Dysregulation of HIF and VEGF is a unifying feature of the familial hamartoma syndromes

James Brugarolas and William G. Kaelin, Jr.*

Dana-Farber Cancer Institute and Harvard Medical School, Howard Hughes Medical Institute, 44 Binney Street, Mayer 457, Boston, Massachusetts 02115
*Correspondence: william_kaelin@dfci.harvard.edu

The LKB1 tumor suppressor protein controls the activity of the TSC1/TSC2 tumor suppressor complex. Mutations in *LKB1* cause Peutz-Jeghers syndrome (PJS), and mutations in either *TSC1* or *TSC2* cause tuberous sclerosis complex—two syndromes characterized by the development of hamartomas. LKB1 activation by energy deprivation activates AMPK, which in turn phosphorylates and activates TSC2. TSC2 activation results in the inactivation of mTOR, a critical regulator of protein translation. How mTOR dysregulation after inactivation of LKB1 or TSC1/2 contributes to hamartoma development is not known. However, hypoxia-inducible factor (HIF) and VEGF are regulated by mTOR and are likely to play a contributory role.

Two recent studies, including one reported in this issue of *Cancer Cell*, link the signaling pathways responsible for the hamartomatous syndromes Peutz-Jeghers and tuberous sclerosis complex (Corrandeti et al., 2004; Shaw et al., 2004a). Both PJS and tuberous sclerosis complex are inherited in an autosomal dominant manner, and both are characterized by multiple hamartomas.

The term hamartoma refers to benign tumors that are composed of multiple cell types native to the tissue from which they arise. Hamartomas can develop in multiple tissues. In PJS, these lesions develop primarily in the intestine, where they grow as polyps. PJS is also characterized by abnormal mucocutaneous pigmentation and an increased risk of malignant tumors in the intestine and elsewhere (Wirtzfeld et al., 2001). In tuberous sclerosis complex, hamartomas primarily develop in the brain, heart, kidney, and skin.

Peutz-Jeghers results from mutations in the *LKB1* tumor suppressor gene (Hemminki et al., 1998; Jenne et al., 1998). LKB1 is a serine-threonine kinase, and most of the *LKB1* mutations linked to PJS are known or predicted to inactivate its catalytic activity (Boudeau et al., 2003). The best-characterized substrate of LKB1 is AMPK (Hawley et al., 2003; Woods et al., 2003; Shaw et al., 2004b). AMPK is a serine-threonine kinase that functions in a pathway that integrates signals from energy stores (Carling, 2004). As cellular ATP decreases and AMP levels rise, LKB1 activates AMPK, which in turn phosphorylates many substrates involved in energy conservation pathways. In this setting, LKB1 can be viewed as an AMPKK.

Tuberous sclerosis complex results from mutations in either the *TSC1* or *TSC2* tumor suppressor gene (Cheadle et al., 2000). The TSC2 protein (also called tuberin) is a substrate of AMPK (Inoki et al., 2003). TSC2 binds TSC1 (also called hamartin) to form a tumor suppressor complex (for simplicity we will refer to the hamartin/tuberin complex as TSC1/2). TSC1/2 functions in a signal transduction pathway that integrates signals from a variety of sources with the protein translation apparatus through the regulation of mTOR. Under conditions that are adverse for proliferation (such as in the absence of nutrients or certain growth factors), TSC1/2 is activated and inhibits mTOR. TSC2 functions as a GAP (GTPase activating protein) toward the small Ras-like GTPase Rheb (Fingar and Blenis, 2004). In its active, GTP bound form, Rheb activates mTOR, through a mechanism that is not completely understood. Phosphorylation of TSC2 by AMPK pursuant to energy starvation leads to enhanced TSC2 function, as determined by enhanced inhibition of mTOR (Inoki et al., 2003).

mTOR is a serine-threonine kinase. The best-characterized substrates of mTOR are S6K1 and 4E-BP1 (Fingar and Blenis, 2004). S6K1 is activated by phosphorylation and, in turn, phosphorylates the 40S ribosomal S6 protein. S6 phosphorylation has been hypothesized to increase the translation of a class of mRNAs that encode components of the protein translation machinery and possess a terminal oligopyrimidine (TOP) tract in their 5′UTRs. Phosphorylation of 4E-BP1 releases the eukaryotic initiation factor eIF-4E, which then recruits other components of the eIF-4 complex to the 5′ end of cap-bearing mRNAs, thus stimulating protein translation.

The most recent findings have now established that LKB1 regulates TSC1/2 through AMPK, thereby linking the *LKB1* tumor suppressor gene to the TSC1/2 tumor suppressor pathway. Activation of LKB1 results in phosphorylation and activation of AMPK. In turn, AMPK phosphorylates and activates TSC2, thereby inhibiting mTOR (Corrandeti et al., 2004; Shaw et al., 2004a). By inhibiting mTOR, AMPK stalls a major energy-consuming process: the translation of proteins and, in particular, the translation of proteins comprising the translational machinery itself.

TSC1/2 function is also regulated by the tumor suppressor protein, PTEN. Germline *PTEN* mutations result in a variety of clinical syndromes. Notably, two of these syndromes, Cowden syndrome and Bannayan-Riley-Ruvalcaba syndrome (BRRS), are also characterized by hamartomas (Eng, 2003). PTEN is a dual specificity phosphatase that catalyzes the conversion of PIP3 (phosphatidylinositol 3,4,5 triphosphate) to PIP2 (Phosphatidylinositol 4,5 biphosphate) (Cantley, 2002). PIP3 is an important second messenger whose levels are tightly regulated. In response to growth factor stimulation, intracellular PIP3 levels rise transiently, leading to activation of multiple effectors, including Akt and PDK1. In the absence of PTEN, increased levels of PIP3 result in an abnormal activation of these effector proteins. Akt directly phosphorylates TSC2, which leads to decreased TSC2 GAP activity (Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002).

Hence, two different tumor suppressor pathways converge

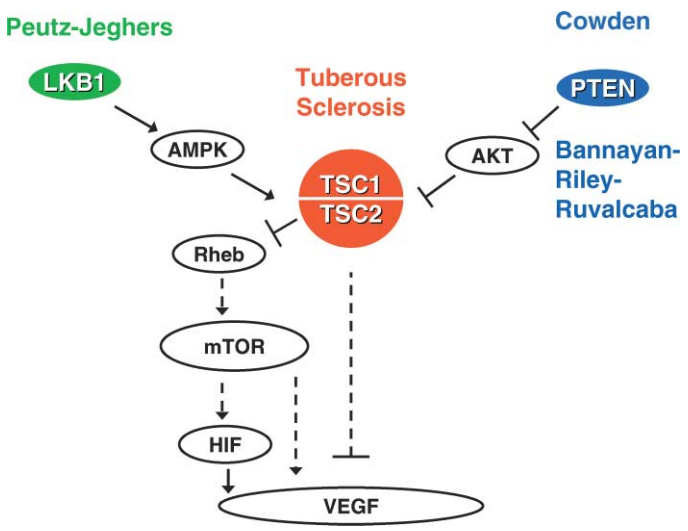

Figure 1. Signaling mechanisms by LKB1, PTEN, and TSC1/2 tumor suppressors

Dashed lines indicate functional interactions with incompletely understood mechanisms.

to regulate TSC1/2 (Figure 1). Loss of LKB1 results in decreased activation of AMPK and the downregulation of TSC1/2 function. Loss of PTEN increases Akt activity, which also downregulates TSC2 function. This may explain the development of hamartomas in Cowden syndrome (and BRRS), PJS, and tuberous sclerosis complex. How inactivation of TSC1/2 leads to hamartoma development is not known. Hamartomas arising in tuberous sclerosis complex patients do show evidence of mTOR activation (El-Hashemite et al., 2003b). Similarly, polyps from *LKB1*$^{+/-}$ mice also exhibit increased phosphorylation of mTOR effectors (Shaw et al., 2004a). These observations are at least consistent with the notion that dysregulation of mTOR contributes to the development of hamartomas.

Precisely how the regulation of mTOR and protein translation is linked to cell proliferation control is an area of intense investigation. It is intriguing, however, that genes encoding translation factors are sometimes amplified in cancers, transform cells in tissue culture, and cooperate with other oncogenes to cause tumors in mice (Holland et al., 2004). The protein elongation factor, eEF-1α2, is amplified in ovarian tumors and, when overexpressed, allows NIH 3T3 cells to grow in anchorage-independent conditions and enhances focus formation (Anand et al., 2002). Ectopic expression of the translation initiation factor eIF-4E allows clonal expansion and anchorage-independant growth of mammary epithelial cells (Avdulov et al., 2004), and inhibition of eIF-4E blocks cell proliferation (De Benedetti and Graff, 2004). eIF-4E also cooperates with Myc to promote tumor development in mice (Ruggero et al., 2004; Wendel et al., 2004).

One of the pathways regulated by changes in mTOR activity involves the hypoxia-inducible transcription factor, HIF (Hudson et al., 2002). HIF is a heterodimeric transcription factor composed of an α and a β subunit that regulates a gene expression program that is important for adaptation to hypoxia. HIF is regulated by oxygen levels through oxygen-dependent hydroxylation of two prolyl residues in the α subunit (Kaelin, 2002). Hydroxylation of these residues targets HIF-α for polyubiquitylation by a multiprotein complex that contains pVHL, which is encoded by the von Hippel-Lindau tumor suppressor gene (Kaelin, 2002). Polyubiquitylation, in turn, earmarks HIF for destruction by the proteasome. The role of HIF in tumorigenesis has been most clearly established in the context of von Hippel-Lindau disease, which is caused by inactivating *VHL* mutations. The tumors that arise in this setting, including blood vessel tumors called hemangioblastomas and clear cell renal carcinomas, exhibit high levels of HIF. Elimination of HIF in pVHL-defective renal carcinoma cells using HIF shRNA, or by reintroducing wild-type pVHL, is sufficient to suppress their ability to form tumors in nude mice (Kondo et al., 2003; Zimmer et al., 2004). Moreover, expression of a stabilized form of HIF that evades pVHL control is sufficient to restore tumorigenesis in pVHL-deficient tumor cells reconstituted with wild-type pVHL (Kondo et al., 2002; Maranchie et al., 2002). Hence, HIF clearly plays a causal role in the development of pVHL-defective tumors.

HIF is thought to contribute to tumor development through increased expression of growth factors such as TGFα, PDGFβ, and VEGF. In addition, HIF also increases the expression of glucose transporters and glucose metabolic enzymes, which might also influence tumor development and have been linked to the characteristic metabolic changes observed in tumors referred to as the "Warburg Effect" (Dang and Semenza, 1999). Regulation of mTOR by HIF is likely to involve changes in HIF stability as well as changes in HIF mRNA accumulation (Brugarolas et al., 2003; Hudson et al., 2002). It is attractive to speculate that regulation of HIF by mTOR is a means of coupling changes in nutrient availability to changes in glucose uptake and metabolism, mitogenesis, and tissue angiogenesis. For example, increased angiogenesis would enhance the delivery of oxygen and nutrients to a tissue undergoing growth and expansion.

In keeping with the above considerations, HIF is regulated by growth factor availability as well as by changes in oxygen. Regulation of HIF by growth factors involves TSC1/2, because TSC2-deficient cells, in contrast to normal cells, fail to downregulate HIF in response to growth factor deprivation (Brugarolas et al., 2003). Failure to downregulate HIF in TSC2-deficient cells has functional consequences as determined by increased expression of HIF target genes such as VEGF (Brugarolas et al., 2003; El-Hashemite et al., 2003a; Liu et al., 2003). Notably, expression of a disease-associated TSC2 mutant (in contrast to wild-type TSC2) in TSC2-deficient cells fails to normalize HIF (and VEGF), suggesting that the regulation of HIF might be important for TSC2 tumor suppression function (Brugarolas et al., 2003; El-Hashemite et al., 2003a). The regulation of HIF by TSC1/2 involves mTOR, because HIF levels in TSC2-deficient cells are normalized by treatment with the mTOR inhibitor rapamycin (Brugarolas et al., 2003).

PTEN is also involved in the regulation of HIF (Zundel et al., 2000), and activation of the PI3 kinase pathway results in the upregulation of HIF and its target genes (Jiang et al., 2001; Mazure et al., 1997; Zhong et al., 2000). Expression of a constitutively active form of Akt in a transgenic mouse model in the prostate increases the expression of HIF and its targets (Majumder et al., 2004). As was true for TSC2-deficient cells, treatment of these mice with a rapamycin analog normalizes the levels of HIF and HIF target genes (Majumder et al., 2004).

Increased HIF and VEGF levels may be a common, and perhaps unifying, feature of the familial hamartoma syndromes

and VHL disease. Rodent cells lacking Tsc1 or Tsc2 overproduce VEGF (Brugarolas et al., 2003; El-Hashemite et al., 2003a; Liu et al., 2003), and VEGF protein levels are increased in angiofibromas from tuberous sclerosis complex patients (Nguyen-Vu et al., 2001). Interestingly, VEGF protein levels are also elevated in LKB1-deficient cells (Ylikorkala et al., 2001). However, while in TSC2-deficient cells VEGF mRNA levels are also elevated, it is unclear whether VEGF mRNA levels are elevated in LKB1-deficient cells. In one study, VEGF mRNA levels were found to be elevated in *Lkb1*$^{-/-}$ mouse embryos by in situ hybridization (Ylikorkala et al., 2001). In another study, however, VEGF mRNA levels in *Lkb1*$^{-/-}$ mouse embryo fibroblasts (MEFs) were unchanged compared to wild-type MEFs by Northern blot (Bardeesy et al., 2002).

The regulation of VEGF by TSC1/2 might involve several pathways (Figure 1). Rapamycin normalizes HIF levels in TSC2-deficient cells but fails to completely normalize VEGF, suggesting that VEGF is regulated through both a rapamycin-sensitive and a rapamycin-insensitive (presumably mTOR-independent) pathway (Figure 1) (Brugarolas et al., 2003). Corrandeti and coworkers likewise found that rapamycin partially downregulated VEGF in LKB1-defective cells, although the effects were not normalized for the effects of rapamycin on global translation. With respect to mTOR, there is evidence that mTOR can signal to VEGF in both HIF-dependent and independent manners (Mamane et al., 2004).

While there are overlapping features among Cowden syndrome (and BRRS), Peutz-Jeghers syndrome, and tuberous sclerosis complex, these diseases differ in many ways, including the cells and organs that primarily give rise to tumors. These differences might be explained, at least partly, if the pathway depicted in Figure 1 is in fact far more complex, with each tumor suppressor protein performing some nonredundant function(s) that is not linearly connected to those performed by the others. Indeed, it has already been shown that LKB1 has other substrates besides AMPK (Lizcano et al., 2004). AMPK phosphorylates other proteins besides TSC2 (Carling, 2004), and PIP3 levels, which increase upon PTEN inactivation, regulate many pathways besides the TSC pathway (Cantley, 2002). It is also unclear whether LKB1 is the only AMPKK. In summary, the relatively linear pathway depicted in Figure 1 is almost certainly part of a more complex network, with each tumor suppressor protein serving as a critical nodal point with multiple branches. Some of these branch points might involve tissue-specific functions that would link these various tumor suppressor proteins to specific tumor types.

An immediate question stemming from these findings is whether rapamycin-like drugs will alter the natural history of the familial hamartoma syndromes. Encouraging in this regard is the fact that rapamycin can downregulate HIF and VEGF in a variety of settings. Rapamycin has been used as an immunosuppressant for years but recently has attracted interest as a potential anticancer agent due to its antiproliferative properties in vitro and the growing appreciation that many cancer-causing mutations (including those described here) directly or indirectly lead to increased mTOR activity (Sawyers, 2003). Interestingly, *PTEN*$^{-/-}$ cells are more sensitive than their wild-type counterparts to the antiproliferative effects of rapamycin, suggesting that cells might become "addicted" to high levels of mTOR activity under certain circumstances (Weinstein, 2002; Sawyers, 2003). Moreover, treatment with rapamycin has little effect on HIF levels in wild-type MEFs and wild-type mouse prostates (in contrast to their Tsc2-deficient and transgenic *Akt* counterparts, respectively) (Brugarolas et al., 2003; Majumder et al., 2004). These observations suggest that rapamycin use might be associated with a therapeutic window despite the central role of mTOR in cellular homeostasis.

Along similar lines, a number of agents that inhibit VEGF or its receptors are currently being tested in man and might theoretically prove useful, alone or in combination with rapamycin, for the treatment of the hamartoma syndromes. Testing these concepts in clinical trials would be congruent with recent reports that have firmly established the importance of using cancer genetics, and knowledge of cancer-relevant molecular pathways, to guide cancer therapy (Kaelin, 2004).

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Selected reading

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