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ORIGINAL ARTICLE

Rheb activates AMPK and reduces p27Kip1 levels in Tsc2-null cells via mTORC1-independent mechanisms: implications for cell proliferation and tumorigenesis

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Tuberous sclerosis complex (TSC) is an autosomally inherited disorder that causes tumors to form in many organs. It is frequently caused by inactivating mutations in the *TSC2* tumor-suppressor gene. TSC2 negatively regulates the activity of the GTPase Rheb and thereby inhibits mammalian target of rapamycin complex 1 (mTORC1) signaling. Activation of mTORC1 as a result of lack of TSC2 function is observed in TSC and sporadic lymphangioleiomyomatosis (LAM). TSC2 deficiency has recently been associated with elevated AMP-activated protein kinase (AMPK) activity, which in turn correlated with cytoplasmic localization of p27Kip1 (p27), a negative regulator of cyclin-dependent kinase 2 (Cdk2). How AMPK in the absence of TSC2 is stimulated is not fully understood. In this study, we demonstrate that Rheb activates AMPK and reduces p27 levels in Tsc2-null cells. Importantly, both effects occur largely independent of mTORC1. Furthermore, increased p27 levels following Rheb depletion correlated with reduced Cdk2 activity and cell proliferation *in vitro*, and with inhibition of tumor formation by Tsc2-null cells *in vivo*. Taken together, our data suggest that Rheb controls proliferation of TSC2-deficient cells by a mechanism that involves regulation of AMPK and p27, and that Rheb is a potential target for TSC/LAM therapy.
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Introduction

Tuberous sclerosis complex (TSC) is an autosomally inherited disorder with benign tumor formation in the brain, kidney, heart, lung, skin and eye (Cheadle *et al*., 2000; Dabora *et al*., 2001). The most frequent manifestation of TSC in the lung is known as lymphangioleiomyomatosis (LAM), a disease that also occurs sporadically (Carsillo *et al*., 2000).

TSC and LAM result from the inactivation of either of the two tumor-suppressor genes, *TSC1* that encodes hamartin (TSC1), and *TSC2* that encodes tuberin (TSC2) (van Slegtenhorst *et al*., 1997; Carsillo *et al*., 2000; Sato *et al*., 2002). TSC1 and TSC2 form an active complex (TSC1/2) that negatively regulates mammalian target of rapamycin complex 1 (mTORC1) signaling, which promotes cell growth (Huang and Manning, 2008). Along with others, we showed that the small GTPase Rheb is the molecular link between TSC1/2 and mTORC1. Rheb activates mTORC1, and TSC2 inhibits Rheb by acting as a GTPase-activating protein (Castro *et al*., 2003; Garami *et al*., 2003; Tee *et al*., 2003; Inoki *et al*., 2003a; Zhang *et al*., 2003b).

The loss of TSC1/2 activity in TSC and LAM results in aberrant mTORC1 activation, thereby contributing to tumor growth (El-Hashemite *et al*., 2003; Choo and Blenis, 2006; Pollizzi *et al*., 2009). Thus, inhibition of mTORC1 with rapamycin may reduce the size of TSC tumors (Easton and Houghton, 2006; Paul and Thiele, 2008). However, a clinical trial with rapamycin suggested that additional targets and therapeutic interventions are needed to efficiently treat TSC and LAM (Paul and Thiele, 2008).

Elevated AMP-activated protein kinase (AMPK) activity has recently been reported in tumors and tumor-derived cells deficient in Tsc2 (Short *et al*., 2008). AMPK is a Ser/Thr kinase activated by changes in the AMP to ATP ratio that occur during metabolic stress. Its primary role is to promote catabolic processes while inhibiting anabolic pathways, thus restoring energy levels. At the molecular level, AMPK is activated by binding to AMP and phosphorylation by LKB1 (Viollet *et al*., 2009; Zhang *et al*., 2009). A consequence

of AMPK activation is the inhibition of cell growth. AMPK phosphorylates TSC2 and the mTOR partner Raptor to inhibit mTORC1 signaling (Inoki *et al*., 2003b; Gwinn *et al*., 2008). However, AMPK and mTORC1 were both activated in Tsc2-null cells (Hahn-Windgassen *et al*., 2005), indicating that AMPK phosphorylation of Raptor is not sufficient to inhibit mTORC1 signaling and cell growth when Tsc2 is absent.

AMPK also phosphorylates the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 (p27) (Liang *et al*., 2007; Short *et al*., 2008). Nuclear p27 inhibits cell cycle progression, whereas cytoplasmic p27 has been suggested to promote cell survival and motility (Assoian, 2004; Wu *et al*., 2006). Regulation of p27 localization is complex and may occur at multiple levels. For example, TSC2 increases p27 stability and nuclear localization by a mechanism that is independent of its effect on mTORC1 (Rosner *et al*., 2006, 2007). Conversely, Akt has been shown to phosphorylate p27 at both T157 and T198, and phosphorylation on these residues has been associated with reduced nuclear localization of p27 (Fujita *et al*., 2002; Vervoorts and Luscher, 2008). However, in TSC2-null cells, Akt activity is repressed by an inhibitory feedback loop by mTORC1-induced activation of p70S6K (Manning, 2004), indicating that Akt does not have a critical role in the regulation of p27 in the absence of TSC2. Instead, elevated AMPK activity controls p27 stability and subcellular localization in Tsc2-null cells, which affects Cdk2 activity and cell survival (Short *et al*., 2008, 2010). These observations suggest that AMPK may contribute to tumor progression in TSC, and underscores the importance of understanding how AMPK is activated in TSC cells.

As Rheb is constitutively activated in Tsc2-null cells (Garami *et al*., 2003; Finlay *et al*., 2007), we sought to determine whether it regulates AMPK activity. Here, we show that Rheb but not mTORC1 activates AMPK. We also demonstrate that Rheb regulates p27 and the proliferation of TSC cells, including those derived from lung specimens of patients with LAM. Our results illustrate the importance of Rheb as a potential therapeutic target for TSC and LAM.

Results

Rheb activates AMPK

We explored whether Rheb regulates AMPK in Tsc2-null ELT3 cells, a tumor-derived rat cell line from the Eker model (Howe *et al*., 1995). As Rheb is constitutively activated in Tsc2-deficient cells (Garami *et al*., 2003; Finlay *et al*., 2007), we depleted Rheb using RNA interference to investigate its role in regulating AMPK activity. Rheb small interfering RNA (siRNA) decreased Rheb mRNA and protein levels (Figure 1a). Rheb depletion inhibited mTORC1 activity, as evidenced by reduced phosphorylation of p70S6K on T389, a residue directly related with p70S6K activation by mTORC1, and by diminished phosphorylation of S6, a p70S6K substrate (Inoki and Guan, 2009) (Figure 1a). We found that depletion of Rheb under serum-free conditions inhibited AMPK activity, as indicated by a reduction of the levels of both phospho-AMPK (p-T172), and phospho-acetyl-CoA carboxylase (p-ACC), an AMPK downstream target (Zhang *et al*., 2009) (Figure 1b). As inhibition of mTORC1 by depletion of Rheb may eliminate negative feedback signaling to Akt and because it was shown that Akt can inhibit AMPK (Hahn-Windgassen *et al*., 2005; Short *et al*., 2008), we analyzed whether Rheb depletion can affect Akt activity, measured by the phosphorylation levels at both S473 and T308. Phosphorylation of Akt was barely detected, and Rheb depletion only modestly affected Akt phosphorylation at T308. Therefore, it is unlikely that Akt has a major role in the regulation of AMPK by Rheb (Figure 1b). To confirm this finding, we investigated the effect of Rheb depletion on AMPK activity in serum-starved *Tsc2*−/− MEFs. Similarly to the ELT3 cells, decreased Rheb protein levels correlated with inhibition of AMPK activity and reduction of the p-ACC levels in the absence of any effect on Akt activity (Figure 1c). These results highlight the role of Rheb in the activation of AMPK in Tsc2-null cells and indicate that Rheb is necessary for AMPK activation in Tsc2-null cells under conditions of cellular stress caused by serum deprivation.

To examine whether mTORC1 was involved in the regulation of AMPK activity, we used the mTORC1 inhibitor rapamycin. As long exposure to rapamycin could affect mTORC2 activity (Sarbassov *et al*., 2006), and eliminate negative feedback signaling to Akt (Manning, 2004), we inhibited mTORC1 for a short time in the absence of serum. Under this experimental condition, we found that while rapamycin was effective in inhibiting phosphorylation of S6, it failed to affect AMPK activity (Figure 1d). Together, these data suggest that Rheb may affect AMPK function in an mTORC1-independent manner.

To confirm the effect of Rheb on AMPK obtained by Rheb depletion, we studied Rheb's contribution to AMPK activation by overexpression of Rheb in *Tsc2*+/+ MEFs. Rheb overexpression increased the phosphorylation levels of AMPK, an effect that was blocked by the AMPK inhibitor *compound C* (Figure 2a). To corroborate that Rheb activates AMPK independently of mTORC1, we treated vector and Rheb transfected *Tsc2*+/+ MEFs with rapamycin. Rapamycin was unable to inhibit the effect of Rheb overexpression on AMPK activation as well as the phosphorylation of the AMPK downstream targets acetyl-CoA carboxylase (ACC) and Raptor (Figure 2b). As control, rapamycin effectively blocked Rheb-induced phosphorylation of S6 (Figure 2b). However, rapamycin was unable to inhibit Rheb-induced phosphorylation of the mTORC1 downstream target 4EBP1. This was consistent with recent findings indicating that 4EBP1 phosphorylation may become rapamycin resistant in some cell types and under certain experimental conditions (Choo *et al*., 2008; Thoreen *et al*., 2009). Therefore, to confirm that Rheb can regulate AMPK activity independently of

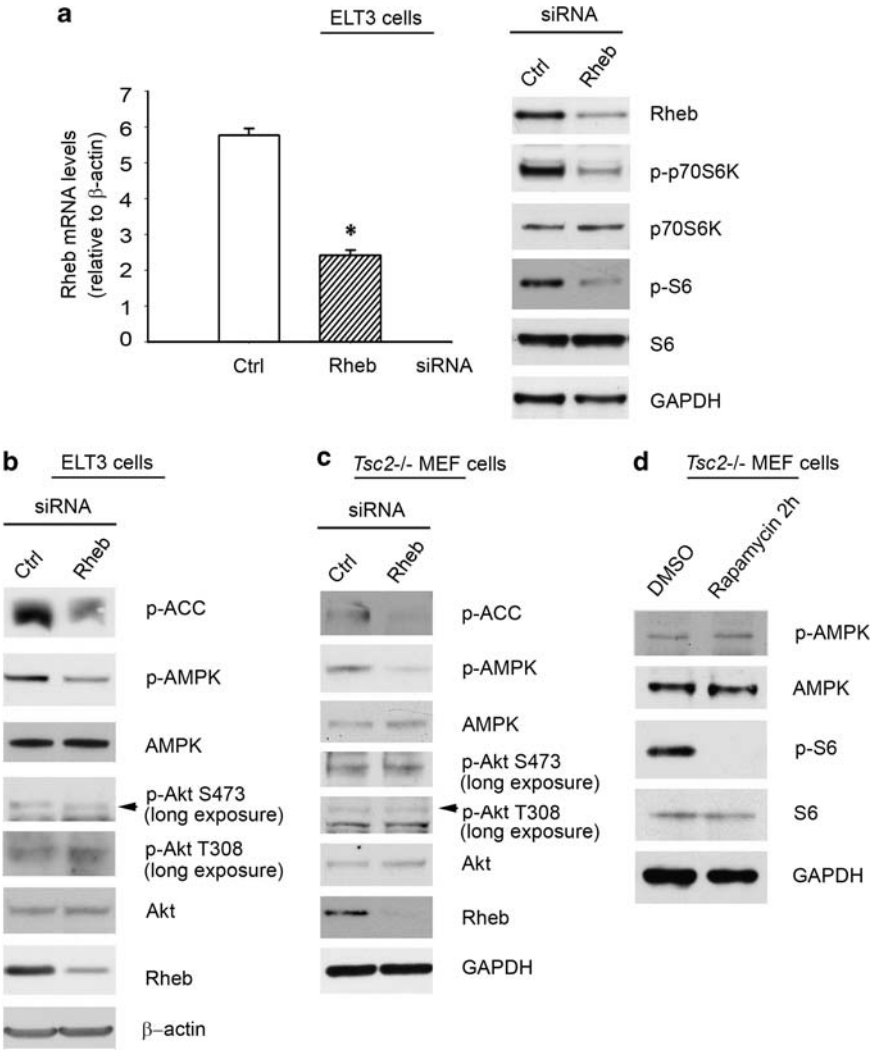


Figure 1 Rheb regulates mTORC1 and AMPK1 activities in Tsc2-null cells. (**a**) Left: Real-time PCR quantification of Rheb mRNA levels from serum-starved ELT3 cells transfected with 50 nM of control (Ctrl) or Rheb siRNA#1. Results are expressed as levels of Rheb mRNA relative to β-actin mRNA levels. Data are the mean ± standard error of the mean (s.e.m.) ($n=3$). *Student's *t*-test ($P<0.05$). Right: The effect of 50 nM of control or Rheb #1 siRNA on expression of endogenous Rheb and of the indicated proteins in serum-starved ELT3 cells was determined by immunoblotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (**b–d**) ELT3 cells (**b**) or *Tsc2*−/− MEF cells (**c**, **d**) were transfected as in (**a**). Lysates from serum-starved cells were characterized by immunoblotting for expression of the indicated proteins. Transfected cells were additionally incubated with the mTORC1 inhibitor rapamycin (20 nM) for 2 h (**d**).

mTORC1, we used Torin1, an ATP-competitive mTOR inhibitor, which effectively inhibits mTORC1 activity (Thoreen *et al*., 2009). As expected, Torin1 inhibited the Rheb-mTORC1-mediated phosphorylation of S6 and 4EBP1, but, unexpectedly, also increased the basal AMPK and ACC phosphorylation levels (Figure 2c). However, it failed to block the Rheb-induced AMPK activation (Figure 2c). Similarly, knockdown of the mTORC1 component Raptor was also unable to block Rheb-induced activation of AMPK (Figure 2d). Together, these results confirmed that Rheb can regulate AMPK activity independently of mTORC1.

Rheb reduces p27 levels

Activated AMPK reduces the nuclear levels of p27 in Tsc2-null cells (Short *et al*., 2008). As Rheb is responsible for AMPK activation in Tsc2-null cells (Figure 1), it may also negatively regulate p27. We confirmed by confocal microscopy that, compared with *Tsc2*+/+ MEFs, *Tsc2*−/− MEFs failed to increase nuclear p27 levels following removal of serum (Figure 3a). We then tested whether Rheb affects nuclear p27 levels in *Tsc2*−/− MEF cells transfected with control or Rheb siRNA. By confocal microscopy, we found that the nuclear levels of p27 were higher in cells treated with Rheb siRNA than in cells treated with control siRNA (Figure 3b). Quantification of the nuclear fluorescence intensity of p27 from another experiment (Supplementary Figure S1) performed as in Figure 3b is shown in Figure 3c. Cell fractionation experiments yielded results consistent with the observations made with confocal microscopy (Figure 3d). Rheb

depletion induced an evident increase of p27 in both cytoplasmic and nuclear fractions. The increase of p27 in both compartments resembles the one resulting from cell cycle arrest on serum removal in NIH3T3 (*Tsc2*+/+) cells (Rosner *et al*., 2007; Short *et al*., 2008). Similar results were also observed on overexpression of TSC2 or phosphatidylinositol 3 kinase/Akt inhibition (Rosner *et al*., 2007; Kim *et al*., 2009). Consistent with the lack of regulation of AMPK activity by mTORC1, we observed that treatment with rapamycin for 2 and 8 h did not alter the nuclear levels of p27 in *Tsc2*−/− MEFs (Figures 3d and e). However, treatment with rapamycin for 8 h increased the cytoplasmic levels of p27, although to a smaller extent than Rheb knockdown (Figure 3e). This effect is likely a consequence of activated Akt by rapamycin-induced increased phosphorylation at S473 (Figure 3e), although phosphorylation levels at T308 were not affected (data not shown). In contrast to *Tsc2*+/+ MEF cells (Figure 2b), phospho-4EBP1 levels were reduced in the *Tsc2*−/− MEFs at both timepoints of rapamycin treatment (Figures 3d and e). In agreement with the results obtained with rapamycin and supporting an mTORC1-independent regulation of p27 by Rheb, treatment with the mTOR inhibitor Torin1 did not antagonized the upregulation of the cytoplasmic and nuclear p27 levels following Rheb knockdown (Figure 3f).

Rheb regulates AMPK and p27 in a subset of human colon cancer cell lines

Mutations that constitutively activate KRAS/extracellular signal-regulated kinase (ERK) signaling, as those found in several cancer types including colon cancer, predict inactivation of TSC2 (Shaw and Cantley, 2006; Ma *et al*., 2007) and thus increased Rheb activity. To investigate whether the effect of Rheb on AMPK and p27 described above extends to cancer cells, we included several human colon cancer cell lines in our study. By using an siRNA sequence different from the studies in the Tsc2-null cells, we found that knockdown of human Rheb in SW620 cancer cells inhibited AMPK phosphorylation, and correlated with upregulation of both cytoplasmic and nuclear p27 (Figure 4a). However,

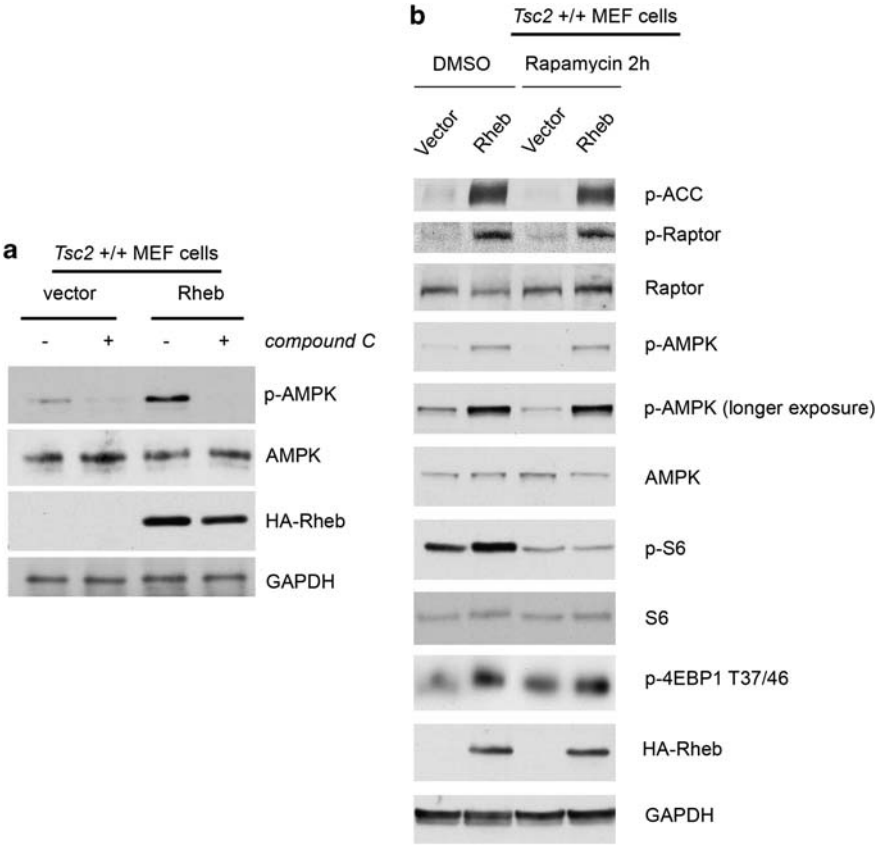
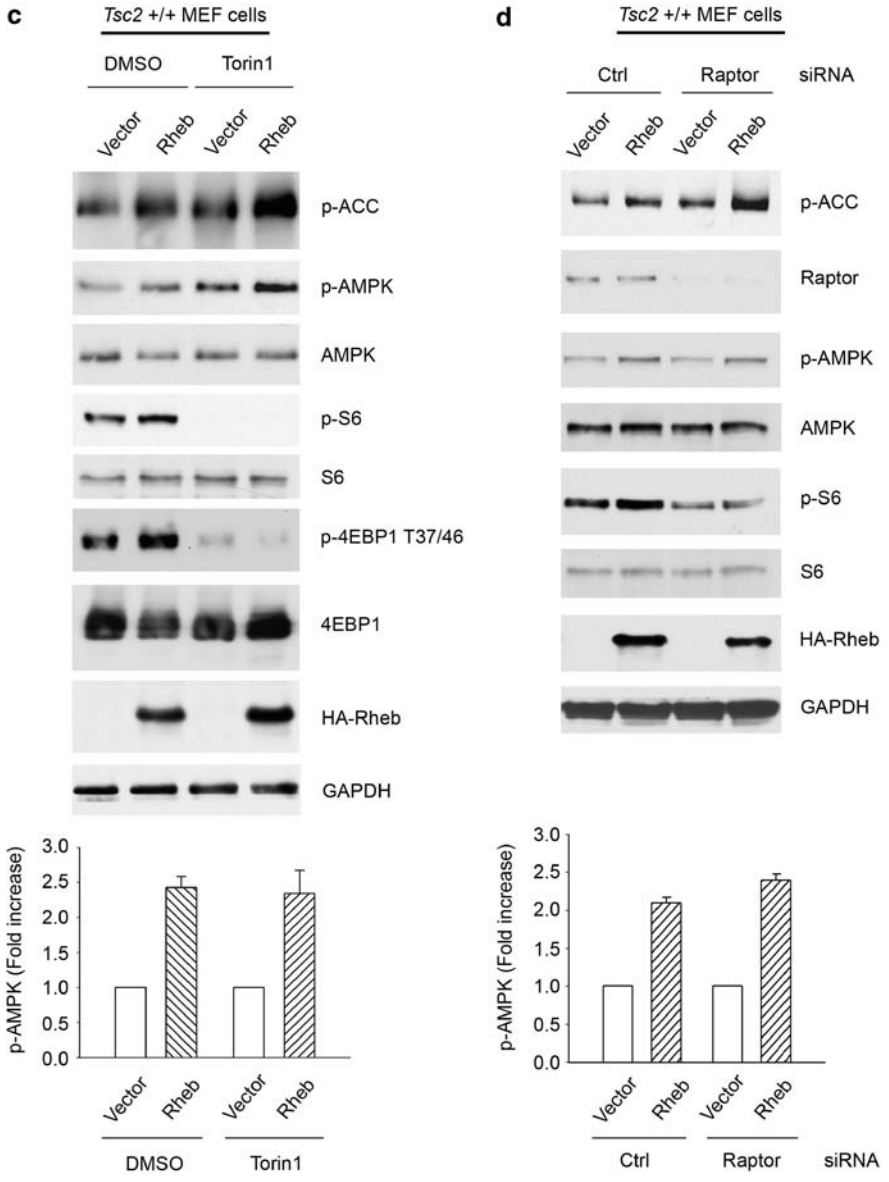


Figure 2 Rheb overexpression activates AMPK1 independently of mTORC1. (**a**) *Tsc2*+/+ MEF cells were transiently transfected with an HA-tagged wild-type Rheb construct or the corresponding empty vector as indicated. Serum-starved cells were incubated with vehicle (dimethylsulphoxide (DMSO)) or the specific AMPK inhibitor *compound C* (20 μM) for 2–4 h. (**b**, **c**) Same as in (**a**), but serum-starved cells were incubated with vehicle (DMSO) or rapamycin (20 nM) for 2 h (**b**) or with vehicle (DMSO) or Torin1 (50 nM) for 2 h (**c**). (**d**) Same as in (**a**), but cells were transiently transfected with empty vector or HA-tagged wild-type Rheb +50 nM of control or raptor SMARTpool siRNA as indicated. Cell lysates from (**a**) to (**d**) were characterized by immunobloting for expression of the indicated proteins. (**c**) and (**d**) Densitometry was used to analyze the data from these experiments. pAMPK band intensities were normalized by total AMPK and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) band intensities. Results are represented as fold increase. Data are the mean ± s.e.m.



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rapamycin neither affected AMPK phosphorylation nor p27 levels (Figure 4a). A similar effect of Rheb depletion on AMPK and p27 was also observed in SW1116 and COLO 320 HSR human colon cancer cells (Figure 4b). These results obtained in human cells parallel those shown above with rat (ELT3) and mouse (MEFs) Tsc2-null cells.

AICAR partially antagonizes Rheb's regulation of p27 levels

As manipulation of Rheb expression induced changes of both AMPK activity and p27 protein levels, we pharmacologically activated AMPK with AICAR to approach whether these Rheb downstream effects are mechanistically linked. We found that AICAR antagonized the increase of the nuclear levels of p27 following Rheb knockdown in *Tsc2*−/− MEFs (Figures 4c and d), suggesting that the effect of Rheb on AMPK affects p27 levels. As expected, the effect of AICAR is accompanied by increased levels of p-AMPK and p-ACC (Figure 4c).

Rheb depletion reduces the proliferative capacity of ELT3 and LAM-derived cells

As p27 negatively regulates cell cycle progression, we speculated that Rheb, through the regulation of p27 levels, affects the proliferation of TSC cells, and therefore is involved in the development of TSC and LAM. To explore this possibility, we used ELT3 cells and characterized primary LAM-derived cells (see Supplementary Figure S2) as model systems. ELT3 cells are tumorigenic in nude mice and represent a well accepted model for TSC/LAM (Howe *et al*., 1995).

To assess the effect of Rheb depletion on the proliferation ability of ELT3 cells, we first estimated

cell numbers by direct cell count and by measuring metabolic cell activity. We found that Rheb depletion significantly reduced the number of ELT3 cells (Figures 5a and b), consistent with inhibition of cell proliferation. Both methods used yielded similar results of inhibition of cell proliferation by Rheb siRNA 2 days after transfection (30.4 and 27.8% inhibition by measuring metabolic cell activity or by cell count, respectively). To assure that the effect of Rheb correlates with inhibition of cell cycle progression, we analyzed DNA synthesis by measuring BrdU incorporation. We showed that Rheb depletion reduced BrdU incorporation, suggesting that Rheb indeed positively regulates cell cycle progression (Figure 5c). To establish whether increased apoptosis could be responsible for a decrease in cell numbers on Rheb depletion, we examined the activity of caspase-3 and caspase-7. We found that cells treated with Rheb siRNA had slightly lower caspase activity than those treated with control siRNA (Supplementary Figure S3), indicating that the observed effect of Rheb depletion on cell number was a consequence of inhibition of cell proliferation rather than of increased apoptosis. Moreover, a pool of four human Rheb-specific siRNAs that efficiently depleted Rheb levels in LAM-derived

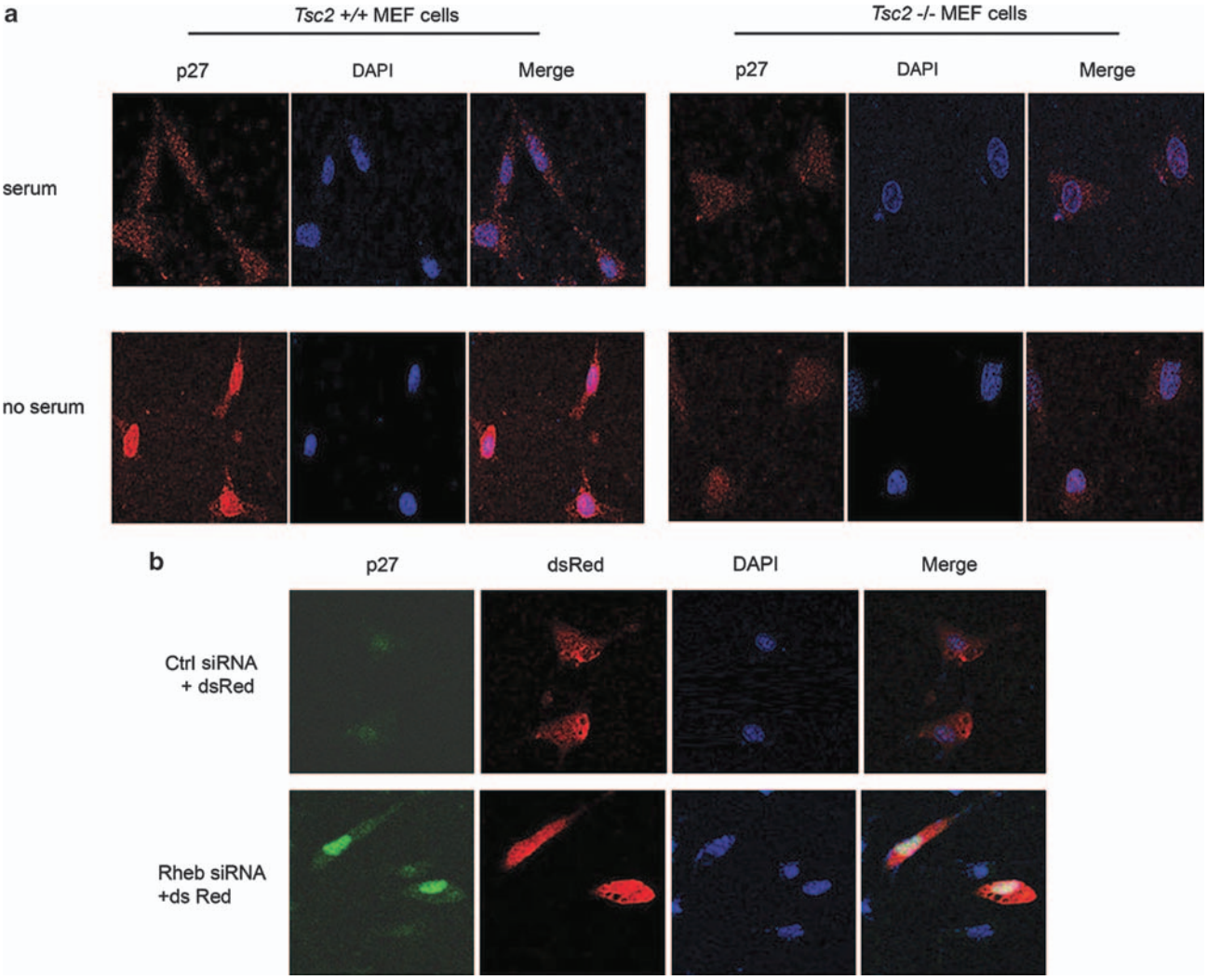
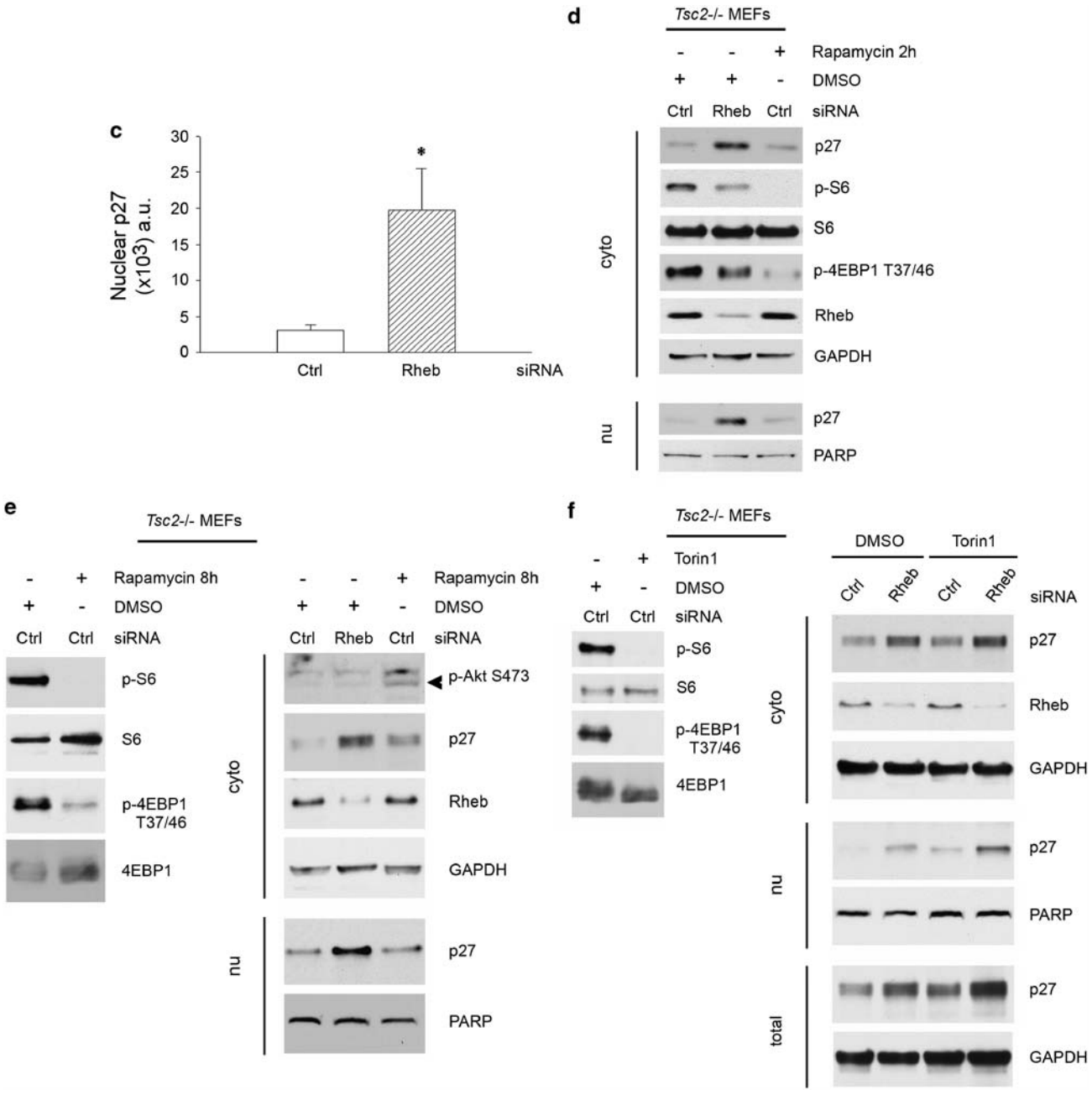


Figure 3 Rheb, but not mTORC1, regulates p27 levels *in vitro*. (**a**) *Tsc2*+/+ and *Tsc2*−/− MEF cells were grown in 10% fetal bovine serum (FBS) medium or serum-starved overnight. Cells were fixed and stained with an anti-p27 antibody followed by an Alexa Fluor 488-conjugated secondary antibody, and analyzed by confocal microscopy. 4,6-Diamidino-2-phenylindole (DAPI) stained the nuclei (blue), thus nuclear p27 appears purple. (**b**) *Tsc2*−/− MEF cells were co-transfected with 50 nM of control or Rheb siRNA#1 and the transfection marker dsRed, and 24 h later, were serum-starved overnight. Confocal microscopy was used to compare levels and localization of p27 (green) between control and Rheb siRNA treatment in cells that expressed dsRed (red cells). (**c**) Quantification of nuclear fluorescence intensity of p27 staining from another experiment performed as in (**b**). Fluorescent imaging data of the latter experiment are shown in Supplementary Figure S3. Data are the mean ± s.e.m. ($n = 14$), and a.u. refers to arbitrary units. *Student's *t*-test ($P < 0.05$). (**d–f**) *Tsc2* −/− MEF cells were transfected with 50 nM of control or Rheb siRNA#1 and 24 h later, were serum-starved overnight. Rapamcyin (**d**, **e**) or Torin1 (**f**) was added for 2 h (**d**), 8 h (**e**) or 2 h (**f**) before harvest. (**d**, **e**) Cells were fractionated and then characterized by immunoblotting for expression of the indicated proteins. To confirm the purity of the cell fractions and equal loading, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for cytoplasmic fractions and PARP for nuclear fractions. (**f**) As in (**d**, **e**) with additional analysis of total cell lysates (GAPDH as loading control).



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cells was able to reduce proliferation of LAM cells derived from three different patients (Figure 5d). Together, these data confirmed that Rheb enhances the proliferation capacity of TSC/LAM cells.

Rheb affects cell proliferation and Cdk2 activity through p27
We next investigated whether the upregulation of p27 when Rheb is depleted (shown in Figure 6a for ELT3 cells) translates into reduced cell proliferation. Using two different p27-specific siRNAs that induced a considerable decrease of p27 expression (Figure 6b), we demonstrated that the inhibition of cell proliferation in Rheb-depleted cells was reversed by co-depletion of p27 (Figure 6c). To confirm a functional role of p27 in the effect of Rheb on TSC cell proliferation, we also measured the activity of the p27 target and G1/S transition-promoting protein Cdk2 (Alkarain and Slingerland, 2004). We showed that the effect of Rheb depletion on cell proliferation correlated with inhibition of Cdk2 activity (Figure 6d), which was blocked by co-depletion of p27 (Figure 6e).

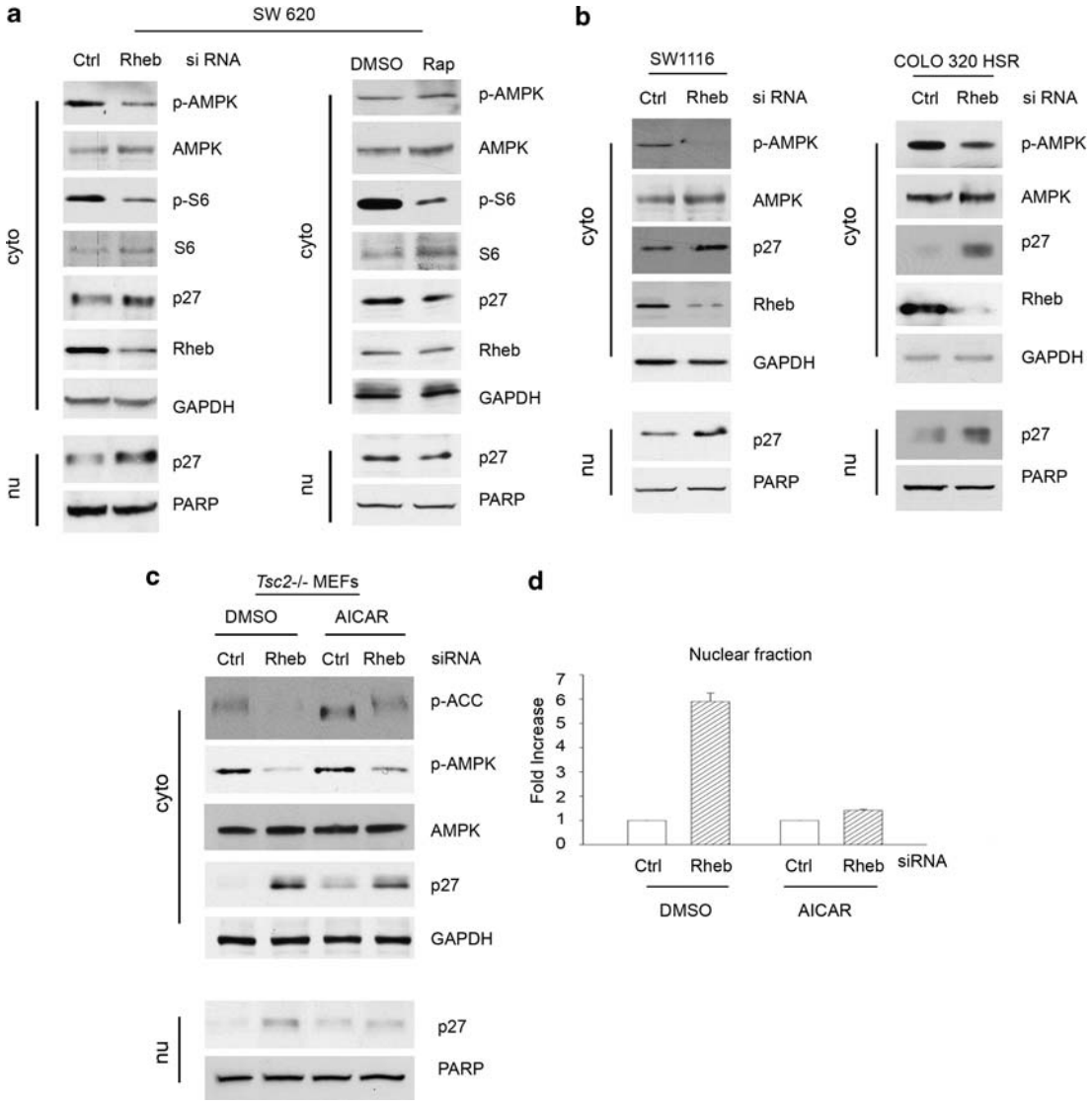

Figure 4 AMPK activation correlates with the Rheb-mediated negative regulation of p27. (**a**) SW620 human colon cancer cells were transfected with 50 nM of control or Rheb siRNA#2 and 24 h later, were serum-starved overnight. Cells were fractionated and then characterized by immunoblotting for p27, S6, phospho-S6 and Rheb expression. Also, SW620 cells were treated with vehicle (dimethylsulphoxide (DMSO)) or rapamycin (20 nM) for 1–2 h before cell fractionation. To confirm the purity of the cell fractions and equal loading, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used for cytoplasmic fractions and PARP for nuclear fractions. (**b**) Same as (**a**), but for human colon cancer cells SW1116 and COLO 320 HSR transfected with control or Rheb siRNA#2. (**c**) *Tsc2*−/− MEF cells were transfected and serum-starved overnight as in (**a**). Indicated cells were incubated with the AMPK activator AICAR (1 mM) for 1–2 h. Cells were fractionated and then characterized by immunoblotting for AMPK, phospho-AMPK, phospho-acetyl-CoA carboxylase (p-ACC), p27, Rheb, GAPDH (loading control) and PARP (loading control). (**d**) Densitometry was used to analyze the data from experiments conducted as described in (**c**). The results illustrate the effect of AICAR on Rheb depletion-induced changes of p27 in nuclear fractions. Results are represented as fold increase. Data are the mean ± s.e.m.

Rheb knockdown reduces in vivo *tumor growth of ELT3 cells, which correlates with increased p27 levels in tumor cells*

To evaluate the relevance of the role of Rheb *in vivo,* we examined the tumorigenic activity of Tsc2-null ELT3 cells that were stably depleted of Rheb by using a short hairpin RNA (shRNA)mir construct targeting a different Rheb mRNA sequence than the one selected for the Rheb siRNAs. We isolated several clones that stably expressed low levels of Rheb and compared their proliferation ability with that of cells transfected with non-silencing control shRNAmir. As shown in Figures 7a and b, we found a correlation between the extent of decrease of cell proliferation and the levels of Rheb expressed. We chose ELT3 clone #6 cells to generate subcutaneous flank tumors in nude mice. As both shRNA-mediated Rheb knockdown and inhibition of *in vitro* cell proliferation were incomplete, clone #6 is more likely representative of a 'clinical' setting. We found that tumors derived from control shRNAmir-transfected ELT3 cells were significantly larger than those derived from Rheb shRNAmir-transfected cells

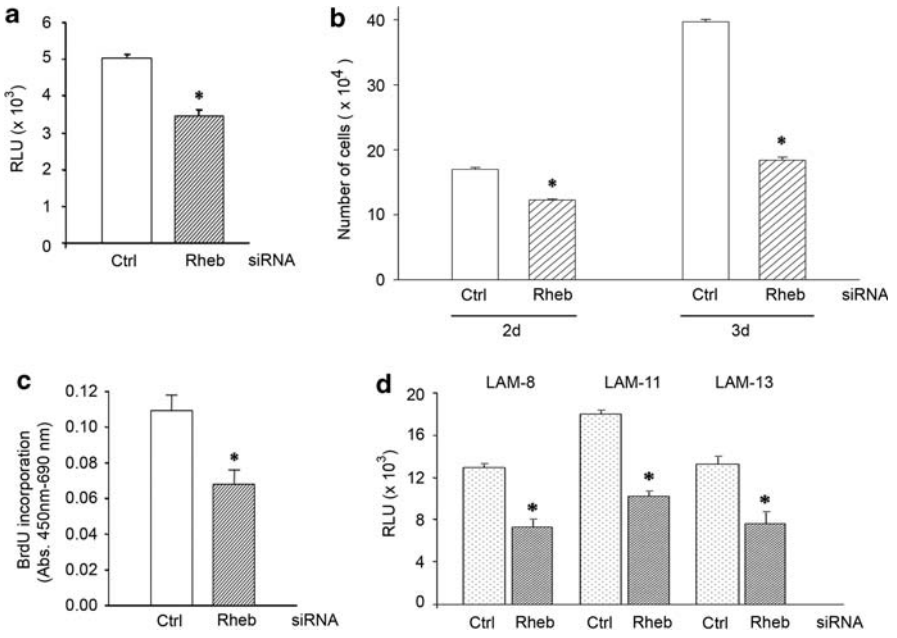

Figure 5 Rheb is important for proliferation of Tsc2-null and LAM-derived cells. (**a**) ELT3 cells were transfected with 50 nM of control or Rheb siRNA#1. Two days after transfection the number of metabolically active cells was estimated by a bioluminescent assay measuring endogenous ATP levels. Results are expressed as relative luminescence units (RLUs). (**b**) Cells were transfected as in (**a**). At 2 (2d) and 3 days (3d) after transfection, cells were counted using a hematocytometer. (**c**) Same as (**a**), but for measurement of BrdU incorporation using a colorimetric assay. Absorbance at 450 nm (reference wavelength 690 nm) represents BrdU incorporation. (**d**) LAM cells from three patients were transfected with 50 nM of control siRNA or Rheb SMARTpool siRNA, and after 24 h, were serum-starved for another 24 h. Proliferation of LAM-derived cells was evaluated by the bioluminescence/ATP method under similar conditions as described above for ELT3 cells. All data are the mean ± s.e.m. *Student's *t*-test ($P<0.05$).

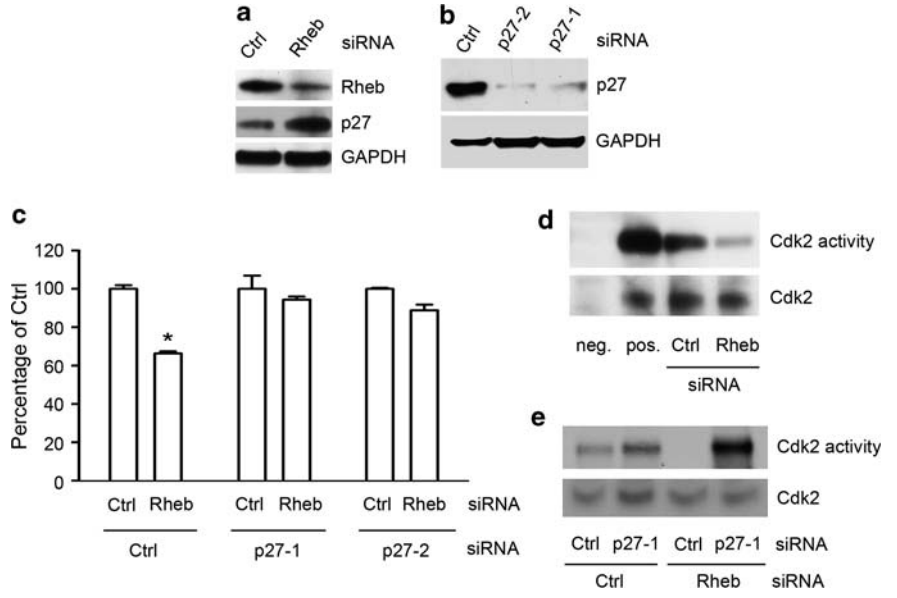

Figure 6 Reduction of p27 expression inhibited the effect of Rheb depletion on cell proliferation and Cdk2 activity. (**a**) ELT3 cells were transfected with 50 nM of control or Rheb siRNA#1, and 24 h later, were serum-starved overnight. Rheb and p27 levels were evaluated by immunoblotting. (**b**) ELT3 cells were transfected with 50 nM of control, p27-1 or p27-2 siRNA. Serum-starved cells were characterized by immunoblotting for p27 expression. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control. (**c**) ELT3 cells were transfected with 50 nM of control, p27-1 or p27-2 siRNA plus 50 nM of control or Rheb siRNA#1, as indicated. After 24 h, the cells were cultured overnight in serum-free medium and the number of metabolically active cells was estimated by a bioluminescent assay measuring endogenous ATP levels. Results are expressed as percentage of the corresponding control siRNA samples. Data are the mean ± s.e.m. of three independent experiments. *Student's *t*-test ($P<0.05$). (**d**) Cdk2 activity was evaluated in ELT3 cells transfected with 50 nM of control or Rheb siRNA#1. Immunoglobulin G (IgG) instead of the Cdk2 antibody was used for the negative control sample (neg.). ELT3 cells stimulated with E2 (estradiol), a promoter of ELT3 cell proliferation (Yu *et al*., 2009) and cyclin E-cdk2 activation (Planas-Silva and Weinberg, 1997), served as positive control (pos.). (**e**) Under identical experimental conditions as in (**c**), Cdk2 activity was evaluated in cells transfected with 50 nM of control or Rheb siRNA#1 in the presence or absence of 50 nM of control or p27-1 siRNA, as indicated.

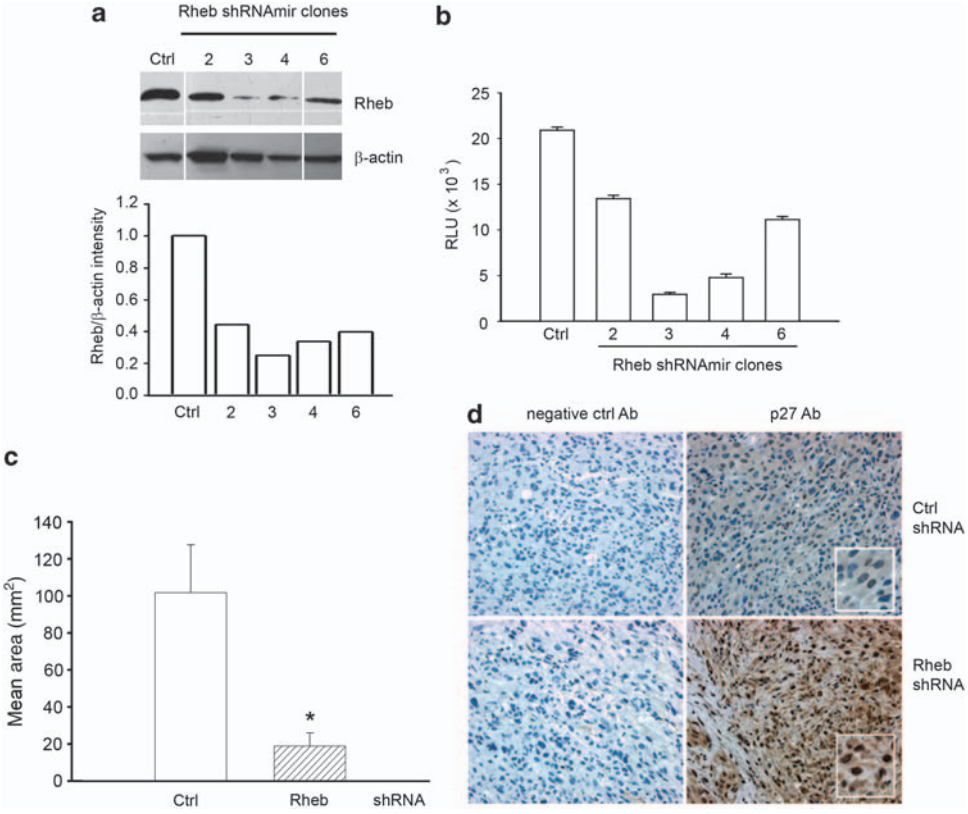

Figure 7 Rheb is essential for p27 expression and the tumorigenic activity of Tsc2-null ELT3 cells *in vivo*. (**a**) Endogenous Rheb levels from ELT3 cell clones with stable expression of control or Rheb shRNAmir were determined by western blot analysis (top) and quantified relative to β-actin by densitometry (bottom). (**b**) Equal numbers of cells from control and ELT3 clones with different levels of Rheb expression were grown in serum-free medium for 3 days. Cell proliferation was evaluated by estimating the number of metabolically active cells. Data are the mean ± s.e.m. (**c**) Growth of flank tumors generated by subcutaneous injection of ELT3 cells stably expressing control or Rheb shRNAmir (clone #6) into nude mice. Data are the mean cross-sectional area ± s.e.m. of tumors derived from five animals per group. *Student's *t*-test ($P<0.05$). (**d**) Immunohistochemistry analysis for p27 expression in subcutaneous tumors derived from mice injected with ELT3 cells stably expressing control or Rheb shRNAmir (clone #6). Brown staining indicates p27 expression.

(Figure 7c). This indicates that shRNA-mediated knockdown of Rheb substantially reduced *in vivo* tumor growth. Immunohistochemistry analysis showed significantly higher p27 levels in tumor cells with stable Rheb knockdown than in control cells (Figure 7d). These findings correlate with the *in vitro* studies and point at the importance of Rheb in the regulation of p27 and tumor growth.

Discussion

A major milestone in understanding TSC pathogenesis is the finding that TSC1/2 negatively controls mTORC1 signaling (Huang and Manning, 2008). As mTORC1 is the main controller of cell growth, its constitutive activation in TSC is believed to contribute to tumorigenesis, and consistently, inhibition of mTORC1 with rapamycin reduces the size of TSC tumors. However, tumor re-growth is observed once rapamycin treatment is discontinued, indicating that mTORC1-independent mechanisms contribute to the survival of tumor cells (Easton and Houghton, 2006; Paul and Thiele, 2008). Recently, elevated AMPK activity has been found in TSC tumors and Tsc2-null cells, and correlated with increased cell survival (Short *et al*., 2008). This observation suggests that AMPK could contribute to the survival of tumor cells on rapamycin treatment or during metabolic stress that is usually associated with the development of solid tumors. However, how AMPK is activated in Tsc2-null cells and whether mTORC1 is involved is unknown. Our study demonstrates that Rheb can control AMPK activity independently of its regulation of mTORC1 signaling. In correlation with these results, we found that Rheb reduces p27 levels independently of mTORC1. We present a model in which Rheb, with AMPK involved, contributes to TSC tumorigenesis by reducing the levels of p27. Consistent with this possibility, we showed that Rheb depletion reduced the tumorigenic activity of Tsc2-null cells, and correlated with an *in vivo* upregulation of p27 levels. Our results agree with previous findings indicating that farnesyl transferase inhibitors and atorvastatin, which block Rheb activity, inhibit the growth of Tsc2-deficient cells (Gau *et al*., 2005; Finlay *et al*., 2007).

Previous studies have shown that Akt inhibits AMPK, likely through a decrease of the AMP to ATP

ratio through activation of glycolysis and oxidative phosphorylation (Gottlob *et al*., 2001; Kovacic *et al*., 2003; Hahn-Windgassen *et al*., 2005). In agreement, overexpression of activated Akt in Tsc2-null cells inhibits AMPK activity (Hahn-Windgassen *et al*., 2005). These studies suggest that the low levels of active Akt found in Tsc2-null cells are responsible for the elevated activity of AMPK. However, because we observed that Rheb depletion reduced AMPK activity without affecting Akt activity, our study implies that Rheb, rather than Akt, is responsible for activating AMPK in Tsc2-null cells.

Although activation of AMPK is generally associated with tumor-suppressor activity, there is evidence that AMPK may also contribute to tumorigenesis. For instance, AMPK activation correlated with survival and Cdk2 activation in Tsc2-deficient cells (Short *et al*., 2008), and with proliferation and survival of prostate cancer cells (Park *et al*., 2009). Furthermore, Liang *et al*. (2007) recently linked activation of AMPK to p27-mediated autophagy-based cell survival under metabolic stress, a mechanism that may be associated with the development of solid tumors under low supply of nutrients and oxygen. Consistent with a tumorigenic role of AMPK, we have demonstrated that Rheb activation of AMPK correlates both with inhibition of nuclear p27 function in Tsc2-null cells and several human colon cancer cell lines, and with increased proliferation of Tsc2-null cells. By demonstrating that Rheb has a critical role in the activation of AMPK, and by showing that mTORC1 is not involved in this process, our study extends the reports by Liang *et al*. (2007) and Short *et al*. (2008). Thus, our findings suggest that Rheb should be considered as a potential therapeutic target in tumors associated with AMPK activation.

In analogy to the negative regulation of Akt by mTORC1/p70S6K signaling, we speculate that Rheb activation of AMPK serves to counteract metabolic cellular stress caused by upregulation of mTORC1. In normal cells or cells with proficient TSC2, Rheb activation of AMPK may serve to stop excessive Rheb and mTOR activation through an AMPK-induced activation of TSC2. In Tsc2-null cells, AMPK cannot regulate Rheb through this mechanism, leading to high levels of Rheb, and therefore, as we have shown here, to high basal levels of activated AMPK.

In Tsc2-null cells, inhibition of AMPK activity increased the levels of nuclear p27, and correlated with inhibition of Cdk2 activity (Short *et al*., 2008). Consistent with a role of Rheb in the regulation of AMPK activity in Tsc2-null cells, Rheb regulates p27 independently of mTORC1. The inability of Akt to respond to Rheb depletion suggests that Akt is also not a component of the signaling pathway by which Rheb regulates p27 in Tsc2-null cells. A potential mechanism is that Rheb stimulates AMPK-mediated nuclear exclusion of p27. However, when Rheb was depleted not only nuclear but also cytoplasmic p27 levels were elevated. As cell cycle inhibition correlates with increased p27 levels (Alkarain and Slingerland, 2004; Rosner *et al*., 2007), the elevation of the cytoplasmic levels of p27 is likely a consequence of an increased nuclear influx of p27 on Rheb knockdown that translated into reduced cell cycle progression and thus enhanced p27 stability. For instance, resembling our results, it was observed that serum removal, which induces cell cycle arrest in normal cells, had induced an increase in nuclear and cytoplasmic p27 levels in NIH3T3 cells (Rosner *et al*., 2007; Short *et al*., 2008). Alternatively, a combined effect of Rheb on p27 stability and subcellular localization may explain the parallel increase of the cytoplasmic p27 levels on Rheb depletion. Similarly, overexpression of TSC2 in HEK293 cells or inhibition of phosphatidylinositol 3 kinase/Akt in renal carcinoma cells, both known regulators of p27 stability and subcellular localization, induced a parallel increase of the nuclear and cytoplasmic p27 levels (Rosner *et al*., 2007; Kim *et al*., 2009).

In support of a link between AMPK and p27 downstream of Rheb, the AMPK activator AICAR blocked the nuclear p27 increase following Rheb depletion (Figure 4c). However, AICAR failed to considerably affect the cytoplasmic p27 increase. This finding is not necessarily surprising because AMPK activation has recently been reported to increase stability (Short *et al*., 2010), and cytoplasmic localization of p27 (Short *et al*., 2008). Alternatively, Rheb expression may be necessary for cytoplasmic degradation of p27 independently of AMPK. Taken together, additional approaches are needed to address whether AMPK is responsible for the Rheb effect on p27.

An mTORC1-independent regulation of p27 is controversial. A previous report indicates that mTORC1 regulates p27 by activating serum- and glucocorticoid-inducible kinase 1 (SGK1) (Hong *et al*., 2008), although a recent study showed that mTORC2, rather than mTORC1, is responsible for SGK1 activation (Garcia-Martinez and Alessi, 2008). Our work cannot conciliate the differences between these two reports, but indicates that mTORC1 is not involved in regulating p27 in Tsc2-null cells. In addition, we could not detect any phosphorylation of SGK1 on T256 (data not shown), which correlates with the activation of the kinase. Thus, we believe that disrupted SGK1 activity in Tsc2-null cells excludes this kinase from regulating p27. In agreement, if mTORC2 is indeed responsible for SGK1 activation, Huang *et al*. (2009) reported reduction of mTORC2 activity in cells lacking TSC1/2, which suggests that SGK1 does not have a role in regulating p27 in *Tsc2−/−* cells.

We found that Rheb's role in regulating AMPK and p27 extends to colon cancer cells. Two of the colon cancer cell lines we analyzed, SW620 and SW1116, carry activating *KRAS* mutations (http://www.sanger.ac.uk/genetics/CGP/CellLines). As activated KRAS may regulate p27 (Vervoorts and Luscher, 2008), our results suggest that Rheb overrides KRAS signaling and has an obligatory role in p27 regulation. These findings may have important implications for treating colon cancer and other types of cancer in which defective or absent p27 function is associated with poor prognosis.

However, the biological consequences of Rheb regulation of AMPK and p27 in cancer need further study. It would also be interesting to establish in what other milieus Rheb has a critical role in the regulation of AMPK and p27, which could help to predict which patients would benefit from targeting Rheb.

In summary, our study highlights Rheb's potential as an mTORC1-independent controller of aberrant cell proliferation under metabolic stress, a condition commonly found in solid tumors. The discovery that, unlike exposure to rapamycin, Rheb depletion represses AMPK activity and induces p27 function indicates that targeting Rheb may be an effective approach for reversing TSC and LAM tumorigenesis. As we showed that Rheb also regulates AMPK in non-cancerous cells, a more complete understanding of how Rheb does this could provide insights into the relevance of Rheb/AMPK signaling in normal physiology and how its disruption could result in pathological conditions. These studies could additionally help us to identify potential novel targets for TSC therapy.

Materials and methods

Further details and additional methods are provided as Supplementary Information.

Gene knockdown reagents and inhibitors

A pool of four human Rheb-specific siRNA duplexes (SMARTpool, M-009692-02), a rat/mouse/human Rheb-specific siRNA (Rheb siRNA#1, 5′-CUAUGGAGUAUGUCUGAGG-3′), a rat/human Rheb-specific siRNA (Rheb siRNA#2, D-099204-02, 5′-GAAAGGGUGAUCAGUUAUG-3′), a pool of four mouse Raptor-specific siRNAs (SMARTpool, M-058754-01) and non-silencing siRNAs controls (siGLO and non-targeting siRNA) were obtained from Dharmacon (Lafayette, CO, USA). Rat p27-specific siRNAs (p27-1, 5′-GAAGCACUGCCGAGAUAUG-3′ and p27-2, 5′-GCUCCGAAUUAAGAAUAAU-3′) and non-silencing control siRNA (*Silencer* Negative Control #1) were from Ambion (Foster City, CA, USA). MicroRNA-adapted shRNA (shRNAmir) specifically targeting rat Rheb and non-silencing control cloned into the retroviral pShag Magic version 2.0 vector (pSM2) were from Open Biosystems (Huntsville, AL, USA). The target sequence of rat Rheb mRNA for shRNAmir was 5′-AUUGUCAAUGAGGACUUUCCCA-3′.

Rapamycin and *compound C* were purchased from Calbiochem (San Diego, CA, USA). AICAR was from Cell Signaling Technology (Beverly, MA, USA). Torin1 was kindly provided by Drs Nathanael S Gray (Dana-Farber Cancer Institute) and David M Sabatini (Whitehead Institute/MIT/HHMI).

Cell culture

Tsc2-null ELT3 (leiomyoma/myosarcoma tumor-derived 3) cells (Howe *et al*., 1995) were kindly provided by Dr C Walker (MD Anderson Cancer Center, Houston, TX, USA), and maintained in DF8 medium supplemented with 10% fetal bovine serum as previously described (Howe *et al*., 1995). Primary cell cultures were established from lung specimens obtained from non-LAM donors or from patients diagnosed with LAM who underwent lung transplantation after institutional approval. We followed the procedures described earlier to establish airway smooth muscle cultures (Halayko *et al*., 1999). *Tsc2*+/+ *p53*−/− and *Tsc2*−/− *p53*−/− mouse embryonic fibroblasts (MEFs) (Zhang *et al*., 2003a) kindly provided by Dr DJ Kwiatkowski (Harvard Medical School, Boston, MA, USA) were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. Human colon cancer cells SW1116 and SW620 were maintained in DME H16 medium, and COLO 320 HSR in RPMI 1640 medium, both media supplemented with 10% fetal bovine serum.

Cell fractionation

Cell fractions were isolated using NE-PER nuclear and cytoplasmic extraction reagents (Pierce/Thermo Fisher Scientific, Inc., Rockford, IL, USA). Briefly, cells were harvested, washed, pelleted by centrifugation, treated with cytoplasmic extraction reagent and spun to pellet intact nuclei. The supernatants (cytoplasmic extracts) were saved and nuclei resuspended in nuclear extraction reagent. The nuclear fractions were recovered after centrifugation.

Cell proliferation assays

The day after transfection with Rheb or control siRNAs, cells were serum-starved overnight, and then BrdU was added to the medium for 3 h. Cells were fixed and BrdU incorporation was measured by a colorimetric enzyme-linked immunosorbent assay. To assess cell proliferation based on bioluminescent measurements of ATP present in all metabolically active cells, a cell proliferation/cytotoxicity kit (Lonza, Walkersville, MD, USA) was used. To assess cell proliferation by cell counting, cell numbers were estimated on the day following seeding (day 0), and on days 2 and 3 using a hematocytometer.

Tumor growth in nude mice

ELT3 cell tumors in female athymic Ncr:nu/nu mice (Taconic Laboratories, Inc., Hudson, NY, USA) ($n=5$ for each experimental group) were established as previously described (Howe *et al*., 1995). Briefly, 7- to 9-week-old mice were subcutaneously inoculated with 2.3×10^6 cells over each flank, and development of tumors monitored weekly after inoculation. Tumor growth was estimated by measuring the mean cross-sectional area. Six weeks after inoculation, animals were euthanized and tumors resected for immunohistochemistry analysis.

Conflict of interest

The authors declare no conflict of interest.

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