

mTOR and HIF-1 α -mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome

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Peutz-Jeghers syndrome (PJS) is a familial cancer disorder due to inherited loss of function mutations in the LKB1/STK11 serine/threonine kinase. PJS patients develop gastrointestinal hamartomas with 100% penetrance often in the second decade of life, and demonstrate an increased predisposition toward the development of a number of additional malignancies. Among mitogenic signaling pathways, the mammalian-target of rapamycin complex 1 (mTORC1) pathway is hyperactivated in tissues and tumors derived from LKB1-deficient mice. Consistent with a central role for mTORC1 in these tumors, rapamycin as a single agent results in a dramatic suppression of preexisting GI polyps in LKB1^{+/-} mice. However, the key targets of mTORC1 in LKB1-deficient tumors remain unknown. We demonstrate here that these polyps, and LKB1- and AMPK-deficient mouse embryonic fibroblasts, show dramatic up-regulation of the HIF-1 α transcription factor and its downstream transcriptional targets in an rapamycin-suppressible manner. The HIF-1 α targets hexokinase II and Glut1 are up-regulated in these polyps, and using FDG-PET, we demonstrate that LKB1^{+/-} mice show increased glucose utilization in focal regions of their GI tract corresponding to these gastrointestinal hamartomas. Importantly, we demonstrate that polyps from human Peutz-Jeghers patients similarly exhibit up-regulated mTORC1 signaling, HIF-1 α , and GLUT1 levels. Furthermore, like HIF-1 α and its target genes, the FDG-PET signal in the GI tract of these mice is abolished by rapamycin treatment. These findings suggest a number of therapeutic modalities for the treatment and detection of hamartomas in PJS patients, and potential for the screening and treatment of the 30% of sporadic human lung cancers bearing LKB1 mutations.

AMPK | FDG-PET | glycolysis | hamartoma | polyposis

Peutz-Jeghers syndrome (PJS) is an inherited autosomal dominant disorder in that patients develop benign hamartomatous polyps in the gastrointestinal tract and are predisposed to developing cancer (1). Inactivating mutations in the LKB1/STK11 tumor suppressor gene underlie PJS and have also been associated with sporadic lung adeno- and squamous carcinomas (2–8). Homozygous deletion of *Lkb1* is embryonic lethal to mice while heterozygous deletion of *Lkb1* results in late onset gastrointestinal polyposis between 6–13 months of age that closely models human PJS (9–13). Gastrointestinal hamartomas are benign tumors that consist of hyperplastic glandular epithelial cells, disorganized tissue architecture, and a characteristic arborizing smooth muscle stalk. Several hamartomatous syndromes involve inactivating mutations in genes that negatively regulate the mTORC1 pathway, which promotes cell growth and proliferation. In addition to PJS, these diseases include Cowden's disease, tuberous sclerosis complex, and neurofibromatosis type I, due to inactivating mutation in the PTEN, TSC1, TSC2, or NF1 genes, respectively (14).

The mammalian target of rapamycin (mTOR) is a central regulator of cell growth in all eukaryotes that is found in 2 functionally distinct multiprotein complexes (15). The mTOR

complex 1 (mTORC1) is composed of mTOR and its scaffolding protein raptor. Signaling from mTORC1 is nutrient-sensitive, acutely inhibited by the bacterial macrolide rapamycin, and controls protein translation, cell growth, angiogenesis, and metabolism. Activation of mTORC1 results in phosphorylation of a number of downstream targets involved in promoting cell growth and proliferation. These substrates include proteins involved in the regulation of protein translation such as the p70 S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4EBP1) (16). Among the mRNAs known to be translationally up-regulated by mTORC1 are a number of key pro-growth proteins including cyclin D1, cyclin D3, Mcl-1, c-myc, and the hypoxia inducible factor 1 alpha (HIF-1 α) (17–21).

Accordingly, mTORC1 is activated by mitogenic stimuli acting through the PI3K/Akt and Erk signaling pathways (15). In contrast, under conditions of low intracellular ATP such as after nutrient deprivation or other stresses, the LKB1 tumor suppressor activates the AMP-activated protein kinase (AMPK), which then rapidly inhibits mTORC1 although phosphorylation of both raptor (22) and the TSC2 tumor suppressor (23, 24). Hence, treatment with AMPK activating drugs, or overexpression of LKB1 or AMPK, results in suppression of mTORC1, whereas targeted deletion of LKB1 in mice leads to increased mTORC1 activity in murine fibroblasts, liver, and in polyps of LKB1-heterozygous mice (24–26).

Despite the common feature of elevated mTORC1 signaling in these hamartoma syndromes, the important targets of mTORC1 in LKB1-deficient tumors remain to be defined. We show here that HIF-1 α and its transcriptional targets in glucose metabolism are up-regulated in LKB1-deficient tumors in mice and human Peutz-Jeghers patients. Increased Glut1 and Hexokinase II expression in these polyps of *Lkb1*^{+/-} mice allows them to be visualized by FDG-PET. Because rapamycin strongly suppresses polyposis in the *Lkb1*^{+/-} mice, mTORC1 inhibitors and FDG-PET imaging may be useful clinically in the treatment of PJS patients.

Results

Rapamycin Reduces Tumor Burden and Proliferation in *Lkb1*^{+/-} Mice.

We investigated the effect of rapamycin on preexisting PJS-like polyps, by treating 9-month-old *Lkb1*^{+/-} or *Lkb1*^{+/+} mice for a period of 2 months with rapamycin or vehicle (Fig. S1A). Our preliminary studies revealed that at 9 months of age, 100% of the *Lkb1*^{+/-} mice have developed multiple gastrointestinal hamartomas, consistent with previous reports (10–13). Both *Lkb1*^{+/+}

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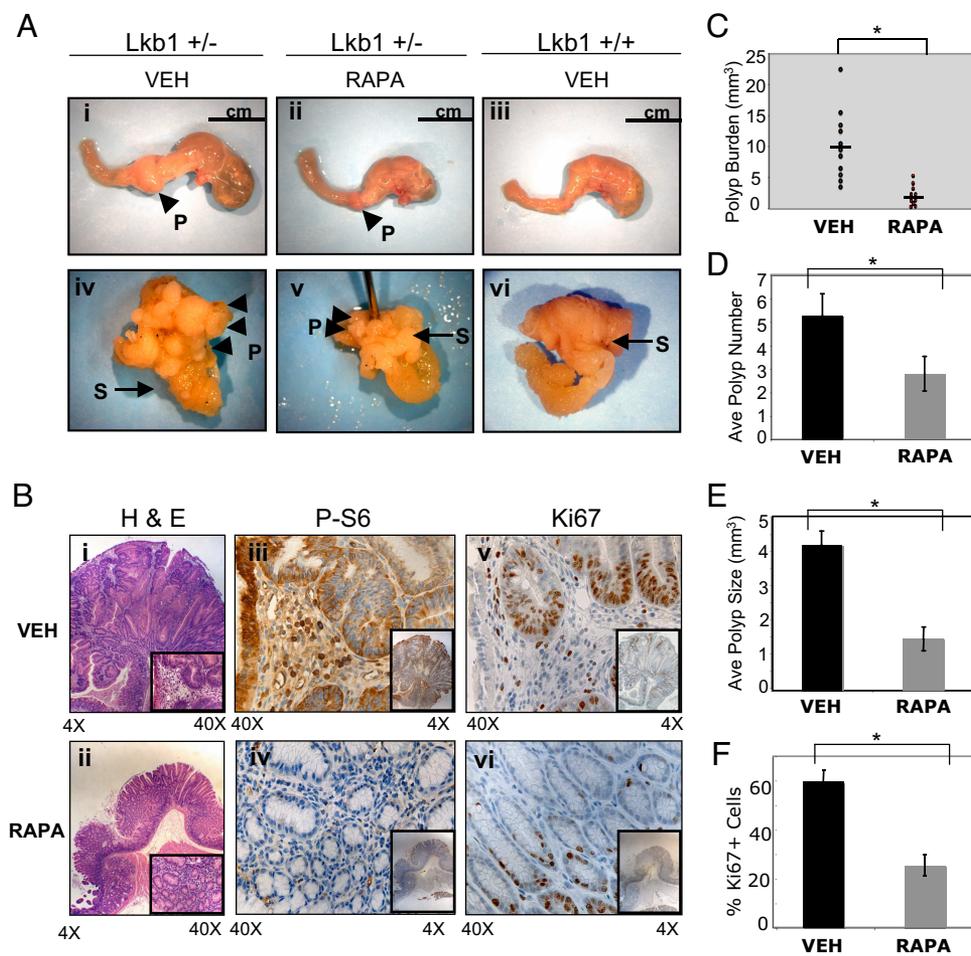


Fig. 1. Rapamycin reduces polyposis, mTORC1 signaling, and proliferation in *Lkb1*^{+/-} polyps. (A) *Top* are images of whole stomach and duodenum and *Bottom* are images of the open stomachs (S) showing the exposed polyps (P) from *Lkb1*^{+/-} mice treated with either vehicle (VEH) (i, iv) or rapamycin (RAPA) (ii, v) and *Lkb1*^{+/+} mice treated with vehicle (VEH) (iii, vi). (B) Immunohistochemical analysis of polyps from VEH- or RAPA-treated *Lkb1*^{+/-} mice: H & E staining (i, ii), P-S6 staining (iii, iv), and Ki67 staining (v, vi). Results are representative of polyps from 5 mice of each treatment group. (C) A graph of the total polyp burden in *Lkb1*^{+/-} mice treated with either VEH (○, *n* = 11) or RAPA (●, *n* = 10). The mean polyp burden for RAPA-treated mice (2.0 ± 1.2) was significantly reduced (*, *P* = 0.00026; Student *t* test, 2 tail) compared with those mice treated with VEH (9.6 ± 5.5). (D) Average polyp number in VEH-treated mice (black bar, *n* = 11) or RAPA-treated mice (gray bar, *n* = 10). Only visible polyps between 1 and ≥5 mm were scored in both VEH- and RAPA-treated mice. The mean polyp number for RAPA-treated mice (2.8 ± 1.4) was significantly reduced (*, *P* = 0.00022; Student *t* test, 2 tail) compared with VEH-treated mice (5.3 ± 1.8). (E) Average polyp size in VEH-treated mice (black bar, *n* = 11) or RAPA-treated mice (gray bar, *n* = 10). The mean polyp size in RAPA mice (1.2 ± 0.9) was significantly reduced (*, *P* < 0.0001; Student *t* test, 2 tail) compared with VEH-treated mice (5.3 ± 1.8). (F) Average percentage of Ki67-positive epithelial cells in VEH-treated mice (black bar, *n* = 5) or RAPA-treated mice (gray bar, *n* = 5). The mean percentage of Ki67-positive cells in RAPA mice (24.5 ± 6.1) was significantly reduced (*, *P* < 0.0002; Student *t* test, 2 tail) compared with VEH-treated mice (59.7 ± 7.3).

and *Lkb1*^{+/-} mice tolerated rapamycin treatment with no obvious cytotoxicity or immunosuppression at the doses used. After 2 months of treatment, polyp size and number in each mouse were quantitated. The wild-type *Lkb1*^{+/+} mice were free of polyps, while all of the *Lkb1*^{+/-} mice treated with vehicle presented severe polyp burden at or before 11–12 months of age, consistent with previous studies of these mice (10–13, 27). These mice had multiple large polyps in the stomach and pylorus and suffered from severe distention of the stomach and anemia (Fig. 1*A i* and *iv*). Histological analysis of H&E-stained polyps from untreated mice were classified as pedunculated, hyperplastic lesions consisting of differentiated glandular epithelium, stroma, and a smooth muscle stalk. In contrast, *Lkb1*^{+/-} mice treated with rapamycin had a dramatic reduction in polyp burden. These mice uniformly had reduced polyp size (Fig. 1*E*) and lower frequency of polyps (Fig. 1*D*), no distention of the stomach (Fig. 1*A ii* and *v*) and appeared active and vigorous at 2 months of treatment. Comparison of the polyp burden between *Lkb1*^{+/-} mice treated or untreated with rapamycin showed an 80% reduction in the overall mass of polyps (Fig. 1*C*). Polyps isolated from treated mice were greatly reduced in size, although still retained some disruption of the normal tissue architecture as shown by H&E staining (Fig. 1*B ii*).

We analyzed mTORC1 signaling in the polyps of *Lkb1*^{+/-} mice to determine whether rapamycin was effectively inhibiting the pathway. Immunohistochemical staining of polyps for phospho-S6 (P-S6) revealed that untreated polyps displayed high levels of P-S6 staining indicative of hyperactive mTOR signaling, while the polyps from rapamycin treated mice were greatly reduced for P-S6 staining indicating successful inhibition of

mTORC1 signaling (Fig. 1*B iii* and *iv*). These results were further corroborated by western blot analysis of polyp lysates (Fig. 1*S1B*).

Rapamycin has been shown to suppress tumor growth and induce apoptosis, resulting in cytostatic or cytotoxic responses in several genetically engineered mouse models with spontaneously arising tumors (15). We sought to examine the mechanism(s) by which rapamycin reduced polyp burden in the *Lkb1*^{+/-} mice. We first analyzed expression of the proliferation marker Ki67 in rapamycin or vehicle-treated polyps. The highest expression of Ki67 was found in proliferating epithelial cells at the base of the crypts. While staining was extensive in the vehicle-treated polyps, rapamycin-treated polyps showed a clear reduction in Ki67 staining (Fig. 1*B v* and *vi* and *F*). However, polyps from treated mice did not show evidence of apoptosis as detected by cleaved caspase-3 protein using immunohistochemistry or immunoblotting. These results suggest that in this tumor model rapamycin may be having a cytostatic effect rather than a cytotoxic effect, consistent with observations in other tumor types (15).

Rapamycin Down-Regulates Expression of HIF-1 α and HIF-1 α Targets.

Activation of mTORC1 results in increased translation of a number of key downstream targets, including cyclin D1 and the hypoxia inducible factor 1 alpha gene (HIF-1 α) (17–20). We examined the protein levels of cyclin D1 and HIF-1 α in the stomachs and polyps of *Lkb1*^{+/+} and *Lkb1*^{+/-} mice by western blot. HIF-1 α but not cyclin D1 levels were elevated in polyps of vehicle-treated *Lkb1*^{+/-} mice. In contrast, the polyps of mice treated with rapamycin showed reduced HIF-1 α levels similar to the basal levels seen in *Lkb1*^{+/+} mice (Fig. 2*A*). Next, we examined protein expression of transcriptional targets of HIF-1 α

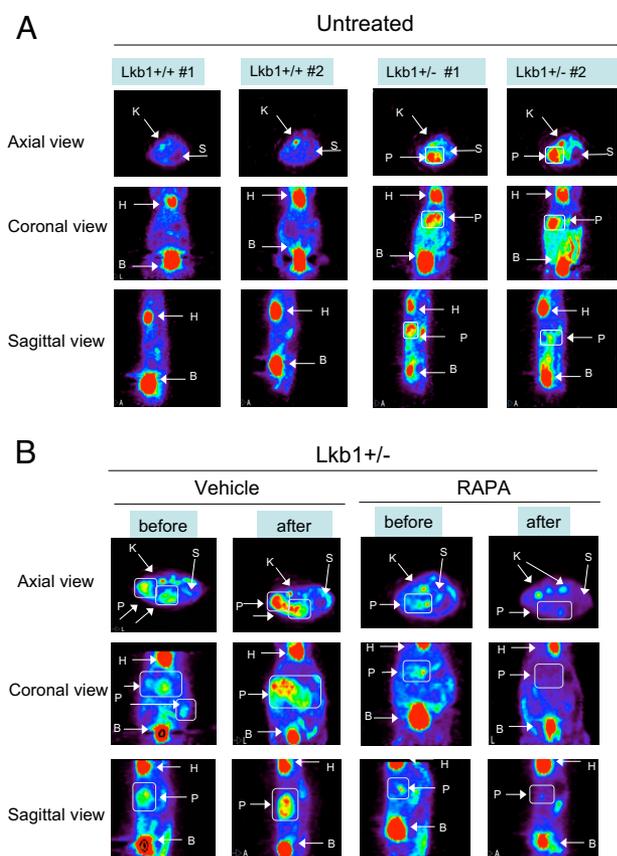


Fig. 3. Polyps from *Lkb1*^{+/-} mice visualized by FDG PET analysis. (A) Left shows FDG PET images of axial, sagittal and coronal views of untreated 12-month-old *Lkb1*^{+/+} mice. Right shows the same views of untreated *Lkb1*^{+/-} mice. The FDG PET images of the mice are labeled accordingly: K, kidney; S, stomach; H, heart; B, bladder; and P, polyp. (B) Left shows FDG PET imaging of axial, sagittal, and coronal views of *Lkb1*^{+/+} and *Lkb1*^{+/-} mice either treated with vehicle or rapamycin at 11 months of age. Right shows the same mice imaged after 1 month of receiving either vehicle or rapamycin. The images of the mice are labeled accordingly: K, kidney; S, stomach; H, heart; B, bladder; and P, polyp.

samples from PJS patients and compared with samples of small bowel and colonic mucosa from normal patient controls. Expression of the mTOR target P-S6 was increased in the epithelium of small bowel of PJS patients compared with that of normal tissue (Fig. 4 A and B). Likewise, strong immunostaining of HIF-1 α and GLUT1 was observed in glandular epithelial cells in 7 of 8 PJP colonic polyp specimens (Fig. 4 D and F). In the normal colonic mucosa specimens, weak immunohistochemical staining of HIF-1 α and GLUT1 was observed relative to the highly elevated levels observed in the PJS samples (Fig. 4 C and E). These results suggest that loss of the LKB1 gene leads to both mTORC1 hyperactivation and increased HIF-1 α and GLUT1 expression in PJS patients in a manner that closely follows the murine model of PJS.

Discussion

Aberrant activation of the mTORC1 pathway has been observed in spontaneously arising tumors in mice genetically engineered for loss of the tumor suppressors *Pten*, *Nf1*, *Tsc2*, or *Lkb1* (32–38). Mutations in these genes are responsible for the inherited cancer syndromes Cowden's disease, neurofibromatosis type I, tuberous sclerosis complex, and Peutz-Jeghers syndrome; collectively referred to as Phakomatoses, and all sharing overlapping clinical features including the development of hamarto-

mas. Biochemical and cell biological studies from the past decade have revealed that these tumor suppressors all are direct components of the mTOR signaling pathway that serve to inhibit mTORC1 activity (15).

The underlying hypothesis is that mutational inactivation of these tumor suppressors in individual cells leads to cell-autonomous hyperactivation of mTORC1, promoting cell growth and ultimately resulting in tumors that are subsequently reliant on mTORC1 signaling for tumor maintenance. Consistent with this possibility, rapamycin analogs have been examined for their therapeutic efficacy in the suppression of tumors that arise in a number of the aforementioned mouse models. The *Pten*^{+/-}, *Nf1*^{+/-}, *Tsc*^{+/-}, *Lkb1*^{+/-}, and activated Akt transgenic mouse models have also proven to be responsive to the mTOR inhibitors rapamycin or rapamycin analogs RAD001 (Novartis), CCI0779 (Wyeth) and AP23573 (Ariad) (34, 39–43). These drugs have been proven to effectively inhibit mTORC1 in vivo and reduce tumor burden through mTORC1 dependent mechanisms, including suppression of cyclin D, Mcl-1, or HIF-1 α and its targets (35, 39–41).

In recent clinical trials, rapamycin and its analog temsirolimus were shown to have palliative success in clinical trials on patients with PTEN-deficient glioblastomas and metastatic renal cell carcinoma (44, 45). Furthermore, in a pair of phase II clinical trials involving tuberous sclerosis (TSC) and lymphangioleiomyomatosis (LAM) patients, partial responses to the rapamycin analog Sirolimus were observed, including regression of angiomyolipomas with continuous therapy (46, 47), consistent with previous clinical observations in TSC patients given rapamycin (48, 49). Combined with data from mouse models, these clinical data suggest that hamartoma syndromes with hyperactivation of mTORC1 may be particularly responsive to rapamycin analogs as a single agent. To date there are no therapies to treat PJS and the only course of treatment is resection of arising gastrointestinal hamartomatous polyps. Consistent with a previous report (34), we found here that rapamycin greatly reduced the polyp burden in the *Lkb1*^{+/-} mouse model of PJS. This suppression was correlated with inhibition of mTORC1 and downregulation of HIF-1 α and its transcriptional targets. While these results are encouraging for the use of rapamycin analogs as therapeutics for PJS, like the recent phase II clinical findings with TSC patients, removal of the drug may result in rapid return of the initial tumor due to the largely cytostatic nature of the response (46). Perhaps new, targeted inhibitors directed at the kinase domain of mTOR will produce greater therapeutic response with targeted cytotoxicity, or perhaps kinase inhibitors that dually inactivate mTOR and PI3K, because PI3K provides a survival signal in most epithelial cell types. As observed in most cancers studied to date, combinations of targeted therapeutics, or of targeted and traditional chemotherapeutics may find the ultimate utility in the treatment of this disease. Importantly, it is worth noting here that rapamycin treatment may not only be therapeutically useful for the hamartomas that arise in Peutz-Jeghers patients, but also in preventing and reducing any secondary malignancies that arise in these patients at additional sites (breast, pancreas, and ovary).

This study also finds the transcription factor HIF-1 α as a relevant target of mTORC1 in LKB1-dependent hamartomas, and the up-regulation of HIF-1 α targets GLUT1 and hexokinase II may be responsible for the ability of these tumors to be visualized by FDG-PET, because both GLUT1 and hexokinase II have been reported as rate-limiting steps for FDG uptake and imaging (31). HIF-1 α has been shown to be an excellent correlate of rapamycin response in a transgenic model of prostate neoplasia dependent on activated Akt, and in VHL-deficient renal cell carcinoma xenografts (20, 39). Consistent with the findings here in spontaneously arising hamartomas in LKB1^{+/-} mice, recent studies using human glioblastoma xenografts and

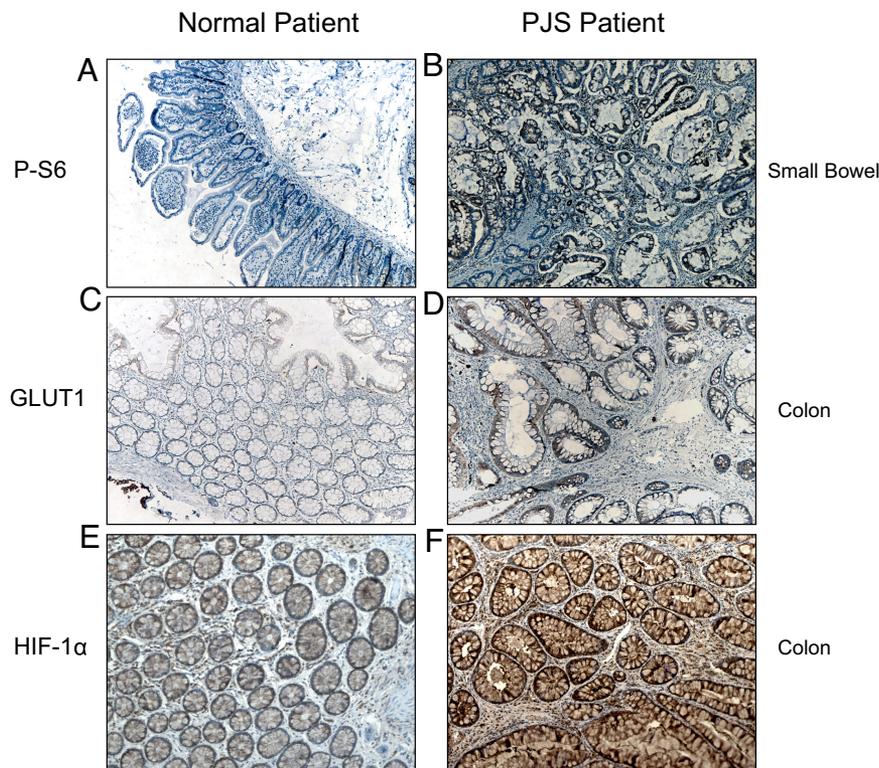


Fig. 4. Polyps from human Peutz-Jeghers patients show increased P-S6, GLUT1, and HIF-1 α expression. *A* and *B* represent immunohistochemistry performed on human small bowel samples from normal patients (*Left*) or Peutz-Jeghers patients (*Right*) that were probed with antibodies against the mTORC1 marker P-S6. *C–F* represent immunohistochemistry performed on normal colonic mucosa (*Left*) and colonic Peutz-Jeghers polyps (*Right*) probed with antibodies against the GLUT1 protein (*C* and *D*) or the HIF-1 α protein (*E* and *F*).

transplanted murine breast carcinomas also found rapamycin-sensitivity of FDG-PET imaging of these tumors (50, 51). Data from LKB1 and AMPK-deficient MEFs demonstrate that HIF-1 α and HIF-1 α targets are dramatically up-regulated in these cells under normoxic conditions, indicating that HIF-1 α up-regulation is not a secondary consequence of other tumor mutations or hypoxia present within the hamartomas. These findings also suggest that AMPK may be a key effector of LKB1 in the suppression of HIF-1 α in the normal gastrointestinal epithelium that when disrupted gives rise to hamartomas. Interestingly, the increase in HIF-1 α was observed in the deficient fibroblasts under conditions of increased cell density when basal AMPK activity is high in the wild-type cells, perhaps due to glucose depletion of the media. In cells that genetically lack the ability to activate AMPK, HIF-1 α is up-regulated under these conditions.

Consistent with these findings, hypoxia-independent up-regulation of HIF-1 α has been observed in murine embryonic fibroblasts deficient in TSC2 or TSC1 (52, 53). Furthermore, evidence from a number of laboratories in the past decade indicates that mTORC1 signaling stimulates increases in HIF-1 α levels independent of VHL and hypoxia-driven stabilization, but instead dependent on increased translation of HIF-1 α due to a functional 5' TOP element in its 5' UTR (20, 39, 52, 54–58). Indeed, fusion of the 5' UTR of HIF-1 α to multiple reporter constructs confers rapamycin-dependent accumulation of the fused cDNA (17, 20, 59).

It will be critical for future studies to define whether the distinct downstream targets of mTORC1 are key for tumorigenesis in different cell types. Preliminary evidence suggests that different effectors may be important, because in a mouse model of Neurofibromatosis type 1, rapamycin dramatically suppressed tumors, although no effects were observed on HIF-1 α or on vascularization; instead, cyclin D appears to be the critical target of mTORC1 in this setting. Notably, no change in cyclin D1 was observed in LKB1 \pm hamartomas, consistent with recent reports on tissue variability of the reliance of cyclin D on mTORC1 (18). Interestingly however, we did observe increases in cyclinD1 in LKB1- and AMPK-deficient fibroblasts parallel-

ing increases in HIF-1 α , suggesting that different cell types exhibit distinct effectors downstream of mTORC1.

Although most often used in the detection of malignant tumors, our findings suggest that FDG-PET may find clinical utility for the identification of polyps in PJS patients. Moreover, FDG-PET may be useful for monitoring the efficacy of treatment or surgical resection of these polyps. It will also be very interesting to determine whether secondary cancers that arise in PJS patients at other sites (breast, pancreas, and endometrium) can also be visualized by FDG-PET, and whether rapamycin analogs or mTOR kinase inhibitors will demonstrate clinical efficacy in the treatment of those tumors. Finally, given the prevalence of somatic LKB1 mutations in sporadic non-small cell lung carcinomas, it will be of great interest to examine if the presence of LKB1 mutations in NSCLC will correlate with the propensity of lung tumors to be imaged with FDG-PET, or their response to mTOR-targeted therapeutics.

Materials and Methods

For immunoblotting, anti-phospho-S6K1 (T389), phospho-ribosomal protein S6 (S235/236), eIF4E, phospho-4ebp1 (Ser-65), hexokinase II (C64G5), phospho-Akt (Thr-308), and bNIP3 antibodies were obtained from Cell Signaling Technology. Antibodies against HIF-1 α (C-term) polyclonal antibody (Cayman Chemicals), GLUT1 (GT11-A Rabbit polyclonal antibody; Alpha Diagnostics Int.), Cyclin D1 (BD PharMingen) and tubulin (Sigma Chemicals) were also used. Rapamycin was obtained from LC Laboratories. A detailed description of all of the other methods appears in *SI Materials and Methods*.

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