The role of mammalian target of rapamycin complex 1 in hepatic physiology and disease

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Abstract

The multi-component kinase mTOR complex 1 (mTORC1) coordinates nutrient and growth factor inputs with numerous downstream processes including protein translation, autophagy, metabolism and cell growth. We have found that inhibition of mTORC1 with rapamycin treatment suppressed whole-body postnatal growth similar to reduced caloric intake. We found that while feeding activated mTORC1 in almost every tissue, there was variability in the upstream activating stimuli. The role of mTORC1 in organ growth was further elucidated by studies we performed using liver-specific mTORC1 gain and loss of function mutants. Confirming our studies with rapamycin, genetic activation or suppression of mTORC1 increased and decreased liver size respectively, and rendered the liver insensitive to nutrients. Rendering the liver insensitive to nutrients also had functional consequences. In response to starvation, the liver shifts to fatty acid catabolism and generates ketone bodies to supplement lowered glucose levels. We find that constitutive activation of mTORC1 prevents the liver from initiating fatty-acid oxidation and ketone production in response to fasting. Many aspects of the hepatic fasting response malfunction in old age including fatty acid catabolism and ketogenesis. We find this aging-dependent process is mediated by mTORC1, and thus loss of mTORC1 function throughout the adult life of the animal prevents the aging induced decrease in hepatic ketogenesis. As such, pharmaceutical inhibition of mTORC1 may be beneficial in battling metabolic disorders due to aging. Pharmaceutical inhibition of mTORC1 is a potential treatment for patients suffering from tuberous sclerosis complex (TSC). The disease TSC in humans is initiated by loss of TSC1 or TSC2, which results in hyper-active mTORC1 activity. The disease can involve development of multiple lesions including brain harmatomas, angiomyolipomas (AMLs), and lymphangioleiomyomatosis (LAM). We have engineered transgenic mice that express Rheb2, an mTORC1 activator, in a doxycycline-inducible manner. Overexpression of Rheb2 led to cystic growths with characteristics of both LAM and AML. We hope this mouse model will be helpful in furthering our understanding of the pathology behind these lesions, and provide a mouse model for therapeutic intervention for TSC.

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Table of Contents

Chapter 1

Introduction

Introduction

In multicellular organisms, it is imperative that organismal growth and metabolism be continually coordinated with nutrient status. As a result, a highly complex multi-layered system has evolved by which nutritional status is sensed, communicated systemically, and buffered throughout the lifespan of an organism. The highly conserved kinase target of rapamycin (TOR) plays an essential role in coordinating cellular growth and metabolism with nutrients and energy. TOR nucleates at least two distinct complexes: complex 1 (TORC1) and complex 2 (TORC2). While insulin and growth factors activate both TORC1 and TORC2, nutrients such as amino acids and glucose activate only TORC1. Both complexes, when active, collaborate to promote pro-growth programs such as mRNA translation. Furthermore, active TORC1 suppresses insulin signaling through a negative feedback loop, while TORC2 promotes the presence of nutrient transporters at the cell surface and cell survival.

In mammals, mutations within the mTORC1 and mTORC1 signaling network result in a wide range of pathologies. Hyper-activation of mTORC1 is the defining insult in Tuberous Sclerosis Complex (TSC). TSC is characterized by benign lesions in multiple tissues that can lead to organ failure and increased mortality. Here we describe a new mouse model for angiomyolipoma and lymphangioleiomyomatosis, two lesions found in the majority of TSC patients. Analysis of the mouse model reveals a role for mast cells in the progression of these devastating lesions.

Perturbations in mTOR signaling also have a large impact on cellular and organismal growth and metabolism. We demonstrate that inhibition of mTORC1 function by treatment with rapamycin inhibits postnatal growth via the same mechanism

as dietary restriction. Rapamycin suppresses the postnatal growth of every tissue measured, and subsequent analysis finds that mTORC1 activity in numerous tissues is sensitive to feeding. Further analysis reveals tissue-dependent diversity in the upstream stimuli that activate mTORC1 upon refeeding.

To understand the role mTORC1 plays in adult organ physiology, we studied mice with both mTORC1 loss and gain of function mutations specifically in the liver. We subjected the mutant mice to fasting and refeeding regimens and examined the effect of perturbing mTORC1 signaling on the hepatic response to both conditions. In response to fasting, the liver produces glucose, ketones and ATP essential for the adaptive response of the entire organism. We find mTORC1 negatively regulates hepatic fatty acid oxidation and subsequent conversion into ketones in response to fasting. The increase in fatty acid oxidation and ketone synthesis in the liver upon fasting is mediated by the actions of the nuclear receptor peroxisome proliferators-activated receptor alpha (PPAR$\propto$). We find that mTORC1 inhibits the transcriptional ability of PPAR$\propto$. As a result, mice with an mTORC1 gain of function mutation in their livers produced dramatically reduced levels of ketones upon fasting.

In rodents, aging induces defects in the hepatic fasting response that are correlated with decreased PPAR$\propto$ transcription and ketone synthesis (Durnas et al., 1990). We find that aging suppresses PPAR$\propto$ via mTORC1. Mice with a hepatic mTORC1 loss of function mutation are protected from the aging-related decline in ketogenesis and PPAR$\propto$ activity.

The use of mouse models to probe mTORC1 biology has revealed an astonishing wide range of functions for the kinase- from disease pathology to lipid metabolism to

regulation of aging. The diversity of results described from our work underscores the need for additional research dissecting tissue-specific roles for mTORC1, and may also help clarify the pleiotropic and inconsistent affects of rapamycin use in the clinic. The following introduction focuses on mammalian TOR (mTOR) biology with emphasis on the role of mTOR Complex 1 (mTORC1) in organismal growth, metabolism and disease.

Discovery of mTOR

Cell growth (an increase in mass) takes precedence over cell proliferation. Genetic mutations that increase cell proliferation but do not promote cell growth lead to smaller and smaller cells. As a result, a cell must always sense its mass to know when to divide. This fundamental aspect of biology, the regulation and sensing of cellular mass, is modulated by the external environment and impinges upon every cellular process. The ancient kinase target of rapamycin (TOR) is a master regulator of cell size, and its relatively recent discovery underscores how little we know about the molecular processes behind the fundamental process of cell growth. Research into TOR function arose from attempts to understand the molecular mechanism behind the drug rapamycin and its anti-fungal (Singh et al., 1979) and anti-tumor properties (Eng et al., 1984). The discovery that rapamycin is also a potent immunosuppressant with low toxicity (Morris et al., 1989) accelerated the search for its cellular target. Treatment with rapamycin leads to a decrease in cell size in both yeast and mammalian cells. It also induces cell cycle arrest in yeast and in some mammalian cells. Using genetic screens in yeast and biochemical purifications in mammalian cells, rapamycin was found to bind the peptidyl-prolyl isomerase FK506 binding protein 12 (FKBP12). The resulting drug/protein complex

binds and allosterically inhibits mTOR kinase activity (Bierer et al., 1990);(Heitman et al., 1991); (Brown et al., 1994); (Sabatini et al., 1994). mTOR is a 289-kDa serine-threonine kinase and belongs to the phosphoinositide-3-kinase (PI3K)-related kinases (PIKK) family along with ATM, ATR, DNA-PK and hSMG1. All kinases have C-terminal kinase domains similar to PI3K, thus giving the family its name. While all members of the family respond to genotoxic stress, mTOR has evolved to also sense hypoxia, nutrients and growth factors.

Components of mTOR complexes

We now group mTOR into two distinct complexes called complex 1 and complex 2. Although both contain mTOR kinase, each complex has unique binding partners that allow for different activating upstream signals and different downstream substrates. In multicellular organisms, both complexes respond to growth factor and hormonal input, while only complex 1 (mTORC1) responds to the presence of nutrients such as amino acids and glucose. mTORC1 is also the direct target for inhibition by rapamycin. mTORC1 has four components in addition to mTOR: regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (mLST8, also known as GβL); proline-rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor). The functional role for each of these proteins within mTORC1 is still being actively researched. Raptor seems to regulate mTORC1 assembly and helps recruit mTORC1 substrates such as S6K (Kim et al., 2002). The role of mLST8 remains unclear, for deletion of the gene has no affect on mTORC1 activity *in vivo* (Guertin et al., 2006). PRAS40 and Deptor directly bind to mTORC1 and inhibit

kinase activity. During activating conditions, mTORC1 phosphorylates both PRAS40 and Deptor; this causes their disassociation and leads to increased mTORC1 catalytic activity (Sancak et al., 2007);(Peterson et al., 2009).

mTORC2 shares several proteins with mTORC1 such as mTOR, mLST8 and Deptor. However, mTORC2 also contains several unique components: rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), and protein observed with Rictor-1 (Protor-1). Rictor and mSIN1 help stabilize each other, and thus may provide structural integrity to mTORC2 (Frias et al., 2006; Jacinto et al., 2006). There has been no clear mTORC2 function ascribed to Protor-1 other than its interaction with Rictor (Thedieck et al., 2007). In contrast to mTORC1, mLST8 is required for mTORC2 catalytic activity *in vivo* (Guertin et al., 2006). However, similar to mTORC1, Deptor also serves as an endogenous inhibitor to mTORC2 (Peterson et al., 2009). The research results described in this thesis involve mTORC1 biology primarily, so the remainder of the introduction will focus on upstream and downstream signaling of mTORC1, as well as its role in growth, metabolism and disease.

Upstream of mTORC1

Four distinct signals are sensed by mTORC1: growth factors, energy status, oxygen and amino acids. All of these inputs, with the exception of amino acids, converge upon the Tuberous Sclerosis Complex (TSC), which is a heterodimer consisting of the proteins TSC1 (also known as hamartin) and TSC2 (also known as tuberin). The TSC1/2 heterodimer acts as a GTPase activating protein (GAP) for the small GTPase Rheb (Ras

homolog enriched in brain) (Tee et al., 2003). Rheb, bound to GTP, positively regulates mTORC1 by direct interaction, although the exact mechanism behind the interaction is unclear (Long et al., 2005; Sancak et al., 2007). As such, active TSC is an inhibitor of mTORC1 by catalyzing the conversion of Rheb-GTP to Rheb-GDP (Inoki et al., 2003).

Growth Factors

Growth factors activate mTORC1 via the canonical PI3K and Ras pathways. Stimulation of these pathways leads to phosphorylation of TSC2 by AKT (Potter et al., 2002), extracellular-signal-regulated kinase 1/2 (ERK1/2) (Ma and Blenis, 2009), and by p90 ribosomal S6 kinase 1 (RSK1) (Roux et al., 2004). All of these phosphorylations inhibit the GAP activity of TSC, thus relieving its inhibition of mTORC1. AKT is also able to activate mTORC1 independently of TSC by phosphorylating and inactiving PRAS40 (Sancak et al., 2007).

The negative feedback loop

The hormone insulin is an important physiologically activator of mTORC1. When insulin binds its cognate tyrosine kinase receptor, insulin receptor substrate 1 (IRS1) is recruited to the receptor and activates phosphoinositide 3-kinase (PI3K). PI3K activation produces phosphatidylinositol (3,4,5)-triphosphate [PtdIns(3,4,5)P_3] which recruits Protein Kinase B (PKB/AKT) to the plasma membrane leading to its activation. mTORC1 is activated by AKT via phosphorylation of TSC and PRAS40. mTORC1 also limits insulin signaling via a negative feedback loop, mediated by the mTORC1 substrate ribosomal S6 kinase 1 (S6K1). S6K1, once activated by mTORC1, can phosphorylate

IRS1, which promotes its degradation (Harrington et al., 2005). Given the role of insulin and insulin-related growth factors in metabolic disorders and cancer, this potent negative feedback loop between mTORC1 and insulin signaling has broad clinical implications. Furthermore, it seems that activated mTORC1 can suppress the activity of other growth factor receptors that do not rely on IRS1, such as platelet-derived growth factor receptor (PDGFR) (Zhang et al., 2007). While the mechanism behind this negative feedback loop is unclear, it indicates that nature has created a paradigm by which mTORC1 not only senses growth factor input, but can also limit the duration of growth factor induced cellular programs.

Energy status

Low ratio of ATP to ADP results in activation of AMP-activated protein kinase (AMPK), a master sensor of intracellular energy status (Hardie, 2007). When activated, AMPK directly phosphorylates TSC2, increasing GAP activity towards Rheb and thus inhibiting mTORC1 (Inoki et al., 2003). In addition, active AMPK can phosphorylate Raptor, thus inhibiting mTORC1 through an unknown mechanism (Gwinn et al., 2008).

Hypoxia

Hypoxia inhibits mTORC1 through multiple mechanisms. Hypoxia reduces ATP levels, thus repressing mTORC1 activity via the mechanism described above (Wouters and Koritzinsky, 2008). Hypoxia also inhibits mTORC1 through transcriptional regulation of DNA damage response 1 (REDD1) (Reiling and Hafen, 2004). REDD1 inhibits mTORC1 via TSC2. REDD1 activates TSC2 by stimulating the its dissociation

from 14-3-3 proteins (DeYoung et al., 2008). Additionally, during hypoxia, promyelocytic leukemia (PML) tumor suppressor and BCL2/adenovirus E1B 19 kDa protein-interacting protein 3 (BNIP3) reduce mTORC1 signaling by disrupting the interaction between mTOR and its positive regulator Rheb (Bernardi et al., 2006).

Amino acids

Amino acid activation of mTORC1 represents a highly conserved role for TOR found in almost all eukaryotes. In keeping with this ancient role, regulation of mTORC1 by amino acids is independent of TSC, for mTORC1 activity remains sensitive to amino acids in cells lacking TSC1 or TSC2 (Nobukuni et al., 2005). The regulation of mTORC1 by amino acids remained elusive until the identification of the role of Rag proteins in mediating amino acid sensing by mTOR (Sancak et al., 2008). The Rag proteins are four small GTPases that form heterodimeric complexes, which are present at endosomal membranes. In response to amino acids, they recruit mTORC1 to the membrane surfaces via interactions with Raptor. This localization is not only necessary for amino acid activation of mTORC1; it is also sufficient to activate mTORC1 in the absence of amino acids. While the interaction between mTORC1 and Rheb is also required for mTORC1 activation, it is unclear how amino acid deprivation leads to dissociation of mTORC1 from Rheb. This disruption between Rheb and mTORC1 due to amino acid deprivation may explain why amino acids are required for growth factor activation of mTORC1 (Sancak et al., 2008).

Other nutrients

In addition to amino acids, glucose has been shown to activate mTORC1 in the β-cells of the pancreatic islets also in an amino-dependent manner. There has also been evidence that mTORC1 may also sense lipid levels. Phosphatidic acid (PA), which can be generated either from hydrolysis of phosphatidylcholine by phospholipase D, acetylation of lysophosphatidic acid (LPA) by LPA acyltransferase (LPAAT), or phosphorylation of diacylglycerol by diacylglycerol kinase can activate mTORC1 in a TSC1/2-independent manner (Foster, 2007). While PA is a ubiquitous metabolite, there is evidence that it is a direct sensor and regulator of phospholipid metabolism and biosynthesis (Loewen et al., 2004). One could even extrapolate and hypothesize that cell size, as a function of membrane content, could be sensed by mTORC1 via PA.

Other cellular conditions

As with all PIKK family of kinases, genotoxic stress activates mTOR, although somewhat indirectly. For instance, the activation of p53 in response to DNA damage rapidly activates AMPK through an unknown process, which in turn phosphorylates and thereby activates TSC2 (Feng et al., 2005). p53 can also increase the transcription of phosphatase and tensin homolog deleted on chromosome 10 (PTEN), an inhibitor of PI3K, and TSC2 (Feng et al., 2005). Pro-inflammatory cytokines such as TNFα activate IκB kinase-β (IKKβ), which in turn physically interacts with and consequently inhibits TSC1 leading to activation of mTORC1 (Lee et al., 2007). This positive relationship between inflammation and mTORC1 activation is thought to be important in tumor angiogenesis (Lee et al., 2007) and in the development of insulin resistance (Lee et al., 2008).

Downstream actions of mTORC1

In response to the wide range of upstream inputs, mTORC1 positively regulates cell growth and proliferation by promoting many anabolic processes, including biosynthesis of proteins, lipids and organelles, and by limiting catabolic processes such as autophagy. The use of rapamycin to mimic loss of mTORC1 function has been an invaluable tool in revealing downstream mTORC1-dependent processes. However, as recent work from our lab and others have demonstrated that rapamycin does not inhibit all functions of mTORC1 (Choo AY, Blenis J; Thoreen CT), additional analysis of genetic mutants of mTORC1 is needed to obtain a full picture of mTORC1 biology.

Protein synthesis

The two best characterized substrates of mTORC1, eukaryotic initiation factor 4E (eIF4E)-binding protein 1 (4E-BP1) and p70 ribosomal S6 kinase 1 (S6K1), have important roles in the positive regulation of protein synthesis by mTORC1. The phosphorylation of 4E-BP1 by mTORC1 prevents its binding to eIF4E, enabling eIF4E to promote cap-dependent translation (Richter and Sonenberg, 2005). The stimulation of S6K1 activity by mTORC1 leads to increases in mRNA biogenesis, cap-dependent translation and elongation, and the translation of ribosomal proteins through regulation of the activity of many proteins, such as S6K1 aly/REF-like target (SKAR), programmed cell death 4 (PDCD4), eukaryotic elongation factor 2 kinase (eEF2K) and ribosomal protein S6 (Ma and Blenis, 2009). The activation of mTORC1 has also been shown to promote ribosome biogenesis by stimulating the transcription of ribosomal RNA through

a process involving the protein phosphatase 2A (PP2A) and the transcription initiation factor IA (TIF-IA) (Mayer et al., 2004).

Autophagy

When nutrient availability is limited, organelles and protein complexes are degraded en masse via autophagy. Autophagy involves the *de novo* synthesis of membrane bound vesicles, termed autophagosomes, which sequester intra-cellular components and deliver them to lysosomes via membrane fusion. Lysosomal degradation of these components liberates biological material to sustain macromolecule biosynthesis and energy production. The inhibition of mTORC1 triggers autophagy, whereas stimulation of mTORC1 reduces this process (Codogno and Meijer, 2005). mTORC1 represses autophagy by phosphorylating multiple components of a protein complex composed of unc-51-like kinase 1 (ULK1), autophagy-related gene 13 (ATG13) and focal adhesion kinase family-interacting protein of 200 kDa (FIP200) (Ganley et al., 2009).

Lipid synthesis

Cell growth and proliferation must be coordinated with lipid and phospholipids biosynthesis. As such, one would expect mTORC1 to regulate these processes as a master regulator of cell growth. However, the role of mTORC1 in lipid metabolism and synthesis has only recently been appreciated. It has been demonstrated that AKT-dependent lipogenesis requires that mTORC1 positively regulates the activity of sterol regulatory element binding protein 1 (SREBP1). Silencing of dSREBP1 in flies results in

reduced cell and organ size which is refractory to dPI3K-induced growth (Porstmann et al., 2008).

mTORC1 inhibits 3T3-L1 preadipocyte differentiation by suppressing the expression and transactivation activity of of peroxisome proliferator-activated receptor γ (PPARγ)- the master regulator of adipogenesis (Kim and Chen, 2004). Kim and Chen found that PPARγ activity is dependent on amino acid sufficiency. This result is consistent with the role of mTORC1 coordinating multiple nutrient inputs with cell growth. Additionally, rapamycin reduces the phosphorylation of lipin-1 (Huffman et al., 2002), a phosphatidic acid (PA) phosphatase that is involved in glycerolipid synthesis and in the coactivation of many transcription factors linked to lipid metabolism, including PPAR∝, PPARγ and PGC1∝. Whether lipin-1 is a direct substrate of mTORC1, and whether it is involved both upstream of mTORC1 through PA production and downstream of mTORC1 in mediating transcription factors is still unclear.

Mitochondrial metabolism and biogenesis

Given that multiple oxidative pathways and the bulk of cellular ATP synthesis occurs in the mitochondria, the discovery that mTORC1 regulates mitochondrial metabolism and biogenesis is not surprising. Inhibition of mTORC1 by rapamycin lowers mitochondrial membrane potential, oxygen consumption and cellular ATP levels, and profoundly alters the mitochondrial phosphoproteome (Schieke et al., 2006). Conversely, hyper-activation of mTORC1 results in increased mitochondrial DNA copy number, as well as the expression of many genes encoding proteins involved in oxidative metabolism (Cunningham et al., 2007). Additionally, conditional deletion of Raptor in

mouse skeletal muscle reduces the expression of genes involved in mitochondrial biogenesis (Bentzinger et al., 2008). Cunningham and colleagues have discovered that mTORC1 controls the transcriptional activity of PPARγ coactivator 1 (PGC1∝), a nuclear cofactor that plays a key role in mitochondrial biogenesis and oxidative metabolism, by directly altering its physical interaction with another transcription factor, namely yin-yang 1 (YY1) (Cunningham et al., 2007).

mTORC1 in the clinic

The mTORC1 inhibitor rapamycin is used successfully in the clinic as a immunosuppressant, and in preventing restenosis following angioplasty. Because, mTORC1 resides within a signaling network littered with tumor suppressors and oncogenes, there has been considerable anticipation for rapamycin as part of anti-tumor therapy. Furthermore, as mTORC1 plays a key role in insulin signaling, rapamycin is also a potential therapeutic for metabolic disorders. Outlined below is a brief description of the uses of rapamycin in these various disorders, and what can be gained from its successes and failures.

Rapamycin as an immunosuppressant

mTOR inhibition by rapamycin has shown promise as a therapeutic strategy for the prevention of transplant rejection and the treatment of autoimmune disease. In renal transplantation, rapamycin is a powerful anti-rejection agent when used in combination with other immunosuppressive agents. Not only does rapamycin have unique anti-atherogenic and anti-neoplastic properties that distinguish it from other anti-rejection

drugs, but it also can promote tolerance and decrease the incidence of chronic allograft nephropathy (Augustine et al., 2007).

The success of rapamycin treatment of transplant patients is due to selective yet broad inhibition of numerous types of immune cells. For example, rapamycin inhibits differentiation of DCs and helps promote their apoptosis (Woltman et al., 2001). Dendritic cells (DCs) are phagocytic cells that reside in almost all tissues. DCs degrade pathogens, release cytokines and activate T-cells. Therefore, rapamycin inhibition of DCs leads to decreased secretion of cytokines and the inability to activate T-cells through presentation of antigens. Rapamycin is also able to interfere with endocytosis in macrophages and DCs, thus interfering with their ability to take up antigens (Hackstein et al., 2002).

In rodents, inhibition of mTOR by rapamycin causes involution of the thymus due to decreased T-cell proliferation (Song et al., 2007). While this decreases overall T-cell output, it does not affect production of T-regulatory cells. This is crucial, for T regulator cells suppress both autoimmune and excessive inflammatory responses. This particular consequence of rapamycin allows for potent immunosuppression without sacrifice of immune surveillance against self-antigens and triggering of excessive inflammation (Sakaguchi et al., 2008). In addition to effector T cells and APCs, rapamycin also inhibits proliferation and/or function of B-cells, natural killer cells, neutrophils, and mast cells. The effect of rapamycin on multiple yet select immune cells allows for broad immunosuppression with limited side effects.

mTOR and cancer

Given its anti-proliferative effects in yeast, there was excitement over the potential of rapamycin as an anti-tumor drug. With the discovery of mTORC1 and its placement downstream of AKT, the excitement increased; many cancers have genetic mutations that upregulate PI3K signaling, and therefore then depend upon activation of the oncoprotein AKT. With mTORC1 activity being regulated by AKT, it was predicted that rapamycin inhibition of mTORC1 would attenuate some of the growth and proliferative promoting actions of AKT. Indeed the use of rapamycin and its analogues has had modest successes in the treatment of mantle cell lymphoma, endometrial cancer, and renal cell carcinoma (Faivre et al., 2006). However, the therapeutic response to rapamycin is highly variable, suggesting that biomarkers capable of predicting cellular responses to rapamycin are needed.

The variable response of tumors to rapamycin has engendered a debate on whether mTORC1 activation or inhibition is more beneficial to cancer cells. The tumor microenvironment is commonly low on nutrients, energy, and oxygen, so cells insensitive to these stresses may have a growth advantage. But does this imply constitutive inactivation or activation of mTORC1? Inactive mTORC1 signaling may initially restrict the growth of transformed cells in a suboptimal environment. Slower growth may allow these cells to acquire other mutations and/or await angiogenesis. Indeed, in poor nutrient conditions, hyper-activation of mTORC1 signaling could lead to unrestricted growth in, causing cells to deplete their energy stores and induce apoptosis. Consistent with this idea, TSC2-deficient cells undergo apoptosis in glucose-free medium, a response suppressed by rapamycin (Inoki et al., 2003). Inhibition of mTORC1 may also help

tumor cells cope with nutrient deprivation by triggering autophagy, thus allowing the cell to obtain nutrients autonomously (Lee et al., 2009). This line of evidence would indicate that inhibition of mTORC1 would actually help tumorigenesis.

The discovery that prolonged exposure to rapamycin decreases AKT S473 phosphorylation in a subset of cancer cells (Sarbassov et al., 2006) changes our interpretation of rapamycin treatment in anti-tumor therapy. In this case, rapamycin blocks the assembly of mTORC2, which in conjuction with phosphoinositide-dependent protein kinase 1 (PDK1) phosphorylates AKT (Sarbassov et al., 2006). However, the mechanism behind why certain cells display this secondary sensitivity to rapamycin and predictive biomarkers for this effect are not known. Regardless, this finding brings up the possibility that the successful clinical responses to rapamycin may reflect the dual inhibition of mTORC1 and mTORC2.

Rapamycin in the treatment of TSC

While the role of mTOR in cancer remains murky, the role of mTORC1 is much clearer in the case of patients with Tuberous Sclerosis Complex (TSC). TSC is an autosomal dominant genetic disease with an incidence of 1/6000 at birth, and is characterized by benign tumors (hamartomas and hamartias) involving multiple organ systems (Kwiatkowski and Manning, 2005). While TSC as a syndrome was described over a century ago, the relatively recent discovery that patients with TSC have inactivating mutations in either TSC1 or TSC2 was a major step forward towards elucidating the mechanism of the disease. There are several hamartoma syndromes that share pathological features with TSC, including Cowden Disease, Peutz-Jeghers

Syndrome, neurofibromatosis and Birt-Hogg-Dube Syndrome. Like TSC1 and TSC2, the tumor suppressor genes linked to these diseases (PTEN, LKB1, NF1 and FLCN respectively) encode proteins that restrict mTORC1 signaling (Corradetti et al., 2004; Shaw et al., 2004). In addition to harmatomas, patients with TSC also develop angiomyolipomas (AMLs) and lymphangioleiomyomatosis (LAM). Unlike harmatomas, both lesions can be highly proliferative with AMLs consisting primarily of smooth muscle cells, fibrous tissue, adipose tissue, and abnormally formed vascular channels (Gomez, 1999). While the prognosis of patients with AMLs can be encouraging given the ability of recurring surgeries to manage the lesions, the prognosis of patients with LAM can be grim with often relentless disease progression. LAM is an infiltrative, proliferative lung lesion composed of aberrant smooth muscle and epithelioid cells with cyst formation and destruction of normal airways (Moss et al., 2001).

The results from clinical trials treating TSC patients with rapamycin are variable. One trial, five of five TSC patients had significant regression of cerebral astrocytomas using an oral rapamycin analogue (Franz et al., 2006). However, rapamycin treatment of 18 TSC patients with renal AMLs only reduced AML volume by a modest 30%, with 17 of 18 patients experiencing tumor regrowth upon termination of the trial. Whether this reduction in AML volume is sufficient to reduce secondary complications and related organ failure is unclear (Bissler et al., 2008). Two independent rapamycin therapy trials of patients with LAM return contradictory results. One trial found a significant improvement in lung function in 70% of patients (Bissler et al., 2008), whereas another independent trial with LAM patients found mild to moderate deterioration of lung function upon rapamycin treatment (Davies et al., 2008). These conflicting results

underscores the need to develop accurate *in vivo* models of TSC in order to learn more about these independent lesions and the roles mTORC1 hyperactivation play in their pathology.

mTORC1 in growth and metabolism

With the ability to respond to numerous growth programs and to sense nutrients and energy, it is expected that mTORC1 plays a fundamental role in cellular, organ and organismal metabolism and growth. In multi-cellular organisms, energy is stored asymmetrically in different tissues, which in turn have different metabolic needs. As a result, mTORC1 will most likely serve unique functions in each tissue; moreover, perturbations of mTORC1 in one tissue could have non-autonomous metabolic consequences. Fortunately, regulation of growth in mammals and insects is well conserved. The TOR field has benefited greatly from genetic analysis of the pathway in flies to inform parallel experiments in mice. Both use insulin and insulin-like growth factors to control rates of cell growth, nutrient use, cell size and body size. Furthermore, steroid hormone–nuclear receptor signaling regulates transitions between life-stages and growth in both. Below is a review of insect and animal models of mTORC1 mutants and their resulting growth and metabolic phenotypes.

TOR and Drosophila

In Drosophila, organismal growth occurs during larval development and is affected by nutrition, temperature and the genetic program. For example, withdrawal of

protein during larval development results in smaller flies (Britton and Edgar, 1998). Larval growth is regulated by an endocrine system based on humoral peptides similar to glucagon (adipokinetic hormone) and insulin (insulin-like peptides (ILPs)) that allows the insect to coordinate rates of cell growth with changes in diet. Similar to mammals, ILPs bind to membrane bound tyrosine kinase receptors and trigger PI3K /AKT/dTOR activation, which, together, compose the insulin/insulin-like growth factor signaling (IIS) system. In the adult fly, in addition to promoting glucose transport and nutrient storage, the IIS system affects feeding behavior, lifespan and reproduction.

Genetic experiments have demonstrated that the IIS system is not only essential for cell and organ growth, but also is sufficient to autonomously increase the growth rate of essentially any cell type (Britton et al., 2002). For example, flies lacking the function of Chico (homologue to IRS-1) are smaller due to decreased cell size and number (Bohni et al., 1999); conversely, flies lacking PTEN have larger cells and enhanced growth (Goberdhan et al., 1999). This pathway is sensitive to nutrient state; starvation represses expression of several ILPs, depletes the second messenger PIP3 and inhibits the activity of the TOR target S6K (Oldham et al., 2000). During larval development, genetically activating IIS or dTOR signaling by overexpression of p110 or Rheb can bypass the known cellular effects of starvation, including the arrest of cell growth and DNA replication in the larval-specific tissues (Saucedo et al., 2003), and the induction of autophagy in the fat body (Scott et al., 2004). These observations indicate that IIS and dTOR activity are suppressed in the larval-specific tissues by starvation, and that this systemically mediates a set of cellular responses that allow insect larvae to cope with fluctuating nutritional conditions.

Nonautonomous dTOR signaling

In flies and mammals, TOR responds to PI3K/AKT activation and amino acid input. However, withdrawal of amino acids does not downregulate PI3K or AKT activities in culture, suggesting that this pathway does not directly respond to nutrient shortage (Hara et al., 2002). Therefore, the question remains of how nutrient availability is linked to humoral and cellular mechanisms that modulate PI3K/AKT activity. To address this question, a recent study tested whether a specific organ, the larval fat body (FB), can function as a nutrient sensor and induce nonautonomous modulation of insulin/IGF growth signaling in peripheral tissues in response to changes in nutrient levels. Analagous to a combination of the vertebrate liver and adipose tissue, the FB during larval stages accumulates stores of proteins, lipids, and carbohydrate, which can then be remobilized to compensate for transitory nutrient shortages. In addition to its storage function, the FB possesses endocrine activity (Britton and Edgar, 1998).

Colombani et al. found that downregulation of an amino-acid transporter (slimfast) in just the fat body reduced the size of the FB and induced a general reduction in the rate of larval growth. The reduction in larval growth correlated to reduced PI3K signaling in peripheral tissues. This effect is specific to the FB, for inhibition of this transporter in other tissues of the fly did not exert this non-autonomous effect (Colombani et al., 2003). Furthermore, inhibition of PI3K activity, which reduces the size of the FB, was not sufficient to phenocopy this result. Importantly, inhibition of dTOR signaling in just the FB by either expression of a dominant negative TOR allele, or overexpression of dTSC1 or dTSC2 did phenocopy the developmental growth reduction

of the larvae seen in the slimfast mutant. To confirm that the phenotype of the slimfast mutant was due to dTOR inhibition, they found that overexpression of dS6K partially rescued the slimfast mutant phenotype. These data indicate that dTOR in the FB senses amino acid levels and can communicate this nutrient signal to the rest of the body by regulating the endocrine function of the FB. This is the strongest evidence to date of a non-autonomous role for TOR in the regulation of organismal growth, although whether this role is conserved in mammals has yet to be determined.

The discovery of new downstream effects of TOR signaling raises the question of which TOR substrate mediates these various downstream pathways. Elegant genetic studies in yeast, flies and mice have allowed researchers to conclude that S6K and 4E-BP1 do not mediate all of the growth-promoting actions of TOR and that there must be additional substrates. For example, whereas homozygote deletion of dTOR in flies results in delayed larval growth with eventual growth arrest at 24% of the mass of wild-type controls, overexpression of dS6K on a dTOR null background only partially rescues larval growth to the pupal stage (Zhang et al., 2000). Consistent with this result, the reduction in growth in dS6K mutant flies is significantly less than in dTOR mutants (Montagne et al., 1999). Taken with the observation that flies lacking d4E-BP have no cell growth changes (Junger et al., 2003), these results raise the distinct possibility that TOR probably has other downstream effectors. Genetic screens in yeast and flies have already begun to uncover new candidates, and further research is needed to establish these new proteins in the TOR signaling network.

mTOR in mice

In mammals, the insulin/IGF-1 and PI3K signaling networks are involved in numerous processes ranging from embryogenesis, postnatal growth, the immune response and aging. As such, derangements in these signaling pathways can result in multiple pathologies including obesity, diabetes, the metabolic syndrome, atherosclerosis and cancer. With the discovery of mTOR and its placement downstream of insulin and PI3K signaling, many researchers have re-examined insulin and PI3K dependent phenotypes in mice and studied the role of mTOR in the manifestation of these phenotypes. In addition, the ability of mTOR to sense nutrients directly positions the kinase to regulate numerous physiological conditions that are sensitive to nutrient fluctuations. Given the wide breadth of biology that could be affected by mTOR activity, the need to study the role of mTOR signaling in the intact animal is pressing. However, much of the data gleaned from such studies is correlative, for genetic proof is difficult to attain given that many of the germ-line deletions of mTOR components are embryonic lethal (Guertin et al., 2006). Furthermore, genetic studies in mice are complicated due to the presence of multiple isoforms for many of the mTOR components that may be able to compensate for deletion of one isoform. As a result, the generation of conditional knockout mice for the various mTOR signaling components provides a valuable tool for the study of temporal and spatial mTORC1 signaling in the postnatal animal. Below I discuss both correlative evidence of mTORC1 signaling in physiological conditions related to growth and metabolism and the data gained from both germ-line and conditional knockout studies of mTORC1 signaling components.

The role of mTORC1 in embryonic development

A low nutrient environment during prenatal development can cause profound developmental and postnatal growth defects. Nutrient deprivation early in development can lead to preferential nutrient consumption by the brain at the expense of other tissues resulting in disproportionality of the fetus. Restriction of nutrients later in development elicits alternations to fetal development such as decreased pancreative beta-cell mass and decreased newborn size (Schwitzgebel et al., 2009). As expected, mTOR is essential to development; deletion of mTOR is embryonic lethal at or around implantation (Gangloff et al., 2004). Explanted mTOR null blastocysts appear normal, but the inner cell mass (ICM) and trophoblast cells failed to expand in culture. However, given that mTOR is contained in at least two distinct complexes, each with different downstream effectors, it is difficult to interpret which branch is responsible for the implantation defect and early embryonic lethality. To test the role of mTORC1 specifically in embryogenesis, Raptor, a necessary component of mTORC1, was deleted. Although they are able to implant, Raptor null embryos fail to expand and differentiate, dying shortly afterwards. Consistent with this result, the ICM and trophoblast cells from explanted Raptor null embryos expand only briefly before detaching and dying (Guertin et al., 2006). Surprisingly, mLST8 null embryos do not phenocopy the mTOR or Raptor null embryo. mLST8 was later found to not be required for the actions of mTORC1 *in vivo* (Guertin et al., 2006); it is required, however, for *in vivo* mTORC2 function (Guertin et al., 2006).

S6K1 knockout mice are viable

While studies with mTOR and Raptor knockouts confirm the essential role for these proteins in embryogenesis, mice null for the mTORC1 substrates S6K and 4E-BP are viable, thus allowing for the study of the role of mTORC1 signaling in the adult

animal. In 1998, the Kozma and Thomas lab published a report describing the S6K1 knockout mouse (Shima et al., 1998). In keeping with the role of mTORC1 in cell size, S6K1 null embryos are smaller *in utero* due to a decrease in cell size, but there are no alterations in cell proliferation. As a result, null mice are born ~20% smaller than their wild-type littermates. Mutant mice continue grow at a slower pace through the first 5-6 weeks of postnatal development. At 11 weeks, mutant mice continue to about 15% smaller than wild-type controls (Shima et al., 1998), with all organs proportional to body size. This growth phenotype confirms that mTORC1 plays a major role in both prenatal and postnatal growth. Whether the control of growth is cell autonomous, non-autonomous, or a combination of the two has yet to be determined.

Given the role of S6K in regulation protein translation and ribosome biogenesis, it was quite a surprise when the researchers found no apparent defect in translation of mRNAs modified by a 5' terminal oligopyrimidine tract (TOP) or in S6 phosphorylation in S6K1 null mice. This unexpected result led directly to the discovery of a second S6 kinase, S6K2 that can compensate for S6K1 in respect to S6 phosphorylation and 5' TOP mRNA translation. However, S6K2 did not compensate for the small size phenotype, implying that the size control exerted by mTORC1 via S6K is through a mechanism separate from S6 phosphorylation (Shima et al., 1998). S6K2 knockout mice were later generated and actually were larger in size than control mice, and S6K2 null cells exerted no proliferation or translational defects. The combined deletion of both S6K1 and S6K2 resulted in perinatal lethality (Pende et al., 2004).

As the S6K1 null mice were further characterized, an aspect of mTORC1 signaling arose which confounds analysis of the pathway in the intact animal. As stated

above, mTORC1 is activated by multiple inputs, such as growth factors, insulin, energy status, oxygen and nutrients. In a process that is not fully understood, mTORC1 phosphorylation of S6K1 not only mediates growth, but also limits growth factor signaling via a negative feedback loop. As a result, a mutation that activates or inactivates mTORC1 activity towards S6K can push insulin signaling in the opposite direction. For example, the loss of S6K1 abrogates the negative feedback loop to insulin signaling, thus making tissues hypersensitive to insulin input. This aspect of mTORC1 signaling is evident in the fact that the S6K1 null mice are not only hypoinsulinemic due to decreased β-cell mass, but are also hypersensitive to insulin signaling (Pende et al., 2000). Moreover, β-cells in mutant mice are disproportionately smaller than other pancreatic endocrine cells as well as other cells in peripheral tissues. The decrease in surface area of the β-cells decreases insulin secretory potential. While mTORC1 regulates β-cell size, it does not seem to impinge upon the ability of β-cells to sense glucose and secrete insulin accordingly (Pende et al., 2000). Whether the decrease in growth rate in S6K1 null mice results from decreased circulating insulin, decreased mTORC1 signaling in tissues, or a combination of the two remains to be determined.

This combination of decreased mTORC1 signaling, decreased insulin levels and hypersensitivity to insulin allows S6K1 null mice to also be resistant to diet and age-induced obesity (Um et al., 2004). At the termination of postnatal growth, whole-body metabolism shifts from a growth-promoting paradigm to an energy storage mode where excess carbohydrates are stored as glycogen and excess fatty acids are stored as triglycerides in adipose tissue. Unlike their wild-type counterparts, adult S6K1 null mice did not exhibit fat storage as they aged. Dissection of mutant mice revealed marked

reduction in both white and brown adipose tissue. Loss of fat is normally associated with the starvation response; however, S6K1 null mice did not eat less, nor did they exhibit other hallmarks of a starvation response, lowered plasma glucose and increased plasma ketone levels. Fat accumulation is dependent on a balance between insulin-stimulated triglyceride accumulation and epinephrine/glucagon induced lipolysis. Compared to age-matched controls, Um et al. found a five-fold increase in the rate of fat lipolysis as well as an increase in metabolic rate as measured by oxygen consumption. This result was independent of epinephrine action. One would expect that increased lipolysis would then translate into increased presence of free fatty acids in the blood. However, S6K1 null mice exhibited comparable levels of plasma free fatty acids and triglycerides to wild-type controls, thus suggesting increased oxidation of fatty acids in mutant animals. Loss of S6K1 converted white adipose tissue into an energy-consuming tissue by increasing mitochondria number and expression of uncoupling protein 1 (UCP1), carnitine palmitoyltransferase 1 (Cpt1) and peroxisome proliferator-activated receptor gamma coactivator 1 (PGC1$\propto$). This change in gene expression also occurred in S6K1 deficient skeletal muscle leading to marked increase in fatty-acid β-oxidation. The increase in oxidative capacity of S6K1 deficient skeletal muscle and white adipose tissue allows mutant mice to resist age and diet-induced obesity (Um et al., 2004).

The role of 4EBPs in mice

This defect in fat accumulation found in S6K1 null mice is reversed in mice deficient for the other well-characterized mTORC1 substrate 4EBP. In mammals, there are three 4EBPs, and phosphorylation by mTORC1 results in decreased binding with and

therefore activation of eukaryotic translation initiation factor 4F. As a result, constitutive activation of eIF4E by deletion of one of multiple 4EBPs mimics a gain of function mutation in regards to mTORC1 signaling. Mice have been generated with either deletion of 4EBP1 alone or in combination with deletion of 4EBP2 (Blackshear et al., 1997; Le Bacquer et al., 2007). Mice lacking 4EBP1 have no obvious abnormalities, possibly indicating compensation by the other 4EBPs. Mutant mice are not larger at birth, develop normally, and have no change in appetite or in lifespan (Tsukiyama-Kohara et al., 2001). However, despite normal plasma insulin levels and presence of glucose transporters, mice are hypoglycemic. As mutant mice developed into adults, both males and females were found to weigh less than age-matched wild-type mice. This difference in weight was not due to changes in organ size, but rather because of a reduction white adipose tissue weight (Tsukiyama-Kohara et al., 2001). While both males and female displayed this phenotype, only males were found to have a decreased metabolic rate as measured by oxygen consumption and CO_2 respiration. Further examination of white adipose tissue in 4EBP1 knockout mice revealed a strikingly similar result found in S6K1 knockout mice: increased levels of PGC1$\propto$ and UCP1, factors converting white adipose tissue into an energy consuming tissue more closely related to brown fat. Whereas there was a transcriptional change in the S6K1 knockout mice, the increased amount of these proteins in the 4EBP1 null mice was due to increased translation (Tsukiyama-Kohara et al., 2001).

These results in white adipose tissue are somewhat paradoxical, for it implies that mTORC1 activation through its downstream substrates S6K1 and 4EBP1 simultaneously represses transcription of oxidative genes while also increasing their translation. Whether

these results reflect a true physiological role for mTORC1 is unclear due to the presence of multiple S6Ks and 4EBPs. To clarify the role of 4EBPs in white adipose tissue, the Sonenberg lab created mice lacking both 4EBP1 and 4EBP2 in the entire body (Le Bacquer et al., 2007). Again, mice were viable, and developed normally, but in contrast to the single 4EBP1 knockout mouse, adult double KO mice gained ~30% more weight under normally fed conditions over the course of 16 weeks (Le Bacquer et al., 2007). This weight gain was primarily a result of increased adipose accumulation. The increase in fat was accompanied by a rise in plasma insulin and cholesterol levels, thus establishing the 4EBP1/2 double KO mouse as a model for the development of obesity. Double KO mice challenged with a high fat diet also gained more weight than control mice under the same diet. The gain in weight was due again to increased presence of fat as well as a greater accumulation of triglycerides in the liver. The increased deposition of fat was due to decreased lipolysis and increased re-esterification of free fatty acids (Le Bacquer et al., 2007). Repression of 4EBP1 and 2 also leads to insulin resistance. Loss of these mTORC1 substrates led to increased phosphorylation of S6K1 by mTORC1, indicating that the 4EBPs and S6Ks compete for catalytic sites in mTORC1. The increased phosphorylation of S6K in 4EBP1/2 null tissues then suppressed insulin signaling through the negative feedback loop discussed earlier. The observations made with these mice mutant for mTORC1 substrates reveal important roles for mTORC1 in key metabolic tissues, and underscore the physiological importance of the negative feedback loop between S6K and insulin and growth factor signaling. However, given the pleiotropic consequence of manipulating fat stores and insulin sensitivity, further

clarification of the physiological roles of mTORC1 will benefit from tissue-specific deletions.

Tissue specific mTORC1 mouse mutants

Tissue-specific genetic mutations in the mTORC1 signaling pathway allow for analysis of tissue-specific roles for mTORC1 as well as possible non-autonomous roles for the signaling pathway. Given the phenotype of S6K1 null β-cells, researchers have examined the consequence of manipulating mTORC1 specifically in the β-cells. Deletion of TSC2 specifically in the β-cells of pancreatic islets result in increased β-cell size and an increase in proliferation. Accordingly, circulating levels of insulin are higher, and consequently blood glucose levels are lower (Rachdi et al., 2008). However, mutant mice exhibit normal body weight and postnatal growth compared to wild-type controls, indicating that such elevated insulin levels in the mutant animals were unable to augment postnatal growth. The mice did possess improved glucose tolerance due, presumably, to increased insulin secretion upon glucose injection (Rachdi et al., 2008). However, the authors were unable to determine whether the mechanism of improved glucose tolerance was only due to increased insulin secretion and not to non-autonomous effects of mTORC1 hyperactive signaling in the β-cells. Although, constitutive mTORC1 signaling led to a sustained suppression of PI3K signaling via the negative feedback loop, there was no deleterious effect within the β-cell arising from decreased PI3K signaling. Indeed, the truly surprising result from these mice was the non-oncogenic increase in β-cell proliferation. Given that patients suffering from any form of diabetes could benefit

from increased capacity to secrete insulin, the knowledge of a pathway that can increase β-cell number and size without forming a tumor is quite promising.

As mentioned above, both the S6K1 and 4EBP mutant mice exhibited significant changes in fat and muscle physiology. Indeed, a role for mTORC1 in fat cell physiology was first suggested by results showing that rapamycin was required for differentiation and maintenance of adipocytes in culture (Bell et al., 2000). Moreover, mouse models for obesity have found increased mTORC1 signaling in white adipose tissue. Such increased mTORC1 signaling led to sustained suppression of insulin signaling and resulted in insulin resistance within the fat (Zheng et al., 2009). To study mTORC1 *in vivo*, a white adipose tissue specific knockout of Raptor, an essential component of mTORC1, was engineered (Polak et al., 2008). Fat-specific Raptor knockout confirms many of the S6K1 null phenotypes. The fat-specific Raptor null mice are lean and are resistant to diet-induced obesity. Loss of raptor led to decreased adipocyte size and number. Similar to the S6K1 knockout mouse, the decrease in fat storage upon loss of Raptor was due to increased oxidative metabolism within the white adipose tissue driven by increased expression of UCP1 (Polak et al., 2008). Despite losing Raptor only in white adipose tissue, there were significant changes to whole body metabolism. Mutant mice had lower circulating levels of insulin, but were more insulin sensitive than wild-type controls resulting in lowered blood glucose and cholesterol levels (Polak et al., 2008). This is surprising, for insulin sensitivity, as measured by glucose tolerance test, is primarily a function of glucose clearance by the muscle and the liver. The authors, perplexed by this change in whole body metabolism, examined insulin-stimulated AKT phosphorylation in peripheral tissues. Comparing control and mutant mice on normal

chow, they found that insulin injection stimulated AKT phosphorylation equally in white adipose tissue and liver, but led to higher levels of phospho-AKT in the muscle of mutant animals (Polak et al., 2008). Hypersensitivity to insulin in the muscle would explain the improved glucose tolerance in the fat-specific Raptor knockout mice. However, it remains unclear how loss of mTORC1 signaling in the fat leads to increased insulin sensitivity in the muscle. Nonetheless, this result may indicate a non-autonomous role for mTORC1 signaling within the fat similar to results found in Drosophila (Colombani et al., 2003). Loss of Raptor in the fat also changed whole body metabolism in response to a high-fat diet. As expected in the Raptor mutant mice, fat deposition was countered by increased oxidative capacity (Polak et al., 2008). Moreover, Raptor mutant mice, as with whole body S6K1 KO mice, continued to be more insulin sensitive under a high fat diet (Polak et al., 2008). Confirming studies performed in obese mice, Polak et al. found hyper-phosphorylation of S6K and decreased AKT phosphorylation in the fat of control mice under a high fat diet due to the negative feedback loop between S6K1 and IRS1. Loss of Raptor in white adipose tissue eliminated the negative feedback loop, and as a result, high-fat diet did not lead to hyper-phosphorylation of S6K1 or to decreased AKT phosphorylation. This could partly explain the increased insulin sensitivity in fat-specific Raptor null mice under a high-fat diet. In summary, nutrient sensitive mTORC1 is required for the important energy storage functions of white adipose tissue; loss of mTORC1 function engenders a brown fat-like phenotype with increased UCP1 expression and fatty acid oxidation. Loss of mTORC1 signaling in white adipose tissue leads to increased insulin sensitivity in muscle and prevents onset of insulin resistance within the adipose tissue on a high-fat diet.

mTORC1 and aging

As more and more studies of mTORC1 within the intact animal are published, a common theme begins to emerge regarding the physiological role for mTORC1: regulation of oxidative metabolism. Loss of mTORC1 function in fat results in increased oxidative capacity due to changes in oxidative gene expression and mitochondria number. Conversely, gain of mTORC1 function due to deletion of 4EBP1 and 2 leads to decreased fatty acid oxidation and sensitivity to diet-induced obesity. In contrast to loss of Raptor in white adipose tissue, loss of Raptor in muscle shows the opposite phenotype: a decrease in oxidative capacity and mitochondrial gene expression (Bentzinger et al., 2008). This latter result is similar to a report where rapamycin treatment or Raptor knockdown in muscle cells and mouse embryonic fibroblasts (MEFs) leads to decreased oxygen consumption and mitochondrial gene expression (Cunningham et al., 2007). Taken together, these results suggest that mTORC1 controls oxidative metabolism and mitochondrial respiration either positively or negatively depending on tissue. Much more work is needed, especially examining mTORC1 function in specific tissues. Given the dramatic metabolic changes caused by manipulation of mTORC1 in fat, muscle and islet cells, it will be interesting to see how mTORC1 signaling impinges upon metabolism of the liver.

Oxidative metabolism and mitochondrial metabolism are important in the adaptive metabolic response to fasting and caloric restriction (CR). Increased mitochondrial activity and number is also at the center of the mechanism by which CR extends lifespan in multiple organisms (Guarente, 2008). CR also inhibits TOR signaling

in multiple organisms and has been shown to extend lifespan in yeast, flies, worms and mice (Kaeberlein et al., 2005; Selman et al., 2009; Vellai et al., 2003). Increased mitochondrial respiration in yeast and mammals is correlated with increased sirtuin levels and activity (Lin et al., 2004). The anti-aging proteins, sirtuins, play a major role in the control of lifespan in response to CR. SIRT1 has also been shown to deacetylate PGC-1∝, thereby increasing its ability to stimulate transcription of nuclear genes encoding mitochondrial proteins as well as mitochondria number (Gerhart-Hines et al., 2007).

As described above, mTORC1 also regulates the levels and activity of PGC-1∝. Whether this marks a convergence point between SIRT and mTORC1 in regulating energy balance and mitochondrial respiration has yet to be determined. However, recent reports have established mTORC1 as an important mediator in extending lifespan in response to CR. The S6K1 full body knockout mouse lives longer and is resistant to age-related pathologies such as bone, immune and motor dysfunction as well as loss of insulin sensitivity (Selman et al., 2009). Interestingly, only female mutant mice exhibited this longevity phenotype, although both males and females had improved metabolic parameters (Selman et al., 2009). Gene expression profiles of liver from fed mutant animals were similar to profiles from livers of wild-type mice subjected to long-term CR. Both profiles exhibited expression changes for genes involved in PPARγ, PGC-1∝ and SIRT1 signaling (Selman et al., 2009). This result is a genetic confirmation of another recent report describing the ability of oral rapamycin treatment to extend lifespan in both male and female mice (Harrison et al., 2009).

Conclusion

In summary, the mTOR signaling network coordinates numerous cellular growth processes with nutrients, energy, oxygen and humoral growth factors. The mTOR field has been driven by advances made in yeast, fly and worm genetics as well as genetic and biochemical studies performed mammalian cells. The last ten years has also seen a plethora of mammalian *in vivo* studies illuminating important autonomous and non-autonomous physiological roles for mTORC1. The challenge now is to delve deeper into mechanistic studies to understand how tissue-specific proteins impinge upon mTORC1 function. Recent exciting studies have outlined how pharmaceutical modulation of mTORC1 can have profound impacts on tumor therapy, metabolic disorders and even aging. However, many of these same studies have also demonstrated very puzzling results that underscore how much more there is to learn about mTORC1 biology.

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Chapter 2

mTOR Complex 1 regulates hepatic ketogenesis in response to fasting and aging

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M.L. assisted with hepatic metabolite measurements, and animal dissections
S.O. assisted with mouse dissections and husbandry

Summary

In fasted animals the liver performs numerous functions that maintain whole-body nutrient homeostasis, including the production of ketone bodies for peripheral tissues to use as energy sources. Aged rodents have a liver autonomous defect in ketogenesis that correlates with a reduction in the activity of PPARα, a master transcriptional regulator of ketogenic genes. Here, we show that the multi-component mTORC1 kinase controls ketogenesis in response to fasting as well as aging. Liver-specific loss of TSC1, an mTORC1 inhibitor, leads to a fasting-resistant increase in liver size and a pronounced defect in fasting-induced ketone production, while the loss of raptor, an essential mTORC1 component, has the opposite effects. Neither a synthetic PPARα ligand nor PPARα overexpression can induce ketone production or ketogenenic gene expression in livers or cultured cells lacking TSC1. mTORC1 inhibits ketogenesis by preventing the fasting-induced nuclear-exit of NCoR1, a negative regulator of PPAR. The deletion of TSC1 does not further decrease ketone production beyond that reduced by aging while, the loss of raptor is sufficient to correct the aging-induced defects in ketogenesis and expression of PPARα target genes. Thus, these findings show that mTORC1 is an important modulator of hepatic ketogenesis and PPARα function and support a role for mTORC1 activity in promoting the aging of the liver.

Introduction

In multi-cellular organisms, evolution has developed a complex interplay between energy consumption, utilization and storage to provide maximal survival to the organism in the face of an uncertain food supply and intermittent fasting. This check and balance system can lead to profound effects such as inhibition of prenatal and postnatal growth when nutrients are limited. Conversely, in the modern era, when food is both plentiful and varied, this same system can lead to pathologies such as obesity, diabetes and atherosclerosis. In addition, the reduced metabolic fitness of many organisms with aging indicates that aging changes the efficacy and sensitivity of many metabolic pathways. As such, elucidating the molecular mechanisms behind these metabolic pathways is important for understanding their regulation of organ and organismal growth, their response to fluctuations in nutrient levels, and pathologies they may produce in response to an overabundance of food and aging.

In mammals, the liver plays a key role in nutrient-regulated organ and organismal growth and in buffering nutrient level due to feeding and fasting cycles. In response to growth hormone (GH), the liver secretes multiple insulin growth factors (IGFs) of which many are required for prenatal and/or postnatal growth (Lupu et al., 2001). Circulating levels of both GH and IGF-1 are reduced under dietary restriction (Shimokawa et al., 2008). In addition to its endocrine roles, the liver is able to store or liberate glucose, and synthesize lipids and cholesterol for transport to peripheral tissues. In periods of extended fasting or in response to dietary restriction, the liver synthesizes glucose and ketone bodies from fatty acids and amino acids liberated from adipose stores and muscle respectively. These liver functions are governed by an interplay between hormones,

transcription factors, and energy substrates, allowing rapid responses to changes in metabolic need (Herzig et al., 2003).

Even the size of the liver is tied to nutrient status. In the rat, a 48-hour fast decreases liver size by 44% as a function primarily of hepatocyte size (Fenn, 1939). The liver also possesses remarkable regenerative capacity, metabolizes large number of drugs and synthesizes bile acids needed for absorption of dietary fats. Interestingly, aging leads to a perturbation in numerous liver functions including regenerative capacity (Timchenko, 2009), drug metabolism (Durnas et al., 1990), bile synthesis from cholesterol (Stahlberg et al., 1991), protein synthesis (Ward and Richardson, 1991), and ketone synthesis (Okuda et al., 1987). Given the myriad of metabolic and growth-related functions carried out by the liver, it is an enormous challenge to decipher all of the various inputs that stimulate, suppress or in general modulate these processes whether they be hormones, growth factors, chemicals or nutrients.

The mTOR complex 1 (mTORC1) is a multi-component kinase that responds to stimuli such as amino acids, growth factors, insulin, stress and hypoxia and regulates cell growth via modifying protein translation, ribosome biogenesis, and autophagy. When active, mTORC1 also suppresses insulin signaling at the level of insulin receptor substrate in a classic negative feedback loop (Harrington et al., 2005). Given its ability to regulate cell size in response to nutrients, growth factors, and insulin we hypothesized that mTORC1 would have an impact on regulating liver size in response to feeding and fasting. What is less clear is what liver functions are regulated by mTORC1. Given that mTORC1 is activated by insulin signaling via PI3K and AKT, initial research studied whether the mTORC1 mediated any insulin-dependent liver functions.

Insulin plays an important role in controlling glucose and lipid homeostasis by affecting multiple organs including muscle, fat and liver. Impairment of insulin signaling pathway plays a key role in the development of type-2 diabetes and the metabolic syndrome. Decreased plasma insulin levels also correlate with lifespan extending caloric restriction regimens. In the liver, insulin stimulates glycogen synthesis, glycolysis and lipogenesis, while suppressing gluconeogenesis and fatty-acid oxidation. Insulin signaling can affect metabolic pathways through signal transduction pathways and by changing patterns of gene expression and mRNA translation. Furthermore, the liver serves to clear insulin via receptor-mediated endocytosis. (Terris et al., 1979).

The physiological role of mTORC1 has been analyzed by full body knockouts of mTORC1 substrates and through the use of the mTORC1 inhibitor rapamycin. Mutant mice null for the mTORC1 substrate S6K1 in all tissues are smaller, hypoinsulinemic, are resistant to diet-induced obesity, and females experience extended lifespan (Selman et al., 2009; Um et al., 2004). Conversely, mice deficient for 4E-BP1 and 4E-BP2, substrates of mTORC1 where phosphorylation leads to inactivation, are sensitive to diet-induced obesity, have increased adipose tissue, and are insulin resistant (Le Bacquer et al., 2007). While the phenotypes of these mutant mice are very compelling, it is difficult to ascertain the individual contribution of mTORC1 in separate tissues to the phenotypes observed. More recently, tissue specific mutations in mTORC1 signaling have been generated in astrocytes (Uhlmann et al., 2002), skeletal muscle (Bentzinger et al., 2008), the beta-cells of pancreatic islets (Rachdi et al., 2008), and fat (Polak et al., 2008), but not liver.

Analysis of mTORC1 signaling kinetics and activity in the liver of obese rats found higher basal activity and accelerated response to insulin, thereby indicating that

metabolic disorders such as obesity are correlated with aberrant hepatic mTORC1 signaling. The role of mTORC1 in insulin mediated liver function was probed through the use of the mTORC1 inhibitor rapamycin. Using isolated rat hepatocytes, researchers found that rapamycin had no effect on insulin stimulated transcription of gluconeogenic genes such as phosphoenolpyruvate carboxykinase (PEPCK) (Liao et al., 1998), or on insulin stimulated glycogen synthesis (Peak et al., 1998). However, recent work from our lab and others has demonstrated that rapamycin does not truly phenocopy loss of mTORC1 function (Thoreen et al., 2009), thereby emphasizing the need to genetically perturb the mTORC1 pathway to truly study its function in hepatic physiology. Here we describe two mouse mutants that allow dissection of both hepatic gain of function and loss of funtion of mTORC1 through the use of conditional alleles. Our analysis confirms that mTORC1 does not play a major rule in regulating hepatic glucose metabolism independent from modulation of the insulin/PI3K/AKT pathway via negative feedback. However, we do uncover a novel role for mTORC1 in the regulation of hepatic fatty acid oxidation and ketone synthesis in response to fasting and aging.

Results

Hepatic mTORC1 signaling responds to feeding, and regulates liver size.

We first established that hepatic mTORC1 is fasting and refeeding sensitive. Upon activation, mTORC1 phosphorylates its downstream substrate S6 kinase, which in turn phosphorylates ribosomal protein S6. Mice fasted for 24 hours exhibited low levels of phosphorylated S6 as determined by immunofluoresence on paraffin-embedded liver sections and western blot analysis on whole-cell lysates from liver (Figure 1A and 1B).

Mice refed with chow displayed an increase in phosphorylated S6 in a time-dependent manner that is blocked by administration of rapamycin via intra-peritoneal injection 2 hours before refeeding (Figure 1A and 1B). Given that mTORC1 activity is modulated by changes in feeding status, we wished to determine which fasting and feeding related liver functions were regulated by mTORC1 activity. To do so, Tsc1 conditional knockout mice were obtained (Uhlmann et al., 2002), and conditional knockout Raptor mice generated. Retro-orbital injection of high-titer adenovirus expressing Cre recombinase resulted in loss of Tsc1 expression specifically in the liver (LiTsc1KO)(Figure 1C). Loss of Tsc1, an inhibitor of mTORC1 activity, resulted in constitutive expression of mTORC1 as exhibited by constant levels of phosphorylated S6 in fed, fasted and refed mice (Figure 1D). Raptor conditional knockout mice did not exhibit sustained loss of Raptor expression upon adenoviral Cre injection (data not shown), so mice were crossed with those expressing Cre from the liver-specific albumin promoter (LiRapKO) (Figure 1C). Loss of hepatic Raptor expression led to low levels of phosphorylated S6 in fed, fasted and refed mice (Figure 1D). Long-term fasting in many mammals induces a profound loss of liver mass due to a decrease in hepatocyte size. LiTsc1KO and LiRapKO mice had larger and smaller livers respectively due to changes in hepatocyte size and did not lose liver mass upon fasting like their wild-type littermates (Figure 1E). Histological comparison with wild-type liver sections uncovered not only changes in hepatocyte size in the mutant mice, but also changes in ploidy (Figure 1F). LiTsc1KO mice possessed larger percentage of hepatocytes with polyploidy or binucleate nuclei, whereas LiRapKO mice possessed a smaller percentage of such hepatocytes

(Figure 1F). The changes in cell size also correlated with changes in protein content consistent with the important role mTORC1 plays in protein synthesis (data not shown).

LiTsc1KO mice have lower plasma ketones than control mice in response to fasting.

In response to fasting, the liver performs numerous functions to maintain essential levels of plasma nutrients. In the initial stages of the fasting response, the liver liberates its carbohydrate stores, and performs gluconeogenesis. As the fasting state progresses, fat stores are lipolyzed and glycerol and free-fatty acids are released into the blood and taken up by the liver to be used as building blocks for glucose and ketone production. It is in the latter stages of a prolonged fast when ketones become increasingly important as a source of acetyl-CoA for peripheral tissues including the brain. Ketones also serve as a major fuel source during sustained periods of dietary restriction.

We sought to determine whether hepatic mTORC1 inhibition was necessary for the execution of the fasting response by the liver. To do so, plasma metabolite levels of LiTsc1KO mice were compared to wild-type mice to determine the consequence of ectopic mTORC1 activation in the liver during a 48 hour fast. The analysis also included mice with a liver-specific deletion of the insulin receptor (LIRKO) to control for the negative feedback loop between the mTORC1 substrate S6K and IRS (Michael et al., 2000), as well as LiRapKO mice. We collected plasma from fed mice and mice fasted for 24 and 48 hours and measured numerous factors including glucose, triglycerides, free-fatty acids (FFA), and ketones (Table 1). In addition, we measured levels of triglycerides and glycogen in the liver (Table 2). All mutant mice were compared to their respective wild-type littermates to control for any differences in strain background. Plasma levels of

many metabolites were similar between control and mutant animals. However, we found that LiTsc1KO had significantly decreased levels of plasma ketones in response to fasting- a phenotype not shared by LIRKO or LiRapKO mice (Table 1 and Figure 2A).

The defect in ketogenesis in LiTsc1KO mice is liver autonomous.

In the adult mouse, ketones are produced in the mitochondria of the liver and kidney from Acetyl-CoA generated primarily by fatty acid oxidation. Given that plasma levels of FFA were unchanged between wild-type and LiTsc1KO mice (Table 1), our first concern was whether there was a defect in FFA uptake by Tsc1 deficient livers. To address this concern, we fasted control and LiTsc1KO mice for 24 hours and injected mice with sodium octanoate- a medium-chain fatty acid that has been previously shown to freely diffuse into mitochondria of livers and serve as a ketogenic substrate ((McGarry and Foster, 1971). Control mice showed a time-dependent increase in plasma ketones after injection, whereas LiTsc1KO mice were unable to generate ketones even when presented with substrate (Figure 1B). Additionally, we performed *ex vivo* hepatic assays measuring rate of fatty acid oxidation from radiolabeled oleic acid. Hepatic fatty acid oxidation was severely blunted in livers lacking Tsc1 compared to control livers (Figure 1C). Thus, we determined that the dramatic decrease in plasma ketones upon fasting found in LiTsc1KO mice were due to a liver autonomous defect in fatty acid oxidation and ketone production.

Given that Tsc1 loss and therefore constitutive activation of mTORC1 inhibited hepatic ketogenesis, we predicted that loss of Raptor in the liver would lead to a constitutive ability to make ketones- even in the fed state. However, the sharp rise in

insulin upon refeeding inhibits lipolysis and leads to decreased plasma FFA levels available to the liver for ketone production. To circumvent the lack of substrate, refed control and LiRapKO mice were given an injection of sodium octanoate upon refeeding following a fast. Whereas refed wild-type animals shut down their ketogenic programs and are unable to produce ketones from injected sodium octanoate, LiRapKO mice were able to produce ketones in the hours post refeeding (Figure 1D).

TSC1 loss suppresses PPARα signaling, whereas Raptor loss ectopically activates PPARα signaling.

Ketogenesis is a multi-component process that is regulated by hormonal input and substrate availability. The nuclear transcription factor peroxisome proliferator-activated receptor-alpha (PPARα) is a necessary component of the ketogenic response. PPARα senses availability of substrate and transactivates numerous enzymes including those involved in fatty acid oxidation and ketogenesis. Endogenous ligand for PPARα is generated from the influx of FFAs into the liver during fasting. Upon ligand binding, PPARα is released from a co-repressor complex and binds to co-activators resulting in transactivation of itself and its targets. PPARα targets include carnitine palmitoyltransferase 1a (Cpt1a), the rate-limiting enzyme for transport of FFAs into the mitochondria; acyl-Coenzyme A oxidase 1 (AOX), an enzyme that involved in beta-oxidation of FFAs to generate Acetyl-CoA, and mitochondrial β-hydroxy-β-methylglutaryl-CoA synthase (HMGCS2) which is the rate limiting step of synthesis of the ketones acetoacetate and β-hydroxybutyrate from Acetyl-CoA (Hsu et al., 2001). In addition to ligand-induced activation of PPARα, numerous papers have reported other

pathways that modulate the PPARα response to fasting and no precise mechanism has been established

Given that the livers of LiTsc1KO mice fail to produce ketones during fasting even when presented with fatty acid substrate, we examined transcript levels of PPARα and many of its targets. Fasting in wild type mice leads to an increase in mRNA levels of PPARα and its targets. However, mRNA levels of these same genes did not increase upon fasting in Tsc1 deficient livers (Figure 2E). As fasting leads to an increase, subsequent refeeding leads to a decrease in mRNA expression of these same enzymes, PPARα, HMGCS2, and CPT1a, within hours. After 48 hours of fasting, LiRapKO mice exhibit similar transcript levels of these enzymes compared to wild-type controls, but after 2 hours of refeeding, while wild-type mice show a significant decrease in these transcript levels, levels remain elevated in LiRapKO livers (Figure 2F).

mTORC1 suppression of PPARα is dominant to administration of exogenous PPARα ligand and to overexpression of PPARα *in vivo* and *in vitro*

To determine whether the inability of LiTsc1KO livers to upregulate PPARα targets are due to defective ligand production, we treated mutant and wild-type mice with the synthetic PPARα agonist WY 14643 for 5 days, and then re-examined their plasma ketone levels and mRNA levels of ketogenic enzymes upon 48 hour fast. We found that 5-day treatment of the PPARα agonist lead to an increase in both plasma ketone levels and transcript levels of all enzymes in wild-type mice. However, agonist treatment failed to restore plasma ketone and ketogenic transcript levels in LiTsc1KO mice upon fasting (Figure 3A and 3B). PPARα and HMGCS2 transcript levels in the intestines of both

control and mutant animals showed a robust increase in response to agonist treatment confirming its efficacy (Figure 3B). To confirm this result, knockdown of Tsc1 or Tsc2 was performed in the transformed mouse hepatocyte cell line AML12. To trigger ketogenesis, cells were placed in a ketogenic media (KM) devoid of insulin and serum, and containing sodium octanoate and WY 14643. Compared to control GFP knockdown cells, reduction in Tsc1 or Tsc2 resulted in a decreased ketone output when placed in KM (Figure 3C, and data not shown). The ketogenic capability of AML12 cells with reduced Tsc1 or Tsc2 levels was restored with rapamycin treatment (Figure 3C, and data not shown). The degree of knockdown for Tsc1 and Tsc2 was validated by RT-PCR (Figure 3C). The cell culture results confirm that the defect in ketogenesis by loss of Tsc1 is cell autonomous and due to modulation of mTORC1.

Fasting leads to an increase in the mRNA of PPAR$\propto$ itself due to PPAR$\propto$ transactivating its own promoter. Mice with hepatic loss of Tsc1 fail to upregulate PPAR$\propto$ mRNA levels upon fasting. To test whether the reduction in PPAR$\propto$ levels in LiTsc1KO livers causes the defect in ketogenesis, mutant and control mice were injected with adenovirus expressing PPAR$\propto$. Hepatic overexpression of PPAR$\propto$ failed to rescue plasma ketone levels upon fasting in LiTsc1KO mice (Figure 3D). This same experiment was repeated in AML12 cells with stable expression of GFP or Tsc1 shRNAs. Again, we find that overexpression of PPAR$\propto$ failed to rescue the defect in ketogenesis in Tsc1 knockdown cell lines placed in KM compared to cells in complete media (CM) (Figure 3E). Overexpression of PPAR$\propto$ was confirmed by RT-PCR (Figure 3D and 3E).

The PPARα corepressor NCoR1 does not disassociate from PPARα target promoters upon fasting in livers from LiTsc1KO mice.

Given that both overexpression of PPARα and presence of excess ligand failed to induce ketogenesis and PPARα transcriptional activity in Tsc1 deficient livers and cells, we next looked at processes that regulate PPARα at the promoter. In the fed state, a large multi-component corepressor complex binds to the PPARα at peroxisome proliferator response elements (PPREs) of target promoters. Promoter repression is accomplished by the histone deacetylase activity of the corepressor complex, which results in a tight chromatin structure. Upon fasting and subsequent ligand binding, PPARα undergoes a confirmational change and the corepressor complex is possibly modified resulting in disassembly of co-repressor proteins and assembly of a series of co-activator proteins. The co-activator complex activates the promoter through histone acetylase and methylase activity and also recruits and binds the transcriptional machinery. Using chromatin immunoprecipitations assays, we measured the degree of chromatin modification in the promoters of PPARα, Cpt1a and HMGCS2. Comparing promoters from fed (repressed) and fasted (activated) livers, we found no significant increase in acetylation of PPREs from Tsc1 null livers in contrast to wild-type controls (Figure 4C). We next examined the switch from corepressor to coactivator complex binding to these promoters in the fed to fasted transition. We see the expected transition from nuclear receptor co-repressor 1 (NCoR1) to coactivator p300 binding at PPREs from wild-type livers in response to fasting. However, we find constitutive binding of the corepressor protein NCoR1 at the PPREs of LiTsc1KO promoters (Figure 4A and 4B).

This result is consistent with the lack of histone acetylation in PPREs of LiTsc1KO promoters.

Knockdown of NcoR1 or inhibition of NCoR1 histone deacetylase activity rescues the ketogenic defect in Tsc1 knockdown cells.

Based on the results obtained from the chromatin immunoprecipitation experiments, we hypothesized that knock down of NCoR1 in Tsc1-knockdown cells should lift the ectopic repression of PPARα transcription. In KM, TSC1/NCoR1 double knockdown cells produce equivalent amount of ketones compared to GFP and NCoR1 knockdown cell lines (Figure 4D). As previously noted, cells with Tsc1 knockdown alone continued to show a dramatic defect in ketogenesis (Figure 4D). We concluded that ectopic activation of mTORC1 under fasting conditions led to a defect in NCoR1 disassociation from the promoters of PPARα target genes. As a result, even in the presence of ligand and increased PPARα, the promoters were constitutively silenced due to the continued histone deacetylase activity of the NCoR1 containing corepressor complex. Therefore, administration of a pharmaceutical HDAC inhibitor such as Trichostatin A (TSA) should phenocopy loss of NCoR. Treating either GFP or Tsc1 knockdown cells with TSA placed in KM exerted no significant effect on control cells, but fully rescued the defect in ketone production in Tsc1 KD cells (Figure 4D).

mTORC1 regulates NCoR1 localization

NCoR1 is a ubiquitous transcriptional repressor and is regulated by multiple paradigms. NCoR1 represses of NFκB signaling, and TNFα stimulation lifts this

inhibition by triggering relocalization of NCoR1 out of the nucleus into the cytoplasm (Espinosa et al., 2002). We tested whether mTORC1 uses this paradigm for regulating NCoR1 activity. Liver sections from fed and 24 hour fasted LiTsc1KO mice were stained with an antibody to NCoR1 and compared to controls. In control mice, fed animals show NCoR1 staining in both the cytoplasm and nucleus, but fasting induces a depletion of NCoR1 staining in the nucleus (Figure 5A). In LiTsc1KO mice however, NCoR1 staining is in the cytoplasm and nucleus under both fed and fasted conditions (Figure 5B). To confirm this regulation of NCoR1 localization by mTORC1, liver sections from LiRapKO mice that were fasted for 24 hours or refed for 1 hour were stained with antibody to NCoR, and compared the staining of liver sections from control mice under the same conditions. Fasted control mice again showed decreased nuclear staining for NCoR, while refeeding for 1 hour resulted in increased NCoR1 nuclear staining (Figure 5C). Liver sections from LiRapKO mice exhibited almost no NCoR1 staining in the nucleus under fasted or refed conditions (Figure 5D). Qualitative observations were confirmed by image analysis measuring pixel intensity of NCoR1 staining along the diameter of the cell (Figure 5A-D).

Aging induced decrease in ketogenesis is due to mTORC1 repression of PPARα signaling

Diminished hepatic ketogenesis and decreased PPARα levels and activity have been previously found to occur in livers from old rodents. We compared old and young control mice and confirmed that upon fasting, old mice had lower plasma ketones and hepatic levels of PPARα compared to young mice (Figure 6A). When old floxed Tsc1

mice were injected with adenovirus-expressing Cre recombinase resulting in loss of Tsc1, further dampening of ketogenesis and PPARα levels was not observed (Figure 6A). This implies that the aging related decline in PPARα activity is refractory to any additional repression caused by constitutive mTORC1 signaling. Accordingly, transcript levels of PPARα and its targets Cpt1a and HMGCS2 are blunted in response to fasting compared to young controls (Figure 6B). Additional loss of Tsc1 has no significant additive effect (Figure 6B). Remarkably, aged LiRapko mice showed no hepatic defects in ketogenesis or PPARα activity compared to young LiRapKO mice (Figure 4C and 4D). NCoR1 staining of liver sections from old fed or fasted mice revealed constitutive nuclear staining raising the possibility that aging may suppress PPARα via NCoR1 (Figure 6E). In response to fasting, mice display "foraging behavior," which is an increase in physical activity. Prolonged fasting eventually leads to onset of torpor- a condition defined by a drop in body temperature and decreased physical movement. Upon fasting, LiTsc1KO mice experience premature onset of torpor, and do not exhibit increased physical activity indicative of foraging behavior (Figure 6F and G).

Discussion

In mammals, evolution has developed a metabolism able to survive long periods of little to no eating. In the fed to fasted transition, there is a gradual shift in whole-body fuel utilization from a combination of carbohydrates and fat to almost exclusively fat. For the brain and red blood cells, which are unable to metabolize fat, this shift is particularly dramatic. Rising ketones levels during fasting supplement endogenously synthesized glucose for use by peripheral tissues. It is estimated that FFA and their ketone body derivatives provide ~80% of caloric requirements after 24 hours of fasting in

adult humans (Berk and Stump, 1999). The majority of this conversion from glucose to fat oxidation for energy occurs in the liver, which plays a central role in the adaptive response to fasting. We find that the multi-component kinase mTORC1 regulates fatty acid oxidation and subsequent conversion into ketone bodies upon fasting.

During a prolonged fast, the body experiences low plasma insulin and high plasma levels of glucagon, epinephrine and glucocorticoids. This combination of hormones and chemicals promotes the lipolysis of triglycerides (TGs) from adipose tissue, thereby increasing the concentration of FFAs in the blood. The liver then takes up the FFAs where they are either re-esterified into triglycerides and secreted as VLDLs or oxidized in peroxisomes and mitochondria via beta-oxidation. The majority of fatty acids are only partially oxidized into acetyl-coenzyme A (acetyl-CoA) which then combines with itself to form ketone bodies. The ATP generated by beta-oxidation of fatty acids is used for hepatic gluconeogenesis from substrates such as glycerol, lactate and amino acids. Therefore, hepatic fatty acid oxidation is required for the metabolic response to fasting.

Peroxisome proliferator-activated receptors (PPARs) play an important role in fatty-acid metabolism (Desvergne et al., 1998) by essentially acting as sensors for fatty acids and their metabolic derivatives (Reddy and Hashimoto, 2001). While there are three isoforms of PPAR, alpha, gamma, and beta, PPAR alpha (PPAR$\propto$) is the master regulator of fatty acid oxidation in the liver (Desvergne and Wahli, 1999). Mice null for PPAR$\propto$ are relatively healthy in the fed state, but become hypoglycemic and hypoketonemic in the fasted state underscoring the requirement of PPAR$\propto$ in the hepatic fasting response (Djouadi et al., 1998; Kersten et al., 1999).

PPARα, irrespective of ligand binding status, remains largely in the nucleus bound to its target promoters as part of a heterodimer with retinoid x receptor-alpha (RXRα) (Guan et al., 2005). In the fed state, these receptors are maintained in a repressed state by co-repressor proteins such as SMRT and NCoR1 (Dowell et al., 1999; Guan et al., 2005). These co-repressors bind multiple nuclear receptors and possess or recruit histone deacetylases or other enzyme activities to tighten chromatin structure thus inhibiting repressing gene transcription (Guan et al., 2005). As FFAs are taken up by the liver upon fasting, certain FAs or FA derivatives directly bind to the ligand-binding domain of PPARα (Forman et al., 1997). Ligand binding contributes towards dissociation of the corepressor protein complex from PPARα. Disassembly of the corepressor complex allows for association of coactivator proteins with PPARα. The coactivator complex recruits or possesses enzyme activity which acetylates histones, thus lifting promoter repression and helping recruit the transcriptional machinery (McKenna and O'Malley, 2002). Just as there are no specific co-repressors for PPARα, the co-activators for PPARα are also shared among many transcription factors that are involved in different nuclear receptor signaling pathways (Glass and Rosenfeld, 2000). There may be upwards of 15 to 30 co-activator proteins that by chromatin remodeling and scaffolding, provide a molecular bridge that transfer the signal of nuclear receptor specific gene activation to basal transcription machinery (Roeder, 2005). Some key co-activators for PPARα include cAMP-response element-binding protein (CREB) binding protein (CBP) and E1A binding protein 300 (p300), three members of the steroid receptor coactivator family (SRC-1/p160), and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 alpha). The coactivators p300 and CBP possess and recruit

histone acetyl transferase activity that activate the promoters. It is unclear what purpose PGC-1 alpha serves in promoting transcription of PPARα targets. While overexpression of PGC-1 alpha can potentiate PPARα transcription, loss of PGC-1 alpha has no deleterious effect (Leone et al., 2005). Given the promiscuity of both the co-repressors and co-activators that regulate PPARα transcriptional activity, we are only beginning to understand how upstream signaling pathways regulate the activity of these corepressor and coactivator complexes in ways that don't have pleiotropic consequences.

Here we find that hyper-activation of hepatic mTORC1 signaling in LiTsc1KO mice inhibits fasting induced ketogenesis by constitutive repression of PPARα transcriptional activity. Our findings suggest that this transcriptional repression is mediated by increased presence of NCoR-1 at the promoter of PPARα and PPARα transcriptional targets. In contrast to livers of control mice, we find that the onset of fasting does not lead to disassociation of NCoR1 and increased association of p300 at the PPARα response elements of promoters for PPARα, HMGCS2 and Cpt1a in LiTsc1KO livers. We functionally verify the defect in this transition in LiTsc1KO livers by detecting no significant increase in histone acetylation at these promoters upon fasting whereas promoters in wild-type livers show robust histone acetylation upon fasting. The increased presence of NCoR1 at promoters of PPARα targets also correlates with increased amount of NCoR1 in the nuclei of fasted hepatocytes from LiTsc1KO mice compared to wild-type mice. We show that either knockdown of NCoR-1 or pharmaceutical inhibiton of histone deacetylase activity is sufficient to lift this repression of PPARα transcriptional activity in hepatocytes with knockdown of Tsc1 or Tsc2.

Although we were unable to elucidate whether mTORC1 is directly or indirectly acting on NCoR, it is not surprising that NCoR1 presence at the PPRE is a target of regulation. In general, the activities of corepressor and coactivator complexes can be regulated by numerous paradigms. For instance, a polymorphic allele of PPAR∝ found in the Asian population enhances recruitment of NCoR, and attenuates response to synthetic PPAR∝ ligands. Silencing NCoR1 or inhibition of HDAC activity restores transactivation activity of this variant allele of PPAR∝ in transfected HeLa cells (Liu et al., 2008). Other signaling pathways modulate NCoR1 mediated repression of transcription by changing its cellular location. Overexpression of p65-NF-kappa-B facilitates notch mediated transcription of multiple targets, including Hes-1, by inducing sequestration of NCoR1 in the cytoplasm through an unknown mechanism. This translocation abrogates repression by NCoR1 of promoters containing SRF or AP-1 sites (Espinosa et al., 2002). Finally, work in neural stem cells shows that direct phosphorylation of NCoR1 by Akt also mediates cellular localization and provides a paradigm by which cytokines promote differentiation of neural stem cells into astrocytes via PI3K/Akt signaling (Hermanson et al., 2002).

When first characterizing the defect in ketogenesis in LiTsc1KO mice, we uncovered previous work describing a similar phenotype in aged rodents. Plasma analysis of ketones, FFAs, and glucagon found a decrease in plasma ketones after a 36 hour fasting period in aged rats compared to young rats, despite higher levels of FFAs and glucagon. This *in vivo* analysis was confirmed through experiments using perfused livers that found that aged livers were not as responsive to glucagon induced ketogenesis as livers from young rats (Okuda et al., 1987). As we continued our analysis and found a

defect in PPARα induction and transcriptional activity, we were also intrigued by previous reports describing an aging related decrease in PPARα levels and DNA binding activity in heart (Iemitsu et al., 2002), spleen (Poynter and Daynes, 1998), kidney (Sung et al., 2004), and liver (Sanguino et al., 2004). Given this compelling data for the affect of aging on PPARα in rodents, we examined whether this novel regulation between mTORC1 and PPARα is also aging dependent. We first confirmed that aged mice do exhibit lower plasma ketones upon fasting compared to young controls. This defect correlates with lowered induction of PPARα and decreased PPARα transcriptional activity. After confirming this link between aging and PPARα, our results demonstrate that loss of hepatic Tsc1 in aged conditional mice did not lead to an additional decrease in fasting ketone levels. In the reverse experiment, we find that aging does not lead to a decrease in fasting ketone levels in LiRapKO mice. We confirmed this resistance to aging by showing no change in PPARα transcriptional activity between old and young LiRapKO mice. This is the first mechanistic description of how mTORC1 could regulate aging in a specific tissue and fits with recent exciting work showing that whole body inhibition of mTORC1 signaling can extend lifespan and provide resistance to aging related metabolic defects (Harrison et al., 2009; Selman et al., 2009)

Experimental Procedures

Materials

Conditional Tsc1 mice were a gift from David Kwiatkowski via Bernardo Sabatini. Raptor conditional mice were developed with the Rippel Transgenic Mouse facility at

MIT. LIRKO mice were a gift from C. Ronald Kahn. Adenovirus was purchased from the Gene Transfer Vector Core at the University of Iowa. Antibodies to phospho-S235/236 S6, phospho-240/244 S6, and total S6 were obtained from Cell Signaling Technology. Antibodies to NCoR1 were purchased from Upstate Biotechnology and Santa Cruz Biotechnology, p300 antibodies were also from Santa Cruz Biotechnology, and all modified histone chip antibodies were from Millipore. Cy3 conjugated secondary antibody was obtained from Invitrogen. WY 14,643 was purchased from Cayman chemicals, rapamycin was purchased from LC Labs, and glucagon, epinephrine, dexamethasone, transferrin, insulin, sodium octanoate, selenium, and oleic acid were purchased from Sigma. Radiolabeled oleic acid was from Perkin Elmer. Adenovirus expression PPAR$\propto$ was purchased from Vector Biolabs. Lentiviral hairpins to TSC1, TSC2, and NCoR1 were from the The RNAi Consortium centered at the Broad Institute.

Animal experiments

Mice were administered adenovirus via retro-orbital injection, and given WY 14,643 via oral gavage or ip injection. Fasting experiments began at lights out and ended at the indicated times. Activity measurements were performed in cages where infrared light beams were placed every 1.5 inches along the length of the cage. Beam breaks were measured using a digital counter. Body temperature was measured using an anal probe accurate to 0.1 degrees Celsius. All experiments were carried out with approval from the Committee for Animal Care at MIT and under supervision of the Department of Comparative Medicine at MIT.

Image analysis

All quantification of fluorescent intensity, pixel location, and hepatocyte size were performed using the NIH software ImageJ

Plasma and hepatic metabolite measurements

Ketones were measured using a colorimetric assay from Wako Chemicals. Serum triglycerides were measured using reagents from Sigma. Non-esterified free fatty acids were measured with a kit from Roche, while plasma insulin was measured using an ELISA kit from DSLabs. Plasma glucose measurements were taken from tail blood using an instant glucometer. All hepatic metabolite measurements were performed using reagents from Sigma.

Quantitative RT-PCR

RNA was isolated from cells and tissues using Qiagen's RNeasy kit. Reverse transcription of RNA into cDNA was performed using reagents from Invitrogen. Primers for rt-pcr were obtained from Integrated DNA Technologies. Reactions were run on an Applied Biosystems Prism machine.

Fatty-acid oxidation assay

Livers were removed from mice and three ~40 mg portions of liver were placed in individual wells of a 24-well plate along with 1 ml of Krebs-Ringer saline and radiolabeled oleic acid. Livers were incubated at 37 degrees Celsium and 5% oxygen for 2 hours. After two hours, the media was removed, and transferred to an eppendorf tube with no cap. Tubes were placed in scintillation vials that contained 2 mls of water, and incubated overnight at 65 degrees Celsius. The next day the vials were cooled down at 4 degrees for 30 minutes, the eppendorf tube was removed, scintillation fluid was added, and then levels of deuterium in the water was measured in a scintillation counter.

In vitro ketogenesis protocol

AML12 cells were passaged in media prescribed by ATCC which contained insulin and serum. For induction of ketogenesis, cells were grown to confluency, washed with PBS, and incubated in media devoid of serum and insulin and containing 50 μM WY 14,643 and 2 mM sodium octanoate.

Chromatin Immunoprecipitation

Liver portions were crosslinked in 1.5% formaldehyde in PBS for 15 minutes at room temperature, and the reaction was quenched with 0.125 M Glycine. Liver cells were disaggregated using 20 gauge syringe needles. Resulting cells were then lysed in 1% SDS lysis buffer for 10 minutes, then sonicated for 30 second intervals for a total of 5 minutes. Resulting lysates were diluted in buffers containing Triton, EDTA, NaCl and Tris-HCl. Diluted lysates were pre-cleared with protein-A agarose/salmon sperm and then incubated with antibody overnight at 4 degrees Celsius. The next day, antibody was precipitated with protein-A agarose/salmon sperm for 1 hour, pelleted and subjected to washes in low salt, high salt, LiCl and TE buffers in that order. The chromatin was eluted from antibody with 1% SDS and 0.1 M $NaHCO_3$, and crosslinks were reversed by heating at 65 degrees Celsius for 4 hours. Resulting chromatin was treated with proteinase K, purified using the High Pure PCR Template Preparation Kit from Roche, and used for PCR analysis.

Immunofluorescence

Immunofluorescence for NCoR1 was performed as follows: Fixed tissue was embedded in paraffin and processed onto slides for immuno-staining. Briefly, paraffin-coated

sections were de-waxed using EZ-DeWax deparrafinization solution (BioGenex), blocked in PBS with 5% goat serum, stained overnight with antibody at 4° C, and then incubated with Cy3-conjugated secondary antibody. Tiled images were obtained from an inverted epifluorescence microscope (Zeiss) and intensity was quantified using ImageJ (NIH).

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Figure Legends

Figure 1. Hepatic mTORC1 is feeding sensitive, and regulates liver size.
(A) Levels of phospho-S6 (ser240/44)) are sensitive to fasting and refeeding with normal chow. Immunofluorescence of phospho-S6 (ser240/44)) (red) and Hoechst (blue) on liver sections from mice. Quantification of fluorescent intensity of the rhodamine channel representing phospho-S6 staining. (B) Western blots of liver protein lysates show an time dependent increase in phospho-(S473)-Akt and phospho-(S240/244)-S6 upon refeeding following a 24 hour fast. Pre-treatment with rapamycin two hours before refeeding blocks the increase in phospho-S6. (C) Western blots of liver protein lysates from Tsc1 and Raptor conditional mice show that presence of Cre recombinase leads to loss of protein due to genetic recombination. (D) Liver knockout of Tsc1 (LiTsc1KO) or Raptor (LiRapKO) leads to insensitive mTORC1 signaling in response to feeding and fasting. Livers from LiTsc1KO mice show constitutive phosphorylation of S6, whereas there is no detectable phospho-S6 in LiRapKO livers. (E) Gross images of increased and decreased liver size in LiTsc1KO and LiRapKO mice respectively. Liver mass from mutant animals does not decrease in response to fasting. Quantification of liver weight results from fed and fasted mice with percent change in weight listed at bottom. (F) The change in liver size is due to changes in cell size. H&E stained sections of wild-type, LiTsc1KO and LiRapKO livers, and quantification of cell size in response to feeding and fasting.

Table 1. Measurement of plasma metabolites reveal lower fasting ketone levels in LiTsc1KO Mice.
Plasma measurements were made from ten mice in each group with averages and standard deviations listed. Fed mice were sacrificed at the beginning of the light cycle, and fasted mice were sacrificed at the same time but after a 24 hour fast. Control[a] mice are Tsc1 conditional mice injected with adenovirus not expressing any recombinant protein. Control[b] mice are insulin receptor conditional mice with no Cre transgene. Control[c] mice are Raptor conditional mice also with no Cre transgene. A * refers to plasma values from mutant mice that are significantly ($p < 0.05$) different compared to the appropriate control.

Table 2. Measurement of liver metabolites reveal a slight insulin resistant phenotype in fed LiTsc1KO mice and differences in hepatic TG levels in both LiTsc1KO and LiRapKO mice.
Hepatic metabolite measurements were made from 5 mice in each group with averages and standard deviations listed. Fed mice were sacrificed at the beginning of the light cycle, and fasted mice were sacrificed at the same time but after a 24 hour fast. Control[a] mice are Tsc1 conditional mice injected with adenovirus not expressing any recombinant protein. Control[c] mice are Raptor conditional mice also with no Cre transgene. A * refers to values from mutant mice that are significantly ($p < 0.05$) different compared to the appropriate control.

Figure2. The defect in ketogenesis found in LiTsc1KO mice is liver autonomous and is due to repression of PPARα transcriptional activity.

(A) LiTsc1KO mice have reduced plasma ketones after 24 hour fast, whereas wild-type, LIRKO and LiRapKO mice exhibit elevated plasma ketones upon fast. (B) Injection with the freely diffusible ketogenic substrate sodium octanoate in fasted mice does not rescue decreased plasma ketone levels in LiTsc1KO mice. (C) *Ex vivo* analysis of liver chunks from fasted LiTsc1KO mice show decreased oleic acid oxidation compared to wild-type control livers. (D) Injection of sodium octanoate LiRapKo mice upon refeeding leads to elevated plasma ketones in contrast to refed control mice. (E) Fasting-induced increased in mRNA levels of PPARα and its targets, Cpt1a, AOX, and HMGCS2 is blunted in LiTsc1KO livers compared to controls. However, levels of PEPCK and PK mRNA are unaffected in LiTsc1KO livers compared to controls. (F) mRNA levels of PPARα, Cpt1a and HMGCS2 do not decrease upon refeeding for 2 hours in LiRapKO compared to control mice.

Figure 3. Exogenous PPARα synthetic ligand or overexpression of PPARα cannot rescue the ketogenic defect in LiTsc1KO mice.
(A) Control and LiTsc1KO mice were administered the PPARα agonist Wy 14,643 via oral gavage for five days consecutively, and then fasted for 24 hours. While synthetic agonist increased plasma ketones in control mice, it had no significant affect on plasma levels of ketones in LiTsc1KO mice. (B) Quantitative RT-PCR of liver RNA reveals no increase in mRNA levels of PPARα or its targets in LiTsc1KO livers compared to control. PPARα and HMGCS2 mRNA levels do increase with agonist treatment in the small intestine of both control and LiTsc1KO mice demonstrating efficacy of the drug. (C) Stable infection of the mouse hepatocyte AML12 cell line with lentivirus expressing short hairpins for GFP and Tsc1 (shGFP and shTSC1), and then incubation in ketogenic media leads to a relatively small amount of ketone body production from shTsc1 infected cells compared to shGFP infected cells. This cellular defect in ketogenesis can be rescued by incubation with rapamycin (20 nM). Lentiviral expression of shTsc1 leads to over a 60% reduction in Tsc1 levels in AML12 cells. (D) Adenoviral mediated overexpression PPARα in mouse livers *in vivo* or in AML12 cells (E) led to an increase in ketone production in control cells, but did not rescue the ketogenic defect in LiTsc1KO mice or in AML12 cells infected with shTsc1. Small graphs show level of PPARα overexpression.

Figure 4. Loss of Tsc1 in the liver leads to constitutive binding of the corepressor protein NCoR1 to the PPRE of PPARα, Cpt1a and HMGCS2 promoters.
(A) Chromatin immunoprecipitation with an antibody to NCoR1 reveals constitutive binding at the PPRE in the promoters of PPARα, Cpt1a, and HMGCS2, whereas NCoR1 is not enriched at the promoters after a 24 hour fast in control livers. (B) ChIP experiments using an antibody to p300 exhibit marked enrichment at PPARα target promoters upon fasting in control mice, but relatively little enrichment at promoters from LiTsc1KO mice under fed or fasted conditions. (C) ChIP assay with an antibody to acetylated histone H4 confirm p300 results by exhibiting dramatic enrichment at PPARα promoters upon fasting in control livers, but little enrichment at promoters from LiTsc1KO mice under fed or fasted conditions. (D) Lentiviral-mediated knockdown of NCoR-1 in shTsc1-AML12 cells rescues the defect in ketone production due to Tsc1 knockdown. Alternatively, treatment of shGFP, shNCoR-1, shTsc1, and

shTsc1/shNCoR1 AML12 cells with the HDAC inhibitor trichostatin A (TSA, 100nm) also rescues the ketogenic defect in Tsc1 knockdown AML12 cells.

Figure 5. mTORC1 regulates cellular location of NCoR.
(A) Immunofluorescent staining for anti-NCoR1 in paraffin-embedded liver sections. In control livers, NCoR1 is found in both the nucleus and cytoplasm in fed animals, but only in the cytoplasm after 24 hour fast. Quantification of NCoR1 intensity across the diameter of hepatocytes. (B) NCoR1 is present in both the nucleus and cytoplasm in fed and fasted livers from LiTsc1KO mice. (C). Wild-type Raptor conditional mice exhibit increased NCoR1 staining within the nucleus upon 1 hour of refeeding compared to fasted mice where NCoR1 is primarily cytoplasmic. (D) The majority of NCoR1 staining in livers from LiRapKO is cytoplasmic in both fasted and refed animals.

Figure 6. mTORC1 regulates aging-dependent changes in ketogenesis.
(A) Fasting of 2-8 month old Tsc1 conditional injected with adeno-cre does not result in increased plasma ketones upon fasting as previously described. Conditional Tsc1 mice 18 months or older also exhibit decreased plasma ketones upon fasting, and injection with cre, and subsequent loss of Tsc1 does not result in an additional decrease in fasting plasma ketones. (B) Quantitative RT-PCR of livers from old and young control and LiTsc1KO mice show that aged mice results in defective PPARα signaling similar to young LiTsc1KO mice, and loss of hepatic Tsc1 in old mice does reduce mRNA levels of PPARα or its targets further. (C) LiRapKO mice aged to 18-24 months do not exhibit lowered plasma ketone levels upon fasting in contrast to aged controls. (D)Quantitative RT-PCR show reveal that aged LiRapKO are protected from the aging-induced defect in PPARα-dependent transcription as seen in control mice. (E) Staining for NCoR1 in liver sections from old mice reveal presence of NCoR1 in the nucleus in sections from both fed and fasted mice in contrast to young mice (see Figure 5). Quantification of NCoR1 staining intensity by ImageJ reveal no significant change in NCoR1 localization upon fasting in aged livers compared to young livers. (F) LiTsc1KO mice do not exhibit fasting-induced hyper-activity unlike their control littermates. Mice were placed in a cage crisscrossed with infrared beams. Beam breaks due to mouse movement were measured for 32 hours. (G) The body temperature of LiTsc1KO mice plummet upon 48 hour fast compared to the modest decrease in body temperature seen in controls.

Figure 1

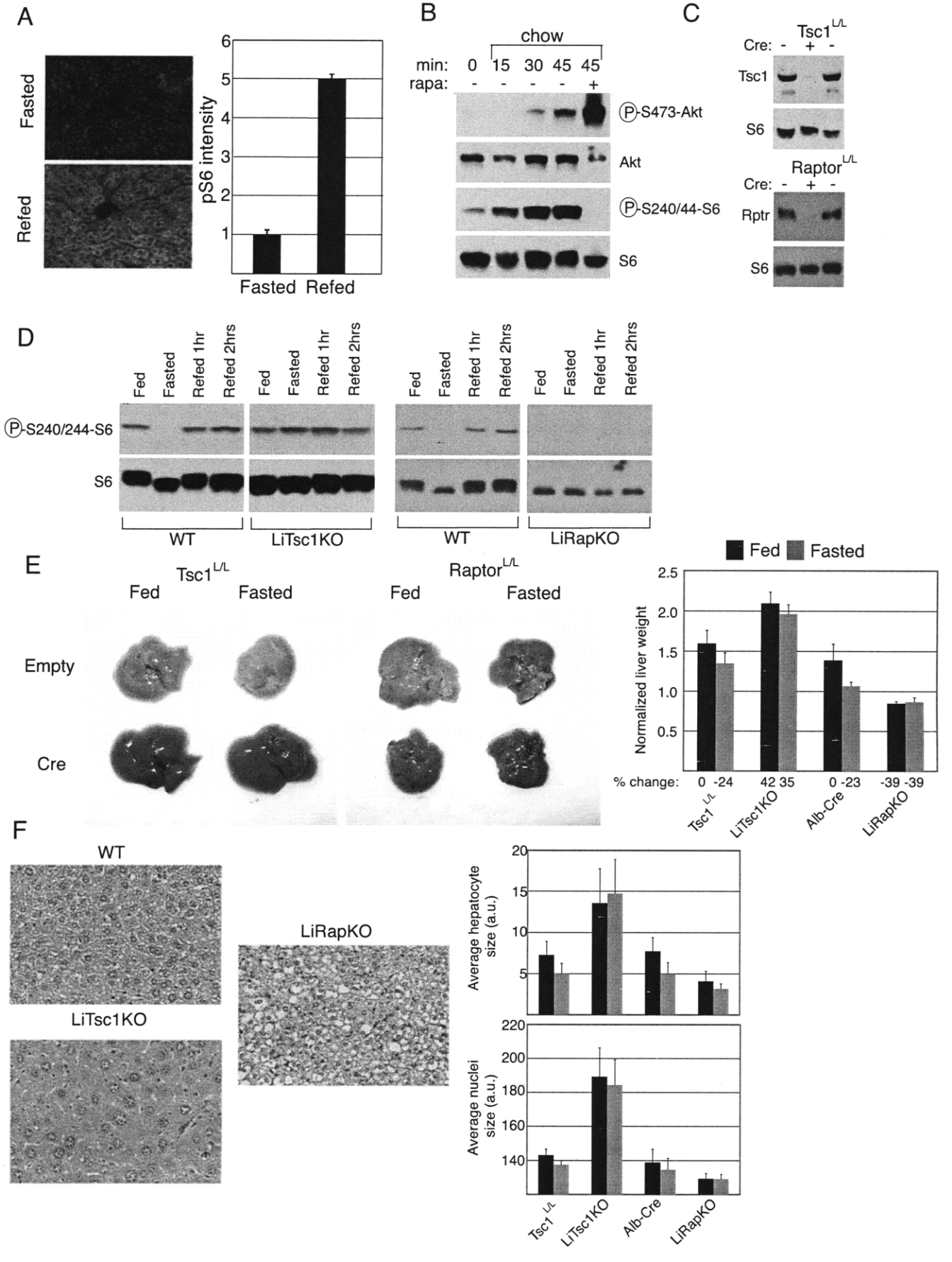

A
Fasted
Refed
pS6 intensity
Fasted Refed
B
chow
min: 0 15 30 45 45
rapa: - - - - +
Ⓟ-S473-Akt
Akt
Ⓟ-S240/44-S6
S6
C
Tsc1L/L
Cre: - + -
Tsc1
S6
RaptorL/L
Cre: - + -
Rptr
S6
D
Fed Fasted Refed 1hr Refed 2hrs
Ⓟ-S240/244-S6
S6
WT
LiTsc1KO
WT
LiRapKO
E
Tsc1L/L
RaptorL/L
Fed Fasted Fed Fasted
Empty
Cre
Fed Fasted
Normalized liver weight
% change: 0 -24 42 35 0 -23 -39 -39
Tsc1 L/L
LiTsc1KO
Alb-Cre
LiRapKO
F
WT
LiTsc1KO
LiRapKO
Average hepatocyte size (a.u.)
Average nuclei size (a.u.)
Tsc1 L/L
LiTsc1KO
Alb-Cre
LiRapKO

Table 1

	Control[a]	LiTsc1KO	Control[b]	LIRKO	Control[c]	LiRapKO
Glucose (mg/dl) Fed	95 ± 10.6	112.3 ± 7.1	96.9 ± 8.6	339 ± 22.7*	102 ± 8.4	94.2 ± 10.1
Fasted	66.8 ± 7.0	63.7 ± 6.8	71.2 ± 4.8	110 ± 15.4*	71.5 ± 12.7	73.5 ± 12.6
Insulin (ng/ml) Fed	3.12 ± 1.4	2.33 ± 1.3	4.10 ± 1.7	64.5 ± 9.5*	2.84 ± 1.5	3.21 ± 1.4
Fasted	0.43 ± 0.27	0.21 ± 0.13	0.52 ± 0.21	3.7 ± 0.86*	0.24 ± .13	0.33 ± .17
Fasting TG (mg/dl)	83 ± 12.3	108 ± 14.8	92 ± 10.4	62 ± 11.2*	108 ± 12.6	77 ± 16.9
Fasting FFA (mM)	0.81 ± 0.07	0.76 ± .05	1.1 ± 0.18	0.64 ± 0.11*	0.34 ± .04	0.31 ± .04
Fasting ketones (mM)	0.96 ± 0.19	0.37 ± 0.12*	1.23 ± 0.29	1.59 ± 0.37	0.91 ± 0.17	0.86 ± 0.13

Table 2

	Control[a]	LiTsc1KO	Control[c]	LiRapKO
Hepatic TG (mg/g) Fed	6.98 ± 0.41	5.39 ± 0.62	5.89 ± 0.52	5.18 ± 1.24
Fasted	20.86 ± 3.4	13.85 ± 1.6*	13.64 ± 2.4	6.56 ± 0.97*
Hepatic Glycogen (ug/mg) Fed	3.26 ± 0.39	1.83 ± 0.21*	2.86 ± 0.34	2.91 ± 0.34
Fasted	1.59 ± 0.17	2.27 ± 0.39	0.82 ± 0.12	0.76 ± 0.18

Figure 2

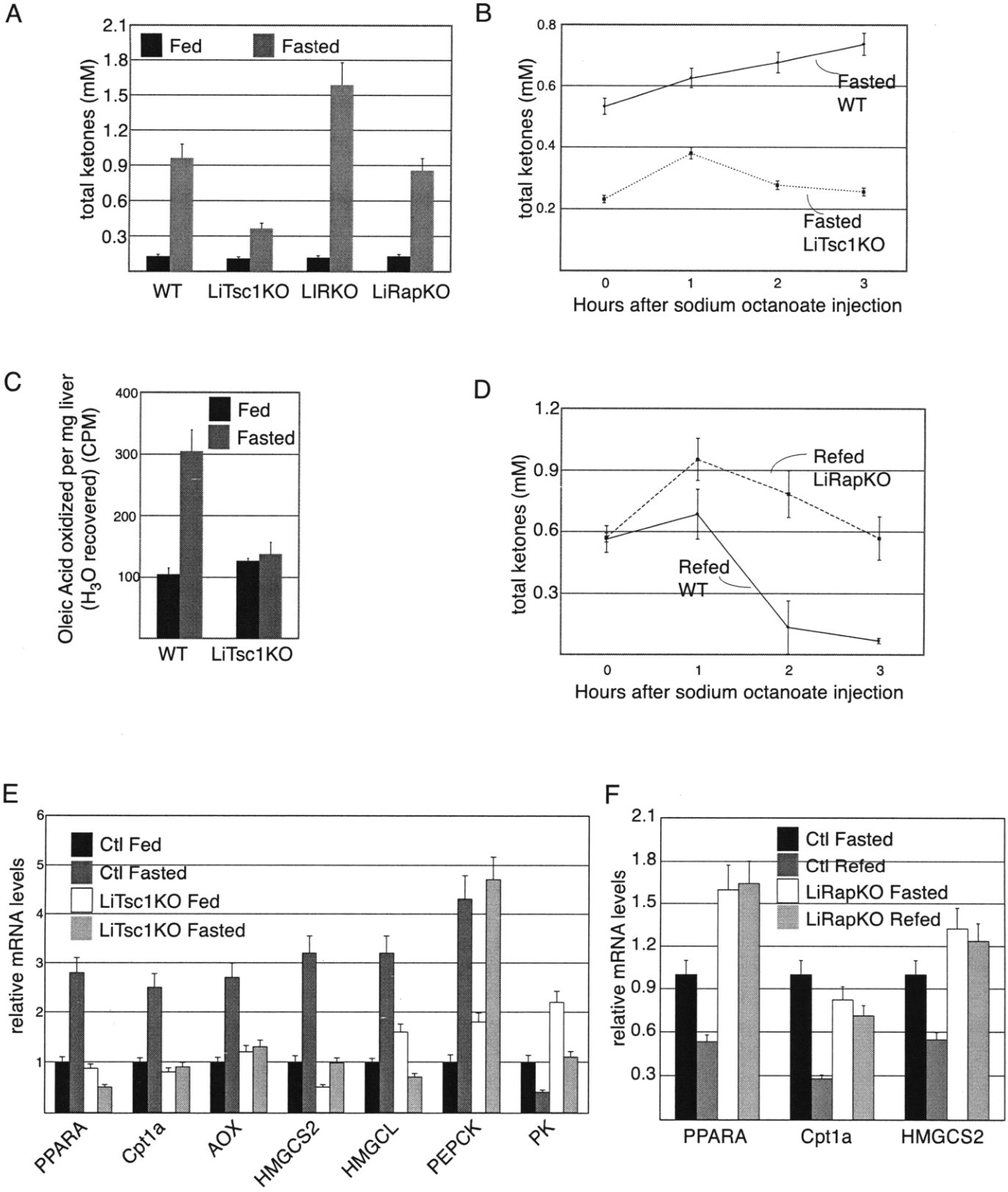

A
total ketones (mM)
Fed
Fasted
WT
LiTsc1KO
LIRKO
LiRapKO
B
total ketones (mM)
Fasted
WT
Fasted
LiTsc1KO
Hours after sodium octanoate injection
C
Oleic Acid oxidized per mg liver
(H_3O recovered) (CPM)
Fed
Fasted
WT
LiTsc1KO
D
total ketones (mM)
Refed
LiRapKO
Refed
WT
Hours after sodium octanoate injection
E
relative mRNA levels
Ctl Fed
Ctl Fasted
LiTsc1KO Fed
LiTsc1KO Fasted
PPARA
Cpt1a
AOX
HMGCS2
HMGCL
PEPCK
PK
F
relative mRNA levels
Ctl Fasted
Ctl Refed
LiRapKO Fasted
LiRapKO Refed
PPARA
Cpt1a
HMGCS2

Figure 3

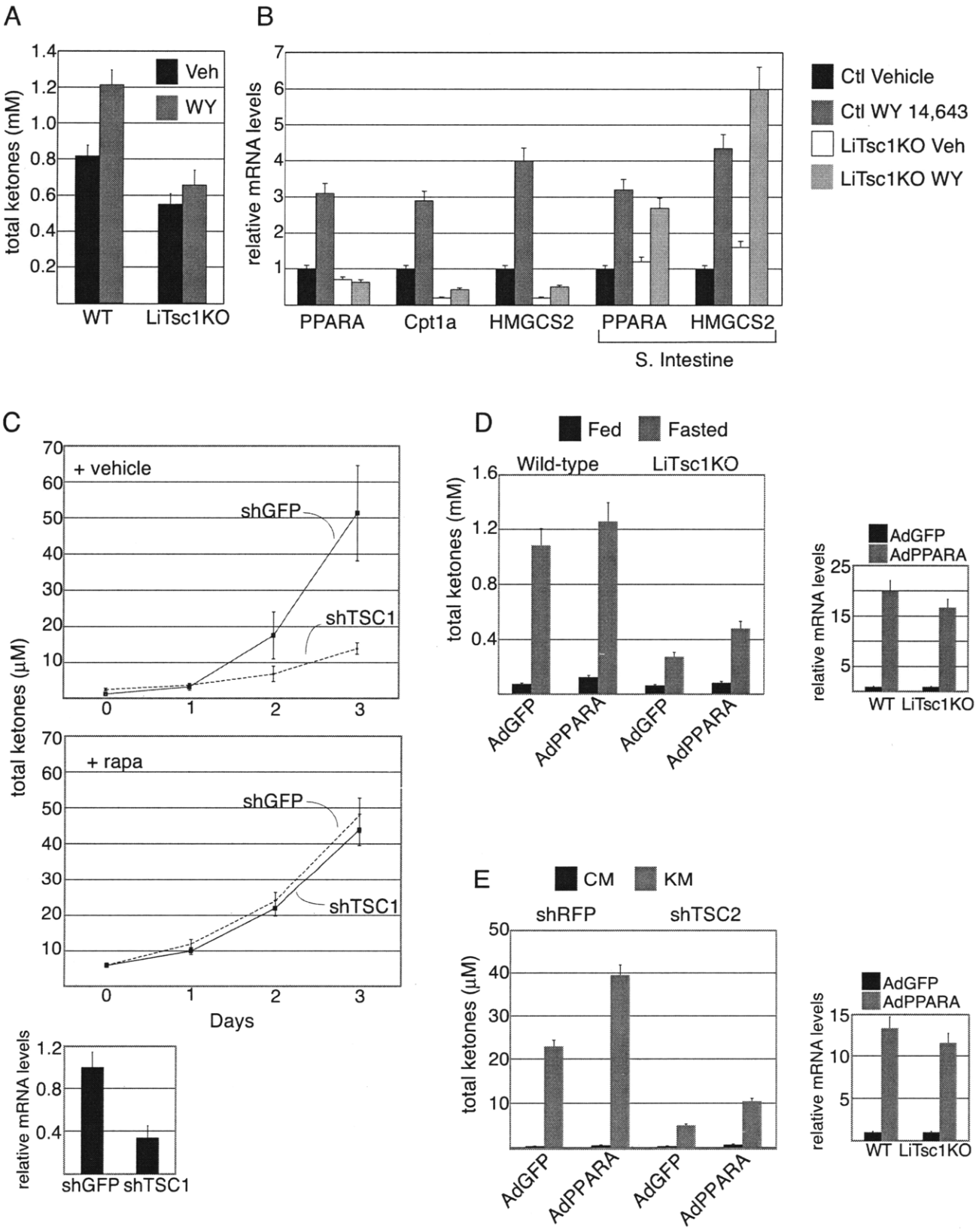
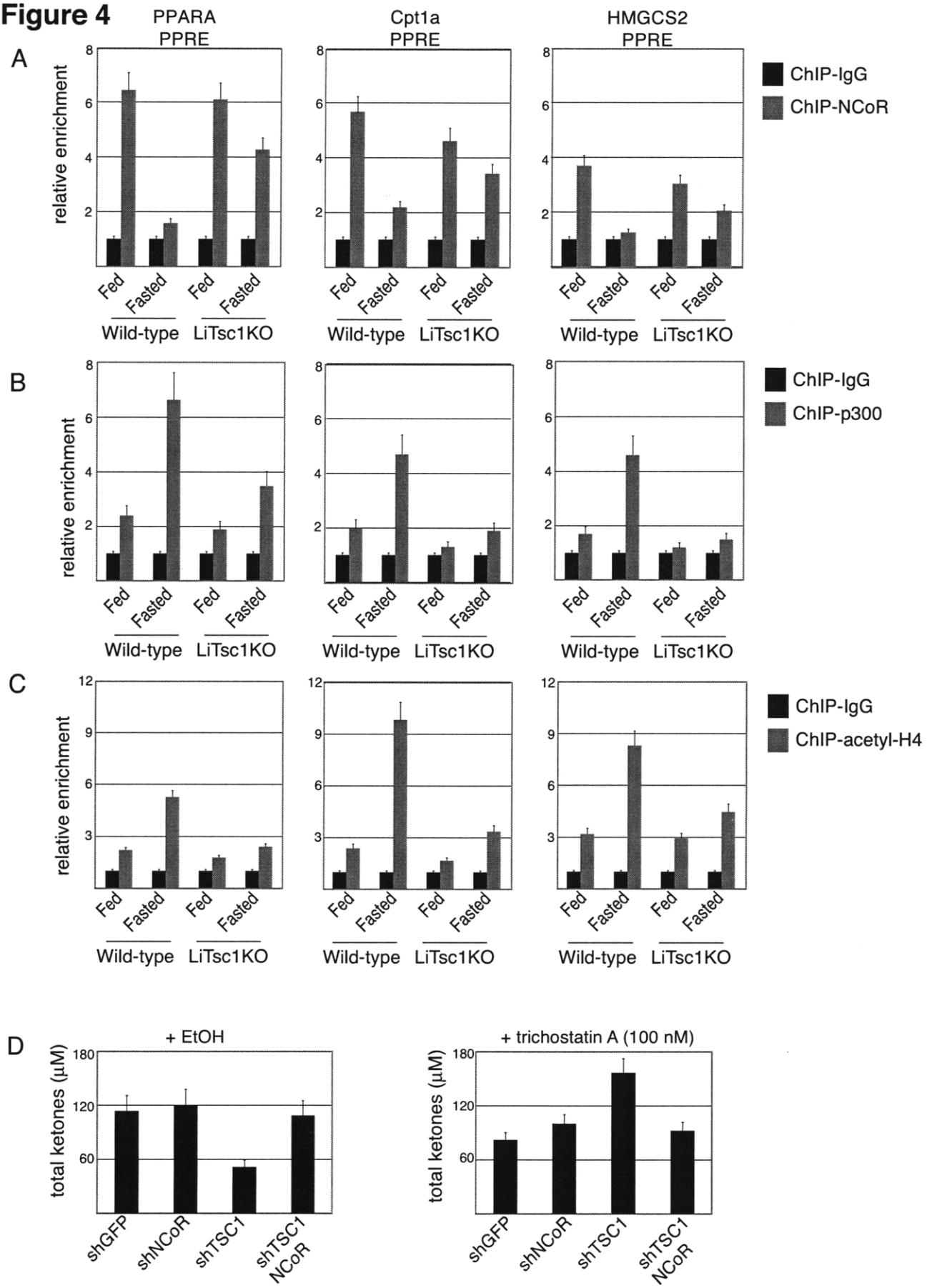

A
B
C
D
E
Veh
WY
total ketones (mM)
WT
LiTsc1KO
relative mRNA levels
PPARA
Cpt1a
HMGCS2
PPARA
HMGCS2
S. Intestine
Ctl Vehicle
Ctl WY 14,643
LiTsc1KO Veh
LiTsc1KO WY
+ vehicle
shGFP
shTSC1
total ketones (μM)
+ rapa
Days
shGFP shTSC1
Fed
Fasted
Wild-type
LiTsc1KO
AdGFP
AdPPARA
CM
KM
shRFP
shTSC2

Figure 4


A
PPARA PPRE
Cpt1a PPRE
HMGCS2 PPRE
relative enrichment
ChIP-IgG
ChIP-NCoR
Fed
Fasted
Wild-type
LiTsc1KO
B
ChIP-IgG
ChIP-p300
C
ChIP-IgG
ChIP-acetyl-H4
D
+ EtOH
+ trichostatin A (100 nM)
total ketones (μM)
shGFP
shNCoR
shTSC1
shTSC1 NCoR

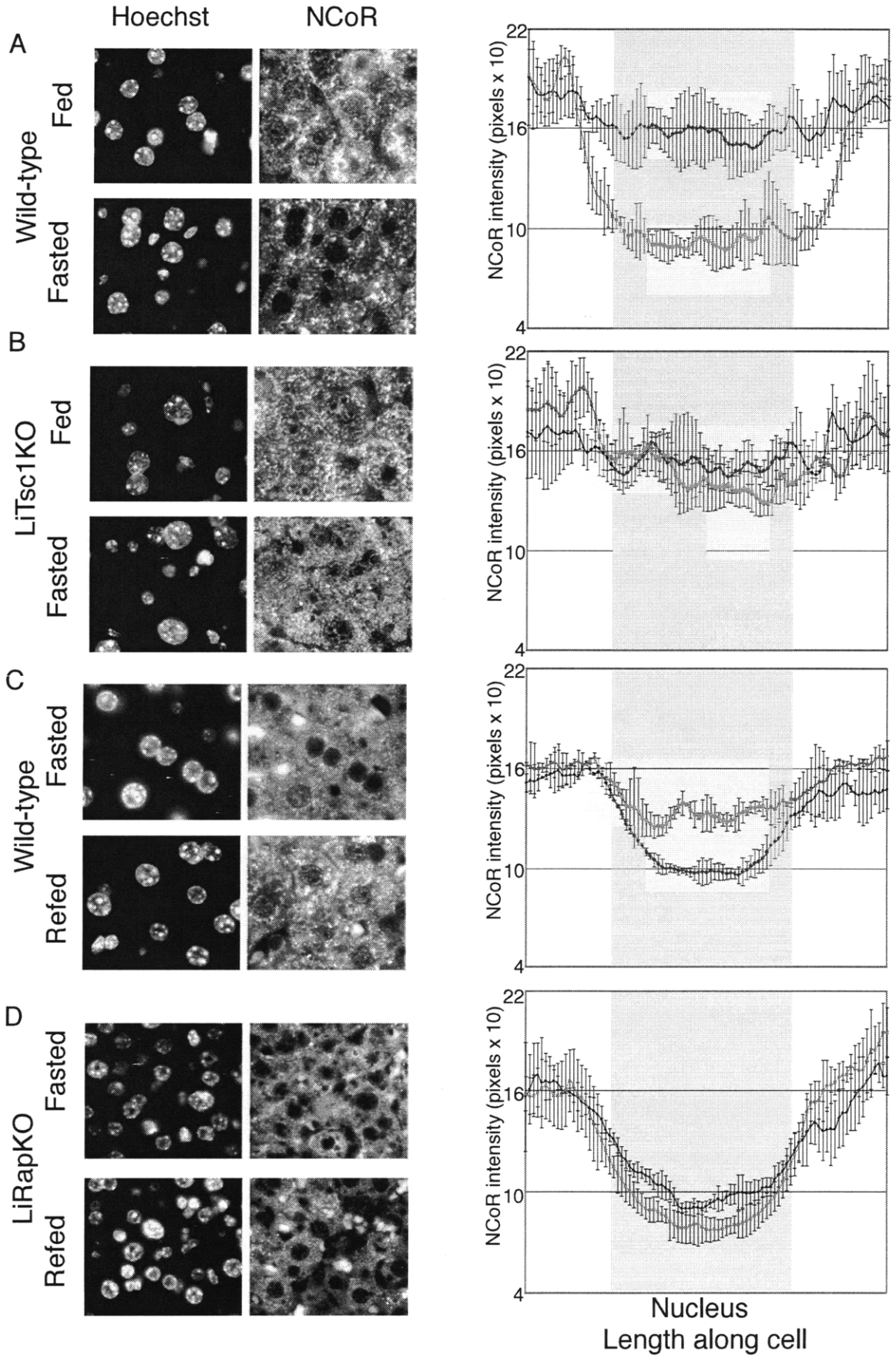
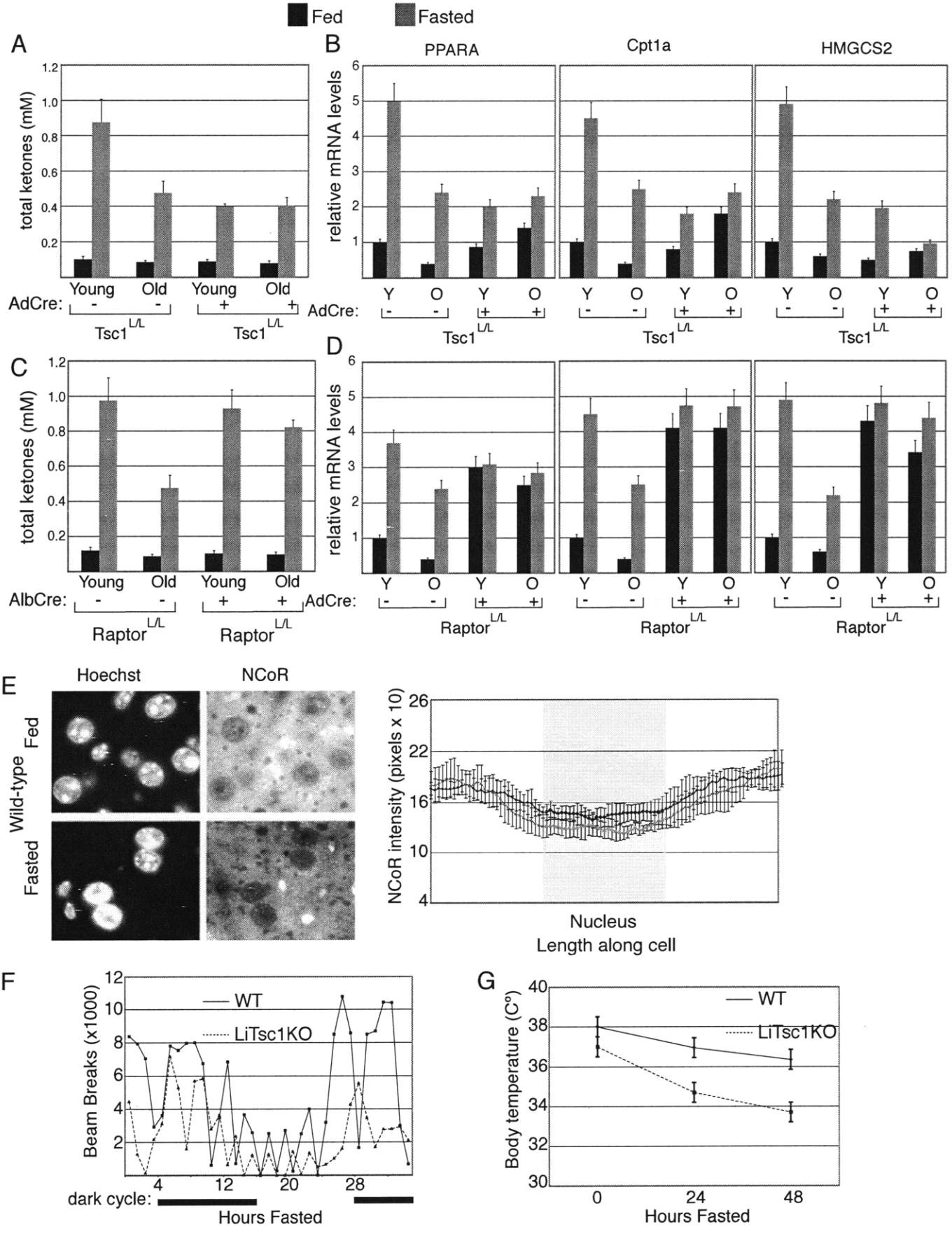

Hoechst
NCoR
A
Wild-type
Fed
Fasted
B
LiTsc1KO
Fed
Fasted
C
Wild-type
Fasted
Refed
D
LiRapKO
Fasted
Refed
NCoR intensity (pixels x 10)
22
16
10
4
Nucleus
Length along cell

Figure 6


Fed
Fasted
A
total ketones (mM)
Young
Old
AdCre:
Tsc1 L/L
B
PPARA
Cpt1a
HMGCS2
relative mRNA levels
Y
O
C
AlbCre:
Raptor L/L
D
E
Hoechst
NCoR
Wild-type
Fed
Fasted
NCoR intensity (pixels x 10)
Nucleus
Length along cell
F
WT
LiTsc1KO
Beam Breaks (x1000)
dark cycle:
Hours Fasted
G
Body temperature (C°)
Hours Fasted

Chapter 3

A novel mouse model for the study of angiomyolipoma and lymphangioleiomyomatosis, lesions found in TSC patients

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All experiments were performed by S.S. and S.O.

Summary

Lymphangioleiomyomatosis (LAM) and angiomyolipomas (AMLs) are unusual and devastating lesions that occur primarily in young women, and are a leading cause of death for patients with tuberous sclerosis complex (TSC). TSC is an autosomal dominant disease that results from inactivating genetic mutations in either Tsc1 or Tsc2. The biochemical consequence of either these mutations is increased mammalian target of rapamycin complex 1 (mTORC1) signaling. AMLs are benign tumors with ectopic proliferation of smooth muscle cells, increased deposition of fibrous tissue, adipose tissue, and abnormally formed vascular channels. LAM patients experience progressive destruction of their lungs by cyst formation and proliferation of smooth muscle-like cells. The majority of LAM patients also have AML, and both lesions are diagnosed by HMB45 positive staining. Here we describe a doxcycyline-inducible transgenic mouse model where overexpression of the mTORC1 positive regulator Rheb2 in the mesentery leads to cystic gowths with internal hemorrhaging, ectopic proliferation of multiple cell types including smooth muscle cells and adipocytes, and expansion of HMB45 positive mast cells. These results indicate that Rheb2 transgenic mice are a novel mouse model to study the pathology of AML and LAM.

Introduction

Tuberous sclerosis complex (TSC) is an autosomal dominant genetic disease with an incidence of one in 6000 at birth, and is characterized by benign tumors (hamartomas and hamartias) involving multiple organ systems (Kwiatkowski and Manning, 2005). Although the disease has been described for over a century, our understanding of the molecular pathogenesis of the syndrome has been limited. A major advance was made with the discovery that TSC patients had inactivating mutations in either TSC1 or TSC2. Work in *Drosophila* and mammalian systems have discovered that TSC1 and TSC2 form an obligate heterodimer with GAP activity towards the small GTPase Rheb (Zhang et al., 2003). When TSC1/2 GAP activity is inhibited by kinase-mediated phosphorylation, Rheb, bound to GTP, activates TOR kinase activity when TOR is contained in complex 1. Three signaling pathways have thus been shown to regulate TSC1/2 GAP activity via phosphorylation: the PI3K-Akt pathway, the ERK1/2-RSK1 pathway, and the LKB1-AMPK pathway. As a result, inactivation of TSC1 or TSC2 in TSC patients leads to constitutive activation of mTORC1 signaling.

The inactivating mutations in either TSC1 or TSC2 found in TSC patients consist of the usual mix of nonsense, missense, insertion and deletion mutations, and show no preference for a particular exon (Kwiatkowski and Manning, 2005). Mutations in TSC2 are fives time more frequent that mutations in TSC1 in TSC patients. However, mutations in either are 100% penetrant. There are, however, differences in the severity of the syndrome as measured by tissue involvement and mortality. Patients with mutations in TSC1 seem to have a more mild form of the disease than those with mutations in

TSC2. Loss of one copy of either gene is sufficient to lead to disease manifestation, although LOH has been found in some TSC tumor samples (Kwiatkowski and Manning, 2005).

TSC harmatomas have been found in patients at all developmental stages in life. For instance, cardiac rhabdomyoma, a benign neoplasm of cardiomyocytes filled with glycogen, is common in perinatal TSC patients but not as frequent in patients older than 5 years old (Isaacs, 2009). Despite having only benign tumors, TSC patients experience accelerated mortality due to growths in the brain, kidney and lung that lead to a failure to thrive, renal failure and respiratory failure respectively. The majority of TSC patients have seizures that have been attributed to cortical tubers and subependymal nodules present in the brains of TSC patients. The tubers of TSC are focal lesions with loss of normal cortical cytoarchitecture and occurrence of abnormal enlarged neurons, glial cells and less differentiated 'giant' cells (Crino and Henske, 1999).

While the brain phenotypes of TSC manifest early in age and sometimes last into adulthood, the major clinical problems in TSC patients post puberty are the progressive development of renal angiomyolipoma (AML) and pulmonary lymphangioleiomyomatosis (LAM). Both proliferative lesions can arise spontaneously but occur more frequently in TSC patients. 80% of TSC patients develop angiomyolipomas (AMLs) by age 15 (Ewalt et al., 1998). AMLs are benign tumors consisting of smooth muscle cells, fibrous tissue, adipose tissue, and abnormally formed vascular channels (Gomez, 1999). AML is found more frequently in women at an incidence of 1/300 compared to 1/5000 of male TSC patients (Ewalt et al., 1998).

Related to AMLs, LAM is an infiltrative, proliferative lung lesion composed of aberrant smooth muscle and epithelioid cells with cyst formation and destruction of normal airways (Moss et al., 2001). Patients with LAM eventually experience respiratory failure as the disease progresses. Histologically, there are two primary components to LAM pathology. Smooth muscle cell proliferation is seen within the lung parenchyma, with cells varying from small round or oval cells, to small to medium spindle-shaped cells to large epithelioid cells (Kelly and Moss, 2001; Sullivan, 1998). Surprisingly, all these cells with variable morphology usually stain positive for smooth muscle actin, and are thought to arise from a common progenitor cell. Second, there is progressive destruction of connective tissue in the lung with the formation of cysts that are found thoughout the lung of LAM patients (Matsui et al., 2000). As much as 63% of women with LAM, sporadic or as part of TSC, also develop angiomyolipoma (Bernstein et al., 1995).

The diagnosis of angiomyolipoma and LAM is usually contingent upon imaging results and positive staining of tumor biopsies with the monoclonal antibody HMB45. The monoclonal antibody HMB45 was originally generated against an extract of human melanoma cells (Hoon et al., 1994). The HMB45 antibody reacts with variably sized proteins of the melanocyte-lineage gene SILV/PMEL17/GP100 (Du et al., 2003). In lymphangioleiomyomatosis and angiomyolipoma cells, the binding sites for HMB45 antibody are cytoplasmic granules that resemble immature melanosomes (Matsumoto et al., 1999). The reason for the occurrence of these HMB45 organelles in LAM and angiomyolipoma cells is unknown.

Beyond the involvement of pathways affected by TSC1 and TSC2, there is limited understanding of the pathogenesis of TSC and the related lesions LAM and AML

due to the difficulty of modeling the disease in rodents. The majority of mouse models focus on the brain related phenotypes of TSC. Homozygote deletion of Tsc1 or Tsc2 cannot be studied in the mouse due to embryonic lethality (Kobayashi et al., 2001). The Eker rat arose as a spontaneous inactivating mutation in Tsc2 and develops brain harmatomas, but with no TSC associated clinical phenotypes (Wenzel et al., 2004; Yeung et al., 1997). Mice with conditional floxed alleles of Tsc1 have also been generated (Uhlmann et al., 2002). Using these mice, Tsc1 has been knocked out specifically in astrocytes leading to the development of seizures and significant brain pathology. However, this model does not produce pathology similar to that seen in TSC cortical tubers (Uhlmann et al., 2002). When Tsc1 is deleted in most neurons beginning at approximately embryonic day 13 (E13), mice experience spontaneous seizures, shortened lifespan, and neuropathological abnormalities including ectopic, enlarged, and aberrant neurons in multiple locations. These abnormal neurons have features similar to the dysplastic neurons seen in TSC cortical tubers (Meikle et al., 2007).

Some mouse models have approximated certain aspects of LAM and AML found in TSC. For example, both Tsc1 and Tsc2 heterozygous mice develop liver hemangiomas consisting of proliferative smooth muscle cells, endothelial cells, and vascular channels. Interestingly, hemangiomas occur more frequently and lead to increased mortality in female mice compared to male mice (Kwiatkowski et al., 2002). Consistent with this sex difference, expression of both the estrogen and progesterone receptor were found in the liver hemangiomas. In the clinic, diagnosis of angiomyolipomas and LAM are contigent upon positive staining with the monoclonal antibody HMB45 by immunohistochemistry. Kwiatkowski et al. were unable to detect

HMB45 positive cells by immunohistochemistry, although they did detect reactivity to the antibody by immunoblotting in some of the hemangiomas. Protein lysate from mouse embryonic fibroblasts null for either Tsc1 or Tsc2 did not show the same result (Kwiatkowski et al., 2002). Therefore, there must be some physiological context that gives rise to this HMB45 positive population in angiomyolipomas and LAM. However, identification of the cell of origin for these growths and the cells that give this unusual antibody reactivity remains elusive. Given that angiomyolipomas in TSC patients heterozygous for either Tsc1 or Tsc2 typically lose the other allele, the heterozygous mouse may be too mild of a mutation to truly mimic the clinical manifestations of angiomyolipoma and LAM.

A major impediment to the development of a mouse model of angiomyolipoma or LAM is pinpointing the cell of origin. Angiomyolipomas have been found in multiple tissues, and it is unclear whether LAM originates from a cell in the lung, or a circulating metastasizing cell. To address these issues, we generated a transgenic mouse that expresses the human Rheb2 gene in response to doxycycline administration. The transgene was placed into the Col2A locus allowing for expression in a wide range of tissues (Beard et al., 2006). Unpublished work from our lab demonstrated that overexpression of the human Rheb2 transgene in mouse cells leads to constitutive mTORC1 signaling similar to loss of Tsc1 or Tsc2. Our goal was to examine the consequence of hyper-activation of mTORC1 in multiple tissues with the hopes of modeling the development of angiomyolipomas and LAM in TSC patients.

Results

Generation and validation of a doxycycline-inducible Rheb transgenic mouse

Human (hs) Rheb2 transgenic mice were engineered by targeting murine embryonic stem (ES) cells with frt homing sites placed in the Col2a locus with a plasmid containing the transgene downstream of a tetracycline operator, and flanked by flp recombinase sites. Additionally, the ES cells were previously engineered to contain the tetracycline-inducible M2rtTA transactivator driven from the endogenous Rosa26 promoter (Beard et al., 2006). Treatment of the resulting ES cells with doxycycline drove expression of hsRheb2 by the Col2a promoter. The engineered ES cells were then used to generate chimera mice that were taken germline with subsequent breeding to wild-type Black6 mice. Mice were bred to homozygosity for both the Col2a-tet-hsRheb2 and Rosa26-M2rtTA loci, and will be referred to as tetRheb2 while control mice only containing the repressor element and will be referred to as M2rtTA.

Resulting tetRheb2 mice were given doxycycline either in their drinking water at a dose of 2mg/ml, via intra-peritoneal (ip) injection once a week at a dose of 20mg/kg or both avenues of doxycycline administration concomitantly. Doxycycline treatment of tetRheb2 mice resulted in approximately 30-fold increase in human Rheb2 mRNA in the intestine (Figure 1A). Due to a lack of commercially available antibodies to Rheb2, antibodies were generated in collaboration with Upstate Biotechnology. None of the antibodies generated were able to detect endogenous human or mouse Rheb2 in multiple human cell lines or mouse tissues. However, one antibody did detect overexpressed Rheb2 in cell culture (Sancak, unpublished data). Using this antibody, overexpression of hsRheb2 upon doxycycline treatment in transgenic mice was detected by immunoblotting

and immunofluorescence in the liver, small intestine, and mesenteric tissue (Figure 1B and 1C). Doxycycline inducible expression of hsRheb2 is also expected in the kidney, testes, lymph nodes, spleen, thymus and the islet cells of the pancreas in transgenic mice (Beard et al., 2006).

Rheb overexpression leads to hyper-activation of mTORC1 and multi-focal cysts within the mesentery

tetRheb2 mice placed under a regimen of doxycycline in the water and via weekly ip injection developed multi-focal cysts originating from the mesenteric tissue within 1 month of treatment (Figure 2A). The development of these cysts was specific to transgene expression as treatment of M2rtTA mice with doxcycyline, or tetRheb2 mice with ampicillin did not lead to cyst development (data not shown). Furthermore, removal of doxycycline after 3 months led to complete regression of cyst formation (Figure 2A). The penetrance of this phenotype varied depending on the sex of the mouse. Female mice developed a large number of cysts, and exhibited poor body condition within 3 months of continuous doxycycline treatment, whereas male mice developed fewer or no cysts and did not exhibit poor body condition within the same time frame (Figure 2B). Mice administered doxycycline via water with no additional ip injections developed cysts as well, but with a latency period of 5-6 months (data not shown), but the sexual dimorphic aspect of the phenotype remained. Rheb2 overexpression also leads to significant edema of the small and large intestines in the majority of transgenic mice, and glomerular nephritis in approximately 20 percent of mutant mice (data not shown). Dissection and subsequent immunofluorescent analysis of the cysts revealed that

hsRheb2 was overexpressed throughout the cyst and surrounding mesenteric tissue (Figure 2C). Staining of phospho-S6 revealed high levels that overlapped with high Rheb2 staining throughout the mesentery and cysts (Figure 2C). These results corroborate that cyst formation is due to overexpression of Rheb2 and hyper-activation of mTORC1.

Cystic lesions in tetRheb2 mice have characteristics of AML and LAM

Histological analysis of the cysts found numerous abnormalities. After one month of doxycycline treatment, the mesenteric tissue of transgenic mice showed increased proliferation of both numerous cell types including mesenteric adipocytes indicative of a lipoma (Figure 3A, top panel). Increased proliferation due to hsRheb2 overexpression was also confirmed by phospho-Histone H3 positivity (Figure 3B). Also present in mesenteric tissue upon doxycycline treatment were greater number of blood vessels, and blood vessels with thicker walls (Figure 3A, middle panel). In contrast to the well-defined and organized lymph nodes present in wild-type mesentery, transgenic mesentery contained disorganized immune cell infiltrates present throughout both cystic and non-cystic portions (Figure 3A, bottom panel). In addition, there was marked expansion of mesenteric smooth muscle cells upon doxycyclin treatment (Figure 3C).

Mesenteric growths from tetRheb2 mice stain positive for HMB45

These histological and immunological findings from the Rheb2-overexpressing mesenteric growths have many similarities with AMLs- benign mesenchymal tumors found commonly in patients with tuberous sclerosis. Loss of function mutations in Tsc1

or Tsc2 is the most common genetic insult found in tuberous sclerosis. Biochemically, we expect that overexpression of the small GTPase Rheb2 to be similar to losing Tsc1 or Tsc2, which as a heterodimer has GTPase activating function towards Rheb1 and Rheb2. Therefore, we stained the cysts with clinical markers used to diagnose AMLs in human patients. Diagnosis of AML depends on imaging and positivity with the melanocytic marker HMB45. However, the exact cell type within the angiomyolipoma that stains positive for HMB45 has not been clearly defined.

Women with tuberous sclerosis also have a high incidence of LAM. LAM is characterized by progressive destruction of the lung by cyst formation and proliferation of cells that stain positive for smooth muscle actin. Given that 50% of LAM patients also have AMLs, it is thought that LAM may be caused by dessimination of AML tissue to the lung. Accordingly, proliferative cells in the lungs of LAM patients also stain positive for HMB45 and other markers of angiomyolipoma. Staining with HMB45 revealed a robust increase in HMB45 positive cells in the mesentery of transgenic mice treated with doxcycycline (Figure 4A and B). HMB45 positive cells were also positive for Rheb2 and contained high levels of phospho-S6 (Figure 4B). Furthermore, the location of HMB45 positive cells was focused near regions of cyst formation. What cell type was staining positive for HMB45?

The HMB45 positive cells are mast cells

The murine mesentery contains numerous different cell types, many of which share characteristics with AML and LAM cells. For example, mouse mesenteric tissue contains pre-adipocytes, smooth muscle cells, mast cells, other immune cells, adipocytes,

stromal and squamous mesothelial cells. Many of the markers specific for each of these cell types such as c-kit, tryptase, vimentin, desmin and smooth muscle actin, are reported to also be present in both AMLs and in the lungs of LAM patients. The morphology of the HMB45 positive cell was very distinct from many of the surrounding adipocytes and stromal cells. Furthermore, HMB45 positive cells were present in wild-type mesentery, although not nearly in the same number compared to transgenic mesentery treated with doxycycline (data not shown). Staining with vimentin confirmed that HMB45 positive cells were of mesenchymal origin, and not epithelial (Figure 4C). As a result, we hypothesized that the HMB45 positive cells were native to the mouse mesentery, and hsRheb2 overexpression leads to expansion of the population. The increase in population of these cells also correlated to cyst formation. Given that previously published work had shown that mast cells also stain positive for HMB45 in certain tumor types (Shidham et al., 2003), we stained hsRheb2 overexpressing mesenteric tissue for mast cell markers. Two common markers used for mast cells are c-kit and tryptase. C-kit (CD177) is a membrane bound tyrosine kinase receptor that is present in melanocytic cells and has also been reported to be diagnostic marker for angiomyolipoma (Makhlouf et al., 2002). Tryptase is an abundant serine protease in mast cells, and staining for tryptase is used as a marker for mast cell activation as well as presence. Of note, tryptase has been characterized as a mitogen that promotes proliferation of fibroblasts and smooth muscle cells.

We found that the HMB45 positive cells found in hsRheb2 over-expressing mesentery co-stained with c-kit (data not shown) and tryptase (Figure 4D). This confirms our initial hypothesis that the HMB45 positive cells in both the wild-type and transgenic

mesentery are mast cells. The mast cells also co-stain with high levels of Rheb2 and phospho-S6 indicating that overexpression of Rheb2 correlates with increased number of mast cells in the mesentery.

In conclusion, we find that overexpression of hsRheb2 in the mesentery of mice leads to cystic growths that can hemorrhage, proliferation of multiple cell types, and expansion of HMB45 positive mast cells. This phenotype is more severe in female mice as judged by both extent of cystic growths and latency of growth formation upon overexpression of hsRheb2. In both male and female mice, these growths require sustained overexpression of hsRheb2, for removal of dox leads to complete remission of cystic growths within 1 month. Given that overexpression of hsRheb2 mimics the genetic insult of losing Tsc1 or Tsc2 as found in patients with tuberous sclerosis, the phenotype of the hsRheb2 transgenic mouse may serve as a clinically important mouse model of tuberous sclerosis. Histological and immunohistochemical features of these hsRheb2 dependent growths have many similar characteristics to angiomyolipomas and LAM such as presence of proliferative blood vessels and proliferative smooth muscle like cells, ectopic fat growth, and presence of HMB45 positive cells. Our identification of these HMB45 cells as mast cells echoes previous findings describing mast cell staining in angiomyolipomas and LAM, and highlights in importance of following up the possible causal role mast cells may play in the development of LAM and angiomyolipomas.

Discussion

LAM is a devastating interstitial lung disease that occurs almost exclusively in young women, and is the 3rd leading cause of death for patients with TSC. The majority

of patients that develop LAM, sporadic or due to having TSC, also develop angiomyolipomas. In these patients, both lesions have identical mutations in either Tsc1 or Tsc2 and approximately 60% exhibit loss of heterozygosity of the remaining functional allele (Carsillo et al., 2000). Furthermore, both lesions share numerous histological characteristics such as presence of smooth muscle-like cells and cells that stain positive for the melanocytic marker HMB45. Here we describe a mouse model where overexpression of Rheb2 in the mesentery, the biochemical consequence of which his similar to loss of Tsc1 or Tsc2, leads to ectopic proliferation of numerous cell types including adipocytes, immune cells and smooth muscle cells. Furthermore, overexpression of Rheb2 leads to increased presence of cells that stain positive for HMB45. Despite being used as a consistent diagnostic marker for both LAM and AMLs, the role of the HMB45 positive cell in the pathology of either lesion remains unclear. Even the cell of origin for each lesion has eluded researchers of these growths. In our transgenic Rheb2 mice, we find that the HMB45 positive cell is most likely a mast cell, or a related cell that shares histological characteristics with mast cells. Our findings support recent evidence of mast cell proliferation within the lungs of LAM patients and highlight the importance of examining their functional role in the pathology of LAM and AMLs.

The etiology of LAM and AMLs has puzzled researchers since the discovery of the disease. Initial thoughts that LAM arose due to aberrant proliferation of a smooth muscle cell native to the lung were challenged when a small percentage of LAM patients who received lung transplants re-developed LAM in the transplanted lung (Karbowniczek et al., 2003). This was also one of the first reports of LAM arising in a patient without an

AML- challenging the perception that LAM could be a benign metastasis of the angiomyolipoma to the lung. Recent work has correlated the multisystem manifestations of LAM, both sporadic and within the TSC patient population, with a metastatic dissemination of LAM cells (Carsillo et al., 2000) Genetic sequencing has allowed researchers to identify cells that are found in renal, lymphatic and lung lesions of LAM and TSC patients as having identical genetic mutations, and later finding these same "LAM" cells in the blood and body fluids of LAM and TSC patients (Karbowniczek et al., 2003). Thus, metastasis seems to be a mechanism by which LAM cells are disseminated but the molecular determinants are unknown. The relationship between mutations in the Tsc genes and many of the key features of LAM cells such as metastatic capabilities, and the presence of smooth muscle-like and melanocytic characteristics is also not clear.

Our finding that the HMB positive cell in the transgenic mesentery may also be a mast cell echoes previous findings from LAM and TSC patients in the clinic. Mast cells are best known for their role in IgE-dependent allergic diseases including asthma (Kirshenbaum et al., 2003). Besides allergens, mast cells are also activated by pathogens such as bacteria through their Toll-like receptors, and by stem cell factor (SCF) via the c-Kit (CD117) receptor. In response to activation, mast cells produce a large array of potent inflammatory mediators by several mechanisms including degranulation of pre-formed compounds and production of numerous cytokines (Beaven and Baumgartner, 1996).

In addition to their role in inflammation, presence of mast cells correlates with increased tissue fibrosis and angiogenesis. Mast cells found in areas of fibrosis and

angiogenesis were found to secrete large amounts of the potent angiogenic and mitogenic polypeptide basic fibroblast growth factor (bFGF) (Qu et al., 1995). This discovery was very relevant for the study of LAM and angiomyolipoma, for bFGF is a powerful mitogenic factor for smooth muscle cells and cells of mesodermal origin (Davis et al., 1997). Mast cells are also the major source of the bFGF found in the lungs of chronic pulmonary fibrosis patients (Inoue et al., 1996). Pulmonary fibrosis, although different in etiology than LAM, shares the pathophysiologic features of SMA-positive cell proliferation and extracellular matrix deposition (Inoue et al., 2002).

Examination by tryptase-positive staining found numerous mast cells within the lungs of LAM patients. The mast cells were localized to the interstitium primarily, and clustered around the spindle, smooth muscle cell-like proliferative LAM cells. In addition, these mast cells were also positive for bFGF (Inoue et al., 2002). Later reports confirmed this initial observation of mast cell presence in lungs of LAM patients. Valencia et al. investigated the role of the renin-angiotensin system in LAM, and found angiotensin II and ACE in proliferative spindle-shaped LAM cells. The authors also found increased number of mast cells in the lungs of LAM patients compared to normal lung, despite any evidence of inflammation within the diseased lung. They hypothesized that chymase, an enzyme secreted by activated mast cells, could lead to formation of angiotensin II, which in turn could stimulate proliferation of the LAM cells (Valencia et al., 2006).

Consistent with our findings that hyper-activation of mTORC1 signaling by overexpression of Rheb2 leads to increased number of mast cells, a recent report found constitutive activation of mTORC1 in mast cells isolated from tumor samples.

Furthermore, they found that rapamycin, an inhibitor of mTORC1 lead to decreased survival and death of mast cells. Biochemical analysis confirmed that activation of mast cells either through their IgE or c-KIT receptor led to activation of mTORC1 and increased mast cell proliferation. This induction was significantly inhibited by rapamycin (Kim et al., 2008). In conclusion, we believe that the Rheb2 transgenic mouse may serve as a useful model of AML and LAM suffered by TSC patients. This model mimics the sexual dimorphism of LAM, exhibits clinical diagnostic markers found in both AML and LAM, and offers an *in vivo* system to study the role of mast cells in stimulating proliferation of cells found in both lesions.

Experimental Procedures

Materials

The antibody to Rheb2 was obtained from Upstate Biotechnology. Antibodies to tryptase and HMB45 were purchased from Abcam. Antibodies to c-kit were from Santa Cruz Biotechnology, to Vimentin from Thermo Fisher, and antibodies to smooth muscle actin were from Vector Labs. Doxycycline was purchased from Sigma.

Animal Experiments

When doxycycline was administered in the water, fresh batches were made once a week, and were not supplemented with any type of sugar. To control for artifacts from weekly ip injections, control mice were injected with PBS. Transgenic mice were generated in collaboration with the Rippel Transgenic Facility with ES cells generously provided by the Caroline Beard from the Jaenisch lab. All experiments were carried out with

approval of the Center for Animal Care at MIT, and under supervision of the Division for Comparative Medicine.

Histology and Immunofluorescence

Tissues were fixed in 10% neutral buffered formalin overnight, and then transferred to 70% ethanol. Tissues were processed, sectioned and stained for hematoxylin and eosin at the Koch Institute histology core at MIT. Additional immunofluorescent staining and microscopic imaging were performed in the following way: Briefly, paraffin-coated sections were de-waxed using EZ-DeWax deparrafinization solution (BioGenex), blocked in PBS with 5% goat serum, stained overnight with antibody at 4° C, and then incubated with Cy3-conjugated secondary antibody. Tiled images were obtained from an inverted epifluorescence microscope (Zeiss) and intensity was quantified using CellProfiler.

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Figure Legends

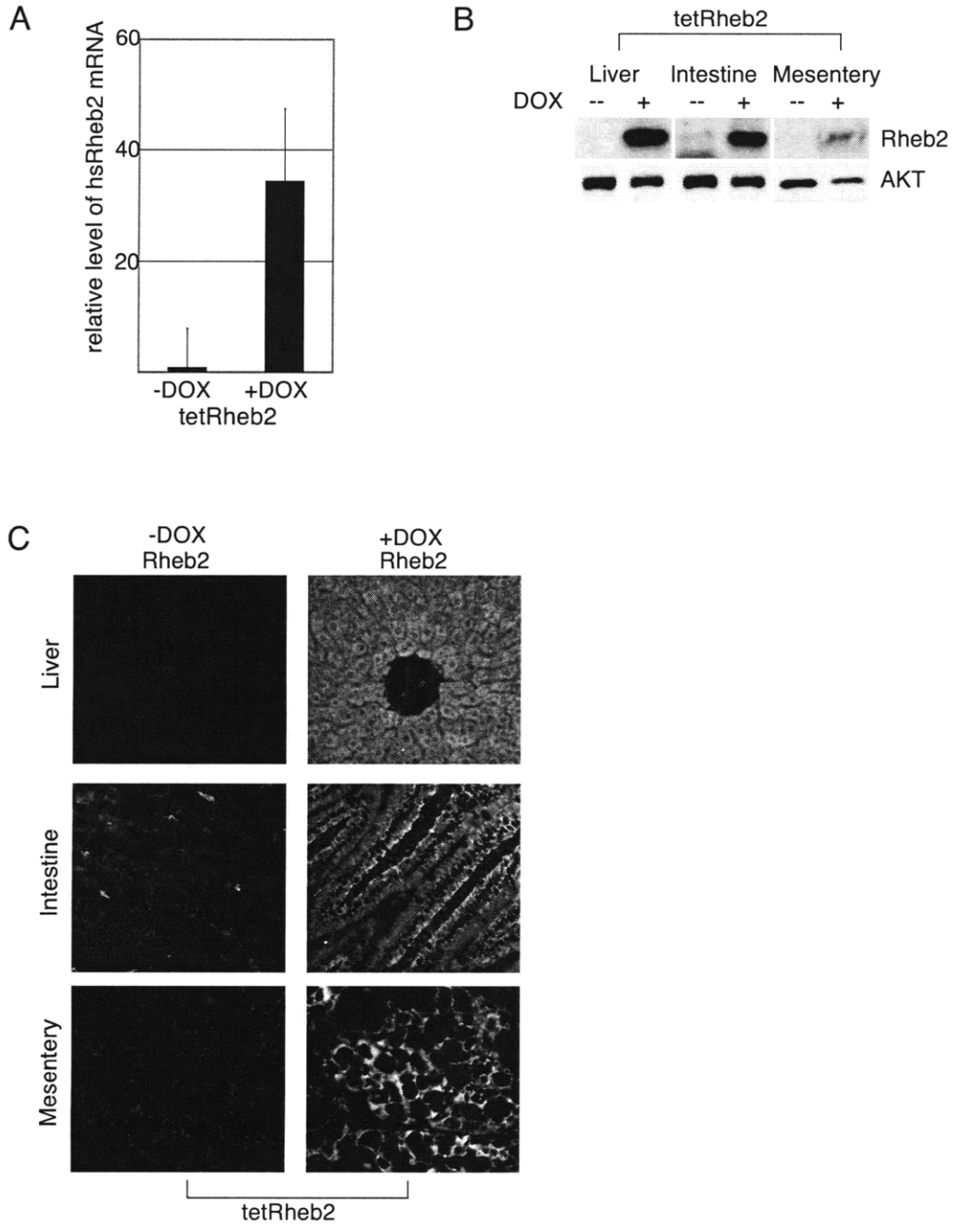
Figure 1. Doxycycline induces overexpression of Rheb2 in multiple tissues.
(A) Quantitative RT-PCR of cDNA isolated from tetRheb2 intestines 6 hours after doxycycline i.p. injection. Primers specific to human Rheb2 were used, and results normalized to RT-PCR measurements of β-actin mRNA. (B) Western blot of lysates from liver, intestine and mesentery probed with anti-Rheb2 and anti-AKT antibodies. (C) Immunofluorescent staining with anti-Rheb2 antibody of tissue sections taken from tetRheb2 mice given normal water and PBS injections, or given doxycycline in the water plus weekly doxycycline injections for 2 weeks.

Figure 2. Continual doxycycline treatment leads to tumor formation in tetRheb2 mice.
(A) tetRheb2 mice develop cystic tumors upon doxcycyline treatment in a time-dependent manner. Removal of doxcycycline for 1 month in tetRheb2 mice with palpable tumors leads to complete regression of tumor growths. (B) Quantification of tumor development by quantification of cyst size. Development of cysts occurs with shorter latency and greater number in female mice compared to male mice. (C) Immunofluorescent staining of tumors reveals high expression of Rheb2 and co-staining with phospho-(S235/236)-S6.

Figure 3. Tumors in tetRheb2 mice show proliferation of adipocytes, blood vessels, and smooth muscle cells.
(A) H&E staining of mesenteric sections of M2rtTA and tetRheb2 mice treated with doxycycline for 3 months. (B) Phospho-Histone H3 staining of mesenteric sections of M2rtTA and tetRheb2 mice treated with doxycycline for 3 months. (C) Smooth muscle actin and phospho-S6 staining of mesenteric sections of M2rtTA and tetRheb2 mice treated with doxycycline for 3 months. Hoechst is used as a nuclear stain for all sections.

Figure 4. Tumors in tetRheb2 mice contain HMB45/Vimentin/tryptase-positive cells.
(A) Immunofluorescent staining of mesenteric tumors from tetRheb2 mice treated with doxycycline for 3 months reveal a population of HMB45/phospho-S6/Rheb2 positive cells. (B) HMB45 positive cells stain positive also for Vimentin. (C) HMB45 positive cells also stain positive for tryptase.

Figure 1


A
relative level of hsRheb2 mRNA
60
40
20
-DOX
+DOX
tetRheb2
B
tetRheb2
Liver
Intestine
Mesentery
DOX
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Rheb2
AKT
C
-DOX
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+DOX
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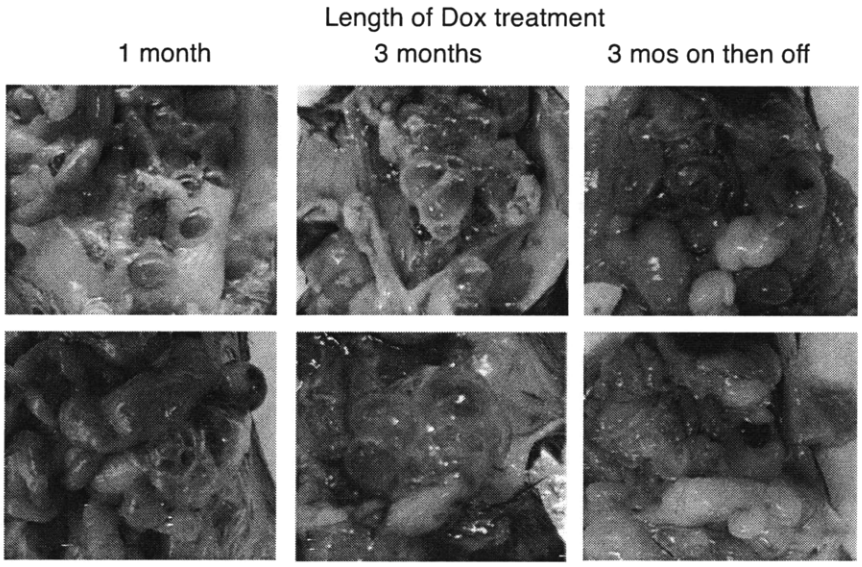
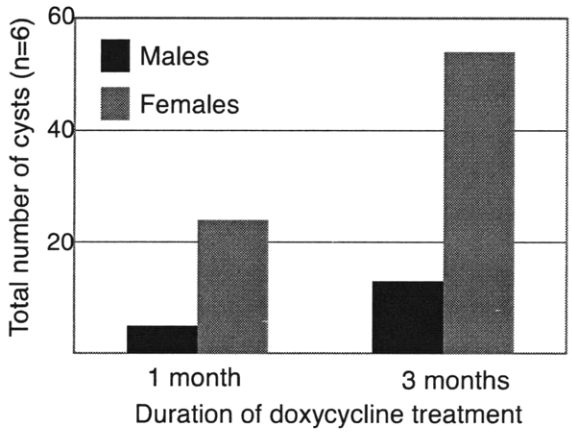
Liver
Intestine
Mesentery
tetRheb2

Figure 2

A


Length of Dox treatment
1 month
3 months
3 mos on then off

B


Total number of cysts (n=6)
60
40
20
Males
Females
1 month
3 months
Duration of doxycycline treatment

C

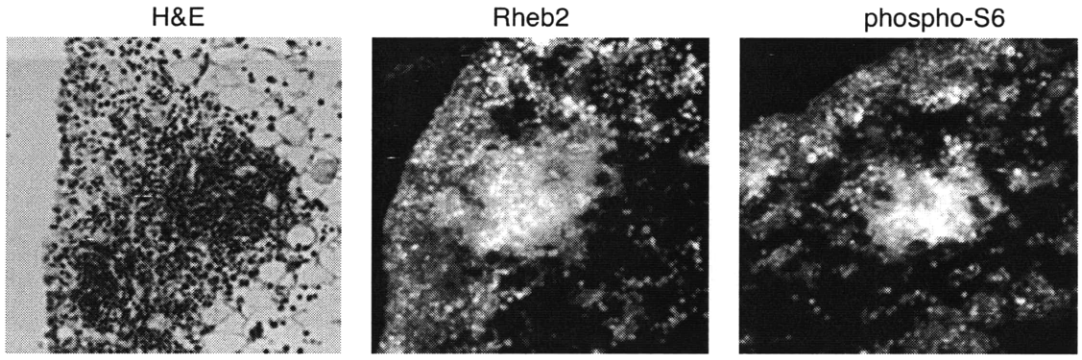
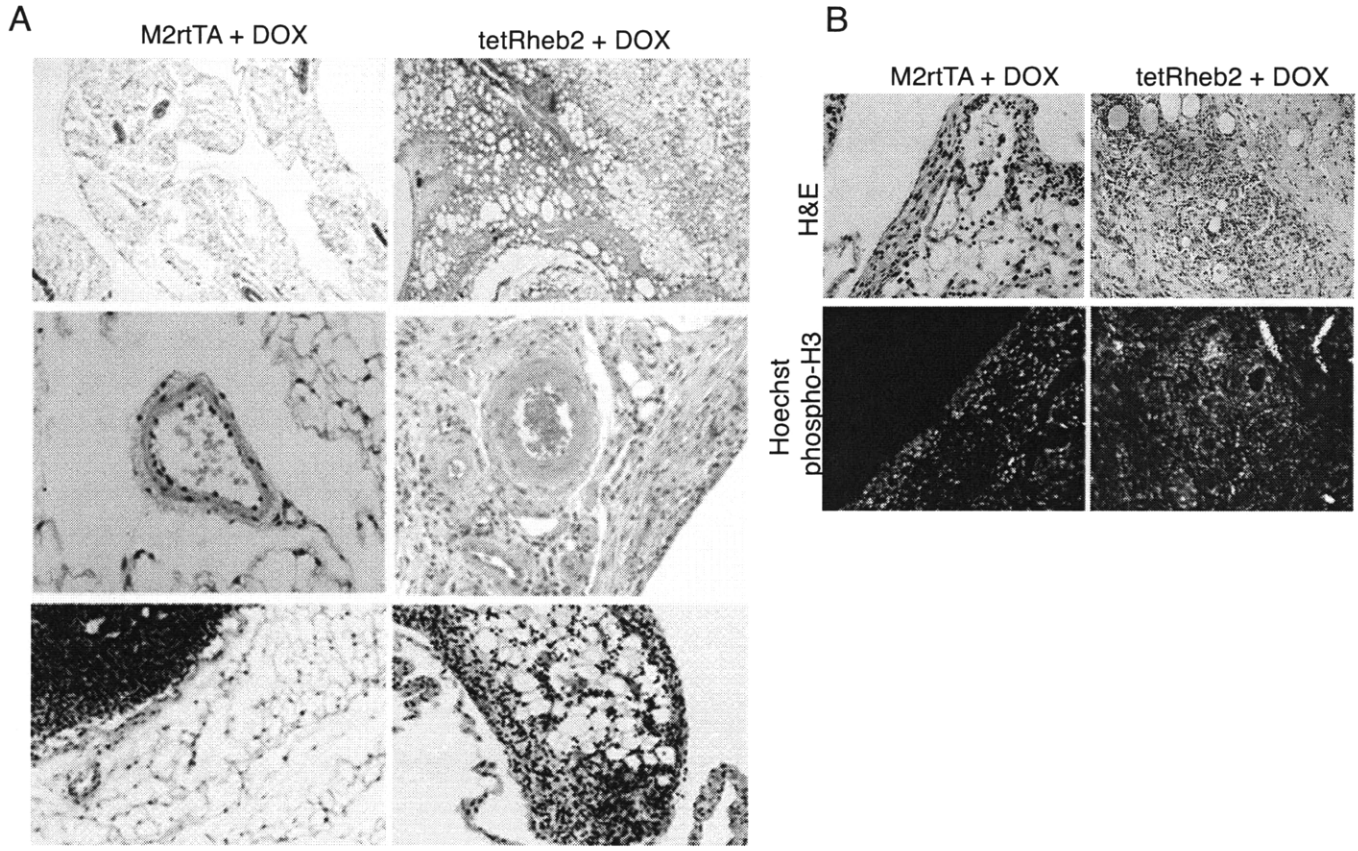

H&E
Rheb2
phospho-S6

Figure 3


A
M2rtTA + DOX
tetRheb2 + DOX
B
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H&E
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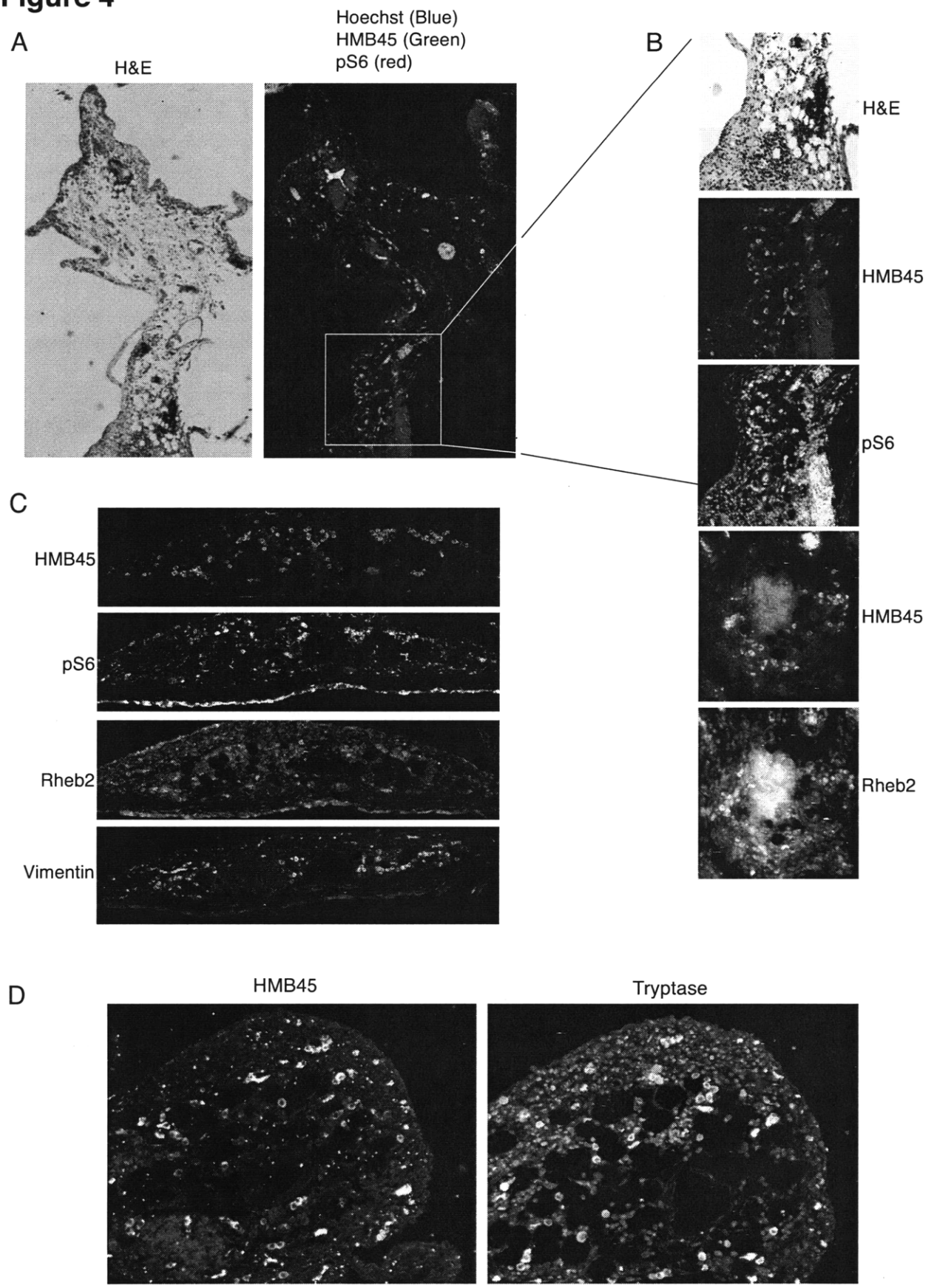
C
Hoechst
SMA
phospho-S6
Merge
M2rtTA + DOX
tetRheb2 + DOX

Figure 4


A
H&E
Hoechst (Blue)
HMB45 (Green)
pS6 (red)
B
H&E
HMB45
pS6
HMB45
Rheb2
C
HMB45
pS6
Rheb2
Vimentin
D
HMB45
Tryptase

Chapter 4

mTOR Complex 1 regulates whole-body growth and nutrient sensing in the postnatal animal

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All experiments were performed by S.S.

Summary

Regulation of cell size impinges upon almost every cellular process, and in multi-cellular organisms, impacts numerous physiological processes including organismal and organ growth. The multi-component kinase mTORC1 coordinates cell size with nutrient intake, stress, growth factor and hormonal input and hypoxia. Reduction in mTORC1 signaling results in reduced organismal size in both Drosophila and mice owing primarily to a defect in cell size. Given the ability to sense nutrients and to regulate organismal growth, we aim to study the affects of mTORC1 on nutrient-regulated postnatal growth and identify tissues that utilize mTORC1 to sense intake of food. We find that inhibition of mTORC1 activity by the drug rapamycin severely diminishes postnatal growth in mice due to a proportional decrease in most organs. This widespread affect is reflected by results indicating that mTORC1 is sensitive to feeding and fasting in almost all tissue types. However, many tissues differ in the nutrient and hormonal stimulus that activate mTORC1 upon feeding. In conclusion, we find that feeding activates mTORC1 in almost every tissue and inhibition of this response in animals leads to pronounced repression in postnatal growth.

Introduction

Both prenatal and postnatal growth are tightly coordinated with nutrient availability. In postnatal growth, the growth hormone (GH)/insulin growth factor-1 (IGF-1) axis is the dominant hormonal controller of body and organ size. While IGF-1 seems to mediate the majority of the growth promoting affects of GH, the two factors do have some independent functions. This is most evident in the fact that a double knockout (KO) of GH and IGF-1 is smaller than either KO alone (Lupu et al., 2001). In addition, the hormone insulin also has growth promoting functions, for deletion of the insulin receptor in specific tissues blocks growth of that respective tissue (Shiojima et al., 2002). Both insulin and IGF-1 levels are affected by dietary intake (Breese et al., 1991), and both activate PI3K signaling via their cognate receptors. The PI3K pathway controls both cell size and number. Down-regulation of the insulin receptor or PI3K in Drosophila results in small flies with fewer and smaller cells (Potter and Xu, 2001), while overexpression or either protein leads to larger flies with more and larger cells (Saucedo and Edgar, 2002). Downstream of PI3K signaling, the control of cell proliferation and size begin to diverge. PI3K activation leads to activation of Akt, mTORC1and numerous other downstream effectors. Manipulation of Akt signaling modulates growth in Drosophila (Verdu et al., 1999), and mice (Chen et al., 2001), although there is tissue-dependent variability effect on cellular proliferation. While the growth promoting actions of Akt is now well established, the contribution of mTORC1 signaling, dependent and independent of Akt, is less clear.

There is increasing evidence for the role of mTORC1 in regulating organ and organismal size. Deletion of the mTORC1 substrate S6 kinase 1 (S6K1) in Drosophila and mice result in decreased body size (Montagne et al., 1999; Shima et al., 1998) due to primarily decreased cell size. Mouse embryonic fibroblasts isolated from S6K1 null embryos exhibited no proliferation defect (Shima et al., 1998) indicating that the control of cell proliferation downstream of PI3K signaling may not go through the mTORC1 branch in most tissues. Whole body deletion of 4E-BP1, another mTORC1 substrate, in mice does not exhibit reduced body or organ size with the exception of adipose tissue (Tsukiyama-Kohara et al., 2001). The results in mice indicate that much of the growth promoting effects of mTORC1 are through its substrate S6K.

As a regulator of cell growth, mTORC1 can respond to not only input from PI3K, but also to nutrients such as amino acids directly. As such, it is important to understand the contribution of each signal to the growth promoting actions of mTORC1 in individual tissues. However, dissecting these multiple inputs into mTORC1 has been a challenge because it was not known which proteins mediate nutrient activation of mTORC1 independent of PI3K activation. As a result, all the mutations in the mTORC1 pathway thus far have targeted components of the pathway that are shared between PI3K and nutrient-stimulated activation. Now, with the discovery that the Rag proteins mediate amino acid stimulation of mTORC1 (Sancak et al., 2008), it will be interesting to see the physiological impact of Rag mutations on postnatal growth.

Here we describe how inhibition of mTORC1 using rapamycin dramatically reduces postnatal growth in mice. However, this reduction in growth by rapamycin is not seen in mice whose postnatal growth has already been blunted due to dietary restriction.

To understand which tissue types mediate the feeding responsive actions of mTORC1, we compared tissues from mice refed for short periods of time following a fast with mice injected with insulin, branched chain amino acids (BAA), glucose or both BAA and glucose following a fast. By quantifying staining of phospho-S6 as a proxy for mTORC1 activity, we were able to determine which tissues activate mTORC1 in response to insulin or nutrients, or a combination of the two. We obtained some surprising and novel results, which allow us to build hypotheses for possible tissue-specific roles for feeding-responsive mTORC1 signaling.

Results

Rapamycin inhibits postnatal growth .

Mice experience postnatal growth from birth to approximately 12 weeks of age, with puberty occurring approximately between week 3 and 4. To determine the consequence of mTORC1 inhibition on early postnatal growth and pubertal growth, rapamycin (10mg/kg in PBS) was administered daily to 3 week old male mice via intra-peritoneal (ip) injection for one entire week. While control PBS-injected mice gained on average 6 grams over the course of the week, rapamycin treated mice only gained an average 3 grams- a 50% decrease (Figure 1A). 3-week-old mice were also placed under dietary restriction for one week, which resulted in no significant weight gain regardless of whether they were treated with rapamycin. This latter result confirms that dietary restriction leads to inhibition of mTORC1 such that rapamycin treatment no longer has an effect on growth (Figure 1A).

After the one-week period, mice were sacrificed and body weight and organs were weighed to measure whether rapamycin inhibited all organ growth proportionally. Rapamycin disproportionately decreases the size of the testes, thymus and spleen compared to PBS-injected controls (Figure 1B). It is unclear whether this reduction in size of these organs is due to cell death, decrease in proliferation, or just decrease in size.

Immunoblots were then performed for phosphorylation of S6, a downstream target of the mTORC1 substrate S6K, to confirm the efficacy of rapamyin inhibition on mTORC1 *in vivo*. As expected, S6 phosphorylation is reduced in all tissues assayed upon rapamycin treatment. Shown here are results for liver, adipose, skeletal muscle, and heart (Figure 1C). When activated, mTORC1 can suppress PI3K signaling as part of a negative feedback loop. As a result, it is expected that rapamycin treatment would abolish this negative feedback loop resulting in increased activation of PI3K signaling as seen by increased phosphorylation of Akt. Consistent with the negative feedback loop, we did see an increase in Akt phosphorylation in many tissues (data not shown). However, in adipose, thymus, lungs and heart, phosphorylation of Akt is blunted upon rapamycin treatment (Sarbassov et al., 2006). This is now understood to be due to chronic rapamycin treatment induced disassembly of mTORC1, which is the hydrophobic kinase for Akt (Sarbassov et al., 2006).

Feeding stimulates mTORC1 in the liver and pancreatic islet cells through different mechanisms.

Having established that mTORC1 inhibition by rapamycin can blunt postnatal growth significantly in essentially every tissue, the question remains- what exactly does

mTORC1 sense in each tissue? Given that mTORC1 has evolved to respond to numerous stimuli such as hypoxia, amino acids, growth factors, and insulin, one may expect that each tissue could utilize mTORC1 sensing and response in a unique manner. To answer this question, we performed a simple screen using wild-type male mice. Mice were fasted for 24 hours, and then refed for 15, 30, and 45 minutes. Tissues were then harvested and stained for phosphorylation of S6- a proxy of mTORC1 activity. As expected, numerous fasted tissues stained for low amount of phospho-S6, and refeeding caused a time-dependent increase in the level of phosphorylation of S6 (Figure 2-4). Pre-treatment with rapamycin before refeeding does block the increase in phospho-S6, thus validating the immunofluorescent staining (data not shown).

To ascertain which aspect of refeeding, the food itself or the subsequent increase in insulin, was directly stimulating mTORC1 in each tissue, fasted mice were given ip injections of glucose, branched-chain amino acids (BAA), both glucose and BAA, or insulin. Mice were then sacrificed at 15, 30 and 45 minutes post injection and levels of phosphorylated S6 were measured in all tissues. The intensity of the phospho-S6 immunofluorscent signal from each treatment was quantified by the image software package CellProfiler (Carpenter et al., 2006), and normalized to levels of phospho-S6 in tissues from fasted mice. We also checked plasma insulin upon injection of BAA, and found levels below 2 ng/ml at all time points, compared to levels near 10 ng/ml reached post refeeding (data not shown). Amino acid injection also did not increase levels of phospho-AKT in all tissues assayed (data not shown). This simple paradigm illuminated a very interesting diversity in stimuli for mTORC1 in individual tissues. In the liver, for example, we find that the refeeding response of mTORC1 can be approximated by either

injection with insulin or BAA, implying that mTORC1 in the liver can respond to both insulin and BAA (Figure 2A). In the pancreatic islet cells by contrast, only a combination of glucose and BAA were able to phenocopy the refeeding-induced increase in phospho-S6, whereas insulin had no effect (Figure 2B).

Feeding stimulates mTORC1 in the small intestine and testes independent of insulin.

The results in the intestine are slightly more complicated. The ip injections of glucose, BAA, the combination of the two, or insulin did not phenocopy the increase in phospho-S6 staining within the epithelial enterocytes upon refeeding (Figure 3A). This result implies that the mTORC1-activating stimulus must either come from the lumen of the intestine, or involve some other aspect of chow not approximated by the injections. However, insulin, glucose, and glucose plus BAA injections did cause an increase in phospho-S6 levels within the paneth cells- found towards the bottom of the intestinal crypts (Figure 3A). This unusual result indicates that the circuitry upstream of mTORC1 can be diverse even within one tissue type. A similar result was seen within the seminiferous tubules of the male testes. Refeeding fasted mice with chow resulted in a robust increase in phospho-S6 levels within the interstitial cells and some of the cells that line the tubules (Figure 3B). The injections were unable to phenocopy this refeeding result, except for a modest increase in phospho-S6 within cells that line the tubules upon BAA injection- with or without glucose (Figure 3B). This finding indicates that the interstitial cells, the majority of which are testosterone-secreting Leydig cells, respond either to a nutrient or combination of nutrients and other factors not mimicked by any of

the injections. This result is particular interesting given the disproportionate reduction in size of the testes caused by rapamycin treatment (Figure 1B).

Feeding stimulation of mTORC1 differs between the spleen and thymus.

Although rapamycin treatment dramatically reduced the size of the thymus and spleen, both tissues seem to regulate mTORC1 differently. In the medulla of the thymus, none of the conditions, with the exception of rapamycin, altered the high level of phospho-S6 staining (Figure 4E). As a result, it seems that mTORC1 phosphorylation of S6K within the thymus is feeding insensitive. The red pulp of the spleen, however, shows dramatic modulation of mTORC1 in response to refeeding following fast. This result can be mimicked by injection of BAA but not glucose or insulin. Similar to the islet cells, it seems that mTORC1 in the spleen is not downstream of insulin signaling, but rather directly or indirectly downstream of amino acid signaling (Figure 4C).

Discussion

The genetic postnatal growth program is tightly coordinated with nutrient intake. Malnutrition leads to stunted growth, whereas an energy rich diet can augment the growth program. Here we find that inhibition of mTORC1, a master regulator of cellular growth, can suppress postnatal growth by mimicking reduced nutrient intake. The reduction in growth affects every tissue, with some tissue being particularly sensitive to mTORC1 inhibition. While we find that eating activates mTORC1 in almost all tissues, there is tissue-to-tissue variability in the activating stimulus. Here we discuss possible functional roles for mTORC1 in tissues where feeding stimulated mTORC1 in unexpected ways.

Pancreatic islet cells

Our findings that glucose and BAA activate mTORC1 in an insulin-independent manner in islet cells confirm earlier reports in both rodent and human islets (Kwon et al., 2004; McDaniel et al., 2002; Xu et al., 1998). However, the role of mTORC1 in islet size and function continues to be actively researched. Mice null for the mTORC1 substrate S6K1 are born with decreased islet mass (Shima et al., 1998). This reduced islet mass correlates with decreased insulin production. Rapamycin inhibits beta-cell proliferation *in vitro* and blocks the effect of Akt activation on beta-cell proliferation (Kwon et al., 2006; Liu et al., 2009). Gain of function mutations in mTORC1 signaling in β-cells leads to hypertrophy, increased insulin synthesis and release, and consequent hyper-insulinemia (Mori et al., 2009). However, mTORC1 does not seem to impinge upon glucose-stimulated insulin secretion. At nanomolar concentrations of rapamycin that inhibit mTORC1 activation by glucose in beta-cells, subsequent insulin secretion is not affected (Kwon et al., 2004). However, rapamycin does not fully inhibit all mTORC1 function (Thoreen et al., 2009), so more research is needed to test the role of mTORC1 in β-cells.

Epithelial cells of the small intestine

The stem cells located within the crypts of the small intestine give rise to 4 different cell lineages. Three of these lineages, absorptive enterocytes, mucus-secreting goblet cells, and hormone-secreting enteroendocrine cells, migrate out of the crypts into adjacent villi and have a relatively short lifespan of 3-5 days. The fourth lineage, paneth cells, migrate to the base of the crypt, survive for over 30 days, and secrete a variety of

anti-microbial peptides and proteins (Huynh et al., 2009). We find that refeeding leads to a robust activation of mTORC1 in all of these cell types. However, the presence of just insulin in the blood is sufficient to activate mTORC1 in the paneth and goblet cells, whereas a luminal signal is probably required for activation of mTORC1 in the absorptive enterocytes. This division in stimuli between the different cell groups could be due to their functional differences. The action of mTORC1 within the absorptive enterocytes could affect nutrient absorption and/or transport of nutrients into the portal circulation. Indeed, rapamycin has been shown to inhibit glucose and fat absorption within the small intestine in rats (Yanchar et al., 1996). The molecular mechanism behind these results is not known. It is also not known whether mTORC1 regulates survival and/or the large amount of protein synthesis found in paneth cells. Recent advances allowing for specific genetic manipulation within the intestinal stem cell niche will hopefully allow more sophisticated analysis of mTORC1 signaling within the small intestine (Barker et al., 2009).

Leydig cells

There have only been a few reports investigating the role of mTORC1 in the male reproductive system. Young male transplant patients who take sirolimus, a rapamycin analogue, have experienced infertility and low sperm count with possible correlation to low testosterone levels. Removal of sirolimus leads to a reverse of all parameters measured, and the accidental pregnancy of the wife of one of the patients (Deutsch et al., 2007). Immunostaining for phospho-S6 was performed in testes of male patients and found positive staining of sertoli and some interstitial cells- staining that was reduced

upon sirolimus treatment (Deutsch et al., 2007). Furthermore, rapamycin can block the testosterone-induced hypertrophy of cardiomyocytes (Altamirano et al., 2009). Metabolic health and reproductive function in mammals are tightly coordinated. As a result, it would not be surprising if mTORC1 played a major role in this coordination- either at the level of reproductive hormones or in the response to androgens.

Thymus/Spleen

The marked reduction in size of the thymus and spleen by rapamycin is not very surprising given the role of rapamycin as a potent immunosuppressant. However, we found the majority of phospho-S6 staining within the red pulp of the spleen- the site of red blood cell filtration and phagocytosis and monocyte reserves (Swirski et al., 2009). Further work is needed to determine how feeding sensitive mTORC1 regulates either of these processes.

In conclusion, we established a potent role for mTORC1 signaling in postnatal growth, and find feeding sensitive mTORC1 signaling in numerous tissues. Administering insulin and nutrients into fasted animals illuminated the wide range of diversity in upstream stimuli for mTORC1 in different tissues. These results are the first step in building hypothesis for tissue specific roles for mTORC1.

Experimental Procedures

Materials

Rapamycin was obtained from LC Labs. Antibodies to phospho-S235/236 S6, phospho-240/244 S6, and total S6 were obtained from Cell Signaling Technology. Cy3 conjugated secondary antibody was obtained from Invitrogen.

Animal work

Rapamycin (1 mg) was dissolved in 20 µl of ethanol, which was then diluted with Ringer's saline solution to a final concentration of 1 mg/ml directly before use. Three-week-old male C57BL/6NTac (Taconic) mice were administered daily intraperitioneal injections of 10 mg/kg rapamycin or the drug vehicle for 7 days. The vehicle- and rapamycin-treated mice ate similar amounts during the 7-day treatment period and at necropsy all mice had evidence of processed food in their stomachs and small intestines. Mice placed under caloric-restriction were fed 60% of their *ad libitum* fed littermates at the onset of the dark cycle each day. For measurement of the refeeding response, mice were fasted for 24 hours, and then refed with normal chow, or injected intraperitoneally with insulin (0.75 U/kg), glucose (750 mg/kg), or branched chain amino acids (Leucine 69 mg, Arginine 30mg, Isoleucine 36 mg per mouse). Mice were then euthanized with C02, and organs were weighed and then fixed in 10% neutral-buffered formaldehyde overnight, or harvested into RIPA buffer and homogenized with mechanical disruption followed by sonication. Lysates from vehicle- and rapamycin-treated organ pairs were normalized for protein content and analyzed by immunoblotting.

Immunofluorescence staining of tissue sections

Fixed tissue was embedded in paraffin and processed onto slides for immuno-staining. Briefly, paraffin-coated sections were de-waxed using EZ-DeWax deparrafinization solution (BioGenex), blocked in PBS with 5% goat serum, stained overnight with antibody at 4° C, and then incubated with Cy3-conjugated secondary antibody. Tiled images were obtained from an inverted epifluorescence microscope (Zeiss) and intensity was quantified using CellProfiler.

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Figure legends

Figure 1. Rapamycin inhibits postnatal growth in a feeding-sensitive manner.
(A) Three-week mice injected daily with rapamycin (10mg/kg) grow only 50% of vehicle-injected control mice over the course of a week ($n=6$, $p < 0.05$). Both groups were allowed to fed *ad libitum* (AL versus AL + Rapa). Age-matched mice fed 60% of fed controls (CR) show no significant gain in weight over the course of the week. Injection of mice under CR with rapamycin (10 mg/kg) results in no significant additional decrease in body weight ($n=5$, $p > 0.05$). (B) Individual organs from vehicle or rapamycin treated AL mice were weighed, and calculated as percent of total body weight. Values obtained from individual mice were averaged and plotted as percent of total body weight on a logarithmic scale. All organs weighed from rapamycin-treated mice maintained their proportionality except for the spleen, thymus and testes. (C) Rapamycin treatment blocks the feeding-induced increase in phospho-S6 in tissues. Protein lysates were generated from tissues from mice fasted 24 hours, and then injected with PBS or Rapamycin two hours before refeeding for 45 minutes. Normalized lysates were immunoblotted for levels of total and phosphorylated S6 (serine 235/236). Shown are representive samples from liver, white adipose tissue (WAT), skeletal muscle, and heart.

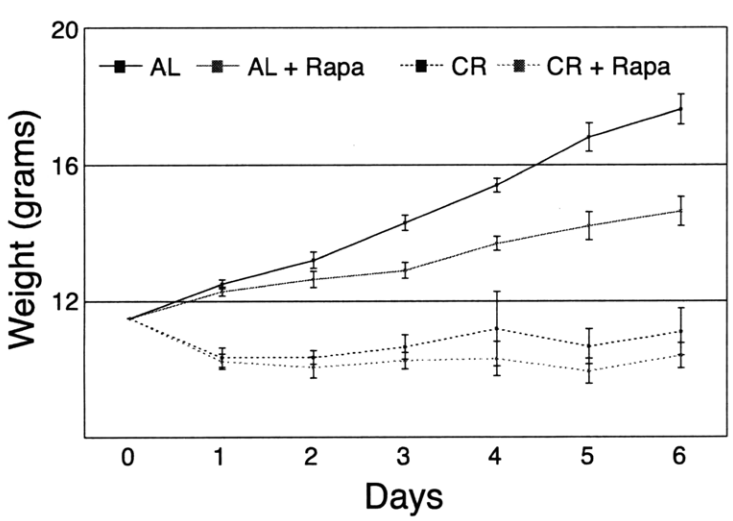
Figure 2. The liver and islet cells of the pancreas upregulate mTORC1 in response to food.
(A) Refeeding following a 24 hour fast leads to a robust increase in levels of phospho-(serine 235/236)-S6 in liver as shown by immunofluorescent staining. Injection of fasted mice with insulin or BAA also leads to a marked increase in phospho-S6 levels, although a bit lower than refeeding. Glucose causes a modest increase in phospho-S6 levels. Quantification of average phospho-S6 intensity over three replicates. (B) The islet cells of the pancreas also show a dramatic increase in phospho-S6 levels upon refeeding. Injection with insulin has no effect on phospho-S6 levels, but the combination of glucose and BAA raises phospho-S6 levels to similar levels as refeeding. Quantification of phospho-S6 immunofluorescent staining is shown.

Figure 3. Enterocytes of the small intestine and cells of the testes show feeding-sensitive mTORC1 activity.
(A) Phospho-S6 levels are undetectable in fasted mice, and increase within 45 minutes or refeeding in all cells of the small intestine. Injection of insulin only increases phospho-S6 in paneth and goblet cells. Injection of glucose mimics the insulin response to a degree. Injection of BAA has no effect on phospho-S6 levels in the small intestine. (B) Interstitial cells of the seminiferous tubules also show a large increase in phospho-S6 upon refeeding. No other treatment mimics this refeeding response. Injection of glucose and BAA activates selected sertoli cells inside the tubules, but has little effect on the interstitial cells.

Figure 4. Quantification of phospho-S6 intensity under different conditions in multiple tissues.
(A) Skeletal muscle and heart (B) both show refeeding sensitive phospho-S6 staining and insulin, glucose and BAA all are able to increase phospho-S6 staining in the fasted animal. (C) The red pulp of the spleen shows an almost 4-fold increase in phospho-S6 staining upon refeeding following fast. Insulin had no effect on phospho-S6 staining compared to fasted mice, whereas BAA with or without glucose increased phospho-S6 staining to levels comparable to refeeding with chow. (D) The cortex of the kidney and the medulla of the thymus (E) both show robust staining of phospho-S6 under all conditions with modest changes induced by other treatments.

Figure 1

A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CR + Rapa
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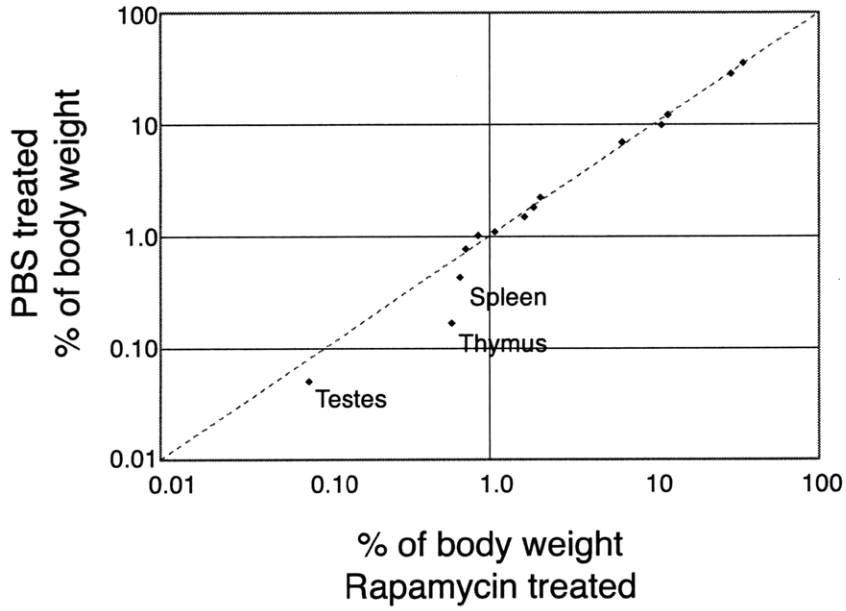
PBS treated
% of body weight
100
10
1.0
0.10
0.01
Spleen
Thymus
Testes
0.01
0.10
1.0
10
100
% of body weight
Rapamycin treated

C

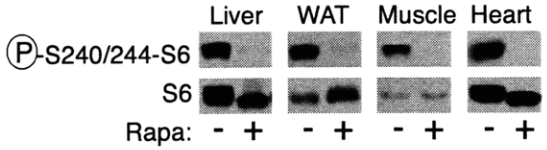
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Figure 2

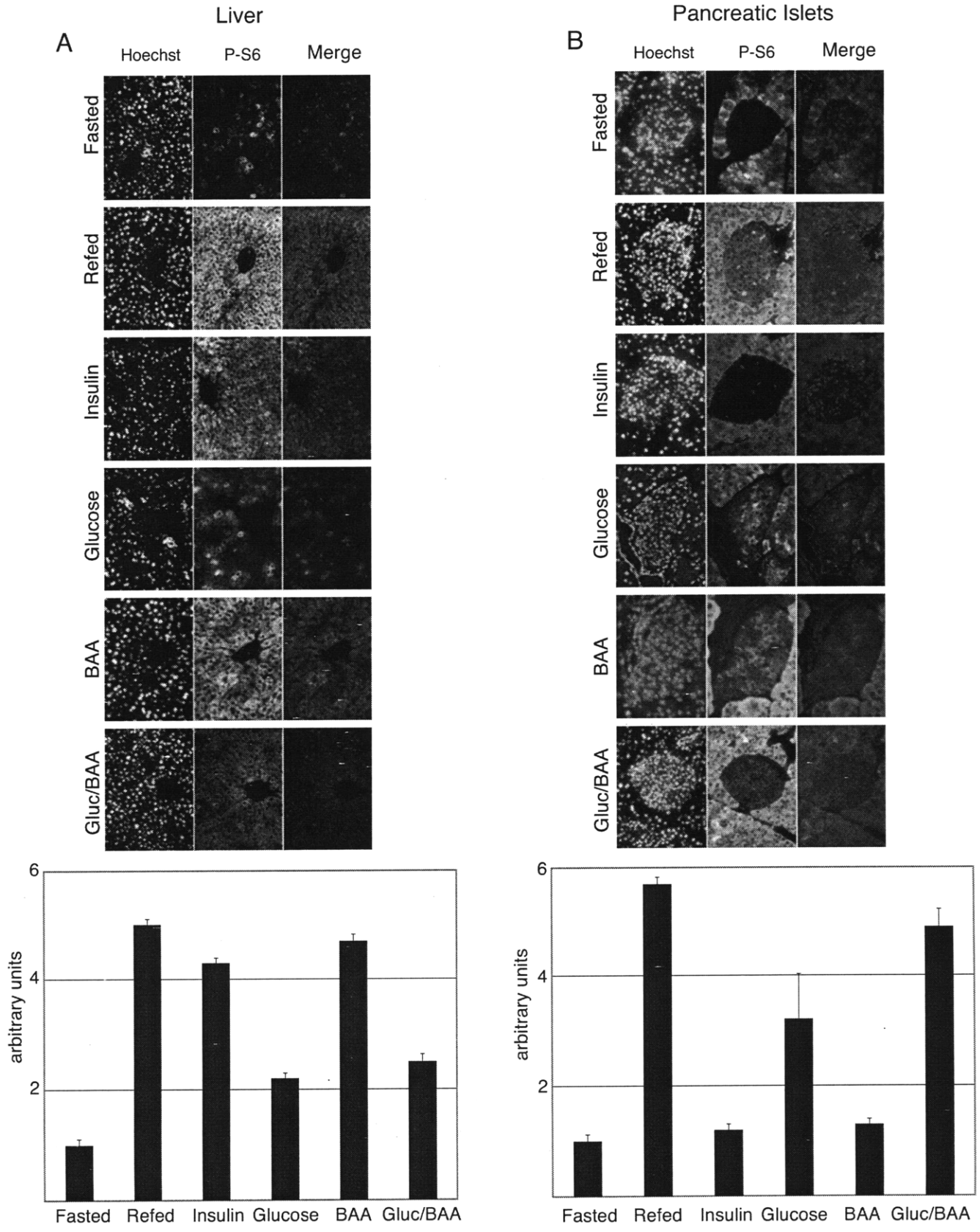
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Figure 3

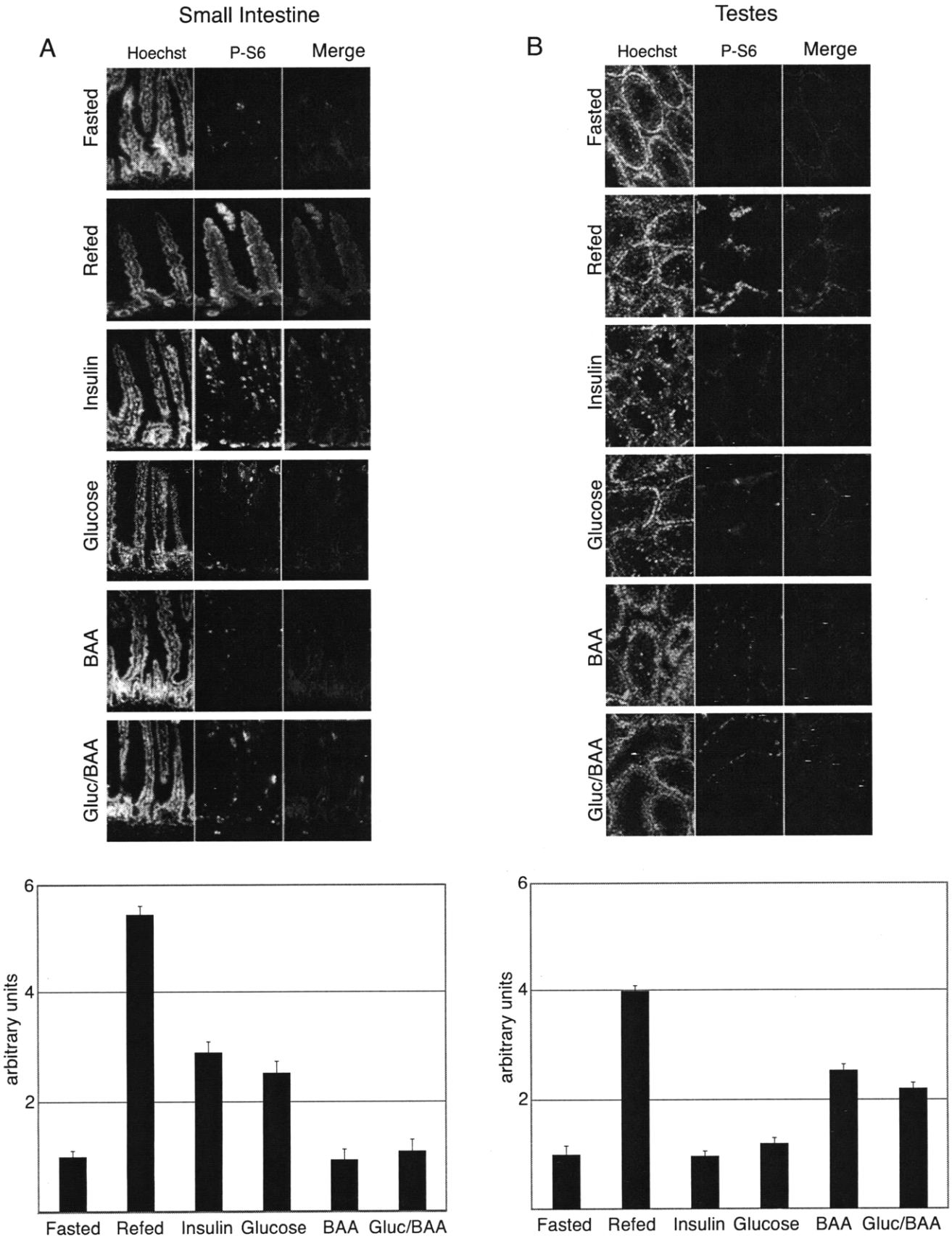
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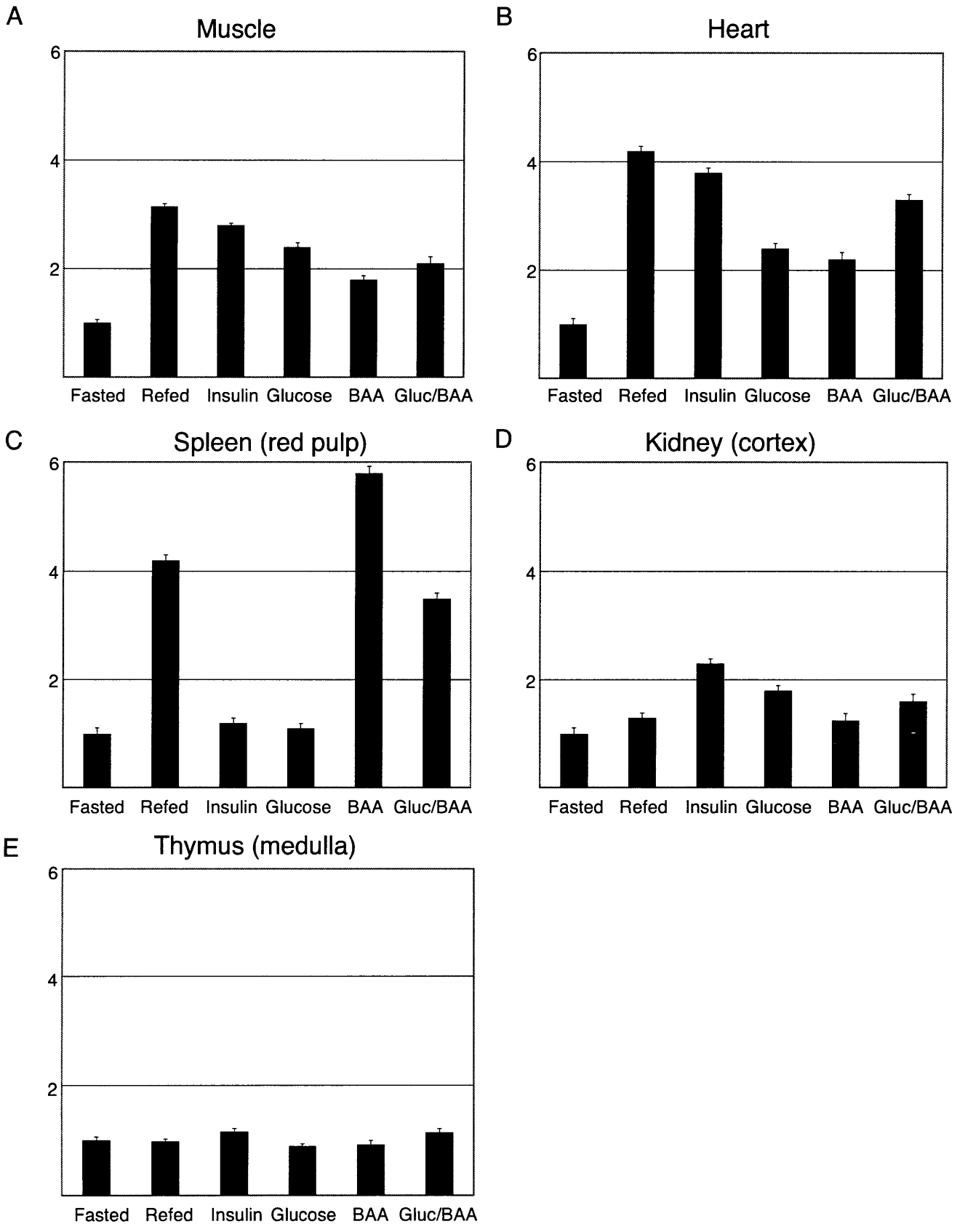
Figure 4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Chapter 5

Discussion and Future Directions

Summary

The multi-component kinase mTORC1 coordinates nutrient and growth factor inputs with numerous downstream processes including protein translation, autophagy, metabolism and cell growth. The work described here focuses on tissue-specific functions of mTOR complex 1 (mTORC1). We found that inhibition of mTORC1 suppresses whole-body postnatal growth similar to the effect of reduced caloric intake. We found that while feeding activates mTORC1 in almost every tissue, the upstream activating stimuli varied from tissue to tissue. Studies we performed using liver-specific mTORC1 gain and loss of function mutants further elucidated the role of mTORC1 in organ growth. Confirming our studies with rapamycin, we found that genetic activation or suppression of mTORC1 in the liver increases and decreases liver size respectively. Moreover, both mutant livers are resistant to fasting-induced reduction in mass. Rendering the liver insensitive to nutrients through liver-specific mTORC1 activation and suppression also had functional consequences. In response to starvation, a normal liver shifts to fatty acid catabolism and generates ketone bodies that supplement lowered glucose levels for use by peripheral tissues. We find that constitutive activation of mTORC1 prevents the liver from initiating fatty-acid oxidation and ketone production in response to fasting. This reduction in fatty acid catabolism and subsequent synthesis of ketones resembles malfunctions in the hepatic fasting response associated with aging. We find this aging-dependent process is mediated by mTORC1 such that loss of mTORC1 function throughout an animal's adult life prevents the aging induced decrease in ketogenesis upon fasting. This finding indicates that pharmaceutical manipulation of

mTORC1 may be beneficial in battling metabolic disorders due to disease and aging. The disease TSC in humans is initiated by loss of TSC1 or TSC2, which also results in hyper-activation of mTORC1. The disease can involve development of multiple lesion types including brain harmatomas, soft tissue angiomyolipomas (AMLs), and the destructive lung lesion lymphangioleiomyomatosis (LAM). We engineered transgenic mice that express Rheb2, an mTORC1 activator, in a doxycycline-inducible manner. Over-expression of Rheb2 led to cystic growths with characteristics of both LAM and AML. We believe this transgenic mouse provides an important platform for studying the pathology behind these lesions and a mouse model for therapeutic intervention for TSC. The following section explores some of the new questions that have emerged from this collective body of work.

Does mTORC1 have non-autonomous roles in postnatal growth?

We find that rapamycin dramatically inhibits the growth of 3 week old male and female mice. All tissues retained the same proportion to whole-body size except the spleen, thymus and testes, which were disproportionately smaller in rapamycin-treated mice. However, the use of rapamycin is problematic, for chronic administration disassembles mTORC2 in some tissues (Sarbassov et al., 2006). As a result, we find lowered phosphorylation of AKT in white adipose tissue, thymus, lung, and heart. Therefore, we cannot rule out the possibility that inhibition of AKT contributed to the growth defect in these tissues. These results raise the question of whether suppression of mTORC1 affects the GH/IGF-1 axis. The levels of both hormones decrease in response to dietary restriction (Breese et al., 1991) through an unknown mechanism. Does

mTORC1 regulate levels of either of these hormones in response to dietary restriction? Given that GH stimulates transcription and secretion of IGF-1 from the liver (Lupu et al., 2001), we examined the effect of TSC1 loss in the liver in 19 day-old animals. After injecting mice with adenovirus expressing cre at day 19, we studied them for 3 weeks, measuring body weight every 3 days. At the end of the 3 week period, mice were weighed and sacrificed. Both female and male LiTsc1KO mice had 40% larger livers than control mice. Loss of hepatic TSC1 in young male mice increased their growth rate by an average of 14% as measured by total weight gain. However, the growth rate did not increase for young female LiTsc1KO mice. Further examination of livers from male LiTsc1Ko mice found no change in transcription of IGF-1, IGF1-binding protein 5 (IGFBP5), or acid labile subunit (ALS). The latter two genes encode for serum IGF-1 binding proteins. Serum levels of IGF-1 between control and young LiTsc1KO mice were also similar. This preliminary finding suggests that hyperactive hepatic mTORC1 signaling may increase postnatal growth rate in a non-autonomous manner. This experiment should be repeated, with a more careful analysis of PI3K signaling in peripheral tissues and a more complete work-up of plasma metabolites.

What is the role of mTORC1 in pubertal growth?

Puberty is controlled by signals from the hypothalamus, pituitary and the gonads making up the gonadotropic axis (Tena-Sempere, 2005). The onset of puberty and the accelerated growth rate associated with puberty are both affected by nutritional status (Leve et al., 2007). For example, dietary restriction in female rodents results in lower luteinizing hormone (LH) and estrogen levels (Roa et al., 2009). Artificial stimulation of

hypothalamic mTORC1 signaling partially rescues decreased (LH) secretion from the pituitary in dietary restricted animals (Roa et al., 2009). We found that rapamycin treatment dramatically reduces testes size in pubescent mice. Additionally, we observe feeding sensitive phospho-S6 staining of the interstitial cells of the seminiferous tubules- the location of testosterone secreting Leydig cells. What is the function of feeding-sensitive mTORC1 signaling within the gonads? Does it coordinate nutrient status with LH stimulated testosterone release from the gonads? Androgens are synthesized from cholesterol, and reports have shown an important role for mTORC1 in both sterol sensing and cholesterol biosynthesis (Porstmann et al., 2008); Peng T, unpublished). The recent development of the Leydig cell line (DuMond et al., 2006) allows for *in vitro* studies of mTORC1 signaling on testosterone release in response to LH. Conditional Raptor or Tsc1 mice crossed with mice expressing cre recombinase from a Leydig cell or ovarian-specific promoter could also genetically test the role of mTORC1 in androgen release. Alternatively, rapamycin therapy in mouse models for hypergonadism could be explored. Data showing that testerone-induced hypertrophy of cardiomyocytes is mediated by mTORC1 would indicate that rapamycin would suppress precocious onset of puberty and accelerated growth rate due to hypergonadism (Altamirano et al., 2009). We hope our preliminary findings coupled with published data will initiate research on how mTORC1 regulates the production and secretion of humoral growth-promoting factors from the liver and gonads during postnatal development.

How does mTORC1 regulate transcriptional changes?

We describe a novel link between mTORC1 and PPARα transcriptional activity. In addition to our work, there are numerous reports linking changes in mTORC1 signaling with transcriptional changes. All of these reports raise the question of how a cytoplasmic kinase complex affects gene expression changes in the nucleus? One possibility is that mTORC1 is not exclusively cytoplasmic, but has a nuclear presence. Research from the Puigserver lab explained rapamycin's reduction of PGC-1α transcriptional activity by describing mTOR and Raptor binding to the transcription factor YY1 (Cunningham et al., 2008). We describe a different mechanism for mTORC1 regulation of transcriptional activity. We find that mTORC1 regulates PPARα transcriptional activity by modulating the localization of its transcriptional co-repressor NCoR from the cytoplasm to the nucleus. However, we were unable to decipher the exact mechanism behind this localization change.

How does aging affect mTORC1 signaling?

We find hyperactivation of NCoR by mTORC1 also occurs naturally in livers from old mice and correlates with decreased PPARα activity. Accordingly, loss of hepatic mTORC1 signaling prevents aging induced malfunctions in liver metabolism. Our data complements recent studies describing extended lifespan in animals via decreased mTORC1 signaling (Harrison et al., 2009; Selman et al., 2009). While our understanding of aging is constantly evolving, the role of aging in inducing epigenetic and gene expression changes is well established (Gravina and Vijg, 2009). Does mTORC1 play a role in these aging induced changes in gene expression and chromatin

remodeling? Although this question can be approached either through a top-down or bottom-up strategy, we chose the latter by first identifying genes whose expression was altered by mTORC1 signaling and then studying proteins that regulate promoter activity of these genes. An alternate approach would be to manipulate downstream substrates of mTORC1. This would require a system where combination genetics could be employed. For example, we could establish AML12 cells with stably expressed shRNAs to both TSC1 and S6K1 and measure induction of PPARα transcriptional activity in our ketogenic media. Restoration of PPARα activity in these double knockout cells would indicate that mTORC1 regulates NCoR localization via S6K1. This method is complicated by the presence of multiple S6Ks and 4EBPs. However, given that deletion of only S6K1 was sufficient to extend lifespan, such an approach could generate interesting results. To further study the molecular mechanism of how mTORC1 regulates aging, cells with premature or advanced aging mutations could serve as a valuable *in vitro* system. For example, numerous studies have been performed in fibroblasts from patients suffering from Hutchinson-Gilford progeria syndrome or restrictive dermopathy. In addition, analogous mutations have also been made in mice to model both of these human diseases. Both of these reagents could provide insight into the role of mTORC1 in aging. Indeed, a report last year reported that the premature aging found in a mouse model of restrictive dermopathy correlates with induction of autophagy due to mTORC1 inhibition (Marino et al., 2008).

Is there crosstalk between mTORC1 and SIRT1?

The sirtuin family of proteins actively regulates aging in yeast, flies and mammals. With work showing the role of mTORC1 in aging in yeast, flies and mammals as well, we believe future research should explore convergence between these two pathways. SIRT1 intersects with PPAR signaling in multiple ways. SIRT1 increases oxidative metabolism in liver by deacetylation of PGC-1∝ and binding with PPAR∝ (Purushotham et al., 2009). Additionally, SIRT1 represses PPARγ signaling in white adipose tissue by docking with NCoR (Picard et al., 2004). The relationship between SIRT1 and mTORC1 regulation of PPAR∝ needs further research. Our analysis of LiTsc1KO mice confirmed earlier reports that loss of Tsc1 results in elevated levels of PGC-1∝ and increased mitochondria number (Cunningham et al., 2008). Given that PGC-1∝ is a well-established co-activator of PPAR∝, we were surprised to find that despite elevated levels of PGC-1∝, PPAR∝ activity was suppressed in Tsc1 null livers. One possible explanation is that mTORC1 suppresses PPAR∝ via inhibition of SIRT1, which results in constitutive NCoR promoter presence. We measured SIRT1 levels in wild-type and Tsc1 null livers under fed and fasted conditions and found no differences. Additionally, we measured levels of acetylated-p53 as a proxy for SIRT1 activity, but the results were inconclusive. Whether overexpression of SIRT1 or treatment with resveratrol, a SIRT1 activator, in AML12 cells deficient for TSC1 rescues the ketogenic defect should be tested.

What is the role of mast cells in LAM?

Our current work describes a correlation between the presence of mast cells and formation of AML and LAM like lesions in our Rheb2 transgenic mice. However, we

are unable to determine whether mast cells contribute to the formation of these growths or are part of an inflammatory response triggered by the growths. To test this, transgenic mice can be crossed to mast cell deficient c-kit$^{W\text{-}sh/W\text{-}sh}$ mice (Wolters et al., 2005). Overexpression of Rheb2 in mast cell deficient mice will answer whether mast cells contribute to the formation and/or progression of the lesions. We could also perform the reciprocal experiment by isolating mast cells from Rheb2 transgenic mice, and injecting them into the mesentery or lungs of immuno-compromised mice (Sonoda et al., 1986). Administration of doxcycyline to the resulting mice to increase Rheb2 expression will determine whether mast cells with hyper-active mTORC1 signaling are sufficient to induce formation of cysts and proliferation of adipose and smooth muscle cells. Finally, there are multiple inhibitors that inhibit mast cell degranulation (Erin et al., 2006). We could test whether inhibition of mast cell degranulation would suppress tumor and cyst formation in the Rheb2 transgenic mice.

Conclusions

The last fifteen years have seen an explosion of research projects aimed at elucidating mTOR biology. Recent research projects examining mTOR function within the intact animal have produced dramatic results and many more questions. With so many cellular processes linked to mTOR activity, it is likely that additional substrates of mTOR have yet to be identified. The search and identification of new direct substrates of mTOR will hopefully answer many mechanistic questions within the field. It may also help identify whether activation of mTORC1 by nutrients extends beyond amino acids. Specifically, given our work and others' on the role of mTORC1 in regulating the PPAR

family of transcription factors, it is apparent that mTORC1 senses fatty acids and lipids as nutrients. If this is true, it brings up numerous questions of how mTORC1 impinges on the numerous functions of fatty acids, lipids and membranes within the cell and within the organism. There are also efforts to generate additional pharmaceutical inhibitors of mTOR. Use of these new compounds in both *in vivo* and *in vitro* studies will be a valuable new tool to probe mTOR function. Every cellular process uses energy; therefore the sensing and communication of energy status is fundamental to all aspects of biology. The study of TOR, a master nutrient sensor, serves as a great vantage point by which to then study the field of biology.

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