

TSC2 regulates VEGF through mTOR-dependent and -independent pathways

James B. Brugarolas,¹ Francisca Vazquez,¹ Archana Reddy,¹ William R. Sellers,¹ and William G. Kaelin, Jr.^{1,2,*}

¹Dana-Farber Cancer Institute and Brigham and Women's Hospital, Harvard Medical School, 44 Binney Street, Boston, Massachusetts 02115

²Howard Hughes Medical Institute

*Correspondence: william_kaelin@dfci.harvard.edu

Summary

Inactivation of the TSC2 tumor suppressor protein causes tuberous sclerosis complex (TSC), a disease characterized by highly vascular tumors. TSC2 has multiple functions including inhibition of mTOR (mammalian target of Rapamycin). We found that TSC2 regulates VEGF through mTOR-dependent and -independent pathways. TSC2 loss results in the accumulation of HIF-1 α and increased expression of HIF-responsive genes including VEGF. Wild-type TSC2, but not a disease-associated TSC2 mutant, downregulates HIF. Rapamycin normalizes HIF levels in TSC2^{-/-} cells, indicating that TSC2 regulates HIF by inhibiting mTOR. In contrast, Rapamycin only partially downregulates VEGF in this setting, implying an mTOR-independent link between TSC2 loss and VEGF. This pathway may involve chromatin remodeling since the HDAC inhibitor Trichostatin A downregulates VEGF in TSC2^{-/-} cells.

Introduction

Tuberous sclerosis complex (TSC; depending on the context, the term "TSC" will be used to refer to the disease tuberous sclerosis complex or as a generic designation for the TSC1 and TSC2 proteins) is a disease characterized by the development of benign tumors, called hamartomas, in multiple tissues including the skin, central nervous system, lung, heart, and kidneys. These tumors are composed of multiple cell types and are frequently highly vascular. TSC patients also appear to be at increased risk for malignant renal carcinomas (RCC) of clear cell histology (Iliopoulos and Eng, 2000). TSC affects approximately 1 in 6000 live births and is linked to germline inactivating mutations of either of two genes, *TSC1* or *TSC2*, with each gene accounting for approximately 50% of the cases (Cheadle et al., 2000).

TSC2 encodes a protein of 200 kDa, TSC2 (also called Tuberlin), which is ubiquitously expressed and localizes to the perinuclear Golgi (The European Chromosome 16 Tuberous Sclerosis Consortium, 1993; Wienecke et al., 1996). TSC2 does not closely resemble other human proteins. TSC2 forms a complex with TSC1 that functions to integrate growth factor signals with the cell growth regulatory apparatus (Marygold and Leevers, 2002). This complex negatively regulates mTOR (mam-

lian target of Rapamycin). In the presence of serum, AKT is activated and phosphorylates TSC2, which disrupts TSC2 binding to TSC1 (Dan et al., 2002; Gao and Pan, 2001; Inoki et al., 2002; Manning et al., 2002; Potter et al., 2002; Radimerski et al., 2002). Dissolution of the TSC2/TSC1 complex allows for activation of mTOR, which phosphorylates 4E-BP and S6 kinase (Tee et al., 2002), ultimately leading to an increase in the rates of protein translation (Gingras et al., 2001). Treatment with Rapamycin, a specific mTOR inhibitor, results in the accumulation of unphosphorylated S6, inhibition of translation, and cell cycle arrest.

Notably, the mTOR pathway is activated in tumors from TSC patients (Goncharova et al., 2002; Kenerson et al., 2002). However, whether inhibition of mTOR fully accounts for the tumor suppressor activities of TSC2 (and TSC1) is unclear (Mommose et al., 2002). TSC2 also functions as a GAP toward the small G protein Rheb, which acts upstream of mTOR (Garami et al., 2003; Saucedo et al., 2003; Stocker et al., 2003). This activity is probably important for TSC function because the TSC2 GAP domain, and the surrounding region, are particularly well conserved in orthologous proteins in *Drosophila* and *C. elegans*, and inactivation of Rheb restores the viability of Tsc-defective *Drosophila* larvae. Moreover, the GAP domain is important for TSC2-dependent tumor suppression in vivo

SIGNIFICANCE

Tuberous sclerosis complex (TSC) is a hereditary cancer syndrome caused by mutations in either of two tumor suppressor genes, *TSC1* or *TSC2*. We found that TSC2, by virtue of its known ability to inhibit mTOR, downregulates the HIF transcription factor. This may explain the phenotypic similarities between TSC and von Hippel-Lindau disease, which is also characterized by excessive HIF. HIF activates a variety of genes implicated in tumorigenesis, including VEGF. VEGF overproduction by TSC2^{-/-} cells was only partially inhibited by the mTOR antagonist Rapamycin, implying an additional, mTOR-independent link between TSC2 and VEGF. The HDAC inhibitor Trichostatin A downregulates VEGF production in TSC2-defective cells, suggesting that mTOR inhibitors and HDAC inhibitors might be beneficial for the treatment of TSC.

(Momose et al., 2002). While expression of a wild-type TSC2 cDNA transgene in the Eker rat, which is a naturally occurring model of TSC caused by an inactivating mutation in *Tsc2*, suppresses tumor formation, a TSC2 deletion mutant lacking the GAP domain does not (Momose et al., 2002).

Eker rats develop a variety of tumors including RCC and splenic hemangiosarcomas (Eker et al., 1981; Yeung et al., 1994). In the mouse, heterozygous mutations in *Tsc2*, or *Tsc1*, result in liver hemangiomas as well as kidney cystic lesions, which occasionally harbor features of malignancy (Kobayashi et al., 1999, 2001; Kwiatkowski et al., 2002; Onda et al., 1999). Homozygous inactivation of either *Tsc2* or *Tsc1* causes embryonic lethality.

While the tumor spectrum of patients with TSC and that of the animal models is quite different, a common feature is their vascular nature. TSC patients develop cutaneous angiofibromas and renal angiomyolipomas, which are highly vascular. The rodents develop RCC, hemangiomas, and hemangiosarcomas. This observation suggests that TSC plays a role in the regulation of angiogenesis. In support of this idea, immunohistochemical analysis of cutaneous angiofibromas from TSC patients reveals increased production of angiogenic factors including basic fibroblast growth factor (bFGF), platelet-derived endothelial cell growth factor (PD-ECGF), angiogenin, and VEGF (Nguyen-Vu et al., 2001). In addition, the conditioned media of *Tsc2*-null fibroblasts from the Eker rat stimulates endothelial cell proliferation to a greater extent than that of wild-type controls (Nguyen-Vu et al., 2001).

Interestingly, animal models of TSC develop phenotypes that are characteristic of Von-Hippel Lindau (VHL) disease in humans. VHL disease, which results from inactivation of the *VHL* tumor suppressor gene, is characterized by highly vascular tumors including RCC (which are often preceded by renal cysts) and hemangiomas (Maher and Kaelin, 1997). These tumor types are typical of animal models of TSC. Moreover, both *Tsc2*^{+/-} (or *Tsc1*^{+/-}) and *Vhl*^{+/-} mice (Haase et al., 2001) develop liver hemangiomas. These data suggest that the products of the *TSC* and *VHL* genes are functionally related.

pVHL is a component of an E3 ubiquitin ligase complex that targets hypoxia-inducible factor (HIF) for degradation (Maxwell et al., 1999). HIF is the generic name for the basic helix-loop-helix heterodimeric transcription factor formed upon binding of a HIF α (HIF-1, -2, and -3) subunit to a HIF β subunit (Kaelin, 2002). In contrast to the β subunit, which is stable and present in the cell in vast excess, the α subunits are labile and highly regulated. In the presence of oxygen, HIF α is hydroxylated by EGLN at specific Prolyl residues creating a high-affinity binding site for pVHL (Epstein et al., 2001; Ivan et al., 2001; Jaakkola et al., 2001; Masson et al., 2001; Yu et al., 2001). Once bound, pVHL directs the polyubiquitylation, and hence proteasomal degradation, of HIF α . Under hypoxic conditions, EGLN activity is diminished, which allows for the accumulation of unmodified HIF α . HIF α , once bound to HIF β , transcriptionally activates a variety of genes involved in acute or chronic adaptation to hypoxia. Among these are genes linked to angiogenesis such as VEGF (vascular endothelial growth factor) and PDGF B (platelet-derived growth factor B). Accordingly, pVHL-defective tumor cells overexpress HIF target genes and are highly angiogenic.

In addition to pVHL, HIF is also regulated by the PI3K pathway. Activation of the PI3K pathway, through growth factor stimulation or deletion of the PI3K inhibitor PTEN, results in HIF

upregulation (Jiang et al., 2001; Laughner et al., 2001; Treins et al., 2002; Zelzer et al., 1998; Zhong et al., 2000; Zundel et al., 2000). HIF upregulation can be blocked by inhibitors of PI3K and to a lesser extent inhibitors of mTOR, but the generalizability of these findings has been questioned (Alvarez-Tejado et al., 2002; Arsham et al., 2002).

Here we show that TSC2 regulates VEGF through both mTOR-dependent and -independent pathways. TSC2 loss results in the accumulation of HIF-1 α (particularly under conditions of low serum and prolonged hypoxia) and increased expression of HIF-responsive genes. Interestingly, the levels of VEGF mRNA are upregulated disproportionately to other HIF-responsive transcripts. Reconstitution with wild-type TSC2, but not a disease-associated mutant, downregulates HIF-1 α . HIF-1 α upregulation associated with TSC2 inactivation is linked to mTOR function and can be inhibited by Rapamycin. Notably, while Rapamycin treatment of TSC2-null cells normalizes the levels of the HIF-1 α and the HIF-regulated protein GLUT-1, Rapamycin only partially downregulates VEGF. These results strongly suggest the existence of an mTOR-independent mechanism of VEGF regulation by TSC2. Importantly, VEGF can be effectively downregulated in TSC2-null cells by the HDAC inhibitor Trichostatin A, but not by a number of other inhibitors of pathways known to regulate VEGF. These results suggest that loss of TSC2 may ultimately increase VEGF levels through alterations in chromatin structure.

Results

Tsc2-null cells exhibit increased levels of Hif-1 α

To determine the effects of *Tsc2* loss on Hif, we analyzed mouse embryo fibroblasts (MEFs) derived from *Tsc2*^{-/-} and *Tsc2*^{+/-} embryos. Loss of *Tsc2* results in lethality during early embryonic development when it is difficult to obtain large numbers of viable fibroblasts (Kobayashi et al., 1999; Onda et al., 1999; Rennebeck et al., 1998). To address this problem, Kwiatkowski and colleagues crossed *Tsc2*^{+/-} mice with *p53*^{-/-} mice to generate MEFs that were also deficient for p53, which enhances the lifespan of the limited number of MEFs that can be obtained (Onda et al., 1999). In the experiments that follow, *Tsc2*^{-/-}; *p53*^{-/-} MEFs were compared to *Tsc2*^{+/-}; *p53*^{-/-} MEFs. For simplicity, these MEFs are referred to as "*Tsc2*^{-/-}" and "*Tsc2*^{+/-}," respectively.

As shown in Figure 1A, Hif-1 α protein levels were modestly elevated in *Tsc2*^{-/-} cells compared to *Tsc2*^{+/-} cells (see also Figures 1B and 1C). Treatment with the hypoxia mimetic Deferoxamine (DFO) resulted in Hif-1 α upregulation in both cell types (Figure 1A). Induction of Hif-1 α under hypoxic conditions (1% O₂) was more pronounced in *Tsc2*-null cells compared to *Tsc2*^{+/-} cells, with respect to both magnitude and duration (Figure 1B). These data indicate that Tsc2 is required for proper downregulation of Hif-1 α levels following prolonged exposure to hypoxia.

Tsc2-null cells fail to downregulate Hif-1 α in low serum

In the absence of growth factors, TSC2 inhibits mTOR leading to the accumulation of unphosphorylated S6. To begin to determine whether HIF-1 α is regulated by TSC2 through mTOR, we examined the effects of serum on Hif-1 α protein levels. As expected, growth of *Tsc2*^{+/-} MEFs in low (0.5%) serum led to decreased S6 phosphorylation (Figure 1C). Incidentally, S6 protein levels also decreased under these conditions. Importantly, Hif-1 α protein levels were also diminished in *Tsc2*^{+/-} cells when

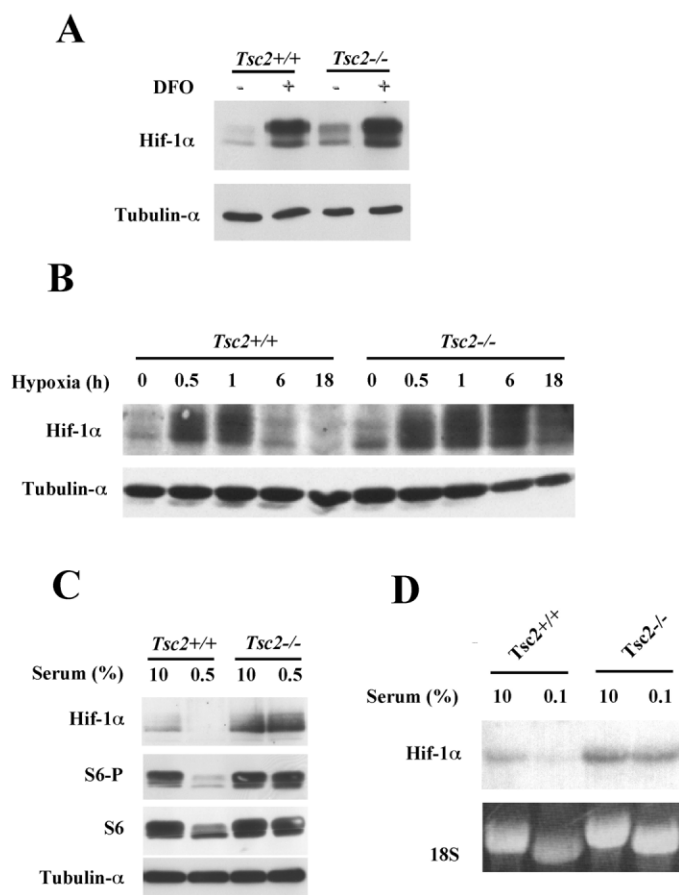


Figure 1. Effect of Tsc2 loss on Hif-1 α

A and **B**: Anti-Hif-1 α and anti-tubulin immunoblot analysis of *Tsc2*^{+/+} and *Tsc2*^{-/-} cells. In **A**, cells were treated with DFO (200 μ M) for $\sim$ 10 hr where indicated by "+." In **B**, cells were exposed to 1% oxygen for the duration indicated prior to analysis.

C: Immunoblot analysis with the indicated antibodies of *Tsc2*^{+/+} and *Tsc2*^{-/-} cells grown in high (10%) or low (0.5%) serum. S6-P refers to an antibody recognizing S6 phosphorylated on Ser 235/236.

D: Hif-1 α Northern blot analysis of *Tsc2*^{+/+} and *Tsc2*^{-/-} cells grown in high (10%) or low (0.1%) serum.

they were grown in low serum (Figure 1C). As previously shown by others (Jaeschke et al., 2002), serum deprivation failed to block S6 phosphorylation in *Tsc2*^{-/-} cells (Figure 1C). Interestingly, the failure to downregulate S6 kinase activity was mirrored by a failure to downregulate Hif-1 α . These results suggest that the mechanism whereby Tsc2 regulates Hif-1 α is sensitive to serum and may involve mTOR. Northern blot analysis revealed that the increased accumulation of Hif-1 α in *Tsc2*^{-/-} cells relative to *Tsc2*^{+/+} cells was at least partly due to changes in Hif-1 α mRNA levels (Figure 1D).

Wild-type Tsc2 inhibits Hif-1 α

To study further the functional relationship between Tsc2 and Hif, *Tsc2*^{-/-} MEFs were transfected with a mammalian expression vector encoding either wild-type TSC2 or a disease-associated TSC2 missense mutant (N1645K) along with a vector encoding enhanced green fluorescence protein. Successfully transfected cells were collected by FACS, replated, and subse-

quently switched to low serum for $\sim$ 18 hr prior to analysis. Both the wild-type and mutant TSC2 proteins were produced at comparable levels and approximated the endogenous Tsc2 levels seen in untransfected *Tsc2*^{+/+} MEFs (Figures 2A and 2B). Reconstitution with wild-type, but not mutant, TSC2 blocked S6 phosphorylation (data not shown) and markedly downregulated Hif-1 α protein levels (Figure 2A). The slight decrease in Hif-1 α protein levels in the cells reconstituted with mutant TSC2 might indicate that this particular missense mutant is not a true-null. Nonetheless, impaired downregulation of Hif-1 α by a disease-associated TSC2 mutant is consistent with the idea that dysregulation of HIF contributes to tumor formation following TSC inactivation.

Downregulation of TSC2 is sufficient for HIF-1 α upregulation

To determine whether Tsc2 downregulation is sufficient to upregulate Hif-1 α , we performed knockdown experiments using siRNA directed against TSC2 or, as a positive control, against the HIF prolyl hydroxylase EGLN1 (Kaelin, 2002). Two different TSC2 synthetic siRNAs were utilized that, in contrast to the EGLN1 siRNA or a scrambled siRNA, markedly reduced TSC2 protein levels when introduced into U2OS osteosarcoma cells (Figure 3A). As expected, EGLN1 siRNA increased HIF-1 α levels, especially in the absence of serum. Cells treated with either of the two TSC2 siRNAs, but not the scrambled siRNA, accumulated HIF-1 α in the absence of serum (Figure 3B). Collectively, these results indicate that TSC2 loss is both necessary and sufficient for HIF-1 α upregulation under conditions of serum deprivation.

Hif-1 α upregulation in *Tsc2*-null cells requires mTOR activity

To ask whether regulation of Hif-1 α by TSC2 is mediated by mTOR, we evaluated the effects of Rapamycin on Hif-1 α protein levels. As shown in Figure 4A, Rapamycin treatment inhibits S6 phosphorylation independently of *Tsc2* status. Treatment of *Tsc2*^{+/+} cells with Rapamycin had no appreciable effect on Hif-1 α protein levels under both serum-rich and serum-poor conditions (Figure 4B). In contrast, Rapamycin treatment decreased Hif-1 α levels in *Tsc2*^{-/-} cells under both serum-rich and serum-poor conditions, resulting in a Hif-1 α profile resembling that observed in *Tsc2*^{+/+} cells (Figure 4B). The changes in Hif-1 α were mirrored by changes in the Glut1 glucose transporter, which is the product of a Hif target gene. These data suggest that Hif-1 α accumulation in *Tsc2*-null cells results from inappropriate activation of mTOR.

Increased Hif transcriptional activity in *Tsc2*-null cells

To evaluate the functional significance of Hif-1 α elevation in *Tsc2*-null cells, Northern blot analyses of *Tsc2*^{-/-} and *Tsc2*^{+/+} MEFs were performed under standard growth conditions (10% serum) using probes for different Hif target genes including *VEGF*, which is involved in angiogenesis, *PGK1* and *Eno1*, which are involved in glucose metabolism, and the abovementioned *Glut1*. While the expression of *Eno1* was similar between the two cell types, the expression of *VEGF*, *Glut1*, and *PGK1* mRNAs was elevated in *Tsc2*^{-/-} cells compared to controls (Figure 5A). These differences were even more pronounced under low serum conditions, in keeping with the differences in Hif-1 α protein levels shown in Figure 1C (data not shown). These data suggest

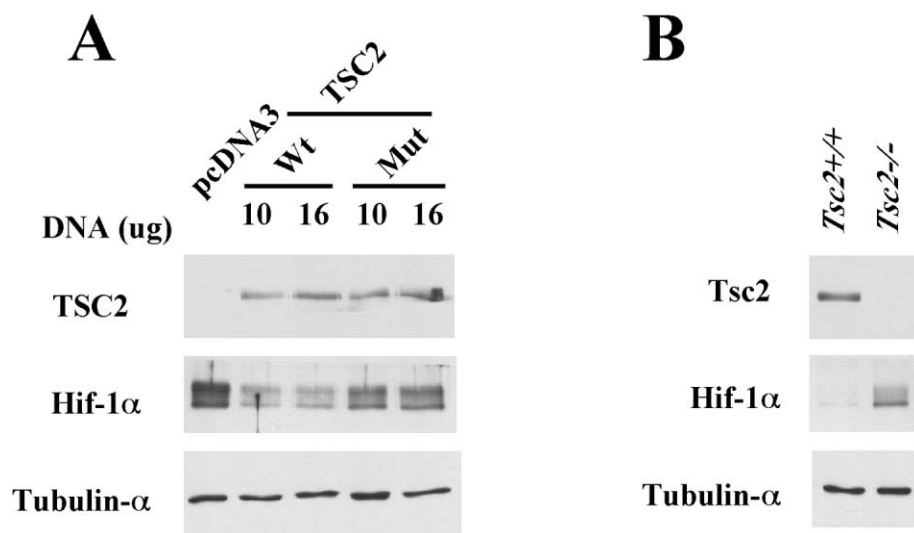


Figure 2. Suppression of Hif-1α after restoration of TSC function

A: *Tsc2*^{-/-} MEFs were transfected with plasmids (10 or 16 μg, as indicated) encoding wild-type ("Wt") or mutant (N1645K; "Mut") TSC2, or the empty vector (pcDNA3), as well as a plasmid encoding enhanced green fluorescent protein (EGFP). GFP-positive cells were isolated by FACS and replated in media containing 0.05% serum. Eighteen hours later, immunoblots were performed with the indicated antibodies.

B: Immunoblots of untransfected *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs grown in 0.1% serum performed under the same experimental conditions as in **A**.

that the modest elevation in Hif-1α levels observed in *Tsc2*^{-/-} cells under serum-rich conditions translates into increased expression of at least a subset of Hif target genes. All four of these mRNAs were induced by hypoxia, in keeping with their being regulated by Hif (Figure 5B). As was observed for Hif protein

(Figure 1B), the accumulation of these transcripts following chronic exposure to hypoxia was more sustained in the *Tsc2*^{-/-} cells relative to controls. Hif-1α mRNA levels, in contrast to Hif-1α protein levels, were not altered by hypoxia in either *Tsc2*^{+/+} or *Tsc2*^{-/-} cells (Figure 5B). The failure of *Tsc2*^{-/-} cells to down-regulate Hif-1α protein levels, and its mRNA targets, with prolonged hypoxia must therefore reflect postranscriptional changes in Hif-1α following TSC inactivation. Of note, the control of HIF target genes in MEFs depends entirely on Hif-1α and not its paralog, Hif2-α (Park et al., 2003).

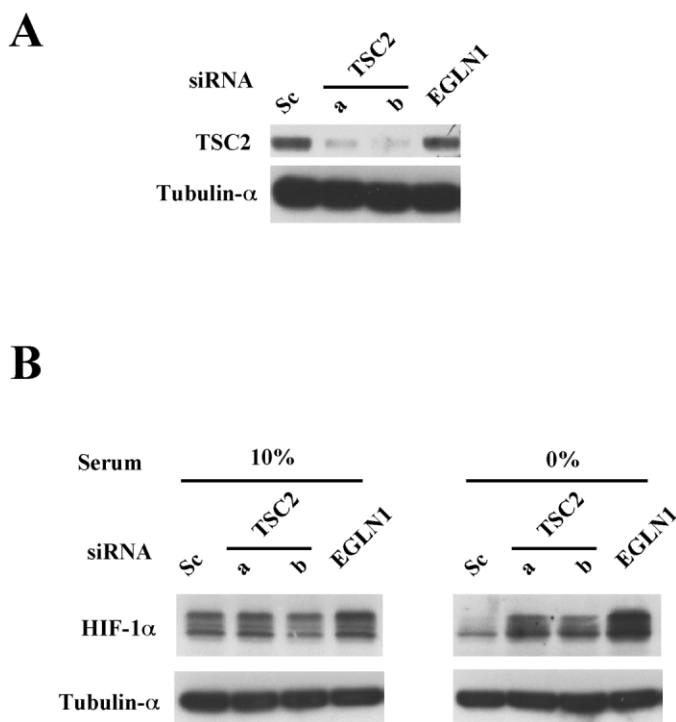


Figure 3. Inhibition of TSC2 is sufficient to enhance HIF-1α accumulation

A: Anti-TSC2 and anti-tubulin immunoblot analysis of U2OS osteosarcoma cells 48 hr after transfection with siRNA directed against EGLN1, against TSC2 (sequence "a" or sequence "b"), or scrambled control ("Sc").

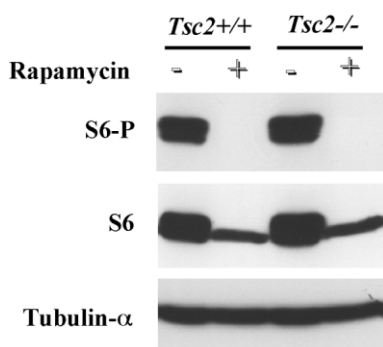
B: Anti-HIF-1α and anti-tubulin immunoblot analysis 6 days after transfection with siRNA used in **A**. Cells were placed in media containing 10% or 0% serum 3 days after transfection as indicated.

VEGF upregulation in *Tsc2*-null cells

VEGF is thought to be important for neoangiogenesis and tumor development (Ferrara, 2002). Moreover, the induction of VEGF mRNA in *Tsc2*-null cells appeared to be disproportionate to the changes seen in other hypoxia-inducible mRNAs (Figure 5A). To assess the significance of VEGF mRNA upregulation, we evaluated VEGF protein levels in the conditioned media of *Tsc2*^{-/-} and *Tsc2*^{+/+} MEFs. Since in serum-rich media the abundance of secreted proteins (such as VEGF) relative to exogenous proteins is very low, cells were grown in synthetic, serum-free media. Conditioned media were concentrated by TCA precipitation, normalized for protein concentration, and immunoblotted with an anti-VEGF antibody. As shown in Figure 5C, the levels of VEGF protein were significantly higher in *Tsc2*-null cells than in controls.

These results were corroborated by performing VEGF ELISAs on conditioned media derived from comparable numbers of *Tsc2*^{-/-} and *Tsc2*^{+/+} cells grown under standard conditions (Figure 5D). To control for any global differences in metabolic rates or rates of cell proliferation, VEGF levels were normalized within each sample to intracellular protein concentration. Intracellular protein levels were typically higher in the *Tsc2*^{-/-} cells compared to controls, but were usually within a factor of 2 (data not shown). Hence, even after normalization, VEGF protein levels in the conditioned media of *Tsc2*^{-/-} cells were markedly elevated compared to controls (see also Figure 6B).

A



B

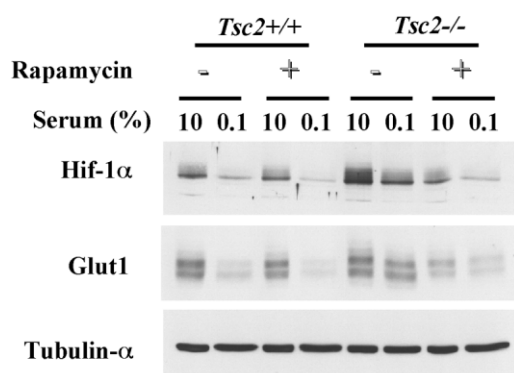


Figure 4. Rapamycin inhibits Hif-1 α accumulation in *Tsc2*^{-/-} cells

A and B: Immunoblot analysis with the indicated antibodies of *Tsc2*^{+/+} and *Tsc2*^{-/-} cells grown in presence ("+") or absence ("—") of 25 nM Rapamycin for 36 hr. In **B**, cells were grown in high (10%) or low (0.1%) serum for 40 hr as indicated.

Rapamycin downregulates VEGF secretion in *Tsc2*-null cells

To evaluate the contribution of mTOR to VEGF upregulation in *Tsc2*-null cells, we repeated these experiments in the presence or absence of Rapamycin. Treatment of *Tsc2*^{-/-} cells with Rapamycin under conditions that blocked S6 phosphorylation (Figure 4A) and normalized Hif-1 α levels (Figure 4B) led to diminished VEGF secretion, albeit to levels that were still markedly elevated compared to *Tsc2*^{+/+} cells (Figure 6A). The effect of Rapamycin was even less pronounced if VEGF levels were normalized for total intracellular protein concentration in an attempt to correct for Rapamycin's known inhibitory effect on global protein translation (Figure 6B). Treatment with Rapamycin using 10-fold higher concentrations did not decrease VEGF levels further (data not shown). Likewise, VEGF continued to be overproduced by *Tsc2*^{-/-} cells that were pretreated with Rapamycin for 24 hr prior to media replacement with fresh, Rapamycin-containing media (data not shown). In contrast, Rapamycin did not affect

Hif-1 α levels (Figure 4B) or VEGF levels (Figure 6B) in *Tsc2*^{+/+} cells. These data indicate that loss of *Tsc2* results in the inappropriate activation of mTOR, mTOR-mediated upregulation of Hif, and the upregulation of VEGF. Importantly, these data also suggest the existence of an mTOR-independent pathway leading to VEGF upregulation in *Tsc2*^{-/-} cells.

Trichostatin A is a potent inhibitor of VEGF

To begin to explore the mTOR-independent pathway, we treated *Tsc2*^{-/-} cells with drugs that inhibit signal transduction pathways known to regulate VEGF (Table 1). Among these were inhibitors of PI3K and Ras pathways, Src, as well as inhibitors of various protein kinases, histone deacetylase, and nitric oxide synthase (NOS) (Table 1). Since many of these drugs can affect cell proliferation, VEGF levels were normalized to intracellular protein concentrations. Of the drugs screened, only Trichostatin A (TSA) consistently downregulated VEGF by >50% (Figure 7). A meaningful analysis of the effects of TSA on *Tsc2*^{+/+} cells was not possible because they produce very low levels of VEGF under basal conditions. Since TSA blocks protein deacetylation, we examined protein acetylation patterns in *Tsc2*^{-/-} and *Tsc2*^{+/+} cell lysates using an antibody directed against acetylated lysine residues. Preliminary results using this approach have not revealed a difference in the global pattern of protein acetylation between these two cell types (data not shown). Finally, the treatment of *Tsc2*^{-/-} cells with both Rapamycin and TSA simultaneously did not have additive effects, compared to treatment with each drug individually (data not shown), suggesting that the pathways regulated by TSA and Rapamycin are not completely independent of each other.

Discussion

Tsc2-null cells have higher levels of Hif-1 α mRNA and protein than *Tsc2*^{+/+} cells. Reintroduction of wild-type TSC2 into *Tsc2*-null MEFs downregulates Hif-1 α . Conversely, downregulation of TSC2 with siRNA upregulates Hif-1 α . These results indicate that TSC2 is an important regulator of HIF. The increase in Hif-1 α levels in *Tsc2*-null cells appears to be functionally important as it is accompanied by increased expression of Hif target genes.

Our results are consistent with a recent study by Liu and coworkers who found that Hif-2 α was elevated in renal carcinoma cell lines derived from tumors arising in the Eker rat (Liu et al., 2003). However, their study did not allow any conclusions regarding the role of *Tsc2* in this process since Hif-2 α was similarly elevated in chemically transformed *Tsc2*^{+/+} rat kidney cell lines used as controls (Liu et al., 2003). One interesting possibility suggested by these authors was that Hif-2 α elevation in the *Tsc2*^{+/+} cell lines reflected mutations at other loci that impinge upon Hif regulation.

Both the *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs used in our studies lacked p53, and both p53 as well as its downstream target MDM2 have been implicated in HIF regulation (Carmeliet et al., 1998; Ravi et al., 2000). Thus, our MEF data might reflect a genetic interaction between *Tsc2* and p53. On the other hand, downregulation of TSC2 in *p53*^{+/+} U2OS osteosarcoma cells also led to the elevation of HIF. Nonetheless, it remains possible that HIF dysregulation following TSC inactivation requires a second "hit" such as p53 loss.

Serum deprivation results in the downregulation of Hif-1 α

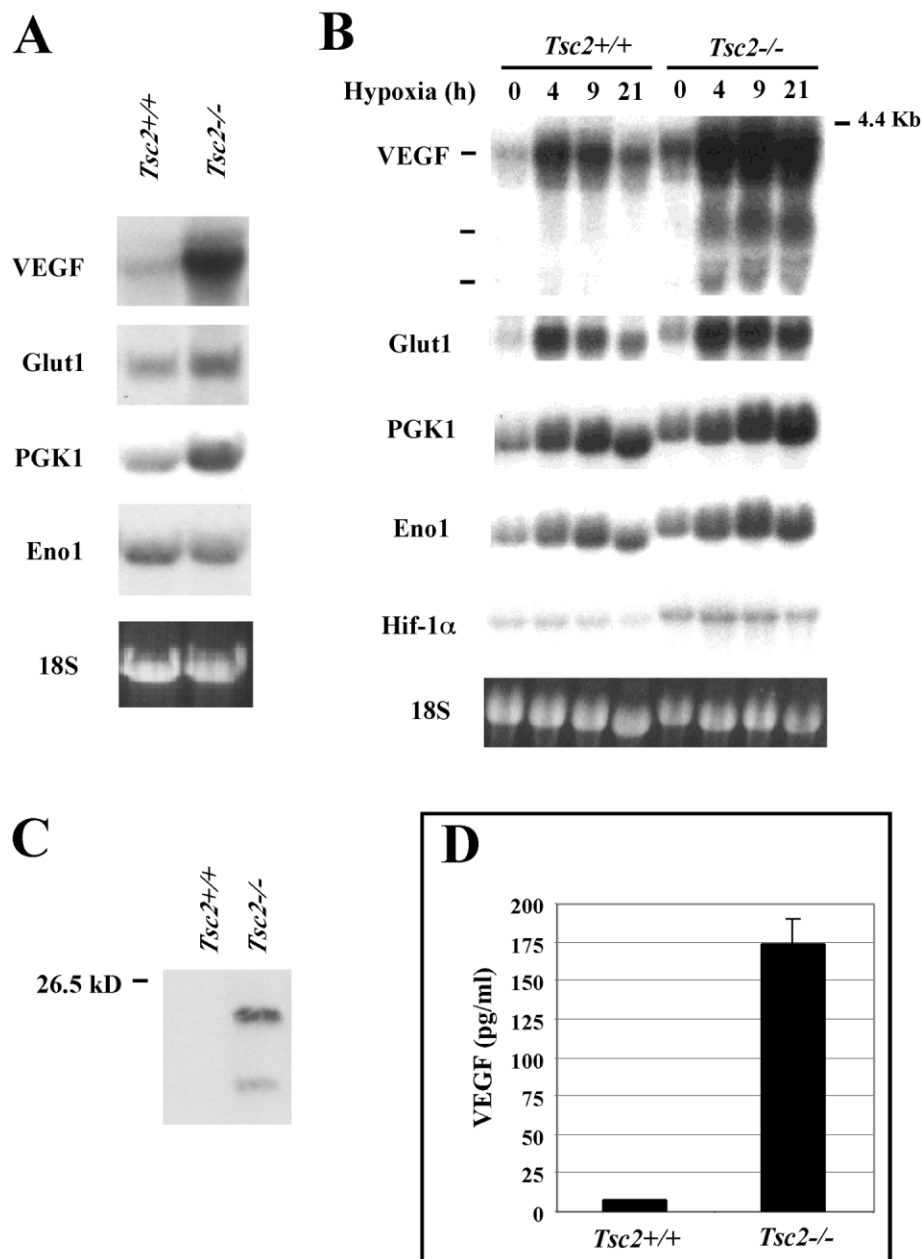


Figure 5. Activation of HIF target genes in *Tsc2*^{-/-} cells

A and **B**: Northern blot analysis of *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs with the indicated probes. In **B**, cells were exposed to 1% oxygen for the duration indicated prior to analysis.

C: Similar numbers of *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs were plated and maintained in synthetic, serum-free media. Thirty-six hours later, conditioned media were immunoblotted for VEGF (after TCA precipitation and normalization for total protein).

D: Similar numbers of *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs were plated and maintained in serum-rich (10% serum) media for 24 hr. VEGF levels were analyzed by ELISA. Error bars equal one standard deviation (*n* = 3).

mRNA and protein levels in *Tsc2*^{+/+} cells but not in *Tsc2*^{-/-} cells. Hence, growth factor deprivation leads to Hif-1α downregulation through a mechanism that requires Tsc2. There is precedence for growth factor signals impinging upon Hif-1α mRNA accumulation (Jiang et al., 1997). Interestingly, the inappropriate elevation of Hif-1α protein in *Tsc2*-null cells can be corrected by Rapamycin. These data are consistent with a model whereby loss of TSC2 results in activation of mTOR, which in turn upregulates HIF-1α. It seems fitting that mTOR, which functions in an insulin-responsive pathway that regulates cell growth, would also activate HIF. In this way, mTOR would couple increased protein translation and cell growth with enhanced glucose uptake, energy generation, and development of an adequate blood supply. Notably, upregulation of HIF by mTOR appears to be a common mechanism in tumorigenesis. Multiple oncogenic

events, including activation of AKT (Treins et al., 2002; Zhong et al., 2000; Zundel et al., 2000), inactivation of PTEN (Jiang et al., 2001), HER2 overexpression (Laughner et al., 2001), and the BCR-ABL (Mayerhofer et al., 2002) also upregulate HIF through mTOR. Based on our findings, it is likely that TSC1, which negatively regulates mTOR in complex with TSC2, also regulates HIF, but this remains to be proven.

The induction of Hif-1α protein and Hif-responsive transcripts by hypoxia is attenuated following prolonged exposure to low oxygen, presumably due to a feedback mechanism. This attenuation, which requires TSC2, appears to be posttranscriptional as it is not accompanied by changes in Hif-1α mRNA levels. Conceivably, mTOR phosphorylates HIF-1α or one of its upstream regulators such as pVHL or EGLN. Interestingly, the primary sequences of both HIF-1α and EGLN1 reveal potential

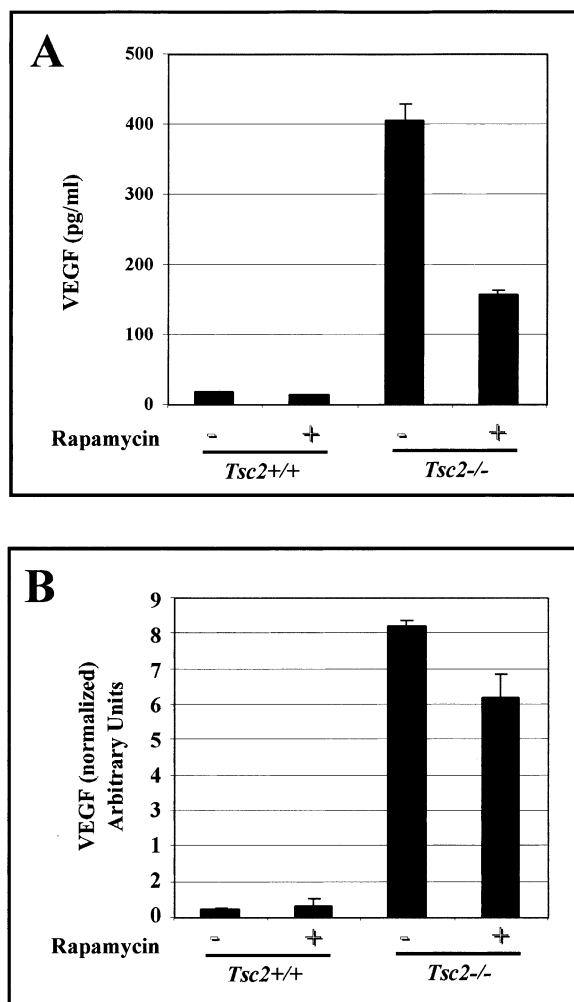


Figure 6. Effects of Rapamycin on VEGF secretion in *Tsc2*-null cells

A and B: Similar numbers of *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs were plated and maintained in serum-rich (10% serum) media in the presence (+) or absence (-) of Rapamycin (25 nM). Thirty-six hours later, conditioned media were analyzed by anti-VEGF ELISA and cell extracts were prepared as in Figure 4A. VEGF values were (B) or were not (A) normalized to total cellular protein. Error bars equal one standard deviation (n = 3).

mTOR phosphorylation sites (Schalm and Blenis, 2002). Moreover, the region of HIF-1 α called the ODD domain (oxygen-dependent degradation domain), which contains the HIF-1 prolyl hydroxylation/pVHL binding site, is also sufficient for HIF-1 α 's Rapamycin sensitivity in the setting of PI3K pathway activation (Hudson et al., 2002). That mTOR signals to HIF-1 α through EGLN1 is supported by our observation that EGLN1 is required for HIF-1 α downregulation by low serum (Figure 3B).

Since a patient-derived TSC2 missense mutant was impaired with respect to downregulation of Hif-1 α , it appears that regulation of HIF by TSC2 is important for its tumor suppressor function. Inappropriate activation of HIF contributes to tumor formation in other settings including tumors, such as renal cell carcinomas and hemangioblastomas, that are linked to inactivation of the *VHL* gene (Maher and Kaelin, 1997). Indeed, HIF dysregulation may underlie the partial phenotypic overlap be-

Table 1. List of drugs used in screen for VEGF inhibitors in *Tsc2*-null MEFs

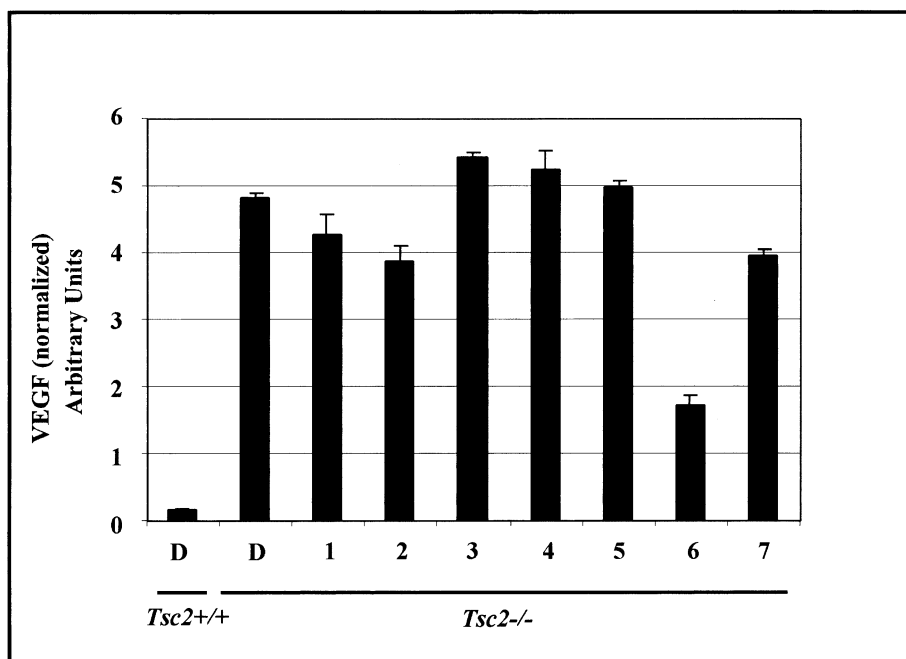
Drug	Target	Conc.	Reference
LY294002	PI3K	20 μ M	(Suzuma et al., 2002)
Wortmannin	PI3K	0.1 μ M	(Suzuma et al., 2002)
PD098059	MEK1	100 μ M	(Pedram et al., 1998), (Milanini et al., 1998)
U0126	MEK1/2	50 μ M	(Stiehl et al., 2002)
SB203580	P38	30 μ M	(Yamamoto et al., 2001)
PD169316	P38	10 μ M	(Yamamoto et al., 2001)
SB202190	P38	0.35 μ M	(Pages et al., 2000)
SP600125	JNK	10 μ M	(Wang et al., 2002)
FTI277	Farnesyl transferase	10 μ M	(Maity et al., 2000)
AG1478	EGFR	10 μ M	(Maity et al., 2000)
H89	PKA	1 μ M	(Ghosh et al., 2001)
Calphostin C	PKC	0.1 μ M	(Friedlander et al., 1995)
Go6983	PKC	0.1 μ M	(Fedorov et al., 2002)
Genistein	SRC	50 μ M	(Suzuma et al., 2002)
Herbimycin A	PTK/SRC	3.4 μ M	(Gruden et al., 1997)
PP2	SRC	50 μ M	(Fredriksson et al., 2000)
H7	S/T Kinase	50 μ M	(Park et al., 2001)
LY83583	Guanylate cyclase	1 μ M	(Chin et al., 1997)
TSA	HDAC	100 ng/ml	(Kim et al., 2001)
L-NAME	NOS	3 mM	(Gavin et al., 2000)

tween TSC and VHL disease. The importance of HIF with respect to VHL-associated tumor development is underscored by the fact that tumor suppression by pVHL can be overridden by HIF variants that do not bind to pVHL (Kondo et al., 2002; Maranchie et al., 2002).

It is noteworthy that kidney cancer is a prominent feature of both VHL disease and the Eker (*Tsc2*^{+/-}) rat. The majority of sporadic clear cell RCC also harbor (somatic) *VHL* mutations and, accordingly, overproduce HIF and its downstream targets (Wiesener et al., 2001). Interestingly, TSC patients are also at an increased risk for clear cell RCC (Walker, 1998), and when tumors arise in this setting, they typically are *VHL*^{+/+} (Parry et al., 2001). One explanation would be that dysregulation of HIF following loss of TSC reduces the selection pressure to eliminate pVHL.

A number of HIF targets have been implicated in tumorigenesis. HIF induces multiple changes in cellular glucose uptake and metabolism that allow for sustained energy generation in a hypoxia environment ("Warburg Effect") (Seagroves et al., 2001). In addition, HIF directs the production of autocrine growth factors (for example, TGF α) that act as mitogens, as well as angiogenic factors (for example, VEGF and PDGF B). The importance of HIF with respect to tumorigenesis is underscored by the finding that genetic disruption of HIF in most (but not all) models tested to date impedes tumor formation in vivo (Pugh and Ratcliffe, 2003).

Upregulation of VEGF following TSC inactivation is striking and seemingly disproportionate to other HIF targets. Moreover, treatment of *Tsc2*^{-/-} cells with Rapamycin at concentrations that are sufficient to inhibit mTOR and normalize Hif-1 α production (as well as the canonical Hif target Glut1) does not completely normalize VEGF. The simplest interpretation of these data is that TSC regulates VEGF through mTOR-dependent and -independent pathways. VEGF regulation is complex and involves both transcriptional and posttranscriptional mechanisms (Robinson and Stringer, 2001). Recent studies are consis-



- D. DMSO**
1. SB203580
2. PD169316
3. SB202190
4. AG1478
5. H7
6. TSA
7. L-NAME

Figure 7. Screen for drugs that downregulate VEGF in *Tsc2*-null fibroblasts (see also Table 1)

Tsc2^{-/-} MEFs were plated and maintained in standard media with 10% serum. Drugs were added to fresh media containing 10% serum for 36 hr prior to analysis of VEGF levels by ELISA. In parallel, *Tsc2*^{+/+} and *Tsc2*^{-/-} cells were treated with DMSO ("D"). Shown are VEGF values normalized to total cellular protein. Error bars equal one standard deviation (n = 3).

tent with the idea that regulation of the VEGF promoter by the PI3K pathway is complex and involves HIF-dependent and -independent pathways (Pore et al., 2003).

Earlier studies indicated that histone deacetylase inhibitors block VEGF overproduction in certain settings (Kim et al., 2001). In keeping with this, we found that VEGF overproduction by *Tsc2*^{-/-} cells is very sensitive to TSA. How TSA affects VEGF production is not yet clear. In one study, downregulation of VEGF with TSA was correlated with increased production of pVHL and p53 (Kim et al., 2001). In any event, our findings suggest that TSC inactivation ultimately impacts upon chromatin structure. Notably, a link between histone acetylation and TSC2 had been previously suggested. TSC2 was found to bind to several nuclear receptors in vitro and to affect their transcriptional transactivation activities in cells (Henry et al., 1998). While TSC2 is thought to be a cytosolic protein (Wienecke et al., 1996), it is possible that a small fraction localizes to the nucleus where it may affect transcription.

Rapamycin has been shown to decrease tumor growth and angiogenesis in vivo (Guba et al., 2002). PTEN loss, which leads to activation of the PI3K pathway and mTOR, sensitizes cells to the antiproliferative effects of Rapamycin in vivo (Neshat et al., 2001; Podsypanina et al., 2001), possibly due to a phenomenon referred to as "oncogene addiction" (Mills et al., 2001; Weinstein, 2002). Based on this observation, one might predict that TSC-

defective cells would likewise be particularly sensitive to Rapamycin. Finally, histone deacetylase inhibitors are also being tested in man and, based on our studies, might be useful adjuncts in this setting.

Experimental procedures

MEF culture

Tsc2^{+/+}; *p53*^{-/-} and *Tsc2*^{-/-}; *p53*^{-/-} MEFs between passages 6 and 8 were obtained from D.J. Kwiatkowski and grown in DMEM supplemented with 10% heat inactivated fetal bovine serum (or lower percentages as indicated) and in a 10% humidified CO₂ incubator. Cells were analyzed up to passage 12. Where indicated, Deferoxamine (DFO) (Sigma) was added to the media from a freshly prepared 100 mM stock solution in sterile water to a final 200 μ M concentration. Where indicated, Rapamycin (Calbiochem) was added to the media from a 25 μ M stock solution in methanol to a final concentration of 25 nM. All other drugs were obtained from Calbiochem and were dissolved in water or DMSO as recommended by the manufacturer.

Protein extraction and immunoblot analysis

Adherent cells were washed with PBS and lysed in 50 mM Tris-HCl (pH 7.4), 250 mM NaCl, and 0.5% NP-40 supplemented with protease inhibitors (Complete Mini, ROCHE) for 30 min at 4°C. Protein concentration was determined by the Bradford Method (Bio-Rad). For each gel, comparable amounts of protein were loaded per lane, electrophoretically resolved, and wet transferred onto a 0.45 μ m Nitrocellulose membrane (Bio-Rad). Membranes were blocked in TBS with 0.1% Tween and 5% nonfat dry milk and probed with the indicated primary antibody (polyclonal HIF-1 α [gift of J. Pouyssegur] [Richard et al., 1999], polyclonal TSC2 [Santa Cruz C-20], polyclonal Phos-

pho-S6 S235/236 [Cell Signaling], polyclonal S6 [Cell Signaling], monoclonal Tubulin- α [Sigma, clone B-5-1-2], or polyclonal hVEGF [R&D AF-493-NA]. Bound antibody was detected with an appropriate HRP-conjugated secondary antibody (Pierce) and chemiluminescence (Pierce).

Transfection and FACS sorting

Tsc2-null MEFs (90% confluent) were transfected with pcDNA3-TSC2, pcDNA3-TSC2 (N1645K) [Inoki et al., 2002], or the backbone vector, along with enhanced green fluorescence protein (EGFP) expression vector, using Lipofectamine Plus (Invitrogen). Eighteen to twenty-four hours later, GFP expression was confirmed under UV light using an ECLIPSE TE 200 microscope (Nikon). Cells were trypsinized, resuspended in a solution containing 66% trypsin in PBS and 15 mM EDTA, and sorted at a rate of 8,000–10,000 events/s using an EPICS ALTRA FACS machine (Beckman Coulter) under sterile conditions. Cells with fluorescence intensity that was 1 log above untransfected controls were collected and aliquoted to 12 well plates (300,000 $\pm$ 5% cells/well). Sorting resulted in an enrichment of EGFP-positive cells from 20%–30% presorted to >95% after sorting (data not shown). After a few hours, cells were washed with PBS and media replaced with DMEM containing 0.05% serum. Eighteen hours later, cell lysates were prepared.

RNAi experiments

U2OS osteosarcoma cells (30%–50% confluence) were grown in 6 well plates. Prior to transfection, cells were washed with PBS and media replaced with 800 μ l of OPTI-MEM I (Invitrogen). In parallel, 4 μ l of Oligofectamine (Invitrogen) was combined with 11 μ l of OPTI-MEM I and incubated at room temperature for 10 min. 1.5 μ g of the indicated duplex siRNA oligonucleotide (Dharmacon) was diluted into 180 μ l of OPTI-MEM I, added to the Oligofectamine/OPTI-MEM I mixture, and incubated at room temperature for 20 min. The siRNA complexes were then added to the cell culture media. After incubation for 3–4 hr in a 5% CO₂ incubator, 0.5 ml of fresh media with 30% serum was added to a final serum concentration of 10%. Forty-eight hours after transfection, cells were either harvested for Western Blot analysis or replated. Twenty-four hours later, cells were switched to media with either 10% or 0% serum and harvested 3 days later for Western blot analysis. Oligonucleotide sequences (sense strand) were as follows: Sc (5'-CAAUGAUAACAAUCCAUAUGA-3'), TSC2 a (5'-AAAGUUCACCUACUGCUGGCA-3'), TSC2 b (5'-CAAUGAGUCACAGUCCUUUGA-3'), EGLN1 (5'-AAAGCUCCUUCUACUGCUGCA-3').

RNA extraction and Northern blot analysis

RNA was extracted from exponentially growing MEFs using a Nucleospin RNA Purification Kit (BD Biosciences) according to manufacturer's recommendations. Twenty to thirty micrograms of total RNA per lane was electrophoretically resolved and transferred to Nylon membrane using a Turbo-blotter apparatus (Schleicher & Schuell) according to manufacturer's recommendations except that transfers took place for 8–10 hr. After UV crosslinking, membranes were hybridized to a dCTP-³²P labeled (Perkin-Elmer) probe generated with a HighPrime (Roche) kit according to manufacturer's recommendations. Probes were purified using a G50 column (Amersham Biosciences), boiled, and added to ExpressHyb solution (BD Biosciences). Membrane prehybridization, hybridization, and washes were carried out per manufacturer's recommendations. Bands were visualized by film autoradiography with an intensifying screen. In some cases, the membranes were stripped by two rinses in 80°C–100°C water and reprobed. The following probes were used: h165VEGF cDNA (EcoR1 fragment), Glut1 (generated by PCR from murine genomic DNA using primers: 5'-TGACAA GACACCCGAGGAGC-3' and 5'-GTCCAGCCCTACAGATTAGC-3'), PGK1 (generated by PCR from murine genomic DNA using primers: 5'-TGG GGTGGATGCTCTCAGC-3' and 5'-TTCTGCAGACTTACAGCTGG-3'), and Enolase1 (generated by PCR from murine genomic DNA using primers: 5'-GAG CAGAGGTTTACCACAACC-3' and 5'-AGGCAGTTGCAGGACTTCTCG-3').

TCA precipitation

Trichloroacetic acid (100% solution) was added to a final concentration of 20% to conditioned media. After a 20 min incubation on ice, the samples were centrifuged at 31,000 g for 20 min (4°C). Pellets were washed with –20°C Acetone, centrifuged at 31,000 g for 30 min (4°C), air dried, and resuspended in protein lysis buffer (described above). Protein concentrations

were estimated using the Bradford Method (Pierce). Immunoblot analysis was performed as described above using 200 μ g protein/lane.

VEGF ELISA

Cells were seeded in 12 well plates (100,000 cells per well). Three independent wells were used for each set of experimental conditions. Once the cells were attached, the media were changed to DMEM with 10% serum and drugs were added as indicated. Twenty-four to thirty-six hours later, VEGF ELISA (R&D Systems) were performed (50 μ l media/well) according to manufacturer's instructions with a Wallac Victor 2 (PerkinElmer). Where stated, VEGF protein levels were divided by the intracellular protein concentrations within each sample for normalization.

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