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The role of the lysosome and transcription factor EB in tuberous sclerosis complex

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SCHOOL OF MEDICINE

Thesis

**THE ROLE OF THE LYSOSOME AND TRANSCRIPTION FACTOR EB
IN TUBEROUS SCLEROSIS COMPLEX**

by

ANNA S. NIDHIRY

B.S., University of California, San Diego 2016

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Approved by

First Reader

Matthew D. Layne, Ph.D.
Associate Professor of Biochemistry

Second Reader

Elizabeth Petri Henske, M.D.
Professor of Medicine
Harvard University, School of Medicine

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IN TUBEROUS SCLEROSIS COMPLEX**

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ABSTRACT

Tuberous Sclerosis Complex (TSC) is a rare, autosomal dominant genetic disease that results from the loss-of-function mutations of either the *TSC1* or *TSC2* genes. It is a multisystemic disorder with manifestations in several organs including the lungs, kidneys, brain, skin, and heart. Loss of either *TSC1* or *TSC2* causes hyperactivation of the mechanistic target of rapamycin complex 1 (mTORC1) pathway resulting in cell proliferation and the continuous activation of multiple anabolic pathways that lead to tumor growth.

Transcription factor EB (TFEB) is one of the many downstream targets of the mTORC1 pathway and is a master transcriptional regulator of lysosomal biogenesis. In the classical paradigm of TFEB-mTORC1 interaction, TFEB is negatively regulated by mTORC1. Interestingly, TFEB is upregulated and fully functional in *Tsc1*- and *Tsc2*-deficient, mTORC1 hyperactivated cells suggesting an alternate regulation involved in the context of TSC. The objective of this research was to investigate how TFEB upregulation and resulting lysosomal dysregulation in *Tsc1*- and *Tsc2*-deficient cells drives the pathogenesis of Tuberous Sclerosis Complex. By western blot analysis, elevated levels of TFEB were detected in *Tsc1*- and *Tsc2*-deficient cells compared to *Tsc1*- and *Tsc2*-expressing cells and multiple lysosomal proteins also showed increased expression in these cell lines. In *Tsc1*- and *Tsc2*-expressing MEFs, TFEB was phosphorylated and retained in

the cytoplasm. Immunofluorescence imaging of TFEB in *Tsc1*- and *Tsc2*-deficient cells demonstrated nuclear localization. This was in contrast to previous studies, in which TFEB was retained in the cytoplasm upon mTORC1 activation. Immunohistochemistry staining of TFEB and the lysosomal protein, NPC1 (NPC Intracellular Cholesterol Transporter 1), in human TSC-associated renal cell carcinoma (RCC) also showed increased staining of TFEB and NPC1 in RCC compared to normal kidney tissue. This histological finding was confirmed in two mouse models of renal TSC, *TSC2* +/- AJ mice and *KSP-CreERT2 Tsc2^{fl/fl}* mice, which revealed strong expression of NPC1 in cyst-lining cells, specifically in the apical region, suggesting increased lysosome number and activity as a contributing factor to cystogenesis.

These results suggest a novel mechanism in the development of Tuberous Sclerosis Complex through the dysregulation of lysosomal activity. Further investigation into the mechanism of nuclear localization of TFEB and its upregulation in *Tsc1*- and *Tsc2*-deficient cells may provide insight into the pathogenesis of the disease and could indicate a new therapeutic target for treating TSC and its associated disorders.

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LIST OF ABBREVIATIONS

AML.....	Angiomyolipoma
BU.....	Boston University
bHLH-LZ.....	Basic helix-loop-helix-leucine-zipper
BSA.....	Bovine Serum Albumin
CLEAR.....	Coordinated Lysosomal Expression and Regulation
CTS K.....	Cathepsin K
DAB.....	3,3'-Diaminobenzidine
DJK.....	David James Kwiatkowski
DMEM.....	Dulbecco's Modified Eagle Medium
DMSO.....	Dimethyl Sulfoxide
FBS.....	Fetal Bovine Serum
GAP.....	GTPase-activating Protein
Gibco.....	Grand Island Biological Company
HEXA.....	Beta-Hexosaminidase A
IHC.....	Immunohistochemistry
IF.....	Immunofluorescence
KO.....	Knock Out
LAM.....	Lymphangi leiomyomatosis
LAMP1.....	Lysosome-associated Membrane Glycoprotein-1
MEF.....	Mouse Embryonic Fibroblast
MiT.....	Microphthalmia-associated Transcription

MITF.....	Melanocyte Inducing Transcription Factor
mTOR	Mechanistic Target of Rapamycin Complex 1
NPC1.....	NPC Intracellular Cholesterol Transporter 1
NPC2.....	NPC Intracellular Cholesterol Transporter 2
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
pS6	Phosphorylated S6 Ribosomal Protein
PVDF	Polyvinylidene Difluoride
qPCR.....	Quantitative Polymerase Chain Reaction
RHEB	Ras Homolog Enriched in Brain
RCC.....	Renal Cell Carcinoma
SEGA	Subependymal Giant Cell Astrocytoma
shRNA.....	Short Hairpin RNA
TFEB.....	Transcription Factor EB
TFEC.....	Transcription Factor EC
TFE3	Transcription Factor E3
TSC	Tuberous Sclerosis Complex
tS6	Total Phosphorylated S6 Ribosomal Protein
WT	Wild Type

CHAPTER 1

INTRODUCTION

Overview

Tuberous Sclerosis Complex (TSC) is a rare genetic disease that is autosomal dominant and caused by a loss-of-function mutation in either the *TSC1* or *TSC2* gene (Crino et al., 2006). It is a multi-systemic disorder that can cause tumors in various organs in the body including the brain, skin, lung, kidney, retina, and heart (**Figures 1 and 2**). In the United States, nearly 50,000 individuals are affected by Tuberous Sclerosis Complex, and it is thought to affect nearly 2 million people worldwide with a prevalence of one in every 6,000-10,000 births (Henske et al., 2016, Hallett et al., 2011).

A diagnosis of TSC can be made prenatally by fetal ultrasound through the detection of cardiac rhabdomyomas or cortical tubers (Wortmann et al., 2008). Other common manifestations of TSC in the brain are subependymal nodules and subependymal giant cell astrocytomas (SEGAs) (Northrup & Krueger, 2013). Subependymal nodules appear in the brain as undifferentiated giant cells and collections of abnormal glial cells, which can calcify into SEGAs (Stein & Reidman, 2016). Congenital SEGAs can be seen in 2.2% of patients and cortical tubers occur in approximately 90% of TSC patients (Kotulska et al., 2014, Stein & Reidman, 2016). Disruption of the brain anatomy by cortical tubers can also induce epilepsy and seizures, which are some of the most common neurological manifestations of TSC, and affect 70-90% of patients in early childhood (Chu-Shore et al., 2010).

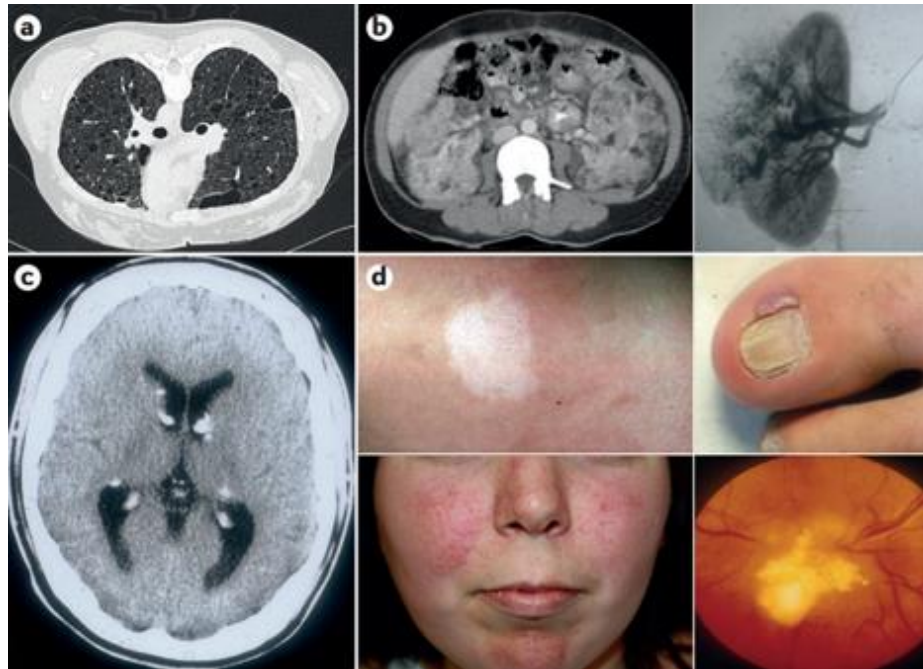


Figure 1. Manifestations of Tuberous Sclerosis Complex in the skin, kidney, and lungs. This figure shows TSC in a clinical context as (a) a chest scan of lymphangiomyomatosis (LAM). Renal manifestations of TSC (angiomyolipomas) are depicted by an (b) abdominal CT scan (left) and a renal angiogram (right). TSC in the brain is shown by (c) a CT scan of subependymal nodules. Skin manifestations of TSC are depicted by (d) four panels showing hypomelanotic macules (top left), subungual fibroma (top right), facial angiofibroma (bottom left), and retinal hamartoma (bottom right) Figure adapted from (Henske et al., 2016).

In women, a highly common pulmonary manifestation of TSC is Lymphangiomyomatosis (LAM), a destructive, cystic lung disease that occurs in nearly 80% of women with TSC. LAM presents symptomatically as chest pain, fatigue, shortness of breath, pneumothorax, and/or chylous pleural effusion (Silva et al., 2019, Moss et al., 2001). LAM is characterized by spindle-shaped cells with smooth muscle-like staining properties, known as LAM cells, that contain mutations in either the *TSC1* or *TSC2* gene (Steagall et al., 2018, Torre et al., 2017). In the kidney, TSC can manifest as renal cell carcinoma, renal cysts, and/or angiomyolipomas (Guo et al., 2014, P. Yang et al., 2014).

Angiomyolipomas (AMLs) are the most common renal manifestations of TSC (70-80%) and are characterized by abnormal blood vessels, the proliferation of fat cells, and immature smooth muscle cells in the kidney (Jinzaki et al., 2017, Brakemeier et al., 2017). TSC can also manifest in a range of lesions on the skin, an important diagnostic criterion of the disease. These lesions include: angiofibromas, unguinal fibromas, forehead plaques, shagreen patches, and hypomelanotic macules (Józwiak et al., 1998).

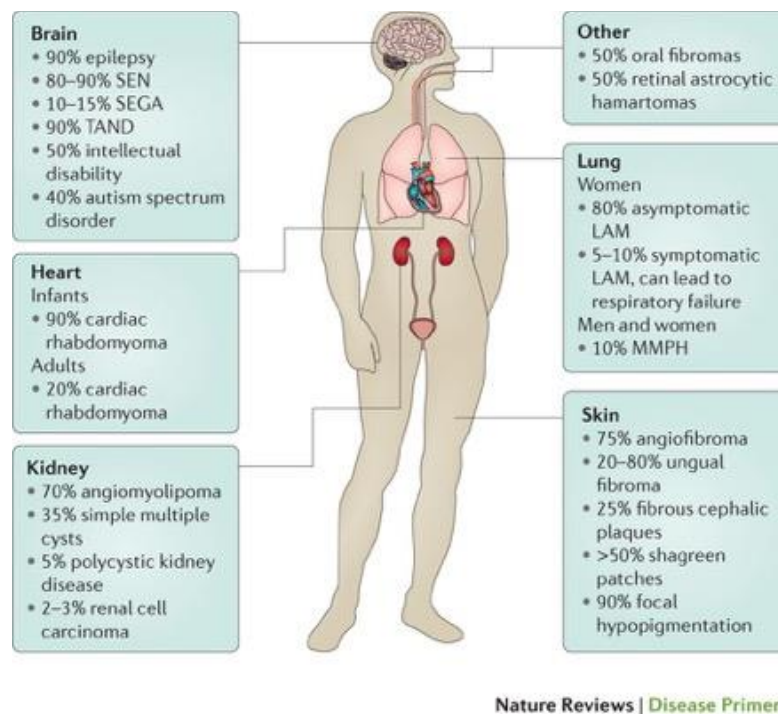


Figure 2. Prevalence of Clinical Manifestations of Tuberous Sclerosis Complex. TSC occurs in the brain, heart, kidney, lung, skin, retina, etc. This diagram illustrates the prevalence of the clinical manifestations of TSC in multiple organs in the body. Figure obtained from (Henske et al., 2016).

A clinical diagnosis of Tuberous Sclerosis Complex is established by the presence of two major features (**Table 1**) or one major feature and two minor features such as

“confetti-like” skin lesions, dental pits, intraoral fibromas, etc. (Northrup & Krueger, 2013). A “probable” diagnosis consists of one major and one minor feature, and a “possible” diagnosis is established by one major or two or more minor features (Northrup & Krueger, 2013).

Table 1. Tuberous Sclerosis Complex (TSC) Diagnostic Criteria. Adapted from (Northrup & Krueger, 2013).

Major Features	Minor Features
Hypomelanotic macule	“Confetti-like” skin lesions
Facial angiofibromas	Dental enamel pits
Ungual fibromas	Intraoral fibromas
Shagreen patch	Retinal achromic patch
Multiple retinal hamartomas	Multiple renal cysts
Cortical tuber	Nonrenal hamartomas
Cerebral white matter radial migration lines	-
Subependymal nodules	-
Subependymal giant cell astrocytoma	-
Cardiac rhabdomyoma	-
Lymphangiomyomatosis	-
Renal angiomyolipomas	-

Genetics of TSC

Tuberous Sclerosis Complex is caused by mutations in either the *TSC1* gene that encodes the protein hamartin or the *TSC2* gene that encodes the protein tuberin, which together form the TSC complex (Jones et al., 1999, Sancak et al., 2005). *TSC1* and *TSC2* are located on chromosome 9 (on position q34) and chromosome 16 (on position p13), respectively (Povey et al., 1994). TSC follows the Knudson two-hit (loss-of-heterozygosity) tumor suppressor model (Knudson, 1971), in which a normal allele of *TSC1* or *TSC2* must be inactivated through a sporadic mutation in a non-germline cell in

order to activate abnormal cellular proliferation (Crino et al., 2006). *TSC1* mutations are generally familial, affecting ~20% of patients, and often present as a milder version of disease compared to *TSC2* mutations (Emmerson et al., 2003). *TSC2* mutations are observed in approximately 70% of TSC patients, and demonstrate higher rates of de novo germline mutations compared to *TSC1* mutations (Emmerson et al., 2003). In 10-15% of patients, a mutation cannot be defined (Camposano et al., 2009). This suggests the presence of mosaicism or intronic mutations contributing to the TSC phenotype (Tyburczy et al., 2015).

mTORC1 Signaling in TSC

The mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) regulates cell growth and proliferation by sensing nutrient fluctuations in the environment through various inputs including amino acids, glucose, growth factors, and cellular stress (Kim et al., 2013). Functionally, the *TSC1*, *TSC2*, and *TBC1D7* proteins make up the TSC protein complex, which negatively regulates the mTOR pathway by inhibiting mTORC1 (Gao et al., 2002). In conditions of high nutrient availability, the TSC complex is inhibited and activates mTORC1 resulting in an increase in anabolic processes such as nucleotide, protein, and lipid biosynthesis (**Figure 3**) (Kim et al., 2013). The TSC protein complex functions as a GTPase-activating protein (GAP) toward a direct upstream regulator of mTORC1, the GTPase Ras homolog enriched in brain (RHEB) (Cao et al., 2017). GTP-bound RHEB is an mTORC1 activator. However, in the absence of nutrient signaling, RHEB is kept in its GDP-bound state by the TSC protein complex and prevents its activation (Dibble & Manning, 2013).

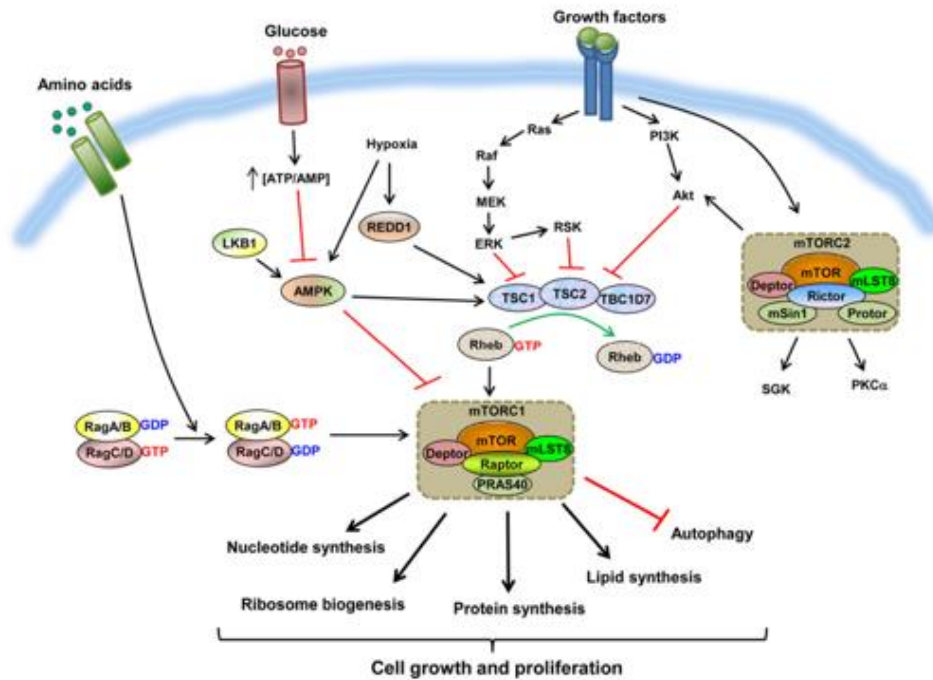


Figure 3. Summary of the mTOR signaling pathway and its regulation. Major cellular components and pathways associated with the mTORC1 and mTORC2 protein complexes are shown. Activation of mTORC1 by amino acids, glucose, and growth factors promotes various anabolic pathways that result in cell growth and proliferation. Figure obtained from (Kim et al., 2013).

In Tuberous Sclerosis Complex, inactivating mutations in the second allele of either the *TSC1* or *TSC2* gene causes hyperactivation of mTORC1 as RHEB remains in its GTP-bound, active state (Cao et al., 2017). Thus, the constitutive activation of mTORC1 results in continuous cell growth and proliferation through the constant activation of downstream anabolic pathways (Jaeschke et al., 2002).

Treatment of Tuberous Sclerosis Complex

Currently, there is no cure for Tuberous Sclerosis Complex. However, there are treatment options for the various symptoms associated with TSC. Seizures are commonly treated with antiepileptic drugs (ex. Vigabatrin) and dietary therapy (Curatolo et al., 2012,

Kossoff et al., 2005). Previously, embolization or surgical resection was the standard of care in treating manifestations of TSC such as angiomyolipomas and SEGAs. A more recent understanding of the involvement of the mTORC1 pathway has led to the use of mTOR inhibitors Rapamycin (Sirolimus) and its derivative, Everolimus, in the treatment of TSC (Franz et al., 2013, Nathan et al., 2015). Unfortunately, serious and potentially life-threatening adverse effects are associated with the use of mTOR inhibitors including respiratory infection and bone marrow suppression (Samuels, 2017). Treatment with Everolimus has also been reported to cause adverse effects related to female fertility such as amenorrhea and irregular menstruation (Davies et al., 2017). Discontinuation of mTOR inhibitor therapy results in the regrowth of TSC-associated tumors requiring life-long use of the treatment (Samuels, 2017). For these reasons, it is critical to identify alternate therapeutic strategies for the treatment of TSC that would benefit patients with a longer and, if possible, complete remission of the disease.

Lymphangiomyomatosis (LAM) a TSC-associated Disease

LAM is a progressive lung disease characterized by cystic destruction, chylous pleural effusion, and the abnormal proliferation of LAM cells in the form of lung nodules (Steagall et al., 2018, Henske & McCormack, 2012). It predominantly affects women and can occur in a sporadic form or in association with TSC (Costello et al., 2000, Henske & McCormack, 2012). LAM cells, which are smooth muscle-like cells, are the pathogenic manifestation of LAM in the lung that causes cystic destruction of lung parenchyma and eventual loss of pulmonary lung function (Krymskaya, 2008). Symptoms of the disease can include pneumothorax and exertional dyspnea (Urban et al., 1999, McCormack, 2008).

Currently, the reason for the predominance of LAM in females and the mechanism of the cystic phenotype of LAM are unclear. However, it is shown in mice that estrogen involvement enhances the survival and proliferation of Tsc2-deficient cells (Yu et al., 2009, Henske & McCormack, 2012).

Studies have reported high expression of Cathepsin K, a lysosomal protease, in LAM cases (Chilosi et al., 2009). Cathepsin K is a protease typically expressed by osteoclasts for bone-remodeling with high matrix-degrading activity (Dongre et al., 2017, Chilosi et al., 2009). Cathepsin K is released extracellularly through lysosomal exocytosis, a calcium-dependent process that enables the lysosome to release its contents into the extracellular space for cellular clearance (Jaiswal et al., 2002, Medina et al., 2011). It is hypothesized that LAM cells play a role in the cystic phenotype of LAM through the release of cathepsin K and other proteases (Dongre et al., 2017). Previous studies have demonstrated a 40-fold higher mRNA expression of cathepsin K in LAM tissue in comparison to normal tissue (Dongre et al., 2017). In the same study, immunohistochemistry experiments confirmed expression of cathepsin K in LAM tissue but not in normal lung tissue (Dongre et al., 2017). Similarly, increased expression of cathepsin K is also evident TSC-associated renal angiomyolipomas (Martignoni et al., 2012). This provides insight into the potential for cathepsin K as a biomarker of TSC and suggests lysosomal activity may be involved in the pathogenesis of LAM and other TSC-derived diseases.

The Role of the Lysosome in TSC

Lysosomes are vesicular organelles with degradative properties that arise from a range of hydrolytic enzymes contained within their membrane (Dielschneider et al., 2017). Lysosomes function to maintain homeostasis through sensing and maintaining nutrient levels in the cell by recycling essential nutrients and degrading waste products via cellular processes that include: autophagy, phagocytosis, and exo- and endocytosis (Dielschneider et al., 2017). Dysregulation of lysosomal function can lead to altered cellular metabolism and signaling that enables the progression and development of cancer (Dielschneider et al., 2017). The TSC protein complex is recruited to and localizes in the lysosomal membrane with mTORC1 during cellular stress to inhibit mTOR signaling in unfavorable conditions (Demetriades et al., 2016). Inactivating phosphorylation of TSC2 by Akt or other upstream kinases causes dissociation of the TSC complex from Rheb at the lysosomal membrane facilitating the activation of mTORC1 (Menon et al., 2014).

Role of TFEB and Lysosomal Biogenesis in TSC

The microphthalmia-associated transcription factor (MiT) family is a group of basic helix-loop-helix-leucine-zipper (bHLH-LZ) transcription factors downstream of mTORC1 that function in maintaining cellular homeostasis and regulating cellular processes (Napolitano & Ballabio, 2016). The MiT family of transcription factors consists of four members (MITF, TFEB, TFE3, and TFEC) that are dysregulated in cancers and function as oncogenes (M. Yang et al., 2018, Ploper & De Robertis, 2015). Specifically, *TFEB* and *TFE3* translocations and consequent hyperactivation are found in a rare subset of sporadic renal cell carcinomas (Argani et al., 2016). Prior data in which all four MiT family

members were analyzed demonstrated that TFEB is most highly expressed in Tsc2-deficient cells. For this reason, this study is focused on TFEB.

TFEB, Transcription Factor EB induces lysosomal gene expression by binding to the promoter regions of several lysosomal genes, known collectively as the Coordinated Lysosomal Expression and Regulation (CLEAR) network (Sardiello et al., 2009). TFEB regulates processes such as autophagy by inducing autophagosome-lysosome fusion and autophagosome biogenesis and initiating lysosomal exocytosis (Settembre et al., 2011). In this way, TFEB promotes intracellular clearance through the regulation of organelle-specific degradation pathways and cellular metabolism to maintain homeostasis (Palmieri et al., 2011).

The Current Paradigm on TFEB-mTORC1 Interaction

Prior research has shown that TFEB is regulated by mTORC1 through control of its phosphorylation state and nuclear localization (Peña-Llopis et al., 2011). TFEB is one of the targets of the mTORC1 pathway, and thus is also capable of responding to environmental cues. Under nutrient-rich conditions, the mTORC1 pathway is active in the lysosomal membrane and directly phosphorylates TFEB causing its cytoplasmic retention (Settembre et al., 2012). Direct phosphorylation of TFEB at Ser211, and potentially Ser142, triggers its binding to 14-3-3 chaperone proteins and prevents the nuclear localization of TFEB (**Figure 4**) thereby reducing its transcriptional activity (Roczniak-Ferguson et al., 2012). However under starvation or stress conditions, the mTORC1 pathway is inhibited, causing the translocation of the unphosphorylated form of TFEB into the nucleus and the induction of lysosomal genes (Settembre et al., 2012).

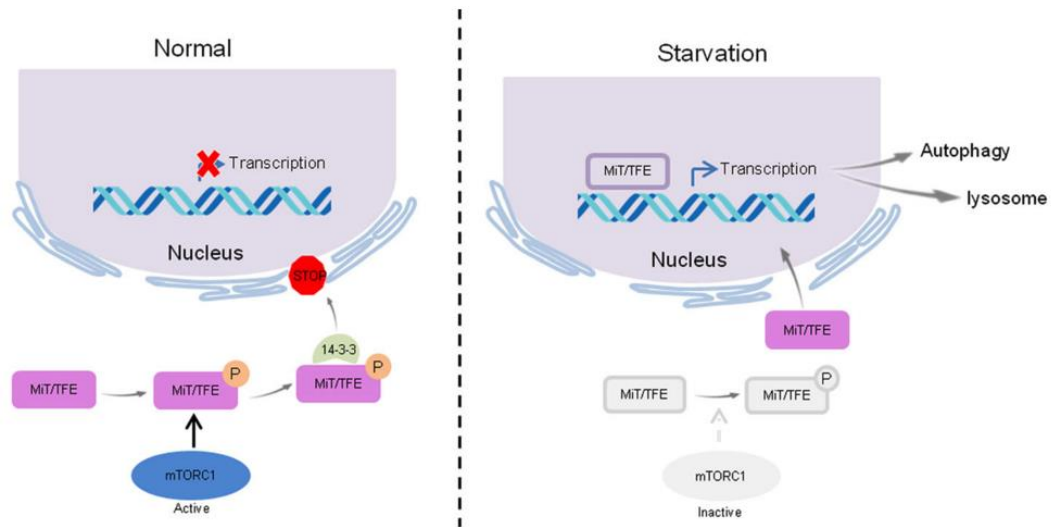


Figure 4. TFEB-mTORC1 interaction and subcellular localization of MiT family transcription factors. mTORC1 is active and directly phosphorylates MiT/TFE transcription factors (TFEB) under nutrient-rich (normal) conditions, which promotes 14-3-3 protein binding and results in cytoplasmic retention. Under nutrient-deprived (starvation) conditions, mTORC1 is inactive and MiT/TFE transcription factors translocate into the nucleus and promote CLEAR network genes transcription. Figure adapted from (M. Yang et al., 2018).

Aims of the Present Study

The aim of this study is to understand the regulation of TFEB in the context of TSC and its role in the pathogenesis of the disease as a positive regulator of lysosomal biogenesis. According to previous studies, mTOR activity induces phosphorylation of TFEB resulting in its cytoplasmic retention and inactivity. However, preliminary data has shown that Tsc1- and Tsc2-deficient cell lines (with high mTORC1 activity) have increased levels and activity of TFEB. This led to the hypothesis that in TSC-null cells, an alternate pathway must be involved that upregulates TFEB either through a decrease in 14-

3-3 proteins, increased calcineurin activity, or another unexplored mechanism. For this reason, 14-3-3 and calcineurin protein expression were examined in both Tsc1- and Tsc2-expressing and Tsc1- and Tsc2-deficient cells.

This study examined the expression of TFEB and lysosomal proteins in both *in vivo* and *in vitro* models of TSC. Tsc1- and Tsc2-deficient as well as Tsc1- and Tsc2-expressing cells were treated with mTOR inhibitors to determine whether increased lysosomal biogenesis is mTOR dependent by examining lysosomal protein and gene expression after treatment. The study also investigated the subcellular localization of TFEB in Tsc1- and Tsc2-deficient cells through immunofluorescence imaging and explored the mechanism of nuclear localization in mTORC1 hyperactivated cells. Through these experiments this project established foundational knowledge to better understand the pathogenesis of TSC and potentially identify a new therapeutic target and biomarker for the disease.

CHAPTER 2

METHODS

Cell Culture

Tsc1^{+/+}, *Tsc1*^{-/-}, *Tsc2*^{+/+}, *Tsc2*^{-/-} mouse embryonic fibroblasts MEFs were provided by David James Kwiatkowski at Brigham and Women's Hospital Boston, MA, US (Zhang et al., 2003). Cell culture was conducted in Dulbecco's Modified Eagle Medium (DMEM, Gibco/Thermo Fisher, Scientific, Waltham, MA, USA) supplemented with either 10% or 0.1% fetal bovine serum (FBS; Atlanta Biologicals, Flowery Branch, GA, USA) and 1% penicillin/streptomycin. Cells were passaged with 0.25% trypsin-EDTA (Life Technologies Corporation, Grand Island, NY, USA) and incubated in a humidity-controlled environment at 37° C with 5% CO₂.

Generation TFEB Knockdown Cell Lines

TFEB MISSION mouse shRNA Lentiviral Transduction Particles (Sigma-Aldrich Corporation, St. Louis, MO, USA) were used to downregulate TFEB in *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs. Cells were transduced with Lentiviral particles for 24 hours with polybrene (10 µg/ml). The infected cells were selected with puromycin at 5 µg/ml in 10% FBS media.

Animal Studies

Animal studies were conducted by Dr. Hilaire Lam and Thomas Hougard according to institutional protocols that were approved by Boston Children's Hospital Animal Care and Use Committee. KSP-CreERT mice that had a tamoxifen-induced Cre

recombinase driven by a kidney-specific cadherin (KSP) promoter were crossed with a *Tsc2^{fl/fl}* mouse to create a KSP-CreERT2 *Tsc2^{fl/fl}* mouse model of TSC (Lam et al., 2017). Tamoxifen treatment at 8 weeks induced recombination, and mice were euthanized at 5 months. TSC2 +/- AJ mice were generated by the Kwiatkowski Lab.

Protein Extraction

For protein extraction and immunoblot, cells were washed with ice-cold PBS, scraped, and lysed on ice with (1x) radioimmunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, Inc., Danvers, MA, USA) in combination with phosphatase and protease inhibitors (Sigma-Aldrich Corp.). Cells were spun in a centrifuge at 10,000 xg for 10 minutes and the supernatant was transferred into a fresh tube. Each sample (1 μ l) was mixed with 1 mL of Bradford reagent (1:10 dilution) (Sigma-Aldrich Corp.) and inserted into an Ultrospec 2000 UV/Visible spectrophotometer (Pharmacia Biotech, Inc., Piscataway, NJ, USA) for protein quantitation.

Western Blot Analysis

Extracted cell lysates containing approximately 20 μ g of protein were normalized to the desired protein concentration through dilution with distilled water. Samples were mixed with reducing agent (10X) and LDS sample buffer (4X) prior to heating at 70°C for 10 minutes. The protein samples were then run on 4-12% Bis-Tris gel (Invitrogen, Waltham, MA, USA) at 150 volts for 120 minutes. The gel was then transferred onto a polyvinylidene difluoride (PVDF) membrane, and the transfer buffer was run for 2 hours

at 100 volts. Membranes were washed with TBST and blocked with 5% non-fat milk or 5% BSA for 1 hour at room temperature. After blocking, membranes were cut and blocked with primary antibodies (amounts below) in 5% BSA overnight at 4° C. The following morning, membranes were washed three times for 10 minutes in TBST, incubated with secondary antibody, anti-rabbit IgG horseradish peroxidase or anti-mouse IgG horseradish peroxidase (1:5000) for 1 hour at room temperature, and then washed again three times as previously described. Chemiluminescence was then visualized with SuperSignal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific, Waltham, MA, USA) on SynGene G:BOX gel documenting system. Protein bands were normalized and evaluated against β -actin (Sigma-Aldrich Corp., A5316). The following antibodies were used at 1:1000 dilution unless otherwise indicated: β -actin (Sigma-Aldrich Corp., A5316), phosphorylated S6 (pS6) ribosomal protein (Thr 389) (Cell Signaling Technology Inc., 9234), total S6 kinase (TS6K) ribosomal protein (Cell Signaling Technology Inc., 2217), TSC2 (Cell Signaling Technology Inc., 4308), TSC1 (Cell Signaling Technology Inc., 4906), TFEB (1:3000) (Bethyl Laboratories, Montgomery, TX, USA, A303-673A), TFEB (Cell Signaling Technology Inc., 32361), Cathepsin K (CTS K) (Abcam, Cambridge, MA, USA, ab19027), LAMP1 (Cell Signaling Technology Inc., 3243), LAMP1 (1:200) (Santa Cruz Biotechnology Inc., Dallas, TX, USA, sc-19992), NPC1 (Abcam, ab134113), NPC2 (Thermo Fisher Scientific, PA5-51463), HEXA (Thermo Fisher Scientific, PA5-45175), Calcineurin (Cell Signaling Technology Inc., 2614), 14-3-3 Family Antibody Sampler Kit (Cell Signaling Technology Inc., 9679) .

mRNA Extraction and Real-Time PCR

For mRNA extraction, cells were first washed two times with ice cold PBS, then resuspended in buffer RLT prior to homogenization using QIASHredder (Qiagen, Hilden, Germany). After homogenization, mRNA was isolated using the RNeasy Plus Micro Kit with on-column genomic DNA-digest (Qiagen) according to the manufacturer's protocol. To generate cDNA from isolated and purified mRNA, Affinity Script quantitative PCR (qPCR) cDNA Synthesis Kit (Agilent Technologies) was used. Real-time PCR was conducted using StepOne Plus Realtime PCR Machine (Applied Biosystems) with TaqMan Real-Time PCR Master Mix (Thermo Fisher Scientific). Gene expression was measured relative to β -actin, which was stably expressed in experimental conditions. The delta delta Ct ($\Delta\Delta$ Ct) method was used to calculate the fold change of the experimental group compared to the control group. TaqMan real-time PCR assays (Thermo Fisher Scientific) with optimized probe/primer sets were used for the following genes: *Npc1* (assay ID: Mm00435300_m1), *Npc2* (assay ID: Mm00499230_m1), *Hexa* (assay ID: Mm00599877_m1), *Ctsk* (assay ID: Mm00484039_m1), *Mitf* (assay ID: Mm00434954_m1), and *Tfe3* (assay ID: Mm01341186_m1).

Immunofluorescence

Tsc1^{+/+}, *Tsc1*^{-/-}, *Tsc2*^{+/+}, *Tsc2*^{-/-} MEFs were plated onto 35 mm glass bottom dishes (Corning, Inc., NY, USA). Cell media was removed using a vacuum and cells were fixed with 2% paraformaldehyde (PFA) for 15 minutes and permeabilized with 0.1% Triton X-

100 (Sigma-Aldrich Corp.) for 5 minutes and washed three times with PBS. Cells were then blocked for 30 minutes in 1% BSA and incubated with primary antibodies, TFEB (1:200) (Cell Signaling Technology Inc., 32361S) and LAMP1 (1:100) (Santa Cruz Biotechnology Inc., catalog sc-19992) in blocking buffer (1% BSA). Cells were then washed three times again with PBS and stained with secondary antibody (1:1000 dilution) anti-Rabbit Alexa Fluor Red 568 (Life Technologies, Eugene, OR, USA), anti-Rat Alexa Fluor Green 488 (Life Technologies), in 1% BSA and kept in the dark for 1 hour. Cells were washed again three times with PBS and mounted with VECTASHIELD Antifade Mounting Medium (Vector Laboratories Inc., Burlingame, CA, USA). DAPI staining (4', 6-diamidino-2-phenylindole) (Sigma-Aldrich Corp.) was used to visualize nuclei. The Olympus Fluoview FV10i confocal microscope was used to image cells with a 60x objective lens.

Immunohistochemistry

Immunohistochemistry (IHC) was performed on mouse kidneys and human tissue samples that were provided formalin-fixed, paraffin-embedded, and sectioned onto slides that were positively charged by Brigham and Women's Hospital. Tissue samples were obtained from patients with written informed consent prior to inclusion in this study and with the approval of Partners Human Research Committee. Gradient ethanol and xylenes were used to deparaffinize sections. Antigen retrieval was performed by a heat-induced process using a microwave. After blocking sections with 5% goat serum, sections underwent incubation overnight with primary antibodies at 4°C. 3,3'-Diaminobenzidine

(DAB) reagent (Vector Laboratories Inc.) was used to detect immunoreactivity followed by hematoxylin counterstain (Dako, Agilent Technologies Company, Santa Clara, CA, USA) Sections were imaged with the Olympus BX63 microscope or Zeiss FSX1000 using a 4.2x or 40x objective lens.

Statistical Analyses

All quantitative, normally distributed data for *in vitro* studies were analyzed for statistical significance using a Student's unpaired t-test with the GraphPad Prism Software (GraphPad Prism version 6 for Mac; GraphPad Software, www.graphpad.com). Statistical significance was defined by $P < 0.05$.

CHAPTER 3

RESULTS

Identifying TFEB Expression Levels in Tsc1- and Tsc2- Deficient Cells

The first aim of the study was to examine TFEB activity in Tuberous Sclerosis Complex in an in vitro model using MEFs. Tsc1- and Tsc2-double knockout MEFs are two well-established models in the field for studying TSC in vitro (Zhang et al., 2003, Huang et al., 2008, Ozcan et al., 2008). Due to the lethality caused by complete loss of Tsc1 and Tsc2 at mid-gestation in mouse models, MEF cultures are commonly used to study the function of TSC1 and TSC2 in the mTOR pathway and were used throughout the project (Rennebeck et al., 1998, Kwiatkowski et al., 2002). Although Tuberous Sclerosis Complex is inherited in an autosomal dominant pattern, TSC follows the Knudson two-hit tumor suppressor model and there is a high rate of spontaneous mutations and resulting loss of heterozygosity (Knudson, 1971, Crino et al., 2006). This loss of heterozygosity through the inactivation of the normal allele of TSC1 or TSC2 is the necessary condition for abnormal cell proliferation and the tumorigenic clinical manifestations of the disease (Crino et al., 2006). Therefore, to confirm prior work (Peña-Llopis et al., 2011), protein expression levels of TFEB in Tsc1- and Tsc2-deficient cells were analyzed by western blot in triplicate in MEFs grown in 10% FBS media (**Figure 5**). Increased TFEB protein expression was observed in Tsc2-deficient cells compared to Tsc2-expressing cells (**Figure 5**). Increased TFEB protein expression was also observed in Tsc1-deficient cells, although to a lesser extent.

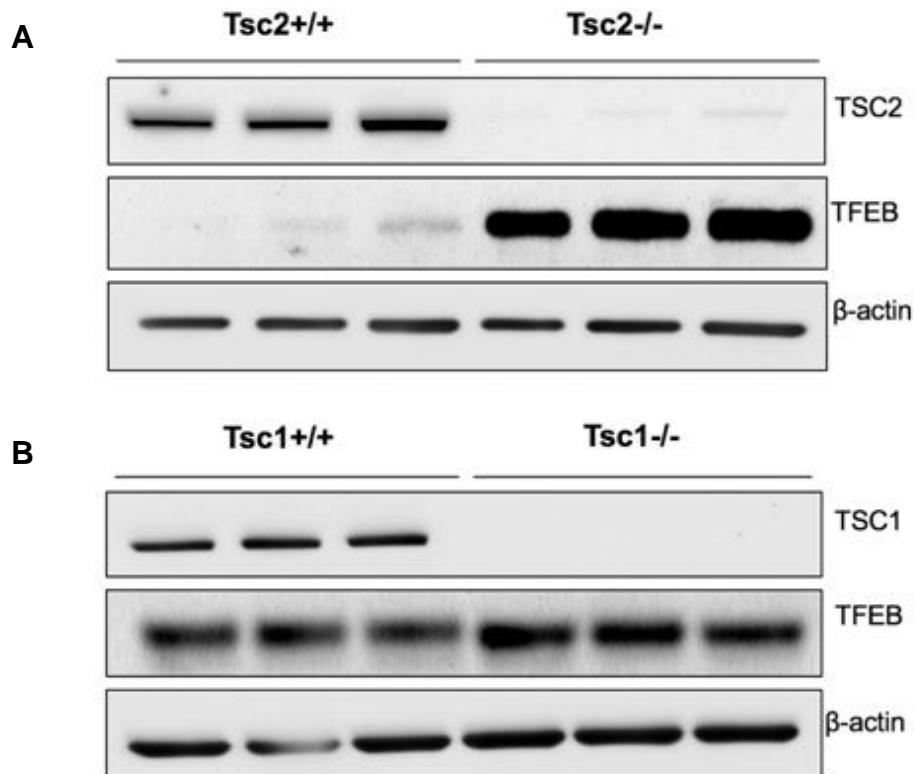


Figure 5. TFEB protein expression is increased in Tsc1- and Tsc2-deficient cells. Western blot of Tsc2-expressing and Tsc2-deficient MEFs (A) and Tsc1-expressing and Tsc1-deficient MEFs (B) in triplicate. MEFs were grown in 10% FBS and β -actin was used as a loading control. Both Tsc1- and Tsc2-deficient cells suggest elevated protein expression of TFEB compared to Tsc1- and Tsc2-expressing cells.

Characterizing the Subcellular Localization of TFEB in TSC

To identify the subcellular localization of TFEB in mTORC1 hyperactive cells, Tsc1- and Tsc2- deficient and expressing MEFs were imaged by immunofluorescence. Cells were stained with antibodies for TFEB and LAMP1 to determine the localization of TFEB in the nuclear or cytoplasmic compartments due to loss of functional TSC1 or TSC2 protein. LAMP1 was used as a lysosomal marker for colocalization since TFEB can be found in the nucleus, in the cytoplasm (bound to 14-3-3 proteins), or on the lysosomal membrane (Martina & Puertollano, 2013). Confocal microscopy of Tsc1- and Tsc2- expressing cells (**Figure 6**) showed localization of TFEB (and expression of LAMP1) in the cytoplasmic compartment. In contrast, Tsc1- and Tsc2-deficient cells revealed nuclear localization of TFEB and expression of LAMP1 in the cytoplasmic compartment (**Figure 6**). Consistent with previous studies, Tsc1- and Tsc2-deficient cells were also larger in cell and nuclei size compared to Tsc2-expressing cells likely due to mTORC1 hyperactivity and resulting metabolic dysregulation (Huang & Manning, 2008, Tapon et al., 2001).

The Effect of mTOR Inhibition on Subcellular Localization of TFEB

Currently rapamycin, which is a known mTORC1 inhibitor, or rapalogs (rapamycin analogs) are used for the treatment of TSC and LAM (Frost & Hulbert, 2015). Both rapamycin and rapalogs have a cytostatic rather than cytotoxic effect, causing tumor regrowth after discontinuation of treatment (Valianou et al., 2019). Torin1 is an inhibitor of both the mTORC1 and mTORC2 pathways. It was hypothesized that treatment of Tsc1- and Tsc2-expressing cells with mTOR inhibitors would localize TFEB to the nucleus while

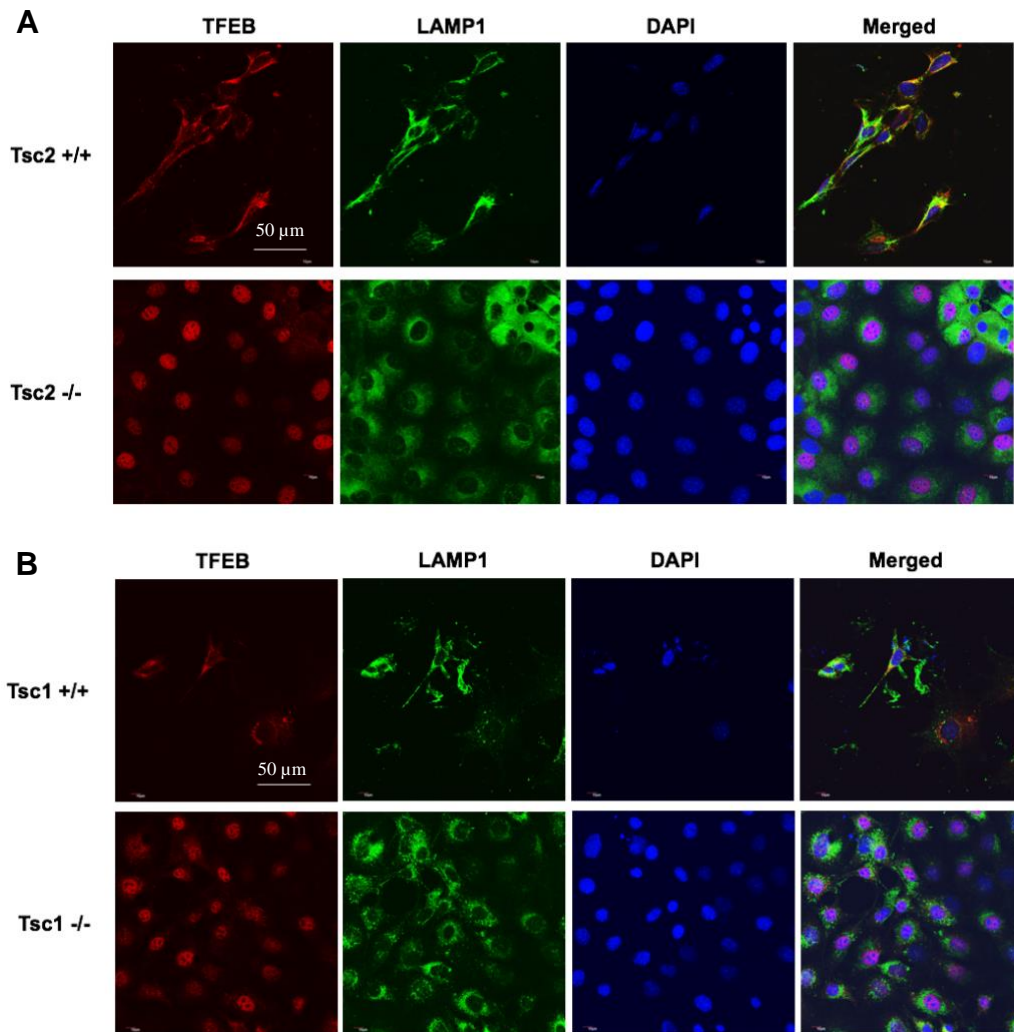


Figure 6. TFEB shows increased nuclear localization in Tsc1- and Tsc2-deficient cells. Immunofluorescence (IF) imaging of Tsc2-expressing and Tsc2-deficient MEFs (A) and Tsc1-expressing and Tsc1-deficient MEFs (B) fixed with 2% PFA and permeabilized in 0.1% Triton. TFEB (red), LAMP1 (green), and DAPI (blue). Both Tsc1- and Tsc2-deficient cells show nuclear localization of TFEB. Images were taken at 60x magnification.

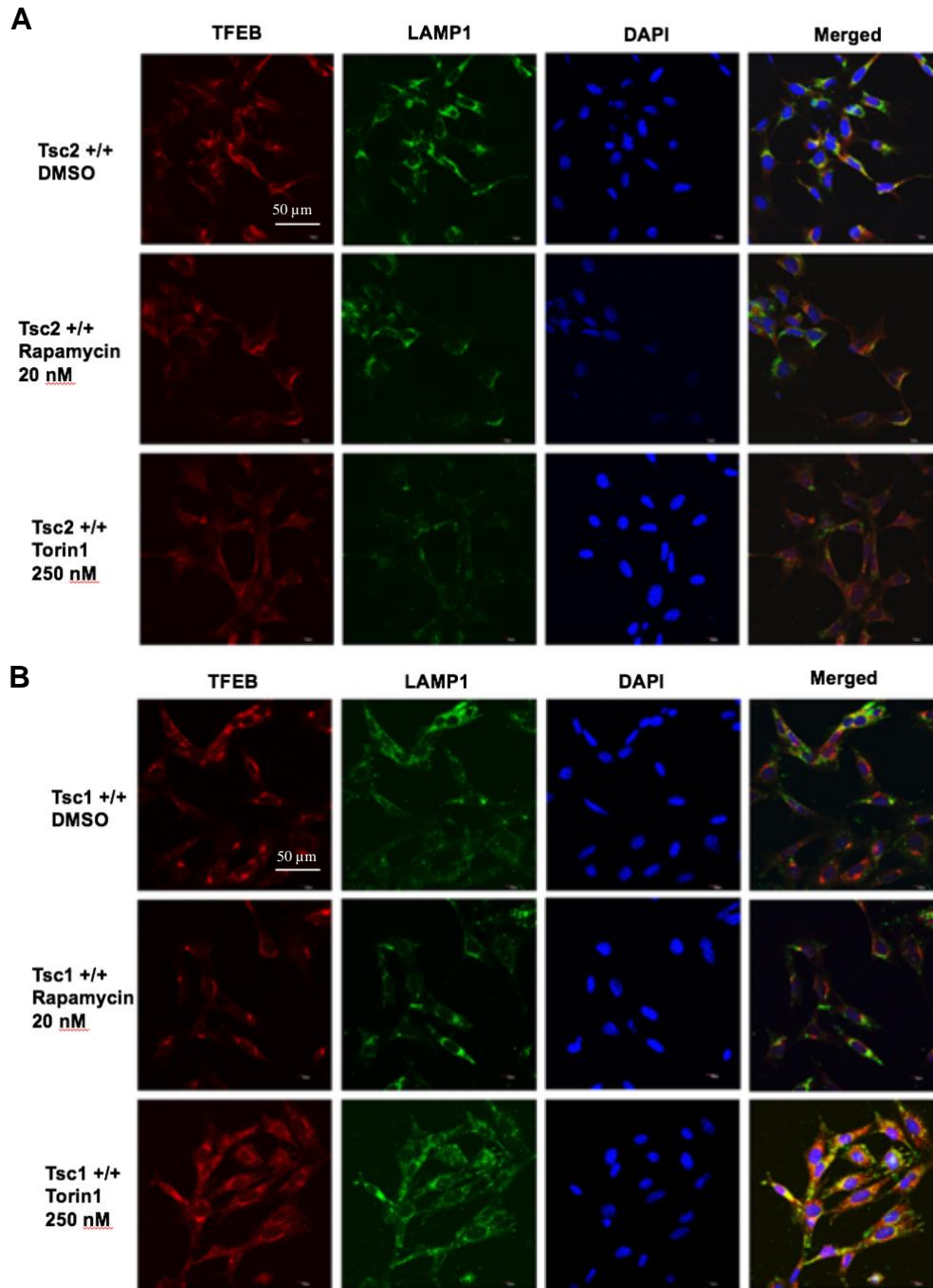


Figure 7. Rapamycin and Torin1 treatment do not show nuclear localization of TFEB in Tsc1- and Tsc2-expressing cells. IF imaging of Tsc1-expressing MEFs (A) and Tsc2-expressing MEFs (B) treated with 20 nM rapamycin, and 250 nM Torin1 for 24 hours and fixed with 2% PFA. TFEB (red), LAMP1 (green), and DAPI (blue). After rapamycin and Torin1 treatment, there is no visible change in the localization of TFEB compared to untreated cells. Images were taken at 60x magnification.

treatment of Tsc1- and Tsc2-deficient cells with mTOR inhibitors would localize TFEB to the cytoplasm. To test this hypothesis, Tsc1- and Tsc2-expressing cells were treated with rapamycin or torin1 at a concentration of 20 nM and 250 nM respectively for 24 hours (**Figure 7**). Upon rapamycin treatment, no substantial difference was observed in the localization of TFEB in Tsc1- and Tsc2-expressing cells consistent with previous literature (Roczniak-Ferguson et al., 2012). Unexpectedly, this result was also shown by mTOR inhibition with torin1, which was expected to have a stronger effect compared to rapamycin, and no apparent change was observed in the localization of TFEB in both Tsc1- and Tsc2-expressing cells.

The effect of mTOR inhibition on the subcellular localization of TFEB was also examined in Tsc1- and Tsc2-deficient cells (**Figure 8**). In Tsc1-deficient cells, rapamycin and torin1 treatment for 24 hours (at the same concentrations) appeared to show localization of TFEB in the cytoplasm, which was more apparent with torin1 compared to rapamycin treatment. Contrary to the hypothesized effect, Tsc2-deficient cells had a much weaker response to mTOR inhibition with only slightly higher expression of TFEB observed in the cytoplasm after torin1 inhibition and even less of an effect with rapamycin treatment.

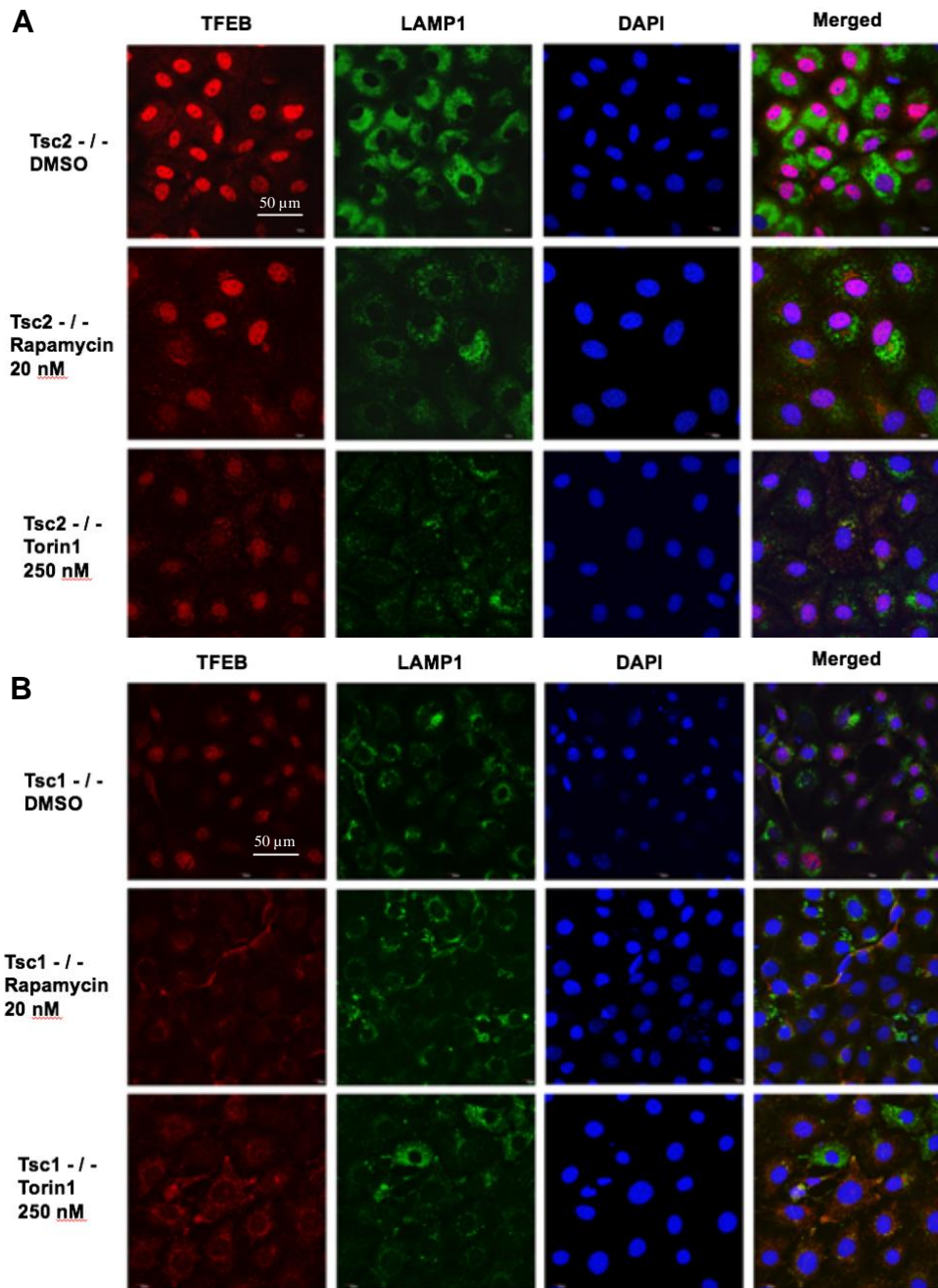


Figure 8. Torin1 treatment localizes TFEB in the cytoplasm in Tsc1- and Tsc2-deficient cells. Immunofluorescence imaging of Tsc1-deficient MEFs (A) and Tsc2-deficient MEFs (B) treated with 20 nM rapamycin, and 250 nM Torin 1 for 24 hours and fixed with 2% PFA. TFEB (red), LAMP1 (green), and DAPI (blue) are shown. After Rapamycin and Torin1 treatment, TFEB appears to localize in the cytoplasmic compartment. Images were taken at 60x magnification.

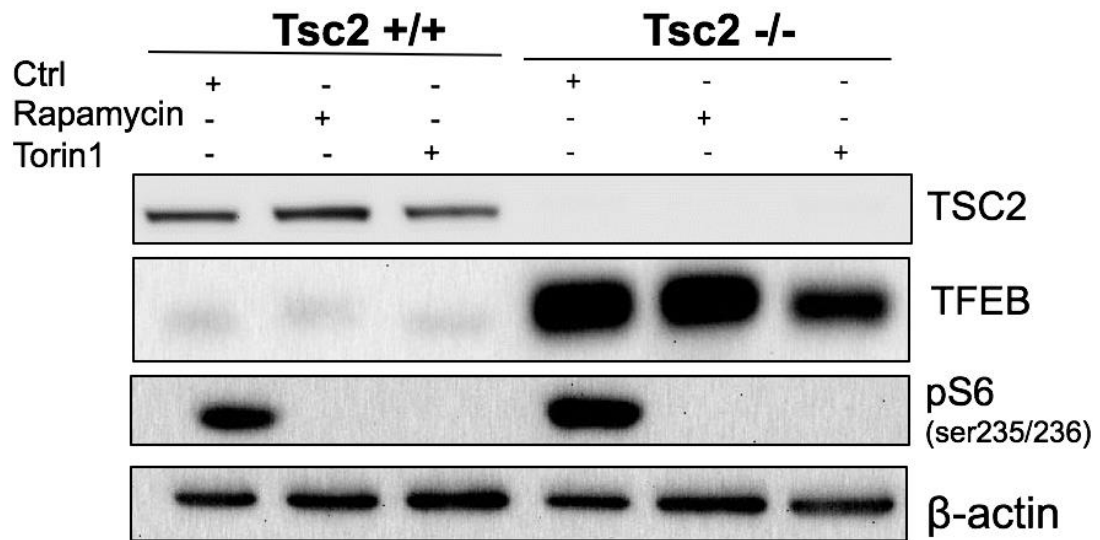


Figure 9. Torin1 treatment reduces TFEB protein expression levels in Tsc2-deficient cells. Western blot of Tsc2-expressing and Tsc2-deficient cells in triplicate in 10% FBS media. Cells were treated with vehicle control, 20 nM rapamycin, and 250 nM Torin1 for 24 hours. Tsc2 *-/-* immunoblot shows decreased expression of TFEB after Torin1 treatment. β -actin was used as a loading control and phospho-S6 was used as a measure of mTORC1 activity and therefore, the effectiveness of mTORC1 inhibitors.

The Effect of mTOR Inhibition on TFEB Protein Expression

To characterize the effect of mTOR inhibition on TFEB protein expression, Tsc2 WT and null MEFs were treated with rapamycin at a 20 nM concentration and torin1 at a 250 nM concentration for 24 hours in media supplemented with 10% FBS and analyzed by western blot (**Figure 9**). TFEB protein expression was analyzed with β -actin as a loading control, and mTORC1 hyperactivation in Tsc2-deficient cells was confirmed by evaluating phospho-S6 expression levels (a known substrate and indicator of mTORC1 activity). After rapamycin treatment, no difference in TFEB protein expression levels in Tsc2-deficient cells was observed. In contrast, torin1 treatment of Tsc2-deficient cells resulted in decreased TFEB protein expression levels.

Lysosomal Gene Expression in Tsc1- and Tsc2-deficient Cells

To investigate whether loss of functional TSC impacts lysosomal gene expression, relative mRNA expression was measured by qRT-PCR in triplicate with three biological replicates per group. Lysosomal genes (*Npc1*, *Npc2*, *Hexa*, *Ctsk*) and the MiTF transcription factor family members, *Mitf* and *Tfe3*, were measured at the mRNA level in MEFs (**Figure 10**).

Compared to Tsc1- and Tsc2-expressing cells, Tsc1- and Tsc2-deficient cells showed significantly increased expression of lysosomal genes *Npc1*, *Npc2*, *Ctsk*, and *Hexa*. The lysosomal gene *Npc1* had a 6-fold increase in mRNA expression in Tsc1-deficient cells. A similar increase was seen in *Ctsk* mRNA expression. Both *Npc2* and *Hexa* were also upregulated in Tsc1-deficient cells, with approximately 1.5-fold and 3-fold increases

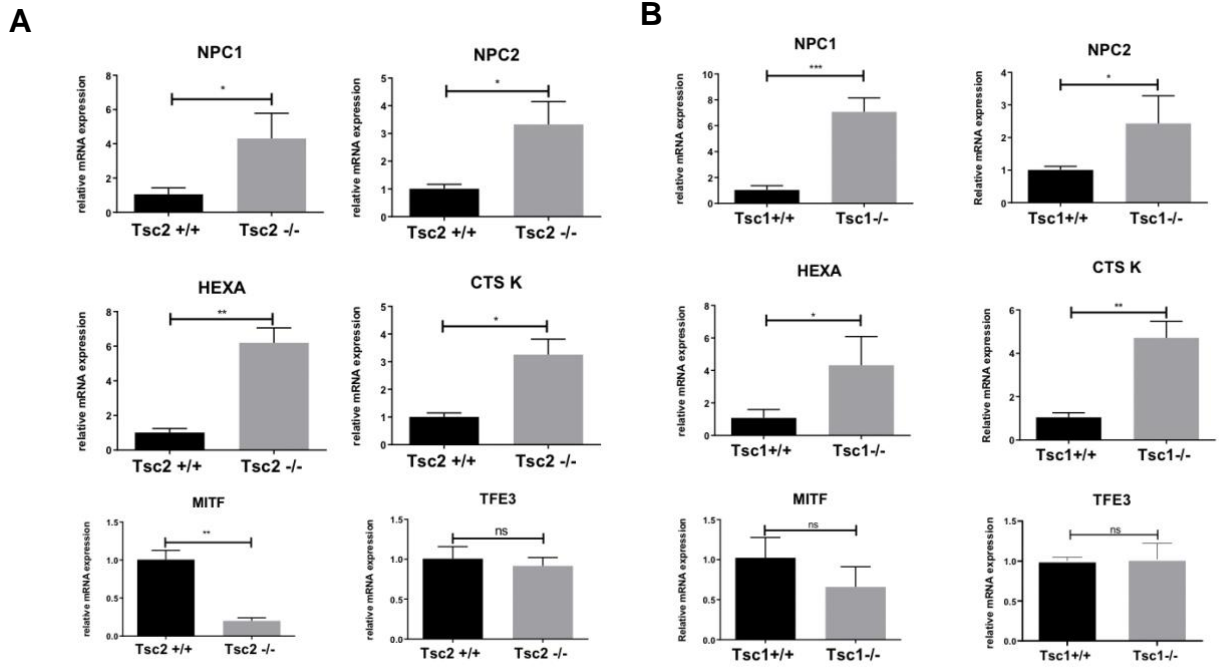


Figure 10. Lysosomal gene expression is increased in Tsc2- and Tsc1-deficient cells. Relative mRNA expression by qRT-PCR of lysosomal genes for Tsc1- and Tsc2-deficient cells compared to wildtype MEFs. Expression of lysosomal genes and MITF transcription factor family members: *Npc1*, *Npc2*, *Hexa*, *Ctsk*, *Mitf*, and *Tfe3* shown in Tsc2 MEFs (A) and Tsc1 MEFs (B). Lysosomal genes have increased expression in Tsc1- or Tsc2-deficient cells. For all groups n=3. *p<0.05, **p<0.01, ***p<0.001.

respectively. Tsc2-null MEFs also showed upregulation of lysosomal gene expression compared to Tsc2-expressing cells. *Npc1* expression increased 3-fold, while *Npc2* and *Ctsk* showed two-fold increases in Tsc2-null cells. Among these cells, *Hexa* demonstrated the largest increase (five-fold). This indicates that there is elevated mRNA expression of lysosomal genes, suggesting dysregulation of lysosomal activity in Tsc-null cells.

Mitf and *Tfe3* levels were also evaluated (**Figure 10**) to examine whether relative mRNA expression of other MITF transcription factor family members were also affected by loss of TSC. Surprisingly, *Mitf* expression levels were significantly reduced in Tsc2-deficient cells but not in Tsc1-deficient cells. *Tfe3* showed no significant change across all four cell lines.

Investigating Lysosomal Protein Expression in Tsc1- and Tsc2-deficient cells

After examining lysosomal gene expression at the mRNA level, lysosomal protein expression was studied. Tsc2-expressing and Tsc2-deficient MEFs were grown under starvation conditions for four days in 0.1% FBS. Previous experiments in the lab have established that these starvation conditions are required to induce lysosomal biogenesis and increase lysosomal protein expression in Tsc1- and Tsc2-deficient MEFs (data not shown). The lysosomal proteins NPC1, HEXA, and CTSK were then examined. Under serum starvation, Tsc2-deficient cells showed increased TFEB and lysosomal protein expression by western blot compared to Tsc2-expressing cells (**Figure 11**). Phospho-S6 (PS6) and total-S6 (TS6) were also examined to confirm mTORC1 activity, and β -actin was used as a loading control.

To further investigate involvement of the mTOR pathway, considering the effect of mTORC1 inhibition in Tsc1- and Tsc2-deficient cells by IF (Figure 8), rapamycin treatment was administered to Tsc2-expressing and Tsc2-deficient cells for 48 hours at a 20 nM concentration in triplicate (**Figure 11A**). Rapamycin inhibition of mTORC1 in Tsc2-null cells inhibited phosphorylation of TFEB (as expected) and increased TFEB protein expression. However, lysosomal protein expression was unchanged by rapamycin treatment, which was consistent with the lower presence of nuclear TFEB in Tsc1- and Tsc2-deficient cells after rapamycin treatment (Figure 8). In fact, CTS K expression levels were slightly reduced with rapamycin treatment in Tsc2-deficient cells. Protein expression levels of NPC1 and HEXA showed no prominent change.

Lysosomal protein expression with mTOR inhibition was examined further in Tsc2-expressing and Tsc2-deficient cells with 24-hour torin1 treatment at a concentration of 250 nM under serum starvation conditions with 0.1% FBS for four days (**Figure 11B**). DMSO treatment was used as a control, and samples were loaded in duplicate. In Tsc2-expressing cells, torin1 treatment increased the expression of lysosomal proteins of HEXA, LAMP1, NPC1, and CTS K, consistent with the localization of TFEB inside the nucleus.

Compared to Tsc2-expressing cells treated with DMSO, DMSO-treated Tsc2-deficient cells also showed increased lysosomal protein expression, suggesting that loss of TSC2 protein causes increased expression of lysosomal genes. However, under torin1 treatment, Tsc2-deficient cells did not increase lysosomal protein expression. Specifically, proteins LAMP1 and CTS K showed downregulation, while both NPC1 and HEXA protein expression appeared unchanged. Phospho-S6 was once again used as a measure of

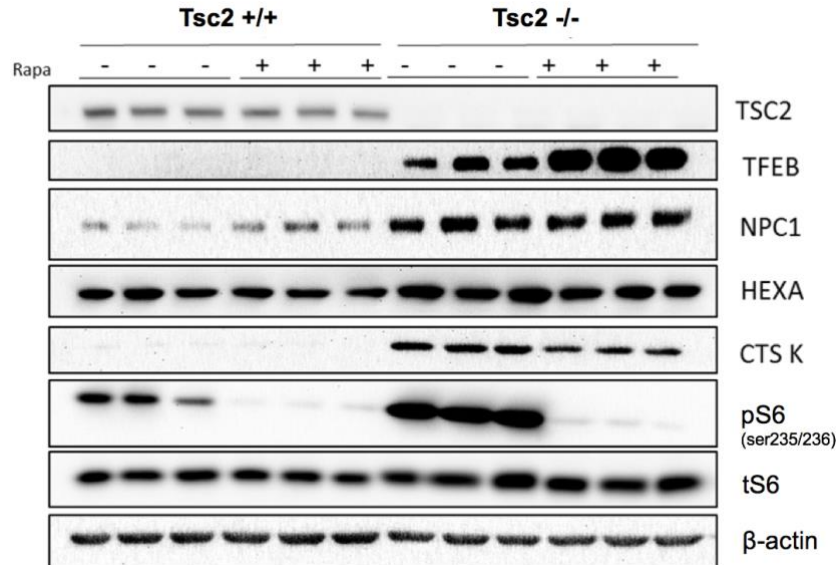
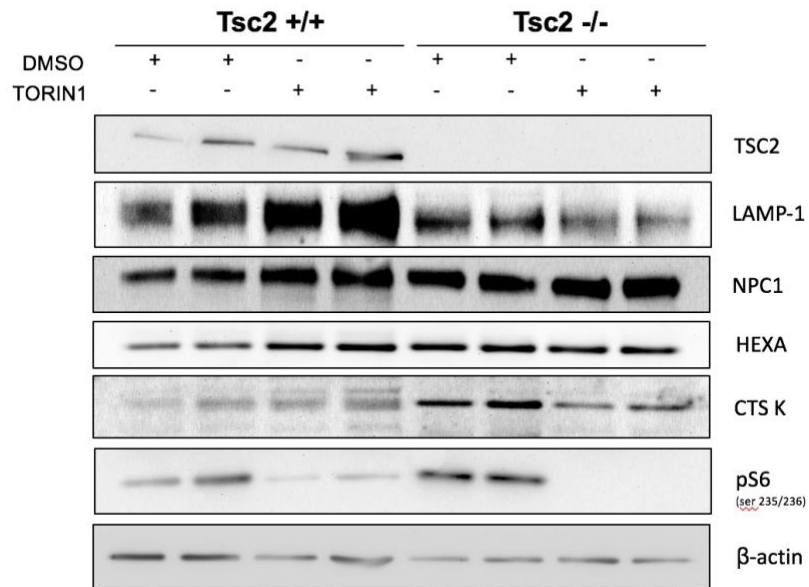
A**B**

Figure 11. mTOR inhibition by rapamycin and Torin1 does not increase in lysosomal protein expression in Tsc2-deficient cells. Western blot of Tsc2-expressing and Tsc2-deficient MEFs grown in 0.1% FBS media for four days. (A) 20 nM rapamycin treatment was administered to cells for 24 hours in triplicate. (B) Cells were treated with 250 nM Torin1 and DMSO (control) in duplicate for 24 hours. Tsc2-deficient cells did not show increased expression of lysosomal proteins after rapamycin or Torin1 treatment. β -actin was used as a loading control and phospho-S6 was used as a measure of mTORC1 activity.

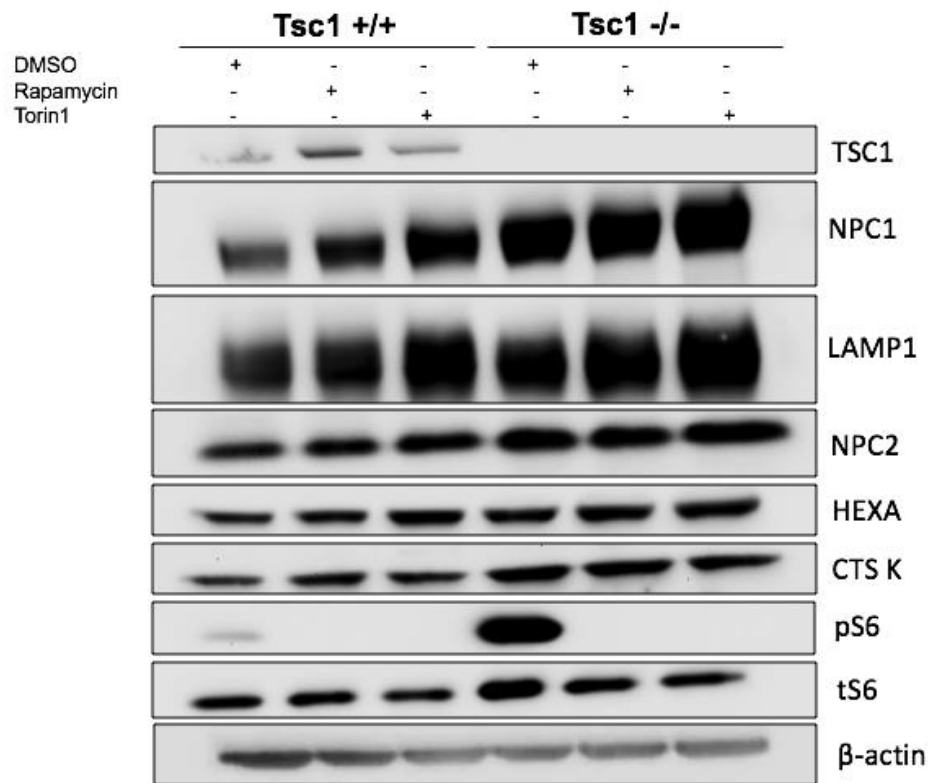


Figure 12. mTOR inhibition suggests increases lysosomal protein expression in Tsc1-expressing cells, but not Tsc1-deficient cells. Western blot of Tsc1-expressing and Tsc1-deficient MEFs grown in 0.1% FBS media for four days. Cells were treated with 20 nM rapamycin and 250 nM Torin1 for 24 hours. Tsc1-expressing cells appear to show increased expression of lysosomal proteins after both rapamycin and Torin1 treatment. Tsc1-deficient cells showed no notable increase in lysosomal proteins after mTOR inhibition. β-actin was used as a loading control and phospho-S6 was used as a measure of mTORC1 activity.

mTORC1 activity and β -actin was used as a loading control. The effect of rapamycin and torin1 treatment were also studied in Tsc1-expressing and deficient MEFs under the same treatment conditions as the previous experiment (**Figure 12**). Overall, Tsc1-deficient cells seemed to display increased lysosomal protein expression across all conditions for NPC1, NPC2, LAMP1, HEXA, and CTS K. In Tsc1-expressing cells, mTOR inhibition with rapamycin and torin1 appeared to increase lysosomal protein expression. However, similar to Tsc2-deficient MEFs, Tsc1-deficient cells did not display increased lysosomal protein expression with rapamycin or torin1 treatment.

Investigating the Effect of TFEB Knockdown on Lysosomal Protein Expression in Tsc1- and Tsc2-deficient Cells

To examine the effect of TFEB downregulation on lysosomal protein expression in Tsc1- and Tsc2-deficient cells, Tsc2-deficient MEFs were transfected with short hairpin RNA (shRNA) against TFEB. Cells were grown under serum starved conditions with 0.1% FBS for four days and evaluated by western blot analysis (**Figure 13**). Transfection with shTFEB resulted in partial downregulation of TFEB in Tsc2-deficient cells. Upon TFEB knockdown, Tsc2-deficient cells showed reduced lysosomal protein expression of NPC1, NPC2, and LAMP1 compared to control Tsc2-deficient cells. However, CTS K protein expression was unchanged between sh-control and TFEB knockdown conditions. This result suggests that lysosomal protein expression in Tsc2-null cells is TFEB-dependent.

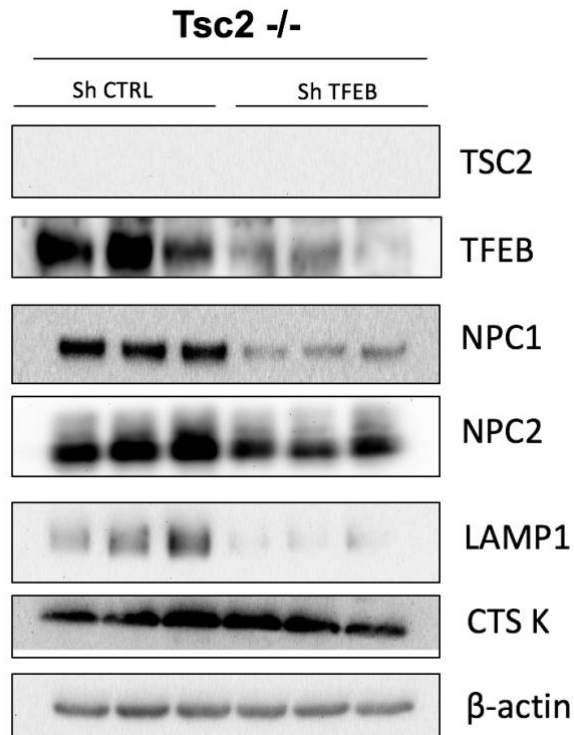


Figure 13. Downregulation of TFEB appears to decrease lysosomal protein expression in Tsc2-deficient cells. Western blot of Tsc2-deficient MEFs transfected with either sh-control or shTFEB (548) and grown in 0.1% FBS media for four days in triplicate using three biological replicates. Knockdown of TFEB in Tsc2-deficient cells decreased the expression of lysosomal genes. β -actin was used as a loading control.

Characterizing Phosphorylated TFEB Expression in Tsc1-deficient Cells

TFEB subcellular localization is known to be regulated through mTORC1 phosphorylation (Peña-Llopis et al., 2011). According to the established paradigm on the regulation of TFEB by mTOR, the mTORC1 complex negatively regulates TFEB via phosphorylation, which serves as a priming event for 14-3-3 chaperone protein binding causing its cytoplasmic retention (Settembre et al., 2012). This establishes mTOR as an inhibitor of TFEB, and phosphorylated TFEB protein expression levels should be elevated with active mTOR. However, in our current model of mTOR hyperactivated cells in Tsc1- and Tsc2-deficient cell lines, TFEB was nuclear-localized suggesting that TFEB phosphorylation may be decreased. Therefore, we hypothesized that decreased phosphorylation of TFEB causes nuclear localization of TFEB in Tsc-null cells. Unfortunately, there are currently no effective phospho-TFEB antibodies for mouse-derived cell lines. For this reason, the migration pattern of TFEB in Tsc1-deficient and Tsc1-expressing cells was examined through western blot analysis as the starting point of further studies into the role of TFEB phosphorylation in the mechanism and pathogenesis of TSC.

Unphosphorylated proteins are known to migrate faster in the blot and appear as a “lowered” band compared to phosphorylated proteins due to decreased molecular weight. To determine whether the use of mTOR inhibitors had an effect on TFEB migration, Tsc1-expressing and Tsc1-deficient MEFs were treated with rapamycin and torin1 for 24 hours (**Figure 14**). Cells were also grown in 0.1% FBS media for four days and phospho-S6 was

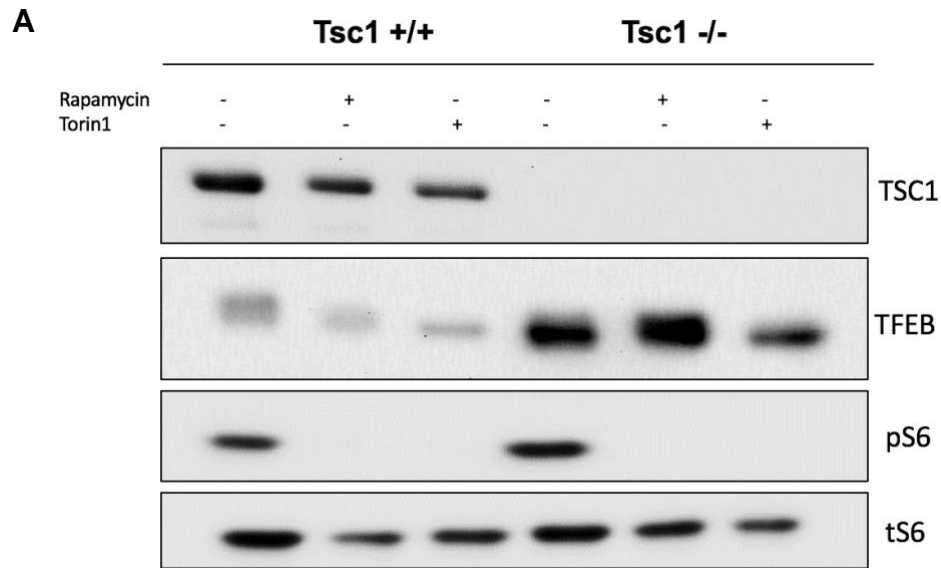


Figure 14. TFEB migration and response to mTOR inhibition is different in Tsc1-deficient cells compared to Tsc1-expressing cells. Tsc1-expressing and Tsc1-deficient cells treated with rapamycin and Torin1 and grown in 0.1% FBS media for four days. TFEB migration pattern appears different in Tsc1-expressing cells compared to Tsc1-deficient cells. Phospho-s6 was used as a measure of mTORC1 activity.

used as a measure of mTOR activity. Western blot analysis showed different migration patterns for TFEB in Tsc1-expressing and Tsc1-deficient cells. In the control condition, the TFEB protein band appeared lower in Tsc1-deficient cells compared to Tsc1-expressing cells suggesting hyposphorylation of Tsc1-deficient cells. No substantial change in migration was observed in Tsc1-expressing and Tsc1-deficient cells after treatment with mTOR inhibitors compared to untreated cells.

Investigating TFEB Nuclear Localization in Tsc1- and Tsc2-deficient Cells

The mechanism of TFEB nuclear localization was further studied by examining protein expression levels of calcineurin and various 14-3-3 binding protein isoforms. Calcineurin is a phosphatase that induces TFEB nuclear localization by dephosphorylating TFEB at the Ser142 and Ser211 residues (Medina et al., 2015). Therefore, increased calcineurin activity might explain the pattern of TFEB nuclear localization observed in Tsc1- and Tsc2-deficient cells. As a preliminary experiment to examine calcineurin activity, protein expression levels were examined in Tsc1- and Tsc2-deficient cells grown in 10% FBS with β -actin as a loading control (**Figure 15**). There was no apparent change in calcineurin expression levels in Tsc1-deficient cells. Tsc2-deficient cells seemed to show a slight decrease in calcineurin protein expression. Further experiments are necessary to determine the activity of this phosphatase.

14-3-3 binding protein expression levels were next evaluated to understand the mechanism of TFEB nuclear localization in Tsc1- and Tsc2-deficient cells in **Figure 16A** and quantitated in **Figure 16B**. 14-3-3 proteins bind to TFEB in a phosphorylation-

dependent manner, ultimately blocking TFEB nuclear localization (Puertollano et al., 2018). Thus, a decrease in 14-3-3 protein levels in Tsc1- and Tsc2-deficient cells might explain the nuclear localization of TFEB. Several isoforms of 14-3-3 binding protein were studied (14-3-3 alpha-beta, 14-3-3 zeta-delta, 14-3-3 tau, 14-3-3 eta, 14-3-3 gamma, and 14-3-3 epsilon) in Tsc1 and Tsc2 MEFs grown in 10% FBS to determine whether isoform-specific reduced expression of 14-3-3 proteins could be responsible for the nuclear localization of TFEB. Interestingly, 14-3-3 protein expression of 3 isoforms: 14-3-3 alpha-beta, 14-3-3 zeta-delta, and 14-3-3 tau decreased in both Tsc1- and Tsc2-deficient cells compared to Tsc1- and Tsc2-expressing cells, while expression of 14-3-3 eta and 14-3-3 epsilon remained unchanged. Contrary to expectation, increased expression of 14-3-3 gamma protein was observed.

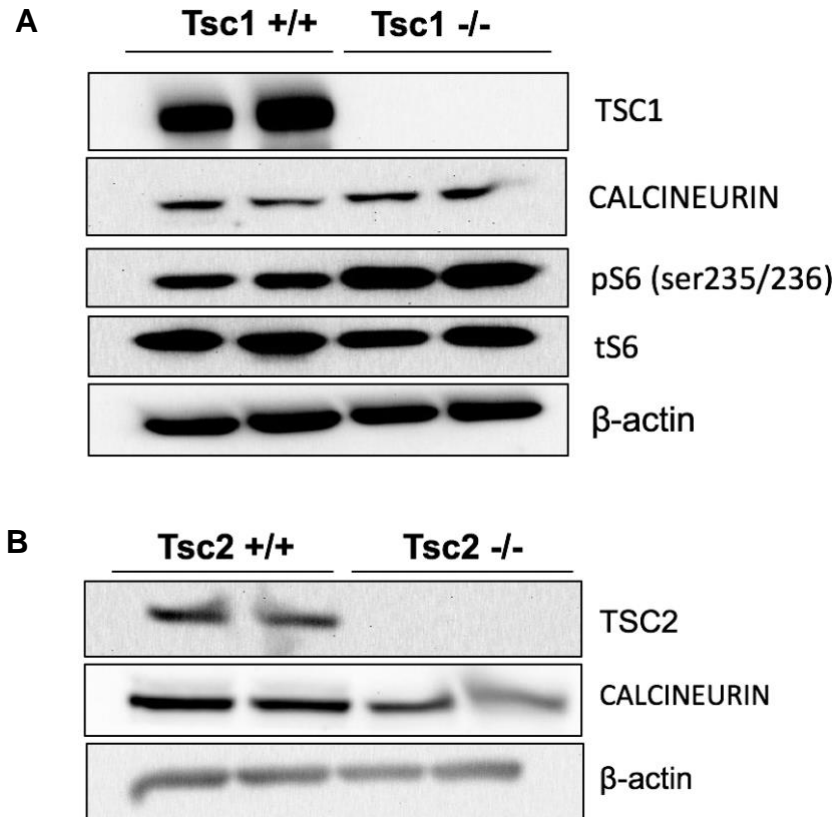


Figure 15. Calcineurin protein expression in Tsc1- and Tsc2-deficient cells. Western blot of (A) Tsc1-expressing cells and Tsc1-deficient cells and (B) Tsc2-expressing cells and Tsc2-deficient cells grown in in 10% FBS media. Calcineurin protein expression does not appear to change in Tsc1- or Tsc2-deficient cells. β -actin is used as a loading control.

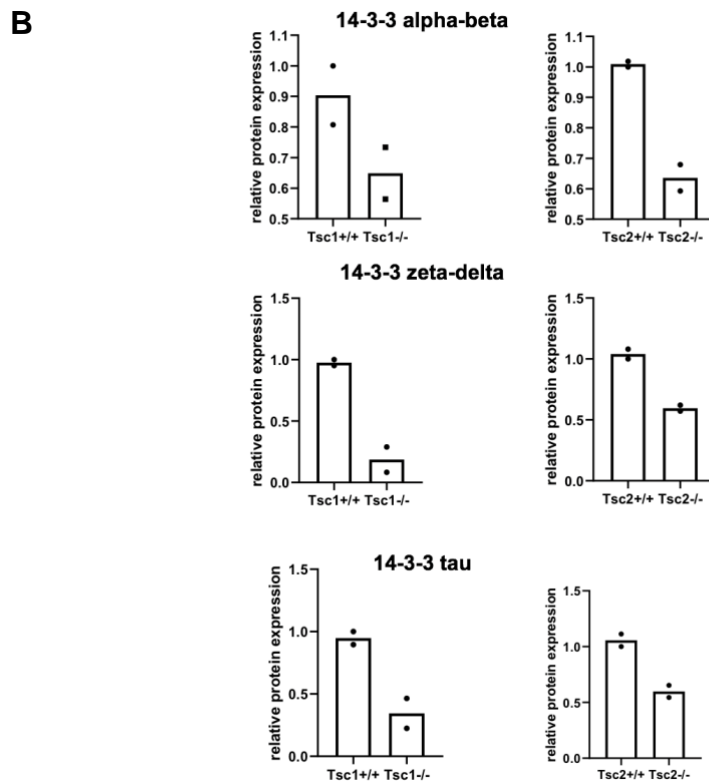
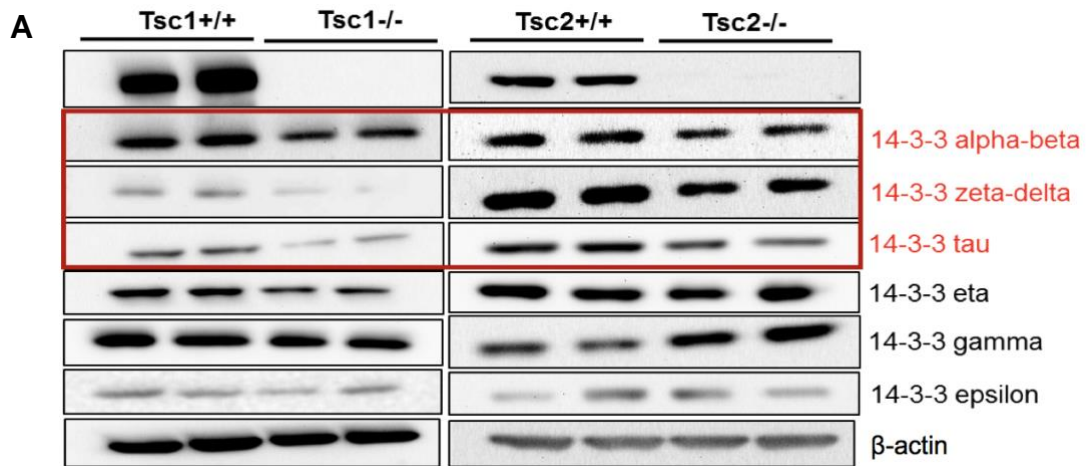


Figure 16. 14-3-3 protein expression displays a decreasing trend in Tsc1- and Tsc2-deficient cells. Western blot of Tsc1 and Tsc2 MEFs grown in 10% FBS media (A) and relative protein expression quantified by ImageJ for 14-3-3 alpha-beta, 14-3-3 zeta-delta, and 14-3-3 tau isoforms (B). Tsc1- or Tsc2-deficient cells appear to show lower expression of 3 out of 6 isoforms of 14-3-3 binding proteins (14-3-3 alpha-beta, 14-3-3 zeta-delta, and 14-3-3 tau) compared to wildtype. β-actin is used as a loading control.

Expression of TFEB and NPC1 in Human Samples of TSC Patients

Immunohistochemistry (IHC) staining was used to characterize the expression of TFEB and lysosomal proteins in an *in vivo* model of TSC. Previous experiments in the lab (not shown) had observed increased expression of TFEB protein in human tissue samples of LAM and AMLs. For this reason, TFEB and lysosomal protein expression was also examined in TSC-associated renal cell carcinoma, another clinical manifestation of TSC. RCC samples were obtained from Brigham and Women's Hospital and stained for TFEB and the lysosomal protein, NPC1 (**Figure 17**). Normal kidney tissue samples were also obtained from the same patients and stained for TFEB and NPC1. IHC staining in renal tumors appeared to show high levels of TFEB expression that was localized in the nucleus. Diffuse positivity of nuclear TFEB observed in the distal and proximal tubules was expected in normal tissue samples since these cells are postulated to be the cells of origin for TSC lesions (Gonçalves et al., 2017, Armour et al., 2012, Bissler et al., 2019). Kidney lesions also stained positively for NPC1 in TSC-associated RCC compared to normal kidney tissue indicating high expression of these specific lysosomal proteins in RCCs. Together, these findings suggest an association between nuclear TFEB and higher expression of its downstream target, NPC1, in TSC-related RCC.

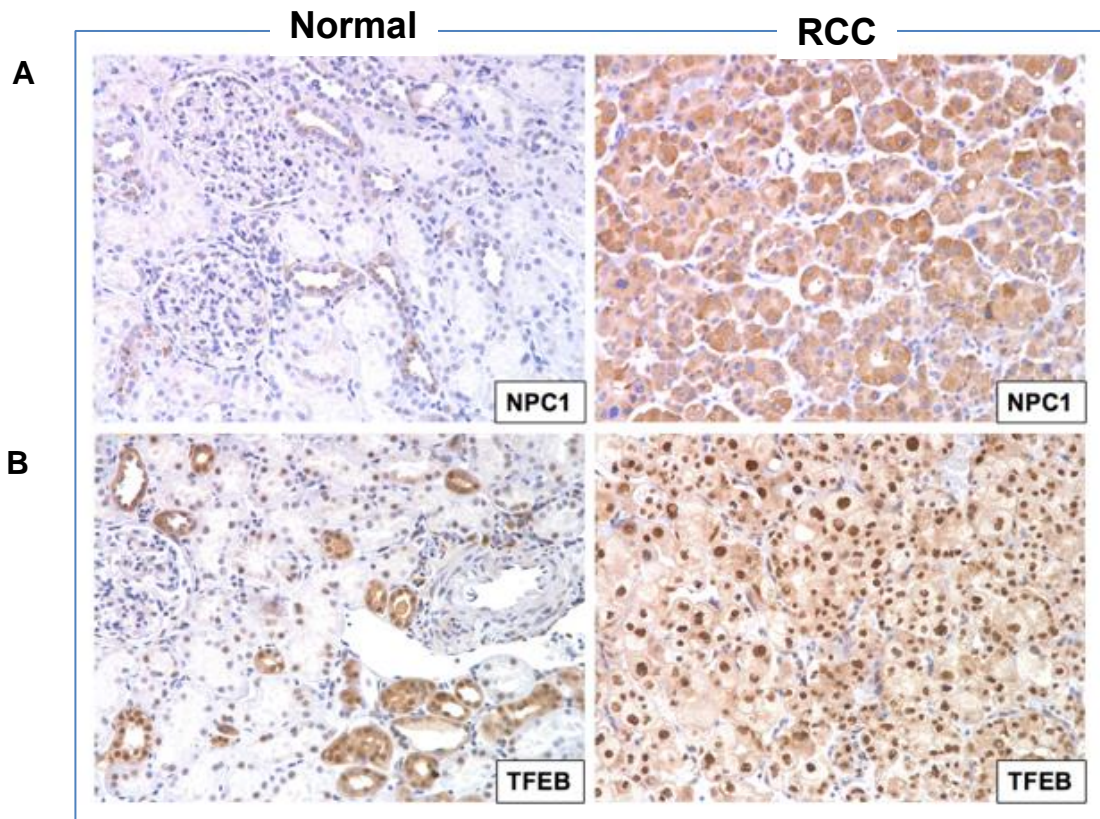


Figure 17. IHC staining of TFEB and NPC1 suggests increased expression in human samples of TSC-associated renal cell carcinoma (RCC). Immunohistochemistry (IHC) staining of kidney tissue samples of human patients with TSC-associated RCC for (A) NPC1 and (B) TFEB. NPC1 and TFEB expression appears to be increased in RCC-tumor samples with strong nuclear localization of TFEB. Samples were obtained from three patients with TSC-associated RCC.

Lysosomal Exocytosis Model of Tuberous Sclerosis Complex

The expression of TFEB and lysosomal protein NPC1 in mTORC1-driven renal cysts was examined *in vivo* in TSC2 +/- AJ and KSP-CreERT2 Tsc2^{fl/fl} mouse models that developed kidney cysts. TSC2 +/- AJ mice were generated by the Kwiatkowski Lab to genetically resemble an individual with Tuberous Sclerosis Complex. These mice present a cystic kidney phenotype, similar to TSC patients. IHC of kidney cysts in this mouse model appeared to stain positive for NPC1 in the apical region of cyst-lining cells (**Figure 18A**). This result suggests a high concentration of lysosomes in the apical region of the cell, which could point toward the possibility of increased lysosomal exocytosis in cyst-lining cells.

To validate the observation made in the previous model, IHC staining for NPC1 was also carried out in KSP-CreERT2 Tsc2^{fl/fl} mice that showed a cystic kidney phenotype (**Figure 18B**). KSP-CreERT mice that carried a tamoxifen-induced Cre recombinase driven by a kidney-specific cadherin (KSP) promoter were crossed with a Tsc2^{fl/fl} mouse to create a KSP-CreERT2 Tsc2^{fl/fl} model generated by Dr. Lam in the Henske Lab (Lam et al., 2017). Cysts in this mouse model appeared to stain positive for NPC1 with the characteristic apical distribution of staining towards the lumen of the cyst. This was in contrast to KSP-Cre control mice which showed NPC1 staining in the basal region of proximal tubule cells (**Figure 18C**).

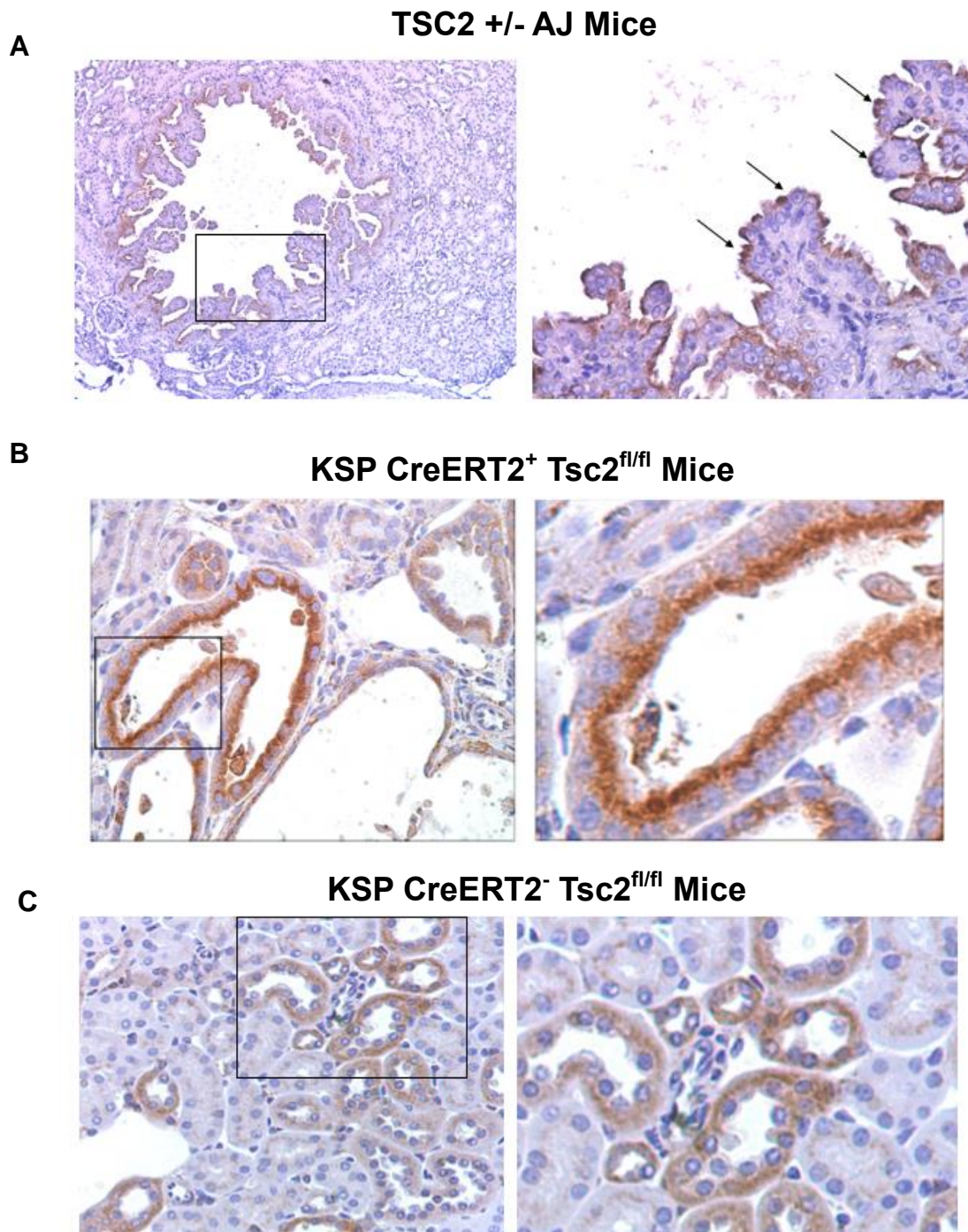


Figure 18. Mouse models of renal TSC show predominantly apical NPC1 staining. Immunohistochemistry (IHC) staining of NPC1 in TSC-associated kidney cysts from two mouse models of renal TSC. (A) TSC2 +/- AJ mice and (B) KSP-CreERT2 Tsc2^{fl/fl} mice show apical staining of NPC1 in cyst-lining cells of the kidney.

Summary

The goal of this project was to examine the regulation of TFEB and the lysosome in a cell model to better understand the pathogenesis of TSC. Increased expression of TFEB in Tsc1- and Tsc2-deficient cells was confirmed by western blot *in vitro* in cell lines as well as by IHC *in vivo* in human and mouse tissue samples of TSC. Immunofluorescence imaging of TFEB showed nuclear localization of TFEB in Tsc1- and Tsc2-deficient cells, in contrast to Tsc1- and Tsc2-expressing cells in which TFEB was localized in the cytoplasmic compartment. This localization pattern was reversed following mTOR inhibition by rapamycin and torin1 treatment. Investigating the mechanism of TFEB nuclear localization, in Tsc-null cells TFEB appeared to migrate farther down the western blot suggesting decreased phosphorylation in these cells. Based on previous published literature, we analyzed calcineurin and 14-3-3 protein expression in order to gain further information about the mechanism of TFEB nuclear localization Tsc1- and Tsc2-deficient cells despite the high mTORC1 activity. Western blot analysis of calcineurin levels in Tsc1- and Tsc2-deficient cells showed no increase in protein expression. Three out of six isoforms of 14-3-3 proteins were reduced in Tsc-null cells, making 14-3-3 a potential candidate for future studies. Further IHC staining of two mouse models of renal TSC showed apical staining for NPC1 in cyst-lining cells.

CHAPTER 4

DISCUSSION

This study sought to investigate the regulation of TFEB in Tsc1- and Tsc2-deficient cells and to examine its role in the pathogenesis of Tuberous Sclerosis Complex (TSC) through both *in vitro* and *in vivo* models. Past studies have shown that mTORC1 negatively regulates TFEB activity in wild-type cells through colocalization on the lysosomal membrane and direct phosphorylation that prevents its nuclear localization (Settembre et al., 2012). Based on this established mechanism, TFEB would be expected to be inactive in a mTORC1 hyperactivated condition. However, prior work had shown that TFEB was upregulated in Tsc2-deficient cells compared to Tsc2-expressing cells (Peña-Llopis et al., 2011). This result was repeated and verified by western blot (Figure 5).

After confirming this increase in TFEB expression, we investigated the subcellular localization of TFEB in Tsc1- and Tsc2-deficient MEFs. Under nutrient deprivation, mTORC1 is inactivated by the TSC protein complex, which allows the recruitment of TFEB into the nucleus for lysosomal genes transcription by binding CLEAR sequences (Ploper & De Robertis, 2015). In this way, mTOR acts as a regulator of TFEB, lysosomal biogenesis, and autophagy to maintain cellular homeostasis. According to this mechanism of TFEB intracellular localization, TFEB would be expected to remain in the cytoplasm in an mTORC1 hyperactivated cell that is Tsc1- or Tsc2-deficient. However, immunofluorescence imaging of both Tsc1- and Tsc2-deficient cells had the opposite effect (Figure 6).

To confirm the role of the mTOR pathway in the subcellular localization of TFEB in our cell lines, Tsc1- and Tsc2-expressing (as well as Tsc1- and Tsc2-deficient cells) were treated with mTOR inhibitors, rapamycin and torin1. Immunofluorescence imaging of Tsc1- and Tsc2-expressing MEFs treated with both rapamycin and torin1, appeared to cause no change in the localization of TFEB. Contrary to our expectation, TFEB remained localized in the cytoplasmic compartment in these cells similar to untreated cells (Figure 7). This was in contrast to previous studies in which mTOR inhibition with torin1 induced strong localization of TFEB in the nuclear compartment, while rapamycin treatment showed no apparent change in localization (Roczniak-Ferguson et al., 2012).

Interestingly, Tsc1- and Tsc2-deficient cells treated with rapamycin and torin1 appeared to induce expression of TFEB in the cytoplasmic compartment although this expression was visible to a lesser extent with rapamycin treatment as expected (Figure 8). This result suggests a rapamycin insensitive mechanism of TFEB subcellular localization in Tsc1- and Tsc2-deficient cells. One possible explanation for why TFEB is nuclear in Tsc1- and Tsc2-deficient (mTOR hyperactive) cells could be the presence of a nuclear mTORC1 complex. Previously, mTORC1 was thought to exist as a single nutrient sensing hub in the cell. However, more recent research has suggested mTOR recruitment to various intracellular compartments in addition to lysosomes including the Golgi apparatus, peroxisomes, and even the nucleus (Goberdhan et al., 2016). Hyperactivation of a nuclear mTOR has also been implicated in prostate cancer, in which nuclear mTOR causes reprogramming of cellular metabolism by altering mTOR-chromatin associations (Audet-Walsh et al., 2017). In the context of TSC, a nuclear mTOR could explain the movement

of TFEB between intracellular compartments. Inhibition of such a nuclear mTOR could prevent phosphorylation of nuclear TFEB and allow it to re-localize to the cytoplasmic compartment as seen in our Tsc1- and Tsc2-deficient cells treated with torin1.

To understand the impact of the upregulation of TFEB and its nuclear localization in Tsc-null cells, lysosomal gene and protein expression was analyzed in Tsc1- and Tsc2-deficient cells (Figure 10-13). Tsc1- and Tsc2-deficient cells showed significant upregulation of lysosomal genes ($p < 0.05$) at the mRNA level compared to Tsc1- and Tsc2-expressing cells (Figure 10). Western blot analysis also showed increased expression of lysosomal proteins in Tsc1- and Tsc2-deficient cells, suggesting lysosomal dysregulation and increased lysosomal biogenesis (Figure 11-12). Inhibition of mTOR activity with torin1 increased lysosomal protein expression in Tsc1-2 expressing cells (as expected). This suggests that inhibition of the mTORC1 complex in Tsc1- and Tsc2-expressing cells increased transcription of lysosomal genes through regulation of TFEB. On the other hand, torin1 treatment in the Tsc1-2 null cells did not change lysosomal protein expression consistent with the localization of TFEB in the cytoplasm after this pharmacologic treatment.

The TFEB-dependence of lysosomal protein expression in Tsc1- and Tsc2-deficient cells was further examined by knockdown of TFEB by shTFEB to confirm whether the increase in lysosomal gene expression in Tsc1- and Tsc2-deficient cells was only TFEB-dependent or due to the parallel upregulation of another similar MiT family transcription factor (Figure 13). Consistent with our hypothesis, downregulation of TFEB appeared to decrease lysosomal protein expression in Tsc2-deficient cells, suggesting that

lysosomal protein expression in Tsc-null cells is, at least in part, TFEB-dependent. Surprisingly, protein expression of the lysosomal protease, cathepsin K, was unchanged by shTFEB knockdown. High expression of cathepsin K has been observed in LAM cases and it is hypothesized that release of cathepsin K by LAM cells could be responsible for the cystic phenotype observed in the TSC-associated disease (Chilosi et al., 2009, Dongre et al., 2017). For this reason, further studies are necessary to evaluate whether cathepsin K overexpression is TFEB-dependent. A follow-up experiment would be to examine the expression of MiT family transcription factors at the mRNA level in response to TFEB knockdown in Tsc1- and Tsc2-deficient cells to determine whether or not compensatory mechanisms are at play in these cells as a result of TFEB downregulation.

Prior studies have reported *TFEB* translocations and consequent upregulation of native TFEB protein in a subset of renal cell carcinomas (Argani et al., 2016). To confirm our *in vitro* findings of TFEB and lysosomal protein expression *in vivo*, immunohistochemistry (IHC) staining was used to characterize TFEB and NPC1 (a lysosomal protein) in human samples of TSC-associated renal cell carcinoma (Figure 17). In previous experiments (data not shown), increased expression of TFEB and NPC1 was observed in tissue samples LAM and AML from TSC patients. Consistent with these findings, we observed an increase in both TFEB and NPC1 expression in TSC-associated kidney tumors compared to normal tissue, which substantiated *in vitro* findings of upregulated TFEB in cell models of TSC. This further suggests the presence of increased lysosomal biogenesis in TSC, which could potentially be a driver in the pathogenesis of the disease.

Previous studies established mTOR as an inhibitor of TFEB nuclear localization through the direct phosphorylation of TFEB in Tsc1- and Tsc2-expressing cells (Settembre et al., 2012). However, the unexpected nuclear localization of TFEB in Tsc1- or Tsc2-deficient, mTOR hyperactivated cells drove the investigation into the phosphorylation status of TFEB. TFEB is known to be phosphorylated by mTORC1 at the S142 and S211 sites (Settembre et al., 2012). Due to a lack of specific antibodies for phosphorylated TFEB at S142 and S211 sites, we compared the migration pattern of TFEB in Tsc1-expressing and Tsc1-deficient cells to identify clues that may suggest a decrease in phosphorylation of TFEB in Tsc1- or Tsc2-deficient cells (Figure 14). Interestingly, western blot results indicated increased mobility of TFEB protein in Tsc1-deficient cells compared to Tsc1-expressing cells suggesting hypophosphorylation of Tsc1-deficient cells. This was similar to results from prior studies that examined the mobility of TFEB by western blot in both Tsc1- and Tsc2-deficient cells and observed strong expression of “fast-migrating” forms of TFEB in Tsc1- and Tsc2-deficient MEFs compared to Tsc1- and Tsc2-expressing MEFs (Peña-Llopis et al., 2011, Roczniak-Ferguson et al., 2012). However, this pattern was not observed in our experiments with Tsc1-deficient cells treated with 10% FBS or in Tsc2-deficient cells treated with both 10% and 0.1% FBS serum and was unique to Tsc1 cells treated with 0.1% serum. In Tsc2-deficient cells, it is possible that this difference in migration is not apparent because of the large difference in TFEB expression compared to Tsc2-expression. Given these results, further experiments using treatment with phosphatase and/or involving phosphoproteomic analysis are necessary to understand the phosphorylation status of TFEB.

To further investigate the mechanism of TFEB nuclear localization, calcineurin expression was examined by western blot in Tsc1- and Tsc2-deficient cells (Figure 15). Lysosomal calcium signaling is known to activate calcineurin, a calcium-dependent protein phosphatase, and subsequently dephosphorylate TFEB to promote its nuclear localization (Medina et al., 2015). Inhibition of calcineurin using siRNA and Cyclosporin A has also been shown to reduce TFEB nuclear localization and suppress lysosomal biogenesis and autophagy (Medina et al., 2015). For this reason, we hypothesized that increased activity of calcineurin could be responsible for TFEB nuclear localization through dephosphorylation. However, our data did not provide supporting evidence to this theory since no change in calcineurin protein expression was observed in Tsc1-deficient cells, and calcineurin appeared to be decreased in Tsc2-deficient cells. Since protein expression levels of calcineurin are not fully representative of its activity in the cell, this approach may not be fitting to determine whether or not calcineurin is involved in the mechanism of TFEB nuclear localization. Thus, a deeper examination into this potential mechanism is necessary before calcineurin can be ruled out.

Another established mechanism for the cytoplasmic retention of TFEB is through the binding of 14-3-3 proteins to phosphorylated TFEB at the S211 site (Roczniak-Ferguson et al., 2012). Binding of phosphorylated TFEB to 14-3-3 proteins masks the nuclear localization signal (NLS) causing its cytoplasmic retention (Roczniak-Ferguson et al., 2012). This nuclear localization signal is thought to be located near the p-S211 site from residues 241 to 252 on the TFEB protein. Binding of 14-3-3 protein to TFEB causes a conformational change that physically disrupts the NLS and blocks the signal (Xu et al.,

2019). We hypothesized that in Tsc1- and Tsc2-deficient cells, isoform-specific reductions in 14-3-3 protein levels could be another reason for the abnormal nuclear localization of TFEB in mTOR hyperactive cells. Western blot of Tsc1- and Tsc2-expressing and Tsc1- and Tsc2-deficient cells for various isoforms of 14-3-3 binding protein showed decreased expression levels of three specific isoforms of 14-3-3 binding protein: alpha-beta, zeta-delta, and tau in Tsc1- and Tsc2-deficient cells (Figure 16). The remaining 14-3-3 isoforms, 14-3-3 eta and epsilon showed no remarkable change, while protein expression of 14-3-3 gamma appeared to increase, specifically in Tsc2-deficient cells. While increased expression of 14-3-3 gamma could influence TFEB nuclear localization in these cells, this is unlikely given that increases were only visible in Tsc2-deficient cells and not Tsc1-deficient cells. These results suggest that reduced protein levels of specific isoforms of 14-3-3 binding proteins could be responsible for TFEB nuclear localization in TSC, however further experiments, including examining total 14-3-3 protein levels, are necessary to understand this mechanism further.

In order to understand the mechanism of TFEB subcellular localization, it would also be interesting to study the regulation of the nuclear export signal (NES) in TFEB. The NES is thought to be regulated by phosphorylation of TFEB at the S142 and S138 site (Li et al., 2018). Phosphorylation of TFEB at S138 by GSK3 β primes TFEB for phosphorylation at S142 by ERK or mTORC1 (Li et al., 2018). The presence of a NES in TFEB offers an alternative explanation to the nuclear mTOR theory that would explain why TFEB localizes to the cytoplasm upon mTOR inhibition. Evidence of an alteration in

the NES of TFEB in Tsc1- or Tsc2-deficient cells compared to WT would provide an explanation for this deviated pattern of TFEB intracellular localization.

Cathepsin K, a protease commonly associated with osteoclasts for bone resorption, is known to be strongly expressed in both LAM cells and renal angiomyolipomas (Chilosi et al., 2009) (Martignoni et al., 2012). Expression of cathepsin K is not normally seen in normal renal or lung tissue indicating that it could potentially be involved in the pathogenesis of LAM and TSC-associated renal diseases. Cathepsin K is typically released by cells into the extracellular matrix (ECM) through lysosomal exocytosis. In the context of TSC, we hypothesized that overexpression of cathepsin K and increased lysosomal exocytosis in LAM cells, AMLs, and TSC-associated RCC could be responsible for cystogenesis and tissue damage in these diseases. To understand the mechanism of the cystic phenotype in TSC-associated diseases, samples from renal TSC mouse models TSC2 +/- AJ and KSP-CreERT2 Tsc2^{f/f} mice were stained for NPC1 (Figure 18). IHC staining of these samples revealed positive expression of NPC1 in the apical region of cyst-lining cells of diseased kidney tissue. This suggests that there could be increased lysosomal exocytosis in these cells that leads to lung destruction and cyst formation in TSC.

Conclusion and Future Directions

The next steps in this project are to continue to explore the mechanism of TFEB nuclear localization in TSC to determine whether lysosomal exocytosis induces pulmonary cyst and renal tumor formation and to develop a therapeutic strategy to combat TSC by targeting TFEB. One possible strategy would be to decrease TFEB activity to baseline

levels similar to Tsc1- and Tsc2-expressing cells in an mTORC1 independent manner, which could potentially result in a reduction of TSC-associated symptoms. However, developing this type of therapeutic agent would require a highly targeted approach and a deeper understanding of the role of lysosomes in the pathogenesis of TSC.

The results from this study suggest that lysosomal biogenesis could play a role in the pathogenesis and progression of tuberous sclerosis complex, specifically through the induction of TFEB and upregulation of lysosomal proteins in Tsc1- and Tsc2-deficient cells. Currently, mTOR inhibitor therapy with rapalogs such Everolimus and Sirolimus is the treatment of choice in TSC. However, its effectiveness is limited in scope due to the apparent regrowth of tumors after cessation of treatment. For this reason, there remains a clinical need for lasting, targeted treatment of TSC that will not only halt the progression of the disease but also prevent its recurrence.

This study identified the potential role of TFEB in the pathogenesis of TSC through the unexpected upregulation and nuclear localization of TFEB in Tsc1- and Tsc2-deficient cells. A deeper understanding of the mechanism of TFEB induction and nuclear localization may close the gap in our knowledge of TSC and potentially identify a new biomarker for the disease. Lysosomal exocytosis is also another promising therapeutic target for TSC in the future as well. Given our knowledge of the calcium-dependent nature of lysosomal exocytosis, the use of FDA-approved calcium channel blockers to inhibit lysosomal exocytosis, such as verapamil, is another therapeutic avenue to be explored.

The findings from this study have many implications in TSC from both a scientific and clinical standpoint, including a novel understanding of the disease pathogenesis as well

as new potential biomarkers and therapeutic targets. Further study into the role of TFEB could provide valuable information into not only the development of TSC and its molecular biology, but also for other cancers and neurodegenerative disorders involving lysosomal dysregulation.

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