DYSREGULATED mTOR SIGNALING AND TISSUE-SPECIFIC PHENOTYPES IN TUBEROUS SCLEROSIS COMPLEX

By

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Dissertation

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

DOCTOR OF PHILOSOPHY

in

Cell and Developmental Biology

December, 2013

Nashville, Tennessee

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To Keith Musolff and Nancy Wall

for their teaching, support, and mentorship

throughout my education

ACKNOWLEGEMENTS

First, I would like to thank my mentor Kevin for the exciting rotation six years ago and accepting me into the lab for the last four. Thank you for being such an encouraging and patient mentor during this training – you've taught me so much about being a physician scientist. You've been there through the early periods of good data, and the dark days of live cell calcium imaging, cilia in zebrafish, and the ups and downs of autophagy. I particularly appreciate your enthusiasm as a PI to dive into the newest results without hesitation or knowing where they will lead. As your first graduate student, I can confidently say you've got the hang of this mentoring thing pretty quickly.

To the current and past members of the Ess lab, thank you for your kindness and support over the last few years. You've always been there to help with a technique, talk about data analysis, or simply grab a coffee. Rob and Cary, thank you for being secondary PI's for me – and for the usually insightful and often humorous un-PC conversations about death, politics, and religion. Peggy, thank you for keeping the mouse colony running so smoothly and the lab in one piece – my kidney project could not have been done without you. Bryan, thank you for also keeping the lab running – and making and caring for those iPSCs, like no one else. Laura, its been great having you in lab and I hope your time here is as excellent for you as it has been for me.

Thank you to all my Vanderbilt friends and members of TDSG that have been here as long, or nearly as long as I have. You've given me lifelong memories and friendships, and have made the work of medical and graduate

school that much more enjoyable. From the science advice and loaned reagents, to the dinners, board game gatherings, nights out on the town, and weddings – the experiences and support from all of you have been so important in my journey through school to this point.

To Keith, Pam, Jenn, Christy, Jeff, Danielle, Melody, Laura and all the rest of my non-science and non-Vanderbilt friends, thank you so much for the chats, dinners, parties, and trips that kept me sane during these years. You kept me connected to the outside word, and all put up with my strange hours and ramblings about mTOR signaling. You've been so important for supporting my education and growth.

Thank you to the Vanderbilt MSTP, for accepting me and giving me the opportunity to learn and grow in this program. To Terry, Larry, Michelle, Melissa, and Jim, your leadership and work makes this program shine, and I wouldn't trade my time here for anything in the world.

To Keith Musolff and Nancy Wall, thank you for being the mentors and teachers I needed throughout different times in my education. Both academically and personally, you have been such a positive force in my life. Thank you for always being there to push me farther in my education and for encouraging me to take leadership roles and be the best in whatever I did. Thank you both for showing me the value of individualized learning. And Nancy, thank you for sharing your love of developmental biology with me.

I'd like to thank my current and past committee members for helping guide my research, through all three model systems, and seemingly ten different

specific aims I pursued. Your insight and questioning has impacted my education immensely much. Thank you for helping me narrow and focus my energies, and not allowing me to spread myself too thin.

To all of my family, thank you so much for a lifetime of encouragement and support. You have made me into who I am today. Mom and dad, thank you for always being there for me and for nurturing my love of science and learning. From fun summer vacations, to working together on school projects, you've given so much to me. Grandma and Grandpa, thank you for always listening to me, even when you might not have understood the bulk of what I was talking about. And when I was a kid, thank you for always making me go grab a map or World Book Encyclopedia to learn new facts and double-check what I thought I already knew.

And finally, to Brandon, thank you for being with me during the duration of my graduate school years. Thank you for waiting patiently in Atlanta while I finish up my time here in Nashville. Thank you for putting up with weekends apart when I had experiments to finish. Thank you for providing a second home for me in Atlanta. The last four years here wouldn't have been possible without you with me. Thank you so much for all of that, and here's to a lifetime of sharing more of these successes, challenges, and celebrations together.

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List of Abbreviations

4E-BP1, Eukaryotic initiation factor 4E-binding protein 1

Aqp1, Aquaporin 1

Aqp2, Aquaporin 2

ASD, Autism spectrum disorders

AML, Angiomyolipoma

BUN, Blood Urea Nitrogen

CKO, Conditional knockout

DCT, Distal convoluted tubule

EEG, Electroencephalogram

eIF4E, Eukaryotic initiation factor 4E

eIF4G, Eukaryotic initiation factor 4G

ESC, Embryonic stem cell

FKBP12, FK506-binding protein of 12 kDa

FXS, Fragile X Syndrome,

GAP, GTPase activating protein

GEF, Guanine nucleotide exchange factor

GFP, Green fluorescent protein

GTP, Guanosine-5'-triphosphate

GDP, Guanosine diphosphate

iPSC, Induced pluripotent stem cell

IQ, Intelligence Quotient

KOSR, Knockout serum replacement

MECP2, Methyl CpG binding protein 2

mGluR5, Metabolic glutamate receptor 5

mLST8, mTOR associated protein, LST8

mRNA, Messenger ribonucleic acid

mTOR, Mechanistic target of rapamycin

mTORC1, Mechanistic target of rapamycin complex 1

mTORC2, Mechanistic target of rapamycin complex 2

Oct4, Octamer-binding transcription factor 4

P, Postnatal day

PCT, Proximal convoluted tubule

PKCα, Protein kinase Cα

PKD, Polycystic kidney disease

RCC, Renal cell carcinoma

Raptor, Regulatory-associated protein of mTOR

RHEB, Ras homologue enriched in brain

Rictor, Rapamycin insensitive companion of mTOR

ROCK, Rho associated coiled-coiled kinase

Sox2, Sex determining region Y-box 2

S6K1, p70-S6 Kinase 1

S6, p70-Ribosomal protein S6

TSC, Tuberous sclerosis complex

CHAPTER I

INTRODUCTION TO TUBEROUS SCLEROSIS COMPLEX AND mTOR SIGNALING

Overview

Tuberous sclerosis complex (TSC) is a multi-system developmental disorder found in approximately 1 in 6,000 people that affects the brain, kidney, skin, heart, and lungs (Crino, Nathanson, and Henske 2006; Curatolo, Bombardieri, and Jozwiak 2008; Osborne, Fryer, and Webb 1991). Affected organs have prominent hamartomas - benign focal lesions containing disorganized tissue that is related to the organ of origin. Hamartomas in TSC include shagreen patches and hypopigmented macules of the skin, angiomyolipomas of the kidney, and cortical tubers in the brain (Crino, Nathanson, and Henske 2006). Patients with TSC can have symptoms arising from dysfunction of any of these organ systems, but neurologic symptoms are usually most severe and include epilepsy, autism, and developmental delay.

While the tuberous sclerosis complex was identified as a unique disease by clinical presentations more than 125 years ago, it was not until the last two decades that advances in genetics allowed the causative genes, *TSC1* and *TSC2,* upstream regulators of the mechanistic target of rapamycin (mTOR) signaling pathway, to be identified (Bourneville 1880; van Slegtenhorst et al. 1997; Consortium 1993). Much research has focused on the contribution of

dysregulated mTOR signaling in the pathogenesis of tuberous sclerosis. The protein products of *TSC1* and *TSC2* form a heterodimer to function as an inhibitor of mTOR complex 1 (mTORC1), a major signaling component in many cellular processes, including protein translation, metabolism, cell size regulation, cellular differentiation and tissue patterning. Current models of TSC propose a "two-hit hypothesis" for disease pathogenesis. In this manner, patients with TSC inherit a mutant copy of either *TSC1* or *TSC2*. The current model predicts the generation of hamartomatous tissue in whichever organ systems there is a loss of heterozygosity at either the *TSC1* or *TSC2* loci. A somatic mutation in the remaining functional copy of either of these genes results in dysregulated mTOR signaling and subsequent hamartoma generation.

My primary research has been focused on how mutations in *TSC1* or *TSC2* result in tissue-specific pathologies characteristic of tuberous sclerosis complex. Specifically, I have explored the mechanisms of tissue-specific disease pathogenesis – with a loss of heterozygosity driving pathogenesis in the kidney and haploinsufficiency possibly driving pathogenesis in neural progenitor cells and neuronal tissues. Chapter I will provide a clinical context to the phenotypes seen in patients with TSC, focusing on the skin, kidney, and neurologic lesions characteristic of TSC. Chapter I will go on to provide background into the current understanding of the genetics and molecular mechanisms of dysregulated mTOR signaling and TSC. Finally I will describe the current model of TSC pathogenesis, following Knudson's two-hit hypothesis while discussing current limitations of this canonical model. Chapter II will describe how the canonical two-hit model of

tuberous sclerosis complex is helpful in understanding the pathogenesis of kidney lesions in this disease. Evidence will be presented that supports increased mTORC1 signaling being responsible for cystogenesis in these patients, possibly through disruption of the primary cilium. Chapter III will demonstrate mTORC1-dependent phenotypes in heterozygous human stem cells derived from TSC patients. These phenotypes include increased pluripotency and cell survival in the absence of a second hit mutation – in contradiction to the classical model. Chapter IV will discuss the importance of these data for understanding a multisystem disease such as TSC as a collection of various tissue-specific pathogenic mechanisms. Chapter IV will highlight possible future directions and extension of this research to further understand the various disease manifestations of TSC. Finally, broader implications of this research to the pathogenesis of autism and epilepsy will be addressed.

Clinical Manifestations of Tuberous Sclerosis Complex

One of the first clinical descriptions of tuberous sclerosis complex was published in 1880 by the neurologist Désiré-Magloire Bourneville, who reported a patient with facial angiofibromas, epilepsy and severe developmental delay (Bourneville 1880). On autopsy, Dr. Bourneville identified many brain lesions that resembled “hard potatoes” in the brain, giving rise to the Latinized name of this disease, “tuberous sclerosis.” Additionally, this initial patient also exhibited hard white lesions on both kidneys. From this initial description, and the accumulation

of reports of other patients with similar symptoms, the most common signs of TSC were codified in 1908 by Heinrich Vogt into a useful diagnostic triad which included epilepsy, developmental delay, and facial angiofibromas (Vogt 1908). Vogt's triad remained the standard of diagnosis for TSC for nearly 60 years. Diagnostic criteria were ultimately revised because, while each member of the triad alone was very common in TSC patients, the complete triad was present in fewer than one third of all patients (Lagos and Gomez 1967). The revised diagnostic criteria opted to focus on the *signs* of pathologic lesions in the organ systems, such as cortical tubers, rather than the *symptoms* these lesions cause, such as epilepsy (**Table 1.1**) (Roach, Gomez, and Northrup 1998; Roach and Sparagana 2004).

Of the pathologies present in TSC, neurological manifestations are highly prevalent and are usually the most concerning symptoms clinically. The most common brain lesions in TSC are cortical tubers, found in more than 90% of patients. These tubers contain disorganized atypical glial and neuronal cell types, usually with accompanying astrocytosis (Yates et al. 2011). Cortical tubers arise very early in brain development. Autopsies of aborted fetuses with TSC display cortical tubers at 19 weeks of gestation, indicating generation of these tubers during periods of neurogenesis and restructuring of the cortex (Park et al. 1997; Wei et al. 2002). Cortical tubers have blurred boundaries of the gray and white matter, irregular arrangement of neurons, diffuse astrocytosis and very large, poorly differentiated cells called "giant" cells (Mizuguchi and Takashima 2001). Within the cortical tubers reside individual cells that express markers of glia,

Category	Major Criteria	Minor Criteria
Brain	Glioneuronal hamartomas Subependymal nodules Subependymal giant cell astrocytoma	Cerebral white matter migration lines Retinal achromic patch
Skin	Facial angiofibromas or forehead plaques Shagreen patch Three or more hypomelanotic macules Ungula or periungual fibromas	Confetti skin lesions Gingival fibromas
Kidney	Renal angiomyolipoma	Multiple renal cysts
Other	Cardiac rhabdomyoma Multiple retinal nodular hamartomas Lymphangioleiomyomatosis	Multiple pits in dental enamel Hamartomatous rectal polyps Nonrenal hamartomas Bone cysts

Table 1.1 Diagnostic criteria for Tuberous Sclerosis Complex.

TSC probability is divided into three categories based on these findings:

Definite TSC: Either 2 major features or 1 major feature with 2 minor features
Probable TSC: One major feature and one minor feature
Possible TSC: Either 1 major feature or 2 or more minor features

Adapted from (Roach, Gomez, and Northrup 1998) and (Roach and Sparagana 2004)

immature neurons, and fully mature neurons, supportive of defects in cellular differentiation and maturation (Crino et al. 1996; Jozwiak, Jozwiak, and Skopinski 2005; Ess et al. 2004). Typical characteristics of a cortical tuber are shown in **Figure 1.1**. Both the numbers of cortical tubers present, as well as the proportion of brain occupied by these lesions, have been correlated with the severity of neurological impairment (Goodman et al. 1997; Jansen et al. 2008). Having more than the median number of tubers is correlated with poor seizure control as well as moderate to severe cognitive impairment (Goodman et al. 1997). Patients with an increased tuber volume-to-brain ratio tend to have earlier onset of epilepsy and decreased cognitive functioning (Jansen et al. 2008). These cortical lesions are thought to be responsible for the most severe neurological phenotypes of TSC, including epilepsy, cognitive deficits, and autism (Ess 2009).

Of the neurological symptoms of TSC, epilepsy is the most prevalent, affecting up to 90% of patients (Yates et al. 2011; McClintock 2002). The majority of TSC patients (60%) will have a seizure in the first year of life and nearly all TSC patients who have a single seizure at some point in life will develop epilepsy (Webb, Fryer, and Osborne 1991; Chu-Shore et al. 2010). The epileptogenic foci can often be localized to one or more cortical tubers in these patients. In cases of medically intractable epilepsy, resection of the localizing tuber is frequently a successful treatment option (Koh et al. 2000; Jansen et al. 2005). Not all cortical tubers, however, are epileptogenic and conversely, abnormal EEG activity can sometimes also be seen from structurally normal areas adjacent to tubers in TSC patients. This suggests that histologically normal brain tissue may still behave

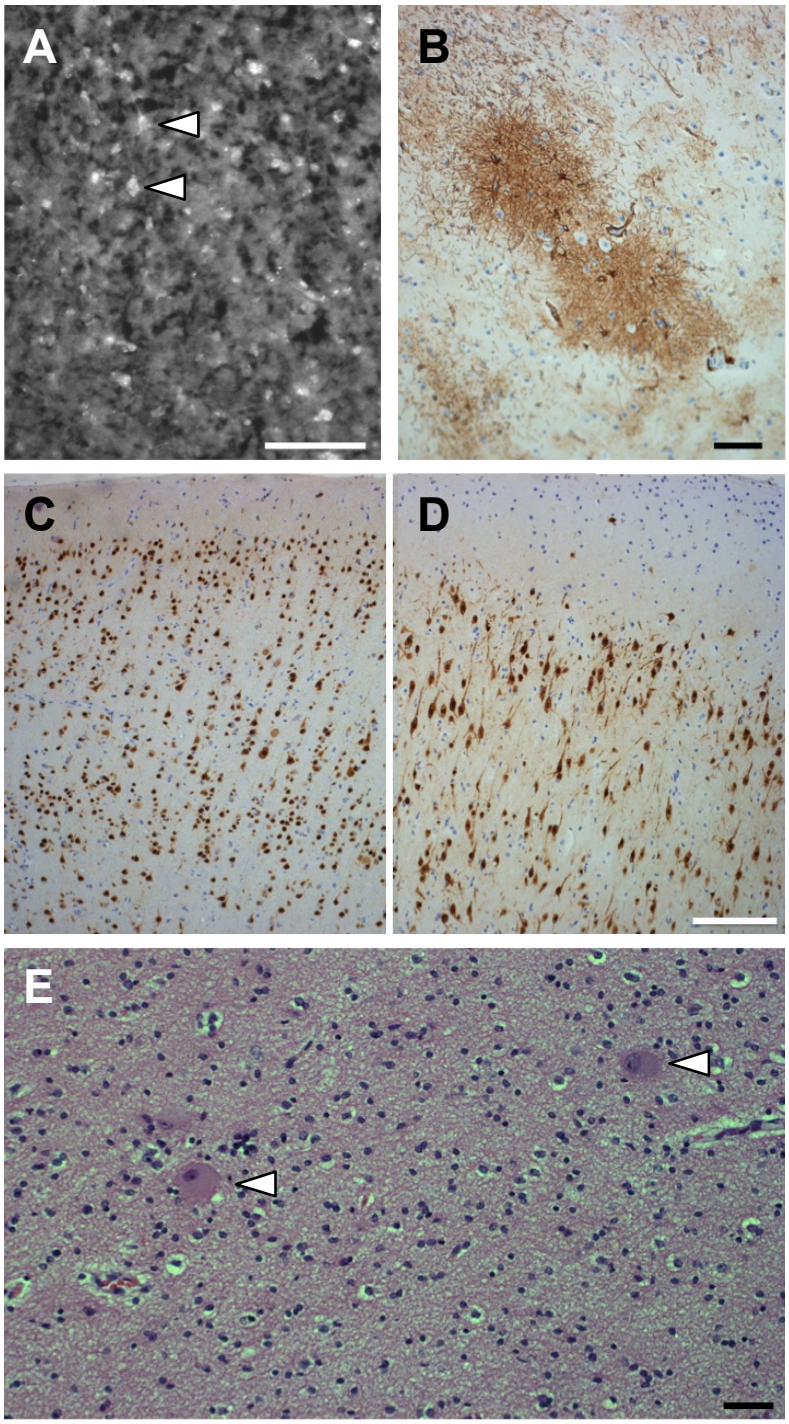


Figure 1.1 Histological features of cortical tubers. (A) Tubers contain immature cells that stain for neuroprogenitor markers such as Pax6. Two such neurons are shown at the arrowheads. (B) Cortical tubers also display diffuse astrocytosis as shown through staining for GFAP, a marker of astrocytes. (C-D) Laminar patterning of the cortex through Tuj1 staining. Neurons lose their laminar organization in cortical tubers (D) as compared to normal brain tissue (C). (E) Large eosinophilic cells called “Giant” Cells are also characteristic of cortical tubers seen in TSC, shown at arrowheads.

abnormally at the molecular level (Thiele 2010; Holmes and Stafstrom 2007; Chugani et al. 1998; Major et al. 2009).

While epilepsy is by far the most common neurological manifestation of TSC, cognitive deficits and autism are also seen in many patients. Cognitive deficits are found in 44-65% of TSC patients and are correlated with severity of epilepsy in these patients (Yates et al. 2011; Joinson et al. 2003). Furthermore, even in patients within the normal range of IQ, patients had on average 10-point lower scores compared to unaffected siblings (Joinson et al. 2003). Autistic behaviors are found in approximately 50% of TSC patients and are a major source of stress for TSC patient caregivers (Kopp, Muzykewicz et al. 2008).

Apart from the neurological manifestations of TSC, renal pathologies are common and diverse. Renal manifestations of TSC include cysts, angiomyolipomas (AML) and rarely, renal cell carcinoma (RCC) (Washecka and Hanna 1991; O'Callaghan et al. 2004). AMLs occur in up to 70% of patients with TSC and cysts occur in approximately 30% of patients (O'Callaghan et al. 2004). Unlike brain lesions, which arise prenatally, many kidney lesions are not present at birth. By 10 years of age, however, 80% of TSC patients have detectable kidney lesions (Ewalt et al. 1998). Many of these lesions remain asymptomatic, but if these lesions progress to affect kidney function, defects are not usually seen until after the second decade of life. Although these lesions are most commonly benign, renal complications are the most common cause of TSC-related mortality (Shepherd et al. 1991).

Angiomyolipomas, common in TSC, are renal hamartomas that are composed of disorganized arrangements of vasculature, smooth muscle, and adipocytes. Due to extensive vascularization of these hamartomas, rupture and bleeding is an important complication of AMLs. TSC patients with symptomatic AMLs often have flank pain and hematuria (Steiner et al. 1993). Rarely, if the rupture is particularly severe, a medical emergency called Wunderlich syndrome can occur. Large ruptures can cause hypovolemic shock and require immediate surgery to stop hemorrhaging (Moratalla 2009).

Renal cysts are both common and generally asymptomatic in TSC. However, a small subset of patients with TSC will develop severe polycystic kidney disease (PKD) (van Slegtenhorst et al. 1997). These patients usually have detectible kidney lesions at birth and progress to renal failure by early adulthood (van Slegtenhorst et al. 1997; O'Callaghan et al. 2004). As described in Chapter II, these patients often have a large deletion in the *TSC2* gene and occasional involvement of the neighboring polycystic kidney disease 1 (*PKD1*) gene.

Unlike neurological and renal pathologies, dermatologic manifestations are less concerning clinically, yet they are very useful diagnostically. Dermatologic manifestations are the most common signs of TSC and are seen in up to 98% of TSC patients (Webb, Fryer, and Osborne 1996). The most common skin manifestations of TSC include hypopigmented macules called ash-leaf spots, angiofibromas of the face, and raised areas of skin, usually on the lower back, called shagreen patches. Hypopigmented macules are often the earliest skin lesions identified. Shagreen patches typically appear later, often around 5 years

of age. Facial angiofibromas become more common during adolescence (Webb et al. 1996).

Mutations in the *TSC1* and *TSC2* genes are causative for TSC

Accurate documentation of TSC cases and the development of Vogt's triad by the early 1900s lead to extensive pedigree analyses for TSC. The first familial case of TSC was identified in 1913 and suggested an autosomal dominant means of inheritance (Berg 1913). However, it was not until advances in molecular biology and genetics that the causative genes for TSC were identified. Linkage analyses identified two distinct loci causative for TSC (Janssen et al. 1991). The genes at these loci were subsequently cloned and named *TSC1* and *TSC2* (Consortium 1993; van Slegtenhorst et al. 1997). *TSC1* was the first gene identified. It is located at 9q34 and encodes a transcript of 8.6kb that contains 23 exons. The second gene identified, *TSC2,* is located at 16p13.3 and encodes a transcript of 5.5 kb that contains 41 exons. The protein products of *TSC1* and *TSC2* are hamartin and tuberin, respectively and have no homology to each other but function together as a heterodimer. Mutations of either gene are causative for TSC. In the current model, hamartin binds to tuberin, thus stabilizing the entire complex. Tuberin then mediates its function through activity of its GAP domain. The individual functions of these two proteins provides some rationale for both the types of mutations in each gene that result in TSC as well as the relative proportion of disease caused by each gene. With a functional

GAP domain, mutations at the *TSC2* locus are more likely to inhibit function of the protein product and cause disease. Causative *TSC2* mutations are most often nonsense mutations, large deletions and missense mutations (within the GAP domain) (Maheshwar et al. 1997). *TSC1,* encoding for a stabilizing protein, is less sensitive to missense mutations disrupting function, and of TSC patients with *TSC1* mutations, most have nonsense mutations or small deletions resulting in a frameshift (Maheshwar et al. 1997). Of TSC patients with identifiable mutations, *TSC1* mutations are found only in approximately 20% of TSC cases; the majority of TSC cases are caused by mutations in *TSC2* (Jones et al. 1999). Generally patients with *TSC2* mutations have more severe phenotypes than those with mutations in *TSC1* (Jones et al. 1997). Up to 15-20% of clinically diagnosed patients do not have any identifiable *TSC1* or *TSC2* mutations (Sancak et al. 2005). Patients with unidentifiable mutations tend to have milder clinical phenotypes, and failure to detect mutation may be due to somatic mosaicim or mutations in non-coding yet important regulatory regions of these two genes (Kwiatkowska et al. 1999).

The hamartin/tuberin complex and mTOR signaling

The protein products of *TSC1* and *TSC2,* hamartin and tuberin respectively, form a complex that regulates signaling through the mechanistic target of rapamycin (mTOR) kinase. The pathogenesis of TSC is thought mainly to result from the dysregulation of the mTOR kinase signaling pathway

secondary to mutations in upstream signaling components. The mTOR signaling pathway has been implicated in the regulation of many cellular processes, including cell growth, proliferation, motility, cell survival, gene transcription and protein synthesis (Inoki, Corradetti, and Guan 2005). mTOR, a serine/threonine kinase, mediates these functions with distinct protein binding partners. Two complexes containing mTOR have been well defined and have specific downstream targets. The mTOR-Raptor-mLST8/GβL complex, or mTOR complex 1 (mTORC1), signals through p70-S6 Kinase 1 (S6K1) and 4E-BP1, and mainly affects translational activity and autophagy (Tee and Blenis 2005). The mTOR-Rictor-GβL-mSin1 complex, or mTOR complex 2 (mTORC2), activates Akt and Protein Kinase Cα (PKCα) and affects cell proliferation and cytoskeletal architecture (Garcia-Martinez and Alessi 2008; Sarbassov et al. 2004). Rapamycin, the chemical compound from which mTOR derives its name is a mTORC1 specific inhibitor. mTORC2, which contains Rictor (rapamycin insensitive companion of mTOR), is largely unaffected by rapamycin treatment (Jacinto et al. 2004). Raptor and Rictor appear to be mutually exclusive binding partners of mTOR, controlling substrate specificity as well as the sensitivity to rapamycin. In normal tissue, the hamartin/tuberin complex regulates mTOR signaling activity with negative regulation of mTORC1 and positive regulation of mTORC2 (Huang et al. 2009).

Hamartin, a 140-kilodalton (kDa) protein, and tuberin, a 200-kDa protein with a C-terminal GTPase activating (GAP) domain, function together but are not homologous. Both proteins are ubiquitously expressed, although highest levels

are found in the brain, kidney, and heart (Plank et al. 1999). The hamartin/tuberin complex is an important signaling hub in the mTOR kinase signaling pathway and differential phosphorylation of the complex integrates cues from upstream signaling components responding to growth factors, stress, energy status, oxygen levels, and amino acid availability. Some of the upstream pathways that signal through the hamartin/tuberin complex include AKT, ERK, WNT, and Rag-GTPases (Manning et al. 2002; Ma et al. 2005; Inoki et al. 2006; Kim, Goraksha-Hicks et al. 2008). Growth factors and mitogen signaling usually cause increased mTORC1 activity through inhibition of the hamartin/tuberin complex. Insulin-like growth factors cause activation of Akt, which phosphorylates tuberin and inactivates its GAP domain (Manning et al. 2002; Inoki et al. 2002). Activation of the Ras/MAPK pathway also results in inhibitory tuberin phosphorylation by ERK. Phosphorylation by ERK causes the hamartin/tuberin heterodimer to dissociate and allows degradation of the individual proteins (Ma et al. 2005). Both of these inhibitory phosphorylation events of tuberin result in increased mTORC1 activity. During cellular stress, mTOR activity is generally decreased, partially due to augmentation of the inhibitory action of the hamartin/tuberin complex. In low glucose environments for example, AMPK phosphorylates tuberin to enhance its GAP domain activity (Inoki, Zhu, and Guan 2003). In hypoxic conditions, upregulation of REDD1 causes sequestered tuberin to be released and promotes hamartin/tuberin complex formation (DeYoung et al. 2008). Through stimulation of GAP domain function and promotion of hamartin/tuberin complex formation, cellular stress pathways can effectively inhibit mTORC1 activity.

Classically, this heterodimer complex is thought to be functional throughout the cytoplasm, but more recently these two proteins have been recognized to preferentially locate to the Golgi apparatus, lysosome, nucleus, and basal body of the primary cilia (Wienecke et al. 1996; York, Lou, and Noonan 2006; Hartman et al. 2009). Differential subcellular localization of these two components may be another way, in addition to differential phosphorylation, that convergent pathways integrate their signal through this complex to control mTOR output.

mTOR Complex 1 (mTORC1)

The upstream regulation and downstream targets of mTOR Complex 1 are the most thoroughly understood components of the mTOR signaling pathway. Upstream of mTORC1, hamartin binds and stabilizes tuberin by preventing ubiquitylation of the complex (Benvenuto et al. 2000). This stabilization allows the C-terminal GAP domain of tuberin to inhibit Ras homologue enriched in brain (RHEB). The hamartin/tuberin complex then induces the conversion of the active RHEB-GTP bound state to the inactive RHEB-GDP state. In its active state, RHEB is a potent stimulator of mTORC1. RHEB-GTP stimulates mTOR signaling through antagonistic binding of FKBP38, an endogenous inhibitor of mTOR activity (Bai et al. 2007). While the hamartin/tuberin complex GTPase activity stimulates a GTP-to-GDP transition and inhibits RHEB activity, there is no established guanine nucleotide exchange factor (GEF) for RHEB to reverse this

process. Regulation of the reactivation of RHEB is still poorly understood, but may be another important means of mTOR regulation.

In normal cells, signals from growth factors, high nutrient availability, or other pathways converge as described earlier to trigger inhibition of the hamartin/tuberin complex, allowing mTORC1 stimulation by RHEB and subsequent activation of cellular processes that result in increased cell growth and metabolism. The two most studied substrates of mTORC1, p70-S6 Kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), both help regulate protein translational machinery (von Manteuffel et al. 1997; Hara et al. 1998). Phosphorylation of S6K1 by mTORC1 results in downstream phosphorylation of 40S ribosomal protein S6 (S6), eukaryotic elongation factor 2 kinase, and a 80-kDa nuclear cap binding protein, the combination of which causes the recruitment of ribosomal machinery (Khaleghpour et al. 1999). Phosphorylation of 4E-BP1 by mTORC1 disrupts 4E-BP1 binding to mRNA. Free mRNA can then bind eukaryotic initiation factor 4G (eIF4G) and recruit the translational machinery activated by S6K1 (Hara et al. 1997). Therefore, both of these targets of mTORC1 cooperatively work to increase cellular protein translation.

While mTORC1 signaling has the greatest effect on translation, there are many other downstream targets of this complex. mTORC1 inhibits activation of autophagy through phosphorylation of ULK1, a protein that plays a role in the formation of autophagic vesicles (Jung et al. 2009). mTORC1 also stimulates *de novo* lipid biogenesis and membrane formation through a PPAR-γ dependent

mechanism (Laplante and Sabatini 2009). Activation of mTORC1 promotes lipogenesis in response to insulin signaling (Li, Brown, and Goldstein 2010). mTORC1 signaling also influences cellular proliferation and survival. Increased lipid biogenesis and increased translation are prerequisites for normal cell proliferation; increased mTORC1 activity provides both new membrane for cell division and the ribosomal machinery needed for cell growth prior to mitosis. It has also been shown that the phosphorylation of the two main downstream targets of mTORC1, S6K1 and 4E-BP1, is required for G_1 phase progression and cell cycle control (Fingar et al. 2004). mTORC1 activity has also been shown to increase levels of cyclins A, D1, and E causing cells to progress more quickly through the cell cycle (Ito and Rubin 1999; Tapon et al. 2001; Vadlakonda, Pasupuleti, and Pallu 2013). While mTORC1 signaling has been most closely associated with translation, effects are seen across other critical cellular processes.

Rapamycin, the drug from which the mechanistic target of rapamycin derives its name, is an mTORC1-specific inhibitor. Rapamycin is a highly selective agent that has minimal off-target effects even at concentrations 10 to 20-fold greater than the levels normally used to completely inhibit mTORC1 in cell culture assays (Davies et al. 2000). Rapamycin binds FK506-binding protein of 12 kDa (FKBP12) (Brown et al. 1994). The FKBP12-rapamycin complex can then cooperatively bind mTOR, restricting access to mTORC1 substrates and propagation of mTOR signaling (Yang et al. 2013). Furthermore FKBP12-

Rapamycin can also sequester mTOR, preventing assembly of mTORC1 (Oshiro et al. 2004).

mTOR Complex 2 (mTORC2)

mTOR can also interact with Rictor to form a second complex that is rapamycin-insensitive and is not regulated through RHEB activity. The upstream regulation and downstream targets of mTORC2 are much less well understood than those of mTORC1. There is evidence that the hamartin/tuberin complex can directly bind and positively regulate mTORC2 (Huang et al. 2009). This is in direct contrast to the negative regulation of the hamartin/tuberin complex on mTORC1. However, control of mTORC2 independent of the hamartin/tuberin complex has also been described, mainly downstream of the phosphoinositide-3 kinase/Akt pathway (Dalle Pezze et al. 2012). While mTORC1 downstream targets mainly include protein translational control, mTORC2 is thought to regulate the actin cytoskeleton and therefore cellular morphology (Jacinto et al. 2004). The two main downstream targets of mTORC2 are Akt and protein kinase Cα (PKCα). mTORC2 phosphorylates Akt, priming Akt for further activation through other pathways. This allows interesting and complicated feedback loops, as Akt signaling is upstream of both mTORC1 and mTORC2 activity (Sarbassov et al. 2005). mTORC2 stimulation of PKCα and reorganization of the actin cytoskeleton is thought to largely underlie the effect of mTORC2 on cellular structure (Sarbassov et al. 2004).

Current model of TSC pathogenesis

Current models of TSC suggest a mechanism akin to Knudson's two-hit hypothesis. In this model initially used to explain the pathogenesis of retinoblastoma, an individual inherits a single mutant copy of a tumor suppressor gene. A malignancy occurs when there is a somatic mutation, or "second hit," in the functioning copy of the gene, resulting in a loss of heterozygosity of that tumor suppressor gene (Knudson 1971). Applied to tuberous sclerosis complex, a patient inherits a mutation in either the *TSC1* or *TSC2* genes. The location and timing of a loss of heterozygosity event in an individual progenitor cell determines where the characteristic lesions of TSC manifest. For example, if a second hit is somatically acquired in a neuroprogenitor population, a cortical tuber forms, while a second hit in epidermal cells would result in a shagreen patch. In this model, loss of heterozygosity at either the *TSC1* or *TSC2* loci would result in the absence of the hamartin/tuberin heterodimer. This leads to dysregulated mTOR signaling, specifically hyperactivation of mTORC1 and downregulation of mTORC2 (**Figure 1.2**). Most work has focused on the role of hyperactivation of mTORC1 in the pathogenesis of hamartomatous tissue in TSC. Consistent with this model, loss of heterozygosity has been identified in many hamartomas, as well as hyperactivation of mTORC1 as detected by hyperphosphorylation of downstream mTORC1 targets (Sepp, Yates, and Green 1996; El-Hashemite et al. 2003). This loss of heterozygosity has been repeatedly identified in renal and

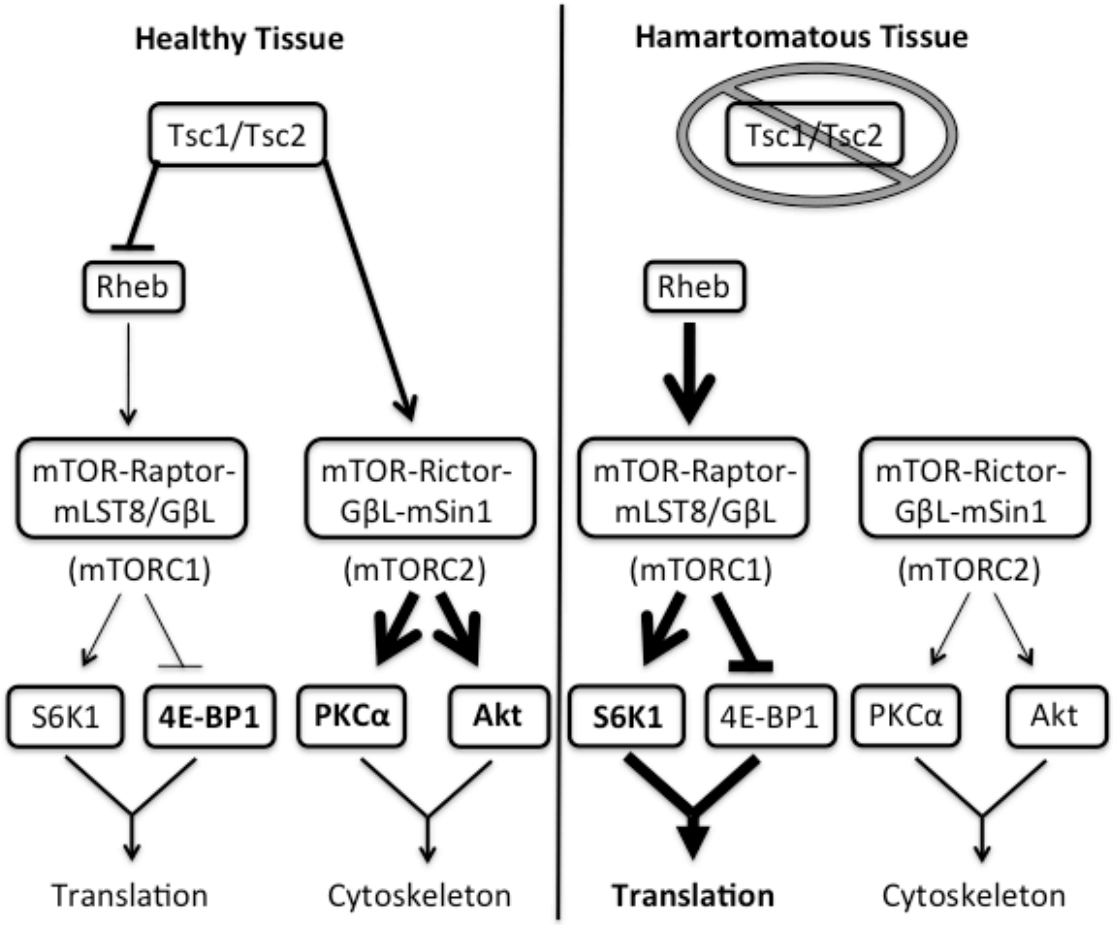


Figure 1.2 Schematized view of the canonical model of mTOR signaling in both healthy and hamartomatous tissue. In normal tissue, TSC1/TSC2 function together to regulate mTOR activity. In current models of TSC, loss of heterozygosity of either *TSC1* or *TSC2* results in the loss of the heterodimer complex and subsequent hyperactivation of mTORC1.

lung manifestations of TSC (Henske et al. 1997; Cai et al. 2010). Additionally, microdissection of these lesions shows the same acquired somatic mutation in all cell types found in the hamartoma, indicating a clonal origin for these tumors (Niida et al. 2001). Finally, in samples from renal and lung manifestations, there is clear loss of protein expression of either hamartin or tuberin, confirming the loss of heterozygosity in human lesions. The high rate of detectable second hits, clonal origin, loss of protein product, and hyperactivation of mTORC1 signaling all support the two-hit hypothesis as a mechanism for TSC pathogenesis.

While the two-hit hypothesis has found much support in non-neuronal tissue, in cortical tubers – the putative cause for the most debilitating symptoms of the disease – second hits have long been undetectable (Henske et al. 1996; Niida et al. 2001). Even with current sequencing techniques, second hits are rarely observed. Whole tuber DNA extraction and sequencing fail to identify acquired mutations. In one of the few reports of detected loss of heterozygosity in cortical tuber samples, identification of a second mutation was achieved through laser dissection of single giant cells from the tuber and then pooling these cells together for sequencing (Crino et al. 2010). These giant cells are electrically silent when studied *ex vivo* (Cepeda, Andre, Vinters et al. 2005; Cepeda, Andre, Flores-Hernandez et al. 2005). This suggests that they are not the primary electrical cause of seizures and other neurological manifestations in TSC. However, even if these very rare acquired mutations play a role in the

pathogenesis of TSC, this is a much different mechanism in hamartoma formation in the brain than in the clonal hamartomas seen in the lungs and kidneys. In cortical tubers, it appears that the vast majority of cells in the hamartomatous lesion are heterozygous for either *TSC1* or *TSC2*. This suggests two possible mechanisms for tuber pathogenesis. First, there could be non-cell-autonomous effects of loss of heterozygosity in the small subset of giant cells with a detected acquired somatic mutation. In this hypothesis, the rare occurrences of second hits result, not in a clonal expansion as seen in kidney and lung lesions, but, in foci which signal to otherwise normally appearing heterozygous cells. These heterozygous cells would then develop abnormally in response to signals from the nearby giant cell that has had a loss of heterozygosity. This results in localized disorganization during the differentiation and migration of these progenitors, leading to the pathologic cortical tubers seen in patients. In the second possibility, neuronal tissue is uniquely sensitive to alterations in hamartin and tuberin levels. In this case, haploinsufficiency of either *TSC1* or *TSC2* results in minor signaling changes due to a dosage change in either hamartin or tuberin. Neuronal tissue may then be uniquely sensitive to minor alterations in mTOR signaling. Unlike in the renal and lung lesions, in this model, loss of both alleles in the brain may then not be necessary for hamartoma pathogenesis. Further supporting haploinsufficiency as a mechanism for neural pathogenesis are clinical observations, where abnormal EEG activity can be seen from structurally normal areas of the brain that do not contain giant cells. These areas, therefore, should not be affected by non-cell-autonomous effects

from this cell population with acquired second hits. (Thiele 2010; Holmes and Stafstrom 2007; Chugani et al. 1998). Cortical tubers can also be electrically silent while the adjacent tissue is hyperexcitable (Major et al. 2009). Additionally, in patients without clear focal lesions in the brain, otherwise structurally normal tissue still can display metabolic abnormalities suggestive of axonal dysfunction and demyelination. Combined, these data suggest that histologically normal brain tissue, without a loss of heterozygosity, may still behave abnormally at the molecular level.

The presence of two very different mechanisms for the pathogenesis of TSC-associated lesions in different tissues presents challenges for relevant modeling of the disease. Furthermore, comparing patients with TSC and animal models is problematic due to species-specific pathologies. In rodent models of TSC, animals with heterozygous deletions of the *Tsc1* or *Tsc2* genes have far lower rates of non-neuronal lesions than that seen in humans, and brain pathologies such as cortical tubers are almost non-existent. The earliest model of TSC is the Eker rat, a spontaneously arising rodent model that features an inactivating mutation of the *Tsc2* gene (Yeung et al. 1994). The pathologies seen in this model are strikingly different than those seen in human patients. While Eker rats almost never have cortical tubers, they have nearly 100% penetrance for bilateral renal cell carcinoma, a finding that is incredibly rare in human patients (Hino et al. 1994). To model cortical tubers specifically, it is necessary to inactivate both copies of either *Tsc1* or *Tsc2.* Conventional knockouts for *Tsc1* and *Tsc2* are embryonic lethal (Rennebeck et al. 1998). To circumvent this

species-specific obstacle, tissue-specific knockouts of either *Tsc1* or *Tsc2* have been generated in mice using Cre-Lox recombination. For example, two complementary models that have been used to understand the role of mTOR signaling during neurodevelopment are dorsal and ventral forebrain-specific knockouts of *Tsc1* (Carson et al. 2012; Fu et al. 2012). Reminiscent of the human disease, these models show atypical giant cells, cortical dyslamination, and decreased seizure thresholds. These models, however, contain large numbers of cells lacking both copies of *Tsc1,* and the disorganization observed is found throughout the entirety of the cortex, in direct contrast to the heterozygous and focal nature of cortical tubers in humans. While conditional knockouts are required to observe cortical lesions in mice, recent evidence suggests that even heterozygous rodents may have subtle learning deficits and increased sensitivity to epilepsy. Eker rats, heterozygous for *Tsc2,* have no cortical tubers present, yet demonstrate abnormal episodic learning and increased sensitivity to repeat chemical induction of seizures (Waltereit et al. 2006). Heterozygous *Tsc2* rats also display decreased long-term potentiation, long-term depression, and synaptic plasticity in the hippocampus (von der Brelie et al. 2006). These very subtle defects in heterozygous rodents are still not as severe nor do they exhibit the variety of symptoms as seen in TSC patients. Therefore, heterozygous rodent models under-represent the neurological phenotypes of *TSC2* mutation. Conditional knockouts for *Tsc1* or *Tsc2*, while closer approximations to the human phenotype, arise from a loss of heterozygosity not detected in human

brain tissue. These limitations of rodent models reveal the need for new ways to explore cortical pathogenesis in TSC.

Rodent models, while insufficient to understand the pathogenesis of cortical tubers, are excellent for understanding non-neuronal phenotypes of TSC. Non-neuronal lesions in TSC are clearly due to clonal expansion of progenitor cells following a loss of heterozygosity at either *TSC1* or *TSC2.* Tissue-specific knockout of either of these genes can mimic the pathogenesis of a variety of lesions. Common TSC-associated lesions of the heart, lung and kidney manifestations have all been well modeled using these methods and have shown that disease pathogenesis is due to dysregulated mTOR signaling (Meikle et al. 2005; Prizant et al. 2013; Armour, Carson, and Ess 2012).

My work demonstrates how dysregulated mTOR signaling contributes to the tissue specific phenotypes seen in tuberous sclerosis complex. In Chapter II, I will show how the canonical two-hit model is instructive to understanding the pathogenesis of kidney disease in TSC. Here I provide evidence that loss of either *Tsc1* or *Tsc2* in a rodent model results in cystogenesis in an mTORC1- but not mTORC2-dependent process. Additionally I will provide evidence that increased mTORC1 activity alters primary cilia, providing a mechanistic link between altered mTOR signaling and a known mediator of renal cystogenesis. In Chapter III, I will highlight both the limitations of the canonical two-hit model in neural tissue as well as the rodent models currently available. Then I will introduce a new model of TSC using patient-derived, human-induced pluripotent stem cell (iPSC). Finally, I will provide evidence that these patient-derived iPSCs

have not undergone loss of heterozygosity, yet still display altered behavior consistent with changes in mTORC1 activity. These phenotypes of patient derived iPSCs include resistance to differentiation and increased cell survival. Together, these data suggest haploinsufficiency as an important mechanism for TSC pathogenesis. Chapter IV will explore how these data explain a multisystem disease, such as TSC, as a collection of various tissue-specific pathogenic mechanisms, and tie these findings to autism and epilepsy more broadly.

CHAPTER II

CYSTOGENESIS AND ELONGATED PRIMARY CILIA IN TSC1-DEFICIENT DISTAL CONVOLUTED TUBULES

This work was published in its entirety under the same title in the August 15, 2012 issue of the American Journal of Physiology Renal Physiology (Armour, Carson, and Ess 2012).

Introduction

Tuberous Sclerosis Complex (TSC) is a multiorgan hamartomatous disease of the brain, skin, and kidney found in approximately 1 in 6,000 live births (Young and Povey 1998). Neurological symptoms of TSC predominate in young patients and include mental retardation, epilepsy, and autism (Crino, Nathanson, and Henske 2006). Renal pathology is less common in young children but is usually seen after the second decade of life. The renal pathology is diverse and includes cysts, angiomyolipomas (AML), and, in rare instances, renal cell carcinoma (O'Callaghan et al. 2004; Washecka and Hanna 1991). AMLs occur in up to 70% of patients with TSC and cysts are seen in ~30% of patients (O'Callaghan et al. 2004). While most kidney lesions are asymptomatic in TSC patients, a subset of patients present with severe polycystic kidney disease (PKD) that causes significant morbidity and mortality (Sampson et al. 1997).

TSC results from a loss of function mutations of either the *TSC1* or *TSC2* genes. In normal tissue, hamartin and tuberin (the protein products of *TSC1* and *TSC2*, respectively) form a complex that regulates mammalian target of rapamycin (mTOR) signaling activity (Consortium 1993; Povey et al. 1994). The

mTOR kinase regulates many critical cellular processes, including control of cell size, proliferation, cell survival, and protein synthesis (Inoki, Corradetti, and Guan 2005). mTOR carries out these functions with specific protein binding partners that associate into two distinct complexes. mTOR complex 1 (mTORC1) contains Raptor/mLST8/GβL, phosphorylates p70-S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), and controls mRNA translation (Tee and Blenis 2005). mTOR complex 2 (mTORC2) contains Rictor/GβL/mSin1, directly phosphorylates and activates Akt and protein kinase Cα, and indirectly activates NDRG1 via SGK1 phosphorylation (Garcia-Martinez and Alessi 2008). In contrast to mTORC1, mTORC2 function is much less well defined but appears to be involved with cytoskeletal regulation (Sarbassov et al. 2004). Raptor and Rictor are mutually exclusive binding partners of mTOR, controlling substrate specificity as well as sensitivity to rapamycin, a selective mTORC1 inhibitor. Current data suggest that the hamartin/tuberin complex is a negative regulator of mTORC1 but a positive regulator of mTORC2 (Huang et al. 2009). Loss of either hamartin or tuberin then leads to increased mTORC1 activity but decreased mTORC2 activity (Huang et al. 2009; Wullschleger, Loewith, and Hall 2006). Current genetic models of TSC support a germline mutation inactivating one allele of either *TSC1* or *TSC2* for each patient. Loss of heterozygosity at the other allele allows constitutive activation of mTORC1 signaling, resulting in phosphorylation and activation of ribosomal S6K1 and other downstream targets and is thought to lead to hamartoma formation (Crino et al. 2010; Sepp, Yates,

and Green 1996). Contributions of decreased mTORC2 activity in TSC have not been well studied but may play an important role in TSC pathogenesis.

Accumulating evidence connects mTOR signaling to kidney development and cystogenesis. Large deletions of both *TSC2* and *PKD1* in a subset of patients with TSC cause TSC and severe kidney disease. Intriguingly, recent reports show a direct physical interaction between tuberin and polycystin-1. This interaction is required for the proper subcellular localization of tuberin as well as mTORC1 inhibition (Dere et al. 2010; Shillingford et al. 2006). Additionally, hamartin has been shown to localize to the basal body of the primary cilia, an organelle the dysfunction of which leads to cystogenesis (Hartman et al. 2009). Furthermore, loss of either *Tsc1* or *Tsc2* in mouse embryonic fibroblasts and knockdown of *tsc1a* in zebrafish cause increased ciliary length (DiBella, Park, and Sun 2009; Hartman et al. 2009; Yuan et al. 2012).

The first animal models of TSC were generated by conventional inactivation of the *Tsc1* or *Tsc2* genes and revealed an absolute requirement of either gene during embryonic development, as homozygous mutants died before embryonic *day 10* (Kobayashi et al. 1999; Kwiatkowski et al. 2002). Rodents heterozygous for *Tsc1* or *Tsc2* develop kidney lesions in adulthood with some kidney lesions progressing to malignancy (Kwiatkowski et al. 2002; Kobayashi et al. 1999; Yuan et al. 2012). To define the role of hamartin in specific organs, conditional knockout (CKO) models of *Tsc1* have been generated to dissect tissue-specific pathogenesis in TSC (Traykova-Brauch et al. 2008; Uhlmann et al. 2002; Wang et al. 2007; Zhou, Brugarolas, and Parada 2009). Conditional loss of

Tsc1 in the proximal convoluted tubule (PCT) resulted in cystic kidneys, although the mechanisms linking dysregulation of mTORC1 and mTORC2 signaling with cystogenesis were not extensively studied (Zhou, Brugarolas, and Parada 2009).

We now show that CKO of the *Tsc1* gene in the distal convoluted tubule (DCT) causes cystogenesis. In these kidneys, there is both increased mTORC1 and decreased mTORC2 signaling. Notably, primary cilia in the DCT are longer than those from control mice, suggesting defects in cilia structure and function. Treatment of these CKO mice with the mTORC1 inhibitor rapamycin prevented cystogenesis and normalized cilia length. However, loss of mTORC2 activity alone, through CKO of the *Rictor* gene in the DCT, did not cause cystogenesis or alter cilia length. Our findings suggest that loss of the *Tsc1* gene in the DCT is sufficient for renal cystogenesis in TSC. This appears to require increased mTORC1 activity, possibly through cilia-dependent mechanisms that may be both cell-autonomous and non-cell-autonomous.

Materials and Methods

Mice: *Emx1-Cre* mice were obtained from Jackson Laboratories (Bar Harbor, Maine; line no. 005628) and maintained on a C57Bl/6 background. Mice with a *Tsc1* "floxed" allele were obtained from Dr. D. Gutmann (Washington University, St. Louis, MO) and maintained on a mixed SV129J/C57Bl/6 background. Mice with the *Rictor* floxed allele were a gift of Dr. Mark Magnuson (Vanderbilt University) and were maintained on a C57Bl/6 background (Shiota et al. 2006).

Through interbreeding, we generated *Tsc1*$^{Flox/Flox}$*;Emx1-Cre* mice (*Tsc1* CKO) and *Rictor*$^{Flox/Flox}$*;Emx1-Cre* (*Rictor* CKO) mice that are homozygous for either the *Tsc1* or *Rictor* floxed allele and heterozygous for *Emx1-Cre*. Heterozygous *Tsc1*$^{Flox/wt}$*;Emx1-Cre* animals were used as controls and were indistinguishable from other control genotypes including *Tsc1*$^{Flox/Flox}$ or *Tsc1*$^{Flox/wt}$ *Emx1-Cre* negative mice. *Rictor*$^{Flox/Flox}$*;Cre*-negative animals were used as controls for *Rictor* CKO mice. Cre reporter strains included Z/EG transgenic mice (Jackson Laboratories; line no. 004178) and *mTomato/mGFP* transgenic mice (Jackson Laboratories; line no. 007676). Animal experiments were approved by the Vanderbilt Institutional Animal Care and Use Committee.

Serum Chemistries: Blood samples were collected at euthanasia through cardiac puncture. Initial blood urea nitrogen (BUN) measurements were performed by the laboratory of Dr. Raymond Harris (Vanderbilt University). Additional BUN and serum chemistries performed by Antech (Southaven, MS).

Rapamycin treatment: Rapamycin (LC Laboratories, Woburn, MA) was dissolved in a stock solution at 30 mg/ml in ethanol. Before use, the stock solution was diluted with vehicle 0.25% Tween 20/0.25% polyethylene glycol in PBS. *Tsc1* CKO mice and control littermates received intraperitoneal injections with either rapamycin (3 mg/kg) or vehicle twice a week, starting at postnatal days (P)13–15.

Antibodies: Arl13b (1:500; gift of T. Caspary, Emory University, Atlanta, GA), aquaporin 1 (AQP1; 1:500; Abcam), AQP2 (1:500; Abcam), calbindin D-28k (1:2,000; Swant), NCC (1:500; Millipore), fluorescein-labeled Lotus Tetragonolobus Lectin (1:2,000; Vector Laboratories), phospho-Akt Serine473 (clone D9E, 1:1,000; Cell Signaling), phospho-S6 Serine235/236 (1:1,000; Cell Signaling), Akt (1:1,000; Cell Signaling), S6 ribosomal protein (clone 5G10, 1:1,000; Cell Signaling), phospho-NDRG1 Thr346 (1:1,000; Cell Signaling), and actin (1:1,000; Cell Signaling).

Immunofluorescence and imaging: Paraffin sections underwent antigen retrieval in 10 mM citrate buffer. Both frozen and paraffin sections were blocked in 5% normal goat serum, incubated in primary antibodies overnight at 4°C, followed by AlexaFluor conjugated secondary antibodies (Invitrogen) for 1 h. Slides were mounted with Vectashield (Vector Laboratories). Photomicrographs were taken using an Olympus BX UCB epifluorescence microscope and a Hamamatsu ORCA-ER CCD camera. Photomicrographs for cilia were taken on a Nikon spinning disk confocal microscope (Quorum Systems) with Metamorph software. Z-stack images were Z-projected, and cilia length was measured using ImageJ Software (Version 1.43S; National Institutes of Health). Cilia length was measured from calbindin-positive tubules in each photomicrograph using sections obtained from three control and three *Tsc1* or *Rictor* CKO mice. Measurements of cilia length were done while blinded to genotypes or treatment

with vehicle or rapamycin. Due to the variability of numbers of visible cilia in each section, between 87 and 150 cilia were measured for each group.

Immunoblotting: Kidneys were snap frozen in liquid nitrogen and homogenized in RIPA buffer containing phosphatase and protease inhibitor cocktails (Sigma-Aldrich). Western blotting was completed using standard conditions. Blots were probed with either horseradish peroxidase-conjugated secondary antibodies (GE Healthcare) or with fluorescent-tagged secondary antibodies, Alexa 680 (rabbit; Invitrogen), and IRDye 800 fluorochromes (mouse and rat; Licor). For horseradish-peroxidase-conjugated secondary antibodies, signal was developed using ECL Western blotting substrate (Pierce, Rockford IL) and visualized on BioMax film (Kodak). Fluorescent-tagged secondary antibodies were visualized using an Odyssey fluorescence scanner. After visualization, digitized band densities were quantitated using ImageJ (National Institutes of Health).

Results

Loss of Tsc1 in the DCT increases kidney size

Patients with TSC have multiorgan manifestations, usually with early neurological involvement followed by renal involvement in older children and adults. To address the dual pathologies of kidney and brain in TSC, we generated *Tsc1* CKO (*Tsc1*$^{Flox/Flox}$*;Emx1-Cre*, CKO) mice. While usually described as a “brain-

specific" gene, the *Emx1* promoter also directs Cre expression in the kidney (Briata et al. 1996; Inoue et al. 2004). *Tsc1* CKO mice are postnatally viable but small, with extensive brain abnormalities, seizures, and complete mortality by P25 (Carson et al. 2012). Because available antibodies are not suitable to determine hamartin levels, we assessed *Tsc1* gene inactivation by isolating genomic DNA from kidneys and used PCR to determine recombination status. As expected, DNA from *Tsc1* CKO kidneys yielded both recombined and unrecombined bands indicating targeted heterogeneity within the kidney, while control kidneys had only the unrecombined *Tsc1* gene (data not shown). By P15, kidneys from *Tsc1 CKO* mice were larger than littermate controls (**Figure 2.1A**). When normalized for total body weight, the difference in kidney size was even more striking (**Figure 2.1B**). Despite size abnormalities in the *Tsc1* CKO kidneys at P15, there was no significant change in blood urea nitrogen (BUN), a marker of kidney function, between *Tsc1* CKO mice and littermate controls (**Figure 2.1C**). This suggests that *Emx1-Cre*-driven loss of *Tsc1* in the kidney leads to kidney size abnormalities but not overt renal failure, suggesting the premature death of *Tsc1* CKO mice is likely due to their extensive brain pathology (Carson et al. 2012).

Increased mTORC1 signaling and mild cystogenesis

To determine if abnormalities of kidney size were associated with increased mTORC1 signaling, we measured levels of phospho-S6 in kidney

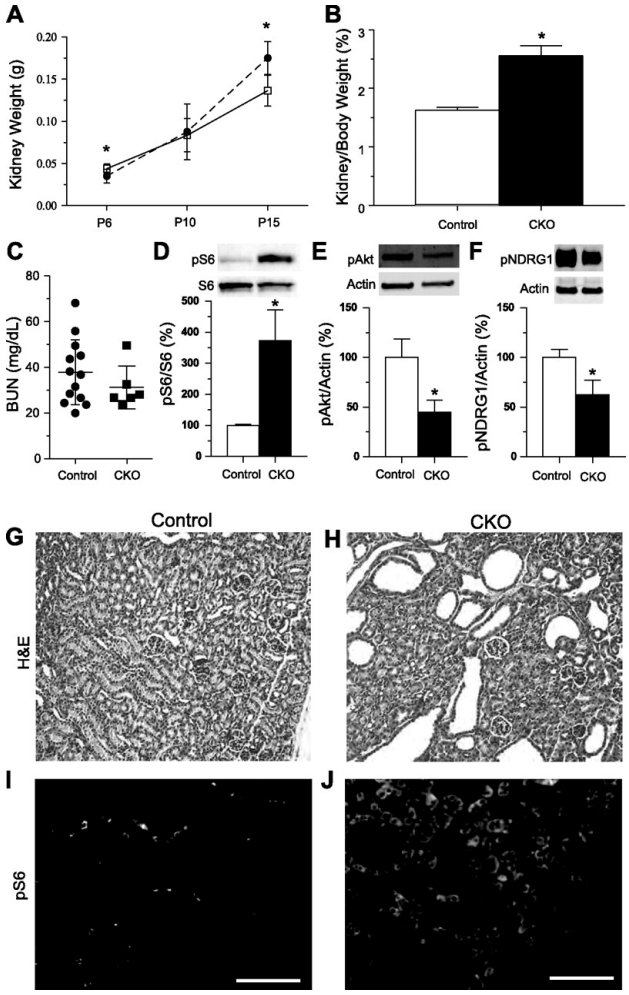


Figure 2.1 Postnatal day (P)15 *Tsc1* conditional knockout (CKO) mice have large kidneys, renal cysts and increased mammalian target of rapamycin complex 1 (mTORC1) signaling. *A*: kidney weights from control (solid line) or *Tsc1* CKO mice (dashed line) at P6 to P15; $n \geq 3$ for each group. *$P < 0.03$. *B*: kidney weight normalized to body weight from P15 control and *Tsc1* CKO mice. (control $n = 9$; CKO $n = 3$). *$P < 0.03$. *C*: no differences in blood urea nitrogen (BUN) levels from control and *Tsc1 CKO* mice. *$P > 0.05$, Student's *t*-test (control $n = 13$; CKO $n = 6$). *D*: immunoblotting for phospho-S6 reveals increased mTORC1 activity. Blots were stripped and reprobed for total S6 levels. *E*: immunoblotting for phospho-Akt (Ser473) reveals decreased mTORC2 signaling. *F*: decreased phospho-NDRG1 (Thr346) further reveals decreased mTORC2 signaling in P15 *Tsc1* CKO kidney compared with littermate controls. Data were analyzed with Student's *t*-test. *$P < 0.001$ for phospho-S6, $P < 0.05$ for phospho-Akt and *$P < 0.005$ for phospho-NDRG1. All graphs are plotted as means ± SD, control extract expression levels were set to 100%. *G* and *H*: hematoxylin and eosin (H&E) staining of kidney sections from P15 control and *Tsc1* CKO mice. Moderate cystic dilations are seen in kidneys from *Tsc1* CKO mice. *I* and *J*: immunofluorescence for phospho-S6 in control and *Tsc1* CKO mice. Phospho-S6 is diffusely expressed in the majority of the *Tsc1* CKO kidney but found only in isolated cells in kidney from control littermates. Scale bars = 100 μm.

extracts from control and *Tsc1* CKO kidneys. P15 CKO kidneys had increased ratios of phospho-S6 to total S6, indicating increased mTORC1 signaling (**Figure 2.1D**). We also examined mTORC2 signaling using phosphorylation of Akt at Ser473, a sensitive and specific readout of mTORC2 activity (Jacinto et al. 2006; Shiota et al. 2006). We found a significant reduction of phospho-Akt in these same kidney samples (**Figure 2.1E**). Additional evidence for decreased mTORC2 signaling is seen by reduced phosphorylation of NDRG1 at Thr346 (**Figure 2.1F**). Histological examination of kidneys from *Tsc1* CKO mice at P15 showed cystic changes when compared with littermate controls (**Figure 2.1G,H**). Immunofluorescence for phospho-S6 again showed increased mTORC1 signaling throughout the kidney in *Tsc1* CKO mice compared with (**Figure 2.1I-J**). Interestingly, both cystic and morphologically normal appearing tubules had increased mTORC1 signaling.

Emx1-Cre expression in the DCT

To determine expression within the kidney, we crossed *Emx1-Cre* mice to reporter animals that express GFP only after exposure to Cre recombinase. Animals transgenic for both *Emx1-Cre* and Z/EG revealed GFP signal within the cortex but not medulla. GFP coexpression was determined within the kidney using markers for the DCT (calbindin and NCC), the PCT (Aqp1), and collecting duct (Aqp2). Abundant GFP coexpression was seen with calbindin (**Figure 2.2A-C**). and similar results were seen with NCC (data not shown). While most cells in

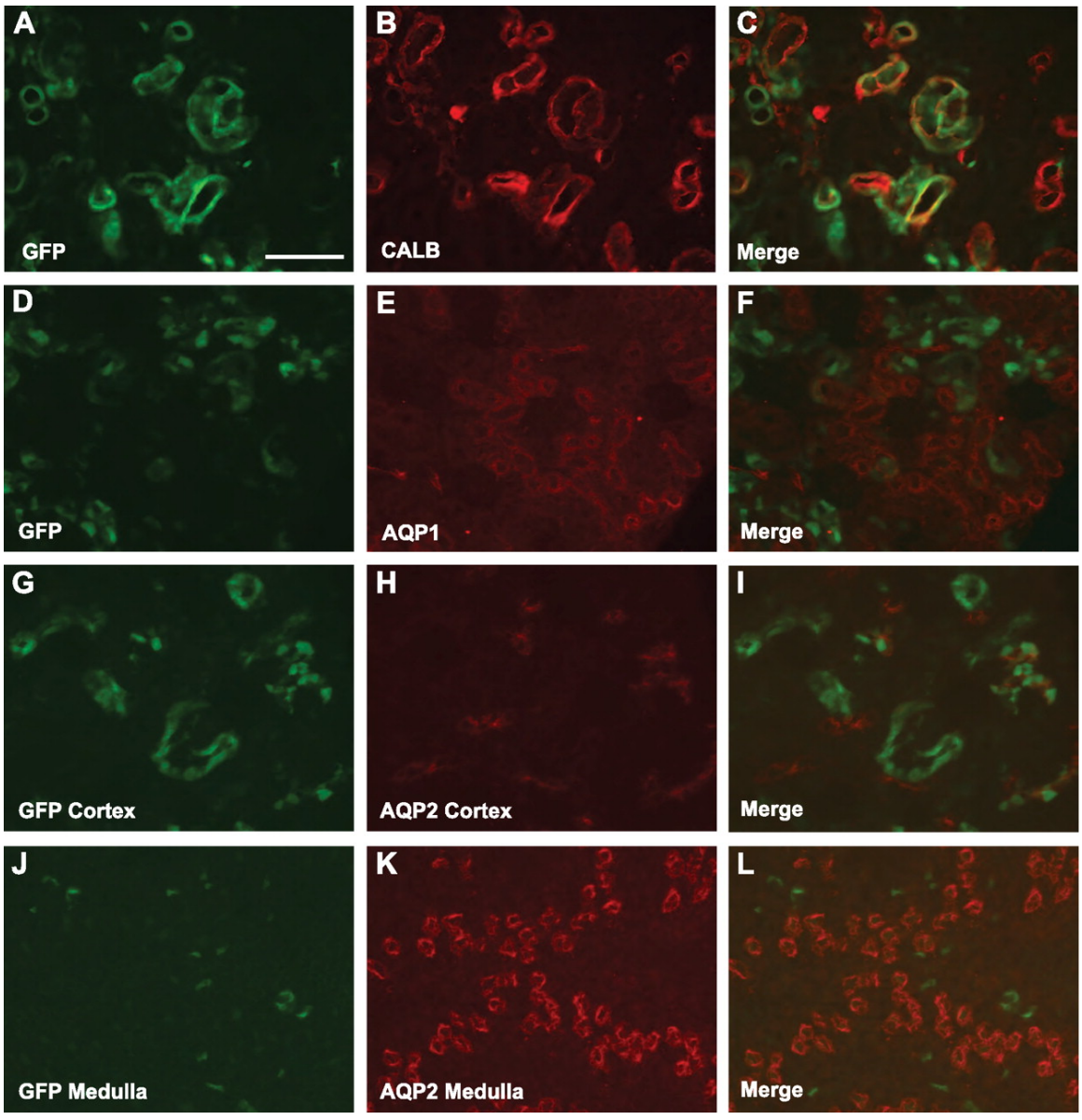
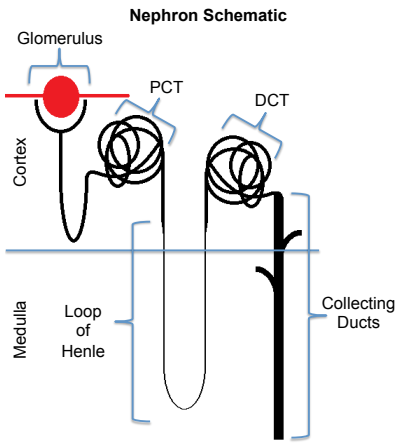


Figure 2.2 *Emx1-Cre* mediates gene recombination in the distal convoluted tubule. *A–C*: GFP expression from Cre recombinase fate mapping (*A*) and calbindin (CALB), a marker of the distal convoluted tubules (DCT; *B*), colocalize in the cortex of the kidney, merged image (*C*). Neither AQP1, a marker of the proximal tubule, nor aquaporin 2 (AQP2), a marker of collecting ducts, colocalize in the cortex with GFP (*D–F* and *G–I*, respectively). Only faint reporter GFP was observed in the medulla, and this signal did not colocalize with AQP2 (*J–L*). Scale bar = 100 μm.



the DCT were double labeled with GFP, a few calbindin- and NCC-positive cells did not double label, suggesting that a subset of DCT cells did not express *Emx1-Cre* and were not targeted. GFP did not coexpress with either Aqp1 or Aqp2 in the kidney (**Figure 2.2D-L**), indicating that *Emx1-Cre* expression was restricted to the DCT.

Rapamycin withdrawal from Tsc1 CKO mice leads to massive kidney enlargement and severe cystogenesis

To see if kidney abnormalities in *Tsc1* CKO mice were dependent on increased mTORC1 signaling, we treated *Tsc1* CKO mice twice weekly with rapamycin starting at P14 and continuing until P90. Using this regimen, we achieved nearly 100% survival of CKO animals at P90, whereas prior natural history studies (Carson et al. 2012) demonstrated complete mortality by P25. A group of control and *Tsc1* CKO animals were euthanized at P90 while on rapamycin, with another group of control and *Tsc1* CKO animals euthanized on P120, 30 days after stopping rapamycin. On rapamycin, kidneys from P90 *Tsc1* CKO mice were slightly larger than kidneys from littermate controls (**Figure 2.3A**). However, in the post-rapamycin group, kidneys from *Tsc1* CKO mice were greatly increased in size compared with control littermates (**Figure 2.3A-B**). While some small cysts were seen in P90 *Tsc1* CKO kidneys on rapamycin (**Figure 2.3C-D**), P120 *Tsc1* CKO mice post-rapamycin exhibited severely dilated cystic-appearing structures (**Figure 2.3E-F**). While *Emx1-Cre* expression

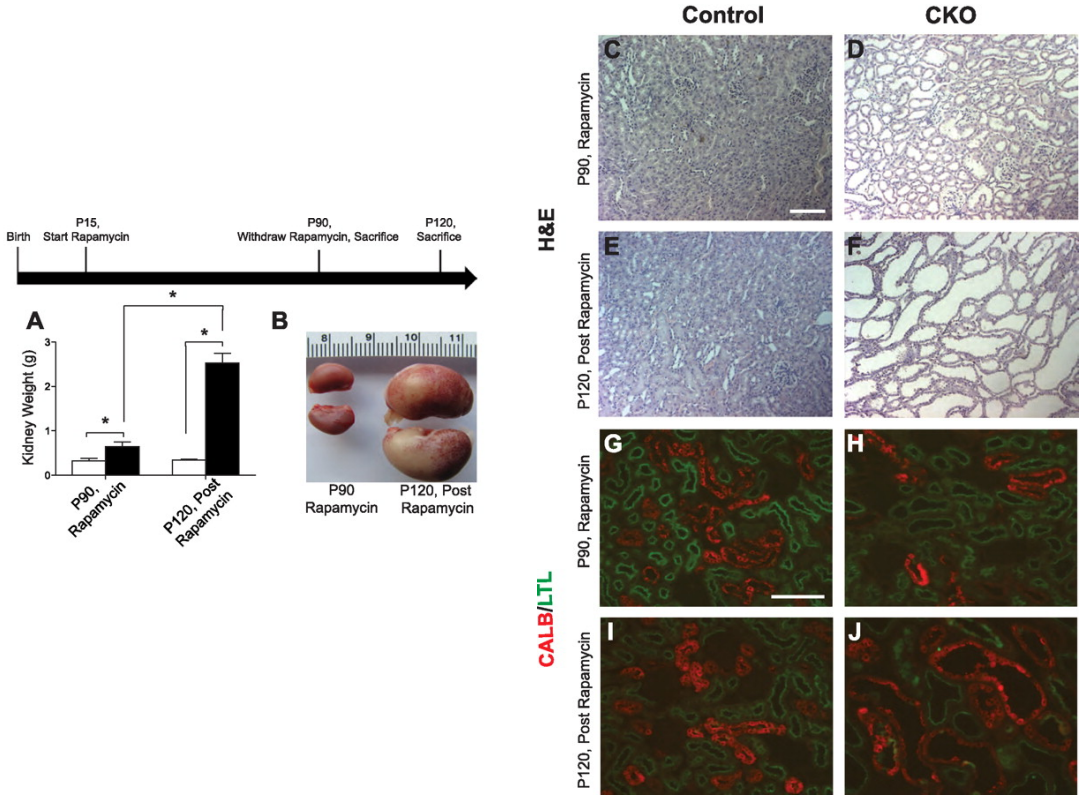


Figure 2.3 Rapamycin treatment partially inhibits cyst formation in *Tsc1* CKO mice and upon withdrawal causes giant cystic kidneys. Two groups of mice were treated with rapamycin from P15 to P90. At P90, kidneys from one group of animals euthanized on rapamycin were analyzed. For the second group of animals, rapamycin treatment was discontinued at P90 and mice were euthanized 30 days post rapamycin treatment at P120. *A*: Rapamycin treatment reduces cystogenesis in *Tsc1* CKO kidneys. After rapamycin withdrawal *Tsc1* CKO kidneys become much larger than those from control littermates ($n \geq 3$ for each group of control and *Tsc1* CKO mice). *B*: gross images of representative *Tsc1* CKO kidneys on rapamycin at P90 (*left*) or 30 days post rapamycin treatment at P120 (*right*). *C–F*: H&E staining of paraffin sections from *Tsc1* CKO and control littermate kidneys on or off of rapamycin. Rapamycin-treated *Tsc1* CKO mice have slightly dilated tubules at P90 (*D*) compared with controls (*C*). At P120, 30 days post rapamycin treatment, kidneys from *Tsc1* CKO show large cystic dilations of all tubules (*F*) compared with littermate controls (*E*). *G–J*: immunofluorescence for LTL [proximal convoluted tubule (PCT) marker, green] and the calbindin (DCT marker, red). Dilations of both the PCT and DCT are observed while on rapamycin (*H*) and post rapamycin treatment (*J*) compared with controls (*G* and *I*, respectively). Graphs are plotted with means ± SD. Data were analyzed with Student's *t*-test. $^*P < 0.001$. Scale bar = 100 μm.

appeared to be restricted to the DCT (**Figure 2.2**), cystic dilatations were seen in *Tsc1* CKO in both the PCT and DCT (**Figure 2.3G-J**), suggesting both cell-autonomous and non-cell-autonomous mechanisms.

To further explore mTORC1 and mTORC2 signaling in the kidney, *Tsc1* CKO and control mice were treated with rapamycin from P14 to P40. Kidneys were removed within 24 h of the last dose of rapamycin or 2 wk following rapamycin cessation. Immunoblots revealed increased mTORC1 signaling as indicated by pS6 levels in *Tsc1* CKO kidneys 2 wk after rapamycin cessation, while levels in *Tsc1* CKO and control kidneys on rapamycin were suppressed to levels below those seen in control mice off rapamycin (**Figure 2.4**). As the *Tsc1*/*Tsc2* genes have been reported to activate mTORC2 signaling, we assessed Akt phosphorylation at Serine473. Using this same group of control and *Tsc1* CKO mice, we found significantly decreased levels of mTORC2 signaling in *Tsc1* CKO mice that were on rapamycin as well those examined after rapamycin withdrawal (**Figure 2.4**). These overall results suggest that renal abnormalities in *Tsc1* CKO mice are mainly due to mTORC1-dependent signaling. However, rapamycin treatment did not completely reverse the pathology, with P90 animals on rapamycin still displaying cystic changes, suggesting other mechanisms including decreased mTORC2 signaling may contribute. Alternatively, rapamycin treatment starting at P14 may simply be too late to reverse kidney pathology. Rapamycin injections at P6 were attempted but hampered by toxicity and mortality in both *Tsc1* CKO and control animals.

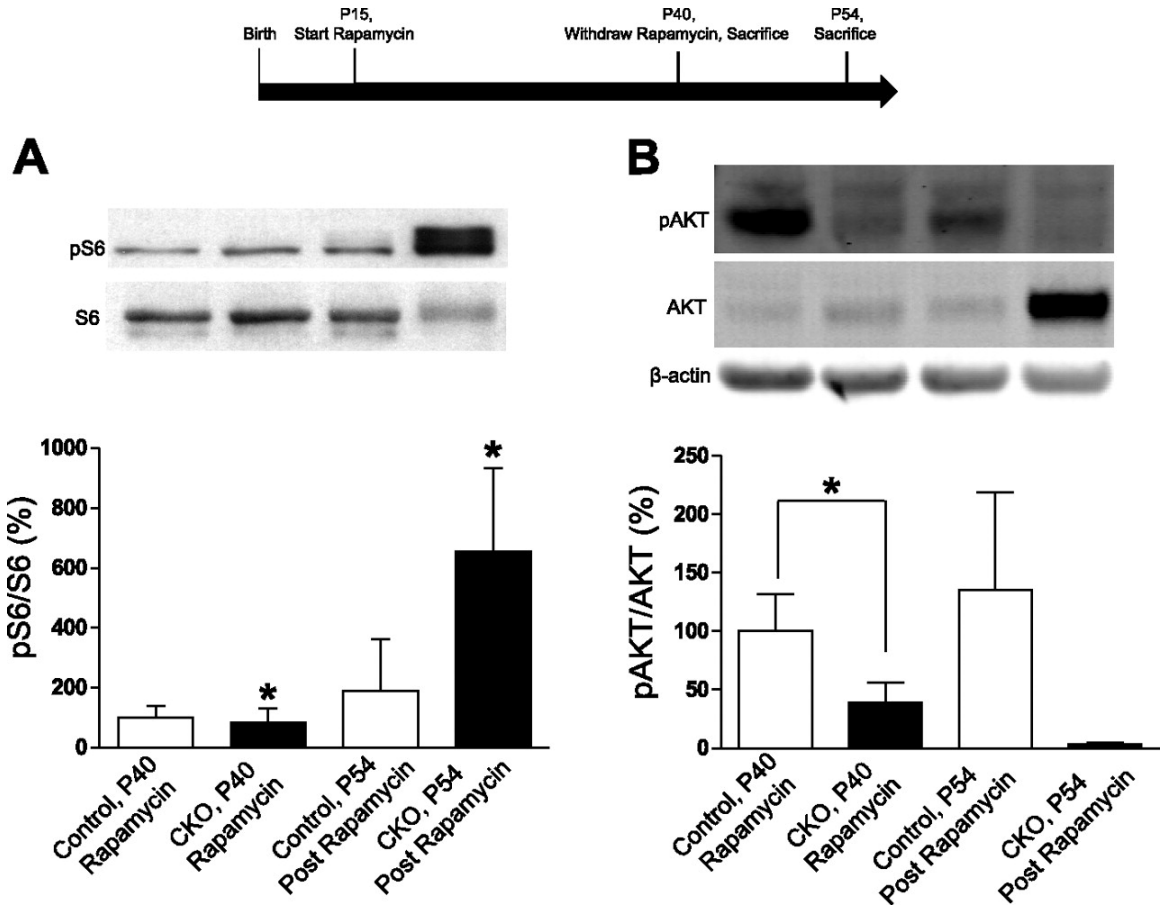


Figure 2.4 Rapamycin treatment normalizes mTORC1 but not mTORC2 signaling in *Tsc1* CKO kidneys. Two groups of animals were treated with rapamycin from P15 to P40. Kidneys were taken from the first group of euthanized animals at P40 (n = 4 for control mice and n = 3 for CKO). Rapamycin treatment was discontinued in the second group of animals for 14 days, from P40 to P54 and protein extracts made from these kidneys at P54 (n = 3 for control and n = 2 for CKO). *A*: mTORC1 signaling, as shown by S6 protein phosphorylation, in *Tsc1* CKO kidneys was decreased to that seen in control animals with rapamycin treatment. Post rapamycin phospho-S6 levels were greatly increased. *P < 0.05. *B*: mTORC2 signaling, as shown by Akt phosphorylation at Serine473, in P40 *Tsc1* CKO kidneys was significantly decreased while on rapamycin. *P = 0.039. While trending towards significance, CKO extracts post rapamycin treatment did not have statistically significant decreases in phospho-Akt (Serine473); P = 0.12.

Loss of Rictor in the DCT is not sufficient to cause overt kidney abnormalities

These findings with rapamycin suggest that reversal of mTORC1 signaling was largely responsible for the kidney pathology in *Tsc1* CKO mice. To isolate the contribution of decreased mTORC2 signaling during kidney development, we developed a *Rictor* CKO mouse model using the same breeding strategy used for *Tsc1* CKO mice. These homozygous *Rictor* floxed mice with a single copy of *Emx1-Cre* (*Rictor* CKO) are viable with no overt brain abnormalities (R. P. Carson and K. Ess, unpublished data). At P15, kidneys from *Rictor* CKO mice also had no noticeable differences in weight compared with control littermates (**Figure 2.5A-B**). As antibodies against Rictor did not give reproducible results, mTORC2 signaling was assessed using levels of phospho-Akt (Serine473). No differences were seen in *Rictor* CKO compared with control mice (**Figure 2.5C**), likely due to decreased mTORC2 signaling being restricted to the DCT. To determine if the *Rictor* gene was inactivated, we extracted genomic DNA from kidneys and used PCR to measure recombination. DNA from *Rictor* CKO mice had unrecombined and prominent recombined bands, indicating *Rictor* inactivation in a subset of the kidney (**Figure 2.5D**). Kidney DNA extracted from a control littermate only showed the unrecombined gene. *Rictor* CKO kidneys appeared normal and indistinguishable from littermate controls (**Figure 2.5E-F**). Coimmunofluorescence for phosphorylated NDRG1, a downstream indicator of

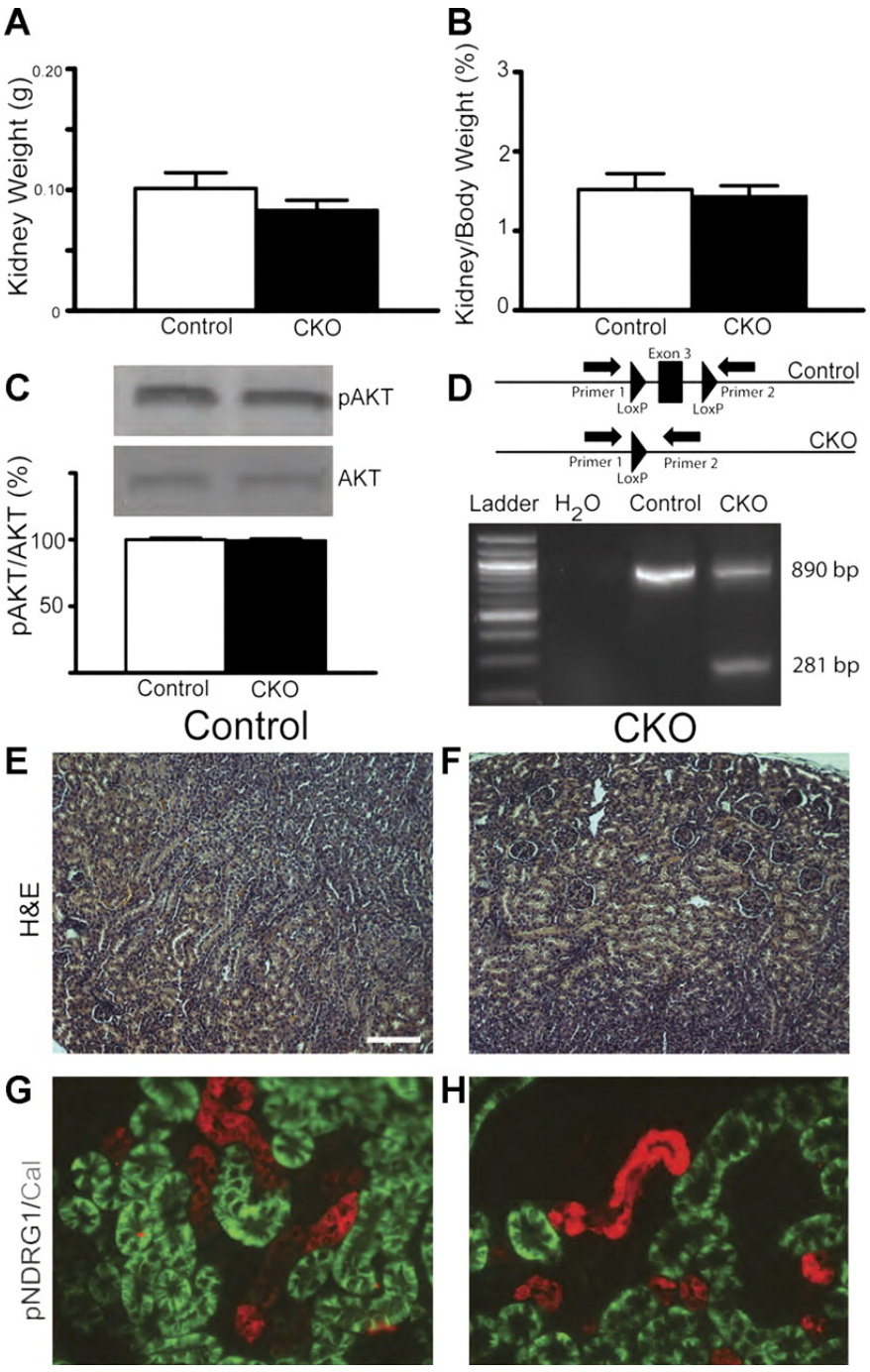


Figure 2.5 *Rictor* CKO kidneys at P15 do not develop cysts. *A* and *B*: kidneys from P15 *Rictor* CKO mice did not show alterations in kidney weight nor in kidney-to-body weight ratios compared with control littermates (control n = 15; CKO n = 7). *C*: no changes in AKT phosphorylation at Serine 473 were detected through western blot analyses of protein extracts from P9-P15 *Rictor* CKO mice. *D*: PCR reveals recombination of exon three of the *Rictor* floxed allele in the kidney of *Rictor* CKO mice. *E* and *F*: H&E staining do not show any appreciable kidney abnormalities. *G* and *H*: immunofluorescence for pNDRG1, a marker of mTORC2 activity, and calbindin, a marker of the DCT, revealed undetectable levels of mTORC2 activity in the DCT in both control and CKO animals.

mTORC2 activity, and calbindin, a marker of the DCT, showed that even in kidneys from control animals the DCT has undetectable levels of endogenous mTORC2 activity (**Figure 2.5G**). Therefore, further decreases of mTORC2 activity in the DCT of *Rictor* CKO animals were not measurable with immunofluorescence (**Figure 2.5H**). These findings suggest that kidney abnormalities seen after the loss of the *Tsc1* gene in the DCT are largely due to increased mTORC1 signaling.

Abnormalities of the primary cilia in the DCT from Tsc1 but not Rictor CKO mice

The precise pathological mechanisms causing cystogenesis or brain malformations in TSC are poorly understood. As other diseases such as Bardet-Biedl Syndrome and Joubert Syndrome also have shared brain and kidney pathology, we hypothesized that abnormalities of the primary cilia may connect TSC to “ciliopathies” (Badano et al. 2006). This hypothesis was bolstered by a recent report (Hartman et al. 2009) that hamartin is expressed in the basal body and *Tsc1*-deficient fibroblasts have abnormalities of cilia length. In addition, zebrafish with Morpholino knockdown of *tsc1a* also have elongated cilia and rapamycin treatment shortened cilia (Yuan et al. 2012). We measured the length of primary cilia in the DCT from P15 *Tsc1* CKO and control mice and found longer primary cilia in the DCT from mutant mice (**Figure 2.6B**)**.** In contrast, P90 mice on rapamycin did not have changes in cilia length. However, the P120 *Tsc1* CKO mice (30 days after stopping rapamycin injections) again had increased cilia

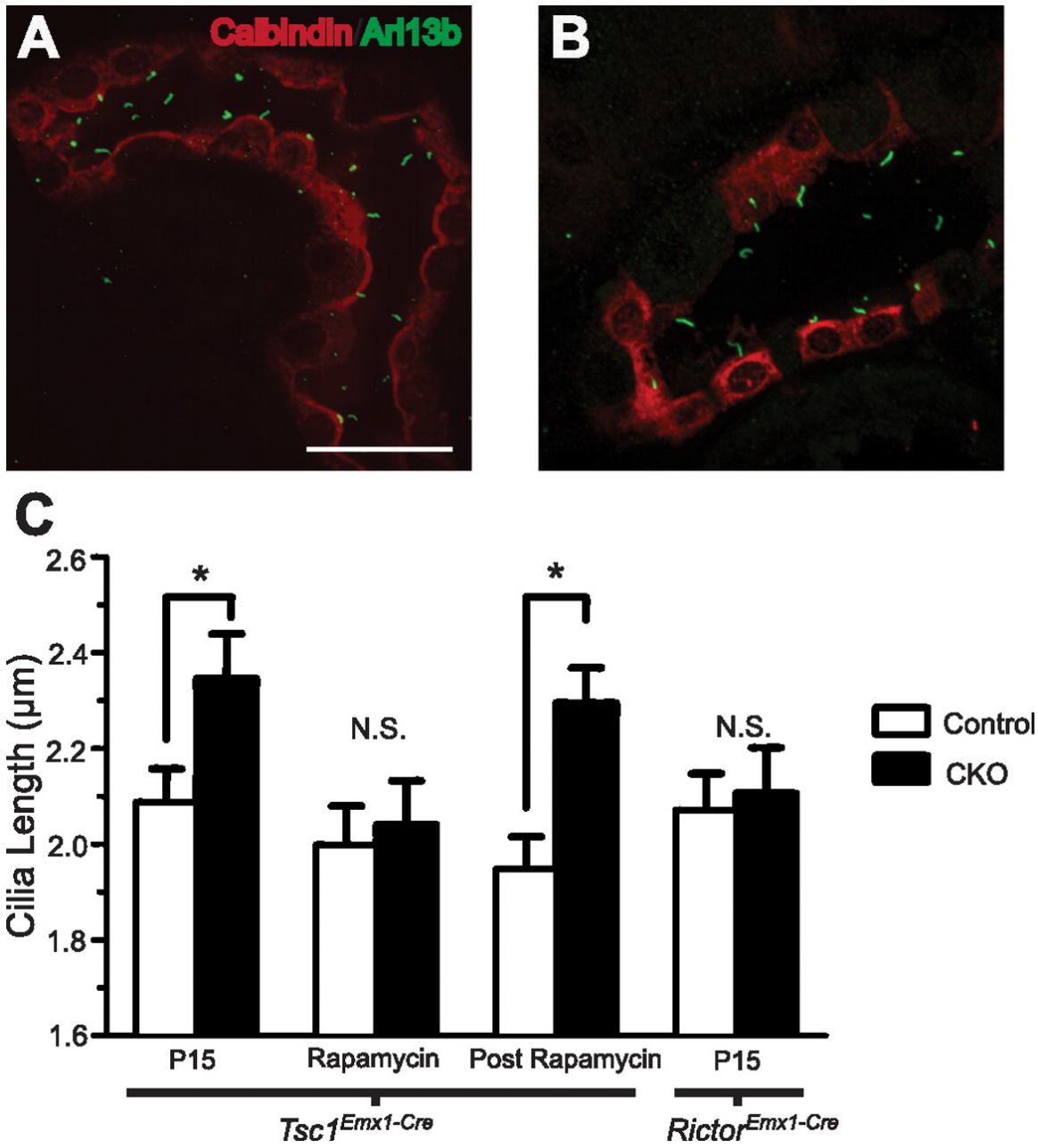


Figure 2.6 Primary cilia are longer in *Tsc1*-deficient but not *Rictor*-deficient tubules. *A* and *B*: representative photomicrographs of cilia in the DCT of *Tsc1* CKO mice on rapamycin at P90 (*A*) or 30 days after rapamycin withdrawal at P120 (*B*). Coimmunofluorescence for Arl13B (a marker of primary cilia) and calbindin (marker of the DCT). *C*: cilia length in untreated mice at P15, during rapamycin treatment at P90 and 30 days post rapamycin withdrawal at P120. Primary cilia in the DCT of kidneys from *Tsc1* CKO mice were longer 30 days after rapamycin cessation. No changes in cilia length were noted between control mice and *Rictor* CKO mice. *$P < 0.05$. Scale bar = 25 μm. N.S, not statistically significant; $n = 3$ for each group of control, *Tsc1* CKO and $Rictor^{Emx1-Cre}$ CKO mice.

length compared with controls (**Figure 2.6**). To assess the potential contribution of decreased mTORC2 signaling to this phenotype, cilia from P15 *Rictor* CKO and control littermates were measured with no alterations in their length seen. Our pharmacologic and genetic data suggest that cilia length abnormalities in the DCT are due to increased mTORC1 but not decreased mTORC2 signaling.

Discussion

Our results demonstrate the formation of dilated cystic tubules from selective loss of *Tsc1* in the DCT. This extends previous studies (Traykova-Brauch et al. 2008; Zhou, Brugarolas, and Parada 2009) that have shown cystogenesis from *Tsc1* inactivation in the PCT or from both the PCT and DCT. Additionally, we demonstrate an integral role for abnormal mTORC1 but not mTORC2 signaling in cystogenesis. This is shown by both pharmacological experiments using rapamycin and from the selective inactivation of the *Rictor* gene in the DCT. Furthermore, we found abnormally long primary cilia in *Tsc1*-deficient but not *Rictor*-deficient DCT. The abnormal cilia length in the DCT of *Tsc1* CKO mice is also reversible by treatment with rapamycin. These findings strongly suggest that increased mTORC1 but not decreased mTORC2 is the main contributor to cyst formation in TSC. Kidney disease initiation and progression in TSC may be due to defects in the structure or function of the primary cilia, linking TSC to other disorders that have dual kidney and brain pathologies.

Intriguingly, cystic changes were not restricted to the DCT. This is shown by increased mTORC1 activity at P15 throughout the kidney and cystic dilations within both the PCT and DCT in the kidneys at P120 post-rapamycin withdrawal (**Figure 2.3**). The presence of cysts in compartments other than *Emx1-Cre* expression domains suggests that non-cell-autonomous processes may be contributing to this cystic phenotype. This may also explain the decrease in mTORC2 signaling seen in *Tsc1* CKO but not *Rictor* CKO kidney. Non-cell-autonomous mechanisms have recently been reported in studies using human samples and from a zebrafish model of TSC (Crino et al. 2010; Kim et al. 2011). Newer therapies for patients with TSC need to consider potential non-cell-autonomous mechanisms when designing therapies to alter the course of renal disease. An alternative explanation is that loss of the *Tsc1* gene may cause altered cell fate, with DCT cells inappropriately expressing markers of the PCT.

The primary cilium is an important mechanistic link between PKD and the mTOR pathway (Ibraghimov-Beskrovnaya and Natoli 2011). PKD is one of several disorders described as a “ciliopathy.” Links between cystogenesis and defects in primary cilium were first identified in a mouse model of PKD that has a mutation of *Ift88*, a gene required for normal primary cilia (Pazour et al. 2000). Other primary cilium-related proteins have been shown to also play a role in cystogenesis (Nauli et al. 2003; Zullo et al. 2010). Previously, longer cilia have also been found after kidney injury (Verghese et al. 2009). Here, our findings of altered cilia length after loss of *Tsc1* in the DCT and normalization with rapamycin treatment link mTORC1 signaling to the primary cilia. Hamartin

expression at the basal body coupled with elongated primary cilia in *Tsc1*- or *Tsc2*-deficient mouse embryonic fibroblasts further supports our findings (Hartman et al. 2009). In addition to these results with cultured cells, the knockdown of *tsc1a* in zebrafish also increased cilia length and caused left-right patterning defects and cystic appearing kidneys (DiBella, Park, and Sun 2009; Yuan et al. 2012). The further use of complementary zebrafish and mouse models will greatly enhance our understanding of the role of the *TSC* genes and mTORC1 in the regulation of primary cilia length and function. Shortened cilia or loss of primary cilia has a clear functional consequence, including altered calcium flux and cell size control in response to flow. However, the functional impact of elongated primary cilia as we have seen here remains to be elucidated. Longer cilia may have disrupted flow-sensing capabilities through stiffening or other mechanical inhibition of cilia flexibility. Alternatively, longer cilia may disrupt normal retrograde and anterograde transport across the length of the cilia, preventing the normal activation and propagation of cilia dependent pathways such as Wnt signaling. Future experiments need to address the relative contributions of these mechanisms to cystogenesis. Insights into the functional impact of elongated cilia will be particularly useful in studying kidney phenotypes in animal models of TSC and understanding the pathogenesis of PKD and other kidney diseases that have altered mTORC1 signaling.

CHAPTER III

Heterozygous Patient Derived iPSC Lines Have Enhanced Cell Survival and Maintenance of Pluripotency

Introduction

As discussed in Chapter I, current models of TSC pathogenesis posit that while patients inherit one mutant copy of *TSC1* or *TSC2*, it is only after a somatic mutation (resulting in loss of heterozygosity at either of these loci) that the pathologic lesions of TSC develop. Loss of heterozygosity leads to a clonal expansion and abnormal cellular differentiation resulting in a hamartoma. Data from both rodent models and TSC-associated human lesions support this two-hit model of hamartoma formation. Loss of heterozygosity at either *TSC1* or *TSC2* has been well described in kidney, lung, and heart lesions from TSC patients (Sepp, Yates, and Green 1996; Henske et al. 1997; Cai et al. 2010; El-Hashemite et al. 2003). In Chapter II, I presented evidence that this two-hit model of TSC progression is important in understanding the pathogenesis of kidney manifestations in TSC.

For neurological pathologies, while rodent models have classically supported allelic loss of heterozygosity as a mechanism for TSC pathogenesis, evidence from human tissue has not. Therefore, the use of these rodent models as an appropriate model for human neuropathology has been called into question. In contrast to both non-neuronal lesions in patients and the various rodent models currently used, cortical tubers in patients rarely display loss of

heterozygosity. As cortical tubers are often epileptogenic foci in patients, neurological surgery and tuber excision is a treatment option for these patients. Tuber resections have provided many samples for DNA extraction and subsequent deep sequencing of these focal lesions. Surprisingly, attempts to identify acquired somatic mutations have revealed that second-hit mutations in cortical tubers are exceedingly rare (Henske et al. 1996; Niida et al. 2001; Qin et al. 2010).

The rarity of detected second-hit mutations in surgically resected TSC patient brain tissue suggests an alternative mechanism to the two-hit hypothesis for the pathogenesis of cortical tubers. We hypothesize that human neuronal tissue is uniquely sensitive to alterations in hamartin and tuberin levels. Specifically, we propose that haploinsufficiency of either *TSC1* or *TSC2* results in minor mTOR signaling changes due to a dosage change in either hamartin or tuberin. In addition to the absence of detected second-hits, otherwise structurally normal tissue can still display metabolic abnormalities providing support for haploinsufficiency. These metabolic changes imply axonal dysfunction and demyelination in areas with only germline mutations in *TSC1* or *TSC2* (Wu et al. 2013). These data further suggest that histologically normal brain tissue, without a loss of heterozygosity, may still behave abnormally at the molecular and cellular levels.

The limitations of rodent models to accurately represent human neurological disease in TSC reveal the need for new ways to explore cortical pathogenesis in TSC. To address these challenges and to better define the role

of heterozygosity of *TSC1* and *TSC2* genes during the pathogenesis of TSC, we generated induced pluripotent stem cells (iPSC) from patients with TSC. These methods have provided the opportunity to directly study disease pathogenesis in human cells. Especially for neurological diseases, where human tissue is especially difficult to obtain, the generation of patient-derived iPSCs has allowed disease-specific neuronal tissue to be generated through directed differentiation of these cells. Additionally, iPSCs also provide a window to early events in embryonic development and cellular differentiation that may affect disease progression.

Stem Cells and Pluripotency

The derivation of embryonic stem cells (ESCs), first isolated from mice in 1981, set a groundwork that allowed the understanding of pluripotency, self-renewal, and differentiation pathways (Evans and Kaufman 1981). ESCs are self-renewing cell populations derived from the inner cell mass of blastula-stage embryos, capable of differentiating into all cell types of the three primary germ layers. The derivation of human ESCs in 1998 allowed further understanding of fundamental stem cell pathways and the identification of species-specific differences in stem cell behavior (Thomson et al. 1998). ESCs revolutionized the study of embryogenesis, provided a source of human tissue to help understand normal function of organs derived from all three germ layers, allowed for rapid drug screening in human cells and sparked new hope regarding regenerative

medicine treatments. However, ethical concerns regarding the derivation and application of these cells have long provided obstacles for their laboratory use and governmental funding of these projects.

Though cells normally progress from stem cells to terminally differentiated states during development, previous experiments using somatic cell nuclear transfer have demonstrated that this progression is not irreversible. Nuclei of differentiated cells retain the ability to generate new cloned animals and initiate normal developmental programs when introduced into enucleated oocytes (Gurdon 1962; Gurdon, Laskey, and Reeves 1975). The ability to “reprogram” these nuclei to an embryonic state in the presence of oocyte cytoplasm suggested that specific factors in the cytoplasm are responsible for this reversal of differentiation.

Much work using ESCs has helped elucidate regulation of the two unique properties of stem cell populations: maintenance of pluripotency and self-renewal. These properties of ESCs are regulated by a complex network of genes. Three of the master regulators of this network are SOX2, OCT4, and NANOG. This regulatory network both promotes self-renewal and causes these cells to resist signaling cascades that induce differentiation. SOX2 and OCT4 cooperatively bind as a heterodimer to regulatory elements for many pluripotency related genes (Rodda et al. 2005). OCT4 (octamer-binding transcription factor 4) is a homeodomain transcription factor encoded by the gene *POU5F1* (Takeda, Seino, and Bell 1992). It is maternally expressed in the oocyte and then is expressed in the embryo, continuing expression through the generation of the inner cell mass

(Scholer et al. 1990). SOX2 (sex determining region Y box 2) is a high motility group domain transcription factor, and is expressed in the developing inner cell mass (Yuan et al. 1995).

OCT4 and SOX2 were identified as complementary transcription factors regulating pluripotency because loss of either OCT4 or SOX2 prevented normal embryogenesis (Avilion et al. 2003; Nichols et al. 1998). Conventional knockout of the *Sox2* gene in mice causes embryonic lethality. In these knockouts, the epiblast does not develop and embryos die at implantation (Avilion et al. 2003). *Oct4*-null embryos progress slightly farther than *Sox2*-null embryos, but die shortly after implantation (Nichols et al. 1998). Furthermore, dosage effects of both genes regulate cell fate decisions of stem cell populations. Decreased Oct4 results in differentiation to trophectoderm cells, while increased Oct4 promotes mesendodermal lineages (Chew et al. 2005; Niwa et al. 2005). Likewise, dosage effects of Sox2 relate to altered cell fates. Increased Sox2 promotes ectodermal and trophectoderm differentiation and prevents endoderm formation (Kopp, Ormsbee et al. 2008). Because both of these transcription factors are required for early embryogenesis and are able to control cell fate determination, much work has been done to understand the mechanisms by which these two important transcription factors influence stem cell behavior.

Maintenance of pluripotency and self-renewal is also controlled through Nanog, a homeodomain transcription factor. Nanog is positively regulated by the binding of Oct4 and Sox2 to the *Nanog* enhancer region, and is required for pluripotency both in the epiblast as well as *in vitro* stem cell lines (Mitsui et al.

2003; Rodda et al. 2005). Like Oct4 and Sox2, loss of Nanog prevents epiblast formation (Mitsui et al. 2003). Initially, Nanog was identified in a screen of factors that could sustain pluripotency in media that promotes differentiation (Chambers et al. 2003). It was subsequently shown to be important for formation of the inner cell mass as well as the generation of stem cell lines from this population (Rossant and Tam 2009; Mitsui et al. 2003). While overexpression of Nanog inhibits differentiation, loss of Nanog causes a loss of pluripotency and differentiation to endodermal lineages (Mitsui et al. 2003; Chambers et al. 2003). Nanog can also bind the promoter regions for both *Oct4* and *Sox2,* providing interesting feedback loops for these three master regulators (MacArthur et al. 2012).

The discoveries of master regulatory transcription factors in both mouse and human ESCs and the complicated networks they control to maintain pluripotency and self-renewal paved the way for the development of techniques for reprogramming differentiated somatic cells towards an embryonic state. This led to the generation of human induced pluripotent stem cells (hiPSC) through the introduction of master regulators and reprogramming of human fibroblasts (Takahashi et al. 2007). The first successful induction of pluripotency from somatic cells was completed with the exogenous addition of *Oct4* and *Sox2* along with *Klf4* and *c-Myc* (Takahashi and Yamanaka 2006). Introduction of these genes caused the cells to be gradually reprogrammed, eventually expressing other pluripotent markers like Nanog. These cells produced

teratomas and had the ability to generate cells from all three germ layers, similar to ESCs.

During the reprogramming process, exogenous genes begin to remodel chromatin structure. Over 1,000 loci experience dramatic changes to chromosomal histone modification, most involving pluripotency-related genes (Koche et al. 2011). Following this remodeling, changes in gene expression at these loci are observed, including reactivation of genes involved in DNA replication and cell cycle progression (Mikkelsen et al. 2008). Reactivation of genes involved in early embryogenesis result in the morphology of cells reverting to more embryonic shapes as well as increased proliferation (Smith et al. 2010). Concurrently, fibroblast-specific genes begin to be silenced and these mesenchymal cells begin to behave as epithelial cells (Li et al. 2010). Finally, endogenous pluripotency genes like *Oct4*, *Sox2*, and *Nanog* begin to be expressed (Brambrink et al. 2008). The re-expression of these endogenous genes allows the reestablishment of pluripotency and self-renewal networks. Autoregulation of these pathways, through positive feedback loops, cause these cells to retain stem cell identity after exogenously introduced vectors are silenced (Kim, Chu et al. 2008; Jaenisch and Young 2008).

Patient derived iPSCs as a model for TSC

The generation of iPSCs is clearly useful in many fields of medicine and science. Patient specific iPSCs are especially promising for regenerative and

transplant medicine because they should minimize the risk of tissue rejection given their autologous nature (Wernig et al. 2008). We are currently utilizing iPSCs derived from both healthy and lesion TSC patient skin samples to model disease pathogenesis and discover underlying mechanisms of this disorder. We hypothesize haploinsufficiency of either *TSC1* or *TSC2* causes minor changes in mTOR signaling. This can prominently impact stem cell populations, as mTOR signaling is connected to many cellular processes crucial for normal stem cell function such as cell proliferation and survival.

Increased mTOR complex 1 (mTORC1) activity signals for increased lipid biogenesis and increased translation, both necessary for cell proliferation (Laplante and Sabatini 2009; von Manteuffel et al. 1997). Increased mTORC1 activity, therefore, provides new membranes for cell division and increases function of ribosomal machinery that is needed for cell growth prior to mitosis. Additionally, the two main downstream targets of mTORC1, S6K1 and 4E-BP1, are required for G_1 phase progression. mTORC1 also increases cyclin D1 levels, promoting cell cycle progression (Fingar et al. 2004; Vadlakonda, Pasupuleti, and Pallu 2013). mTORC2 activity has long been connected to cell-survival pathways such as FOXO signaling and p53 through Akt activation (Birkenkamp and Coffer 2003; Zhou et al. 2001; Sarbassov et al. 2005). However, mTORC1 activity has also been linked to cell survival, specifically after loss of cell-cell contact and anchorage depletion (Arakawa-Takeuchi et al. 2010).

While connections between mTOR signaling and both proliferation and cell survival have been previously established, the relationship between mTOR

and pluripotency has only recently been explored. First, regulation of *Nanog* is controlled through the Akt signaling pathway, which is both upstream and downstream of mTOR activity (Niwa et al. 2009; Kim et al. 2010). Akt activation causes increased expression of *Nanog* that, in turn, through a positive feedback loop, increases expression of *Sox2* and *Oct4.*

More recent work has revealed a more direct connection between mTORC1 signaling and the complex network controlling the generation of stem cell lines and the maintenance of pluripotency. Both increased and decreased mTORC1 activity affects generation of pluripotent stem cell lines. Hyperactivation of mTOR signaling in *Tsc2*$^{-/-}$ mouse embryonic fibroblasts (MEF) almost completely prevents reprogramming to iPSCs, while inhibition of mTORC1 activity through rapamycin treatment can augment reprogramming efficiency in these cells (He et al. 2012). Conversely, greatly decreased mTOR activity also prevents maintenance of pluripotency. Conventional knockout of the *mTOR* gene prevents the expansion of embryonic stem cells (ESCs) from blastula-stage embryos to pluripotent self-renewing stem cell lines (Gangloff et al. 2004). Maintenance of pluripotency is also affected through altered mTORC1 activity. High doses of rapamycin push existing stem cells to differentiate to mesodermal and endodermal lineages (He et al. 2012; Zhou et al. 2009). Removal of knockout serum replacement (KOSR), a potent mTOR stimulator and a crucial component of stem cell maintenance media, also decreases maintenance of pluripotent cells (Zhou et al. 2009).

While extreme hyperactivation or hypoactivation of mTOR signaling can deplete stem cell populations or decrease somatic cells reprogramming efficiency, smaller changes to mTOR signaling can enhance maintenance of pluripotency. While high dose (10 nM) rapamycin treatment decreases reprogramming efficiency of somatic cells, low dose (0.3 nM) treatment may enhance reprogramming efficiency (Chen et al. 2011). Furthermore, *Tsc2* siRNA knockdown and mTORC1 activation in existing murine stem cell lines prevent differentiation of the cells. This effect is phenocopied in knockdowns of *Flcn* and *Fnip1/2,* two putative downstream targets of mTORC1. Both Flcn and Fnip1/2 appear to function by inhibiting the translocation of the transcription factor Tfe3 to the nucleus. This translocation is important in maintenance of pluripotency (Betschinger et al. 2013). In this model, increased mTORC1 activity inhibits Flcn and Fnip1/2 to allow Tfe3, a pluripotency-promoting transcription factor to translocate to the nucleus and activate gene expression.

Overall, these data suggest that mTORC1 activity is crucial to reprogramming, maintenance of pluripotency, and cell survival iPSCs. In this chapter we provide evidence that TSC patient-derived iPSCs heterozygous for *TSC1* or *TSC2* have alterations in mTORC1 controlled cellular functions. We first demonstrate the absence of loss of heterozygosity in our iPSC lines. We then show patient-derived iPSCs have increased survival when artificially stressed. Lastly, we provide evidence that these stem cells maintain pluripotency at higher rates than control iPSC lines. These data are consistent with moderately

increased mTORC1 activity, providing evidence that haploinsufficiency may be a mechanism for TSC pathogenesis.

Materials and Methods

Primary Fibroblast Cultures and iPSC Reprogramming:

Primary fibroblasts from TSC patients (TSP12, 16, 20) and control volunteers (CA, CD, CE, and CF) were obtained by punch biopsy. The punch biopsy sample was cultured in DMEM (Gibco) supplemented with 10% fetal bovine serum (Sigma),10 units/mL penicillin and 10 ug/mL streptomycin. Fibroblasts from culture were then reprogrammed to iPS cells.

Fibroblasts from TSP16, TSP20, CD, CE, and CF were reprogrammed using a plasmid transfection protocol as previously described (Okita et al. 2011). Briefly, 6.0 x 10^5 fibroblasts from each line were collected and suspended in 130uL of electroporation buffer containing 1 ug/uL of each of three plasmid expression vectors. Combined the 3 vectors contain *OCT4*, *SOX2*, *KLF4*, *L-MYC*, *LIN28*, and shRNA to *p53*. Cells were electroporated using a Neon cell transfection system (Invitrogen). These cells were transferred to 10 mL DMEM (Gibco) supplemented with 10% FBS (Sigma) without antibiotics in 10 cm cell culture dishes (TPP). The following day, media was changed to DMEM (Gibco) supplemented with 10% fetal bovine serum (Sigma),10 units/mL penicillin and 10 ug/mL streptomycin. After 7 days, cells were trypsinized and 1.0 x 10^5 cells were

replated on 10 cm gelatin-coated plates with $1.8x10^6$ mitomycin C (Sigma) treated SNL cells. The following day media was changed to human embryonic stem cell media containing DMEM/F-12 with Glutamax (Gibco), Knockout Serum Replacement (Gibco), 1X non-essential amino acids (Sigma), 10 units/mL penicillin and 10 ug/mL streptomycin (MediaTech), 4 ng/mL bFGF (Promega) and 0.1 mM β-mercaptoethanol (Sigma). Media was replaced daily for 2 weeks. As iPS colonies appeared, colonies were isolated and plated onto Matrigel (BD Biosciences) coated plates in the human embryonic stem cell media as described above supplemented with LIF (Millipore) and ROCK Inhibitor (Y-27632) (Millipore). iPSC lines were then expanded in mTESR1 media (StemCell Technologies) and used for further experiments.

TSP12 fibroblasts were transduced with lentiviral vectors expressing *KLF4, OCT4*, and *SOX2 (*viPS Vector Kit, Open Biosystems), centrifuged at 800 x g for 45 minutes at room temperature (Paya et al. 2006). CA fibroblasts were transduced with lentiviral vectors expressing mouse *Slc7a1*. Cells were then transfected with a mouse retrovirus to express human *KLF4, OCT4*, and *SOX2.* Six days later 36,000 fibroblasts were plated onto SNL feeder cells, in human embryonic stem cell media as described above. Colonies were picked after 2 weeks and cell lines expanded as described above on Matrigel coated plates in mTESR1 media.

DNA Sequencing and *TSC1/2* Mutation Identification:

Genomic DNA for sequencing was isolated using DNeasy Blood and Tissue Extraction Kit (Qiagen). Exon-containing DNA was captured using SureSelect XT (Agilent). Next generation sequencing was done on an Illumina HiSeq2000 (Solexa) through the Vanderbilt Genome Sciences Resource. FASTQ files were processed by the Vanderbilt Genome Science Resource to variant call files. Putative causative mutations for TSC at the *TSC1* and *TSC2* loci were verified in the variant call files through identifying the same mutation in DNA samples from both fibroblasts and multiple iPSC lines from each patient.

Single Cell Suspension Experiments:

iPSCs from various cell lines were dissociated into a single-cell suspension by incubation at 37°C for 5 minutes in 0.5% Trypsin-EDTA (Gibco). Trypsin was diluted using mTESR1 media and cells were collected through centrifugation at 200 x g for 5 minutes. The supernatant was removed, and cells were washed in PBS and centrifuged again. iPSC were resuspended in mTESR1 media, cells were counted and 50,000 cells were plated per well of a 24-well plate. Cells were allowed to grow for 12 hours for immunofluorescence experiments or 72 hours for alkaline phosphatase. Cells were treated with 20 nM rapamycin (LC Laboratories) dissolved in DMSO (Sigma). Vehicle treated cells were treated with DMSO only.

Alkaline Phosphatase Detection:

Cells were fixed using a citrate buffer/acetone solution. A Fast Violet B capsule (Sigma Aldrich) was dissolved in deionized water. The fast Violet B solution was applied to cells and incubated in the dark at room temperature for 15 minutes. Fast Violet B solution was removed and cells were washed twice with distilled water.

Immunohistochemistry:

Media was removed and cells were washed twice with warm PBS. Cells were fixed with ice-cold 100% methanol at -20°C for 10 minutes. Cells were washed in PBS and blocked in PBS with 5% Normal Goat Serum (Sigma) for 1 hour. Cells were incubated with Nanog primary antibody in block (1:200, Cell Signaling) overnight at 4°C. Cells were washed three times and incubated in a secondary antibody Alexa Fluor-568 (1:500, Invitrogen) for 1 hour at room temperature. Photomicrographs were obtained using a Zeiss Axiovert 200M microscope and a Hamamatsu camera. Images as a group were adjusted for contrast and brightness and counted in ImageJ (NIH).

Immunoblotting:

iPSC lines were lysed in RIPA buffer containing protease and phosphatase inhibitors (Sigma) for 10 minutes on ice. Cellular debris was removed through centrifugation and cellular lysates were stored at -80°C. For nuclear and cytoplasmic fractionation, a NE-PER Nuclear Cytoplasmic Extraction

kit was used (Thermo Scientific). Standard techniques were used for immunoblotting and quantification. Blots were probed using primary antibodies overnight at 4°C. Primary antibodies used include hamartin (1:1000, Cell Signaling), Tuberin (1:1000, Cell Signaling), S6 (1:1000, Cell Signaling), phospho-S6 -Serine 240/244 (1:1000, Cell Signaling), Histone H3 (1:1000, Cell Signaling), β-actin (1:10,000) and Tfe3 (1:500 Sigma). Blots were incubated in secondary antibodies for 1 hour at room temperature. Secondary antibodies (1:10,000, Li-COR) were imaged using the Odyssey Imaging system and quantified with ImageJ (NIH).

Statistical Analyses:

Experiments were done in triplicate. Student's t-test was used to determine significance, p-values as indicated in figure legends. Statistical analysis and graph generation were completed using Prism 5 software (GraphPad).

Results

Generation of Heterozygous iPSC Lines from TSC Patients

We generated iPSCs from multiple patients (TSP12, 16, 20) in addition to healthy volunteers as controls (CA, CD, CE, CF). The pluripotency of cell lines derived from both TSC patients and controls were validated first through expression of various stem cell markers including alkaline phosphatase, OCT4,

and Nanog as shown through both quantitative PCR and immunofluorescence (Cawthon et al. 2013). The ability to differentiate to cells from all three primary germ layers was demonstrated through spontaneous differentiation of embryoid bodies from all stem cells lines and subsequent staining for cell types from all three germ layers. Finally, to ensure cellular stability of these lines, karyotypes obtained from all cell lines displayed no chromosomal abnormalities (Cawthon et al. 2013).

DNA samples from 15 patients, from both iPSCs as well as primary fibroblast cultures from which the iPSCs were derived, were sequenced using highly redundant (>80X coverage for *TSC1* and *TSC2*) exome sequencing to determine causative mutations in these patients and to identify any second-hit mutations. Single mutations were identified in eight of the 15 patients (**Table 3.1**). Consistent with previously described frequencies, of the eight detected mutations, only one patient had a mutation in *TSC1*, with the remaining 7 mutations located in *TSC2.* The location and nature of these mutations are described in **Table 3.1**. The majority of these mutations are frameshift or nonsense mutation. In no patients were two distinct mutations detected, suggesting that these cell lines are heterozygous for either *TSC1* or *TSC2*. The absence of detected mutations in the remaining seven patient samples is likely due to limitations of the sequencing modality used, which is less likely to identify large insertion or deletions. To fully identify mutations in this patient population and further confirm the absence of second-hit mutations in these cell lines, other modes of mutation detection, such

Patient	Gene	Chromosome	Chromosome Location	Mutation	Type of Mutation
TSP8	Tsc2	16	2131735	C to G	Nonsense
TSP20	Tsc2	16	2106206	T to A	Nonsense
TSP21	Tsc2	16	2113023	TTG to T	Frameshift
TSP22	Tsc1	9	135796754	G to A	Nonsense
TSP23	Tsc2	16	2129160	C to T	Nonsense
TSP24	Tsc2	16	2138045	CCA to C	Deletion of Splice Site AG Acceptor
TSP30	Tsc2	16	2121871	C to T	Missense
TSP31	Tsc2	16	2134478	C to T	Nonsense

Table 3.1 Identified mutations of *TSC1* or *TSC2* in 8 patients with TSC

as multiplex ligation-dependent probe amplification should be used in future studies.

Despite this limitation of our sequencing technique, immunoblotting for hamartin and tuberin gives further evidence for heterozygosity in these TSC patient-derived iPSCs. Most *TSC1* mutations result in nonsense-mediated RNA decay, while most *TSC2* mutations result from nonsense or frameshift mutations that result in a premature stop codon (Jeganathan et al. 2002; Maheshwar et al. 1997). Therefore, if a loss of heterozygosity has occurred at either of these loci, we would expect to see an absence of hamartin or tuberin on western blot. Specifically to rule out the possibility of detecting a truncated N-terminal segment of tuberin lacking a functional GAP domain, we probed for tuberin using an antibody directed towards a C-terminal epitope. Immunoblot analysis revealed the presence of both hamartin and tuberin in our cell lines, consistent with a heterozygous nature of these cells (**Figure 3.1A**).

Additionally, cells without either functional copy of either *TSC1* or *TSC2* should display gross hyperactivation of the mTORC1 pathway. Instead, immunoblots for phospho-S6, a downstream readout of mTORC1 activity, show that patient-derived iPSCs have similar levels of mTORC1 activity as compared to controls (**Figure 3.1 B**). The combination of immunoblot analysis and deep sequencing data provides strong evidence that these TSC patient-derived iPSC are heterozygous for either the *TSC1* or *TSC2* loci.

While these TSC patient-derived iPSCs appear to have normal mTORC1 activity (as measured by pS6 levels with immunoblotting), patient-derived iPSCs

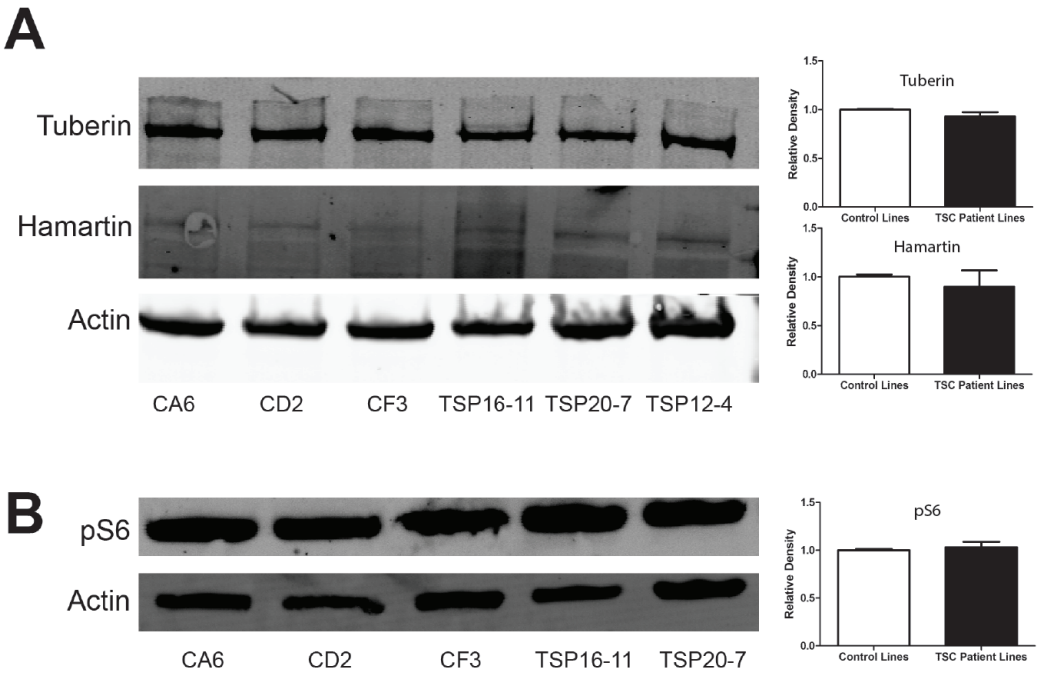


Figure 3.1 TSC Patient Derived iPSCs express both *TSC1* and *TSC2* and do not have hyperactivation of mTORC1 activity. (A) Three patients derived iPS lines (TSP12-4, 16-11, and 20-7) have detectable tuberin and hamartin. (B) Patient lines (TSP16-11 and TSP20-7) do not have gross hyperactivation of mTORC1 as shown through S6 phosphorylation.

have phenotypes consistent with increased mTORC1 signaling, such as increased cell size and proliferation (Cawthon et al. 2013). In addition, treatment with the mTORC1 inhibitor rapamycin slows proliferation and decreases the cell size towards normal, further implicating more subtle changes in mTORC1 signaling as underlying the phenotype of TSC patient-derived cells (Cawthon et al. 2013).

TSC iPSC Have Increased Cell Survival and Maintenance of Pluripotency

We next wanted to explore whether heterozygous patient iPS lines demonstrated increased cell survival and resistance to differentiation. To test this hypothesis, we developed a technique to stress iPSC through single-cell dissociation and sparse plating. It is well established that, unlike murine cells, human stem cells do not grow well when dissociated into single-cell suspensions (Fong et al. 2004; Pyle, Lock, and Donovan 2006) Loss of cell-cell adhesions cause human ES cells to undergo apoptosis and spontaneously differentiate (Watanabe et al. 2007; Narumiya, Ishizaki, and Uehata 2000; Amit et al. 2000). Cells enzymatically dissociated, using trypsin or Accutase, are stressed at higher levels than mechanically separated cells and have plating efficiencies of less than 3% (Holm et al. 2013; Hasegawa et al. 2006). Therefore, to maximize cell plating and pluripotency after passaging, cells are often mechanically separated and allowed to remain in larger aggregations (Sjogren-Jansson et al. 2005;

Rosler et al. 2004; Carpenter et al. 2004). In cases where cells are enzymatically dissociated, treatment with a Rho-associated coiled-coiled kinase (ROCK) inhibitor is required to prevent premature differentiation and apoptosis (Holm et al. 2013; Watanabe et al. 2007).

To test the cell survival and maintenance of pluripotency of our TSC patient lines, we stressed the cells using enzymatic dissociation. Single-cell suspensions from multiple control and patient lines were prepared with trypsinization followed by resuspension in stem cell media, and were plated at 50,000 cells/well of a 12-well dish. Cells were allowed to grow for three days, and a colorimetric substrate was used to detect alkaline phosphatase activity, which is a cell surface marker of pluripotent colonies (O'Connor et al. 2008). After cell dissociation and plating, TSC patient-derived cells expanded to fill the entire well, with the vast majority of these cells positive for alkaline phosphatase (**Figure 3.2 B**). Conversely, control cell lines had much less densely plated cells with only a few colonies staining positive for alkaline phosphatase (**Figure 3.2A**). To test whether the increased survival and maintenance of pluripotency of TSC patient-derived iPSC was mTORC1 dependent, we treated these single-cell suspensions with rapamycin (20 nM) 1 hour before and during plating. Rapamycin greatly reduced the number of alkaline phosphatase-positive cells (**Figure 3.2C**).

To determine whether this was simply a trypsin specific sensitivity, we completed the same experiment with a ‘gentler’ method of disassociating iPSCs, using Accutase to enzymatically dissociate the cells (Bajpai et al. 2008). The results were the same as seen previously with trypsin, with TSC patient-derived

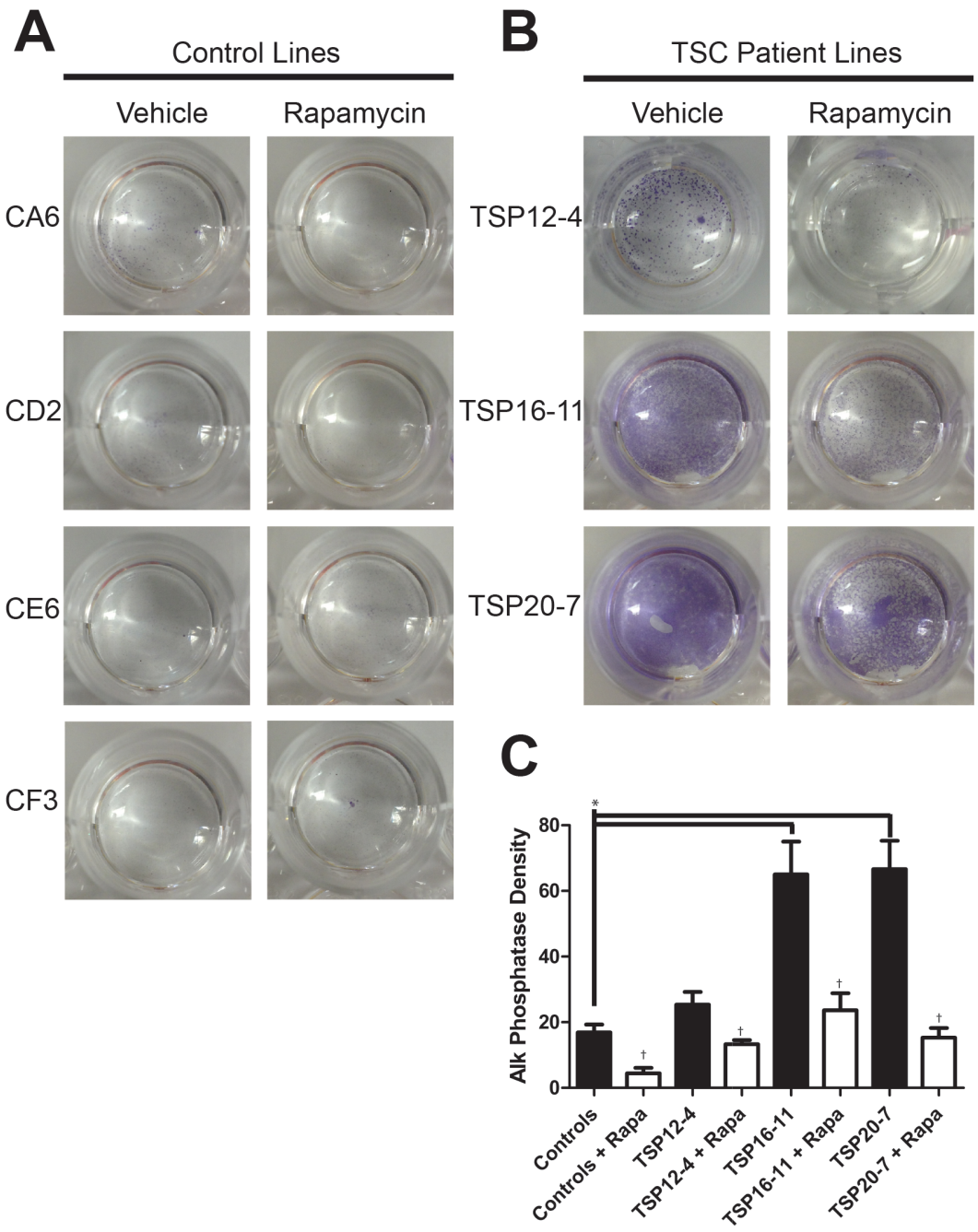


Figure 3.2 TSC Patient Derived iPSCs have increased survival and maintenance of pluripotency 3 days after single cell suspension. Control (A) and patient lines (B) were enzymatically dissociated and plated at 50,000 cells per well of a 24-well plate. 3 days after plating, cells were stained for alkaline phosphatase activity. Density of the colorimetric assay were measured. 2 of the 3 patient cell lines were significantly different than control lines (* $p < 0.05$). Density of all cell lines treated with rapamycin were significantly decreased compared to the same line treated with vehicle only († $p < 0.05$).

lines having increased survival and expression of pluripotency markers much more than controls (Data not shown).

To evaluate the possibility that differences in the doubling times of the cell lines accounted for some of the dramatic results seen 72 hours after plating, we dissociated and plated the cells through the same methods as before, but analyzed the cells 12 hours later. Of note, alkaline phosphatase is a cell surface marker and is digested through enzymatic dissociation techniques and does not recover within this 12 hour timeframe. Instead, to measure pluripotency at 12 hours we used immunofluorescence for Nanog to determine the relative number of pluripotent cells. Patient-derived iPSCs had a dramatically increased percentage of Nanog-positive cells as compared to controls (**Figure 3.3A-C**). Additionally, a much larger number of patient-derived iPSCs survived single-cell suspension as compared to control lines (**Figure 3.3D**).

After these results, we were interested in the mechanism of increased pluripotency of these cells. A recent paper using mouse ESCs showed that hyperactivation of mTOR signaling after *Tsc2* knockdown resulted in the translocation of the transcription factor Tfe3 from the cytoplasm to the nucleus (Betschinger et al. 2013). This intracellular translocation is required for maintenance of pluripotency. However, knockdown of *Tsc2* in this model resulted in detectable hyperactivation of mTORC1, which we have not been able to demonstrate in our iPSCs by immunoblotting (**Figure 3.1**). Preliminary analyses suggest increased TFE3 in the nuclear fractions of our patient cells compared to controls (**Figure 3.4**). While confirmatory experiments are required, this suggests

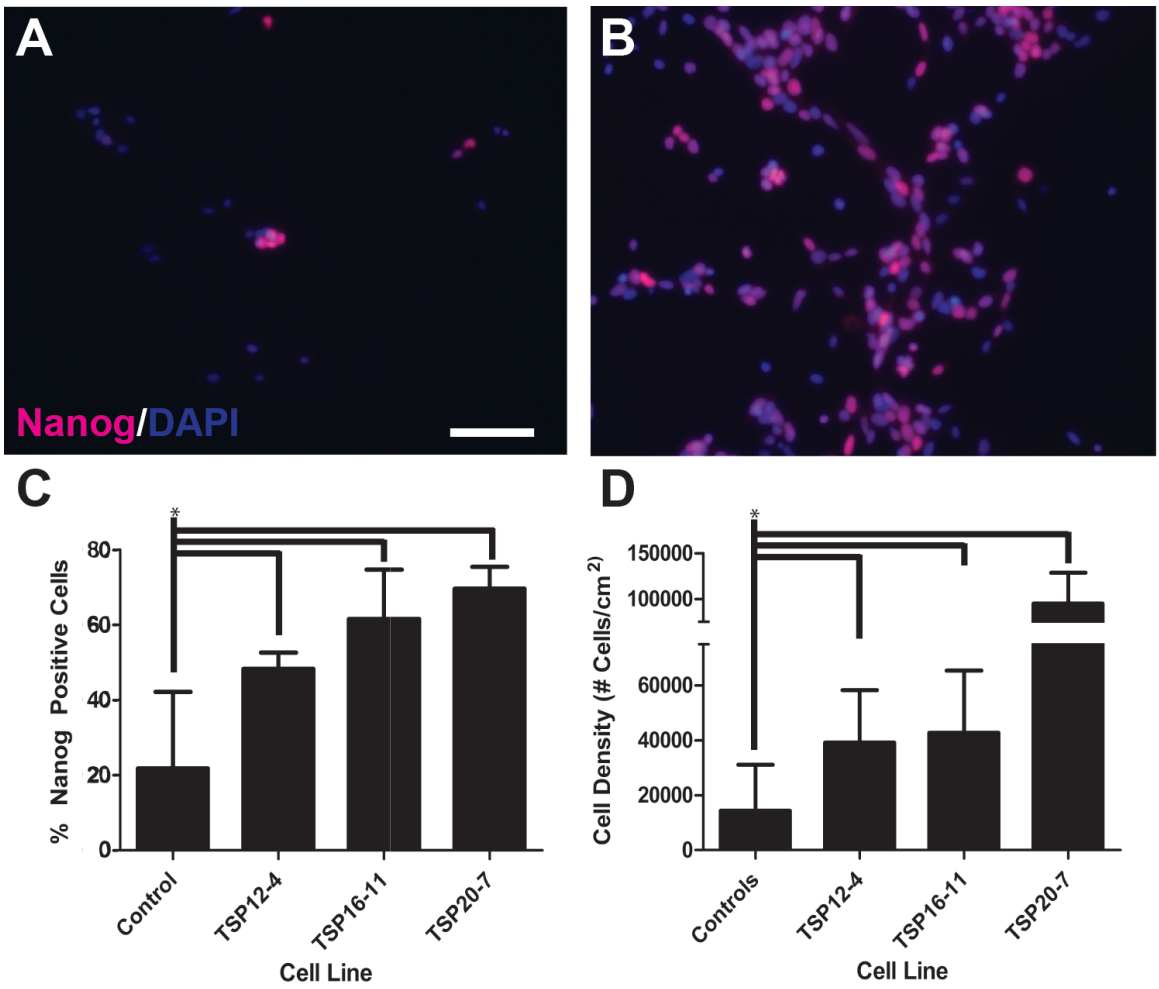


Figure 3.3 TSC Patient iPSCs have increased survival and maintenance of pluripotent markers 12 hours post single cell suspension and plating. Cell lines were enzymatically dissociated and plated at 50,000 cells per well of a 24-well plate. (D) More patient cells adhered and survived than controls. (C) A larger percentage of cells retained staining of the marker of pluripotency Nanog as well. Representative images from controls (A, CD2) and TSC patient lines (B, TSP16-11) are shown. * $p < 0.05$.

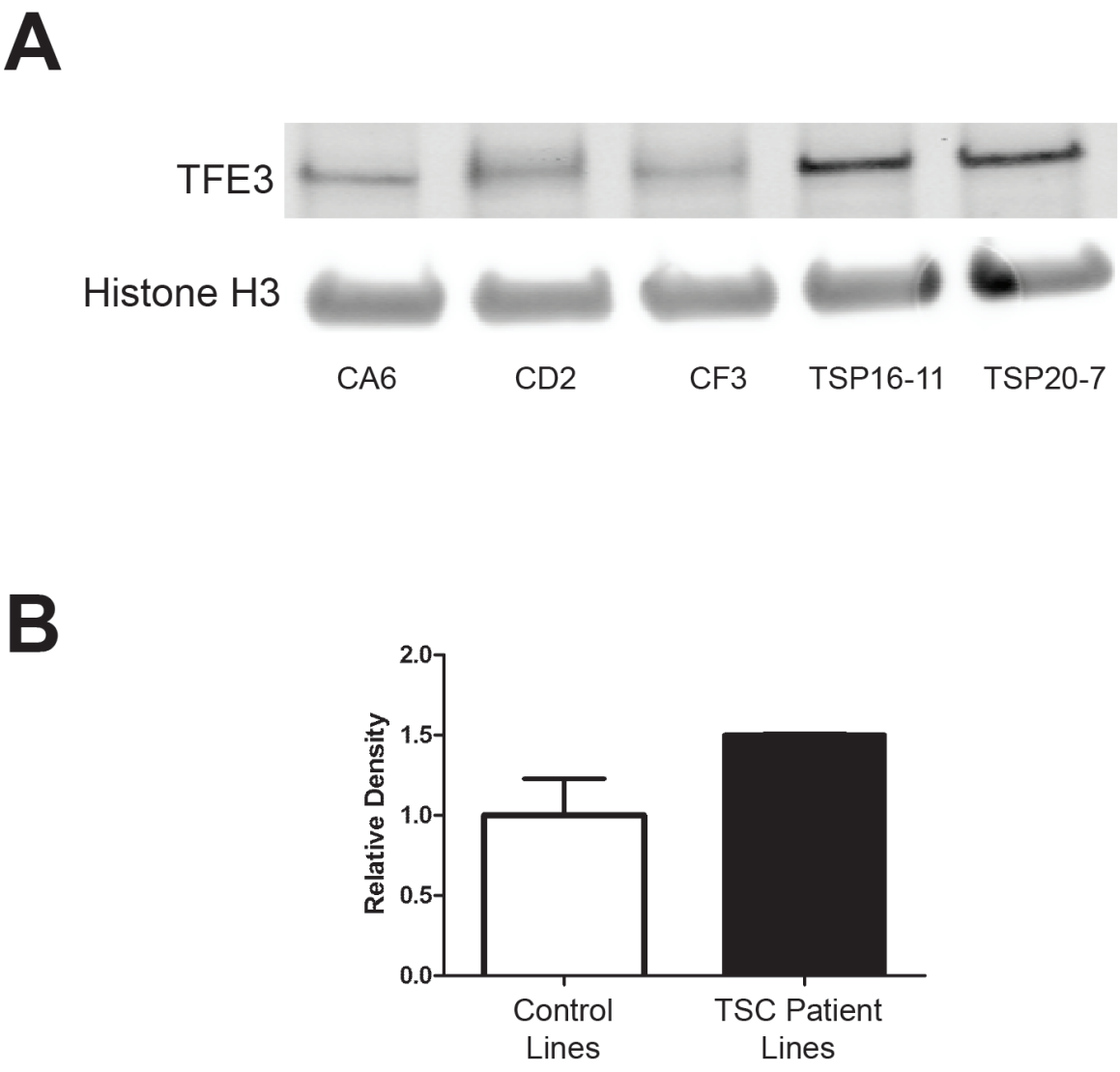


Figure 3.4 TFE3 is increased in the nucleus of TSC patient derived iPSCs
A) Western blot of nuclear extracts from control and TSC patient derived iPSCs show increased nuclear TFE3 in patient lines. B) Relative levels of TFE3 on western blot, normalized to Histone H3 levels.

that increased TFE3 translocation to the nucleus may contributes to the increased pluripotency we have observed. Additionally, this suggests that subtle changes in mTOR signaling are sufficient for TFE3 translocation.

Discussion

These data, generated using patient-derived iPSCs, have provided novel insight into the mechanism of TSC pathogenesis and implicated pathways of cell survival and pluripotency. These patient lines are the first use of iPSCs to dissect the pathogenesis of TSC. TSC patient derived iPSCs are heterozygous for *TSC1* or *TSC2* and are therefore a more accurate representation of the neuronal lesions seen in the disease. We propose that these phenotypes of patient-derived iPSCs are due to haploinsufficiency of either *TSC1* or *TSC2*. These data demonstrate human iPSCs may be an important tool in studying neurological manifestations of TSC, because other heterozygous models, including rodents, have minimal brain pathology.

Interestingly, we have shown that these heterozygous cells have abnormalities consistent with altered mTORC1 activity, indicating that a loss of heterozygosity may not be necessary for disease phenotypes in the context of specific organ systems. Previously, we have shown these cells to be larger than and proliferate more quickly than controls, consistent with increased mTORC1 activity. Furthermore, these deficits can be reversed through rapamycin treatment and inhibition of mTORC1 activity.

We have continued to build on these observations, and now have intriguing data that TSC patient derived iPSCs show increased survival and pluripotency. This increased survival may be due to an inhibition of apoptosis. Many cell types need cell-cell adhesions and proper anchorage to an extracellular matrix to survive. When these contacts are lost, cells go through cell cycle arrest and activate caspase-3 and other factors to induce apoptosis (Pelizon et al. 2002; Chiarugi and Giannoni 2008). The signal transduction between loss of anchorage and cell cycle arrest and apoptosis is mediated through ROCK1-dependant inhibition of mTORC1 signaling (Arakawa-Takeuchi et al. 2010; Park et al. 2011). Therefore, we suspect the increased survival in our single-cell suspension experiments is due to increased mTORC1 signaling. This is further supported through the reversal of this phenotype with mTORC1 inhibition with rapamycin. To confirm this, activation of apoptotic signaling in dissociated cultures should be further examined in our TSC patient-derived iPSCs.

These patient derived cells also maintained pluripotency more frequently than control cells. Our experiments indicate increased pluripotency may be due to increased levels of nuclear TFE3, though further work is needed to verify the mechanisms responsible for these phenotypes. The regulatory network connecting mTOR signaling to this translocation has recently been explored, but additional work needs to be done to determine whether putative mTOR targets involved in pluripotency such as Flcn1/2 and Fnip1 are truly substrates of mTORC1 (Betschinger et al. 2013). Additional studies are also needed to

determine whether these cells are resistant to directed differentiation towards specific cell types, such as toward neuronal fates.

Overall, my data are consistent with the following model of TSC pathogenesis **(Figure 3.5)**. Increases in mTORC1 signaling prevent transduction of pro-apoptotic and cell cycle arrest signals through ROCK1. Normally, cellular stress and anchorage depletion should cause ROCK1 to decrease mTORC1 signaling through phosphorylation and activation of tuberin. Instead, in our TSC patient derived iPSCs, low levels of constitutively active mTORC1 blunt this response and instead promote cell survival. These same low levels of constitutively active mTORC1 permit the nuclear translocation of TFE3 to the nucleus via phosphorylation of FLCN1. Accumulation of TFE3 results in increased pluripotency of these cells. In keeping with this model, low dose rapamycin should reverse these phenotypes through inhibition of this subtle increase in mTORC1.

The combination of increased survival and pluripotency, combined with previous data showing increased proliferation and cell size may be instructive to understanding the formation of cortical tubers. First, cortical tubers contain giant cells that may be mechanistically related to the cell size phenotype of these iPSCs. Increased proliferation and cell survival can easily manifest in solid organs as disorganized patterning. Without proper neurogenesis, focal lesions could form, comprised of improperly arranged cells. Additionally, increased pluripotency and cell survival could account for the abnormally differentiated and immature cells present in the cortical lesions. Progenitor populations may be

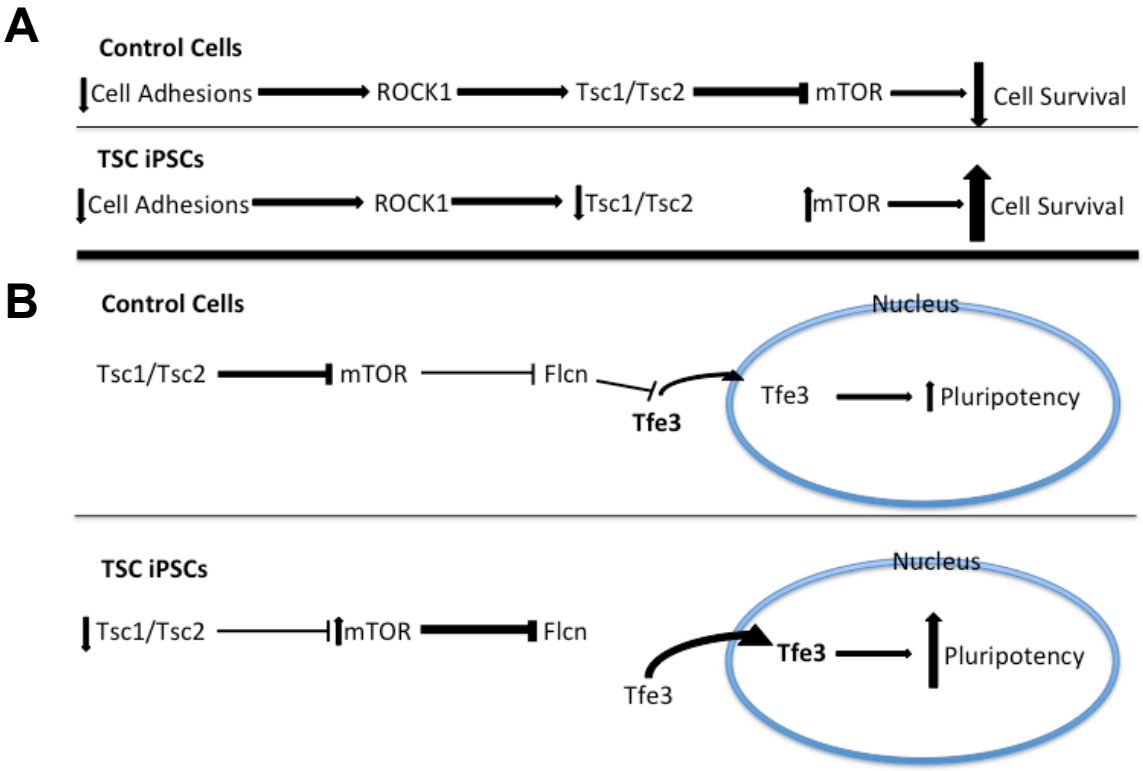


Figure 3.5 Proposed models of increased survival and pluripotency in TSC iPSCs. A) Patients lines have increased survival through blunted responses to ROCK1 signaling in response to single cell suspension techniques. B) Patient lines have slightly increased mTOR activity that promotes TFE3 translocation to the nucleus.

resisting normal differentiation cues in the context of subtle mTORC1 activation, thus remaining in a relatively undifferentiated state. This could account for the abnormal cells that display markers of both neurons and astrocytes. Future work will explore both the response of these iPSCs to differentiation cues and properties of differentiated neurons from these lines. These experiments will further define the pathogenesis of neuronal phenotypes of TSC.

CHAPTER IV

FUTURE DIRECTIONS: TISSUE SPECIFIC PHENOTYPES AND BROADER IMPLICATIONS FOR HUMAN HEALTH

The work presented in this dissertation describes the characterization of TSC related phenotypes in both kidney and stem cell populations. In the kidney, we have shown that loss of *Tsc1* and hyperactivation of mTORC1 signaling results in cystic kidneys, possibly through a ciliary related mechanism. In stem cell populations, I have identified that cell survival and maintenance of pluripotency are altered in cell lines heterozygous for *TSC1* or *TSC2*. Here, I will discuss the relevance of the data presented. I have broken this chapter into three parts. In Part I, I will discuss why tissue specific pathogenesis occurs in TSC. Part II will discuss how understanding the mechanisms of pathogenesis in TSC provides a foothold to a better understanding of autism and epilepsy. In Part III, I will conclude with future directions of the experiments presented in Chapters II and III.

Part I: Tissue Specificity of Pathogenesis

One of the most important topics explored in TSC is how a germline heterozygous mutation contributes to focal, organ specific phenotypes. This is a pressing question in many diseases, whether it is other hamartomatous disorders

such as PTEN Hamartoma Tumor Syndrome or cancer syndromes such as retinoblastoma. My work in kidney and stem cell populations strongly suggests that separate mechanisms of disease pathogenesis can occur in different organ systems. Specifically in TSC, we observe loss of heterozygosity driving the pathogenesis of non-neuronal lesions, while neurologic phenotypes can occur with only heterozygous mutations of either *TSC1* or *TSC2.* In TSC, tissue sensitivity to mutations in these genes is likely a combination of 1) differential levels of hamartin and tuberin in different tissue types, 2) the tissue specific functions controlled by mTOR signaling, and 3) expression of effector and modifier genes that can compensate for *TSC1* or *TSC2* disruption.

The location and timing of hamartin and tuberin expression may provide clues to why only certain organ systems are affected in TSC. While hamartin and tuberin appear to be ubiquitously expressed, certain organs have higher levels of these proteins than others. Early in development hamartin and tuberin levels are high throughout the embryo, but levels are especially high and expressed very early in cardiac tissue. Other areas of high expression include the kidney and brain (Murthy et al. 2001). Organs with the highest levels of protein are likely the organs that would be most affected by a loss of these proteins. High expression in these organs embryonically, therefore, are not surprising, as these three organs have high rates of TSC associated pathologies. Furthermore, while levels of hamartin and tuberin decrease dramatically throughout the rest of the body postnatally, levels of these two proteins remain high, well into adulthood, in the brain. This suggests an important ongoing role in neurological tissue, which may

suggest higher sensitivity to subtle changes in the levels of these proteins.

The distinct tissue specific cellular functions controlled through mTOR signaling may also play a role in determining which cell types are most sensitive to changes in hamartin and tuberin levels. Brain tissue dynamically regulates mTOR signaling to properly modulate many tightly regulated neuronal processes including axon growth, dendritic arborization, synaptogenesis, and protection of neurons from toxicity through regulation of autophagy (Jaworski et al. 2005; Kumar et al. 2005; Ravikumar et al. 2004; Miller and Kaplan 2003). These processes are controlled through an intricate signaling pathway and subtle perturbations can be magnified into large phenotypes. In the kidney, however, mTOR serves as a more blunt signal for cellular growth and provides a cellular mechanism to respond to injury and increased filtration demand in cases such as diabetic nephropathy or loss of contralateral kidney function (Nagai et al. 2005; Cleper 2012). This response is initiated primarily to increase organ mass to accommodate increased demand on the organ. This organ-wide response is in contrast to the fine alterations seen in neuronal tissue, such as synaptic changes in individual neurons. These differences of mTOR function in the brain and kidney may explain why brain tissue is sensitive to heterozygosity in *TSC1* or *TSC2*, while kidney lesions require loss of both copies of either gene.

Finally, effector genes or non-genetic environmental ‘second hits’ may be playing a role in the focal nature of lesions in the brain. Patients with TSC, while having similar causative mutations in *TSC1* or *TSC2* have wide ranging clinical severity. This is likely due to multiple confounding variables such as

environmental causes and mutations in modifier genes (Nadeau 2005). Perhaps other, yet unidentified, genes may be partially compensating for heterozygous loss of either of these genes in the brain, as is seen in other neurologic disease models (Tanabe, Martin, and Dauer 2012). Somatic mutations in progenitor cells may create a localized population of cells heterozygous for these yet unidentified compensatory genes. This may provide a specific region where the cells in TSC, already abnormal due to slight alterations in mTOR signaling, combined with altered compensation processes can initiate cortical tuber formation. Advances in sequencing should allow the comparison of genomic variation between skin and tuber samples from the same patients, allowing possible local modifier genes to be identified. We cannot rule out, however, the possibility that rare second hit mutations in *TSC1* or *TSC2* may generate organizing foci for tuber formation (Crino et al. 2010; Qin et al. 2010). As seen in our kidney model in Chapter II, loss of heterozygosity in the DCT can affect neighboring cells in the PCT. Non-cell-autonomous effects of the rare giant cells with a somatic second hit, may still affect the location of tubers and severity of disease. While these cells appear to be electrically silent and may not be integrated into neuronal circuitry, they may be affecting neighboring cells through cell-cell contact or soluble factors (Cepeda, Andre, Flores-Hernandez et al. 2005; Cepeda, Andre, Vinters et al. 2005). Studies in kidney as well as the brain can help identify non-cell-autonomous mechanisms of altered mTOR signaling. iPSCs modified with knockdown of *TSC1* and *TSC2* grown in co-culture with cells heterozygous for the same gene could help tease out the role of non-cell-autonomous changes in TSC

pathogenesis. While the focal nature of hamartomas and the organs in which they occur are still poorly understood, the combination of iPSCs and rodent models should greatly enhance our knowledge of this phenomenon. iPSCs are an especially important tool as they are the first model that display readily apparent phenotypes in heterozygous cells. This tool will specifically help us understand the neurological signs of TSC, like cortical tubers, as well as neurological symptoms of TSC, such as autism and epilepsy.

Part II: mTOR Signaling in Autism and Epilepsy

Autism spectrum disorders are neurological diseases characterized by the three core behavioral abnormalities of impaired social interactions, communication deficits, and repetitive behaviors (Association 2000). Often these characteristic behaviors are present along with other comorbid conditions like epilepsy and cognitive disability, as is seen in TSC (Matson, Matson, and Beighley 2011). Autism is a growing problem, and current prevalence of the disease is estimated at 1% (Kogan et al. 2009). While much research has looked into the etiology of autism, no single cause has emerged. However, twin studies have suggested a largely genetic basis for the disease. Identical twins have up to a 90% concordance rate for the disorder (Bailey et al. 1995; Ronald and Hoekstra 2011). While many genes contribute to the pathogenesis of autism, TSC and other genetic syndromes have proved very useful tools in understanding this condition. Syndromes causing ASD with a single genetic

cause are specifically useful, because identification of mutated genes and their associated pathways provides a foothold to understand the etiology of the larger collection of disorders. While only 10% of all ASD patients have such neurodevelopmental syndromes, research into their associated pathologies has proven invaluable to understanding ASD as a whole (Hampson, Gholizadeh, and Pacey 2012). TSC related behaviors of autism including impaired social interactions, stereotypical behaviors and communication deficits were identified early in the 20th century, long before autism was described as a diagnosis (Critchley and Earl 1932; Kanner 1968). TSC remains one of the best-understood monogenetic causes of autism to date. In addition to TSC, Rett Syndrome, Fragile X syndrome, and PTEN hamartoma tumor syndrome are all single gene genetic disorders that cause autism. TSC and dysregulated mTOR signaling have been linked to autism, but recent work on these other monogenetic causes of autism has begun to reveal mTOR as signaling hub for ASD. Therefore understanding upstream control and downstream signaling mechanisms of mTOR may be crucial in identifying targets for autism treatments in the future.

Rett syndrome is a developmental disorder caused by mutations in the Methyl-CpG-binding protein 2 (*MECP2)* gene. This gene is found on the X chromosome. Males with mutations are thought to have embryonic lethal phenotypes and is thus only found in females. Patients with Rett syndrome develop normally until around 6 to 18 months of age. At onset, there is a developmental regression, specifically in motor development. Hand wringing behaviors become especially prominent (Davis 2011). Children with Rett

syndrome have severe neurological deficits including autism, epilepsy, and cognitive impairment (Chahrour and Zoghbi 2007). The *MECP2* gene is a transcriptional regulator that works as through modifying chromatin structure (Nan et al. 1998). Rodent models have shown that *Mecp2* regulates dendrite and spine morphogenesis as well as altering synaptic plasticity (Boggio et al. 2010; Kishi and Macklis 2005). Interestingly, these changes in neuronal function seem to be mediated through mTOR signaling. Rodent models with a loss of *Mecp2* show markedly decreased mTOR signaling, and levels continue to decrease as the disease progresses (Ricciardi et al. 2011). Furthermore, the loss of *Mecp2* and associated decrease in mTOR signaling results in decreased protein synthesis in these neurons (Ricciardi et al. 2011). Changes in protein translation have been clearly linked to altered synaptic activity and neuronal morphology, so it is likely decreased mTOR signaling and associated decrease in mTORC1-dependant translation is responsible for these effects (Jaworski and Sheng 2006; Hoeffer and Klann 2010). Decreased mTOR activity in Rett Syndrome is particularly interesting, because it is the opposite direction of change as observed in TSC. This suggests that precise regulation of mTOR is crucial for normal neuronal function, and either activation or inhibition of the pathway may be detrimental to developmental disorders.

Fragile X syndrome (FXS) is another neurodevelopmental disorder characterized by cognitive impairment and autism, as well as macrocephaly. This disorder is a trinucleotide repeat disorder, where expansion of a trinucleotide causes silencing of *FMR1* gene (Verkerk et al. 1991). Loss of *FMR1* expression

causes increased protein synthesis, possibly through increased activation of metabolic glutamate receptor 5 (mGluR5) (Bear, Huber, and Warren 2004). Treatment with mGluR5 antagonists has shown some promise in reversing increased protein synthesis and FXS associated phenotypes (Krueger and Bear 2011). Interesting, loss of *FMR1* seems to increase synthesis of mTOR signaling components. Additionally, mGluR5 activation may be signaling directly through mTOR pathways to increase protein synthesis. Loss of *FMR1* causes increased production of the catalytic subunit of PI3K, and subsequent hyperactivation of this kinase, an upstream regulator of mTOR signaling (Gross et al. 2010). Further experiments have shown that loss of *FMR1* also increased assembly of mTOR Complex 1 and increased activity through S6 and 4E-BP1 phosphorylation (Sharma et al. 2010). This provides further genetic evidence that dysregulation of mTOR signaling may underlie some of the phenotypes in ASD.

PTEN hamartoma tumor syndrome is an autosomal dominant disorder caused by mutations in the phosphatase and tensin homolog (*PTEN*) gene and is characterized by hamartomatous growths in various organs including the skin, breasts and GI tract. While most of these lesions are benign, some will progress to malignancy. Patients with PTEN hamartoma tumor syndrome have approximately a 90% probability of cancer during their lifetime, most commonly of breast tissue (Riegert-Johnson et al. 2010). This syndrome encompasses many distinct sub-syndromes such as Cowden syndrome, Lhermitte-Duclos disease, and Proteus syndrome with similar causative mutations, but not completely overlapping pathologies (Salmena, Carracedo, and Pandolfi 2008). The protein

product of the causative gene, PTEN, is a negative regulator of the mTOR signaling pathway. Loss of PTEN results in the hyperactivation of mTOR signaling and formation of the characteristic hamartomas similar to TSC (Kwon et al. 2006). Finally, mouse models have shown that loss of *Pten* results in macrocephaly and autistic behaviors subsequent to this hyperactivation of mTOR signaling (Page et al. 2009).

In addition to TSC, it is interesting that this collection of the four most common monogenetic causes of autism all have disruption of mTOR signaling as a common component in disease pathogenesis. Understanding the regulation of this pathway and its downstream targets will be crucial to provide a foothold into the causative mechanisms of the varied causes of autism. In addition to these monogenetic causes, there is some clinical evidence that mTOR signaling may also be implicated in sporadic autism. Up to 30% of ASD patients have macrocephaly (Lainhart et al. 2006). This is similar to macrocephaly seen in TSC rodent models as well as patients with either PTEN hamartoma tumor syndrome or Fragile X syndrome. These macrocephaly inducing disorders all have identified increases in mTOR activity, and alterations of protein synthesis, cell growth, differentiation, and cell survival. Dysregulation of any of these cellular processes could manifest as increased brain volume. The high prevalence of macrocephaly in non-syndromic forms of ASD provides further support alterations in the mTOR signaling pathway in the pathogenesis of autism.

With these data implicating dysregulated mTOR as important in the pathogenesis of autism, pharmacologic modifiers of the mTOR signaling pathway

have begun to be explored as a therapeutic approach for autism. As the role of mTOR signaling is most closely related to TSC pathologies, initial studies have focused on treating TSC rodent models, as well as patients, with mTORC1 inhibitors such as rapamycin. In mouse models, autistic-like behaviors in mice heterozygous for *Tsc2* are reversed through mTOR inhibition (Sato et al. 2012). These promising results have spurred clinical trials in TSC patients, currently ongoing, exploring whether similar inhibitors can reverse autistic behaviors in humans (Sahin 2011).

While inhibition of mTOR signaling may be therapeutic in TSC, other autistic disorders may need a more targeted approach for therapy. For example, while Fragile X syndrome has increased mTOR signaling similar to TSC, this increased activity has the opposite downstream effects in the pathogenesis of each disease. Fragile X Syndrome shows activation of mTOR signaling downstream of mGluR5 activation (Sharma et al. 2010). This causes increased protein synthesis at the synapse and increased synaptic plasticity (Sharma et al. 2010; Huber et al. 2002). Conversely, while protein synthesis is also increased in TSC, rodent models of TSC have impaired synaptic plasticity (von der Brelie et al. 2006). In TSC, while cellular wide protein synthesis is increased, it appears that protein synthesis is, in fact, reduced in the synaptic compartment of these neurons (Auerbach, Osterweil, and Bear 2011). Therefore, while these two monogenetic disorders both have increased mTOR signaling, this dysregulation has opposite effects on synaptic plasticity. Surprisingly, double mutants heterozygous for both *Tsc2* and *Fmr1* do not display synaptic phenotypes in

contrast to each mutation alone. Presumably, the opposite effects on synaptic plasticity correct each other, resulting in properly functioning neurons (Auerbach, Osterweil, and Bear 2011).

Understanding intricacies of mTOR signaling and disease specific differences should lead to insights in pharmacological targets. In Fragile X syndrome, specific inhibition of the mTOR pathway through targeting of mGluR5, upstream of the pathway, has been promising in rodent models of the disease (Krueger and Bear 2011; Vinueza Veloz et al. 2012). Downstream targets of mTOR signaling have also been modified to correct phenotypes in rodent models of autism. Downstream of mTORC1, the target of 4E-BP1, eukaryotic initiation factor 4E (eIF4E) has been linked to autism as well (Neves-Pereira et al. 2009). Fragile X syndrome, through increased mTORC1 and inhibition of 4E-BP1, has increased activation of eIF4E (Wang, Kim, and Zhuo 2010). The synaptic dysfunction in models of Fragile X syndrome is phenocopied when eIF4E is either overexpressed or disinhibited through the removal of binding partners (Gkogkas et al. 2013). Furthermore, pharmacological inhibition of eIF4E corrects the autistic behaviors seen in these mice (Gkogkas et al. 2013). The effective modulation of both upstream effectors, such as mGluR5, and downstream targets of mTOR signaling, such as eIF4E, to treat mouse models of ASD suggest that further understanding of the mTOR pathway should prove instructive for drug development. Elucidating the differential effects of altered mTOR signaling on various downstream targets such as synaptic protein synthesis and synaptic plasticity is important for identifying viable treatment

options for the many separate etiologies of ASD.

Our results in TSC patient derived iPSCs will augment the identification of these potential treatment targets for ASD. Patient derived iPSCs have increased cell size, proliferation, maintenance of pluripotency and cell survival, providing interesting clues to targetable downstream pathways for the treatment of TSC. As mentioned previously, macrocephaly is found in many of the monogenetic disorders causing autism as well as sporadic ASD cases. Our findings in human iPSCs help provide a basis for understanding this phenotype. Increased numbers of cells in the brain due to increased proliferation and survival, and the subsequent larger size of terminally differentiated cells can clearly result in a large brain phenotype. Additionally, we have shown increased pluripotency and cell survival in our TSC iPSC lines. Persistence of a progenitor pool, due to resistance to differentiation, will result in a longer period of neurogenesis and subsequently more cells in the brain. Further, proper neurodevelopment is dependant on an extensive period of pruning, with loss of synaptic connections as well as a large number of neurons. Increased survival of our iPSC lines suggest that this normal pruning process may be disrupted in TSC and result in more cells and inappropriate connections between cells. These mechanisms may also be seen in other monogenetic forms of autism that have altered mTOR signaling, as well as less well understood sporadic cases. Identification of pharmacological targets in the pathways of pluripotency (TFE3) or cell survival (ROCK) may be helpful in the treatment of TSC as well as other autism spectrum disorders.

Another important neurological symptom of TSC is epilepsy. Like autism, better understanding the mechanisms of epileptogenesis in TSC may provide clues to the cause and pathogenesis of epilepsy generally, as well as help identify targets for new antiepileptic drugs. Epilepsy is present in up to 90% of patients with TSC (Yates et al. 2011; McClintock 2002). Outside of TSC patients, epilepsy is still a relatively common disorder, affecting approximately 1% of the general population (Russ, Larson, and Halfon 2012). While current treatments for epilepsy focus on reducing excitatory circuits and enhancing inhibitory circuits in the brain, there is a paucity of treatments that target the molecular mechanisms underlying this disorder. mTOR signaling has been implicated in epileptogenesis and mTOR inhibitors have shown promise in treating the disease. Therefore, understanding of TSC may provide insight into how dysregulated mTOR signaling results in epilepsy.

The process of epileptogenesis begins with some injury to neurological tissue. In the case of TSC, this injury is due to genetic and molecular causes. In many other cases, while genetic predisposition is likely important, the injury might be due to a traumatic or infectious insult to the tissue. Whatever the initiating insult, a silent phase of molecular changes eventually results in the presentation of multiple spontaneous seizures (Pitkanen and Lukasiuk 2011). During the silent phase, molecular changes result in neurogenesis, astrocytosis, and synaptic modifications (Pitkanen and Lukasiuk 2009). As mentioned earlier, current antiepileptic drugs can change the excitatory and inhibitory electrical pathways in the brain, managing the recurrence of seizures. These drugs, however are

largely ineffective at delaying or preventing the generation of seizures after an initial insult (Temkin 2009). Understanding the molecular changes during this silent phase will be instructive to identifying drugs that can inhibit the generation of epilepsy rather than just treatment of symptoms. As the cortical tubers in TSC display astrocytosis and alterations in neurogenesis, similar to acquired epilepsy, it is likely that understanding the pathogenesis of TSC will provide insight to epilepsy as a whole. Furthermore, as described earlier, mTOR signaling plays a major role in synapse formation and function. Alteration in synapses and the electrical circuitry is crucial in epileptogenesis (Dudek and Shao 2004). Additionally, mTOR is a major regulator of axon and dendritic growth and neuronal survival, both processes with roles in epilepsy (Kumar et al. 2005). These observations suggest mTOR signaling may mediate some of these changes during the silent phase of epileptogenesis. Interestingly, the ketogenic diet has been used for more than a decade in the treatment of epilepsy. While difficult for many patients to adhere to, this diet can be effective at controlling refractory epilepsy (Freeman, Kossoff, and Hartman 2007). Recently it has been shown this high fat, low carbohydrate diet limits circulating glucose, which in turn decreases mTOR activity (McDaniel et al. 2011). Decreased mTOR activity may be mediating the antiepileptogenic effect of this diet, further linking mTOR activity to epilepsy. Therefore, better understanding of the upstream control and downstream targets of mTOR signaling through models of TSC may lead to new treatments for epilepsy.

Increased mTORC1 signaling in TSC patients and the increased sensitivity of rodent models to seizures, linked mTOR dysregulation to clinical epilepsy. This prompted mTOR inhibitors, such as rapamycin to be tested as a possible treatment to prevent epileptogenesis, first in rodent models and then in human patients. In both cases, mTORC1 inhibitors were able to decrease seizure generation (Meikle et al. 2008; Muncy, Butler, and Koenig 2009). Additionally, in the mouse models, rapamycin treatment prevented the cellular changes associated with epileptogenesis including changes in synaptogenesis, cell survival, and astrocytosis (Meikle et al. 2008; Ehninger et al. 2008; Zeng et al. 2008). Furthermore, during epileptogenesis, abnormal mTOR activity may cause detrimental changes to preventative inhibitory circuitry in the brain, and inhibition of mTOR may preserve these inhibitory circuits (Fu et al. 2012; Buckmaster and Wen 2011). Similar prevention of epileptogenesis and associated molecular and cellular changes with rapamycin treatment has been shown in numerous mouse models (Sunnen et al. 2011; Huang et al. 2010; Anderl et al. 2011).

While these findings suggest mTOR signaling is important in the pathogenesis of epilepsy, the upstream signals from injury promoting mTORC1 activity and the substrates of this complex that mechanistically mediate progression of the disorder remains to be determined. The findings presented in Chapters II and III may provide insight into the mechanisms that underlie epileptogenesis. Synaptic plasticity and function have been shown to be important mediators of epileptogenesis. Subtle changes in mTOR signaling and downstream protein synthesis can effect synapse function and perhaps lead to

the hyperexcitable state seen in epilepsy. Changes in cell survival and resistance to differentiation could also bias the brain towards seizure activity. If there are neuronal subtype specific sensitivities to altered mTOR activity, the relative balance between excitatory and inhibitory circuitry could be shifted, resulting in a hyperexcitable state. Soluble factors can also affect surrounding cells, contributing to epileptogenesis. These soluble factors can cause astrocytosis and the influx of immune cells (Vezzani et al. 2011). Insights into the non-cell-autonomous mechanisms in TSC as demonstrated in the kidney may provide insight into how altered mTOR signaling can contribute to these pathogenic mechanisms in the brain. Furthermore, proper function of excitatory synapses is partially regulated by primary cilia, so ciliary changes in the brain may also alter these circuits (Kumamoto et al. 2012). Use of patient derived iPSCs should further the understanding of these epileptogenic mechanism to more broadly understand acquired epileptic disorders. Indentifying these mechanistic links between mTOR activity and epileptogenesis is a crucial step to development of eventual therapeutics.

Part III: Future Directions

Kidney Manifestations and Primary Cilia

The work described in Chapter II showed that dysregulated mTORC1, but not mTORC2 causes cystogenesis in the kidney, possibly through dysregulation

of the primary cilia. Furthermore, loss of *Tsc1* in the distal convoluted tubule has both cell-autonomous and non-cell-autonomous effects in cystogenesis. This work suggests future directions to establish functional changes in elongated primary cilia, explore primary cilia defects in other organ systems, and to determine the mechanism of non-cell-autonomous changes due to altered mTOR signaling.

Elongated primary cilia provide an interesting mechanistic link between mTOR signaling and TSC kidney pathologies. As discussed in Chapter II, links between cystogenesis and defects in primary cilium have long been identified (Pazour et al. 2000; Nauli et al. 2003; Zullo et al. 2010). In the renal tubule, cilia protrude into the lumen in order to sense fluid flow (Pazour et al. 2002). Fluid flow in the tubule results in bending of the primary cilia, which in turn activates calcium channels and increases intracellular calcium levels (Praetorius and Spring 2001; Nauli et al. 2003). Changes in intracellular calcium in response to flow are thought to regulate cell proliferation and the plane of cell division (Luyten et al. 2010). Bending of the primary cilia also alters Wnt signaling, an important mediator of planar cell polarity (Sugiyama et al. 2011; Corbit et al. 2008). When ciliary structure or function is altered, dysregulated proliferation and plane of cell division, secondary to changes in calcium influx and Wnt signaling, results in cystogenesis. While shorter primary cilia have clear defects in transduction of signals detecting flow, it remains to be seen whether elongated primary cilia display similar deficits.

Future work should address the functional consequences of elongated primary cilia in the kidney tubules. Experiments should address whether *Tsc1* deficient cells have abnormal responses to flow, including changes in calcium influx, Wnt signaling, and planar cell polarity. Additionally while TSC related changes in cilia are due to mTORC1 activation as my work has shown, it remains to be seen if inhibition of mTORC1 can alter ciliary flow-sensing functions. Furthermore, it should be addressed whether non-mTOR related changes to cilia length could help correct the kidney phenotypes in these TSC models. Alteration of cilia stabilizing proteins such as DCDC2 to shorten elongated cilia and return them to normal lengths or pharmacological elongation of cilia in control animals with lithium may provide support for this model (Miyoshi et al. 2009; Massinen et al. 2011). Approaches using both genetic and pharmacological methods will provide evidence whether cystic phenotypes seen in TSC are due to structural constraints of longer cilia, or more subtle changes in ciliary signal transduction. Demonstrating changes in these processes will be crucial to understanding the mechanistic link between mTOR signaling and kidney pathology.

The identification of altered primary cilia in TSC may provide insight into other tissue specific phenotypes of the disease. Ciliopathies, such as Bardet-Biedl Syndrome, Meckel Syndrome, Joubert Syndrome and Oral-Facial-Digital Syndrome, are all caused by ciliary dysfunction and have dual brain and kidney involvement, similar to TSC (Quinlan, Tobin, and Beales 2008). The multi-system involvement and prominent effects in the brain and kidney of these ciliopathies should raise suspicion that TSC may also be part of this class of disorders.

Primary cilia are important in many aspects of neurodevelopment including establishment of progenitor populations, progenitor cell fate determination, and synaptic integration (Chizhikov et al. 2007; Han et al. 2008; Gorivodsky et al. 2009; Kumamoto et al. 2012). As the primary cilium is crucial for normal neuronal development, it would be interesting to explore whether ciliary dysfunction is contributing to neurological phenotypes in TSC. Future work initially needs to identify whether changes in primary cilia are seen in both adult neurons as well as progenitor populations in TSC models. If elongated cilia are present in the brain as well, functional changes should be assessed. Cilia are specifically connected to neural plate polarization, neuronal migration, synapse formation and maintenance (Kumamoto et al. 2012; Higginbotham et al. 2012; Higginbotham et al. 2013). Changes in neuronal migration and synaptic function are thought to contribute to the pathogenesis of both autism and epilepsy. Therefore altered cilia may be an important link between mTOR signaling and these neurological manifestations of TSC.

Additionally, abnormal cilia may also be responsible for some of the phenotypes seen in our patient derived iPSCs. While elongated cilia have not yet been observed in these lines, cilia may contribute to the cell proliferation abnormalities of these cells. In most non-dividing cells, cilia are present on the apical surfaces. However, in cells undergoing cell division, the primary cilium is present during gap phases of the cell cycle. The primary cilium must dissemble and release the basal body for cells to undergo mitosis and progress through the cell cycle (Quarmby and Parker 2005; Tucker, Scher, and Stiles 1979). Therefore,

alterations in cilia stability influence progression through the cell cycle and overall proliferation rates (Goto, Inoko, and Inagaki 2013). Changes in primary cilia during the gap phases in our stem cell lines may affect cell cycle progression and the increased proliferation we observe in TSC patient derived iPSCs. Future work should first identify whether TSC patient derived iPSCs have elongated primary cilia. Live cell imaging to observe cell cycle associated ciliogenesis and deciliation may prove insightful into the proliferation phenotypes.

Finally, while loss of *Tsc1* in the mouse kidney was restricted to the DCT, cystogenesis and altered mTORC1 activity was not restricted to this region. Future work should begin to explore the mechanism of non-cell-autonomous effects of altered mTOR signaling. Further evidence for non-cell-autonomous mechanisms in TSC have also been reported in studies using human samples and from a zebrafish model of TSC (Crino et al. 2010; Kim et al. 2011). Future directions will focus on how loss of mTOR affects neighboring cells, specifically identifying which soluble or cell surface molecules influence surrounding tissue. Alternatively, changes in the DCT may be causing decreased flow and increased pressure in more proximal portions of the nephron. In this case, it will be necessary to see if increased pressure or decreased flow secondary to cystogenesis in the DCT is indirectly injuring proximal tubules. The increase in diameter of the PCT and mTOR signaling changes may not be due to cellular signals from *Tsc1* null cells but the result of a more global kidney injury. *In vitro* models of mixed populations of mutant and wild type primary cultured tubule epithelial cells as well as other non-TSC models of cystogenesis should be able

to address these possibilities. While rapamycin and other pharmacological inhibitors are useful for cell autonomous changes in mTORC1 signaling, newer therapies should consider potential non-cell-autonomous mechanisms when designing therapies to alter the course TSC pathologies. If soluble or cell-surface markers mediate these changes, targeting these molecules may protect neighboring tissue without a loss of heterozygosity from further damage and disease progression.

TSC Patient Derived iPS Cells and Altered Survival and Pluripotency

The experiments described in Chapter III established TSC patient derived iPS line specific phenotypes in cells heterozygous for *TSC1* or *TSC2.* Previously we have shown that these heterozygous iPSCs are larger and proliferate more rapidly than controls, consistent with alterations in mTOR signaling. Here we have shown abnormalities in cell survival and maintenance of pluripotency as well. These phenotypes have provided many avenues for future experiments.

First, the collective phenotypes seen in these cells all point to increased mTORC1 activity as a mechanistic mediator of their pathogenesis. Immunoblot analysis to assay mTORC1 activity has not been able to show changes in downstream targets of mTORC1 in these patient derived iPSCs. We hypothesize that the heterozygous nature of these cells results in subtle increases in mTORC1 signaling. To detect this, more sensitive techniques than immunoblot analysis are needed to measure mTORC1 activity such as ELISA or mass

spectrometry that allow highly accurate quantification of phospho-species of mTORC1 targets. These methods can be used to identify more precisely the activity of the mTOR kinase. Alternatively, alterations in these cells could be due to other non-mTOR related functions of the hamartin/tuberin complex. While the majority of work has implicated mTOR signaling as the major downstream target of the hamartin/tuberin complex there remains the possibility that this heterodimer has mTOR-independent functions as well. While not fully understood, there is some evidence that the hamartin/tuberin complex and Rheb can regulate p42/44 MAPK phosphorylation and B-Raf kinase activity independent of effects on mTOR activation (Karbowniczek et al. 2004). B-Raf and MAPK signaling are crucial for normal cellular differentiation (Rauen 2013). Hamartomas in TSC could be partially due to abnormalities in this pathway independent of mTOR signaling. The TSC patient derived iPSCs will be helpful in identifying whether B-Raf and MAPK signaling is dysregulated by the heterozygosity of *TSC1* or *TSC2.* Furthermore*,* more work needs to explore whether other, currently unidentified, non-mTOR mediated changes can result from dosage changes in either hamartin or tuberin.

The alterations in pluripotency seen in these iPSCs deserve more exploration to determine the causative mechanism of this phenotype. Further experiments will support the observed increase in nuclear TFE3 in these cells. More complete separation of nuclear and cytoplasmic fractions should allow a clear analysis of this phenomenon. Increased nuclear TFE3 has been linked to increase mTORC1 activity (Betschinger et al. 2013). If increased mTORC1

activity is indeed responsible for the increased nuclear TFE3 in the heterozygous iPSCs, then inhibition of mTORC1 with rapamycin should return nuclear TFE3 levels to normal. However, as discussed earlier, there remains the possibility of possible non-mTOR related functions of hamartin and tuberin. The connections between mTORC1 and the regulation of TFE3 localization are not yet well described. Future projects need to explore the kinases responsible and function of the phosphorylation of the upstream regulators of TFE3, such as FLCN and FNIP1/2. While there is evidence that FLCN is a substrate of mTORC1, it is unclear what role this phosphorylation event has on the function of this protein (Baba et al. 2006; Piao et al. 2009). Other parallel pathways or other kinases may also be affecting FLCN function. The functional consequences of increased pluripotency in these iPSCs should also be addressed. Time course experiments using directed differentiation should be done to identify the ability of these iPSCs to leave a pluripotent state, as well as the rate of progression through different developmental stages towards terminally differentiated cells. Alterations in pluripotency may cause an expansion of progenitor populations, or prevent maturation of functional neurons contributing to the neurological phenotypes in TSC. Finally, the resistance to differentiation of these cells should be explored in novel rodent models as well. In particular, examination of embryonic neurogenesis in animal models may identify changes in both the size of progenitor populations and altered rates of neuron production or synaptic integration. Increased pluripotency may also affect adult neurogenesis. To address this possibility, hippocampal and other adult progenitor populations

should be examined. The increased pluripotency of these cells could be a mechanism that generates the immature neurons and glia identified in cortical tuber tissue. Treatment that “resensitizes” these cells to normal differentiation signals may reduce the size of cortical tubers and ameliorate neurological symptoms.

Altered cell survival in patient derived iPSCs implicates new pathways to explore as mediators of the pathogenesis of TSC. mTORC1 activation promotes cell survival, regulates cell cycle progression, and inhibits apoptosis in response to cellular stress (Park et al. 2011). Cell-cell contact promotes survival signals through ROCK1 to activate mTORC1 signaling (Arakawa-Takeuchi et al. 2010; Park et al. 2011). Increased survival in cell suspension is clearly increased in *TSC1* or *TSC2* null cells as well as our heterozygous iPSC lines. Further effort will identify whether the iPSCs have decreased activation of pro-apoptotic machinery in suspension. Additionally, the mechanism of increased cell survival in our iPSC should be explored to determine whether the heterozygosity of these cells is working through a ROCK1-mTORC1 axis or another parallel pathway. Improper regulation of cell survival may contribute to the pathogenesis of TSC through survival of abnormally differentiated cells, as well as persistence of normal cells usually undergoing apoptosis during neurodevelopment. As apoptosis is crucial in the pruning of excess neurons during development, these changes in cell survival can explain some of the alterations in patterning seen in TSC. Further elucidating the mechanisms of cell survival should help to better understand the development of cortical tubers.

Conclusion

A better understanding of the molecular mechanism of TSC will provide new avenues for treatments, both for the disease itself as well as the broader categories of autism and epilepsy. Our rodent models and patient derived iPSCs allow the identification of downstream mediators of mTOR signaling disrupted in TSC. Elucidating the pathways regulated through mTOR such as protein translation, ciliary function, cell survival, and maintenance of pluripotency will not only help in the understanding of normal developmental process but also impact human health by identifying targets for pharmacological intervention. The TSC patient derived iPSCs provide an ideal opportunity for drug screening to identify small molecules that regulate the various aspects of this pathway. iPSCs can be used to identify molecules that correct the phenotypes of increased pluripotency and cell survival. Additionally, through directed differentiation towards neurons, small molecules can be tested at different time points during neurogenesis to identify therapeutic windows for treatment of TSC, as well as broader autism and epilepsy disorders. The generation of rodent models and TSC patient derived iPSCs are crucial to both understanding the mechanisms underlying the pathogenesis of TSC. Furthermore, these models provide a testable system for potential pharmaceutical treatments of the disease. Ultimately, the tissue specific phenotypes and underlying mechanisms identified in this work will hopefully lead to future treatments for TSC and its associated comorbidities.

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