Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex

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Mammalian target of rapamycin (mTOR) is a central regulator of protein synthesis whose activity is modulated by a variety of signals. Energy depletion and hypoxia result in mTOR inhibition. While energy depletion inhibits mTOR through a process involving the activation of AMP-activated protein kinase (AMPK) by LKB1 and subsequent phosphorylation of TSC2, the mechanism of mTOR inhibition by hypoxia is not known. Here we show that mTOR inhibition by hypoxia requires the TSC1/TSC2 tumor suppressor complex and the hypoxia-inducible gene *REDD1/RTP801*. Disruption of the TSC1/TSC2 complex through loss of TSC1 or TSC2 blocks the effects of hypoxia on mTOR, as measured by changes in the mTOR targets S6K and 4E-BP1, and results in abnormal accumulation of Hypoxia-inducible factor (HIF). In contrast to energy depletion, mTOR inhibition by hypoxia does not require AMPK or LKB1. Down-regulation of mTOR activity by hypoxia requires de novo mRNA synthesis and correlates with increased expression of the hypoxia-inducible *REDD1* gene. Disruption of REDD1 abrogates the hypoxia-induced inhibition of mTOR, and REDD1 overexpression is sufficient to down-regulate S6K phosphorylation in a TSC1/TSC2-dependent manner. Inhibition of mTOR function by hypoxia is likely to be important for tumor suppression as TSC2-deficient cells maintain abnormally high levels of cell proliferation under hypoxia.

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Tuberous sclerosis complex, a disease characterized by benign tumors in multiple tissues, results from mutations in either Tuberous Sclerosis Complex 1 (*Tsc1*) or 2 (*Tsc2*) (Cheadle et al. 2000). Tsc1 (also called hamartin) and Tsc2 (also called tuberin) form a protein complex (van Slegtenhorst et al. 1998) that integrates signals from a variety of sources, including growth factors (Gao and Pan 2001; Potter et al. 2001; Tapon et al. 2001) and energy stores (Inoki et al. 2003b), with the protein translation apparatus. Tsc2 functions as a GTPase-activating protein (GAP) toward the small G protein Rheb, which through a poorly understood mechanism controls mammalian target of rapamycin (mTOR), a central regulator of protein translation (Castro et al. 2003; Garami et al. 2003; Inoki et al. 2003a; Saucedo et al. 2003; Stocker et al. 2003; Tee et al. 2003; Y. Zhang et al. 2003).

Regulation of mTOR by growth factors has been intensively studied. In response to growth factor stimulation, phosphatidylinositol 3-kinase (PI3K) is activated, leading to the generation of phosphatidylinositol-3,4,5-triphosphate and the recruitment of Akt to the plasma membrane where it is activated by phosphorylation (Cantley 2002). Akt is a serine/threonine kinase that phosphorylates many effectors including Tsc2 (Dan et al. 2002; Inoki et al. 2002; Manning et al. 2002; Potter et al. 2002). The mechanism whereby Akt phosphorylation regulates Tsc2 function is controversial, but it is thought ultimately to lead to its inactivation, thereby allowing the accumulation of Rheb-GTP and the activation of

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mTOR. In support of the idea that Tsc1/Tsc2 plays a critical role in mTOR regulation by growth factors, cells deficient for Tsc1 or Tsc2 fail to down-regulate mTOR function in response to growth factor deprivation (Gao et al. 2002; Inoki et al. 2002; Jaeschke et al. 2002; Kwiatkowski et al. 2002; H. Zhang et al. 2003).

Recent studies have also shed light on how Tsc1/Tsc2 regulates mTOR function in response to changes in energy availability. AMP-activated protein kinase (AMPK) is a master regulator of energy metabolism that is activated in response to energy deprivation (Carling 2004). AMPK functions as a serine/threonine kinase and directly phosphorylates Tsc2 (Inoki et al. 2003b). Cells deficient for Tsc1/Tsc2, or producing a TSC2 variant that can not be phosphorylated by AMPK, fail to down-regulate mTOR in situations of energy deprivation (Inoki et al. 2003b). Signaling by energy depletion also involves the Lkb1 tumor suppressor protein, which is inactivated in Peutz-Jeghers syndrome. Lkb1 is a serine/threonine kinase that phosphorylates a variety of substrates, including AMPK (Hawley et al. 2003; Woods et al. 2003; Shaw et al. 2004b). AMPK activation with consequent Tsc1/Tsc2-mediated inhibition of mTOR by energy depletion requires Lkb1 (Corradetti et al. 2004; Shaw et al. 2004b). Thus under conditions that are adverse for growth, such as in the presence of reduced energy stores, mTOR function is inhibited, thereby down-regulating protein synthesis and conserving energy.

mTOR function is also regulated by amino acid availability (Gingras et al. 2001). Whereas mTOR regulation by growth factors and energy stores requires an intact Tsc1/Tsc2 complex, amino acids seem to regulate mTOR function through both Tsc1/Tsc2-dependent and independent pathways (Gao et al. 2002; H. Zhang et al. 2003).

mTOR is a conserved serine/threonine kinase that phosphorylates a series of substrates involved in protein translation including 4E-BP1 and S6K (Gingras et al. 2001). 4E-BP1 binds the translation initiation factor eIF-4E, preventing its interaction with other members of the eIF-4 complex and thereby inhibiting translation initiation of 5′ cap (7-methyl GTP) mRNAs. 4E-BP1 phosphorylation by mTOR, as well as other kinases in the PI3K pathway, relieves this inhibition, thereby promoting mRNA translation (Gingras et al. 2001).

In mammals, there are two *S6K* genes, *S6K1* and *S6K2*, and each has two different splice forms. While S6K2 and the long form (~85 kDa) of S6K1 localize to the nucleus, the short form of S6K1 (~70 kDa) is cytoplasmic. S6K is thought to exist in an inactive, closed conformation, resulting from an intramolecular interaction between the catalytic domain and a pseudosubstrate domain (Fingar and Blenis 2004). There are at least eight phosphorylation sites in S6K1 and its activation requires a complex process of phosphorylation involving mTOR as well as other PI3K effectors. Phosphorylation in the linker region, at Ser 371 and Thr 389, is essential for S6K1 activation (Dufner and Thomas 1999). Thr 389 is the major rapamycin-sensitive site and can be phosphorylated by mTOR in vitro, suggesting that it is a direct mTOR target (Dufner and Thomas 1999). Phosphorylation at this site has also been shown to be regulated in response to changes in Tsc1/Tsc2 levels (Inoki et al. 2002; Jaeschke et al. 2002; Kwiatkowski et al. 2002; Manning et al. 2002; H. Zhang et al. 2003).

The best-characterized substrate of S6K1 is the ribosomal protein S6. S6 is an integral component of the 40S subunit that is required for cell proliferation but whose precise function is unclear (Volarevic et al. 2000). S6 is thought to be regulated primarily through successive phosphorylation events beginning at S236 and S235. Phosphorylation at these sites is rapamycin-sensitive and largely mediated by S6K (Dufner and Thomas 1999). Indeed, cells deficient for both S6K1 and S6K2 have profoundly reduced levels of S6 S235/236 phosphorylation (Pende et al. 2004). Thus, S235/236 phosphorylation provides an accurate readout for endogenous S6K activity.

The Hypoxia-inducible factor (Hif) has also been shown to be regulated by mTOR (Hudson et al. 2002). Hif is a heterodimeric transcription factor composed of a stable β and a labile α subunit whose levels are controlled by oxygen tension (Semenza 2000). In the presence of oxygen, Hif-α subunits are hydroxylated at specific prolyl residues and targeted for degradation by an E3 ubiquitin ligase that contains the von Hippel-Lindau tumor suppressor protein (pVHL) (Kaelin 2002). pVHL inactivation in patients with von Hippel-Lindau disease results in Hif up-regulation and the development of tumors. As in pVHL-deficient cells, Tsc2-deficient cells harbor increased levels of Hif-α relative to wild-type cells, especially under growth factor poor conditions (Brugarolas et al. 2003). Hif up-regulation in Tsc2-deficient cells is likely to result from increased mTOR activity as mTOR increases Hif stability and increased Hif levels in Tsc2-deficient cells can be normalized by treatment with rapamycin (Hudson et al. 2002; Brugarolas et al. 2003).

It was recently shown that mTOR function is regulated by hypoxia (Arsham et al. 2003). Hypoxia down-regulates 4E-BP1 phosphorylation and increases 4E-BP1 binding to eIF-4E at 5′ cap structures. Similarly, hypoxia down-regulates S6K phosphorylation at multiple sites including T389 and inhibits S6 phosphorylation. Hypoxia-induced inhibition of mTOR is dominant over mTOR activating signals from growth factors and nutrients and occurs independently of Hif-1α (Arsham et al. 2003). However, how mTOR function is regulated by hypoxia is not known.

Here we show that mTOR inhibition by hypoxia requires an intact Tsc1/Tsc2 complex. Furthermore, down-regulation of mTOR function by hypoxia requires de novo transcription and the expression of the hypoxia-inducible *Redd1/RTP801* gene.

Results

Tsc1/Tsc2 complex is required for mTOR regulation by hypoxia

To examine the contribution of the Tsc1/Tsc2 complex to the regulation of mTOR function by hypoxia, the ef-

fects of hypoxia on MEFs derived from *Tsc2*-deficient embryos were analyzed. Loss of Tsc2 results in lethality at approximately day 10.5 of gestation when it is difficult to obtain a large number of fibroblasts (Rennebeck et al. 1998). To extend the life span of the few fibroblasts that can be obtained from these embryos, mice were intercrossed with mice carrying mutations in *p53*, which enhances the life span of the limited number of MEFs that can be obtained (H. Zhang et al. 2003). In the experiments that follow, $Tsc2^{-/-};p53^{-/-}$ MEFs were compared with $Tsc2^{+/+};p53^{-/-}$ MEFs. For simplicity, these MEFs are referred to as "$Tsc2^{-/-}$" and "$Tsc2^{+/+}$," respectively.

Tsc1/Tsc2 complex regulates mTOR in response to growth factors. As shown before, serum deprivation inhibits mTOR, as evidenced by decreased phosphorylation of the mTOR effector S6K (T389) (Fig. 1A). S6K T389 is the major rapamycin-sensitive site and is required for S6K activity (Dufner and Thomas 1999). The down-regulation of S6K phosphorylation is associated with a decrease in S6 phosphorylation (S235/236). We also confirmed that mTOR inhibition by serum deprivation requires an intact Tsc1/Tsc2 complex and is abrogated in $Tsc2^{-/-}$ MEFs (Fig. 1A) (Jaeschke et al. 2002; H. Zhang et al. 2003). Rapamycin inhibits S6K phosphorylation and activity regardless of Tsc2 status, supporting the concept that Tsc1/Tsc2 functions upstream of mTOR (Fig. 1A).

$Tsc2^{+/+}$ MEFs down-regulated S6K phosphorylation and activity in response to hypoxia (Fig. 1A), in keeping with an earlier study using transformed human embryonic kidney (HEK293) cells (Arsham et al. 2003). Down-regulation of S6 phosphorylation in response to hypoxia was also demonstrable in mice exposed to 6% oxygen, suggesting that this phenomenon is not restricted to tissue culture (Supplementary Fig. 1). In contrast, hypoxia failed to down-regulate S6K and S6 phosphorylation in $Tsc2^{-/-}$ MEFs (Fig. 1A) and $Tsc1^{-/-}$ mouse 3T3 cells (Fig. 1B), indicating that mTOR inhibition by hypoxia requires an intact Tsc1/Tsc2 complex. Likewise hypoxia promoted the binding of 4E-BP1 to 7-methyl GTP (7mGTP) in $Tsc2^{+/+}$ cells but not $Tsc2^{-/-}$ cells, consistent with Tsc2-dependent inhibition of 4E-BP1 phosphorylation by mTOR in response to hypoxia (Fig. 1C). Taken together, these data indicate that mTOR inhibition by hypoxia requires a functional Tsc1/Tsc2 complex.

We previously showed that Hif-α (hereafter referred to as Hif) levels are regulated in response to growth factors through a mechanism that involves the Tsc1/Tsc2 complex and mTOR (Brugarolas et al. 2003; see also Fig. 1A). As $Tsc2^{-/-}$ MEFs failed to down-regulate mTOR activity in response to hypoxia, we postulated that Hif levels might also be affected. $Tsc2^{-/-}$ MEFs were not impaired in the up-regulation of Hif in response to hypoxia. However, whereas $Tsc2^{+/+}$ MEFs down-regulated Hif with prolonged hypoxia, Hif levels remained elevated in $Tsc2^{-/-}$ MEFs (Fig. 1D). Consistent with the idea that increased Hif levels in $Tsc2^{-/-}$ cells under prolonged hypoxia result from increased mTOR activity, treatment

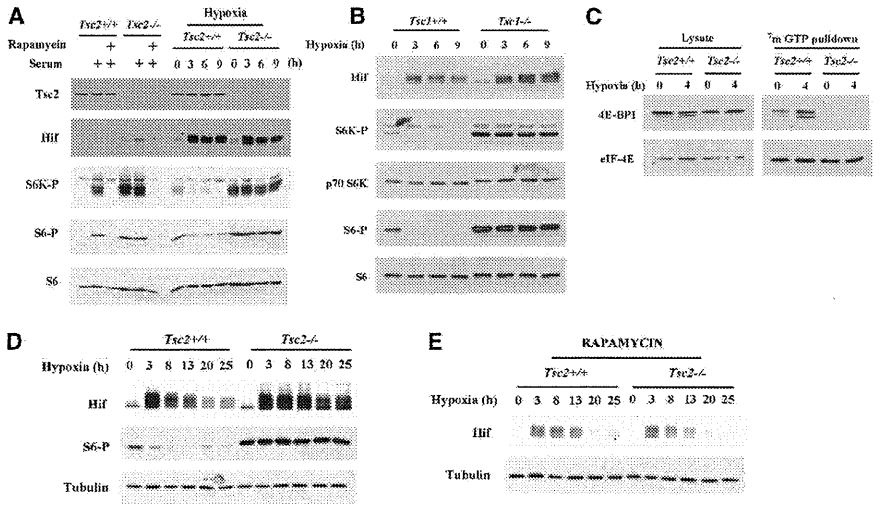

Figure 1. Tsc2 regulates mTOR in response to hypoxia. (*A*) Western blot analysis of $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEFs. (Hif) Hif-1α and/or Hif-2α; (S6K-P) S6K phosphorylated T389; (S6-P) S6 phosphorylated on S235/236. *Left* panel shows MEF in 0.05% serum or following serum addition (10% serum for 45 min) pretreated or not with rapamycin (1.5 h prior to serum addition). *Right* panel shows MEFs exposed to hypoxia for the indicated periods of time. (*B*) Western blot analysis of $Tsc1^{+/+}$ and $Tsc1^{-/-}$ mouse 3T3 cells treated with hypoxia for the indicated periods of time. (*C*) Western blot analysis of input (*left*) and ^{7}mGTP-bound (*right*) proteins from extracts of $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEFs exposed to hypoxia for the indicated periods of time. (*D*,*E*) Western blot analysis of extracts from $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEFs exposed to hypoxia for the indicated periods of time. In *E* all the cells were treated with rapamycin for 26 h prior to lysis regardless of the duration of hypoxia.

with rapamycin restored the down-regulation of Hif normally observed after prolonged hypoxia (Fig. 1E).

The importance of Tsc2 in the regulation of mTOR by hypoxia is further supported by reconstitution experiments. *Tsc2*$^{-/-}$ MEFs were infected with a retrovirus encoding epitope-tagged human Tsc2. Retrovirally transduced *Tsc2*$^{-/-}$ MEFs achieved Tsc2 protein levels that were similar to endogenous levels in *Tsc2*$^{+/+}$ MEFs (Fig. 2A). Reintroduction of Tsc2 into *Tsc2*$^{-/-}$ MEFs restored the down-regulation of S6 phosphorylation by hypoxia (Fig. 2A).

To determine the generalizability of our observations, the role of Tsc2 in mTOR regulation by hypoxia was examined in other cell types. Tsc2 knockdown with two different Tsc2 siRNAs in HEK293 and HeLa cervical carcinoma cells blocked the down-regulation of S6 phosphorylation by hypoxia (Fig. 2B; Supplementary Fig. 2), indicating that Tsc2 is required for mTOR regulation by hypoxia in multiple cell types.

Tsc2 inactivation confers a proliferative advantage under hypoxia

Taken together, these data establish the importance of Tsc1/Tsc2 in the regulation of mTOR by hypoxia. As Tsc1/Tsc2 functions as a tumor suppressor, we asked whether disruption of this complex would affect cell proliferation under hypoxic conditions. In keeping with previous observations, the rates of cell proliferation of

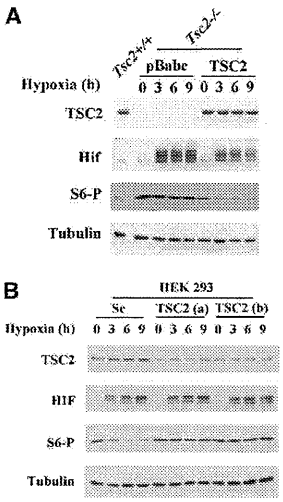

Figure 2. Tsc2 is both necessary and sufficient for the regulation of S6 phosphorylation by hypoxia. (*A*) Western blot analysis of *Tsc2*$^{-/-}$ MEFs retrovirally transduced with either a Tsc2 expression vector or an empty vector and treated with hypoxia for the indicated periods of time. *Tsc2*$^{+/+}$ MEFs are included as controls. (*B*) Western blot analysis of HEK293 cells transfected with two different synthetic Tsc2 siRNAs (a and b) or a scrambled siRNA (Sc) and exposed to hypoxia for the indicated periods of time.

Tsc2$^{-/-}$ and *Tsc2*$^{+/+}$ MEFs were very similar under normoxic conditions for several days (Fig. 3A; H. Zhang et al. 2003). After 4 d in culture the rate of proliferation of *Tsc2*$^{+/+}$ cells began to decline relative to *Tsc2*$^{-/-}$ cells, possibly due to depletion of nutrients and growth factors from the media. Consistent with this idea, *Tsc2*$^{-/-}$ but not *Tsc2*$^{+/+}$ MEFs proliferate under conditions of serum deprivation (H. Zhang et al. 2003). We next measured the proliferation of *Tsc2*$^{-/-}$ and *Tsc2*$^{+/+}$ cells under hypoxic conditions. To avoid confounding effects from media depletion, the media was changed daily. Under hypoxic conditions *Tsc2*$^{-/-}$ MEFs exhibited a marked proliferative advantage compared with *Tsc2*$^{+/+}$ MEFs (Fig. 3B). Increased proliferation of *Tsc2*$^{-/-}$ MEFs under hypoxic conditions correlated with persistently elevated levels of S6 phosphorylation (Fig. 3C) and was abrogated by treatment with rapamycin (Fig. 3B). These data suggest that failure to down-regulate mTOR in response to hypoxia in *Tsc2*$^{-/-}$ MEFs confers a growth advantage that might contribute to tumor formation in TSC patients. It should be noted that the growth inhibitory effect of hypoxia on *Tsc2*$^{+/+}$ MEFs in these assays is p53 independent since both the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs used here lack p53.

Tsc1/Tsc2 regulation by hypoxia is AMPK and Lkb1 independent

Energy depletion results in Tsc1/Tsc2-mediated mTOR inhibition through a mechanism that involves Lkb1 and AMPK (Inoki et al. 2003b; Corradetti et al. 2004; Shaw et al. 2004a). Since hypoxia might also affect cellular energy stores, we sought to determine whether hypoxia regulated mTOR through a similar pathway. AICAR (5-aminoomidazole-4-carboxyamide), a cell-permeable AMPK agonist, inhibited mTOR and down-regulated S6 phosphorylation (Fig. 4A; Kimura et al. 2003). As previously reported (Shaw et al. 2004b), *Tsc2*$^{-/-}$ cells failed to down-regulate S6 phosphorylation in response to AICAR despite robust activation of AMPK as determined by AMPK phosphorylation in the T loop and the phosphorylation of its substrate acetyl-CoA carboxylase (ACC) (Fig. 4A), thereby justifying the use of AICAR as an AMPK perturbant in the experiments described below.

To ask whether hypoxia signals are transduced through an energy depletion pathway in our system, we first examined the effects of hypoxia on AMPK activity. After 4 h of hypoxia, S6 phosphorylation was markedly down-regulated without a detectable increase in AMPK activity, as measured by ACC phosphorylation (Fig. 4B). The ability to detect ACC phosphorylation in response to AMPK activation was confirmed by studying cells treated with AICAR in parallel (Fig. 4B). Furthermore, in vitro kinase assays with AMPK immunoprecipitates from the same lysates used for Figure 4B indicated that hypoxia, in contrast to AICAR, did not increase and may have decreased AMPK kinase activity (Fig. 4C). These results are consistent with a recent study of HEK293 cells exposed to hypoxia for 30 min (Arsham et al. 2003).

We next asked whether AMPK activity is necessary for

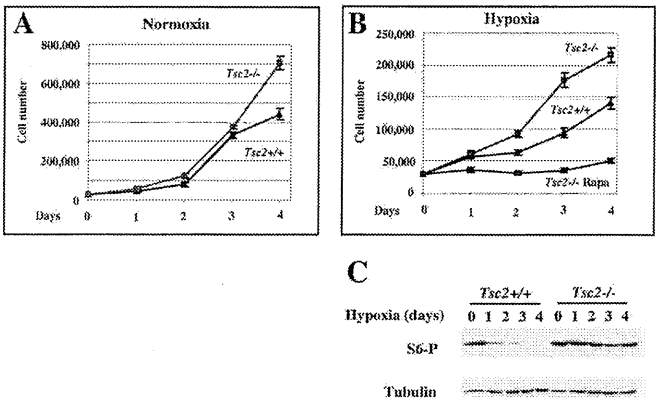

Figure 3. Tsc2 loss confers a proliferative advantage under hypoxic conditions. (*A*) Proliferation rates of $Tsc2^{-/-}$ and $Tsc2^{+/+}$ MEFs under normoxic conditions. (*B*) Proliferation rates under hypoxic conditions of $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEFs (treated or not with rapamycin). Error bars for *A* and *B* equal one standard deviation ($n = 3$). Note different *Y*-axis scales in *A* and *B*. (*C*) Western blot analysis of $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEFs cultured in parallel under hypoxic conditions for the indicated number of days.

the hypoxia-induced down-regulation of mTOR function. Pharmacological inhibition of AMPK with compound C (Inoki et al. 2003b) at doses sufficient to block the down-regulation of S6 phosphorylation by AICAR did not block the down-regulation of S6 phosphorylation by hypoxia (Fig. 4D). Thus AMPK is required for AICAR signaling but not for hypoxia signaling.

Next we examined the requirements for the AMPK kinase Lkb1. Consistent with previous studies, $Lkb1^{-/-}$ MEFs failed to down-regulate S6 phosphorylation in response to AICAR (Fig. 4E; Shaw et al. 2004b). In contrast, we found that Lkb1 loss did not affect the down-regulation of S6 phosphorylation by hypoxia (Fig. 4F). In addition, HeLa cells, which are defective for Lkb1 (Tiainen et al. 1999), also down-regulated S6 phosphorylation in response to hypoxia but not to AICAR (Supplementary Fig. 3) indicating that Lkb1, like AMPK, is dispensable for hypoxia signaling. We noted, however, that ACC and AMPK do become phosphorylated in HeLa cells after prolonged hypoxia, possibly due to a non-Lkb1 AMPK kinase (Supplementary Fig. 3). Nonetheless, inhibition of S6 phosphorylation occurred prior to appreciable AMPK activation in these cells, again in keeping with the idea that the acute down-regulation of mTOR in response to hypoxia is AMPK independent. It is possible that signaling to Tsc1/Tsc2 in response to chronic hypoxia involves both Lkb1/AMPK-dependent and independent pathways since prolonged hypoxia would predictably lead to ATP depletion and accumulation of AMP.

Regulation of mTOR by hypoxia requires Redd1 induction

To begin to unravel the signaling pathway whereby hypoxia regulates Tsc1/Tsc2, we asked whether this mechanism required de novo transcription. Provocatively, inhibition of transcription with actinomycin D blocked the down-regulation of S6 phosphorylation by hypoxia, indicating that de novo transcription is necessary for hypoxia signaling (Fig. 5A). As a control actinomycin D alone did not affect baseline S6 phosphorylation (Fig. 5A).

Recently, the *Drosophila* ortholog of the hypoxia-inducible *Redd1* gene was isolated in a screen for suppressors of insulin signaling (Reiling and Hafen 2004). *Redd1* was originally identified by several groups as a gene that is induced at the mRNA level in response to stresses such as hypoxia or DNA damage (Ellisen et al. 2002; Shoshani et al. 2002). *Redd1* encodes a protein with a predicted MW of 25 kDa that lacks any known functional domains (Ellisen et al. 2002; Shoshani et al. 2002). Redd1 belongs to a family of highly conserved proteins that includes Redd2, which might also be regulated by hypoxia (Cuaz-Perolin et al. 2004). Redd orthologs have been shown to inhibit insulin signaling in *Drosophila* and epistasis analysis suggests that they act upstream of Tsc1/Tsc2 (Reiling and Hafen 2004).

To ascertain whether Redd1 was involved in the regulation of the mTOR by hypoxia, we first examined Redd1 induction in response to hypoxia in MEFs. Consistent with previous data, *Redd1* mRNA levels were induced by hypoxia (Fig. 5B; Shoshani et al. 2002). *Redd2* expression, however, was not detected in MEFs (data not shown).

To ask whether Redd1 is necessary for hypoxia-induced down-regulation of mTOR function, we obtained $Redd1^{-/-}$ MEFs (K. Lei and L.W. Ellisen, in prep.). In contrast to $Redd1^{+/+}$ MEFs, $Redd1^{-/-}$ MEFs failed to down-regulate phosphorylation of S6K and S6 in response to hypoxia (Fig. 5C). These data indicate that Redd1, like Tsc1 and Tsc2, is necessary for the hypoxia-induced down-regulation of mTOR signaling. This requirement was specific because $Redd1^{-/-}$ MEFs, in contrast to $Tsc2^{-/-}$ MEFs, down-regulated S6 phosphorylation in response to serum deprivation (Fig. 6A,B). These data indicate that Redd1 regulates mTOR signaling only in response to certain stimuli, such as hypoxia.

Redd1 is sufficient to down-regulate S6K1 phosphorylation

We next asked whether Redd1 is sufficient to down-regulate mTOR function. For this purpose, the effects of Redd1 expression on S6K phosphorylation were determined. HEK293 cells were transfected with HA-tagged

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