GLUCOSE STARVATION INDUCES APOPTOSIS OF TSC-/- CELLS IN A P53-DEPENDENT MANNER

by

Chung-Han Lee

A dissertation submitted in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
(Biological Chemistry)
in The University of Michigan
2007

Doctoral Committee:

Professor Kunliang Guan, Chair
Professor Tom K. W. Kerppola
Professor Benjamin L. Margolis
Professor Cun-Yu Wang
Associate Professor Anne B. Vojtek

Table of Contents

List of Figures

Chapter I: The mTOR Pathway

Regulation of cell size is a fundamental question in biology. Cell size monitoring and regulation are still not well understood mechanisms, but it is clear that these processes are tightly regulated, as under normal conditions specific cell types within a tissue are rather uniform in size. Recent work has shown that one of the pathways integral to the regulation of cell size is the mammalian target of rapamycin (mTOR) pathway. Activation of this pathway leads to increases in cell size, while inhibition of this pathway leads to decreases in cell size. Consequently, changes in size at the cellular level lead to changes at the macroscopic level, which can manifest as either hypertrophy or atrophy. This review covers some of the biochemical regulation of the mTOR pathway which may be important to the regulation of cell size, and it will present several potential clinical applications where the control of cell size may be biologically significant, such as muscle development and diabetes progression.

Pathway overview of mTOR/Raptor regulation

mTOR serves as a signal integrator of several upstream signals including growth factors, nutrients, energy levels, and stress(Inoki et al. 2005). Consequently, one of the critical functions of mTOR is to integrate these signals into a decision to positively or negatively influence cellular growth and proliferation, in other words size and rate of replication.

Tumor Suppressors TSC1 and TSC2 on mTOR

Most upstream regulators of mTOR appear to function through the tumor suppressors Tuberous Sclerosis Complex 1 (TSC1) and Tuberous Sclerosis Complex 2 (TSC2). TSC1 and TSC2 form both a physical and functional complex, where mutation of either protein is sufficient to release mTOR from negative regulation. Functionally, TSC1 is thought to be the regulatory component, while TSC2 is thought to be the catalytic component. TSC1 has no obvious catalytic domain, but it contains a coiled-coiled domain (van Slegtenhorst et al. 1997). TSC2, on the other hand, shows a c-terminal homology with Rap1GAP(Wienecke, Konig & DeClue 1995). In $TSC1^{-/-}$ MEFs, TSC2 levels are substantially decreased(Kwiatkowski et al. 2002); however, $TSC2^{-/-}$ MEFs do not show significant reductions in TSC1 levels(Zhang et al. 2003). It has been suggested that TSC1 levels are not significantly affected by the loss of TSC2 because TSC1 is capable of forming stable homodimers, while TSC2 does not(Nellist et al. 1999). A possible mechanism by which TSC1 may stabilize TSC2 is through exclusion of the ubiquitin E3 ligase HERC1(Chong-Kopera et al. 2006). HERC1 binds to TSC2 and destabilizes it; however, in the presence of

TSC1, HERC1 is unable to bind to the TSC1/TSC2 complex. Consequently, the stability of TSC2 is increased in the presence of TSC1. Another potential E3 ligase for TSC2 is protein-associated with Myc (PAM), which associates with TSC1/TSC2 in neurons and contains a Ring Zinc Finger. Although PAM negatively regulates TSC1/TSC2, it has not been shown that PAM specifically modulates TSC2 stability(Murthy et al. 2004). In *Drosophila* S2 cells, knockouts of *TSC1* also demonstrate significant reductions in TSC2 levels; however, unlike the results seen in mice, knockout of *TSC2* also decreases the levels of TSC1(Gao et al. 2002). Despite the differences seen in mice and *Drosophila*, it is clear that both proteins are necessary for the proper regulation of mTOR. Therefore, loss of either TSC1 or TSC2 is generally considered to have similar effects on mTOR regulation.

Rheb GTPase

Although mTOR is tightly regulated by TSC1/TSC2, this regulation is indirect. Instead, TSC1/TSC2 regulates mTOR via the Ras-like GTPase, Rheb (Ras homolog enriched in brain). Rheb is a member of the Ras superfamily of GTPases; however, it is unique because it has low intrinsic GTPase activity. Therefore, the majority of Rheb is found in the GTP-bound form. Biochemically, TSC2 negatively regulates mTOR by functioning as a GTPase Activating Protein (GAP) for Rheb, thereby inactivating it(Castro et al. 2003, Garami et al. 2003, Inoki et al. 2003, Li, Inoki & Guan 2004, Tee et al. 2003, Zhang et al. 2003). However, the relationship between Rheb-GTP and its effector mTOR is unique because both Rheb-GDP and the nucleotide-free form of Rheb bind to mTOR

more strongly than Rheb-GTP(Long et al. 2005a, Long et al. 2005b, Smith et al. 2005). Consequently, it is still a matter of debate whether the activation of mTOR by Rheb is direct. Recently, it has been suggested that Rheb may directly activate mTOR. By using mutants of Rheb with different GTP loading percentages, it was shown that although nucleotide-free Rheb bound to mTOR more strongly than wild-type Rheb, the bound mTOR displays low in vitro kinase activity against S6 Kinase 1 (S6K1), a direct target of mTOR. On the other hand, a Rheb mutant that was almost entirely associated with GTP showed greater in vitro mTOR activity against S6K1 than wild-type Rheb(Long et al. 2005a). In *S. pombe*, it was shown that hyperactive mutants of Rheb with high GTP binding are able to induce a phenotype similar to loss of TSC1 or TSC2. Compared to wild-type Rheb, these mutants had enhanced affinity for tor2, one of the two yeast homologues of mTOR(Urano et al. 2005). However, it has yet to be demonstrated that these Rheb interactions occur in a similar fashion in vivo in higher eukaryotes. Additionally, a guanine nucleotide exchange factor (GEF) for Rheb still remains to be identified; however, it has also been suggested that on account of Rheb's low intrinsic GTPase activity, it is possible that Rheb may not have an associated GEF(Li et al. 2004, Manning, Cantley 2003).

AKT as an upstream regulator of mTOR

Both growth factor and energy level stimulation influence mTOR activity through TSC2-Rheb(Garami et al. 2003, Tzatsos, Kandror 2006). Growth factor stimulation such as insulin and IGF-1 primarily regulates mTOR signaling through PI3K-AKT. Activation of the insulin receptor leads to the activation of

phosphatidylinositol 3-kinase (PI3K), which increases levels of PIP_3 and leads to activation of AKT. Overexpression of either active PI3K or myr-AKT, an active form of AKT, leads to increased phosphorylation of both eukaryotic Initiation Factor 4E Binding Protein 1 (4EBP-1) and S6K1, which are the two major targets of mTOR in translation regulation. However, this phosphorylation of 4EBP-1 and S6K1 can be inhibited by rapamycin, which is a specific inhibitor of mTOR. This inhibition by rapamycin can be rescued by coexpression of rapamycin-resistant mTOR mutants(Gingras et al. 1998). The regulation of mTOR by PI3K-AKT occurs primarily through the phosphorylation of TSC2. The loss of the AKT phosphorylation sites on TSC2 increases the ability of TSC2 to inhibit mTOR, and consequently leads to increased S6K phosphorylation(Inoki et al. 2002, Manning et al. 2002, Potter, Pedraza & Xu 2002). Phosphorylation of TSC2 by AKT increases mTOR activity, and prevention of TSC2 GAP activity toward Rheb is necessary for the activation of mTOR. However, it remains to be shown that phosphorylation by AKT directly modulates TSC2 GAP activity. A recent report suggests that AKT regulates TSC2 activity by altering its localization. In its hypophosphorylated form, TSC2 is associated with TSC1 at the membrane; however, upon phosphorylation by AKT, it is translocated away from the membrane without changing its intrinsic GAP activity toward Rheb. Bound by 14-3-3 proteins, AKT-phosphorylated TSC2 localizes to the cytosol, where physical separation prevents the inactivation of Rheb that is membrane associated(Cai et al. 2006). It is also interesting to note that conflicting reports exist regarding the role of TSC2 in mediating signaling between AKT and dTOR.

In one report, TSC2 does not appear to be important for AKT to regulate dTOR. In *Drosophila* S2 cells, phospho-mimetic mutations of the AKT phosphorylation sites on TSC2 (AA and DE) had no effect on binding to TSC1 or activation of S6K in response to insulin stimulation as compared to wild-type TSC2. Additionally, mutation of the AKT phosphorylation sites on TSC2 had no effects on *Drosophila* development(Dong, Pan 2004). However, another group has suggested that AKT, TSC2, and dTOR behave more similarly to their mammalian counterparts, where phosphorylation by AKT changes TSC2 localization and affinity for TSC1. Additionally they also showed that the AKT phosphorylation sites are important for the regulation of cell size in the *Drosophila* eye(Potter, Pedraza & Xu 2002); therefore more studies are needed to better understand the relationship between AKT and TSC2 in the regulation of the mTOR pathway.

Downstream Targets of mTOR

Downstream of mTOR, two of the most well characterized targets are S6K1 and 4EBP-1(Inoki et al. 2005). As a result of tight regulation of these two proteins by mTOR, they are often used as functional readouts of mTOR activity. S6K1, which is phosphorylated and activated by mTOR, phosphorylates the ribosomal S6 protein. S6 is a component of the 40S ribosomal subunit. Activation of S6 leads to increased ribosomal biogenesis; however, interestingly enough, mutational loss of the S6K1 phosphorylation sites on S6 leads to increased global protein translation without increasing the percentage of ribosomes engaged in the polysomes(Ruvinsky et al. 2005). On the other hand, 4EBP-1 is inactivated by mTOR phosphorylation. 4EBP-1 in its

hypophosphorylated form binds to and inactivates eIF4E, which is responsible for CAP-dependent translation(Gingras et al. 2001). Therefore, inactivation/phosphorylation of 4EBP-1 by mTOR increases CAP-dependent protein translation.

Raptor as an essential component of the TORC1

In order for efficient phosphorylation of S6K1 and 4EBP-1, these downstream targets must associate with raptor, a scaffolding protein. However, the precise mechanism by which raptor mediates efficient phosphorylation between mTOR and its downstream targets is still not completely understood. Two models have been proposed to explain the mechanism by which raptor mediates signaling downstream of mTOR. The first model suggests that raptor and mTOR associate in two states with varying affinities, one that binds tightly and one that binds loosely. The loose-binding complex is the active complex and promotes efficient phosphorylation of mTOR targets; however, the tight-binding complex is formed in nutrient-poor conditions and inhibits mTOR kinase activity. Furthermore, overexpression of raptor increases the amount of mTOR found in the tight-binding complex, thereby explaining the observation that overexpression of raptor inhibits mTOR activity. However, it is interesting to note that rapamycin is able to disrupt the raptor-mTOR interaction regardless of nutrient status(Kim et al. 2002), but it is phosphate-dependent. The second model suggests raptor simply acts as a scaffolding protein. However, raptor preferentially binds to unphosphorylated forms of mTOR targets and recruits the substrates to the mTOR complex for phosphorylation. Stimulation by insulin decreases the

amount of raptor that can be co-immunoprecipitated with 4EBP-1, and mutation of the mTOR phosphorylation sites on 4EBP-1 to alanines increases the binding of 4EBP-1 to raptor, while mutation to glutamic acid reduces the binding to raptor(Hara et al. 2002). Two motifs on the substrates are important for activation by mTOR, the Tor Signaling (TOS) motif and the RAIP (named by its sequence) motif. The TOS motif is believed to be the site by which the mTOR/raptor complex interacts with its downstream target(Nojima et al. 2003, Schalm et al. 2003, Schalm, Blenis 2002) However, it appears that the RAIP motif operates via promotion of mTOR dependent phosphorylation(Beugnet, Wang & Proud 2003, Tee, Proud 2002).

Disruption of mTOR/raptor by rapamycin

The study of the mTOR pathway has been greatly facilitated by the availability of a specific and potent inhibitor, rapamycin. Rapamycin was originally identified in *Streptomyces hygroscopicus*, and to date there are no other known targets for rapamycin. This specificity is conferred by the use of an intermediary to inhibit mTOR. Rapamycin first complexes with the immunophillin FK506 binding protein 12 (FKBP12), and the rapamycin-FKBP12 complex binds and inhibits mTOR(Sabatini et al. 1994). As the name suggests, FKBP12 also binds to FK506, an inhibitor of the calcineurin pathway. In the presence of both FK506 and rapamycin, there is competition for binding to FKBP12; therefore, in large excess of FK506, rapamycin is unable to inhibit mTOR. It is believed that the rapamycin-FKBP12 complex prevents the association between mTOR and raptor; therefore, downstream targets which depend on raptor binding are

specifically inhibited(Hara et al. 2002, Kim et al. 2002). The downstream targets S6K1 and 4EBP-1 are two such targets which depend on raptor for efficient phosphorylation by mTOR, and thereby their phosphorylation is inhibited in the presence of rapamycin. However, rapamycin has little effect on intrinsic mTOR kinase activity(Peterson et al. 2000).

Regulation of the translation initiation complex via eIF3

Recently, it has also been suggested that mTOR's role in translation initiation can be mediated through the eIF3 translation initiation complex. eIF3 is one of largest initiation factors, with at least 12 different subunits(Mayeur et al. 2003/10). eIF3 binds to the 40S ribosomal subunit, to which S6 is a component. Binding of eIF3 to the 40s subunit inhibits premature association with the 60S ribosomal subunit. In addition, eIF3 also enhances initiation by increasing the binding of the ternary complex(Gingras, Raught & Sonenberg 2001). Under serum starvation or rapamycin inhibition, S6K1 binds tightly to eIF3; however, upon insulin stimulation, S6K1 dissociates from eIF3. This association with eIF3 is disrupted by the phosphorylation of the hydrophobic motif on S6K1(T389); thus, either phosphorylation by mTOR or phosphomimetic mutation seems to be sufficient to decrease the binding affinity between S6K1 and eIF3(Holz et al. 2005). Upon release, S6K1 can be further phosphorylated and activated by PDK1 in a manner dependent on the hydrophobic motif phosphorylation. The fully activated S6K1 is then free to phosphorylate downstream targets(Collins et al. 2003, McManus et al. 2004).

On the other hand, the association between eIF3 and mTOR changes with

activation or inhibition of mTOR. Serum starvation and rapamycin treatment reduce the binding affinity between eIF3 and mTOR/raptor, while insulin stimulates binding between eIF3 and mTOR/raptor. Increases in interaction between mTOR/raptor and eIF3 by insulin stimulation may also help mediate efficient phosphorylation of 4EBP-1 by bringing the translation initiation machinery into proximity of the mTOR complex(Holz et al. 2005).

Additionally, insulin may stimulate the association of eIF3 with eIF4G in an mTOR-dependent manner. eIF4G is a scaffold protein that helps the formation of the eIF4F complex. The eIF4F complex binds to the 5'CAP on mRNAs to promote efficient translation, and it consists of eIF4E, eIF4A, and eIF4G. Although mTOR regulates eIF4E through 4EBP-1, it appears that binding between eIF3 and eIF4G is independent of eIF4E. Insulin is able to stimulate the binding of eIF3 and eIF4G in the absence of eIF4E binding. Although it has been reported that eIF4G is phosphorylated in a rapamycin-reversible fashion on three phosphorylation sites(S1108, S1148, and S1192)(Raught et al. 2000), binding to eIF3 is not correlated to the phosphorylation of S1108; however, correlation to the other phosphorylation sites is still unknown(Harris et al. 2006). Although it appears that eIF3 binds to eIF4G in an mTOR dependent fashion, the specifics of this regulation are yet to be elucidated. For example it still remains to be determined the mechanism by which mTOR regulates eIF3 and eIF4G binding, and whether phosphorylation of eIF4G is of any physiological significance.

Negative Feedback of the mTOR pathway via phosphorylation of IRS-1

Regulation of the AKT-TSC2-mTOR pathway has been further complicated

by the discovery of feedback inhibition on the pathway by both S6K1 and mTOR on insulin receptor substrate 1(IRS-1). IRS-1 and IRS-2 are responsible for conveying downstream signaling upon stimulation of the insulin receptor(IR). When fed a high fat diet, wild-type mice showed increased activation of S6K1 but decreased phosphorylation of AKT in response to insulin; however, in $S6K1^{-/-}$ mice, a high fat diet did not lead to insulin resistance. In wild type mice fed the high fat diet, phosphorylation on IRS-1 was also increased, which was absent in $S6K1^{-/-}$ mice(Um et al. 2004). It was also shown that activation of PI3K by insulin was dependent on TSC2. In the $TSC2^{-/-}$ MEFs, S6K1 activity is highly upregulated, and IGF-1 stimulation yields a muted AKT phosphorylation. However, the phosphorylation of AKT in response to IGF-1 could be restored in the $TSC2^{-/-}$ MEFs by prolonged pretreatment with rapamycin(Harrington et al. 2004, Shah, Wang & Hunter 2004). IRS-1 was identified as a novel S6K1 target in vitro, and inhibition of IRS-1 phosphorylation could be seen in vivo with the addition of rapamycin or RNAi of S6K1 but not S6K2. Phosphorylation of IRS-1 by S6K1 blocks its function. In addition to phosphorylation of IRS-1 by S6K1, IRS-1 mRNA is also decreased in $TSC2^{-/-}$ MEFs, and treatment with rapamycin or RNAi of either S6K1 or S6K2 can restore IRS-1 mRNA(Harrington et al. 2004). However, IRS-1 protein levels in $S6K1^{-/-}$ and wild-type mice are similar (Um et al. 2004). In addition to phosphorylation by S6K1, IRS-1 can also be directly phosphorylated by mTOR/raptor on sites differing from the S6K1 phosphorylation site. However, phosphorylation by mTOR/raptor also decouples IRS-1 from insulin signaling. IRS-1 is phosphorylated in vitro by the immunoprecipitated

mTOR/raptor complex, which may also contain S6K1. In vivo, this phosphorylation could be inhibited by cotransfection of either kinase-dead mTOR or kinase-dead S6K1. However, even in the presence of rapamycin-resistant S6K1, which does not need mTOR/raptor for activation, phosphorylation of the putative mTOR sites can still be inhibited in a rapamycin-dependent manner. Furthermore, phosphorylation of those sites is eliminated by knockdown of raptor even in the presence of rapamycin-resistant S6K1. Together, this suggests that IRS-1 may also be a direct target of mTOR(Tzatsos, Kandror 2006). It is possible that the phosphorylation on the S6K1 dependent site may influence subsequent phosphorylation by mTOR/raptor.

TORC2: An mTOR/rictor complex

Recently, the understanding of mTOR signaling was greatly enhanced by the discovery of a new mTOR binding partner which displaces raptor and changes its downstream specificities. The identification of rictor (rapamycin-insensitive companion of mTOR) demonstrated new functions of the mTOR pathway which were not originally recognized due to the insensitivity to rapamycin inhibition; however, a recent report has suggested that this may not necessarily be the complete story(Sarbassov et al. 2006). It appears that the effect of rapamycin on mTOR-rictor may depend on both cell type and duration of treatment. However, mTOR-rictor is resistant to short term (<2 hr) rapamycin treatment; therefore, unless otherwise noted, the rapamycin effects on mTOR-rictor refer to short term rapamycin treatment (see later for the exception). Under certain lysis conditions, it was shown that rapamycin can specifically disrupt the mTOR-raptor interaction

without disrupting the mTOR-rictor interaction. When treated with rapamycin, immunoprecipitation of FKBP12 is capable of co-immunoprecipitating mTOR/raptor but not mTOR/rictor(Jacinto et al. 2004). In addition to rapamycin resistance, the rictor-mTOR interaction is also unaffected by leucine levels and mitochondrial inhibition, which modulate S6K1 and 4EBP-1 phosphorylation and change the raptor-mTOR interaction. Not only does mTOR/rictor not phosphorylate S6K1 and 4EBP-1, but binding of raptor or rictor is mutually exclusive. This implies that there might be a degree of competition between raptor and rictor for mTOR. With the discovery of rictor, mTOR signaling can now draw greater analogy to yeast TOR signaling. In *Saccharomyces cerevisiae* two distinct *Tors* and TOR complexes (TORCs) exist(Loewith et al. 2002). Either Tor1 or Tor2 can be used to form TORC1, which is sensitive to rapamycin inhibition, but only Tor2 can be used to form TORC2, which is resistant to rapamycin inhibition. Although in mammals there is only one mTOR, it is now understood that mTOR participates in two distinct functional complexes, mammalian TORC1 (mTORC1) and mammalian TORC2 (mTORC2). Both mTORC1 and mTORC2 share mTOR and mLST8; however, their downstream specificities are predicted by association with raptor and rictor, respectively. In the case of mTORC1, raptor is responsible for binding to mTORC1 substrates; however, it is still unreported whether rictor is responsible for mTORC2 substrate specificity.

Downstream targets of mTORC2

The downstream functions of mTORC2 are less well-characterized than

mTORC1. Two such effects are actin cytoskeleton regulation and AKT regulation. However, studies on regulation of the actin cytoskeleton have yielded conflicting reports as depending on context knockdown of rictor can either stimulate or inhibit actin cytoskeleton organization. In yeast, rapamycin-resistant TORC2 regulates the actin cytoskeleton through PKC1. It was reported that RNAi knockdown of rictor in HeLa cells changed the cell morphology and induced actin stress fiber formation. Additionally, knockdown of rictor also changed the localization of paxillin, an adaptor protein found at the actin/plasma membrane junction. However, although knockdown of PKCα, the mammalian homologue of PKC1, yields a similar actin morphology to the rictor knockdown, the stress fibers in the PKCα knockdown appeared better organized(Sarbassov et al. 2004). It was also shown that reintroduction of serum to serum-starved cells induces the formation of stress fibers and induces cell spreading, which is not preventable by pretreatment with rapamycin. In this situation, knockdown of rictor but not raptor by RNAi reduced tyrosine phosphorylation on paxillin and reduced cell spreading and stress fiber formation. The effect of rictor RNAi could be reversed by active Rac-GTP(Jacinto et al. 2004). Similarly in yeast, disruption of the TORC2 complex leads to actin depolymerization(Loewith et al. 2002).

It has been convincingly demonstrated that mTORC2 acts as the PDK-2 on AKT to allow full activation of AKT. This was shown by decreased phosphorylation of AKT on S473 by RNAi of rictor but not raptor in both mammalian and *Drosophila* cells. Additionally, AKT is phosphorylated in vitro by immunoprecipitated mTOR/rictor complexes but not mTOR/raptor

complexes(Sarbassov et al. 2005). Consequently, this implies that mTOR may influence the growing list of AKT functions, which include anti-apoptotic, cell proliferative, and metabolic roles(Hresko, Mueckler 2005). However, since AKT is a one of the important activators of TORC1, it is no longer accurate to think of AKT as an upstream regulator of mTOR. Instead, AKT exists both upstream and downstream of mTOR. The significance of this mutual regulation between AKT and mTOR is still not fully understood and is undergoing further study. Furthermore, knocking down the rictor complex decreases AKT activity but not S6K1 phosphorylation. This provides evidence against the assertion that AKT functions upstream of mTORC1. Although it is possible that nutrient-dependent activation of mTORC1 can compensate for decreases in PI3K-AKT signaling. Additionally, it is still not clear whether mutual regulation of mTORC1 and mTORC2 is dominated by direct competition for mTOR or dominated by negative feedback via IRS-1.

It has also been proposed that the substrate specificity for mTORC1 and mTORC2 is partially mediated by sequences on the substrate including the TOS motif and the c-terminus, which in the case of S6K1 is different from other AGC family members. S6K1, which is usually an mTORC1 target, can be made resistant to rapamycin by deletion of both the TOS motif and the c-terminus after the hydrophobic motif. The TOS motif is thought to facilitate mTORC1 binding, while the c-terminus protects the substrate from phosphorylation by mTORC2. Interestingly, deletion of the c-terminus of S6K1 renders the c-terminal end of S6K1 very similar to AKT. It has been proposed that other TORC2 substrates

will have c-terminal ends similar to AKT and the truncated S6K1; however, these other targets have yet to be identified in mammals(Ali, Sabatini 2005). However, in yeast, Ypk2 has been identified as a direct target of Tor2. Ypk2 is homologous to Serum/Glucocorticoid Kinase (SGK), which is closely related to AKT and a member of the AGC kinase family. The AGC kinase family also includes S6K1. Truncation of the autoinhibitory domain of Ypk2 is able to suppress both the actin polymerization defects and lethality associated with loss of Tor2. However, Ypk2 is unable to restore defects conferred by loss of Tor1. Together, this suggests that Ypk2 may be an important downstream target of TORC2 in yeast; however, it has yet to be shown that SGK plays a similar role in higher eukaryotes(Kamada et al. 2005).

It is apparent that rictor is critical for TORC2 function, but it is still unclear how rictor influences TORC2 activity. Unlike raptor, it has yet to be shown how rictor mediates the phosphorylation of downstream substrates by mTOR. In addition, rictor is also phosphorylated in a PI3K-dependent manner; however, it is unknown what role phosphorylation of rictor may play in the mTOR pathway(Sarbassov et al. 2004).

Long term rapamycin treatment negatively regulates TORC2

Recently, it was reported that prolonged treatment with rapamycin inactivates TORC2 in addition to TORC1. Rapamycin inhibition of TORC1 occurs within 30 minutes. This inhibition is associated with both decreases in phosphorylation of TORC1 targets such as S6K1, and disruption of the mTOR/raptor complex. However, in certain cell types, rapamycin can also inhibit

TORC2, as seen by decreased phosphorylation of the TORC2 target AKT and disruption of the mTOR/rictor complex. This inhibition occurs on the order of 24 hrs, which suggests that the mechanism by which rapamycin inhibits TORC2 may not be identical to the mechanism for TORC1 inhibition. FKBP12/rapamycin is unable to bind to mTORC2; however it is capable of binding free mTOR. It has been proposed that association of free mTOR with FKBP12/rapamycin may preclude formation of mTORC2; however, FKBP12/rapamycin does not disrupt the intact mTORC2. Therefore, inhibition of mTORC2 by rapamycin may require the turnover of existing mTORC2 before mTORC2 levels drop below the threshold necessary for AKT phosphorylation; however, this model has yet to be conclusively shown. Alternative explanations for this effect include transcriptional or translational regulation of an integral TORC2 component or the induction of an inhibitor of rictor by rapamycin treatment; however this all is merely speculation. Interestingly enough, both HeLa cells and HEK293 cells, which are commonly used for many over-expression experiments in the field, are resistant to inhibition of TORC2 by prolonged rapamycin. However, a variety of cell types are sensitive, including lymphocyte cells lines (BJAB, U937, Jurkat, SKW3), a glioblastoma cell line (U87), a melanoma cell line (UACC-903), a muscle tubule cell line (C2C12), endothelial cell line (HUVEC), and a prostate cancer cell line (PC3). The mechanisms for this cell type specificity are still unknown and would be of great interest(Sarbassov et al. 2006).

Cell size control mechanisms

Cell size vs Cell cycle control

An interesting idea that has been suggested is that the role of mTOR on cell growth is independent of its role in proliferation. In other words, cell cycle and cell size may be controlled independently. PTEN is a tumor suppressor that is commonly mutated in many hamartoma syndromes, such as Cowden disease, Lhermitte-Duclos disease, Bannayan-Zonana syndrome, and Proteus syndrome, and several malignant cancers, such as glioblastomas, endometrial carcinomas, melanomas, and advanced prostate adenocarcinomas. Biochemically, PTEN functions as a lipid phosphatase and dephosphorylates PIP_3. Consequently, PTEN negatively regulates AKT, which in turn can lead to negative regulation of mTOR, thereby providing a possible etiology for the hamartoma syndromes. It was observed that in human cancers cells where PTEN was knocked out ($PTEN^{-/-}$), cells underwent a rapid increase in size after irradiation as compared to wild-type counterparts ($PTEN^{+/+}$). Interestingly enough, irradiation led to cell cycle arrest in both $PTEN^{+/+}$ and $PTEN^{-/-}$ cells via P53 dependent pathways; however, cell size was only increased in the $PTEN^{-/-}$ cells. Implication of mTOR's role in the decoupling of cell size and cell cycle in PTEN mutants is shown by pharmacological recovery of cell size control. Both inhibition of PI3K via wortmannin and inhibition of mTOR via rapamycin lead to decreases in cell size in the irradiated $PTEN^{-/-}$ cells. It is worth noting that wortmannin treatment was able to reduce cell size to the wild-type levels; however, rapamycin only led to a partial recovery in cell size. This may imply that the sensing necessary for PTEN mediated cell size control may be predominantly due to PIP_3 regulation; however,

execution of cell size control is only partially mediated through rapamycin sensitive mTOR targets. Furthermore, it was also shown that cells subjected to TSC2 RNAi were of similar size to cells subjected to PTEN RNAi; however, the reduction in TSC2 and PTEN levels were not dramatic enough to draw firm conclusions(Lee, Kim & Waldman 2004). In the brain, it appears that the relationship between mTOR and PTEN in cell size regulation is variable. In $PTEN^{-/-}$ mice, both the soma in the dentate gyrus and the cerebellum show increased cell size compared to $PTEN^{+/+}$ mice; however, low dose treatment with a rapamycin analogue reduced the cell size in the dentate gyrus to wild-type levels, but it had little effect on the cell size in the cerebellum. Further treatment at high doses helped reduced the size of cerebellar soma, but not to the size of wild-type mice. This variability may be the consequence of differences in bioavailability, as levels of S6 phosphorylation were not reduced as significantly in cerebellum(Kwon et al. 2003). On the other hand, in *Drosophila*, knockdown of either dPTEN or dTSC1 is sufficient to increase cell size; however, a double knockdown of dPTEN and dTSC1 has additive effects on cell size regulation. This further suggests that in *Drosophila*, the pathways may have independent components in the regulation of cell size(Gao, Pan 2001). It may also highlight the differences in the regulation of TSC2 by AKT in *Drosophila* as seen by mutations of the AKT phosphorylation sites on TSC2(Dong, Pan 2004). Loss of either dPTEN or dTSC1 can lead to increases in cell size; however, a report has suggested that only knockdown of dTSC1 leads to increases in dS6K(Radimerski et al. 2002), while other reports have also seen increases in dS6K with the

knockdown of dPTEN(Sarbassov et al. 2005, Yang et al. 2006). It is possible that dTSC1 regulates cell size in a dTOR-dependent manner, while dPTEN partially regulates cell size in a dTOR-independent manner(Radimerski et al. 2002).

Cell size control Downstream of mTOR

Roles of the mTOR-S6K pathway in cell size regulation

It is quite clear that mTOR is important for cell size regulation, as seen by both genetic perturbation of mTOR and pharmacologic inhibition of mTOR. Shown more directly, loss of dTOR leads to a decrease in larvae size; however, the larvae fail to mature and die before reaching adulthood. In mosaic *Drosophila*, loss of dTOR leads to a decrease in cell size while maintaining the general organization of the tissue(Oldham et al. 2000, Zhang et al. 2000). However, it is less clear how cell size is regulated downstream of mTOR. One of the most potent candidates in this regulation is S6K. In *Drosophila*, knockout of *S6K* results in high rates of embryonic lethality. In the surviving adults, however, there is a decrease in body size. On the other hand, two S6K homologues exist in mammals, namely S6K1 and S6K2. Although the two homologues seem to be regulated in a similar fashion including rapamycin sensitivity, they exhibit different localizations; S6K1 is primarily cytoplasmic, while S6K2 is primarily nuclear(Lee-Fruman et al. 1999). Either homologue of S6K is sufficient for phosphorylation of S6; however, full phosphorylation of S6 requires both S6K1 and S6K2. Additionally, knockout of either *S6K1* or *S6K2* also has different effects on animal size. Knockouts of *S6K1* results in animals of decreased cell

size as compared to wild-type; however, knockouts of *S6K2* results in animals slightly larger than wild-type. Double knockouts of *S6K1* and *S6K2* yield animals sizes similar to the single knockouts of *S6K1*; however, double knockouts also experience high levels of embryonic lethality. Surprisingly, in the double knockouts for *S6K*, phosphorylation the S6K sites on S6 still can be seen. This may be due to redundant regulation by the RSK pathway on S6(Pende et al. 2004).

Role of downstream proteins of S6K in cell size control

Characterization of S6 has also yielded more information about the regulation of cell size. Mice with a knock-in of S6 to which the phosphorylation targets of S6K were mutated to alanines ($S6^{p-/-}$) were characterized. When compared to wild-type MEFs, $S6^{P-/-}$ MEFs were significantly smaller; however, unexpectedly, these cells also showed increased rates of protein synthesis and cell division. Additionally, to distinguish between the possibilities that the reduction in cell size could be due to either a failure to grow or a secondary effect of accelerated cell cycle progression, cells were arrested by blocking DNA synthesis with aphidicolin. Cell cycle arrest was unable to eliminate the difference in cell sizes. From this data it was apparent that the decrease in cell size was independent of cell cycle progression; thus, this implies that elimination of the S6 phosphorylation sites affected the ability of the cells to grow. To further separate the effects of cell growth and proliferation, the $S6^{p-/-}$ cells were treated with rapamycin. The $S6^{p-/-}$ cells failed to undergo any further reduction in cell size when treated with rapamycin; however, they experienced a decreased rate of

cell cycle progression. These experiments imply that mTOR-mediated cell size regulation functions primarily through S6(Ruvinsky et al. 2005). However, the role of S6 in mediating ribosome biogenesis may be distinct from its role in regulating cell size. In mouse T-cells with only one copy of the S6 allele, ribosome biogenesis is inhibited; however, stimulation of the T-cells by anti-CD3 and anti-CD28 leads to no difference in the increases in cell size in T-cells with one copy of S6 vs. wild-type. However, loss of one copy of S6 leads to a failure to proliferate by activation of a P53-dependent checkpoint(Sulic et al. 2005). It is not yet clear whether this phenomenon will also be seen in other cell types.

Although reports suggest S6 plays a major role for controlling cell size regulation by mTOR, SKAR (S6K1 Aly/REF-like target), another target of S6K, is also involved in cell size regulation. RNAi of SKAR leads to a reduction in cell size; however, the reduction in cell size is not as dramatic as RNAi of S6K1. It has not yet been determined whether this difference in cell size is due to efficiencies in RNAi or due to other molecules being involved in cell size signaling. As mentioned earlier, knockouts of *S6K1* are smaller; however, knockouts of *S6K2* show little change in cell size. It was also shown that SKAR binds and is phosphorylated by S6K1 but not S6K2, which provides circumstantial evidence that it might be involved in mediating S6K1's regulation of cell size(Richardson et al. 2004).

Despite these studies, its still not fully understood how the downstream targets of S6K regulate cell size. It is likely that both SKAR and S6 play a role in cell size regulation; however, SKAR probably still needs to be further

characterized in order to better understand its functional role in cell size regulation. On the other hand, it still remains to be reconciled how phosphorylation of S6 by S6K1 yields a phenotypic outcome different from phosphorylation by S6K2.

Clinical Correlations

As a mediator of cell size and cell growth, the mTOR pathway has many functions in cellular homeostasis. However, it is also quite interesting that mTOR has a functional role in the physiological modulation of cell size. The physiological manipulation of cell size is perhaps most apparent in the development of muscle to which loads and strains often lead to increases in muscle mass to compensate for increases in demand.

Skeletal muscles

In skeletal muscle models, it has been shown that IGF-1 has a role in myogenic induction that is independent of IGF-1's role in maturation(Musaro, Rosenthal 1999). Additionally, IGF-1 also plays a role in the prevention of skeletal muscle atrophy induced by angiotensin II(Song et al. 2005). However, only more recently has the molecular mechanism for this induction been better characterized. Since IGF-1 induced muscle hypertrophy could be blocked by Cyclosporin A (CsA), a calcineurin inhibitor, it was believed that the calcineurin pathway was responsible for muscle hypertrophy. However, calcineurin is also required for myocyte development; therefore, the calcineurin pathway may not be the sole mediator of skeletal muscle hypertrophy. Recent studies have implied that the AKT-TSC-mTOR pathway may also play a role in skeletal muscle hypertrophy. Treatment of mature myocytes with IGF-1 leads to the induction of

the mTOR pathway, and conversely over-expression of AKT also leads to myocyte hypertrophy(Rommel et al. 2001). To further distinguish whether IGF-1-associated skeletal muscle hypertrophy was due to calcineurin or AKT/mTOR, animals were treated with CsA or rapamycin. At concentrations sufficient to inhibit cardiac hypertrophy, CsA was unable to inhibit functional overload-induced skeletal muscle hypertrophy. However, treatment with rapamycin was able to inhibit the compensatory hypertrophy. Additionally, muscle recovery after induced atrophy was inhibited by rapamycin but not CsA (Bodine et al. 2001). Further study of the roles of calcineurin and AKT-mTOR has shown that signaling through calcineurin predominately affects the types of muscle fibers generated, while it has little effect on the size of the muscle fibers. On the other hand, activation of AKT by transfection of myr-AKT or innervation and electrical stimulation of regenerating muscles has little effect on the specification of muscle fiber type; however, it leads to increases in muscle fiber size, which can be inhibited with rapamycin. This suggests that the hypertrophic effect on muscle fibers by AKT predominately signals through mTOR(Pallafacchina et al. 2002).

Clinically, the effects of mTOR activation can be seen in differences in exercise training. Endurance training generally promotes mitochondria biogenesis and fiber switch from fast twitch to slow twitch fiber types, while resistance training has little effect on fiber type selection; however, there is a stimulation of protein synthesis. The effects of exercise training can be mimicked by electrical stimulation ex vivo. Low frequency electrical stimulation

(LFS) for long periods of time has been shown to promote muscle changes similar to endurance training, while, short intermittent higher frequency electrical stimulation (HFS) has been shown to cause muscle changes similar to resistance training. It was shown that HFS induces the activation of the mTOR pathway as seen by phosphorylation on AKT, TSC2, mTOR, S6K1, and 4EBP-1; however, LFS caused no change in the phosphorylation of AKT, mTOR, and S6K and perhaps also a modest decrease in the phosphorylation of TSC2 and 4EBP-1. Therefore, it is possible that resistance training may cause differential activation of the mTOR pathway in order to generate the physiological consequences of muscle hypertrophy(Atherton et al. 2005).

Similar to MEFs derived from knockout mice, $S6K1^{-/-}$ myocytes also show decreases in cell size, while $S6K2^{-/-}$ myocytes have sizes similar to that of wild-type cells, and double knocks of S6K1 and S6K2 yield myocytes of similar size to $S6K1^{-/-}$ cells. Despite decreases in cell size, the cell number per muscle fiber remains unchanged in the $S6K1^{-/-}$ cells. Also similar to the manipulation of AKT, knockout of either S6K1 or S6K2 had little effect on the type of muscle fiber specified, only the cell size(Ohanna et al. 2005).

Clinically, it has been suggested that age-related muscle loss may be due to decreased activation of S6K1. When comparing different age groups, younger (mean age = 25) individuals have high protein synthesis in muscles compared to older (mean age = 72) individuals when the muscles were stimulated by infusion of insulin and amino acids. Moreover, insulin and amino acids stimulation lead to phosphorylation of the AKT, mTOR, 4EBP-1, and S6K1 in

younger individuals; whereas in older individuals the response with similar except S6K1 was not phosphorylated(Guillet et al. 2004). Although this data suggests an attractive model for age-related muscle atrophy, the correlation is still rather tenuous. Further study is needed to firmly establish that failure to activate S6K1 is responsible for age-related muscle atrophy.

Cardiac muscles

Similar to the effects seen in skeletal muscle, rapamycin may also have an effect on cell size in cardiac myocytes. In response to increased load and demand on the heart, the cardiac myocytes often increase in size and lead to hypertrophy of the heart. Although these compensatory measures help the heart gain physiological function in the short term, cardiac hypertrophy leads increased morbidity and mortality; therefore, treatments such as beta-blockers, which decrease effort exerted by the heart, have been standard of care to prevent further exacerbation of heart disease. Like skeletal myocytes, cardiac myocytes that have been treated with rapamycin also show decreased cell size in response to growth factors. More importantly perhaps is that cardiac hypertrophy due to increased load on the heart may also be inhibited by rapamycin. In mice, cardiac hypertrophy due to increased load can be induced by ligation of the aorta. Ligation of the aorta leads to increased S6 phosphorylation, which returns to basal levels after a week post-operation. This upregulation of S6 phosphorylation is also coupled with cardiac hypertrophy as measured by heart weight; however, treatment with rapamycin prior to ligation is able to decrease cardiac hypertrophy in the ligated animals(Shioi et al. 2003). Additionally, in

mice with preexisting cardiac hypertrophy due to aortic ligation, rapamycin was also able to significantly decrease the heart weight to body weight ratio. Although rapamycin significantly reduced the size of both compensated hypertrophy and decompensated hypertrophy, the reduction was more dramatic in the case of compensated hypertrophy (40% vs. 70% reduction respectively). In decompensated hypertrophy, the mice are showing signs of heart failure. However, neither the etiology of compensated vs. decompensated hypertrophy nor the mechanism for differences in rapamycin response are well understood. Additionally, in mice with decompensated hypertrophy, rapamycin treatment helped regain heart function as seen by decreased left ventricular end-systolic dimensions, increased fractional shortening, and increased ejection fraction(McMullen et al. 2004).

Even though the mTOR pathway seems to be implicated in cardiac hypertrophy, it would be unfair to neglect mentioning that perhaps the pathway most well characterized for its role in cardiac hypertrophy is the MAPK pathway. Activation of the MEK/ERK MAPK pathway seems to be critical for cardiac hypertrophy as a response to activation by phenylepherine(PE). S6K2, which is activated by insulin signaling, is also activated by PE. It has also been shown that rapamycin treatment is capable of significantly reducing protein synthesis induced by PE, but the inhibition is not complete, which implies that PE may stimulate protein synthesis by both mTOR-dependent and mTOR-independent pathways(Wang, Gout & Proud 2001). Additionally, the activation of protein synthesis by PE occurs in a PI3K/AKT-independent manner(Wang, Proud 2002).

Since the MAPK-activated Kinase RSK1 was shown to phosphorylate TSC2 and thereby inhibit its activity(Roux et al. 2004), it was proposed that inactivation of TSC2 by RSK1 was the mechanism by which PE promotes mTOR-dependent protein synthesis(Rolfe et al. 2005). However, it is also possible that PE promotes mTOR-dependent protein synthesis via ERK, as phosphorylation by ERK also inactivates TSC2(Ma et al. 2005). Taken together, this cross-talk between the MAPK pathway and the mTOR pathway may be important for the induction of cardiac hypertrophy.

Smooth muscles

Angiotensin II (Ang II) has been shown to be important for the induction of smooth muscle hypertrophy and proliferation. However, the effects of Ang II are not generalizable across all smooth muscle vessels. The effect of Ang II on blood vessels and airways seem to be dependent on species and vessel of origin. Consequently, this complexity of regulation has added difficulty in the study of smooth muscle proliferation, but conversely this variability in response may one day prove to be useful for targeted therapies. In human coronary arteries and the saphenous vein, it has been shown that Ang II is capable of inducing smooth muscle hypertrophy independent of cell proliferation. However, in rats, Ang II stimulated smooth muscle hypertrophy in the aorta but also induced cell proliferation in arterioles. To date, the etiology of these differences is not clearly understood. However, it has been suggested that the differences between the growth vs. proliferation response may be due to relative activation of mTOR vs. ERK pathways in response on Ang II. Treatment of human saphenous vein

cultures with Ang II leads to hypertrophy without increases in proliferation, which was coupled with poor activation of ERK. However, PDGF, which strongly activates ERK in saphenous vein cultures, induced cell proliferation. In rat aortic smooth muscle cells, treatment with Ang II led to increased protein synthesis and phosphorylation of S6K1, both of which could be decreased by similar levels when using equal amounts of rapamycin(Giasson, Meloche 1995). Similarly, in human coronary artery smooth muscle cells, Ang II leads to increased protein synthesis, which is indicative of cellular hypertrophy, which may be a cause of vascular wall thickening. This increase in protein synthesis was associated with activation of the AKT-mTOR signaling pathway, which thereby showed inhibition by both rapamycin and PI3K inhibitors(Hafizi et al. 2004).

It is therefore attractive to postulate that inhibition of protein synthesis is the mechanism by which rapamycin-eluding stents are capable of preventing restenosis. In the European arm of a double-blind study, it was shown that stenting of small coronary arteries with rapamycin-eluding stents as compared to bare wire stents led to patients with larger minimum lumen sizes at 8 months (2.22 mm vs. 1.33 mm), less major cardiac events at 9 months (8% vs. 22.6%), and lower need for revascularization (4% vs. 20.9%)(Schofer et al. 2003). Published concurrently, the American arm of the study showed similar efficacy of rapamycin eluding stents(Moses et al. 2003). However, critics of the study have suggested that the end point used for efficacy, major cardiac events, is based on the need for revascularization, which skews the endpoint toward the measurement of lumen size as opposed to clinical efficacy(Silberberg 2003). The two year follow up of

the study demonstrated the effects on restenosis were maintained after two years; however, despite preventing the need for revascularization, rapamycin eluding stents had no effect on mortality or incidence of myocardial infarction(Weisz et al. 2006). From this study it is not apparent whether this is due to lack of statistical power or lack of clinical efficacy, so larger studies must be done to clarify these findings.

Asthma is a disease of broncho-constriction and inflammation, which can be further characterized into two subtypes. Type I asthma shows smooth muscle hyperplasia around the central bronchi. Type II asthma shows only mild hyperplasia around the central bronchi and involves smooth muscle hypertrophy throughout the bronchioles(Ebina et al. 1993). Treatment with a rapamycin analogue has shown some efficacy in the treatment of severe asthma; however, this has mostly been attributed to the anti-inflammatory effect of rapamycin(Fujitani, Trifilieff 2003). More recently, it has been suggested that effectiveness of rapamycin analogues on asthma may involve more than rapamycin's immunosuppressive role. When S6K1 is activated in the absence of serum, smooth muscle cells increase in cell size and levels of smooth muscle Myosin Heavy Chain(smMHC) also increases. Conversely, inhibition by rapamycin or a PI3K inhibitor leads to decreases in development of long contractile smooth muscles.

The role of the mTOR pathway has been of particular interest in regards to smooth muscle proliferation because the clinical presentation of lymphangioleimyomatosis (LAM) in Tuberous Sclerosis (TSC) patients. In

addition to TSC, mutations of TSC2 have also been connected to lymphangioleimyomatosis. This rare lung disease results in the invasion and proliferation of LAM nodules in the lungs. These nodules contain a mixture of both smooth muscle and melanocytes, and consequently, this overgrowth of cells leads to severe dyspnea and decreased pulmonary function. In primary cultures of LAM nodules, either adding back of wild-type TSC2 or treatment with rapamycin can decrease aberrant phosphorylation of mTOR targets and decrease the mutation-associated increases in DNA synthesis (Goncharova et al. 2002, Goncharova et al. 2006). It is attractive to speculate whether rapamycin may also have a beneficial role in the treatment of smooth muscle hypertrophy associated with LAM.

Beyond Hypertrophy

Rapamycin analogues

In addition to the traditional use of rapamycin as an agent for immunosuppression, recently, many clinical studies have also been conducted on the use of rapamycin and its analogues as an anti-neoplastic agent. Perhaps the best characterized rapamycin analogues include CCI-779(Wyeth Ayerst), RAD001(Novartis Pharma), and AP23573(Ariad Pharma). Although all of these compounds are effective at inhibiting mTOR, the new analogues that have been developed have favorable pharmacologic properties that may prove to be useful for therapy. Interestingly, intermittent administration of these analogues did not result in immunosuppression. However, most clinical trials involving rapamycin and its analogues are still in either phase I or phase II. The studies that have

already reached phase III include the following studies. On Aug 18, 2005, AP23573 was approved for the treatment of soft-tissue and bone sarcomas by the FDA. Additionally, a phase III clinical trial on the concurrent use of CCI-779 with interferon therapy on advanced renal carcinomas has just been completed; however, the results have not yet been published. A phase III study of CCI-779 in addition to letrozole as first line hormone therapy for metastatic breast cancer was terminated before completion. Currently, patients for phase III clinical studies are being recruited to study the effects of CCI-779 on mantle cell lymphoma and also to see the secondary effects on skin cancer in kidney transplant recipients who received rapamycin as therapy. It has been suggested that tumors with mutated PTEN may show increased sensitivity to rapamycin(Guertin, Sabatini 2005). However, this has not been shown conclusively yet.

Diabetes and cell size

As a downstream target of insulin signaling and as a regulator of cell size, the PI3K-mTOR-S6K1 pathway is important in metabolic disorders such as obesity, insulin resistance, and diabetes. In addition to reduced body size, S6K1-deficient mice show hypoinsulinemia and glucose intolerance, which is not due to loss of glucose sensing mechanisms or insulin production capability. Instead, this change is due to reductions in pancreatic endocrine mass, which is accounted for by a selective decrease in β-cell mass and size. Intriguingly, this phenotype is only observed in β-cells and not in other endocrine cells such as α-cells and adrenal cells. These observations clearly demonstrate that S6K1

activity is essential for maintenance of β-cell growth and insulin secretion(Pende et al. 2000). Consistent with this observation, β-cell specific knockouts of PDK1, which is an activator of S6K1, also shows decreases in β-cell mass(Hashimoto et al. 2006). Despite the reduction of circulating insulin levels in S6K1-deficient mice, these mice are resistant to the development of obesity by enhanced β-oxidation and to the development of insulin resistance in both fat and muscle tissue by inhibition of negative feedback on IRS-1(Um et al. 2004).

In addition to regulation of β-cell mass, the mTOR pathway may also play a role in the regulation of kidney hypertrophy. Kidney hypertrophy is a compensatory measure for loss of kidney function. This can be seen in hypertrophy of the remaining kidney in the event of a unilateral nephrectomy. This hypertrophy of the kidney can be prevented in mice by treatment with rapamycin(Chen et al. 2005). Similarly, in mouse models of early diabetic nephropathy induced by streptozotocin, renal hypertrophy can also be seen. This early hypertrophy is mainly due to hypertrophy of the proximal tubules, and is associated with increased S6K1 phosphorylation. Treatment of the mice with rapamycin decreased both kidney hypertrophy due to diabetes and S6K1 phosphorylation. Cultures of tubular cells also showed that overexpression of active S6K1 increased cell size, while overexpression of dominant negative S6K1 decreased the size(Sakaguchi et al. 2006). Another report suggests that rapamycin can reduce glomerular hypertrophy and prevent further progression to kidney disease. Glomerular hypertrophy is believed to be one of the hallmarks for progression to diabetic nephropathy. Moreover, in streptozotocin-induced

diabetic nephropathy, rapamycin treatment attenuated albuminuria, a marker of diminishing renal function in early nephropathy, (Lloberas et al. 2006, Nagai et al. 2005). Although rapamycin might interfere with the development and growth of pancreatic β-cells, the above studies suggest that rapamycin could be a potential therapeutic agent for diabetic complications.

Regulation of Autophagy in Huntington's disease

As seen in myocyte, high levels of amino acids are capable of stimulating myocyte hypertrophy in an mTOR-dependent manner. Conversely, low levels of amino acids are able to inhibit mTOR activity(Kim et al. 2002). During amino acid starvation, cells undergo autophagy, which breaks down cytoplasmic organelles and proteins. Recently, it has been shown that in *Drosophila* inactivation of dTOR by overexpression of TSC1/2, loss of dTOR, or rapamycin treatment stimulated the formation of autophagic vesicles that were not dependent on S6K(Rusten et al. 2004, Scott, Schuldiner & Neufeld 2004). It is attractive to speculate that this mechanism is also involved in atrophy; but, this has yet to be shown clearly. However, the stimulation of autophagy by rapamycin may have interesting clinical consequences. Huntington's disease is an autosomal dominant disease associated with the expansion of the trinucleotide-repeat CAG. This leads to the accumulation of aggregates with expanded polyglutamine tracts within neurons. Although the role of these neuronal aggregates is still unclear, it has been suggested that these aggregates may have toxic effects on the cells, and that they are subjected to clearance by the autophagic system. Additionally, mTOR seems to be sequestered by these

aggregates, which leads to decreases in mTOR activity. This may be a compensatory mechanism, as activation of mTOR activity by overexpression of Rheb increases the toxicity of Huntington repeats in *Drosophila* models(Ravikumar et al. 2004). Furthermore, treatment with rapamycin decreased the neuronal death associated with Huntington's repeats in *Drosophila* models and improved symptoms of Huntington's in mouse models(Berger et al. 2006, Ravikumar et al. 2004). Therefore, it has been suggested that rapamycin can be useful in the treatment of Huntington's by increasing autophagy of protein aggregates. Although Huntington's aggregates decrease upon rapamycin treatment, it is not clear whether this is due to the inhibitory effect on protein synthesis or the stimulatory effect on autophagy by rapamycin. Furthermore, it is also not clear whether the rapamycin effects are due to changes of the protein aggregates because in certain conditions rapamycin protects cells from apoptotic insults. One possible mechanism for this protection is stimulation of the autophagic processing of mitochondria which would prevent subsequent cytochrome C release(Ravikumar et al. 2006). However, the relationship between apoptosis and the mTOR pathway is very complicated, and it has yet to be shown what is the dominant mechanism mediating the anti-apoptotic effects of rapamycin.

Conclusion

In recent years, the complexity of mTOR signaling has exploded, and it is clear that it touches many different pathways both upstream and downstream. With two different TOR complexes and the feedback inhibition of TORC2 by

TORC1 activation, the simplification to linear pathway regulation is impossible. Additionally, as the biochemical regulation of mTOR has increased in complexity, the relevance to clinical processes has also followed. In addition to roles in cell size regulation, the mTOR pathway has also been implicated in tumorigenesis and cell survival. With the myriad of functions and multitude of potential targets in pathogenesis, clear clinical relevance for these targets still remains to be shown. Although it is likely that inhibition of mTOR may be clinically useful for treatment of diseases such as TSC which directly involve misregulation of mTOR, more needs to be done to better understand the importance of mTOR signaling in a more complicated physiological context as seen by various disease states.

Acknowledgments

Foremost, we would like to apologize to all the authors whose primary research we were unable to cite. Additionally, we would like to thank Jeffery Lee, Qian Yang, Eunjung Kim, and Xiaoming Zhao for their critical reading and comments on the manuscript, and funding by grants from NIH and Department of Defense (KLG).

Chapter II: Glucose starvation induces rapamycin reversible apoptosis in TSC-/- cells

The mammalian Target of Rapamycin (mTOR) is a highly conserved serine/threonine kinase in the phosphatidylinositol kinase-related kinase (PIKK) family, which is involved in a diverse set of cellular processes. mTOR exists in one of two distinct functional complexes, TOR complex 1 (TORC1) and TORC2. The study of TORC1 has been greatly facilitated by the availability of the potent and specific inhibitor rapamycin, of which mTOR is the only known target. TORC1 has been implicated as a rapamycin-sensitive regulator of cell size, autophagy, ribosome biogenesis, protein translation, transcription, and cellular viability (Lee, Inoki & Guan 2007). The function of TORC2 is less well characterized, but it is important for cytoskeletal regulation (Jacinto et al. 2004, Loewith et al. 2002, Sarbassov et al. 2004) and AKT activation (Ali, Sabatini 2005, Hresko, Mueckler 2005, Sarbassov et al. 2005). TORC2 may also have some sensitivity to rapamycin; however, both the concentration and duration necessary for TORC2 inhibition greatly exceed what is necessary for TORC1 inhibition. Furthermore, sensitivity of TORC2 to rapamycin is also cell-type specific and likely due to an indirect effect on TORC1 (Sarbassov et al. 2006). For the experiments in this paper, references to mTOR inhibition correspond to the inhibition of TORC1.

It has been established both genetically and biochemically that the tumor

suppressors TSC1 and TSC2 negatively regulate mTOR (Inoki et al. 2002). Loss of either TSC1 or TSC2 results in the autosomal dominant hamartoma syndrome Tuberous Sclerosis Complex (TSC), which is characterized by benign tumors formation in multiple organs including the kidney, liver, lung, spleen, heart, and brain (Young, Povey 1998). Loss of either tumor suppressor is sufficient to induce TSC because TSC1 and TSC2 exist as both a physical and functional complex (van Slegtenhorst et al. 1998). It has been further shown that TSC2 has GAP activity against the small GTPase Ras-homology enriched in brain (Rheb), and that Rheb-GTP stimulates mTOR activity; therefore, loss of either TSC1 or TSC2 leads to over-activation of mTOR(Castro et al. 2003, Garami et al. 2003, Inoki et al. 2003, Li, Inoki & Guan 2004, Tee et al. 2003, Zhang et al. 2003). Therefore, TSC1 is thought to be the regulatory component, while TSC2 serves as the catalytic component to enhance Rheb GTP hydrolysis.

The connection between mTOR and cell viability has been of great interest because mTOR appears to play an important role in cell growth and cell death. Current evidence suggests that mTOR functions both pro-apoptotically and anti-apoptotically. For example, an anti-apoptotic role for mTOR can be seen by the original use of rapamycin as an immunosuppressant, its recent submission for FDA approval as an anti-neoplastic agent, and the recommendation of an mTOR inhibitor as a first line therapy for renal cell carcinomas with poor prognosis (Cho et al. 2007, Hudes et al. 2006). Therefore, in certain situations, mTOR inhibition can sensitize cells to death. On the other hand, TSC tumors, which have high mTOR activity, are benign and highly apoptotic (Wataya-Kaneda et al. 2001),

suggesting that mTOR activation can also sensitize cells to death. However, the mechanism by which this occurs is not well understood. Furthermore, it is still unclear how cellular context influences whether mTOR functions in a pro-apoptotic or anti-apoptotic role. Together, this suggests that mTOR activity must be kept in a tight balance to ensure cellular growth and viability in response to a variety of environmental conditions.

We and others have observed that energy starvation induces cell death in TSC1-/- and TSC2-/- cells in a rapamycin-reversible manner (Inoki, Zhu & Guan 2003, Shaw et al. 2004). This identifies an environmental context where mTOR activation is pro-apoptotic, suggesting a role for mTOR inhibition in low energy survival. As a result, we sought to elucidate the mechanism by which energy starvation preferentially induces cell death in TSC1-/- and TSC2-/- cells. This would provide greater insight into the relationship between mTOR and viability as well as the compensatory mechanisms necessary during energy starvation.

Energy starvation is a key cellular signal that shifts cellular function from anabolic to catabolic processes. Under low energy conditions, ATP is consumed, and ADP accumulates. In order to regenerate ATP and maintain energy balance, two molecules of ADP are converted to ATP and AMP. As AMP accumulates, the intracellular ratio of AMP to ATP increases. This ratio reflects the energy status of the cell, and the increase in the AMP to ATP ratio leads to the activation of AMP-activated protein kinase (AMPK) by making it a more favorable substrate for its upstream kinase, LKB1 (Hardie 2007, Shaw 2006).

AMPK is thought to be the key regulator of low energy response. Direct

phosphorylation of numerous physiological substrates by AMPK shuts down many anabolic processes and activates many catabolic processes (Hardie, Carling & Carlson 1998, Kahn et al. 2005). Consequently, in low energy conditions, AMPK activation is critical for maintaining cellular energy homeostasis and viability. Furthermore, its upstream kinase, LKB1, has tumor suppressive properties through the mechanistic linkage between mTOR and AMPK through direct phosphorylation of TSC2 by AMPK (Corradetti et al. 2004, Shaw et al. 2004).

Here we show that the cell death associated with glucose starvation of TSC1-/- cells is apoptotic and not necrotic. Furthermore, glucose starvation induced apoptosis in TSC-/- cells is dependent on activation of Caspase 3, 9, and 12, and inactivation of mTOR by rapamycin blocks activation of these Caspases.

Results

Constitutive activation of mTOR leads to increases sensitivity to energy starvation

TSC1-/- MEFs, which have impaired ability to inhibit mTOR, underwent massive rapamycin-reversible cell death in response to glucose starvation. However, the TSC1+/+ MEFs remained viable without visible changes in morphology (Fig 1A). Similarly, TSC2-/- LEFs, derived from Eker rat kidney tumors, also underwent energy starvation induced cell death, which was prevented by either treatment with rapamycin or reintroduction of TSC2 (Fig 1B). There are two types of cell death, necrosis and apoptosis. Since it is difficult to visually determine the type of cell death, FACS analysis with Annexin V/Propidium Iodide (PI) double staining was used to distinguish between necrosis and

apoptosis. TSC1-/- MEFs were glucose starved both in the presence and absence of rapamycin. FACS analysis demonstrated that glucose starvation increased Annexin V-Flous staining without increases in PI staining as seen by increased cells in the lower right quadrant (Fig 2). This demonstrated that glucose starvation induced predominately apoptosis as opposed to necrosis. Furthermore, rapamycin was also seen to decrease apoptosis in response to glucose starvation.

Energy starvation induces activation of caspases 9 and 12, but not caspase 8 in TSC cells

Activation of either extrinsic or intrinsic apoptotic pathways can lead to apoptosis; therefore, to determine which pathway was responsible for glucose starvation induced apoptosis, activation of initiator Caspases was determined by cleavage/loss of full length Caspases. Caspases 12 and 9 form part of the intrinsic pathway and respond to cellular stresses, while Caspase 8 forms part of the extrinsic pathway and responds to external death signals, such as FAS ligand (Bao, Shi 2007, Szegezdi, Fitzgerald & Samali 2003). Mitochondrial stress leads to the release of cytochrome C and the eventual cleavage of Caspase 9, while endoplasmic reticulum (ER) stress induces the cleavage of Caspase 12, which then cleaves Caspase 9. Glucose starvation is known to induce ER stress because lack of glucose prevents proper glycosylation of proteins and results in the accumulation of misfolded proteins in the ER and Golgi.

In the TSC1-/- MEFs, glucose starvation induced the intrinsic death pathways as seen by disappearance of full-length Caspases 9 and 12 (Fig 3A). However, in the TSC1+/+ MEFs neither Caspase 12 nor 9 was cleaved. In the

TSC1-/- MEFs, the executioner Caspase 3 was activated as seen by the loss of the full length Caspase 3 and the accumulation of cleaved Caspase 3 in response to glucose starvation. However, glucose starvation did not activate Caspase 3 in the TSC1+/+ MEFs. These results further confirmed activation of apoptosis by glucose starvation (Fig 2), demonstrating that loss of TSC1 during energy starvation can induce damage that triggers the intrinsic pathway Caspase cleavage.

To show that activation of mTOR is responsible for glucose starvation-induced Caspase cleavage in the TSC1-/- MEFs, mTOR was inhibited by rapamycin during glucose starvation. Glucose starvation induced the cleavage of Caspase 12 and 9; however, rapamycin treatment prevented cleavage of Caspases 12 and 9. In contrast, glucose starvation had no effect on Caspase 8 (Fig 3B). The prevention of Caspase cleavage by rapamycin suggested that constitutive mTOR activation during glucose starvation contributed to cell death.

Inhibition of either Caspase 12 or 9 activation prevents Caspase 3 activation during glucose starvation

During late stages of apoptosis, Caspases can be non-specifically cleaved. In order to demonstrate the importance of Caspase 9 and Caspase 12 cleavage as triggers of apoptosis during glucose starvation, various inhibitors were used to inhibit Caspase 9 and Caspase 12. The inhibitor Z-LEHD-FMK was used to specifically inhibit Caspase 9 activity (Ozoren et al. 2000). When Z-LEHD-FMK was added prior to glucose starvation, cleavage of Caspase 3 was prevented (Fig 4A). However, events upstream of Caspase 9 activation, such as cleavage of

Caspases 9 and 12, were not inhibited by Z-LEHD-FMK (data not shown). Since a pharmacological inhibitor of Caspase 12 was not readily available, we instead used the calpain inhibitors ALLN and ALLM. It has been reported that calpain activation is important for the cleavage and activation of Caspase 12 (Nakagawa, Yuan 2000). When TSC1-/- MEFs cells were treated with either ALLN or ALLM, the glucose starvation induced cleavage of Caspase 3 was significantly compromised (Fig 4B). The above data suggested that cleavage of both Caspase 9 and 12 were important for Caspase 3 activation during glucose starvation and were not secondary to changes during late apoptosis.

Discussion

We have shown that proper down regulation of the mTOR pathway is critical for cell survival, when challenged with glucose starvation. This was seen by increased sensitivity of the TSC1-/- MEFs to glucose starvation, and the protection against glucose starvation, when the tumor derived TSC2-/- LEFs were infected by TSC2. Furthermore, the role of mTOR in protecting the cell against energy starvation induced apoptosis was seen by the protective effects against cell death with the inhibition of mTOR by rapamycin, a potent and specific inhibitor of mTOR.

The gross cell death seen during glucose starvation was primarily apoptotic as opposed to necrotic. This was shown both by Annexin/PI double staining and by the activation of Caspase 3. Upstream of Caspase 3, glucose starvation activated branches of the intrinsic pathway, including Caspases 12 and 9, while not affecting the extrinsic pathway. The activation of these pathways

suggests that inhibition of mTOR may play a critical role in maintaining cellular homeostasis during energy crisis. Activation of mTOR leads to increases in cell growth and proliferation. These two activities are both highly energy intensive; therefore, it is likely that during a stress such as energy starvation, growth and proliferation must be inhibited to save resources for more immediate needs.

In addition to activation of an energy crisis, glucose starvation is also known to activate ER stress by preventing proper glycosylation of proteins. In order to protect against ER stress, the early unfolded protein response (UPR) inhibits translation to prevent further build up of proteins in the ER. Since mTOR in a major regulator of protein translation, it is attractive to speculate, that the mTOR pathway may be involved in shutting down translation during the early UPR, and that signals from the UPR pathway may be upstream regulators of mTOR.

Together this data suggest that although mTOR is an activator of growth and proliferation, these activities come at a price. Improper downregulation of mTOR during energy stress speeds cells toward death by eliminating a mechanism for coping with the challenge. Since the various cells within a tumor often experience a heterogeneous environment with areas of nutrient and energy deprivation, the increased sensitivity to energy stress may help explain the highly apoptotic nature of TSC tumors.

Methods

Antibodies and materials

Anti-Caspase 12, anti-Caspase 9, anti-Caspase 3, anti-cleaved Caspase 3,

anti-AMPK, and anti-phospho AMPK (T172) antibodies were obtained from Cell Signaling (Beverly, MA). Anti-Actin, anti-β-tubulin, anti-Caspase 8, antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated IgG secondary antibodies were obtained from Amersham (Buckinghamshire, UK).

The AMPK inhibitor, commonly known as compound C, was obtained from Merck (Whitehouse Station, NJ) and was described previously (Zhou et al. 2001). Cells were treated with 10 μM of the compound suspended in DMSO. Rapamycin was purchased from Cell Signaling and was suspended in methanol at 20 nM. The Caspase 9 inhibitor Z-LEHD-FMK and pan-Caspase inhibitor Z-VAD-FMK were purchased at R&D systems, and used at 20 μM 1 hour prior to and during glucose starvation. The calpain inhibitors, ALLN and ALLM, were purchased at EMD Biosciences, and treatment with 10 μM started 1 hour prior to and during glucose starvation. Protein stability was assayed with cycloheximide from Sigma and used at 50 ng/mL. Etoposide was purchase from Sigma and used at 6 μg/mL.

Cell culture and transfection

MEF cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 50 μg/mL penicillin/streptomycin (P/S). $TSC2^{-/-}$ LExF2 cells (LEF) were maintained in DMEM/F12 (Invitrogen) containing 10% FBS and 50 μg/mL P/S. Glucose starvation was performed with Glucose Free DMEM (Invitrogen) containing 25 mM HEPES, 10% dialyzed FBS (Invitrogen), and 50 μg/mL P/S.

Annexin V/PI staining

Annexin V/Propidium Iodide (PI) double staining was done with Annexin V and PI (BD Biosciences) as per manufacturer's protocol, and samples were analyzed via BD FACScalibur (BD Biosciences).

Acknowledgments

We are indebted to Dr. R.S. Yeung for providing EEF126 cell lines and the LExF2 cell line; Dr. D.J. Kwiatkowski for $TSC1^{-/-, +/+}$ MEFs; Dr. G. Zhou for the AMPK inhibitor. This research would not have been possible without the constant scientific guidance of Ken Inoki. We would also like to thank Jeffery Lee, Grant Yang, Eunjung Kim, Qian Yang, and Xiaoming Zhao for their critical reading and comments on the manuscript, and funding by grants from NIH and Department of Defense (KLG).

Figures

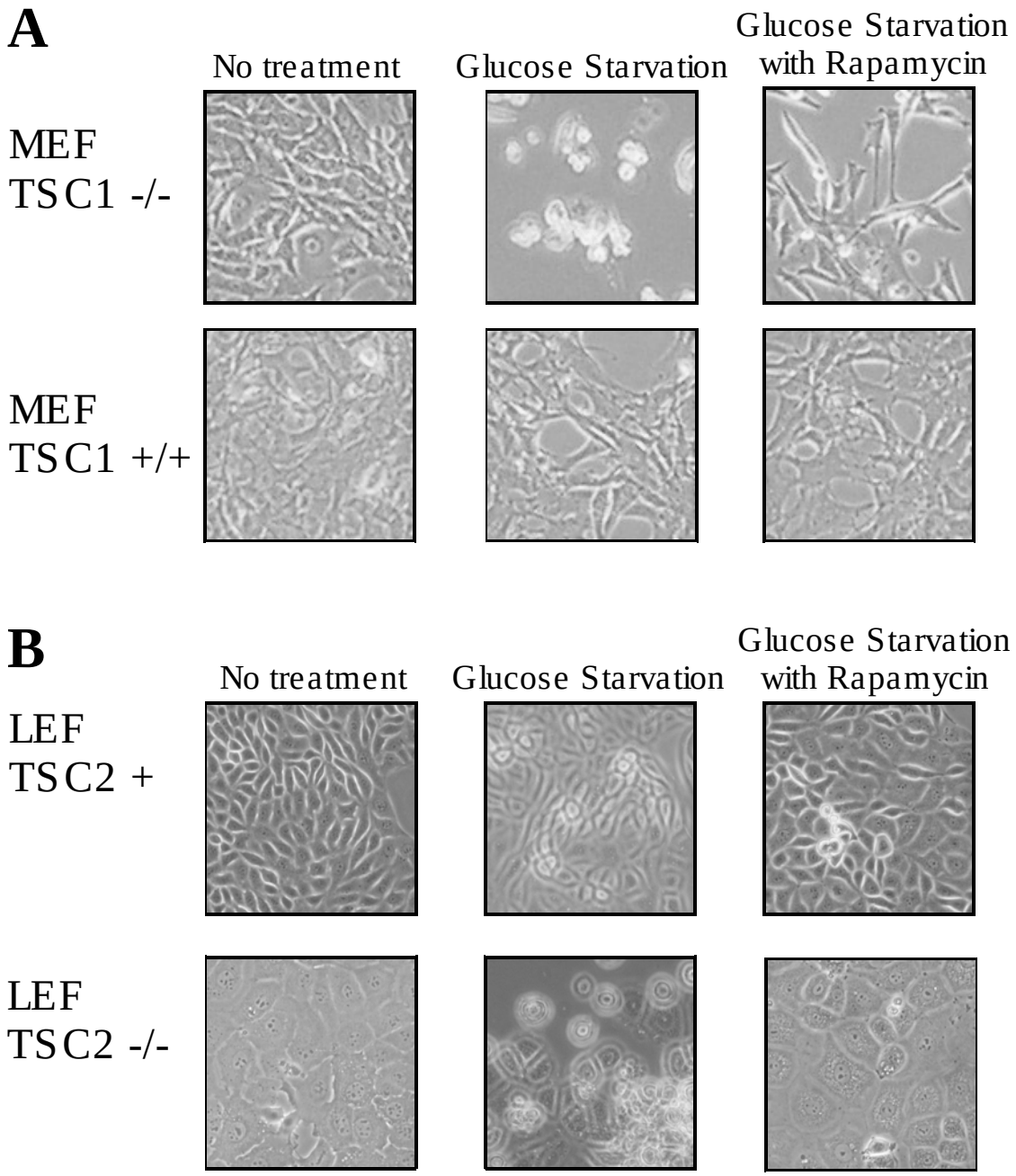

Fig 1 Glucose Starvation induces rapamycin reversible apoptosis in both TSC1-/- and TSC2-/- cells

(A) TSC1-/- MEFs challenged with glucose starvation (15 hrs) were more prone to death, which was protected against by rapamycin treatment (20 nM). (B) LEF TSC2-/- cells were sensitized to glucose starvation (36 hrs), and add back of TSC2 eliminated sensitivity.

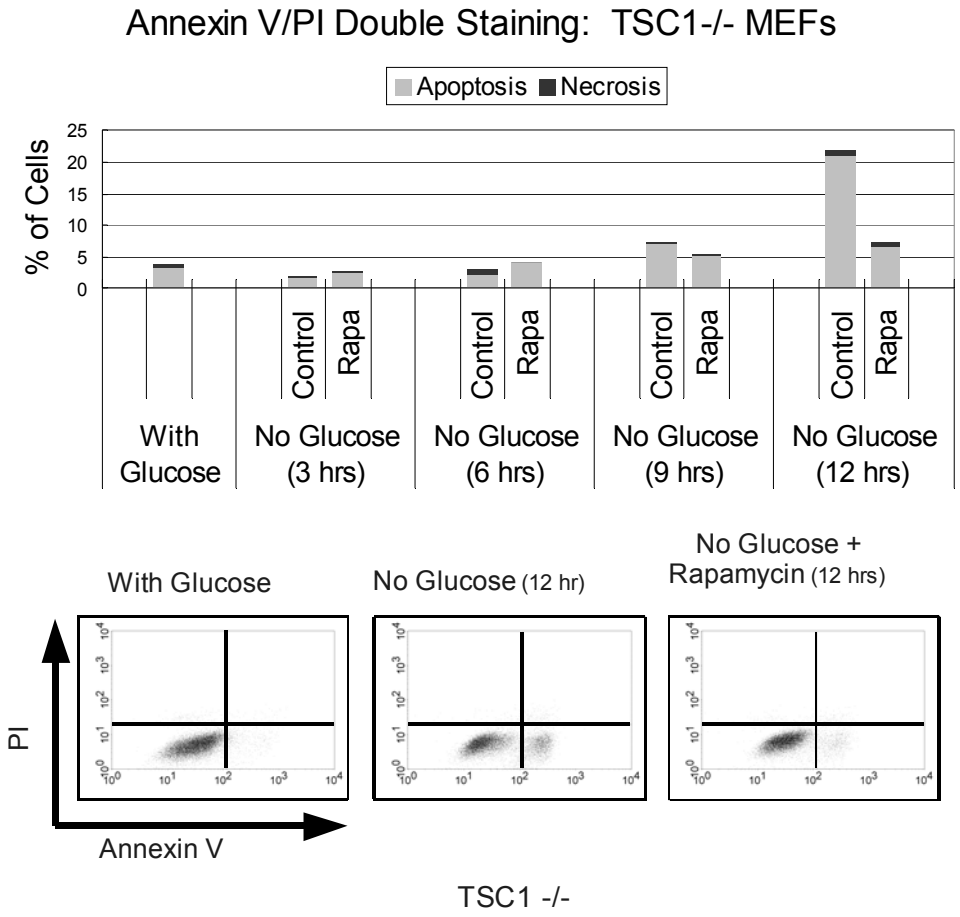

Fig 2 Glucose starvation induces apoptosis in TSC1-/- MEFs

Annexin V (X-axis) / Propidium Iodide (Y-axis) double staining showed glucose starvation (12 hrs) induced cells death predominately through apoptosis as opposed to necrosis in TSC1-/- MEFs. Early apoptotic cells can be stained by Annexin V, which binds to phosphotidyl-serines normally found in the inner-aspect of the cell membrane, but can be found on the outer-aspect of the cell membrane in apoptotic cells. On the other hand, during early apoptosis, PI, which stains DNA, and is excluded from the nucleus, so staining does not occur. During necrosis and late apoptosis, membrane integrity is compromised, and cells are stained by both Annexin V and PI.

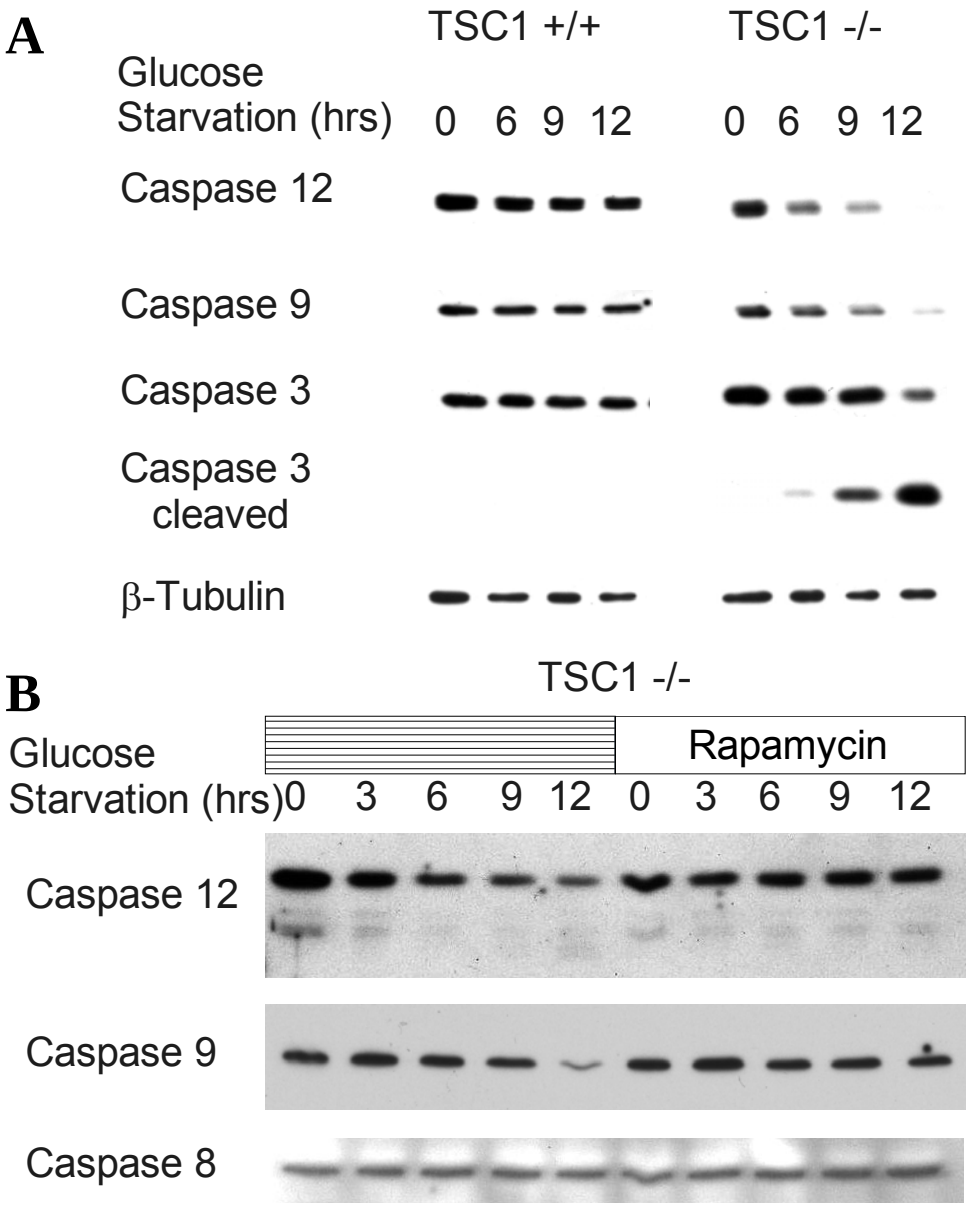

Fig 3 Glucose starvation induces rapamycin reversible activation of Caspases 3, 9, and 12.

(A) Glucose starvation induced cleavage of Caspases 12, 9, and 3 in TSC1-/- MEFs but not in TSC1+/+ MEFs. (B) Rapamycin treatment during glucose starvation prevented Caspase 12 and 9 cleavage. Caspase 8 was not cleaved by glucose starvation.

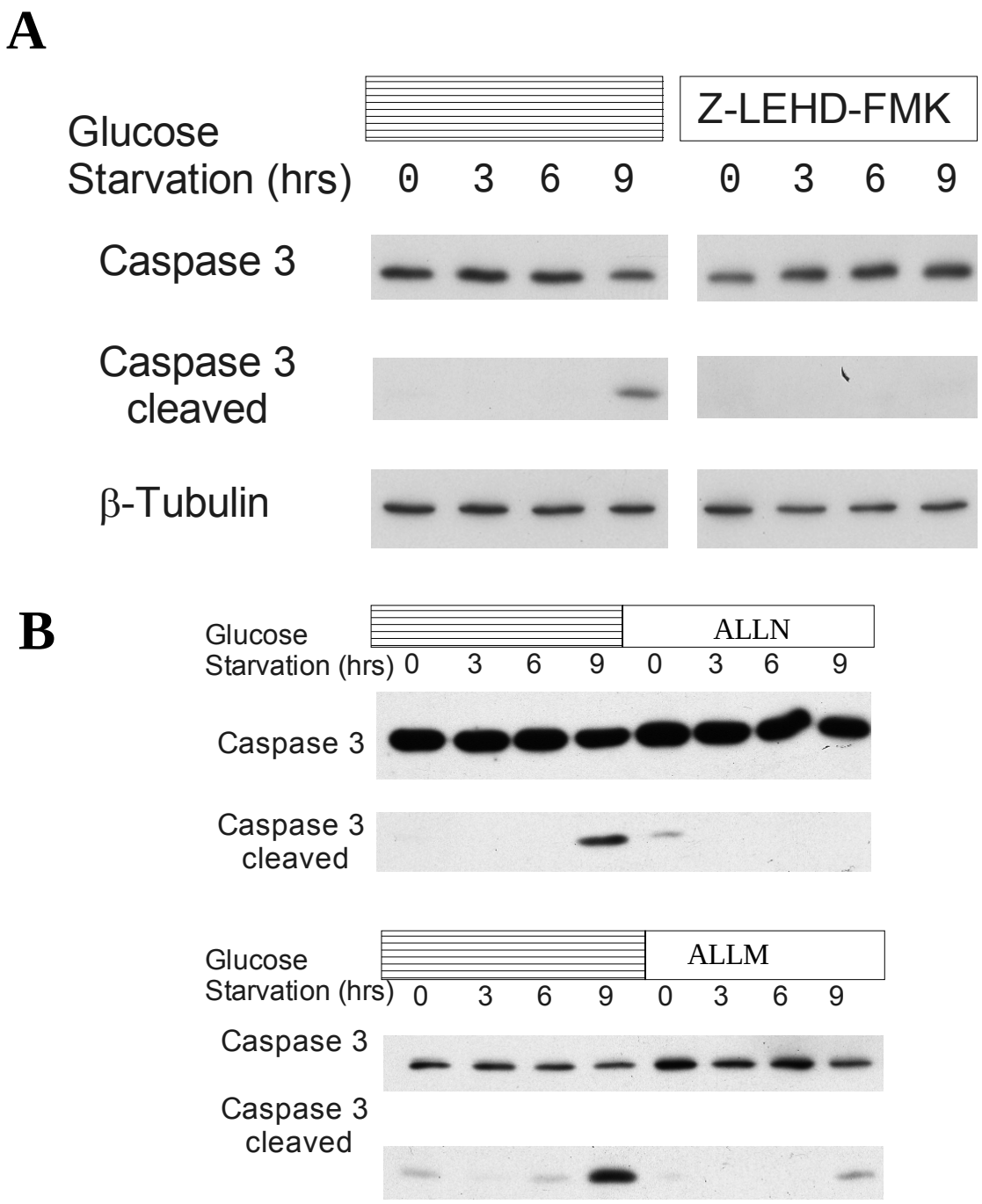

Fig 4 Blocking Caspase 9 or 12 inhibits Caspase 3 activation

(D) 1 hr pretreatment with Caspase 9 inhibitor Z-LEHD-FMK (20 μM) prevented Caspase 3 cleavage during glucose starvation of TSC1-/- MEFs. (E) 1 hr pretreatment with calpain inhibitors ALLN (10 μM) and ALLM (10 μM) reduced Caspase 3 cleavage during glucose starvation of TSC1-/- MEFs.

Chapter III: Loss of TSC induces misregulation of p53 during glucose starvation

Among the functions of AMPK during stressed conditions is to arrest protein synthesis and cell cycle progression. In normal cells, energy starvation induces the phosphorylation of TSC2 by AMPK and subsequently, the inactivation of mTOR. This phosphorylation of TSC2 has a protective role against apoptosis (Inoki, Zhu & Guan 2003). In the presence of TSC1/2, cells undergo cell cycle arrest. However, loss of either TSC1 or TSC2 prevents the inactivation of mTOR during energy starvation, leading to cell death. Inhibition of mTOR by rapamycin, however, can prevent this death. Therefore, mTOR may play a role in determining whether cells undergo cell cycle arrest or cell death in response to energy stress. However, the exact molecular mechanism for this response is not yet known. In normal cells, it has been shown that energy starvation induced cell cycle arrest by the phosphorylation and stabilization of the p53 tumor suppressor protein by AMPK (Jones et al. 2005). We thus set out to determine whether the two pronged regulation of p53 by AMPK and mTOR could account for the differences in cellular viability.

Here we show that glucose starvation of TSC-/- cells dramatically induces p53, a transcription factor important for inducing cellular senescence, cell cycle arrest, and apoptosis. Loss of either TSC1 or TSC2 increases the sensitivity of the cell to energy starvation; however, in the absence of p53, loss of TSC2 no

longer preferentially kills these cells in response to energy starvation. This occurs through a stabilizing phosphorylation of p53 by AMPK and the unabated p53 synthesis by constitutive activation of mTOR. Furthermore, immunohistochemical staining of angiomyolipomas illustrate in vivo that when TSC2 is lost, and mTOR activity is elevated, tumors have increased levels of p53. These results may also explain why the TSC tumors are highly apoptotic and benign.

Results

p53 is necessary for apoptosis of TSC-/- cells in response to energy starvation

To test whether p53 is important for regulating cellular viability in the context of constitutive mTOR activation, TSC2-/- p53-/- and TSC2+/+ p53-/- MEFs were challenged with energy starvation. Both TSC1-/- MEFs, which have an intact p53 gene, and TSC2-/- p53 -/- MEFs have impaired ability to down-regulate mTOR in response to glucose starvation. However, unlike the TSC1-/- MEFs, which showed gross signs of cell death after 15 hours of glucose starvation, both the TSC2-/- p53-/- MEFs and the TSC2+/+ p53-/- MEFs appeared viable at 15 hours (Fig 5A). Furthermore, rapamycin had no effect on either cell type. FACS analysis also showed that TSC2-/- p53-/- and TSC2+/+ p53-/- MEFs were equal resistant to glucose starvation (Fig 5B). To exclude the possibility that the TSC2-/- MEFs had a delayed apoptotic response, Annexin V/PI doubling staining was also done after 24 hours of glucose starvation. Again, the TSC2-/- p53-/- cells did not show enhanced sensitivity to glucose starvation (data not shown). In comparison, in the TSC1-/- MEFs apoptosis could be detected by FACS at 12

hours (Fig 2). Moreover, the TSC2-/- LEF cells, which are positive for p53, showed enhanced sensitivity to glucose starvation (Fig 1B). Together, this data suggests that p53 is necessary for glucose starvation induced cell death, when TSC is lost.

Energy starvation induces p53 phosphorylation and accumulation

The previous results, which suggested that glucose starvation induced apoptosis via the intrinsic pathway (Fig 3A, 3B), made it intriguing to determine the mechanism by which over-activation of mTOR triggered this response. p53 is a potent activator of the intrinsic apoptotic pathway. Furthermore, the observation that loss of p53 reduced sensitivity to energy starvation in TSC cells (Fig 1A vs. Fig 5A) and that the absence of p53 also eliminates the preferential death of TSC2-/- p53-/- MEFs (Fig 5B) suggested that p53 activation could be important for mediating mTOR's pro-apoptotic role during glucose starvation. To test this possibility, p53 activation in the TSC1-/- MEFs was determined by phosphorylation and accumulation. In addition to Caspase cleavage, glucose starvation also induced the phosphorylation of p53 on Ser15 (mouse p53 Ser18) and the accumulation of p53 protein (Fig 6A). However, the phosphorylation and accumulation of p53 could be prevented by inhibition of mTOR or AMPK with rapamycin or compound C, respectively. Rapamycin completely prevented p53 accumulation, but compound C only partially inhibited p53 accumulation. In comparison, glucose starvation did not induce the phosphorylation and accumulation of p53 in the TSC1+/+ MEFs. However, both TSC1+/+ and TSC1-/- MEFs responded to DNA damage. Treatment with the DNA damaging

agent etoposide induced p53 phosphorylation and accumulation in both cell types; therefore, it is unlikely that the TSC1+/+ MEFs are defective in their ability to activate p53 (Fig 6A). Furthermore, prevention of p53 activation in the TSC1-/- MEFs by rapamycin suggests that constitutive activation of mTOR was responsible for the difference in p53 accumulation between the TSC1-/- and TSC1+/+ MEFs. It is interesting to note that treatment with rapamycin reduced total p53 below basal levels.

In order to verify that the p53 response was not limited to just the TSC1-/- MEFs, the TSC2-/- LEFs, which also die preferentially in comparison to their rescued counterparts in response to glucose starvation, were also tested. Similarly in the TSC2-/- LEFs, glucose starvation also induced activation of p53 as seen by the phosphorylation of p53 Ser15 and the accumulation of p53 (Fig 6B). The activation of p53 was also eliminated by inhibition of mTOR by rapamycin. On the other hand, the rescued counterpart TSC2+ LEFs did not respond to glucose starvation with p53 activation (Fig 6B). This indicated that the relationship between glucose starvation and p53 activation depends on mTOR activation during the stress but not the specific cause of mTOR activation.

Since glucose starvation induced both p53 activation and apoptosis, it could be possible that the activation of p53 was secondary to activation of apoptosis. To rule out this possibility, the pan-Caspase inhibitor Z-VAD-FMK was used to block apoptosis. Pre-treatment by the pan-Caspase inhibitor with the concurrent challenge of glucose starvation did not prevent p53 phosphorylation and accumulation in the TSC1-/- MEFs (data not shown). This demonstrated

that p53 activation by glucose starvation was not secondary to Caspase cleavage, but rather contributes to apoptosis.

p53 activation is complicated in that phosphorylation on Ser15 can potentiate phosphorylation on other sites on p53 because Ser15 phosphorylation induces dissociation between p53 and its ubiquitin E3 ligase Mdm2. Furthermore, p53 Ser15 phosphorylation alone is insufficient to induce p53 DNA binding, which is induced by phosphorylation on p53 Ser392 (Kapoor et al. 2000). Therefore, multiple phosphorylations are also necessary to fully activate p53. To see whether glucose starvation of the TSC1-/- MEFs can fully activate p53, other phosphorylation sites on p53 were also examined. Glucose starvation induced phosphorylation on several sites including Ser6, Ser9, Ser20, and Ser392 (Fig 6C). Furthermore, phosphorylation on those sites was eliminated by the addition of rapamycin. However, the p53 protein level was also inhibited by rapamycin, therefore, it is possible p53 phosphorylation decreased indirectly by decreasing total p53 protein.

p53 is stabilized by energy starvation

To better understand the role that energy starvation may have in p53 activation, the effects of energy starvation on p53 synthesis and degradation were examined in the TSC1-/- MEFs. To test the effect of energy starvation on p53 stability, we first starved TSC1-/- MEFs for 9 hours to accumulate p53. The cells were then maintained in either glucose-free media or switched to glucose-containing media. Cycloheximide was also included to block further p53 synthesis. As Fig 7A shows, p53 protein was less stable in glucose-rich

conditions than in glucose-poor conditions. This decrease in p53 stability was correlated with the inactivation of AMPK (Fig 7A). Similar results were seen in the absence of cycloheximide; however, the change in p53 stability was partially masked by continued p53 synthesis (data not shown). Furthermore, glucose starvation-induced phosphorylation of p53 Ser15 was also eliminated by the switch to glucose rich media (Fig 7A). However, the concurrent change in p53 protein level confounded conclusions about AMPK and p53 Ser15 phosphorylation with this experiment.

To test the effect of AMPK activity on p53 phosphorylation during glucose starvation, TSC1-/- MEFs were initially glucose starved for 6 hours, and the cells were treated with MG132, a proteosome inhibitor, to prevent p53 degradation. At the same time, the AMPK inhibitor Compound C was also used to treat the cells. Interestingly, Compound C treatment was able to reduce p53 Ser15 phosphorylation in the absence of p53 degradation, which suggested that activation of AMPK was responsible for p53 Ser15 phosphorylation during energy starvation (Fig 7B).

To rule out the possibility that accumulation of p53 protein during energy starvation was due to changes in p53 protein synthesis, we treated TSC1-/- MEFs with MG132 in the presence or absence of glucose. By blocking p53 degradation, we indirectly measured p53 synthesis by observing its rate of accumulation. In the absence of glucose, the rate of p53 accumulation was actually slightly lower; therefore, it was unlikely that the increase in p53 protein during energy starvation was due to increased synthesis (Fig 7C).

Inhibition of mTOR decreases p53 synthesis without increasing degradation

Rapamycin treatment decreases p53 levels in TSC1-/- MEFs. This change in p53 can be due to decreased synthesis, increased degradation, or a combination of both. To test the effect of mTOR activity on p53 phosphorylation during glucose starvation, TSC1-/- MEFs were initially glucose starved for 6 hours, and p53 degradation was then blocked by MG132. The TSC1-/- MEFs were treated with rapamycin to inhibit mTOR. Even after 4 hours of rapamycin treatment, p53 Ser15 phosphorylation was not decreased in comparison to untreated cells (Fig 8A). This suggested that mTOR was not responsible for p53 Ser15 phosphorylation during energy starvation.

To further confirm this, TSC1-/- MEFs were initially glucose starved for 9 hours, and then the cells were treated with cycloheximide to block protein translation. To examine the effects of mTOR inhibition on p53 stability, rapamycin was added 30 minutes prior to the cycloheximide treatment. Thirty minutes of rapamycin treatment is sufficient to completely eliminate mTOR-dependent phosphorylation of ribosomal S6 kinase 1 (S6K) (data not shown). In the presence of cycloheximide, phosphorylation on p53 Ser15 was still maintained, and rapamycin had no significant effect on the half-life of p53 protein (Fig 8B). This suggests that rapamycin did not destabilize p53 after prolonged glucose starvation, and further demonstrates that phosphorylation on p53 Ser15 is not due to mTOR.

To more directly test the effect of rapamycin on p53 stability, p53 was labeled with ^{35}S-methionine in glucose containing media, and then it was chased with cold methionine. Consistent with the results observed by cycloheximide

treatment, rapamycin did not significantly reduce the half-life of p53 (Fig 8C). The addition of excess methionine during the cold chase also had no effect on mTOR activity, as assayed by S6K1 phosphorylation (data not shown). Together this suggested rapamycin did not stimulate p53 degradation; therefore, the protective role of rapamycin during glucose starvation was not at the level of p53 stability.

In order to compare the effects of mTOR activity on p53 accumulation, MG132 was used to block p53 degradation in both TSC1-/- and TSC1+/+ MEFs. In the TSC1+/+ MEFs, glucose starvation inhibited p53 synthesis as p53 accumulation was detected only in the presence but not the absence of glucose. In contrast, MG132 cause p53 accumulation in TSC1-/- MEF even in the absence of glucose. Therefore, the TSC1-/- MEFs are unable to shut down p53 synthesis in response to glucose starvation (Fig 9A). This indicates that constitutive mTOR activation in TSC cells contributes to high levels of p53 accumulation under energy starvation condition.

To demonstrate that inhibition of mTOR in the TSC1-/- MEFs can indeed reduce the accumulation of p53, TSC1-/- MEFs were pretreated with rapamycin before the addition of MG132. Pretreatment with rapamycin decreased the accumulation of p53, which suggests that rapamycin was capable of reducing p53 synthesis. Furthermore, this decrease in accumulation of p53 was independent of the presence of glucose in the media (Fig 9B). Therefore, inhibition of mTOR seems to be critical for affecting global p53 synthesis.

The effect of mTOR inhibition on p53 regulation was not limited to energy

stress. When rapamycin was added concurrently with the DNA damaging agent etoposide, which causes p53 stabilization, the level of p53 levels was also decreased. Moreover, in the absence of any stresses, basal levels of p53 were also decreased by rapamycin (Fig 9C). Together this suggests that mTOR positively regulates p53 synthesis, and the effects of mTOR are independent of which stresses lead to p53 stabilization.

Together, inhibition of mTOR did not destabilize p53, but decreased p53 synthesis. This suggested that during glucose starvation of TSC1-/- MEFs, inhibition of p53 accumulation by rapamycin was due to decreased p53 synthesis. Furthermore, it also suggested that the lack of robust p53 accumulation in response to glucose starvation in the TSC1+/+ MEFs could be explained by inactivation of mTOR by AMPK-dependent phosphorylation of TSC2 because inhibition of mTOR activity in these cells would decrease p53 synthesis (Inoki, Zhu & Guan 2003).

mTOR regulates the association of p53 mRNA with polysomes

mTOR plays a role in the regulation of both transcription and translation; therefore, to clarify the mechanism by which mTOR affects p53 synthesis, both p53 transcription and translation were examined. In order to determine the effects of mTOR inhibition on TP53 transcription, Quantitative RT-PCR (qRT-PCR) was used to determine p53 mRNA level. After 6 hours of glucose starvation or rapamycin treatment, the level of p53 mRNA was determined and normalized to either actin mRNA or hypoxanthine-guanine phosphoribosyltransferase (HPRT) mRNA (Fig 10). Our data indicate that neither rapamycin nor glucose starvation

significantly changed p53 mRNA levels.

Polysome fractionation was used to examine the effect of mTOR on p53 translation. Lysates were fractionated in a sucrose gradient, and mRNA was collected and analyzed by qRT-PCR to determine the relative distribution of the mRNA. In the untreated TSC1+/+ MEFs, the p53 mRNA was predominately associated with the polysome fractions, represented by fractions 8-12 (Fig 10B). However, treatment with rapamycin decreased the fraction of p53 mRNA in the polysome associated fractions and increased the fraction of p53 mRNA in the non-polysome fractions. This shift in p53 mRNA indicated that rapamycin treatment was able to decrease the fraction of p53 mRNA being actively translated (Fig 10B). However, this decrease in p53 translation may not be specific to p53 because rapamycin is known to affect partition of mRNA in polysome of other genes. Together, the lack of change in p53 mRNA and the shift of p53 mRNA away from the polysome by rapamycin suggested that regulation of p53 protein levels by mTOR is primarily due to decreased translation.

p53 accumulation associated with energy stress in angiomyolipomas

Angiomyolipomas are benign tumors consisting of smooth muscle cells, adipose tissue, and blood vessels of which both the stromal cells and the vasculature demonstrate loss of hetrozygosity for either TSC1 or TSC2, and thusly, mTOR activation(Karbowniczek, Yu & Henske 2003). To further confirm the results seen in the TSC1-/- MEFs, both sporadic and TSC-associated angiomyolipomas were stained for p53 and VEGF. It has been shown that VEGF

expression can be induced by either hypoxia or loss of TSC (Brugarolas et al. 2003, El-Hashemite et al. 2003); therefore, VEGF staining may indicate areas of energy stress or TSC loss. In patient 774, which is a sporadic angiomyolipoma, both neoplastic and normal tissue can be compared (Fig 11A). Patients with sporadic angiomyolipomas do not have associated Tuberous Sclerosis disease, but they have Loss of Hetrozygosity of TSC2; therefore, they show upregulated mTOR (Henske et al. 1995). In normal kidney cells, both VEGF and p53 staining are very low. It is interesting to note that there are small areas of VEGF upregulation, which may reflect areas of energy stress; however, p53 levels are universally low. In comparison, within the angiomyolipoma, both VEGF expression and p53 levels are correspondingly elevated. Consistently, in patient 663, which has a TSC-associated angiomyolipoma, both VEGF and p53 are elevated (Fig 11B). Furthermore, the distribution of p53 and VEGF upregulation are also strikingly similar. Together, co-elevation of p53 and VEGF in angiomyolipomas and the lack of elevation of p53 in normal tissue may suggest that loss of TSC1/2 may also contribute to p53 accumulation during energy stress in vivo.

Discussion

We have shown that mTOR regulates p53 synthesis and the proper coordination of p53 synthesis with stabilization during energy starvation is necessary insure cellular viability. We propose a model that energy starvation activates AMPK, which phosphorylates and stabilizes p53 (Fig 12). However, the AMPK-dependent increase of p53 is controlled because AMPK activation also

inhibits mTOR and thereby inhibits p53 translation. The stabilization of p53 by AMPK phosphorylation is more prominent than the inhibition of p53 translation by AMPK-induced mTOR inhibition. With both aspects of p53 regulation intact, energy starvation of wild type cells only initiates a limited elevation of p53, which induces cell cycle arrest and protects the cells from unfavorable conditions. However, loss of TSC1 or TSC2 results in a dramatic elevation of p53 protein in response to energy starvation because in TSC cells, p53 translation cannot be inhibited by energy starvation. This is due to elevated mTOR activity. The dramatic p53 accumulation in TSC cells is caused by a combined effect of stabilization by AMPK-dependent phosphorylation and constitutive translation by active mTOR. The high level of p53, therefore, induces apoptosis in TSC cells under energy starvation conditions and may contribute to the highly apoptotic and benign nature of TCS tumors.

Although activation of AMPK by hypoxia and AICAR are the same in TSC+/+ and TSC-/- cells (Brugarolas et al. 2004), the effects of energy starvation on cell survival are different. We showed that in the absence of proper down-regulation of mTOR in response to AMPK activation, cells rapidly undergo apoptosis. This was seen by Annexin V staining and cleavage of Caspases 3, 9, and 12 but not 8. This suggests that glucose starvation induced apoptosis of the TSC1-/- MEFs involves activation of the intrinsic apoptotic pathway, but not the extrinsic pathway. Moreover, apoptosis did not occur when mTOR was down-regulated in response to energy starvation, as seen in the case of the TSC1+/+ MEFs and the TSC1-/- MEFs treated with rapamycin. Treatment with

rapamycin pharmacologically mimicked the normal function of the AMPK-TSC2-mTOR pathway that was defective in the TSC1-/- MEFs. This suggests that down-regulation of mTOR during glucose starvation is critical for maintaining cell viability.

Consistent with the model that constitutive p53 translation by elevated mTOR induced apoptosis during glucose starvation, loss of p53 reduced sensitivity to glucose starvation in TSC cells. In the absence of p53, TSC2-/- p53-/- MEFs were resistant to glucose starvation induced cell death; however, prolonged exposure to starvation eventually leads to cell death. p53 is not only a potent activator of the intrinsic apoptotic pathway, but it is also critical regulator for cell cycle arrest and senescence; therefore, proper regulation of p53 is necessary for coordination of these two cell fates. Although loss of p53 protects TSC cells against energy starvation induced apoptosis, loss of p53 can also have deleterious effects on survival during energy starvation. Previous reports suggested that complete loss of p53 sensitizes cells to glucose starvation-induced cell death by the loss of a cell cycle checkpoint (Imamura et al. 2001). In the presence of an intact TSC-mTOR pathway, activation of AMPK induces a p53-dependent arrest in G1 phase, through direct phosphorylation of p53 Ser 15 (Jones et al. 2005). Therefore, via the p53 pathway, cells cease to growth while maintaining viability. Consequently, during glucose starvation in order to ensure proper survival, p53 levels must be modestly elevated to induce cell cycle arrest but not dramatically, which would induce apoptosis.

In normal cells, activation of AMPK not only activates p53 by direct

phosphorylation, but it also inactivates mTOR via TSC2 (Fig 12). This antagonism of mTOR activity by AMPK may be necessary for directing p53 toward cell cycle arrest instead of apoptosis, as loss of TSC sensitizes cells to apoptosis. Although sensitivity to energy starvation requires both mTOR and AMPK activation, mTOR and AMPK play different roles in regulating p53. Under unstressed conditions, p53 was kept at low levels by proteosome-mediated degradation subsequent to ubiquitination by the E3 ligase Mdm2 (Vogelstein, Lane & Levine 2000). AMPK activation functions predominately through stabilization of p53 after direct phosphorylation by AMPK; however, in the absence of TSC2, AMPK does not affect p53 synthesis. Inhibition of mTOR by rapamycin does not lead to dephosphorylation of p53 nor does it destabilize p53. mTOR, on the other hand, facilitates the accumulation of p53 by regulating its translation.

We have also shown that the effects of mTOR inhibition on p53 levels are independent of the insults that stabilize p53. This is reflected by the ability of rapamycin to decrease both basal p53 levels and p53 that is stabilized by either MG132 or DNA damage (Fig 9B, 9C). Furthermore, we have previously shown that in LKB1-/- MEFs, which have impaired ability to activate AMPK, mTOR also remains active in response to energy starvation (Corradetti et al. 2004). These MEFs also undergo rapamycin reversible glucose starvation induced apoptosis; however, since AMPK cannot be activated by LKB1, other kinases may also play a role in stabilizing p53 during glucose starvation. Consistently, when we treated TSC1-/- MEFs with the AMPK inhibitor Compound C, we only see partial

protection against p53 Ser 15 phosphorylation (Fig 6A). In addition, the lack of AMPK activation in LKB1-/- cells will affect the cellular ATP homeostasis under starvation. Therefore, the mechanism of glucose starvation induced cell death in LKB1-/- may not be exactly same as that in the TSC cells. Consistent with mTOR's role in regulating p53 synthesis, it has also been reported that loss of PTEN, which activates mTOR by activation of AKT, also increases p53 expression and upregulation of p53 gene targets (Kim et al. 2007). It has also been reported that treatment with rapamycin reduces p53-dependent apoptosis by HIV infection (Castedo et al. 2001) and ionization radiation (Tirado et al. 2003). Taken together, it is possible that mTOR is critical for modulating the effects of p53 during a variety of stresses.

From these experiments we have identified a novel mechanism by which mTOR regulates p53 to maintain cell viability during energy starvation. This provides new insights into the pro-apoptotic role of mTOR and may help explain the benign nature of TSC hamartomas. Immunohistochemical staining of TSC tumors showed concurrent staining by VEGF and p53, which indicates that in tumors lacking TSC p53 levels are substantially elevated (Fig 11A, 11B). This data indicates that our model of over-accumulation of p53 by disruption of mTOR pathway is not limited to just cell culture, but may also play a role in vivo. Given the fact that the TP53 gene encodes the most commonly mutated tumor suppressor in human cancers, our study suggests that p53 status is important for determining whether mTOR inhibitors are effective against various neoplasms.

Methods

Antibodies and materials

Anti-p53, anti-phospho p53 (S6, S9, S15, S20, S392), anti-AMPK, and anti-phospho AMPK (T172) antibodies were obtained from Cell Signaling (Beverly, MA). Anti-Actin, anti-β-tubulin, anti-Caspase 8, antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA). Horseradish peroxidase-conjugated IgG secondary antibodies were obtained from Amersham (Buckinghamshire, UK).

The AMPK inhibitor, commonly known as compound C, was obtained from Merck (Whitehouse Station, NJ) and was described previously (Zhou et al. 2001). Cells were treated with 10 μM of the compound suspended in DMSO. Rapamycin was purchased from Cell Signaling and was suspended in methanol at 20 nM. The proteosome inhibitor MG132 was obtained from Sigma and used at 20 μM. Protein stability was assayed with cycloheximide from Sigma and used at 50 ng/mL. Etoposide was purchase from Sigma and used at 6 μg/mL.

Cell culture and transfection

MEF cells were cultured in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen) and 50 μg/mL penicillin/streptomycin (P/S). $TSC2^{-/-}$ LExF2 cells (LEF) were maintained in DMEM/F12 (Invitrogen) containing 10% FBS and 50 μg/mL P/S. Glucose starvation was performed with Glucose Free DMEM (Invitrogen) containing 25 mM HEPES, 10% dialyzed FBS (Invitrogen), and 50 μg/mL P/S.

Annexin V/PI staining

Annexin V/Propidium Iodide (PI) double staining was done with Annexin V

and PI (BD Biosciences) as per manufacturer's protocol, and samples were analyzed via BD FACScalibur (BD Biosciences).

^{35}S labeling

^{35}S Pulse/Chase labeling was done with 0.2 mCi/mL of ^{35}S-Met/Cys Trans label in DMEM (-Met/-Cys) containing 10% dialyzed FBS for 1 hr prior to chase with DMEM containing 10% dialyzed FBS, 18 mg L-Cys / 100 mL media, and 9 mg L-Met / 100 mL media. p53 was immunoprecipitated with anti-p53 antibodies and resolved by SDS-PAGE.

^{35}S Pulse labeling for assay of p53 synthesis was performed with labeling media as described earlier but only incubated for 8 minutes prior to immunoprecipitation by anti-p53 antibodies and resolution by SDS-PAGE.

qRT-PCR

10 cm dishes of TSC1-/- or TSC1+/+ MEFs were glucose starved or rapamycin treated for 6 hours prior to lysis with 1 mL Trizol (Sigma), and the aqueous layer was collected after addition of 200 µL chloroform. mRNA was precipitated with 1 volume isopropanol, and isopropanol was removed with a 70% ethanol wash, and the RNA pellet was air dried. Reverse transcription was performed with the Superscript First Strand Synthesis System for RT-PCR (Invitrogen) as per manufacturer's protocol.

Quantitative PCR was performed using Power SYBR Green PCR Master Mix (Applied Biosystems). p53 was amplified using the forward primer 5'-AACCGCCGACCTATCCTTAC-3' and the reverse primer 5'-CTTCTGTACGGCGGTCTCTC-3'. HPRT was amplified using the forward

primer 5'-TCATTATGCCGAGGATTTGGA-3' and the reverse primer 5'-GCACACAGAGGGCCACAAT-3'. Actin was amplified using the forward primer 5'-CCGGGAGAAGATGACTCAAA-3' and the reverse primer 5'-CCAGAATCCAACACGATGC-3'. Samples were done in triplicate to calculate averages and standard deviations.

Polysome fractionation

Mouse embryonic fibroblasts were cultured in DMEM, supplemented with 10% fetal bovine serum, penicillin (100 units/mL) and streptomycin (100 µg/mL). MEFs were seeded in 150 mm Petri dishes (5 x 10^6 cells/dish) and collected 24 hours later. Prior to harvesting, cells were treated with cycloheximide (100 µg/mL) for 10 minutes. Cells were then washed twice with 5 ml of PBS (containing 100 µg/mL), collected by scraping and pelleted at 500 x g for 5 minutes. Cells were lysed in 0.8 ml of extraction buffer (5mM Tris (pH 7.5), 2.5 mM MgCl2, 1.5mM KCl, 100 µg/mL cycloheximide, 2mM DTT, 0.5% Triton-X 100, and 0.5% sodium deoxycholate). Extracts were cleared by centrifugation at 13 000 x g for 2 minutes and then loaded on 11ml sucrose gradients (10-50%) buffered in 20mM HEPES (pH 7.6), 100 mM KCl, 5mM $MgCl_2$. Gradients were subjected to centrifugation using a Beckman SW40Ti Rotor at 38,000 rpm for 2.2 hours at 4^{o}C. Gradients were then fractionated (from the lightest to the heaviest fraction) into 24 fractions (12 drops per fraction; approximately 0.5 ml) while monitoring the optical density at 254 nm. Adjacent fractions were pooled to yield a total of 12 fractions for qPCR.

Immunohistochemistry

Four-micron sections were deparaffinized in xylene and rehydrated in a gradient series of ethanol. For antigen retrieval, sections were boiled in Citric Buffer (10mM sodium citrate- trisodium salt dehydrate, Sigma, St Louis, MO), pH 6.0, for 10 minutes. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in methanol for 15 minutes at room temperature. Non-specific background was eliminated by incubating the tissue with normal goat serum for 10 minutes at room temperature (Zymed, San Francisco, CA). The sections were then incubated in a humidified chamber with mouse monoclonal antibody against p53 (1C12), dilution 1:100, (Cell Signaling Technology, Beverly, MA) or prediluted rabbit monoclonal antibody against VEGF (SP28), (Abcam Inc, Cambridge, MA) overnight at 4ºC. The slides were then washed, incubated with biotinylated affinity purified secondary antibodies (Zymed, San Francisco, CA) for 10 minutes at room temperature, then washed and incubated with enhanced horseradish peroxidase conjugated streptavidin (Zymed) for 10 minutes at room temperature. After washing, the slides were developed using AEC Chromogen Solution (Zymed), lightly counterstained with Hematoxylin (Biomeda, Foster City, CA) and mounted using GelMount (Biomeda).

Acknowledgments

We are indebted to Dr. R.S. Yeung for providing EEF126 cell lines and the LExF2 cell line; Dr. D.J. Kwiatkowski for $TSC1^{-/-, +/+}$ MEFs; Dr. G. Zhou for the AMPK inhibitor. This research would not have been possible without the constant scientific guidance of Ken Inoki. We are also grateful for the help of Emmanuel Petroulakis and Magdalena Karbowniczek, who did the polysome

fractionation and immunohistochemistry respectively. We would also like to thank Jeffery Lee, Grant Yang, Eunjung Kim, Qian Yang, and Xiaoming Zhao for their critical reading and comments on the manuscript, and funding by grants from NIH and Department of Defense (KLG).

Figures

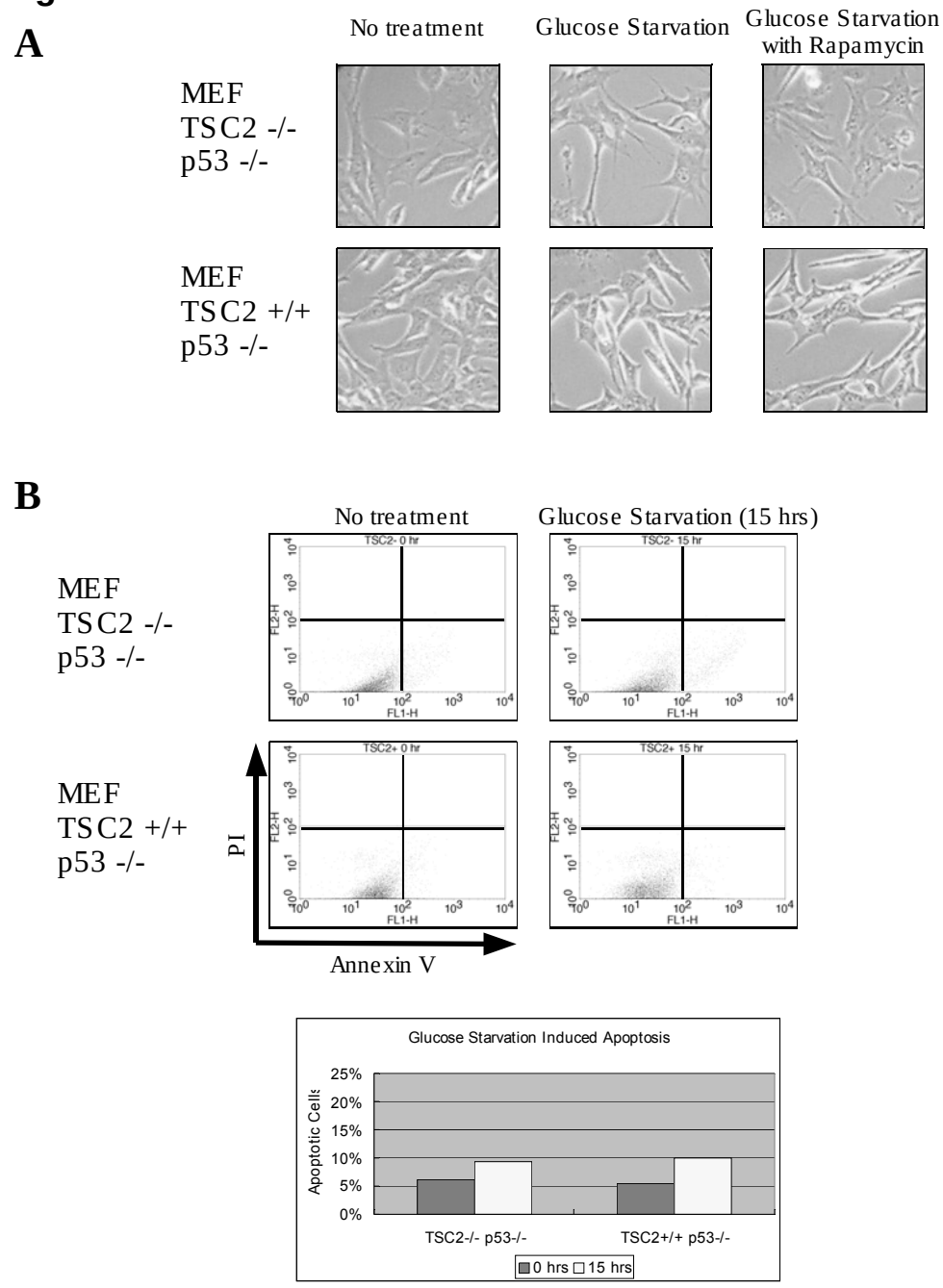

Fig 5 Increased sensitivity to energy starvation in TSC2-/- cells requires p53.

(A) TSC2-/- p53-/- MEFs and TSC2+/+ p53-/- MEFs were resistant to glucose starvation (15 hrs). (B) FACS analysis showed that TSC2-/- p53-/- and TSC2+/+ p53-/- MEFs were equally resistant to energy starvation (15 hrs).

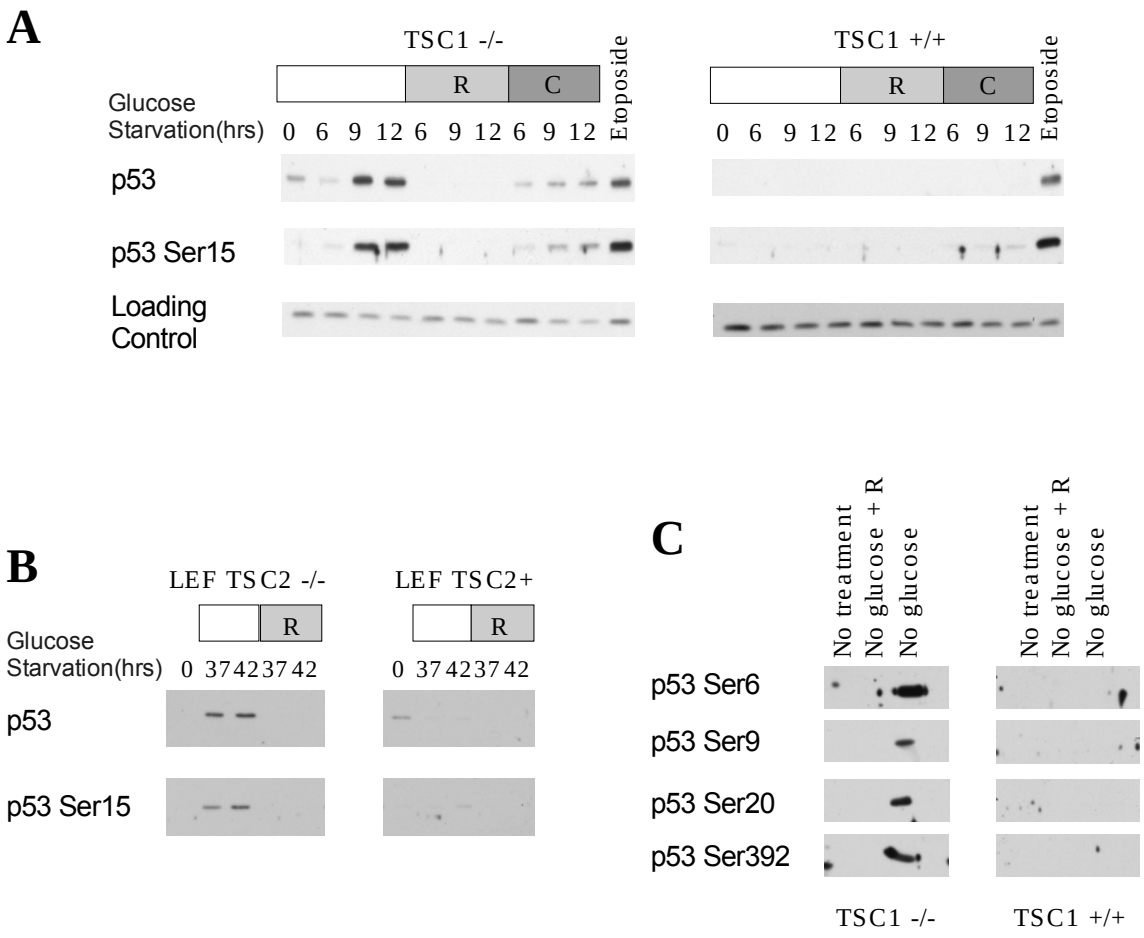

Fig 6 Glucose Starvation induces rapamycin reversible p53 activation.

(A) Glucose starvation time course showed both p53 Ser15 phosphorylation and p53 accumulation in TSC1-/- MEFs, which was reversed by rapamycin (R) and partially reversed by compound C (C, 10 μM); however, this was not seen in TSC1+/+ MEFs. Etoposide treatment for 6 hours (6 μg/mL) induced p53 in both TSC1-/- and TSC1+/+ MEFs. (B) Glucose starvation induced p53 Ser15 phosphorylation and p53 accumulation in TSC2-/- LEFs, which was reverse by rapamycin treatment; however, TSC2+ LEFs did not show induction of p53. (C) Glucose starvation of TSC1-/- MEFs also increased phosphorylation on p53 Ser6, Ser9, Ser20, and Ser392, which was reverse by rapamycin. However, TSC1+/+ MEFs did not show p53 phosphorylation during glucose starvation.

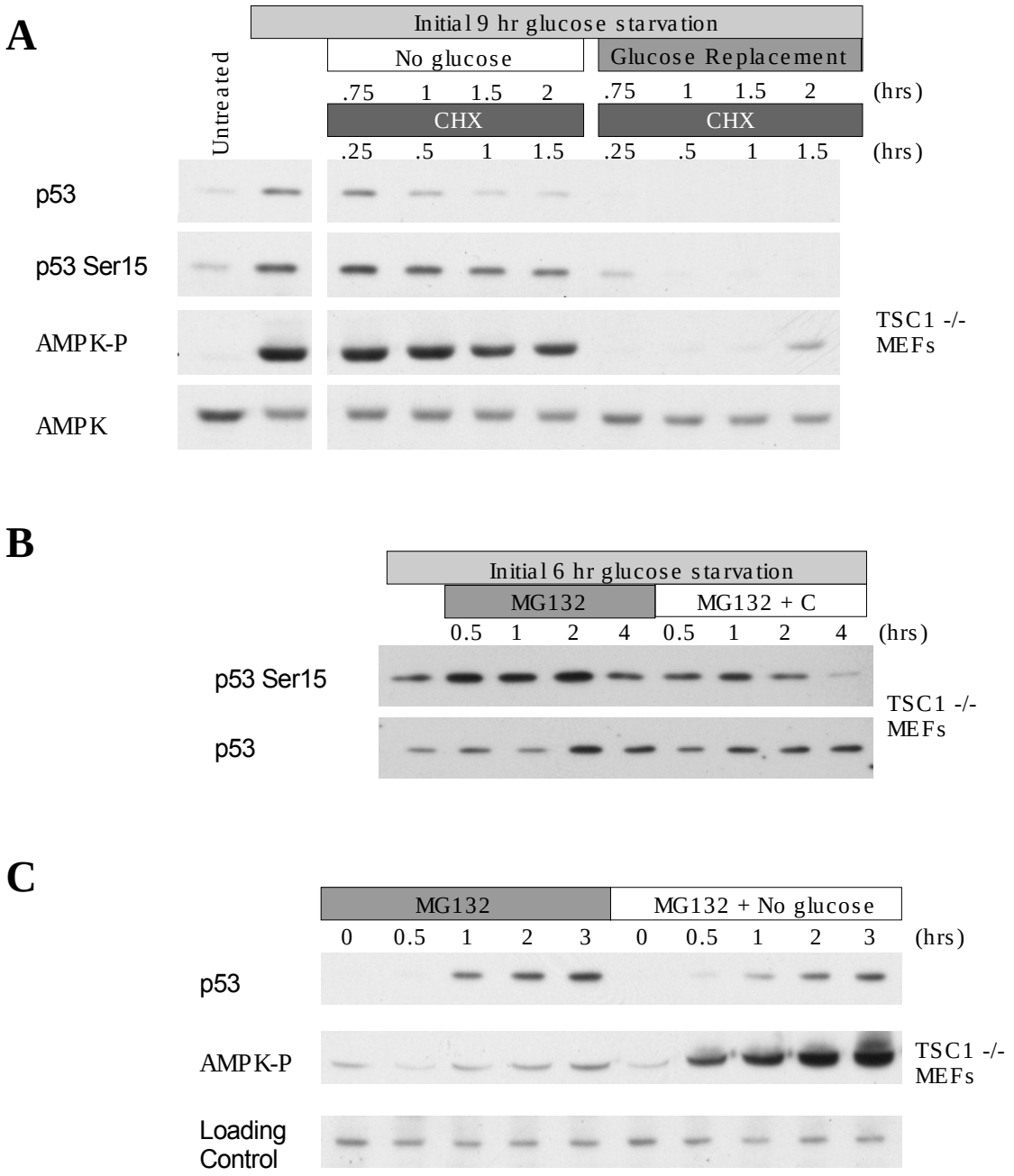

Fig 7 Stabilization of p53 during glucose starvation is due to AMPK.

(A) p53 was stabilized by glucose starvation in TSC1-/- MEFs, and further synthesis was blocked by cycloheximide (50 ng/mL). Reintroduction of glucose decreased p53 stability. (B) p53 was accumulated with glucose starvation in TSC1-/- MEFs, and degradation was blocked by MG132 (20 μM). Compound C decreased phosphorylation on p53 Ser15, when degradation of p53 was blocked. (C) Degradation of p53 was blocked by MG132 in TSC1-/- MEFs. Glucose starvation did not increase p53 synthesis.

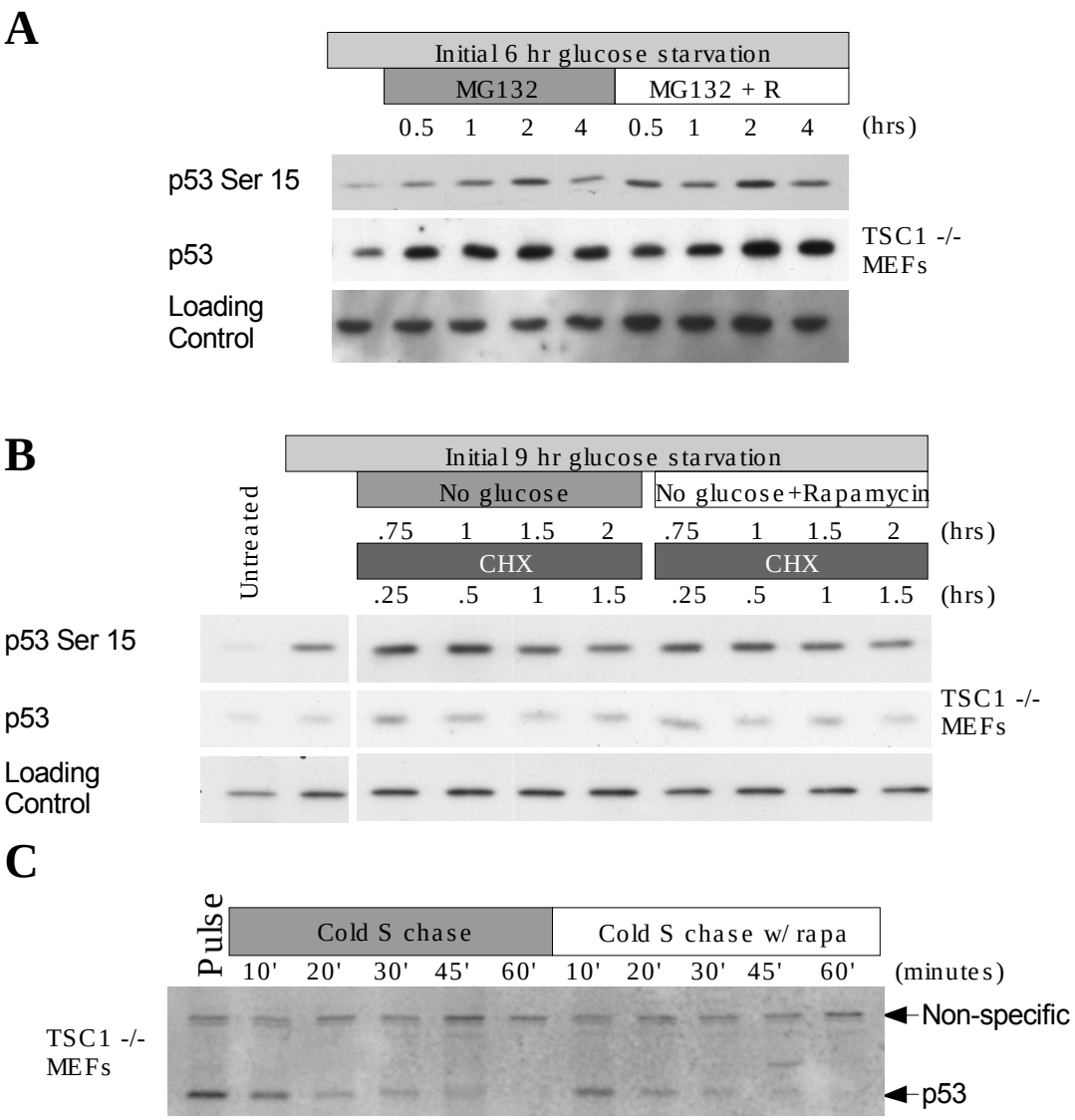

Fig 8 Rapamycin does not affect p53 stability or phosphorylation.

(A) p53 was accumulated with glucose starvation in TSC1-/- MEFs, and degradation was blocked by MG132 (20 μM). Rapamycin did not decrease phosphorylation on p53 Ser15, when degradation of p53 was blocked. (B) p53 was stabilized by glucose starvation in TSC1-/- MEFs, and further synthesis was blocked by cycloheximide (50 ng/mL). Treatment with rapamycin did not affect p53 stability or phosphorylation on Ser15. (C) ^{35}S Pulse-chase both in the presence and absence of rapamycin in glucose-rich media of TSC1-/- MEFs. Rapamycin did not enhance the degradation of p53.

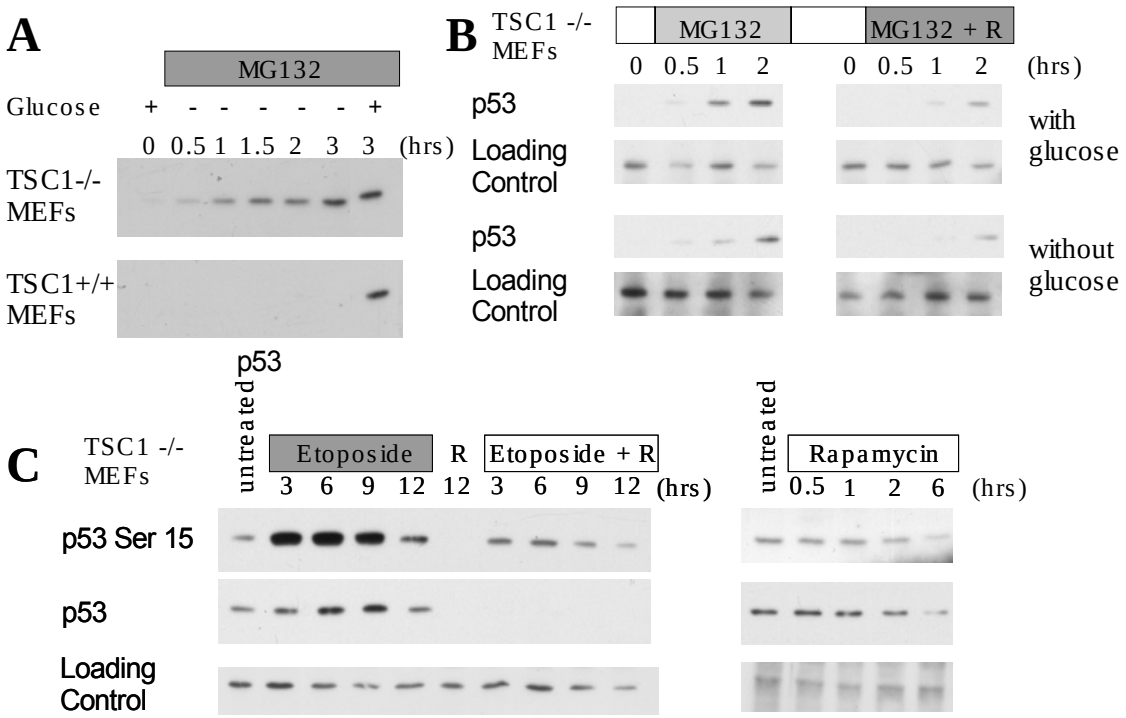

Fig 9 Inhibition of mTOR decreases p53 synthesis.

(A) p53 degradation was blocked by MG132. Accumulation of p53 was examined under various conditions in TSC1-/- and TSC1+/+ MEFs. In TSC1-/- MEFs, glucose starvation is unable to shut down p53 synthesis. In TSC1+/+ MEFs, glucose starvation decreases rate of p53 synthesis. (B) TSC1-/- MEFs were pretreated with rapamycin for 6 hours prior to MG132 treatment. Accumulation of p53 was decreased by rapamycin pretreatment regardless of whether glucose was present. (C) p53 phosphorylation and accumulation was stimulated by etoposide. Concurrent rapamycin treatment decreased p53 protein levels, and detected p53 Ser15 phosphorylation. Rapamycin treatment alone also decreased basal p53 levels.

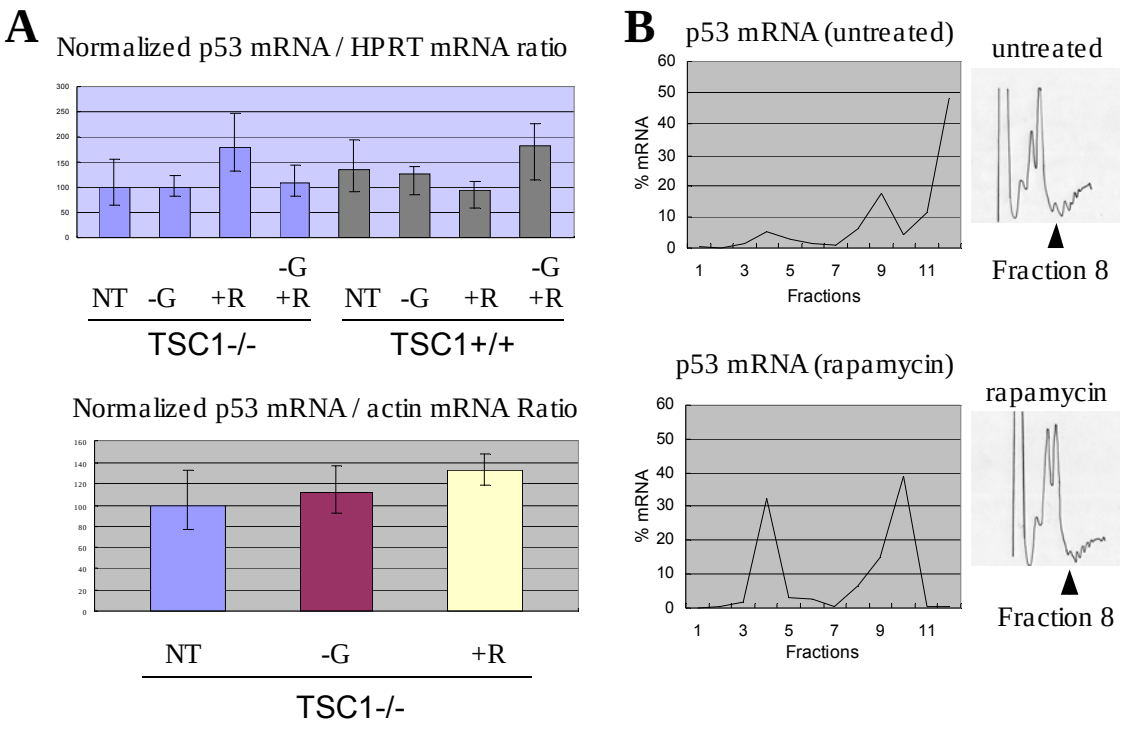

Fig 10 Inhibition of mTOR decreases p53 translation.

(D) p53 mRNA was normalized to either Actin mRNA or HPRT mRNA in TSC1-/- and TSC1+/+ MEFs. Neither glucose starvation nor rapamycin treatment had significant effects on p53 mRNA level. (E) p53 mRNA was fractionated over a sucrose gradient in WT MEFs to examine the p53 mRNA association with polysomes. Fractions 8-12 represent polysome associated fractions. Rapamycin decreased polysome association of p53 mRNA.

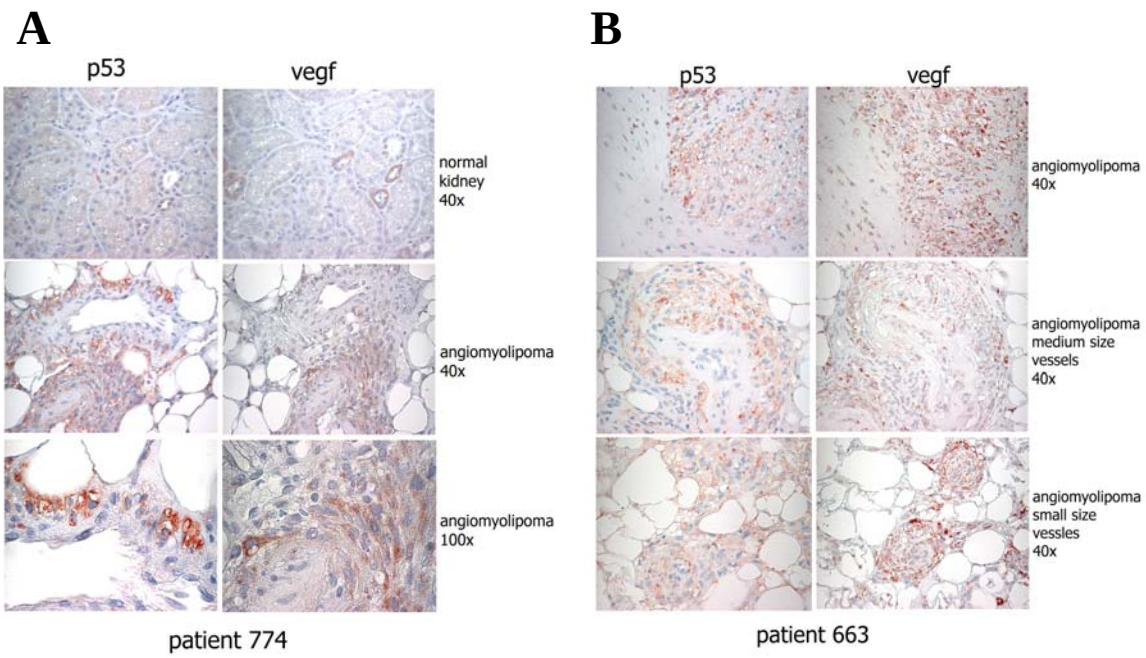

Fig 11 Energy stress in angiomyolipomas is associated with p53 upregulation and model of p53 activation by energy starvation in TSC-/- cells.

(A) Tissues from both normal kidney and sporadically arising angiomyolipomas were stained for p53 and VEGF. Normal tissue showed little upregulation of either p53 or VEGF, while in the angiomyolipoma, both p53 and VEGF staining were dramatically increased. (B) Tissues from TSC patient derived angiomyolipomas were stained for p53 and VEGF. Both p53 and VEGF were correspondingly increased.

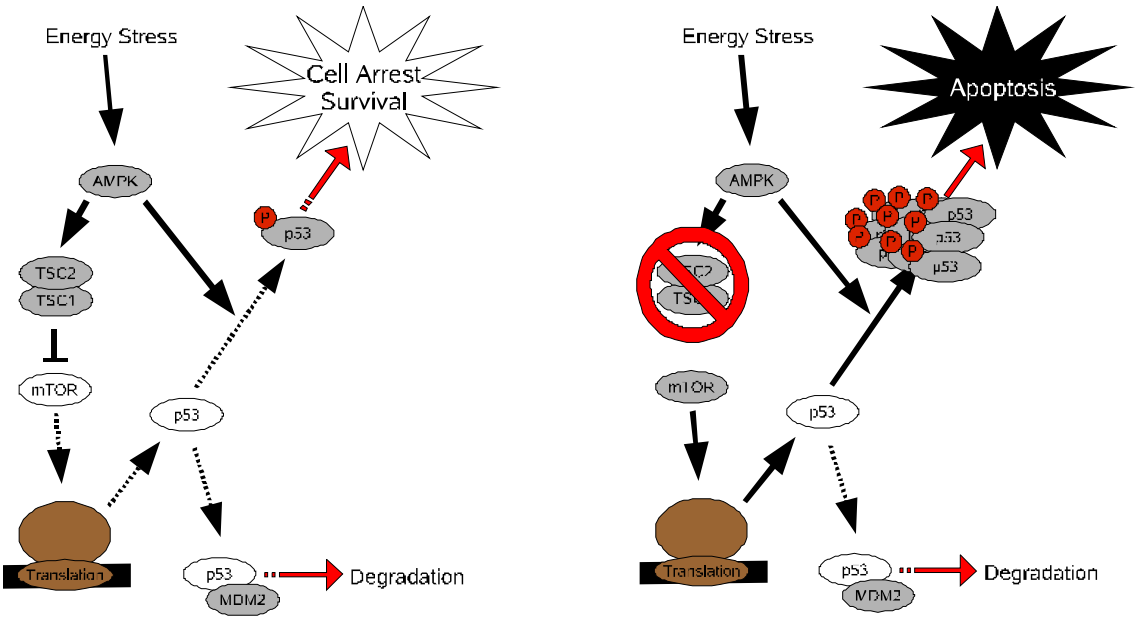

Fig 12 Model

Model for negative regulation of p53 by mTOR to promote survival during energy stress. When the mTOR pathway is intact, AMPK activation down regulates p53 synthesis via the mTOR pathway and stabilizes p53 via phosphorylation. However, in the absence of TSC, p53 synthesis cannot be down regulated; therefore, when AMPK stabilizes p53, p53 is greatly elevated, and apoptosis is induced.

Chapter IV: Future Directions

Together these studies have established a mechanism by which over-activation of mTOR leads to increased cell death via the p53 pathway. This increase in cell death occurs because mTOR is a translational regulator of p53. However, the mechanism by which mTOR regulates p53 translation is not clear.

The delayed effect of mTOR inhibition on p53 synthesis may also imply that the mechanism by which mTOR regulates p53 synthesis is not direct. Rapamycin decreases phosphorylation on S6K and 4EBP within 30 minutes; however, the effects on p53 synthesis were not readily apparent until several hours subsequent to rapamycin inhibition. It is possible that inhibition of mTOR induces the synthesis of a translation factor that represses p53 synthesis, or mTOR inhibition leads to the degradation of a factor that usually stimulates p53 synthesis. Recent reports have suggested that p53 is not only regulated post-translationally but also at the level of translation. p53 can be translated by either cap-dependent or internal ribosomal entry site-dependent mechanisms (Ray, Grover & Das 2006, Yang, Halaby & Zhang 2006). Furthermore, both the 5' and 3' UTR of the p53 mRNA have sequences that are important for regulating of p53 translation (Fu, Benchimol 1997, Fu, Ma & Benchimol 1999, Schumacher et al. 2005, Takagi et al. 2005/10/7, Zou et al. 2006), so it is possible that mTOR may regulate proteins that bind to those regions.

The delayed effects of mTOR inhibition on p53 synthesis may also be important for proper p53 activation and regulation, and thus be physiologically important for cell cycle arrest. Like other groups, we observed a very rapid but transient phosphorylation of p53 in response to energy starvation (< 3 hrs) (Feng et al. 2005); however, only at later time points (> 6 hrs) did an additional unrelenting rise in p53 phosphorylation and protein levels occur in the TSC1-/- MEFs. It is attractive to speculate that the prolonged time necessary for mTOR to act on p53 synthesis may account for the transient effect of AMPK activation on p53. In normal cells, energy starvation induces the activation of AMPK, which activates p53 and inhibits mTOR. Therefore, during this early period, the effect of stabilization is dominant; p53 is activated and starts to accumulate. However, as inhibition of mTOR continues, the effect of mTOR on p53 synthesis becomes more apparent, and p53 cannot be elevated further. In the absence of TSC1/2, cells are no longer capable of shutting down p53 synthesis; therefore, p53 accumulation becomes unrelenting and eventually lead to apoptosis. Furthermore, in addition to p53 being a downstream target of mTOR, it has also been reported that p53 can activate AMPK, which is an upstream regulator of mTOR (Feng et al. 2005). Through the AMPK-TSC-mTOR pathway, p53 is able to form a negative feedback loop to keep its own synthesis in check.

Finally, our discovery of a novel regulation of p53 by mTOR may also have broader clinical implications. Currently mTOR is under study for it's efficacy as an anti-neoplastic agent, and our discovery that mTOR may regulate p53 gives clues that p53 status may be important for determining the sensitivity to mTOR

inhibition. Furthermore, since p53 is also activated by many chemotherapeutics, it is possible that concurrent treatment with mTOR inhibitors may decrease the effectiveness of other chemotherapeutics. However, this is still subject to speculation; therefore, further study would be necessary to validate these possibilities.

Bibliography

Ali, S.M. and Sabatini, D.M. 2005. Structure of S6 kinase 1 determines whether raptor-mTOR or rictor-mTOR phosphorylates its hydrophobic motif site. *J. Biol. Chem.* 280: 19445-19448.

Atherton, P.J., Babraj, J., Smith, K., Singh, J., Rennie, M.J. and Wackerhage, H. 2005. Selective activation of AMPK-PGC-1alpha or PKB-TSC2-mTOR signaling can explain specific adaptive responses to endurance or resistance training-like electrical muscle stimulation. *FASEB J.* 19: 786-788.

Bao, Q. and Shi, Y. 2007. Apoptosome: A platform for the activation of initiator caspases. *Cell Death Differ.* 14: 56-65.

Berger, Z., Ravikumar, B., Menzies, F.M., Oroz, L.G., Underwood, B.R., Pangalos, M.N., Schmitt, I., Wullner, U., Evert, B.O., O'Kane, C.J. and Rubinsztein, D.C. 2006. Rapamycin alleviates toxicity of different aggregate-prone proteins. *Hum. Mol. Genet.* 15: 433-442.

Beugnet, A., Wang, X. and Proud, C.G. 2003. Target of rapamycin (TOR)-signaling and RAIP motifs play distinct roles in the mammalian TOR-dependent phosphorylation of initiation factor 4E-binding protein 1. *J. Biol. Chem.* 278: 40717-40722.

Bodine, S.C., Stitt, T.N., Gonzalez, M., Kline, W.O., Stover, G.L., Bauerlein, R., Zlotchenko, E., Scrimgeour, A., Lawrence, J.C., Glass, D.J. and Yancopoulos, G.D. 2001. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. *Nat. Cell Biol.* 3: 1014-1019.

Brugarolas, J., Lei, K., Hurley, R.L., Manning, B.D., Reiling, J.H., Hafen, E., Witters, L.A., Ellisen, L.W. and Kaelin, W.G.,Jr 2004. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. *Genes Dev.* 18: 2893-2904.

Brugarolas, J.B., Vazquez, F., Reddy, A., Sellers, W.R. and Kaelin, W.G.,Jr 2003. TSC2 regulates VEGF through mTOR-dependent and -independent pathways. *Cancer. Cell.* 4: 147-158.

Cai, S.L., Tee, A.R., Short, J.D., Bergeron, J.M., Kim, J., Shen, J., Guo, R., Johnson, C.L., Kiguchi, K. and Walker, C.L. 2006. Activity of TSC2 is inhibited

by AKT-mediated phosphorylation and membrane partitioning. *J. Cell Biol.* 173: 279-289.

Castedo, M., Ferri, K.F., Blanco, J., Roumier, T., Larochette, N., Barretina, J., Amendola, A., Nardacci, R., Metivier, D., Este, J.A., Piacentini, M. and Kroemer, G. 2001. Human immunodeficiency virus 1 envelope glycoprotein complex-induced apoptosis involves mammalian target of Rapamycin/FKBP12-rapamycin-associated protein-mediated p53 phosphorylation. *J. Exp. Med.* 194: 1097-1110.

Castro, A.F., Rebhun, J.F., Clark, G.J. and Quilliam, L.A. 2003. Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. *J. Biol. Chem.* 278: 32493-32496.

Chen, J.K., Chen, J., Neilson, E.G. and Harris, R.C. 2005. Role of mammalian target of rapamycin signaling in compensatory renal hypertrophy. *J. Am. Soc. Nephrol.* 16: 1384-1391.

Cho, D., Signoretti, S., Regan, M., Mier, J.W. and Atkins, M.B. 2007. The role of mammalian target of rapamycin inhibitors in the treatment of advanced renal cancer. *Clin. Cancer Res.* 13: 758s-63s.

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

Collins, B.J., Deak, M., Arthur, J.S., Armit, L.J. and Alessi, D.R. 2003. In vivo role of the PIF-binding docking site of PDK1 defined by knock-in mutation. *EMBO J.* 22: 4202-4211.

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

Dong, J. and Pan, D. 2004. Tsc2 is not a critical target of akt during normal drosophila development. *Genes Dev.* 18: 2479-2484.

Ebina, M., Takahashi, T., Chiba, T. and Motomiya, M. 1993. Cellular hypertrophy and hyperplasia of airway smooth muscles underlying bronchial asthma. A 3-D morphometric study. *Am. Rev. Respir. Dis.* 148: 720-726.

El-Hashemite, N., Walker, V., Zhang, H. and Kwiatkowski, D.J. 2003. Loss of Tsc1 or Tsc2 induces vascular endothelial growth factor production through mammalian target of rapamycin. *Cancer Res.* 63: 5173-5177.

Feng, Z., Zhang, H., Levine, A.J. and Jin, S. 2005. The coordinate regulation of the p53 and mTOR pathways in cells. *Proc. Natl. Acad. Sci. U. S. A.* 102: 8204-8209.

Fu, L. and Benchimol, S. 1997. Participation of the human p53 3'UTR in translational repression and activation following γ-irradiation. *EMBO J.* 16: 4117-4125.

Fu, L., Ma, W. and Benchimol, S. 1999. A translation repressor element resides in the 3' untranslated region of human p53 mRNA. *Oncogene.* 18: 6419-6424.

Fujitani, Y. and Trifilieff, A. 2003. In vivo and in vitro effects of SAR 943, a rapamycin analogue, on airway inflammation and remodeling. *Am. J. Respir. Crit. Care Med.* 167: 193-198.

Gao, X. and Pan, D. 2001. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. *Genes Dev.* 15: 1383-1392.

Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R.S., Ru, B. and Pan, D. 2002. Tsc tumour suppressor proteins antagonize amino-acid-TOR signalling. *Nat. Cell Biol.* 4: 699-704.

Garami, A., Zwartkruis, F.J., Nobukuni, T., Joaquin, M., Roccio, M., Stocker, H., Kozma, S.C., Hafen, E., Bos, J.L. and Thomas, G. 2003. Insulin activation of rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. *Mol. Cell.* 11: 1457-1466.

Giasson, E. and Meloche, S. 1995. Role of p70 S6 protein kinase in angiotensin II-induced protein synthesis in vascular smooth muscle cells. *J. Biol. Chem.* 270: 5225-5231.

Gingras, A.C., Raught, B. and Sonenberg, N. 2001. Regulation of translation initiation by FRAP/mTOR. *Genes Dev.* 15: 807-826.

Gingras, A.C., Kennedy, S.G., O'Leary, M.A., Sonenberg, N. and Hay, N. 1998. 4E-BP1, a repressor of mRNA translation, is phosphorylated and inactivated by the akt(PKB) signaling pathway. *Genes Dev.* 12: 502-513.

Gingras, A.C., Raught, B., Gygi, S.P., Niedzwiecka, A., Miron, M., Burley, S.K., Polakiewicz, R.D., Wyslouch-Cieszynska, A., Aebersold, R. and Sonenberg, N. 2001. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. *Genes Dev.* 15: 2852-2864.

Goncharova, E.A., Goncharov, D.A., Eszterhas, A., Hunter, D.S., Glassberg, M.K., Yeung, R.S., Walker, C.L., Noonan, D., Kwiatkowski, D.J., Chou, M.M., Panettieri, R.A.,Jr and Krymskaya, V.P. 2002. Tuberin regulates p70 S6 kinase activation and ribosomal protein S6 phosphorylation. A role for the TSC2 tumor suppressor gene in pulmonary lymphangioleiomyomatosis

(LAM). *J. Biol. Chem.* 277: 30958-30967.

Goncharova, E.A., Goncharov, D.A., Spaits, M., Noonan, D.J., Talovskaya, E., Eszterhas, A. and Krymskaya, V.P. 2006. Abnormal growth of smooth muscle-like cells in lymphangioleiomyomatosis: Role for tumor suppressor TSC2. *Am. J. Respir. Cell Mol. Biol.* 34: 561-572.

Guertin, D.A. and Sabatini, D.M. 2005. An expanding role for mTOR in cancer. *Trends Mol. Med.* 11: 353-361.

Guillet, C., Prod'homme, M., Balage, M., Gachon, P., Giraudet, C., Morin, L., Grizard, J. and Boirie, Y. 2004. Impaired anabolic response of muscle protein synthesis is associated with S6K1 dysregulation in elderly humans. *FASEB J.* 18: 1586-1587.

Hafizi, S., Wang, X., Chester, A.H., Yacoub, M.H. and Proud, C.G. 2004. ANG II activates effectors of mTOR via PI3-K signaling in human coronary smooth muscle cells. *Am. J. Physiol. Heart Circ. Physiol.* 287: H1232-8.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J. and Yonezawa, K. 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. *Cell.* 110: 177-189.

Hardie, D.G. 2007. AMP-activated protein kinase as a drug target. *Annu. Rev. Pharmacol. Toxicol.* 47: 185-210.

Hardie, D.G., Carling, D. and Carlson, M. 1998. The AMP-activated/SNF1 protein kinase subfamily: Metabolic sensors of the eukaryotic cell? *Annu. Rev. Biochem.* 67: 821-855.

Harrington, L.S., Findlay, G.M., Gray, A., Tolkacheva, T., Wigfield, S., Rebholz, H., Barnett, J., Leslie, N.R., Cheng, S., Shepherd, P.R., Gout, I., Downes, C.P. and Lamb, R.F. 2004. The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. *J. Cell Biol.* 166: 213-223.

Harris, T.E., Chi, A., Shabanowitz, J., Hunt, D.F., Rhoads, R.E. and Lawrence, J.C. 2006. mTOR-dependent stimulation of the association of eIF4G and eIF3 by insulin. *EMBO J.*

Hashimoto, N., Kido, Y., Uchida, T., Asahara, S., Shigeyama, Y., Matsuda, T., Takeda, A., Tsuchihashi, D., Nishizawa, A., Ogawa, W., Fujimoto, Y., Okamura, H., Arden, K.C., Herrera, P.L., Noda, T. and Kasuga, M. 2006. Ablation of PDK1 in pancreatic beta cells induces diabetes as a result of loss of beta cell mass. *Nat. Genet.* 38: 589-593.

Henske, E.P., Neumann, H.P., Scheithauer, B.W., Herbst, E.W., Short, M.P. and Kwiatkowski, D.J. 1995. Loss of heterozygosity in the tuberous sclerosis (TSC2) region of chromosome band 16p13 occurs in sporadic as well as

TSC-associated renal angiomyolipomas. *Genes Chromosomes Cancer.* 13: 295-298.

Holz, M.K., Ballif, B.A., Gygi, S.P. and Blenis, J. 2005. mTOR and S6K1 mediate assembly of the translation preinitiation complex through dynamic protein interchange and ordered phosphorylation events. *Cell.* 123: 569-580.

Hresko, R.C. and Mueckler, M. 2005. mTOR.RICTOR is the Ser473 kinase for Akt/protein kinase B in 3T3-L1 adipocytes. *J. Biol. Chem.* 280: 40406-40416.

Hudes, G., Carducci, M., Tomczak, P., Dutcher, J., Figlin, R., Kapoor, A., Staroslawska, E., O'Toole, T., Kong, S. and Moore, L. 2006. A phase 3, randomized, 3-arm study of temsirolimus (TEMSR) or interferon-alpha (IFN) or the combination of TEMSR + IFN in the treatment of first-line, poor-risk patients with advanced renal cell carcinoma (adv RCC). *J Clin Oncol (Meeting Abstracts).* 24: LBA4.

Imamura, K., Ogura, T., Kishimoto, A., Kaminishi, M. and Esumi, H. 2001. Cell cycle regulation via p53 phosphorylation by a 5′-AMP activated protein kinase activator, 5-aminoimidazole- 4-carboxamide-1-β--ribofuranoside, in a human hepatocellular carcinoma cell line. *Biochemical and Biophysical Research Communications.* 287: 562-567.

Inoki, K., Li, Y., Xu, T. and Guan, K.L. 2003. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. *Genes Dev.* 17: 1829-1834.

Inoki, K., Li, Y., Zhu, T., Wu, J. and Guan, K.L. 2002. TSC2 is phosphorylated and inhibited by akt and suppresses mTOR signalling. *Nat. Cell Biol.* 4: 648-657.

Inoki, K., Ouyang, H., Li, Y. and Guan, K.L. 2005. Signaling by target of rapamycin proteins in cell growth control. *Microbiol. Mol. Biol. Rev.* 69: 79-100.

Inoki, K., Zhu, T. and Guan, K.L. 2003. TSC2 mediates cellular energy response to control cell growth and survival. *Cell.* 115: 577-590.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Ruegg, M.A., Hall, A. and Hall, M.N. 2004. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. *Nat. Cell Biol.* 6: 1122-1128.

Jones, R.G., Plas, D.R., Kubek, S., Buzzai, M., Mu, J., Xu, Y., Birnbaum, M.J. and Thompson, C.B. 2005. AMP-activated protein kinase induces a p53-dependent metabolic checkpoint. *Mol. Cell.* 18: 283-293.

Kahn, B.B., Alquier, T., Carling, D. and Hardie, D.G. 2005. AMP-activated protein kinase: Ancient energy gauge provides clues to modern understanding of metabolism. *Cell Metab.* 1: 15-25.

Kamada, Y., Fujioka, Y., Suzuki, N.N., Inagaki, F., Wullschleger, S., Loewith, R., Hall, M.N. and Ohsumi, Y. 2005. Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. *Mol. Cell. Biol.* 25: 7239-7248.

Kapoor, M., Hamm, R., Yan, W., Taya, Y. and Lozano, G. 2000. Cooperative phosphorylation at multiple sites is required to activate p53 in response to UV radiation. *Oncogene.* 19: 358-364.

Karbowniczek, M., Yu, J. and Henske, E.P. 2003. Renal angiomyolipomas from patients with sporadic lymphangiomyomatosis contain both neoplastic and non-neoplastic vascular structures. *Am. J. Pathol.* 162: 491-500.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D.M. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. *Cell.* 110: 163-175.

Kim, J., Lee, C., Bonifant, C.L., Ressom, H. and Waldman, T. 2007. Activation of p53-dependent growth suppression in human cells by mutations in PTEN or PIK3CA. *Mol. Cell. Biol.* 27: 662-677.

Kwiatkowski, D.J., Zhang, H., Bandura, J.L., Heiberger, K.M., Glogauer, M., el-Hashemite, N. and Onda, H. 2002. A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells. *Hum. Mol. Genet.* 11: 525-534.

Kwon, C.H., Zhu, X., Zhang, J. and Baker, S.J. 2003. mTor is required for hypertrophy of pten-deficient neuronal soma in vivo. *Proc. Natl. Acad. Sci. U. S. A.* 100: 12923-12928.

Lee, C., Kim, J.S. and Waldman, T. 2004. PTEN gene targeting reveals a radiation-induced size checkpoint in human cancer cells. *Cancer Res.* 64: 6906-6914.

Lee, C.H., Inoki, K. and Guan, K.L. 2007. mTOR pathway as a target in tissue hypertrophy. *Annu. Rev. Pharmacol. Toxicol.* 47: 443-467.

Lee-Fruman, K.K., Kuo, C.J., Lippincott, J., Terada, N. and Blenis, J. 1999. Characterization of S6K2, a novel kinase homologous to S6K1. *Oncogene.* 18: 5108-5114.

Li, Y., Corradetti, M.N., Inoki, K. and Guan, K.L. 2004. TSC2: Filling the GAP in the mTOR signaling pathway. *Trends Biochem. Sci.* 29: 32-38.

Li, Y., Inoki, K. and Guan, K.L. 2004. Biochemical and functional characterizations of small GTPase rheb and TSC2 GAP activity. *Mol. Cell. Biol.* 24: 7965-7975.

Lloberas, N., Cruzado, J.M., Franquesa, M., Herrero-Fresneda, I., Torras, J.,

Alperovich, G., Rama, I., Vidal, A. and Grinyo, J.M. 2006. Mammalian target of rapamycin pathway blockade slows progression of diabetic kidney disease in rats. *J. Am. Soc. Nephrol.*

Loewith, R., Jacinto, E., Wullschleger, S., Lorberg, A., Crespo, J.L., Bonenfant, D., Oppliger, W., Jenoe, P. and Hall, M.N. 2002. Two TOR complexes, only one of which is rapamycin sensitive, have distinct roles in cell growth control. *Mol. Cell.* 10: 457-468.

Long, X., Lin, Y., Ortiz-Vega, S., Yonezawa, K. and Avruch, J. 2005a. Rheb binds and regulates the mTOR kinase. *Curr. Biol.* 15: 702-713.

Long, X., Ortiz-Vega, S., Lin, Y. and Avruch, J. 2005b. Rheb binding to mTOR is regulated by amino acid sufficiency. *J. Biol. Chem.*

Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P. and Pandolfi, P.P. 2005. Phosphorylation and functional inactivation of TSC2 by erk implications for tuberous sclerosis and cancer pathogenesis. *Cell.* 121: 179-193.

Manning, B.D. and Cantley, L.C. 2003. Rheb fills a GAP between TSC and TOR. *Trends Biochem. Sci.* 28: 573-576.

Manning, B.D., Tee, A.R., Logsdon, M.N., Blenis, J. and Cantley, L.C. 2002. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. *Mol. Cell.* 10: 151-162.

Mayeur, G.L., Fraser, C.S., Peiretti, F., Block, K.L. and Hershey, J.W.B. 2003/10. Characterization of eIF3k: A newly discovered subunit of mammalian translation initiation factor eIF3. *European Journal Of Biochemistry / FEBS.* 270: 4133-4139.

McManus, E.J., Collins, B.J., Ashby, P.R., Prescott, A.R., Murray-Tait, V., Armit, L.J., Arthur, J.S. and Alessi, D.R. 2004. The in vivo role of PtdIns(3,4,5)P3 binding to PDK1 PH domain defined by knockin mutation. *EMBO J.* 23: 2071-2082.

McMullen, J.R., Sherwood, M.C., Tarnavski, O., Zhang, L., Dorfman, A.L., Shioi, T. and Izumo, S. 2004. Inhibition of mTOR signaling with rapamycin regresses established cardiac hypertrophy induced by pressure overload. *Circulation.* 109: 3050-3055.

Moses, J.W., Leon, M.B., Popma, J.J., Fitzgerald, P.J., Holmes, D.R., O'Shaughnessy, C., Caputo, R.P., Kereiakes, D.J., Williams, D.O., Teirstein, P.S., Jaeger, J.L., Kuntz, R.E. and SIRIUS Investigators. 2003. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. *N. Engl. J. Med.* 349: 1315-1323.

Murthy, V., Han, S., Beauchamp, R.L., Smith, N., Haddad, L.A., Ito, N. and Ramesh, V. 2004. Pam and its ortholog highwire interact with and may negatively regulate the TSC1.TSC2 complex. *J. Biol. Chem.* 279: 1351-1358.

Musaro, A. and Rosenthal, N. 1999. Maturation of the myogenic program is induced by postmitotic expression of insulin-like growth factor I. *Mol. Cell. Biol.* 19: 3115-3124.

Nagai, K., Matsubara, T., Mima, A., Sumi, E., Kanamori, H., Iehara, N., Fukatsu, A., Yanagita, M., Nakano, T., Ishimoto, Y., Kita, T., Doi, T. and Arai, H. 2005. Gas6 induces Akt/mTOR-mediated mesangial hypertrophy in diabetic nephropathy. *Kidney Int.* 68: 552-561.

Nakagawa, T. and Yuan, J. 2000. Cross-talk between two cysteine protease families: Activation of caspase-12 by calpain in apoptosis. *J. Cell Biol.* 150: 887-894.

Nellist, M., van Slegtenhorst, M.A., Goedbloed, M., van den Ouweland, A.M., Halley, D.J. and van der Sluijs, P. 1999. Characterization of the cytosolic tuberin-hamartin complex. tuberin is a cytosolic chaperone for hamartin. *J. Biol. Chem.* 274: 35647-35652.

Nojima, H., Tokunaga, C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino, K., Hara, K., Tanaka, N., Avruch, J. and Yonezawa, K. 2003. The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. *J. Biol. Chem.* 278: 15461-15464.

Ohanna, M., Sobering, A.K., Lapointe, T., Lorenzo, L., Praud, C., Petroulakis, E., Sonenberg, N., Kelly, P.A., Sotiropoulos, A. and Pende, M. 2005. Atrophy of S6K1(-/-) skeletal muscle cells reveals distinct mTOR effectors for cell cycle and size control. *Nat. Cell Biol.* 7: 286-294.

Oldham, S., Montagne, J., Radimerski, T., Thomas, G. and Hafen, E. 2000. Genetic and biochemical characterization of dTOR, the drosophila homolog of the target of rapamycin. *Genes Dev.* 14: 2689-2694.

Ozoren, N., Kim, K., Burns, T.F., Dicker, D.T., Moscioni, A.D. and El-Deiry, W.S. 2000. The caspase 9 inhibitor Z-LEHD-FMK protects human liver cells while permitting death of cancer cells exposed to tumor necrosis factor-related apoptosis-inducing ligand. *Cancer Res.* 60: 6259-6265.

Pallafacchina, G., Calabria, E., Serrano, A.L., Kalhovde, J.M. and Schiaffino, S. 2002. A protein kinase B-dependent and rapamycin-sensitive pathway controls skeletal muscle growth but not fiber type specification. *Proc. Natl. Acad. Sci. U. S. A.* 99: 9213-9218.

Pende, M., Kozma, S.C., Jaquet, M., Oorschot, V., Burcelin, R., Le

Marchand-Brustel, Y., Klumperman, J., Thorens, B. and Thomas, G. 2000. Hypoinsulinaemia, glucose intolerance and diminished beta-cell size in S6K1-deficient mice. *Nature.* 408: 994-997.

Pende, M., Um, S.H., Mieulet, V., Sticker, M., Goss, V.L., Mestan, J., Mueller, M., Fumagalli, S., Kozma, S.C. and Thomas, G. 2004. S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. *Mol. Cell. Biol.* 24: 3112-3124.

Peterson, R.T., Beal, P.A., Comb, M.J. and Schreiber, S.L. 2000. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. *J. Biol. Chem.* 275: 7416-7423.

Potter, C.J., Pedraza, L.G. and Xu, T. 2002. Akt regulates growth by directly phosphorylating Tsc2. *Nat. Cell Biol.* 4: 658-665.

Radimerski, T., Montagne, J., Hemmings-Mieszczak, M. and Thomas, G. 2002. Lethality of drosophila lacking TSC tumor suppressor function rescued by reducing dS6K signaling. *Genes Dev.* 16: 2627-2632.

Raught, B., Gingras, A.C., Gygi, S.P., Imataka, H., Morino, S., Gradi, A., Aebersold, R. and Sonenberg, N. 2000. Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI. *EMBO J.* 19: 434-444.

Ravikumar, B., Berger, Z., Vacher, C., O'Kane, C.J. and Rubinsztein, D.C. 2006. Rapamycin pre-treatment protects against apoptosis. *Hum. Mol. Genet.* 15: 1209-1216.

Ravikumar, B., Vacher, C., Berger, Z., Davies, J.E., Luo, S., Oroz, L.G., Scaravilli, F., Easton, D.F., Duden, R., O'Kane, C.J. and Rubinsztein, D.C. 2004. Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of huntington disease. *Nat. Genet.* 36: 585-595.

Ray, P.S., Grover, R. and Das, S. 2006. Two internal ribosome entry sites mediate the translation of p53 isoforms. *EMBO Rep.* 7: 404-410.

Richardson, C.J., Broenstrup, M., Fingar, D.C., Julich, K., Ballif, B.A., Gygi, S. and Blenis, J. 2004. SKAR is a specific target of S6 kinase 1 in cell growth control. *Curr. Biol.* 14: 1540-1549.

Rolfe, M., McLeod, L.E., Pratt, P.F. and Proud, C.G. 2005. Activation of protein synthesis in cardiomyocytes by the hypertrophic agent phenylephrine requires the activation of ERK and involves phosphorylation of tuberous sclerosis complex 2 (TSC2). *Biochem. J.* 388: 973-984.

Rommel, C., Bodine, S.C., Clarke, B.A., Rossman, R., Nunez, L., Stitt, T.N., Yancopoulos, G.D. and Glass, D.J. 2001. Mediation of IGF-1-induced skeletal myotube hypertrophy by PI(3)K/Akt/mTOR and PI(3)K/Akt/GSK3 pathways. *Nat. Cell Biol.* 3: 1009-1013.

Roux, P.P., Ballif, B.A., Anjum, R., Gygi, S.P. and Blenis, J. 2004. Tumor-promoting phorbol esters and activated ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. *Proc. Natl. Acad. Sci. U. S. A.* 101: 13489-13494.

Rusten, T.E., Lindmo, K., Juhasz, G., Sass, M., Seglen, P.O., Brech, A. and Stenmark, H. 2004. Programmed autophagy in the drosophila fat body is induced by ecdysone through regulation of the PI3K pathway. *Dev. Cell.* 7: 179-192.

Ruvinsky, I., Sharon, N., Lerer, T., Cohen, H., Stolovich-Rain, M., Nir, T., Dor, Y., Zisman, P. and Meyuhas, O. 2005. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. *Genes Dev.* 19: 2199-2211.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P. and Snyder, S.H. 1994. RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. *Cell.* 78: 35-43.

Sakaguchi, M., Isono, M., Isshiki, K., Sugimoto, T., Koya, D. and Kashiwagi, A. 2006. Inhibition of mTOR signaling with rapamycin attenuates renal hypertrophy in the early diabetic mice. *Biochem. Biophys. Res. Commun.* 340: 296-301.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P. and Sabatini, D.M. 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. *Curr. Biol.* 14: 1296-1302.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L. and Sabatini, D.M. 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. *Mol. Cell.* 22(2): 159-168.

Sarbassov, D.D., Guertin, D.A., Ali, S.M. and Sabatini, D.M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. *Science.* 307: 1098-1101.

Schalm, S.S. and Blenis, J. 2002. Identification of a conserved motif required for mTOR signaling. *Curr. Biol.* 12: 632-69.

Schalm, S.S., Fingar, D.C., Sabatini, D.M. and Blenis, J. 2003. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and

function. *Curr. Biol.* 13: 797-806.

Schofer, J., Schluter, M., Gershlick, A.H., Wijns, W., Garcia, E., Schampaert, E., Breithardt, G. and E-SIRIUS Investigators. 2003. Sirolimus-eluting stents for treatment of patients with long atherosclerotic lesions in small coronary arteries: Double-blind, randomised controlled trial (E-SIRIUS). *Lancet.* 362: 1093-1099.

Schumacher, B., Hanazawa, M., Lee, M.-., Nayak, S., Volkmann, K., Hofmann, R., Hengartner, M., Schedl, T. and Gartner, A. 2005. Translational repression of C. elegans p53 by GLD-1 regulates DNA damage-induced apoptosis. *Cell.* 120: 357-368.

Scott, R.C., Schuldiner, O. and Neufeld, T.P. 2004. Role and regulation of starvation-induced autophagy in the drosophila fat body. *Dev. Cell.* 7: 167-178.

Shah, O.J., Wang, Z. and Hunter, T. 2004. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. *Curr. Biol.* 14: 1650-1656.

Shaw, R.J. 2006. Glucose metabolism and cancer. *Curr. Opin. Cell Biol.* 18: 598-608.

Shaw, R.J., Bardeesy, N., Manning, B.D., Lopez, L., Kosmatka, M., DePinho, R.A. and Cantley, L.C. 2004. The LKB1 tumor suppressor negatively regulates mTOR signaling. *Cancer Cell.* 6: 91-99.

Shioi, T., McMullen, J.R., Tarnavski, O., Converso, K., Sherwood, M.C., Manning, W.J. and Izumo, S. 2003. Rapamycin attenuates load-induced cardiac hypertrophy in mice. *Circulation.* 107: 1664-1670.

Silberberg, J. 2003. What's major about adverse cardiac events? *Lancet.* 362: 1860.

Smith, E.M., Finn, S.G., Tee, A.R., Browne, G.J. and Proud, C.G. 2005. The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. *J. Biol. Chem.* 280: 18717-18727.

Song, Y.H., Li, Y., Du, J., Mitch, W.E., Rosenthal, N. and Delafontaine, P. 2005. Muscle-specific expression of IGF-1 blocks angiotensin II-induced skeletal muscle wasting. *J. Clin. Invest.* 115: 451-458.

Sulic, S., Panic, L., Barkic, M., Mercep, M., Uzelac, M. and Volarevic, S. 2005. Inactivation of S6 ribosomal protein gene in T lymphocytes activates a p53-dependent checkpoint response. *Genes Dev.* 19: 3070-3082.

Szegezdi, E., Fitzgerald, U. and Samali, A. 2003. Caspase-12 and ER-stress-mediated apoptosis: The story so far. *Ann. N. Y. Acad. Sci.* 1010: 186-194.

Takagi, M., Absalon, M.J., McLure, K.G. and Kastan, M.B. 2005/10/7. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell.* 123: 49-63.

Tee, A.R., Manning, B.D., Roux, P.P., Cantley, L.C. and Blenis, J. 2003. Tuberous sclerosis complex gene products, tuberin and hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward rheb. *Curr. Biol.* 13: 1259-1268.

Tee, A.R. and Proud, C.G. 2002. Caspase cleavage of initiation factor 4E-binding protein 1 yields a dominant inhibitor of cap-dependent translation and reveals a novel regulatory motif. *Mol. Cell. Biol.* 22: 1674-1683.

Tirado, O.M., Mateo-Lozano, S., Sanders, S., Dettin, L.E. and Notario, V. 2003. The PCPH oncoprotein antagonizes the proapoptotic role of the mammalian target of rapamycin in the response of normal fibroblasts to ionizing radiation. *Cancer Res.* 63: 6290-6298.

Tzatsos, A. and Kandror, K.V. 2006. Nutrients suppress phosphatidylinositol 3-kinase/Akt signaling via raptor-dependent mTOR-mediated insulin receptor substrate 1 phosphorylation. *Mol. Cell. Biol.* 26: 63-76.

Um, S.H., Frigerio, F., Watanabe, M., Picard, F., Joaquin, M., Sticker, M., Fumagalli, S., Allegrini, P.R., Kozma, S.C., Auwerx, J. and Thomas, G. 2004. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. *Nature.* 431: 200-205.

Urano, J., Comiso, M.J., Guo, L., Aspuria, P.J., Deniskin, R., Tabancay, A.P.,Jr, Kato-Stankiewicz, J. and Tamanoi, F. 2005. Identification of novel single amino acid changes that result in hyperactivation of the unique GTPase, rheb, in fission yeast. *Mol. Microbiol.* 58: 1074-1086.

van Slegtenhorst, M., de Hoogt, R., Hermans, C., Nellist, M., Janssen, B., Verhoef, S., Lindhout, D., van den Ouweland, A., Halley, D., Young, J., Burley, M., Jeremiah, S., Woodward, K., Nahmias, J., Fox, M., Ekong, R., Osborne, J., Wolfe, J., Povey, S., Snell, R.G., Cheadle, J.P., Jones, A.C., Tachataki, M., Ravine, D., Sampson, J.R., Reeve, M.P., Richardson, P., Wilmer, F., Munro, C., Hawkins, T.L., Sepp, T., Ali, J.B., Ward, S., Green, A.J., Yates, J.R., Kwiatkowska, J., Henske, E.P., Short, M.P., Haines, J.H., Jozwiak, S. and Kwiatkowski, D.J. 1997. Identification of the tuberous sclerosis gene TSC1 on chromosome 9q34. *Science.* 277: 805-808.

van Slegtenhorst, M., Nellist, M., Nagelkerken, B., Cheadle, J., Snell, R., van den Ouweland, A., Reuser, A., Sampson, J., Halley, D. and van der Sluijs, P. 1998.

Interaction between hamartin and tuberin, the TSC1 and TSC2 gene products. *Hum. Mol. Genet.* 7: 1053-1057.

Vogelstein, B., Lane, D. and Levine, A.J. 2000. Surfing the p53 network. *Nature.* 408: 307-310.

Wang, L., Gout, I. and Proud, C.G. 2001. Cross-talk between the ERK and p70 S6 kinase (S6K) signaling pathways. MEK-dependent activation of S6K2 in cardiomyocytes. *J. Biol. Chem.* 276: 32670-32677.

Wang, L. and Proud, C.G. 2002. Ras/Erk signaling is essential for activation of protein synthesis by gq protein-coupled receptor agonists in adult cardiomyocytes. *Circ. Res.* 91: 821-829.

Wataya-Kaneda, M., Kaneda, Y., Hino, O., Adachi, H., Hirayama, Y., Seyama, K., Satou, T. and Yoshikawa, K. 2001. Cells derived from tuberous sclerosis show a prolonged S phase of the cell cycle and increased apoptosis. *Arch. Dermatol. Res.* 293: 460-469.

Weisz, G., Leon, M.B., Holmes, D.R.,Jr, Kereiakes, D.J., Clark, M.R., Cohen, B.M., Ellis, S.G., Coleman, P., Hill, C., Shi, C., Cutlip, D.E., Kuntz, R.E. and Moses, J.W. 2006. Two-year outcomes after sirolimus-eluting stent implantation: Results from the sirolimus-eluting stent in de novo native coronary lesions (SIRIUS) trial. *J. Am. Coll. Cardiol.* 47: 1350-1355.

Wienecke, R., Konig, A. and DeClue, J.E. 1995. Identification of tuberin, the tuberous sclerosis-2 product. tuberin possesses specific Rap1GAP activity. *J. Biol. Chem.* 270: 16409-16414.

Yang, D.-., Halaby, M.-. and Zhang, Y. 2006. The identification of an internal ribosomal entry site in the 5′-untranslated region of p53 mRNA provides a novel mechanism for the regulation of its translation following DNA damage. *Oncogene.* 25: 4613-4619.

Yang, Q., Inoki, K., Kim, E. and Guan, K.L. 2006. TSC1/TSC2 and rheb have different effects on TORC1 and TORC2 activity. *Proc. Natl. Acad. Sci. U. S. A.*

Young, J. and Povey, S. 1998. The genetic basis of tuberous sclerosis. *Mol. Med. Today.* 4: 313-39.

Zhang, H., Cicchetti, G., Onda, H., Koon, H.B., Asrican, K., Bajraszewski, N., Vazquez, F., Carpenter, C.L. and Kwiatkowski, D.J. 2003. Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-akt signaling through downregulation of PDGFR. *J. Clin. Invest.* 112: 1223-1233.

Zhang, H., Stallock, J.P., Ng, J.C., Reinhard, C. and Neufeld, T.P. 2000. Regulation of cellular growth by the drosophila target of rapamycin dTOR. *Genes Dev.* 14: 2712-2724.

Zhang, Y., Gao, X., Saucedo, L.J., Ru, B., Edgar, B.A. and Pan, D. 2003. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. *Nat. Cell Biol.* 5: 578-581.

Zhou, G., Myers, R., Li, Y., Chen, Y., Shen, X., Fenyk-Melody, J., Wu, M., Ventre, J., Doebber, T., Fujii, N., Musi, N., Hirshman, M.F., Goodyear, L.J. and Moller, D.E. 2001. Role of AMP-activated protein kinase in mechanism of metformin action. *J. Clin. Invest.* 108: 1167-174.

Zou, T., Mazan-Mamczarz, K., Rao, J.N., Liu, L., Marasa, B.S., Zhang, A.-., Xiao, L., Pullmann, R., Gorospe, M. and Wang, J.-. 2006. Polyamine depletion increases cytoplasmic levels of RNA-binding protein HuR leading to stabilization of nucleophosmin and p53 mRNAs. *J. Biol. Chem.* 281: 19387-19394.