The LKB1 tumor suppressor negatively regulates mTOR signaling

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Summary

Germline mutations in LKB1, TSC2, or PTEN tumor suppressor genes result in hamartomatous syndromes with shared tumor biological features. The recent observations of LKB1-mediated activation of AMP-activated protein kinase (AMPK) and AMPK inhibition of mTOR through TSC2 prompted us to examine the biochemical and biological relationship between LKB1 and mTOR regulation. Here, we report that LKB1 is required for repression of mTOR under low ATP conditions in cultured cells in an AMPK- and TSC2-dependent manner, and that Lkb1 null MEFs and the hamartomatous gastrointestinal polyps from Lkb1 mutant mice show elevated signaling downstream of mTOR. These findings position aberrant mTOR activation at the nexus of these germline neoplastic conditions and suggest the use of mTOR inhibitors in the treatment of Peutz-Jeghers syndrome.

Introduction

The LKB1 tumor suppressor is a serine/threonine kinase that is mutationally inactivated in the autosomal dominant Peutz-Jeghers syndrome (Boudeau et al., 2003), as well as in some sporadic lung adenocarcinomas (Sanchez-Cespedes et al., 2002; Carretero et al., 2004). In the mouse, Lkb1 nullizygosity is embryonic lethal (circa E9–E11) due to vascular and neural tube defects (Ylikorkala et al., 2001), and *Lkb1* heterozygosity engenders sporadic hamartomatous gastrointestinal polyps which phenocopy those of PJS patients (Bardeesy et al., 2002; Miyoshi et al., 2002; Jishage et al., 2002; Rossi et al., 2002). Hamartomas are benign tumors consisting of normal cellular differentiation but disorganized tissue architecture, and are present in several inherited tumor syndromes, including Cowden's disease/Bannayan-Zonana syndrome and tuberous sclerosis complex, which possess germline-inactivating mutations in the tumor suppressors PTEN and either TSC1 or TSC2, respectively.

TSC1 and TSC2 gene products—hamartin and tuberin, respectively—are components of a complex that negatively regulates mTOR signaling and, correspondingly, inactivation of TSC1 or TSC2 is associated with elevated mTOR activity (reviewed in Manning and Cantley, 2003). The PTEN tumor suppressor encodes a lipid phosphatase that serves to dephosphorylate the D3 position of phosphatidylinositol-3,4,5-trisphosphate and thus biochemically directly opposes the activity of phosphoinositide 3-kinase (PI 3-kinase) and its downstream effectors. Key among downstream PI3-kinase effectors is the Akt serine/threonine kinase, which mediates cell proliferation, survival, and growth. Inactivating mutations in PTEN in the context of inherited syndromes, or in sporadic tumors, such as those of the prostate, breast, and glia, thus cause elevated Akt activity (Cantley and Neel, 1999). Akt activates mTOR signaling by directly phosphorylating and inactivating the TSC2 tumor suppressor tuberin (Manning et al., 2002; Inoki et al., 2002). Furthermore, tuberin is constitutively phosphorylated at the same sites in cells derived from tumors lacking PTEN (Manning et al., 2002). Activation of the mTOR pathway has been previously documented in *Tsc2*- and *Pten*-deficient tissues and tumors in vivo (Kwiatkowski et al., 2002; Zhang et al., 2003; Stambolic et al., 1998; Ramaswamy et al., 1999; Sun et al., 1999). Moreover, aberrant cell growth in these systems is specifically suppressed by treatment with mTOR inhibitors, such as rapamycin (Neshat et al., 2001; Podsypanina et al., 2001;

SIGNIFICANCE

LKB1 mutations are associated with the Peutz-Jeghers syndrome (PJS), consisting of pigmentation anomalies, benign gastrointestinal polyps (hamartomas), and predisposition to a range of malignant tumor types. This study forges a biochemical and genetic link from Lkb1 to mTOR signaling via the sequential activation of AMPK and the TSC2 tumor suppressor. This interrelationship, particularly hyperactive mTOR signaling, provides a rational explanation for the shared features of three human disorders characterized by the development of hamartomas (PJS, Cowden's disease, and tuberous sclerosis complex). mTOR inhibitors, including rapamycin analogs, which are currently under clinical trials for a number of cancers, may be effective in the treatment and possible prevention of Peutz-Jeghers polyps and sporadic tumors that show LKB1 loss.

Shi et al., 2002; Kenerson et al., 2002; Jaeschke et al., 2002; Onda et al., 2002; Kwon et al., 2003).

mTOR is a highly conserved serine/threonine kinase that regulates protein synthesis and cell growth in all eukaryotes (Shamji et al., 2003; Harris and Lawrence, 2003). Two well-characterized targets of mTOR signaling are S6K1 and 4E-BP1 (Fingar and Blenis, 2004). S6K1 is activated downstream of mTOR and insulin signaling by multisite phosphorylation, which stimulates its kinase activity toward downstream substrates, such as the ribosomal protein S6. mTOR has been shown to be necessary for the phosphorylation of Thr389 in human S6K1. S6K1 is a critical modulator of cell growth in mammalian cells, although the mechanism by which this is mediated is currently unknown. 4E-BP1 is similarly regulated by multisite phosphorylation downstream of mitogen-stimulated mTOR. When hypophosphorylated, 4E-BP1 acts as a repressor of cap-dependent translation by binding to eIF4E and preventing proper formation of the eIF4F translation initiation complex at the 5′ end of mRNAs. Following growth factor stimulation, 4E-BP1 is sequentially phosphorylated on a number of sites, causing its dissociation from eIF4E, thereby promoting cap-dependent translation.

mTOR signaling is inhibited under conditions of low nutrients, such as glucose and amino acids and low intracellular ATP levels (Shamji et al., 2003). While mTOR was presumed to serve as the direct cellular sensor for ATP levels (Dennis et al., 2001), mounting evidence has implicated AMP-activated protein kinase (AMPK), a well-characterized sensor of intracellular ATP/AMP ratios, in the regulation of mTOR activity (Kimura et al., 2003). Specifically, enforced expression of activated AMPK in cells not undergoing energy stress showed inhibition of mTOR signaling, whereas kinase-dead AMPK dominantly inhibited mTOR signaling in the setting of energy stress (Kimura et al., 2003). The precise target of AMPK that regulates mTOR signaling has recently come to light in studies establishing that AMPK directly phosphorylates and enhances the ability of tuberin to inhibit mTOR signaling (Inoki et al., 2003). Indeed, cells lacking TSC2 are unable to downregulate mTOR following energy stress. However, to date, the role of AMPK in the regulation of mTOR has yet to be validated by genetic means.

The LKB1 tumor suppressor gene encodes a serine/threonine kinase shown recently to be the major upstream kinase of AMPK (Hawley et al., 2003; Woods et al., 2003; Shaw et al., 2004). LKB1 directly phosphorylates Thr172 in the activation loop of AMPK, a modification that is absolutely required for AMPK catalytic activity (Stein et al., 2000). AMPK exists as a heterotrimer in cells, composed of a catalytic kinase subunit (α) and two regulatory subunits (β and γ) (Hardie et al., 2003). The γ subunit contains a series of CBS domains that bind AMP (Scott et al., 2004) and, once bound to AMP, induces a conformation change in the AMPK heterotrimer which allows it to serve as a better substrate for upstream activating kinases such as LKB1 (Kemp et al., 2003). In mammalian cells, there are two catalytic α subunits with some distinct and some overlapping functions, as evidenced by germline inactivation of each gene in the mouse (Viollet et al., 2003; Jorgensen et al., 2004), although mice and cells doubly deficient for both α subunit genes, and hence genetically devoid of AMPK activity, have yet to be reported. The presence of 2 β subunit genes and 3 γ subunit genes has similarly precluded a definitive genetic assessment of AMPK deficiency in mammalian cells.

In addition to the two AMPKs, LKB1 has been shown to activate 11 other AMPK-related kinases, similarly through direct phosphorylation of their activation loop threonines (Lizcano et al., 2004). The kinase activity of LKB1 or any of the AMPK-related kinases is not directly regulated by AMP (Hawley et al., 2003; Shaw et al., 2004; Lizcano et al., 2004; Sakamoto et al., 2004). These data raise the possibility that, among LKB1-regulated kinases, the α1 and α 2 AMPK subunits are uniquely responsive to energy stress, probably reflecting a unique ability to associate with the AMP binding γ subunits. Whether the activities of the AMPK-related kinases are regulated in response to other stimuli is not known, however, in LKB1-expressing cells, several of these kinases appear to be constitutively activated.

Given that LKB1 serves to activate AMPK, and AMPK serves to negatively regulate mTOR in some contexts, we sought to determine whether mTOR signaling was aberrantly elevated in Lkb1 deficient cells as well as in gastrointestinal polyps derived from Lkb1 mutant mice. Additionally, we sought to determine whether LKB1 and TSC2-deficient cells shared other biochemical and physiological properties.

Results

To examine the role of AMPK in the regulation of mTOR signaling under physiological conditions, we evaluated mTOR activity in wild-type and Lkb1-deficient mouse embryonic fibroblasts (MEFs). In normal growth media, Lkb1$^{+/+}$ and $^{-/-}$ MEFs show low AMPK activity, as evidenced by lack of phosphorylation of the critical AMPK effector acetyl CoA carboxylase (ACC) (Figure 1A). Under these conditions, phosphorylation of S6K1 and its substrate ribosomal S6 were readily detectable, consistent with high levels of mTOR activity. On the other hand, in response to treatment with 5-aminoimidizole-4-carboxamide riboside (AICAR), which serves as a cell permeable AMP mimetic (Hardie et al., 2003), AMPK is activated and S6K1 is inhibited in wild-type cells. Strikingly, in the Lkb1-deficient fibroblasts, AMPK activation by AICAR is severely attenuated, and levels of active S6K1 remain high (Figure 1A). In contrast, growth factor signaling to GSK-3, reflective of Akt activity in these cells, is unaffected by AICAR-induced energy stress. We obtained similar results following treatment with the AMPK agonist phenformin (data not shown). To ensure that sustained elevation of mTOR activity was not a secondary consequence of LKB1 deficiency, we stably reconstituted immortalized Lkb1$^{-/-}$ MEFs with retroviruses encoding wild-type or kinase-dead Lkb1. As seen in Figure 1B, wild-type but not kinase-dead Lkb1 restored the sensitivity of S6K1 activity to energy stress. A modest, but reproducible, effect on S6 phosphorylation was also seen under unstimulated conditions in wild-type Lkb1-reconstituted cells (Figure 1B, lane 2).

To address whether other known effectors of mTOR were similarly deregulated by Lkb1 loss, or if S6K1 was specifically affected, we examined the regulation of 4E-BP1. The mTOR-dependent phosphorylation of 4E-BP1 is known to inhibit its association with eIF4E and the 5′-cap complex. The components of endogenous cap complexes can be assayed using 7-methyl GTP sepharose as an affinity reagent (Fingar et al., 2002). As seen in Figure 1C (left panel), 4E-BP1 exists as a number of species with different mobilities on SDS-PAGE due to differences in phosphorylation, with the slowest mobility form being the hyper-phosphorylated form. In growing cells, 4E-BP1 is hyperphosphorylated and very little copurifies in cap com-

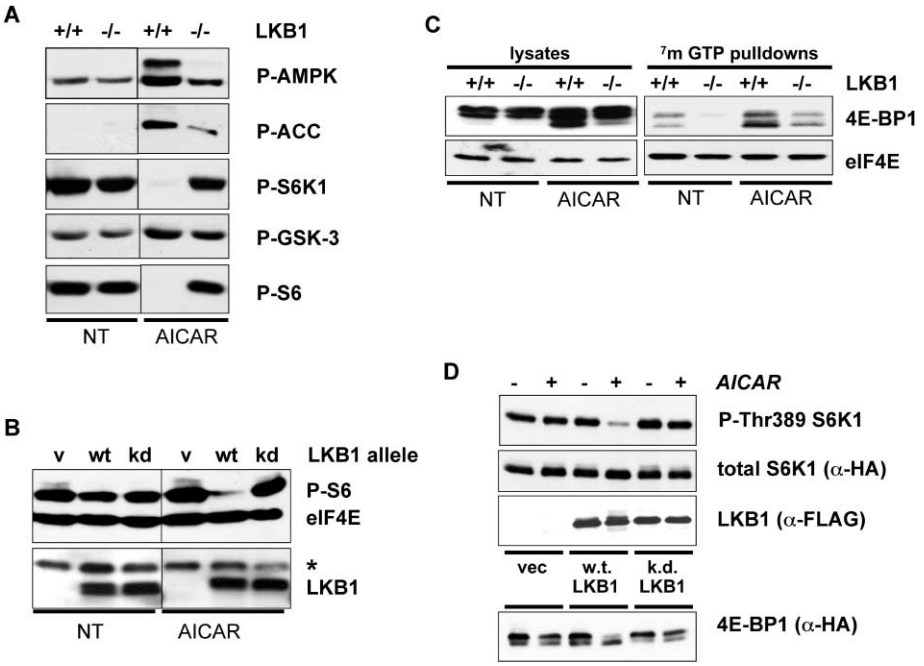

Figure 1. mTOR signaling is not inhibited following energy stress in LKB1-deficient cells

A: Subconfluent cycling littermate-matched primary MEFs of the indicated genotype were left untreated (NT) or were treated with 2 mM AICAR for 2 hr. Total cell extracts were immunoblotted for phospho-Thr172 AMPK (P-AMPK), phospho-Ser79 ACC (P-ACC), phospho-Thr389 S6K1 (P-S6K1), phospho-Ser9 GSK-3b (P-GSK-3), and phospho-Ser235/236 S6 ribosomal protein (P-S6).

B: An immortalized LKB1$^{-/-}$ MEF cell line was reconstituted with vector (v), wild-type (wt), or kinase-dead (kd) human LKB1-expressing retroviruses. Cells were treated as in **A**. eIF4E was used as a loading control. * represents a crossreactive band with the anti-LKB1 antisera as characterized previously (Shaw et al., 2004).

C: Endogenous 5′ cap complexes were isolated from LKB1 primary MEFs as in **A**. 10% of the total cell lysate is shown in the left panels; cap complexes purified on 7-methyl sepharose are shown in right panels. Each was immunoblotted with total 4E-BP1 and eIF4E antibodies. eIF4E is shown as a loading control.

D: HeLa cells (which are LKB1-deficient) were transiently transfected with vector, FLAG-tagged wild-type, or kinase-dead LKB1 along with an HA-tagged S6K1 or an HA-tagged 4E-BP1 construct, then treated where indicated with 2 mM AICAR for 2 hr. S6K1 immunoprecipitates were immunoblotted for activating phosphorylation of S6K1 as before, as well as with anti-HA to control for levels. Lysates were immunoblotted with anti-FLAG to detect levels of LKB1 and with anti-HA to detect phosphorylation-induced changes in 4E-BP1 mobility on SDS-PAGE.

plexes, and that which does is restricted to the hypophosphorylated forms. Notably, treatment of wild-type MEFs with AICAR significantly increases the level of the hypophosphorylated 4E-BP1 and concomitantly leads to increased 4E-BP1 association with the cap complex. Interestingly, Lkb1-deficient cells displayed less 4E-BP1 associated with the cap complex, both under basal conditions and, more markedly, following AICAR treatment (Figure 1C). This is also reflected in the near absence of hypophosphorylated 4E-BP1 following AICAR treatment in the total cell lysates (Figure 1C, top left panel).

To determine whether LKB1 deficiency impacts broadly on mTOR signaling, we tested the LBK1-mTOR link in a distinct cell type of human origin—HeLa cells were selected as they are known to lack LKB1 expression (Tiainen et al., 1999). Following transduction of wild-type or kinase-dead LKB1 along with HA-tagged S6K1 or 4E-BP1 constructs, we assayed mTOR activation of S6K1 by immunoblotting HA immunoprecipitates with anti-phosphoT389 antisera and mTOR phosphorylation of 4E-BP1 by mobility shift on SDS-PAGE. As seen in Figure 1D, introduction of wild-type, but not kinase-dead, LKB1 into HeLa cells restores AICAR-dependent inhibition of S6K1 and 4E-BP1 phosphorylation in response to energy stress, indicating that LKB1-dependent mTOR regulation is conserved in humans and in different cell types.

Recent studies have demonstrated that the TSC2 tumor suppressor protein, tuberin, is a direct target of AMPK and a critical mediator of AMPK-dependent inhibition of mTOR signaling (Inoki et al., 2003). We sought to examine whether LKB1 can regulate tuberin phosphorylation via AMPK, and whether AMPK itself is critical for the effects of LKB1 in downregulating mTOR signaling. First, we examined the ability of purified recombinant AMPK preactivated in vitro with LKB1 to directly phosphorylate tuberin. As seen in Figure 2A, AMPK activated by wild-type LKB1 directly phosphorylates tuberin in vitro. Moreover, we noticed that when tuberin was stoichiometrically phosphorylated by AMPK, it underwent a mobility shift when resolved on low percentage SDS-PAGE gels (Figure 2B). This mobility shift in tuberin was not observed when tuberin was phosphorylated by active recombinant Akt, even at levels of tuberin phosphorylation comparable to those induced by AMPK (B.D.M., unpublished data). To assess this phosphorylation in vivo, we cotransfected HeLa cells with FLAG-tagged tuberin with or without LKB1. We then examined the mobility of tuberin on SDS-PAGE following treatment with AICAR. HeLa cells expressing wild-type LKB1 induced a significant reduction in tuberin mobility after treatment of the cells with AICAR (Figure 2C), suggesting LKB1-induced AMPK activation and subsequent phosphorylation of tuberin. AICAR treatment in the absence of LKB1 expression had no effect on tuberin mobility in HeLa cells (data not shown).

In order to more rigorously examine the requirement of AMPK for LKB1-mediated inhibition of mTOR signaling, we introduced wild-type LKB1 into HeLa cells with or without two different dominant-negative AMPK constructs—a kinase-dead α2 allele and a nonactivatible T172A α1 allele (Crute et al., 1998). As seen in Figure 2D, coexpression of either dominant-negative AMPK α allele blocked the ability of LKB1 to downregulate S6K1 in AICAR-treated HeLa cells.

We next wished to determine whether other known stresses that inhibit mTOR activity might also function through LKB1/AMPK. For these experiments, we compared the response of littermate-matched Lkb1$^{+/+}$ and $^{-/-}$ MEFs to littermate-matched p53-deficient Tsc2$^{+/+}$ and $^{-/-}$ MEFs. Tsc2$^{-/-}$ MEFs have been reported to display aberrantly high levels of mTOR signaling in

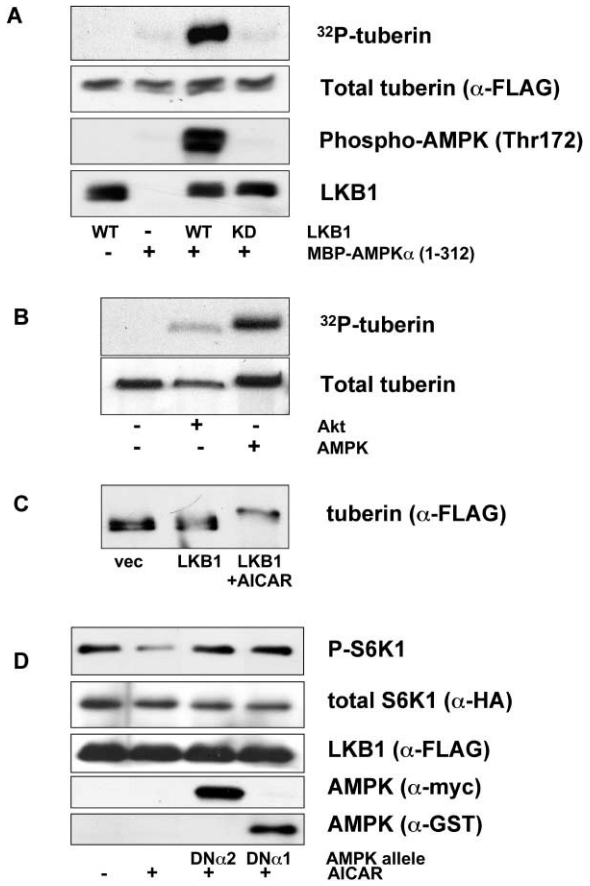

Figure 2. LKB1 activation of AMPK stimulates phosphorylation of tuberin in vitro and in vivo and is required for LKB1-mediated inhibition of S6K1 activity

A: In vitro kinase assay using FLAG-tagged tuberin as a substrate for LKB1-activated AMPK. Purified *E. coli*-produced recombinant AMPK was activated by an in vitro kinase assay with wild-type LKB1, kinase-dead LKB1, or untransfected control immunoprecipitates, then isolated and assayed for its ability to phosphorylate tuberin in vitro.

B: Comparison of recombinant Akt-mediated tuberin phosphorylation in vitro to recombinant AMPK phosphorylation in vitro. Note that only AMPK-mediated phosphorylation induces a significant mobility shift in tuberin on SDS-PAGE.

C: HeLa cells were cotransfected with FLAG-tagged tuberin and either empty vector or wild-type LKB1 and treated with 2 mM AICAR where indicated. Total cell lysates were then immunoblotted with anti-FLAG antisera to detect the introduced tuberin. Note the significant mobility shift induced by AICAR treatment of LKB1-expressing cells. AICAR treatment of cells not expressing wild-type LKB1 had no effect on tuberin mobility (data not shown).

D: HeLa cells were transfected with HA-tagged S6K1 and FLAG-tagged wild-type LKB1 along with empty vector, or dominant negative AMPK α1 (GST-tagged) or dominant negative AMPK α2 (myc tagged). Cells were treated with 2 mM AICAR for 2 hr where indicated. HA immunoprecipitates were immunoblotted with phospho-Thr389 S6K1 antisera to detect activated S6K1 and reprobed for total HA-S6K1 levels. Lysates were immunoblotted with anti-FLAG to detect LKB1 levels and with anti-myc and anti-GST to detect expression of the dominant negative AMPK alleles.

response to a wide variety of cellular stresses (Zhang et al., 2003). As seen in Figure 3A, Lkb1$^{-/-}$ cells fail to downregulate S6K1 activity following AICAR treatment, as shown above. However, LKB1-deficient cells quite effectively downregulate S6K1 activity following amino acid and growth factor deprivation. In contrast, in Tsc2$^{-/-}$cells, S6K1 is not inhibited in response to any of these stimuli (Figures 3A and 3B). Importantly, rapamycin inhibited S6K1 activity in all cell types. In order to examine whether other mitogenic signaling pathways are affected by the loss of Lkb1, we used phospho-specific antibodies that recognize activated forms of Erk and Akt. We found that neither Erk nor Akt activation was enhanced in Lkb1-deficient cells following any of the aforementioned cellular stresses. In fact, Akt activation was reduced in LKB1-deficient cells as compared to wild-type controls under several conditions, including serum deprivation, serum stimulation, and AICAR treatment (Figure 3). Similarly, as seen previously (Kwiatkowski et al., 2002; Jaeschke et al., 2002), Akt activity is dramatically attenuated in the Tsc2-deficient cells following all stimuli, a phenomenon which has previously been attributed to a negative feedback loop inhibiting PI3K/Akt signaling in a number of systems (Garami et al., 2003).

Given these similarities in intracellular signaling, we examined whether Lkb1$^{-/-}$ MEFs might share any biological properties with TSC2$^{-/-}$ MEFs. One property of LKB1-deficient cells that we previously characterized was their sensitivity, relative to wild-type cells, to undergo apoptosis following treatment with AICAR or other AMPK agonists (Shaw et al., 2004). Notably, Tsc2$^{-/-}$ MEFs were recently found to undergo apoptosis under conditions of glucose deprivation, which also serves to activate AMPK (Inoki et al., 2003). Interestingly, this property of Tsc2-deficient MEFs was rescued by concurrent treatment with rapamycin. We therefore investigated whether glucose deprivation would also selectively lead to the apoptosis of Lkb1-deficient MEFs and whether this effect could be rescued by rapamycin treatment. First, we characterized that AMPK signaling and mTOR was aberrant in LKB1$^{-/-}$ cells following glucose deprivation, as seen in Figure 3C. As expected, in the LKB1$^{-/-}$ cells, phospho-S6 was elevated and phospho-ACC was decreased, indicating a defect in AMPK signaling in these cells following glucose deprivation. Using caspase activation—as detected by PARP cleavage in lysates—as a measure of apoptosis (Shaw et al., 2004; Inoki et al., 2003), we observed that Lkb1$^{-/-}$ MEFs show elevated sensitivity to apoptosis induced by glucose withdrawal as compared to their wild-type counterparts, and that rapamycin potently inhibited this apoptotic phenotype (Figure 3C). The PARP cleavage in lysates was mirrored in direct assays of cell viability (Figure 3D). These results confirm the sensitivity of LKB1$^{-/-}$ MEFs to glucose deprivation-induced cell death and the suppression of this effect by rapamycin.

The regulation of mTOR by LKB1 in vivo is intriguing in light of the established role of mTOR activation in the pathogenesis of human tumors. We wished to begin to address the potential role of mTOR deregulation in the pathogenesis of the hamartomatous polyps that characterize LKB1-deficiency in both humans and mice. We therefore investigated the status of the mTOR pathway in hamartomatous gastrointestinal polyps arising in *Lkb1*$^{+/-}$ mice. Western blot analyses of lysates from polyps and adjacent gastrointestinal epithelium revealed a prominent elevation in phospho-S6 and phospho-S6K1 (Thr389) levels in all polyps analyzed (Figure 4A). Furthermore, we detected elevated levels of phospho-4E-BP1 (Ser65), indicating that mTOR-dependent signaling is elevated in the polyps. This property appears to be specific to Lkb1 deficiency rather than secondary to the deregulated growth state of the polyps, since other growth-associated markers, such as phospho-Erk (Figure 4A) and phospho-Akt levels (data not shown), did not show

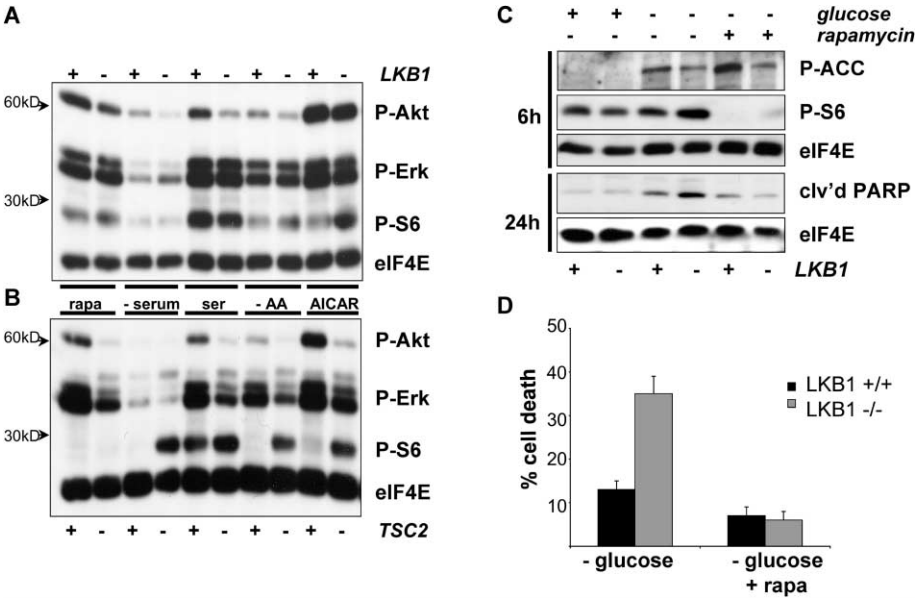

Figure 3. LKB1- and TSC2-deficient MEFs show similar aberrant deregulation of intracellular signaling in response to specific environmental stresses

A and B: Littermate-matched LKB1- or TSC2-deficient MEFs were compared to wild-type MEFs in their response to a number of stress stimuli known to inhibit mTOR signaling (wild-type cells denoted +, −/− cells simply −). mTOR signaling was examined by immunoblotting for phospho-S6 as in Figure 1. Activation of Akt and Erk were examined using their specific activation state phospho-specific antibodies (Ser473 for Akt, Thr202/Tyr204 for Erk). As before, eIF4E is used as a loading control. All MEFs were serum-deprived in DMEM alone for 24 hr. Indicated samples were then placed in fresh media containing 10% serum, with or without 20 nm rapamycin or 2 mM AICAR, all for 90 min. Amino acid deprivation (−AA) was performed by placing the serum-starved cells into D-PBS with 10% dialyzed serum for 90 min.

C: LKB1-deficient MEFs are hypersensitive to apoptosis induced by glucose deprivation, and rapamycin rescues this apoptosis. LKB1-deficient MEFs exhibit defective AMPK activation and mTOR inhibiton following glucose deprivation. Prolonged treatment in glucose-free media leads apoptosis, as indicated by the extent of caspase-mediated PARP cleavage in total cell lysates. Cells were placed in normal media, glucose-free media with 10% dialyzed serum, or glucose-free media with 10% dialyzed serum containing 20 nM rapamycin for 6 or 24 hr as indicated. LKB1 genotype indicated at bottom. eIF4E is used as a loading control.

D: Cells were treated for 24 hr as in **C**, and viability was quantified by MTT assays performed in triplicate on cells of the indicated genotypes. Cell death is expressed as a percentage of the untreated controls.

consistent differences between polyp and adjacent normal tissue. These results were extended by immunohistochemical analyses. While both the normal gastrointestinal epithelium adjacent to the polyp tissue and the polyp stroma showed minimal immunoreactivity to anti-phospho-S6 antisera, the polyp epithelium showed intense cytoplasmic staining (Figures 4B–4D). The activation of S6K1 in the polyps was further confirmed and extended by additional immunohistochemical studies that revealed strong staining for phospho-S6K1 (Thr421/Ser424) specifically in the polyp epithelium (Figure 4E). The staining pattern showed both nuclear and cytoplasmic localization consistent with increased phosphorylation of both the p70 and p85 S6K isoforms. Importantly, the epithelial cells within the polyp that contain elevated levels of phospho-S6 and -S6K1 are thought to be the neoplastic component of the polyp (Hemminki et al., 1997; Wang et al., 1999, Entius et al., 2001; Bardeesy et al., 2002).

Discussion

The development of hamartomas is a stereotypical feature of a number of clinically related human tumor disorders, including Peutz-Jeghers syndrome, Cowden's disease (and related Pten-deficiency disorders), and tuberous sclerosis complex (Gomez et al., 1999; Cantley and Neel, 1999; Devroede et al., 1988). As previously demonstrated for Pten- and Tsc2-deficient tumors in vivo, LKB1-deficient tumors are shown here to exhibit elevated levels of S6K1 activity and phospho-4EBP1, indicative of hyperactivation of mTOR signaling, establishing hyperactivate mTOR signaling as a common theme across these distinct tumor suppressor disorders (Figure 5). In this study, we further demonstrate that, in cell culture-based systems, LKB1 modulation of mTOR signaling is mediated through LKB1-dependent phosphorylation of AMPK in response to energy stress, and that phosphorylation of tuberin by AMPK is a likely mechanism to explain the effects on mTOR signaling. These results provide strong biochemical and genetic evidence that endogenous AMPK mediates mTOR inhibition in response to decreased intracellular ATP.

Consistent with mTOR as a key effector in the biology of LKB1, $Tsc2^{-/-}$ and $Lkb1^{-/-}$ MEFs share a number of properties that are uncharacteristic of cells lacking most tumor suppressors. Both cell types are hypersensitive to apoptosis induced by energy stress, and this adverse response can be rescued by treatment with the mTOR inhibitor rapamycin. Both also display attenuated Akt activation under most conditions, which is in stark contrast to loss of Pten, which causes hyperactivation of Akt. Finally, immortalized MEFs deficient for either TSC2 or LKB1 proliferate more slowly than their wild-type littermate-derived counterparts (R.J.S, N.B., and B.D.M., unpublished data). Aberrant mTOR signaling might also explain two major molecular markers of LKB1 loss previously characterized. VEGF has been shown to be upregulated in embryos and MEFs deficient for LKB1 (Ylikorkala et al., 2001; Bardeesy et al., 2002) or TSC2 (El-Hashemite et al., 2003; Brugarolas et al., 2003). Of note, the increase in VEGF protein levels in LKB1-deficient MEFs is not associated with increased VEGF mRNA levels (Bardeesy et al., 2002), a finding consistent with data demonstrating VEGF to be one of several genes whose oncogene-induced recruitment of mRNA to polyribosomes is critically governed by mTOR signaling (Rajasekhar et al., 2003). Another marker of LKB1 loss is the expression of IGFBP5, the single most upregulated transcript in $LKB1^{+/-}$ mouse GI polyps as compared to normal surrounding tissue. IGFBP5 mRNA levels are also aberrantly

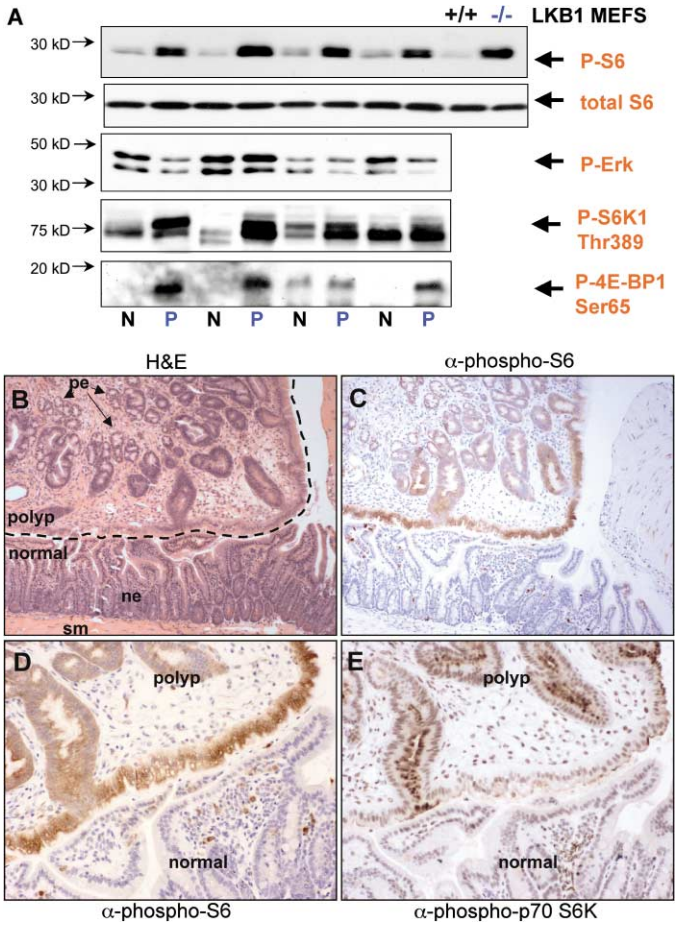

Figure 4. S6K1 activity is dramatically increased in GI polyps from LKB1$^{+/-}$ mice as compared to surrounding normal tissue

A: Lysates were made from polyps or adjacent normal tissue and subjected to immunoblotting with anti-phospho-S6, phospho-Erk, phospho-S6K1 (Thr389), phospho-4E-BP1 (Ser65), or total S6 as a loading control. AICAR-treated LKB1 MEFs of the indicated genotype are in the rightmost two lanes as a comparison (analogous to those in Figure 1A).

B: Hematoxylin and eosin (H&E)-stained section shows a polyp (upper) arising at the pyloric/duodenal junction and the adjacent normal duodenal tissue (lower). The glandular structures of polyp epithelium (pe, arrows) are embedded in the polyp stroma. The normal epithelium of the duodenum (ne) and the submucosa (sm) are indicated.

C: Immunohistochemistry of a section adjacent to the tissue shown in **B** using anti-phospho-S6 antisera shows strong staining in the polyp epithelium and weak or absent staining in the polyp stroma and the normal duodenal tissue.

D: Higher-power view of the image shown in **C** demonstrates cytoplasmic localization phospho-S6 staining in the polyp epithelium. The scattered immunoreactive cells in the normal epithelium have a morphology suggestive of plasma cells.

E: Immunohistochemistry with anti-phospho-S6 kinase antisera shows elevated cytoplasmic and nuclear staining in the polyp epithelium.

high in the LKB1-deficient MEFs (Bardeesy et al., 2002). The transcription of IGFBP5 has previously been shown to be critically dependent on mTOR signaling and is inhibited by rapamycin (Duan et al., 1999). Furthermore, IGFBP5 translation is also dependent on mTOR (Rajasekhar et al., 2003). Thus, hyperactivation of mTOR signaling in the LKB1-deficienct MEFs, polyps, and embryos is likely to explain their previously noted elevated levels of VEGF and IGFBP5.

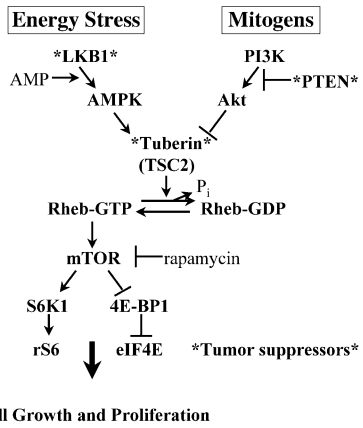

Figure 5. Model describing upstream regulation of mTOR signaling by tumor suppressors mutated in hamartoma syndromes

In response to peptide growth factors and mitogenic stimuli, PI 3-kinase is activated, resulting in the activation of the Akt serine/threonine kinase. Akt directly phosphorylates and inactivates the TSC2 tumor suppressor protein tuberin. Tuberin serves as a GTPase-activating protein (GAP) for the small Ras-like GTPase Rheb. When tuberin is inactivated by Akt, Rheb-GTP levels increase, leading to increased mTOR activity via an unknown mechanism. The Pten tumor suppressor serves to biochemically antagonize PI-3 kinase activity; hence, in Pten-deficient cells, Akt activity and mTOR activity are high. In response to energy stress, cells activate AMPK downstream of the LKB1 tumor suppressor. AMPK directly phosphorylates and activates tuberin, overriding the mitogenic signal from Akt. In the absence of LKB1, AMPK cannot be activated, nor can mTOR be inactivated, via tuberin, in response to energy stress.

The multiple overlapping clinical features between Peutz-Jeghers syndrome and Cowden's disease (CD) caused us to originally hypothesize that LKB1, like PTEN, might negatively regulate PI3-kinase signaling (Cantley and Neel, 1999). However, we have observed in a number of biological settings that Akt activity is not increased in LKB1-deficient cells, and, if anything, is decreased (see Figure 3A). This reduced Akt activation in the LKB1-deficient cells can also account for the previous observation of decreased phospho-GSK3 (Ser9) in LKB1-deficient MEFs (Ossipova et al., 2003). These results suggest that the common biochemical basis underlying the PJS and CD syndromes is not due to a shared activation of PI3K/Akt signaling but rather to upregulation of mTOR signaling in both LKB1- and Pten-deficient cells.

One significant question that remains is whether AMPK is the only kinase downstream of LKB1 that can downregulate mTOR via TSC2 phosphorylation or other mechanisms. LKB1 has been demonstrated to activate 11 AMPK-related kinases in an energy stress-independent manner (Lizcano et al., 2004; Sakamoto et al., 2004). In different cellular contexts, these kinases may phosphorylate TSC2 or other shared substrates with AMPK. Indeed, we have observed that LKB1 can regulate basal mTOR signaling under nonstressed conditions (e.g., Figure 1C). Additional work will be needed to determine whether any of the other AMPK-related kinases can regulate TSC2/mTOR, and to determine which kinase contributes to mTOR regulation in the pathogenesis of Peutz-Jeghers polyposis. Additionally, phosphorylation of TSC2 may not be the only way in which AMPK, or related kinases, negatively regulates mTOR. The recent report that AMPK can directly phosphorylate mTOR itself, and does

so under conditions of energy stress, offers another possible inhibitory signal (Cheng et al., 2004). Therefore, while the lack of response to energy stress in TSC2-deficient cells argues that tuberin is important, it may not be the only target of AMPK in mTOR regulation.

Recently, LKB1 has been demonstrated to be necessary and sufficient for polarity of single intestinal epithelial cells (Baas et al., 2004). LKB1 kinase activity was shown to be necessary for this effect, although which of its 13 downstream effector kinases might play a role in this process has not been delineated. Moreover, LKB1 is the mammalian homolog of Par-4 in *C. elegans*, which was one of the original *par* mutants identified in a screen for early embryonic polarity and partitioning defects (Watts et al., 2000). The *Drosophila* ortholog of LKB1 is also essential for early embryonic polarity (Martin and St Johnston, 2003), and some of its role in that process is likely due to its activation of the Par-1 kinase homolog, which is one of the 11 kinases related to AMPK that have recently been demonstrated to be activated by LKB1 (Lizcano et al., 2004). Thus, LKB1 uniquely regulates both central metabolism, through AMPK, and at least some aspects of cell polarity via Par-1 homologs. Therefore, disruption of LKB1 in the gastrointestinal epithelium would potentially simultaneously result in aberrant mTOR activation and defects in cell polarity. Whether LKB1-mediated inhibition of mTOR plays a role in the establishment of cell polarity remains to be determined. However, it has been demonstrated that in yeast, TOR signaling is critically involved in cell polarity (Loewith et al., 2002; reviewed in Harris and Lawrence, 2003).

Together, with the results shown here, it is now evident that dysregulated activation of mTOR is a common biochemical feature of several autosomal dominant syndromes characterized by the occurrence of hamartomas. These results also suggest that rapamycin and its analogs may be useful for the treatment of polyps arising in PJS patients and possibly in the subset of lung adenocarcinomas that lack LKB1. The fact that rapamycin can promote survival of LKB1-deficient cells under conditions of energy stress, as previously reported for TSC-deficient cells, suggests that there may be certain cell types that are sensitive to growth inhibition by rapamycin, while others may be refractory. The biological response to mTOR inhibition via rapamycin (growth arrest versus apoptosis) is also known to depend on other factors such as p53 status (Huang et al., 2003). While rapamycin might allow LKB1-deficient cells to survive in response to energy stress in vitro, it is unlikely that these cells would continue to proliferate in the presence of rapamycin. Of course, additional studies will be required to validate these possibilities, but the similarity of the polyps developed in the LKB1 heterozygous mice to those in PJS patients suggests that these mice represent an ideal tool to examine this potential therapeutic regiment.

Experimental procedures

Reagents and cell lines

Anti-phospho-AMPK (T172), anti-phospho-ACC (S79), anti-phospho-S6K1 (T389), anti-phospho-S6K1 (T421/S424), anti-phospho-GSK-3 (S9), anti-phospho ribosomal protein S6 (S235/236), anti-ribosomal protein S6, anti-eIF4E, anti-phospho-Akt (S473), anti-phospho-Erk (T202/Y204), anti-4E-BP1, and cleaved PARP (mouse-specific) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Anti-LKB1 antiserum was previously described (Bardeesy et al., 2002). Anti-Flag antibodies (M2 monoclonal and Flag polyclonal) were from Sigma. Anti-HA probe polyclonal antibody was from Santa Cruz. AICAR was obtained from Toronto Research Chemicals (Downsview, ON, Canada). Glucose-free media, D-PBS (containing 1 g/l D-glucose) and dialyzed serum was from Gibco/BRL. Rapamycin was from Calbiochem. 7-methyl GTP sepharose was obtained from Amersham Pharmacia. MBP-AMPKα (1–172) fusion protein was a generous gift of Drs. L. Witters and R. Hurley (Dartmouth Medical School, Hanover, NH). Active recombinant Akt and AMPK used in Figure 2B were purchased from Upstate Biotech (Lake Placid, NY) Constructs used: Flag-wild-type LKB1. Flag-kinase dead (K78I) LKB1 and Flag-tuberin were described previously (Shaw et al., 2004, Manning et al., 2002). HA-4E-BP1 and HA-S6K1 were kind gifts of J. Blenis (Harvard Medical School, Boston, MA). pEBG- AMPK α1 T172A was a kind gift of Dr. L. Witters (Dartmouth Medical School, Hanover, NH). pCDNA3-AMPKα2 (K45R) kinase-dead was a kind gift of M. Birnbaum (University of Pennsylvania Medical School, Philadelphia, PA). Littermate-derived LKB1$^{+/+}$ and $^{-/-}$ MEFs were prepared as described previously (Bardeesy et al., 2002) TSC2$^{-/-}$ p53$^{-/-}$ and control p53$^{-/-}$ littermate MEFs were obtained from Dr. D. Kwiatkowski as previously described (Zhang et al., 2003). HeLa cells were obtained from ATCC.

Cell culture

Cells were cultured and retrovirally infected as previously described (Shaw et al., 2004). HeLa cells were transiently transfected using HeLaMonster transfection reagent according to manufacturer's suggestions (Mirus, Madison, WI). Cells were serum-starved in DMEM without serum 14 hr post-transfection, then 8 hr later the media was replaced with fresh DMEM with 10% FBS or DMEM with 10% FBS and 2 mM AICAR. For the MEF experiments in Figure 1, cells were plated at 1×10^6 cells/10 cm dish, then the next day the media was replaced with fresh DMEM with 10% FBS or DMEM with 10% FBS and 2 mM AICAR. For the MEF experiments in Figures 3A and 3B, 1×10^6 cells of each genotype were plated and the next day serum-starved for 24 hr. Cells were either lysed as such or their media replaced with fresh DMEM with 10% FBS, DMEM with 10% FBS and 2 mM AICAR, DMEM with 10% FBS and 20 nM rapamycin, or D-PBS with 10% dialyzed FBS for 90 min. For Figures 3C and 3D, cells were plated and the next day placed in glucose-free DMEM with 10% dialyzed FBS, with or without 20 nM rapamycin. Cells were kept in glucose-free media with or without rapamycin for 24 hr. For MTT assays, cells were plated in 12-well dishes in triplicate for each condition. MTT assays were performed as previously described (Shaw et al., 2004).

Biochemistry

MEFs were lysed in boiling SDS-lysis buffer (10 mM Tris [pH 7.5], 100 mM NaCl, 1% SDS) after the indicated treatments. After trituration, lysates were equilibrated for protein levels using the BCA method (Pierce) and resolved on 6%–12% SDS-PAGE gels, depending on the experiment. Gels were transferred to PVDF and Western blotted according to the antibody manufacturer's suggestions. Immunoprecipitations, kinase assays, and 7-methyl GTP pulldowns were performed as previously described (Shaw et al., 2004, Manning et al., 2002, Fingar et al., 2002).

Mice colony monitoring and tissue isolation

Lkb1$^{+/-}$ mice, maintained on an FVB/N genetic background, were monitored for the development of gastrointestinal polyps as described (Bardeesy et al., 2002). Mice with clinical signs of disease were euthanized and autopsied. The mean latency, distribution of polyps, and polyp phenotype were comparable to previous studies (Bardeesy et al., 2002). Polyps and adjacent tissue were harvested immediately and either processed for histological analysis or snap frozen in liquid nitrogen for molecular studies. These samples were then placed frozen into Nunc tubes and homogenized in lysis buffer (20 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM pyrophosphate, 50 mM NaF, 5 mM β-glycero-phosphate, 50 nM calyculin A, 1 mM Na3VO4, 10 mM PMSF, 4 μg/ml leupeptin, 4 μg/ml pepstatin, 4 μg/ml aprotinin) on ice for 30 sec using a tissue homogenizer.

Histology and immunohistochemistry

Tissues were fixed in ice-cold 10% formalin overnight and embedded in paraffin. For immunohistochemistry, slides were deparaffinized in xylene and rehydrated sequentially in ethanol. Antigen retrieval using Antigen Unmasking Solution (Vector) was performed according to the manufacturer's instructions. Slides were quenched in hydrogen peroxide (0.3%–3%) to

block endogenous peroxidase activity and then washed in Automation Buffer (Biomeda). Slides were blocked in 5% normal serum for 1 hr at room temperature. Slides were incubated at 4°C overnight with primary antibody diluted in blocking buffer. The avidin-biotin peroxidase complex method (Vector) was used, and slides were counterstained with hematoxylin. Slides were dehydrated sequentially in ethanol, cleared with xylenes, and mounted with Permount (Fisher). The anti-phospho ribosomal protein S6 antibody (S235/236) and anti-phospho-S6K1(T421/S424) were diluted according to manufacturer's suggestions (Cell Signaling Technology, Beverly, MA).

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