 digest, positively identified by 2 DNA fragments and analyzed by DNA sequencing with the upstream T7 promoter (Eurofins). The resulting vector we cloned contains four alanine residues and one glutamic acid residue at amino acid 126, two Brsb1 cut sites in the backbone and a C-terminal EGFP tag (Appendix 4E).



Appendix 4. Site-Directed Mutagenesis and cloning of novel Cyclin B1 NES vector, pCMX-Cyclin B1-4A (A126E). (A) pCMX-Cyclin B1-5A DNA was utilized as the parent vector. (B) It was subjected to BrsbI restriction enzyme digest. (C) Clean parent vector was subjected to SDM PCR with the mutation designed oligo (blue DNA fragment). (D) PCR product was screened by BrsbI digest to assess site silent destruction of the third BrsbI site. (E) A depiction of newly cloned DNA vector pCMX-Cyc B1-4A (A126E) after DNA sequencing results.

REFERENCES

(1993). Identification and characterization of the tuberous sclerosis gene on chromosome 16. Cell *75*, 1305-1315.

Aicher, L. D., Campbell, J. S., and Yeung, R. S. (2001). Tuberin phosphorylation regulates its interaction with hamartin. Two proteins involved in tuberous sclerosis. J Biol Chem *276*, 21017-21021.

Alers, S., Loffler, A. S., Wesselborg, S., and Stork, B. (2012). Role of AMPK-mTOR-Ulk1/2 in the regulation of autophagy: cross talk, shortcuts, and feedbacks. Mol Cell Biol *32*, 2-11.

Arellano, M., and Moreno, S. (1997). Regulation of CDK/cyclin complexes during the cell cycle. Int J Biochem Cell Biol *29*, 559-573.

Assoian, R. K., and Zhu, X. (1997). Cell anchorage and the cytoskeleton as partners in growth factor dependent cell cycle progression. Curr Opin Cell Biol *9*, 93-98.

Astrinidis, A., and Henske, E. P. (2005). Tuberous sclerosis complex: linking growth and energy signaling pathways with human disease. Oncogene *24*, 7475-7481.

Bai, X., Ma, D., Liu, A., Shen, X., Wang, Q. J., Liu, Y., and Jiang, Y. (2007). Rheb activates mTOR by antagonizing its endogenous inhibitor, FKBP38. Science *318*, 977-980.

Barnes, E. A., Kenerson, H. L., Jiang, X., and Yeung, R. S. (2010). Tuberin regulates E-cadherin localization: implications in epithelial-mesenchymal transition. Am J Pathol *177*, 1765-1778.

Benvenuto, G., Li, S., Brown, S. J., Braverman, R., Vass, W. C., Cheadle, J. P., Halley, D. J., Sampson, J. R., Wienecke, R., and DeClue, J. E. (2000). The tuberous sclerosis-1 (TSC1) gene product hamartin suppresses cell growth and augments the expression of the TSC2 product tuberin by inhibiting its ubiquitination. Oncogene *19*, 6306-6316.

Betz, C., and Hall, M. N. (2013). Where is mTOR and what is it doing there? J Cell Biol *203*, 563-574.

Beugnet, A., Tee, A. R., Taylor, P. M., and Proud, C. G. (2003). Regulation of targets of mTOR (mammalian target of rapamycin) signalling by intracellular amino acid availability. Biochem J *372*, 555-566.

Bhatia, B., Northcott, P. A., Hambardzumyan, D., Govindarajan, B., Brat, D. J., Arbiser, J. L., Holland, E. C., Taylor, M. D., and Kenney, A. M. (2009). Tuberous sclerosis complex suppression in cerebellar development and medulloblastoma: separate regulation of mTOR activity and p27Kip1 localization. Cancer Res *69*, 7224-7234.

Borgne, A., Ostvold, A. C., Flament, S., and Meijer, L. (1999). Intra-M phase-promoting factor phosphorylation of cyclin B at the prophase/metaphase transition. J Biol Chem *274*, 11977-11986.

Cai, S. L., Tee, A. R., Short, J. D., Bergeron, J. M., Kim, J., Shen, J., Guo, R., Johnson, C. L., Kiguchi, K., and Walker, C. L. (2006). Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J Cell Biol *173*, 279-289.

Carnero, A., and Hannon, G. J. (1998). The INK4 family of CDK inhibitors. Curr Top Microbiol Immunol *227*, 43-55.

Chang, D. C., Xu, N., and Luo, K. Q. (2003). Degradation of cyclin B is required for the onset of anaphase in Mammalian cells. J Biol Chem *278*, 37865-37873.

Chong-Kopera, H., Inoki, K., Li, Y., Zhu, T., Garcia-Gonzalo, F. R., Rosa, J. L., and Guan, K. L. (2006). TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase. J Biol Chem *281*, 8313-8316.

Ciemerych, M. A., and Sicinski, P. (2005). Cell cycle in mouse development. Oncogene *24*, 2877-2898.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., Hsu, P. D., Wu, X., Jiang, W., Marraffini, L. A., and Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science *339*, 819-823.

Corradetti, M. N., Inoki, K., Bardeesy, N., DePinho, R. A., and Guan, K. L. (2004). Regulation of the TSC pathway by LKB1: evidence of a molecular link between tuberous sclerosis complex and Peutz-Jeghers syndrome. Genes Dev *18*, 1533-1538.

Curatolo, P., Bombardieri, R., and Jozwiak, S. (2008). Tuberous sclerosis. Lancet *372*, 657-668.

Dabora, S. L., Jozwiak, S., Franz, D. N., Roberts, P. S., Nieto, A., Chung, J., Choy, Y. S., Reeve, M. P., Thiele, E., Egelhoff, J. C., *et al.* (2001). Mutational Analysis in a Cohort of 224 Tuberous Sclerosis Patients Indicates Increased Severity of TSC2, Compared with TSC1, Disease in Multiple Organs. Am J Hum Genet *68*, 64-80.
Dan, H. C., Sun, M., Yang, L., Feldman, R. I., Sui, X. M., Ou, C. C., Nellist, M., Yeung, R. S., Halley, D. J., Nicosia, S. V., *et al.* (2002). Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin. J Biol Chem *277*, 35364-35370.
Dann, S. G., Selvaraj, A., and Thomas, G. (2007). mTOR Complex1-S6K1 signaling: at the crossroads of obesity, diabetes and cancer. Trends Mol Med *13*, 252-259.
de Vries, P. J., and Howe, C. J. (2007). The tuberous sclerosis complex proteins--a GRIPP on cognition and neurodevelopment. Trends Mol Med *13*, 319-326.
Demetriades, C., Doumpas, N., and Teleman, A. A. (2014). Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. Cell *156*, 786-799.
Draviam, V. M., Orrechia, S., Lowe, M., Pardi, R., and Pines, J. (2001). The localization of human cyclins B1 and B2 determines CDK1 substrate specificity and neither enzyme requires MEK to disassemble the Golgi apparatus. J Cell Biol *152*, 945-958.
Dunphy, W. G., and Kumagai, A. (1991). The cdc25 protein contains an intrinsic phosphatase activity. Cell *67*, 189-196.
Ehninger, D., de Vries, P. J., and Silva, A. J. (2009). From mTOR to cognition: molecular and cellular mechanisms of cognitive impairments in tuberous sclerosis. J Intellect Disabil Res *53*, 838-851.
Fidalgo da Silva, E., Ansari, S. B., Maimaiti, J., Barnes, E. A., Kong-Beltran, M., Donoghue, D. J., and Porter, L. A. (2011). The tumor suppressor tuberin regulates mitotic onset through the cellular localization of cyclin B1. Cell Cycle *10*, 3129-3139.
Fidalgo da Silva, E., Botsford, S., and Porter, L. A. (2014). Derivation of a novel G2 reporter system. Cytotechnology.
Fingar, D. C., Salama, S., Tsou, C., Harlow, E., and Blenis, J. (2002). Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev *16*, 1472-1487.
Ford, J. C., al-Khodairy, F., Fotou, E., Sheldrick, K. S., Griffiths, D. J., and Carr, A. M. (1994). 14-3-3 protein homologs required for the DNA damage checkpoint in fission yeast. Science *265*, 533-535.
Franz, D. N. (2013). Everolimus in the treatment of subependymal giant cell astrocytomas, angiomyolipomas, and pulmonary and skin lesions associated with tuberous sclerosis complex. Biologics *7*, 211-221.
Franz, D. N., Bissler, J. J., and McCormack, F. X. (2010). Tuberous Sclerosis Complex: Neurological, Renal and Pulmonary Manifestations. Neuropediatrics *41*, 199-208.
Gao, X., and Pan, D. (2001). TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev *15*, 1383-1392.
Garami, A., Zwartkruis, F. J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S. C., Hafen, E., Bos, J. L., and Thomas, G. (2003). Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell *11*, 1457-1466.
Girard, F., Strausfeld, U., Fernandez, A., and Lamb, N. J. (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. Cell *67*, 1169-1179.
Glotzer, M. (2001). Animal cell cytokinesis. Annu Rev Cell Dev Biol *17*, 351-386.
Glotzer, M., Murray, A. W., and Kirschner, M. W. (1991). Cyclin is degraded by the ubiquitin pathway. Nature *349*, 132-138.
Goncharova, E. A., Goncharov, D. A., Eszterhas, A., Hunter, D. S., Glassberg, M. K., Yeung, R. S., Walker, C. L., Noonan, D., Kwiatkowski, D. J., Chou, M. M., *et al.* (2002). Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis (LAM). J Biol Chem *277*, 30958-30967.
Green, A. J., Johnson, P. H., and Yates, J. R. (1994a). The tuberous sclerosis gene on chromosome 9q34 acts as a growth suppressor. Hum Mol Genet *3*, 1833-1834.

Green, A. J., Smith, M., and Yates, J. R. (1994b). Loss of heterozygosity on chromosome 16p13.3 in hamartomas from tuberous sclerosis patients. Nat Genet *6*, 193-196.
Hagting, A., Jackman, M., Simpson, K., and Pines, J. (1999). Translocation of cyclin B1 to the nucleus at prophase requires a phosphorylation-dependent nuclear import signal. Curr Biol *9*, 680-689.
Hagting, A., Karlsson, C., Clute, P., Jackman, M., and Pines, J. (1998). MPF localization is controlled by nuclear export. EMBO J *17*, 4127-4138.
Han, J. M., and Sahin, M. (2011). TSC1/TSC2 Signaling in the CNS. FEBS Lett *585*, 973-980.
Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., and et al. (1995). Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell *6*, 387-400.
Hengst, L., Gopfert, U., Lashuel, H. A., and Reed, S. I. (1998). Complete inhibition of Cdk/cyclin by one molecule of p21(Cip1). Genes Dev *12*, 3882-3888.
Hengst, L., and Reed, S. I. (1998). Inhibitors of the Cip/Kip family. Curr Top Microbiol Immunol *227*, 25-41.
Henske, E. P., Wessner, L. L., Golden, J., Scheithauer, B. W., Vortmeyer, A. O., Zhuang, Z., Klein-Szanto, A. J., Kwiatkowski, D. J., and Yeung, R. S. (1997). Loss of tuberin in both subependymal giant cell astrocytomas and angiomyolipomas supports a two-hit model for the pathogenesis of tuberous sclerosis tumors. Am J Pathol *151*, 1639-1647.
Hershko, A. (1999). Mechanisms and regulation of the degradation of cyclin B. Philos Trans R Soc Lond B Biol Sci *354*, 1571-1575; discussion 1575-1576.
Huang, J., and Manning, B. D. (2008). The TSC1-TSC2 complex: a molecular switchboard controlling cell growth. Biochem J *412*, 179-190.
Ianzini, F., and Mackey, M. A. (1997). Spontaneous premature chromosome condensation and mitotic catastrophe following irradiation of HeLa S3 cells. Int J Radiat Biol *72*, 409-421.
Ichikawa, T., Wakisaka, A., Daido, S., Takao, S., Tamiya, T., Date, I., Koizumi, S., and Niida, Y. (2005). A case of solitary subependymal giant cell astrocytoma: two somatic hits of TSC2 in the tumor, without evidence of somatic mosaicism. J Mol Diagn *7*, 544-549.
Inoki, K., Li, Y., Zhu, T., Wu, J., and Guan, K. L. (2002). TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol *4*, 648-657.
Inoki, K., Zhu, T., and Guan, K. L. (2003). TSC2 mediates cellular energy response to control cell growth and survival. Cell *115*, 577-590.
Izumi, T., and Maller, J. L. (1991). Phosphorylation of Xenopus cyclins B1 and B2 is not required for cell cycle transitions. Mol Cell Biol *11*, 3860-3867.
Jackman, M., Lindon, C., Nigg, E. A., and Pines, J. (2003). Active cyclin B1-Cdk1 first appears on centrosomes in prophase. Nat Cell Biol *5*, 143-148.
Jeffrey, P. D., Russo, A. A., Polyak, K., Gibbs, E., Hurwitz, J., Massague, J., and Pavletich, N. P. (1995). Mechanism of CDK activation revealed by the structure of a cyclinA-CDK2 complex. Nature *376*, 313-320.
Jones, A. C., Shyamsundar, M. M., Thomas, M. W., Maynard, J., Idziaszczyk, S., Tomkins, S., Sampson, J. R., and Cheadle, J. P. (1999). Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis. Am J Hum Genet *64*, 1305-1315.
Jones, K. A., Jiang, X., Yamamoto, Y., and Yeung, R. S. (2004). Tuberin is a component of lipid rafts and mediates caveolin-1 localization: role of TSC2 in post-Golgi transport. Exp Cell Res *295*, 512-524.
Jozwiak, J., Jozwiak, S., Grzela, T., and Lazarczyk, M. (2005). Positive and negative regulation of TSC2 activity and its effects on downstream effectors of the mTOR pathway. Neuromolecular Med *7*, 287-296.
Kim, D. H., Sarbassov, D. D., Ali, S. M., King, J. E., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell *110*, 163-175.
Kim, J., Kundu, M., Viollet, B., and Guan, K. L. (2011). AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1. Nat Cell Biol *13*, 132-141.

Klionsky, D. J., Abeliovich, H., Agostinis, P., Agrawal, D. K., Aliev, G., Askew, D. S., Baba, M., Baehrecke, E. H., Bahr, B. A., Ballabio, A., *et al.* (2008). Guidelines for the use and interpretation of assays for monitoring autophagy in higher eukaryotes. Autophagy *4*, 151-175.
Kohrman, M. H. (2012). Emerging treatments in the management of tuberous sclerosis complex. Pediatr Neurol *46*, 267-275.
Kwiatkowski, D. J., Palmer, M. R., Jozwiak, S., Bissler, J., Franz, D., Segal, S., Chen, D., and Sampson, J. R. (2015). Response to everolimus is seen in TSC-associated SEGAs and angiomyolipomas independent of mutation type and site in TSC1 and TSC2. Eur J Hum Genet.
Lee, M. H., Reynisdottir, I., and Massague, J. (1995). Cloning of p57KIP2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. Genes Dev *9*, 639-649.
Lew, D. J., and Kornbluth, S. (1996). Regulatory roles of cyclin dependent kinase phosphorylation in cell cycle control. Curr Opin Cell Biol *8*, 795-804.
Li, J., Meyer, A. N., and Donoghue, D. J. (1997a). Nuclear localization of cyclin B1 mediates its biological activity and is regulated by phosphorylation. Proc Natl Acad Sci U S A *94*, 502-507.
Li, J., Meyer, A. N., and Donoghue, D. J. (1997b). Phosphorylation makes cyclin (B)eeline for the nucleus. Trends Cell Biol *7*, 181.
Li, Y., Inoki, K., Vikis, H., and Guan, K. L. (2006). Measurements of TSC2 GAP activity toward Rheb. Methods Enzymol *407*, 46-54.
Liu, F., Stanton, J. J., Wu, Z., and Piwnica-Worms, H. (1997). The human Myt1 kinase preferentially phosphorylates Cdc2 on threonine 14 and localizes to the endoplasmic reticulum and Golgi complex. Mol Cell Biol *17*, 571-583.
Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P., and Pandolfi, P. P. (2005). Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell *121*, 179-193.
Ma, L., Teruya-Feldstein, J., Bonner, P., Bernardi, R., Franz, D. N., Witte, D., Cordon-Cardo, C., and Pandolfi, P. P. (2007). Identification of S664 TSC2 phosphorylation as a marker for extracellular signal-regulated kinase mediated mTOR activation in tuberous sclerosis and human cancer. Cancer Res *67*, 7106-7112.
Maheshwar, M. M., Cheadle, J. P., Jones, A. C., Myring, J., Fryer, A. E., Harris, P. C., and Sampson, J. R. (1997). The GAP-related domain of tuberin, the product of the TSC2 gene, is a target for missense mutations in tuberous sclerosis. Hum Mol Genet *6*, 1991-1996.
Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., Norville, J. E., and Church, G. M. (2013). RNA-guided human genome engineering via Cas9. Science *339*, 823-826.
Manning, B. D., and Cantley, L. C. (2003). United at last: the tuberous sclerosis complex gene products connect the phosphoinositide 3-kinase/Akt pathway to mammalian target of rapamycin (mTOR) signalling. Biochem Soc Trans *31*, 573-578.
Manning, B. D., Tee, A. R., Logsdon, M. N., Blenis, J., and Cantley, L. C. (2002). Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell *10*, 151-162.
Menon, S., Dibble, C. C., Talbott, G., Hoxhaj, G., Valvezan, A. J., Takahashi, H., Cantley, L. C., and Manning, B. D. (2014). Spatial control of the TSC complex integrates insulin and nutrient regulation of mTORC1 at the lysosome. Cell *156*, 771-785.
Miloloza, A., Rosner, M., Nellist, M., Halley, D., Bernaschek, G., and Hengstschlager, M. (2000). The TSC1 gene product, hamartin, negatively regulates cell proliferation. Hum Mol Genet *9*, 1721-1727.
Mizushima, N. (2010). Autophagy. FEBS Lett *584*, 1279.
Mizushima, N., and Levine, B. (2010). Autophagy in mammalian development and differentiation. Nat Cell Biol *12*, 823-830.
Morgan, D. O. (1995). Principles of CDK regulation. Nature *374*, 131-134.
Munkley, J., Rajan, P., Lafferty, N. P., Dalgliesh, C., Jackson, R. M., Robson, C. N., Leung, H. Y., and Elliott, D. J. (2014). A novel androgen-regulated isoform of the TSC2 tumour suppressor gene increases cell proliferation. Oncotarget *5*, 131-139.

Nellist, M., Burgers, P. C., van den Ouweland, A. M., Halley, D. J., and Luider, T. M. (2005a). Phosphorylation and binding partner analysis of the TSC1-TSC2 complex. Biochem Biophys Res Commun *333*, 818-826.
Nellist, M., Sancak, O., Goedbloed, M., Adriaans, A., Wessels, M., Maat-Kievit, A., Baars, M., Dommering, C., van den Ouweland, A., and Halley, D. (2008). Functional characterisation of the TSC1-TSC2 complex to assess multiple TSC2 variants identified in single families affected by tuberous sclerosis complex. BMC Med Genet *9*, 10.
Nellist, M., Sancak, O., Goedbloed, M. A., Rohe, C., van Netten, D., Mayer, K., Tucker-Williams, A., van den Ouweland, A. M., and Halley, D. J. (2005b). Distinct effects of single amino-acid changes to tuberin on the function of the tuberin-hamartin complex. Eur J Hum Genet *13*, 59-68.
Nellist, M., Verhaaf, B., Goedbloed, M. A., Reuser, A. J., van den Ouweland, A. M., and Halley, D. J. (2001). TSC2 missense mutations inhibit tuberin phosphorylation and prevent formation of the tuberin-hamartin complex. Hum Mol Genet *10*, 2889-2898.
Norbury, C., and Nurse, P. (1992). Animal cell cycles and their control. Annu Rev Biochem *61*, 441-470.
Numis, A., Major, P., Montenegro, M., Muzykewicz, D., Pulsifer, M., and Thiele, E. (2011). Identification of risk factors for autism spectrum disorders in tuberous sclerosis complex. Neurology *76*, 981-987.
Nurse, P. M. (2002). Nobel Lecture. Cyclin dependent kinases and cell cycle control. Biosci Rep *22*, 487-499.
O'Callaghan, F. J., Noakes, M. J., Martyn, C. N., and Osborne, J. P. (2004). An epidemiological study of renal pathology in tuberous sclerosis complex. BJU Int *94*, 853-857.
Ohtsubo, M., Theodoras, A. M., Schumacher, J., Roberts, J. M., and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. Mol Cell Biol *15*, 2612-2624.
Orlova, K. A., and Crino, P. B. (2010). The tuberous sclerosis complex. Ann N Y Acad Sci *1184*, 87-105.
Parkhitko, A., Myachina, F., Morrison, T. A., Hindi, K. M., Auricchio, N., Karbowniczek, M., Wu, J. J., Finkel, T., Kwiatkowski, D. J., Yu, J. J., and Henske, E. P. (2011). Tumorigenesis in tuberous sclerosis complex is autophagy and p62/sequestosome 1 (SQSTM1)-dependent. Proc Natl Acad Sci U S A *108*, 12455-12460.
Paulovich, A. G., and Hartwell, L. H. (1995). A checkpoint regulates the rate of progression through S phase in S. cerevisiae in response to DNA damage. Cell *82*, 841-847.
Payne, D. M., Rossomando, A. J., Martino, P., Erickson, A. K., Her, J. H., Shabanowitz, J., Hunt, D. F., Weber, M. J., and Sturgill, T. W. (1991). Identification of the regulatory phosphorylation sites in pp42/mitogen-activated protein kinase (MAP kinase). EMBO J *10*, 885-892.
Pearson, G., Robinson, F., Beers Gibson, T., Xu, B. E., Karandikar, M., Berman, K., and Cobb, M. H. (2001). Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr Rev *22*, 153-183.
Peng, C. Y., Graves, P. R., Thoma, R. S., Wu, Z., Shaw, A. S., and Piwnica-Worms, H. (1997). Mitotic and G2 checkpoint control: regulation of 14-3-3 protein binding by phosphorylation of Cdc25C on serine-216. Science *277*, 1501-1505.
Pines, J. (1995). Cyclins and cyclin-dependent kinases: theme and variations. Adv Cancer Res *66*, 181-212.
Pines, J., and Hunter, T. (1994). The differential localization of human cyclins A and B is due to a cytoplasmic retention signal in cyclin B. EMBO J *13*, 3772-3781.
Polyak, K., Lee, M. H., Erdjument-Bromage, H., Koff, A., Roberts, J. M., Tempst, P., and Massague, J. (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. Cell *78*, 59-66.
Porter, L. A., and Donoghue, D. J. (2003). Cyclin B1 and CDK1: nuclear localization and upstream regulators. Prog Cell Cycle Res *5*, 335-347.
Potter, C. J., Pedraza, L. G., and Xu, T. (2002). Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol *4*, 658-665.
Rechsteiner, M., and Rogers, S. W. (1996). PEST sequences and regulation by proteolysis. Trends Biochem Sci *21*, 267-271.

Rennebeck, G., Kleymenova, E. V., Anderson, R., Yeung, R. S., Artzt, K., and Walker, C. L. (1998). Loss of function of the tuberous sclerosis 2 tumor suppressor gene results in embryonic lethality characterized by disrupted neuroepithelial growth and development. Proc Natl Acad Sci U S A *95*, 15629-15634.
Rosner, M., Freilinger, A., Hanneder, M., Fujita, N., Lubec, G., Tsuruo, T., and Hengstschlager, M. (2007a). p27Kip1 localization depends on the tumor suppressor protein tuberin. Hum Mol Genet *16*, 1541-1556.
Rosner, M., Freilinger, A., and Hengstschlager, M. (2006). The tuberous sclerosis genes and regulation of the cyclin-dependent kinase inhibitor p27. Mutat Res *613*, 10-16.
Rosner, M., Freilinger, A., and Hengstschlager, M. (2007b). Akt regulates nuclear/cytoplasmic localization of tuberin. Oncogene *26*, 521-531.
Rosner, M., Fuchs, C., Siegel, N., Valli, A., and Hengstschlager, M. (2009). Functional interaction of mammalian target of rapamycin complexes in regulating mammalian cell size and cell cycle. Hum Mol Genet *18*, 3298-3310.
Rosner, M., Hanneder, M., Siegel, N., Valli, A., and Hengstschlager, M. (2008). The tuberous sclerosis gene products hamartin and tuberin are multifunctional proteins with a wide spectrum of interacting partners. Mutat Res *658*, 234-246.
Rosner, M., and Hengstschlager, M. (2007). Cytoplasmic/nuclear localization of tuberin in different cell lines. Amino Acids *33*, 575-579.
Sancak, O., Nellist, M., Goedbloed, M., Elfferich, P., Wouters, C., Maat-Kievit, A., Zonnenberg, B., Verhoef, S., Halley, D., and van den Ouweland, A. (2005). Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype--phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex. Eur J Hum Genet *13*, 731-741.
Sander, J. D., and Joung, J. K. (2014). CRISPR-Cas systems for editing, regulating and targeting genomes. Nat Biotechnol *32*, 347-355.
Sarbassov, D. D., Ali, S. M., Kim, D. H., Guertin, D. A., Latek, R. R., Erdjument-Bromage, H., Tempst, P., and Sabatini, D. M. (2004). Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol *14*, 1296-1302.
Sartor, H., Ehlert, F., Grzeschik, K. H., Muller, R., and Adolph, S. (1992). Assignment of two human cell cycle genes, CDC25C and CCNB1, to 5q31 and 5q12, respectively. Genomics *13*, 911-912.
Sengupta, S., Peterson, T. R., and Sabatini, D. M. (2010). Regulation of the mTOR complex 1 pathway by nutrients, growth factors, and stress. Mol Cell *40*, 310-322.
Shaw, R. J. (2009). LKB1 and AMP-activated protein kinase control of mTOR signalling and growth. Acta Physiol (Oxf) *196*, 65-80.
Shaw, R. J., Bardeesy, N., Manning, B. D., Lopez, L., Kosmatka, M., DePinho, R. A., and Cantley, L. C. (2004a). The LKB1 tumor suppressor negatively regulates mTOR signaling. Cancer Cell *6*, 91-99.
Shaw, R. J., Kosmatka, M., Bardeesy, N., Hurley, R. L., Witters, L. A., DePinho, R. A., and Cantley, L. C. (2004b). The tumor suppressor LKB1 kinase directly activates AMP-activated kinase and regulates apoptosis in response to energy stress. Proc Natl Acad Sci U S A *101*, 3329-3335.
Sherr, C. J. (1994). G1 phase progression: cycling on cue. Cell *79*, 551-555.
Sherr, C. J., and Roberts, J. M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev *9*, 1149-1163.
Sherr, C. J., and Roberts, J. M. (1999). CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev *13*, 1501-1512.
Short, J. D., Dere, R., Houston, K. D., Cai, S. L., Kim, J., Bergeron, J. M., Shen, J., Liang, J., Bedford, M. T., Mills, G. B., and Walker, C. L. (2010). AMPK-mediated phosphorylation of murine p27 at T197 promotes binding of 14-3-3 proteins and increases p27 stability. Mol Carcinog *49*, 429-439.
Soucek, T., Pusch, O., Wienecke, R., DeClue, J. E., and Hengstschlager, M. (1997). Role of the tuberous sclerosis gene-2 product in cell cycle control. Loss of the tuberous sclerosis gene-2 induces quiescent cells to enter S phase. J Biol Chem *272*, 29301-29308.
Tanida, I. (2011). Autophagosome formation and molecular mechanism of autophagy. Antioxid Redox Signal *14*, 2201-2214.

Tanida, I., Ueno, T., and Kominami, E. (2008). LC3 and Autophagy. Methods Mol Biol *445*, 77-88.
Tanida, I., and Waguri, S. (2010). Measurement of autophagy in cells and tissues. Methods Mol Biol *648*, 193-214.
Tee, A. R., Anjum, R., and Blenis, J. (2003a). Inactivation of the tuberous sclerosis complex-1 and -2 gene products occurs by phosphoinositide 3-kinase/Akt-dependent and -independent phosphorylation of tuberin. J Biol Chem *278*, 37288-37296.
Tee, A. R., Fingar, D. C., Manning, B. D., Kwiatkowski, D. J., Cantley, L. C., and Blenis, J. (2002). Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling. Proc Natl Acad Sci U S A *99*, 13571-13576.
Tee, A. R., Manning, B. D., Roux, P. P., Cantley, L. C., and Blenis, J. (2003b). Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol *13*, 1259-1268.
Toyoshima-Morimoto, F., Taniguchi, E., Shinya, N., Iwamatsu, A., and Nishida, E. (2001). Polo-like kinase 1 phosphorylates cyclin B1 and targets it to the nucleus during prophase. Nature *410*, 215-220.
van Eeghen, A. M., Black, M. E., Pulsifer, M. B., Kwiatkowski, D. J., and Thiele, E. A. (2012a). Genotype and cognitive phenotype of patients with tuberous sclerosis complex. Eur J Hum Genet *20*, 510-515.
van Eeghen, A. M., Chu-Shore, C. J., Pulsifer, M. B., Camposano, S. E., and Thiele, E. A. (2012b). Cognitive and adaptive development of patients with tuberous sclerosis complex: a retrospective, longitudinal investigation. Epilepsy Behav *23*, 10-15.
van Eeghen, A. M., Pulsifer, M. B., Merker, V. L., Neumeyer, A. M., van Eeghen, E. E., Thibert, R. L., Cole, A. J., Leigh, F. A., Plotkin, S. R., and Thiele, E. A. (2013). Understanding relationships between autism, intelligence, and epilepsy: a cross-disorder approach. Dev Med Child Neurol *55*, 146-153.
van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, D., van den Ouweland, A., Halley, D., Young, J., *et al.* (1997). Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. Science *277*, 805-808.
van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J., Snell, R., van den Ouweland, A., Reuser, A., Sampson, J., Halley, D., and van der Sluijs, P. (1998). Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. Hum Mol Genet *7*, 1053-1057.
Vermeulen, K., Van Bockstaele, D. R., and Berneman, Z. N. (2003). The cell cycle: a review of regulation, deregulation and therapeutic targets in cancer. Cell Prolif *36*, 131-149.
Walker, D. H., and Maller, J. L. (1991). Role for cyclin A in the dependence of mitosis on completion of DNA replication. Nature *354*, 314-317.
Wataya-Kaneda, M., Tanaka, M., Hamasaki, T., and Katayama, I. (2013). Trends in the Prevalence of Tuberous Sclerosis Complex Manifestations: An Epidemiological Study of 166 Japanese Patients. PLoS One *8*.
Webb, D. W., Fryer, A. E., and Osborne, J. P. (1991). On the incidence of fits and mental retardation in tuberous sclerosis. J Med Genet *28*, 395-397.
Wienecke, R., Konig, A., and DeClue, J. E. (1995). Identification of tuberin, the tuberous sclerosis-2 product. Tuberin possesses specific Rap1GAP activity. J Biol Chem *270*, 16409-16414.
Wienecke, R., Maize, J. C., Jr., Shoarinejad, F., Vass, W. C., Reed, J., Bonifacino, J. S., Resau, J. H., de Gunzburg, J., Yeung, R. S., and DeClue, J. E. (1996). Co-localization of the TSC2 product tuberin with its target Rap1 in the Golgi apparatus. Oncogene *13*, 913-923.
Wiles, M. V., Qin, W., Cheng, A. W., and Wang, H. (2015). CRISPR-Cas9-mediated genome editing and guide RNA design. Mamm Genome *26*, 501-510.
Wullschleger, S., Loewith, R., and Hall, M. N. (2006). TOR signaling in growth and metabolism. Cell *124*, 471-484.
Yang, J., Bardes, E. S., Moore, J. D., Brennan, J., Powers, M. A., and Kornbluth, S. (1998). Control of cyclin B1 localization through regulated binding of the nuclear export factor CRM1. Genes Dev *12*, 2131-2143.
York, B., Lou, D., and Noonan, D. J. (2006). Tuberin nuclear localization can be regulated by phosphorylation of its carboxyl terminus. Mol Cancer Res *4*, 885-897.

Yu, Z., Zhang, X., Guo, H., and Bai, Y. (2014). A novel TSC2 mutation in a Chinese family with tuberous sclerosis complex. J Genet *93*, 169-172.
Zeng, L. H., Rensing, N. R., Zhang, B., Gutmann, D. H., Gambello, M. J., and Wong, M. (2011). Tsc2 gene inactivation causes a more severe epilepsy phenotype than Tsc1 inactivation in a mouse model of Tuberous Sclerosis Complex. Hum Mol Genet *20*, 445-454.
Zeng, L. H., Xu, L., Gutmann, D. H., and Wong, M. (2008). Rapamycin Prevents Epilepsy in a Mouse Model of Tuberous Sclerosis Complex. Ann Neurol *63*, 444-453.
Zhang, J., Kim, J., Alexander, A., Cai, S., Tripathi, D. N., Dere, R., Tee, A. R., Tait-Mulder, J., Di Nardo, A., Han, J. M., *et al.* (2013). A tuberous sclerosis complex signalling node at the peroxisome regulates mTORC1 and autophagy in response to ROS. Nat Cell Biol *15*, 1186-1196.

VITA AUCTORIS

Jessica Morgan Dare-Shih was born in Windsor Ontario in 1990. She began her undergraduate degree in 2008 at the University of Windsor and graduated with honours in Biological Sciences with Thesis in 2012. She is currently a candidate for a Master's degree in Biological Sciences at the University of Windsor and plans to continue on to a PhD in Biological Sciences at the University of Windsor in 2016.