

Unrestrained Mammalian Target of Rapamycin Complexes 1 and 2 Increase Expression of Phosphatase and Tensin Homolog Deleted on Chromosome 10 to Regulate Phosphorylation of Akt Kinase^{*§}

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Background: Tumor suppressors PTEN and TSC2 act upstream of mTOR kinase.

Results: An mTOR-mediated increase in Hif1 α protein contributes to PTEN transcription.

Conclusion: Both TORC1 and TORC2 up-regulate PTEN levels.

Significance: An increased PTEN level in TSC2-deficient cells may contribute to reduced malignant potential of these cells.

Tuberous sclerosis complex 2 (TSC2) and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) function to block growth factor-induced mammalian target of rapamycin (mTOR) signaling and are mutated in autosomal dominant hamartoma syndromes. mTOR binds to a spectrum of common and different proteins to form TOR complex 1 (TORC1) and TORC2, which regulate cell growth, division, and metabolism. TSC2 deficiency induces constitutive activation of mTOR, leading to a state of insulin resistance due to a negative feedback regulation, resulting in reduced Akt phosphorylation. We have recently described an alternative mechanism showing that in TSC2 deficiency, enhanced PTEN expression contributes to reduced Akt phosphorylation. To explore the mechanism of PTEN regulation, we used rapamycin and constitutively active mTOR to show that TORC1 increases the expression of PTEN mRNA and protein. We found that in TSC2^{-/-} mouse embryonic fibroblasts expression of a kinase-dead mutant of mTOR, which inhibits both TORC1 and TORC2, decreases the expression of PTEN via transcriptional mechanism. Furthermore, kinase-dead mTOR increased and decreased phosphorylation of Akt at catalytic loop site Thr-308 and hydrophobic motif site Ser-473, respectively. Moreover, inhibition of deregulated TORC1 in TSC2-null mouse embryonic fibroblasts or in 293 cells by down-regulation of raptor decreased the levels of the

transcription factor Hif1 α and blocked PTEN expression, resulting in enhanced phosphorylation of Akt at Thr-308 and Ser-473. Finally, knockdown of rictor or mSin1 attenuated the expression of Hif1 α , which decreased transcription of PTEN. These results unravel a previously unrecognized cell-autonomous function of TORC1 and TORC2 in the up-regulation of PTEN, which prevents phosphorylation of Akt and may shield against the development of malignancy in TSC patients.

Phosphatase and tensin homolog deleted on chromosome 10 (PTEN)⁵ represents the most frequently deleted phosphatase and second most frequently deleted tumor suppressor gene in cancer (1). In fact, 50–70% of sporadic tumors including prostate tumors, endometrial tumors, and glioblastomas as well as 30–50% of lung, breast, and colon cancers show loss of one allele of PTEN (2). Complete loss of PTEN in endometrial tumors, glioblastomas, and BRCA1-deficient breast cancer is associated with advanced metastasis (2). Furthermore, germ line mutation of PTEN is associated with autosomal dominant developmental disorders, neurological deficiencies, and hamartoma syndromes including Cowden disease, Bannayan-Riley-Ruvalcaba syndrome, and Lhermitte-Duclos disease, which show a high frequency of cancer predisposition (3, 4).

Growth factor-stimulated class I phosphatidylinositol (PI) 3-kinase family member or its mutated constitutively active catalytic subunit produces phosphatidylinositol 3,4,5-trisphosphate (PIP₃), which regulates a myriad of cellular functions such as cell migration, polarity, proliferation, and survival (5, 6). PIP₃ produced in the plasma membrane recruits proteins con-

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⁵ The abbreviations used are: PTEN, phosphatase and tensin homolog deleted on chromosome 10; TSC, tuberous sclerosis complex; mTOR, mammalian target of rapamycin; TORC, TOR complex; MEF, murine embryonic fibroblast; PI, phosphatidylinositol; PIP₃, phosphatidylinositol 3,4,5-trisphosphate; Rheb, Ras homolog enriched in brain; raptor, rapamycin-sensitive adaptor protein of mTOR; rictor, rapamycin-insensitive companion of mTOR; 4EBP, eukaryotic initiation factor 4E-binding protein; KD, kinase-dead; CA, constitutively active; TOP, terminal oligopyrimidine tract.

taining pleckstrin homology domains such as Akt and PDK-1. Akt activation occurs via phosphorylation by PDK-1 and mTORC2 at two sites, Thr-308 and Ser-473, respectively (7). Structurally, PTEN shares identity with other protein phosphatases in its catalytic domain (8). Although PTEN has been infrequently reported to dephosphorylate protein substrates, its phosphatase activity toward PIP_3 represents an important mechanism for its physiological tumor suppressor function (9–13). Thus, PTEN acts as a negative regulator of PI 3-kinase signal transduction, significantly attenuating the biological activity of Akt. In PTEN-negative cancer cells, Akt is constitutively activated and regulates cell growth, proliferation, angiogenesis, and metabolism via phosphorylation of a number of substrates including tuberous sclerosis complex 2 (TSC2) and PRAS40, inactivation of both of which increases rapamycin-sensitive mTORC1 activity (7, 14).

Mutation in either TSC1 or TSC2 gene contributes to the development of TSC, which manifests as disorders involving pulmonary lymphangiomyomatosis, facial angiosarcomas, and renal angiomyolipomas (15). In addition, TSC patients often display neurological disorders including mental retardation, epilepsy, and autism (16). Clinically, mutations in TSC2 locus contribute more significantly to the manifestation of TSC compared with TSC1 mutation (17). Functionally, TSC1 and TSC2 exist as a heterodimer of which TSC2 contains a GTPase-activating protein domain. The TSC1-TSC2 complex exerts its GTPase-activating protein activity on the small GTPase Ras homolog enriched in brain (Rheb) and blocks mTOR activity (18, 19). Genetic studies in *Drosophila* and in mammalian cells place TSC2 as a signal integration hub in the PI 3-kinase/Akt/mTOR pathway (19, 20). Activated Akt and other mitogenic kinases phosphorylate TSC2 at distinct sites, leading to its dissociation from TSC1 and inactivation (21–24). Thus, inactivated TSC2 maintains elevated levels of GTP-bound Rheb, which activates TORC1 to promote tumorigenesis. Enhanced TORC1 activity is manifested in pathologic specimens of TSC hamartomas (25, 26). Similarly, in PTEN-deficient tumors, increased Akt activity phosphorylates TSC2, resulting in its inactivation, leading to activation of mTOR (7, 27).

TOR exists in two evolutionary conserved complexes, TORC1 and TORC2; the former is more sensitive to rapamycin (28, 29). TORC1 and TORC2 contain two distinct proteins, rapamycin-sensitive adaptor protein of mTOR (raptor) and rapamycin-insensitive companion of mTOR (riCTOR), respectively (28, 30, 31). Both these complexes bind mLST8/G β L and deptor, whereas TORC1 contains PRAS40, and TORC2 contains protor and mSin1 (28, 32, 33). TORC1 directly phosphorylates the eukaryotic initiation factor 4E-binding proteins (4EBPs) and the ribosomal protein S6 kinase and increases ribosomal biogenesis to elicit cell growth, proliferation, and metabolism (32, 34). On the other hand, TORC2 phosphorylates Akt at the hydrophobic motif site Ser-473 to fully activate its anti-apoptotic function (35, 36).

The wide spectrum of hamartomas elicited by loss of TSC2 rarely progress to malignancy. Lack of TSC2 leads to inhibition of Akt activity, which may contribute to reduced malignancy of TSC tumors (37, 38). Multiple mechanisms have been postulated for the down-regulation of Akt phosphorylation in TSC2-

null cells. Disruption of TSC2 produces a state of insulin resistance by a rapamycin-sensitive TORC1-mediated negative feedback loop through insulin receptor substrate 1 (37, 38). A second level of negative regulation on PI 3-kinase/Akt signaling is achieved by down-regulation of PDGF receptor- β in TSC2-null murine embryonic fibroblasts (MEFs) via an unknown mechanism (39, 40). We have recently reported that in TSC2-null MEFs, the level of PTEN is significantly elevated, which also contributes to decreased phosphorylation of Akt (41). The signal transduction mechanism by which PTEN is up-regulated in TSC2-disrupted cells is not known.

In the present study, we found that the enhanced TORC1 activity present in the TSC2-null MEFs regulates the levels of PTEN mRNA and protein via transcriptional mechanism. Similarly, TORC1 controls expression of PTEN in normal cells. Furthermore, we show that along with TORC1 TORC2 also contributes to PTEN expression. Finally, we demonstrate that both TORC1 and TORC2 up-regulate Hif1 α protein, which enhances the transcription of PTEN. Thus, up-regulation of PTEN in TSC patients may attenuate the malignant potential of the tumors despite increased mTOR activity.

EXPERIMENTAL PROCEDURES

Cell Culture—TSC2^{+/+} and TSC2^{-/-} MEFs were grown as described previously in DMEM with low glucose in the presence of 10% fetal bovine serum (41). 293 cells were grown in DMEM with high glucose in 10% fetal bovine serum. The cell stocks were maintained in Plasmocin and Primocin. When necessary, the cells were incubated in serum-free medium for 24 h before the experiment.

Antibodies and Reagents—TSC2, PTEN, Myc, and Hif1 α antibodies were obtained from Santa Cruz Biotechnology. Phospho-Akt antibodies recognizing either Ser-473 or Thr-308 and raptor, rictor, mTOR, phospho-S6 kinase (Thr-389), phospho-4EBP-1 (Thr-37/46), phospho-4EBP-1 (Ser-65), S6 kinase, 4EBP-1, eIF4E, eIF4G, and Akt antibodies were purchased from Cell Signaling Technology. Actin and HA antibodies were purchased from Sigma and Covance, respectively. mSin1 antibody recognizing mSin1.1 and mSin1.2 was obtained from Bethyl Laboratories. shRNA expression plasmids for human raptor (shRaptor 1 and shRaptor 2), human rictor (shRictor 1 and shRictor 2), human mSin1 (mSin1 sh1 and mSin1 sh2) targeting both mSin1.1 and mSin1.2, and scrambled vector were obtained from Addgene. Pooled siRNAs against mouse raptor and rictor and human raptor and rictor and scrambled RNAs were obtained from Santa Cruz Biotechnology. siRNA for mouse mSin1 was obtained from Sigma. Hif1 α 5' terminal oligopyrimidine tract (TOP)-Lux reporter plasmid in which the 5'-untranslated region (5'-UTR) of Hif1 α flanks the luciferase gene was a kind gift from Dr. John Blenis (Harvard Medical School), who obtained it from Dr. Charles Sawyers, University of California at Los Angeles (42). The RNA isolation kit RNazol and OneStep RT-PCR kit were purchased from Invitrogen. Primers to detect mouse and human PTEN mRNAs were obtained from SuperArray Bioscience. The luciferase assay kit was purchased from Promega. Expression plasmids for Myc-tagged raptor, rictor, mSin1.1, mSin1.2, and kinase-dead mTOR (mTOR KD) were obtained from Addgene. Constitu-

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tively active mTOR expression vector (SL1+I2017T) was kindly provided by Dr. Tatsuya Maeda, Institute of Molecular Biology, The University of Tokyo, Tokyo, Japan. In this constitutively active mutant, four amino acids of mTOR are changed (I2017T, V2198A, L2216H, and L2260P) (supplemental Fig. S2) (43). We refer to this mutant as constitutively active mutant of mTOR (CA mTOR).

Cell Lysis and Immunoblotting—MEFs and 293 cells treated with rapamycin (25 nM) or transfected cells were washed with PBS and harvested in radioimmune precipitation assay buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mM Na_3VO_4 , 1 mM PMSF, 0.1% protease inhibitor mixture, and 1% Nonidet P-40) (41, 44, 45). The crude cell extracts were centrifuged at $12,000 \times g$ at 4 °C for 30 min. Cleared cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred to a PVDF membrane. Immunoblotting was performed using the indicated primary antibodies, and the protein bands were developed with HRP-conjugated secondary antibody using ECL reagent as described (41, 44, 45).

RNA Extraction and Semiquantitative RT-PCR—Total RNAs were prepared from TSC2^{-/-} MEFs and 293 cells using the RNazol reagent according to the vendor's instructions. Reverse transcription-PCR was carried out using the OneStep RT-PCR kit as described previously (41).

Transfection and Luciferase Assay—The cells were transfected with the indicated plasmids or siRNAs using FuGENE HD according to the manufacturer's protocol (41, 44, 45). For determining PTEN transcription, a reporter plasmid containing the PTEN promoter driving the luciferase cDNA was transfected along with the indicated expression vector or siRNAs. The luciferase activity was determined using an assay kit according to the vendor's instructions (41, 44, 45).

Statistics—Statistical significance of the data was determined by paired *t* test or analysis of variance using GraphPad Prism 4 software as described previously (41, 44, 45). The significance level was considered at a *p* value <0.05.

RESULTS

mTOR Regulates PTEN Expression in TSC2^{-/-} MEFs—In TSC2-null cells, a negative feedback loop involving enhanced TORC1 activity inhibits phosphorylation of Akt (37, 38, 46). The tumor suppressor protein PTEN represents an important mechanism by which the PIP₃ level, which regulates Akt phosphorylation, is maintained (11, 12). We have recently shown that increased expression of PTEN in the TSC2-null cells also contributes to the reduced phosphorylation of Akt (supplemental Fig. S1) (41). GTPase-activating protein activity of TSC2 blocks TORC1 activity (18, 20). Indeed, TORC1 is constitutively active in TSC2-deficient MEFs as confirmed by the increased phosphorylation of p70 S6 kinase at Thr-389 (supplemental Fig. S1). To examine the role of TORC1 in PTEN expression in TSC2^{-/-} MEFs, we used rapamycin, a potent inhibitor of TORC1 activity (29). Rapamycin significantly reduced the expression of PTEN mRNA in TSC2-null MEFs concomitantly with a reduction in the PTEN protein level (Fig. 1, A and B). Inhibition of TORC1 activity was confirmed by the loss of phosphorylation of S6 kinase in response to rapamycin (Fig. 1B) (37, 41, 46). The reduction of the PTEN protein level

resulted in the up-regulation of Akt phosphorylation in these cells (Fig. 1B). These findings suggest that TORC1 regulates the expression of PTEN in TSC2-deficient cells. To obtain more insight into this mode of regulation of PTEN expression, we extended these studies in growing human 293 cells, which express active TSC2, in the presence of serum. Incubation of these cells with rapamycin showed a reduction of PTEN mRNA and protein that was concomitant with the inhibition of TORC1 (Fig. 1, C and D). Similar to the observation obtained with TSC2-null MEFs, rapamycin increased the phosphorylation of Akt at Ser-473 and Thr-308 (Fig. 1D).

Rapamycin has been shown to significantly inhibit TORC1 activity, although prolonged incubation (more than 12 h) with this macrolide blocked TORC2 activity in some cancer cells (29). The results described above were obtained with cells incubated with rapamycin for 24 h (Fig. 1, A–D). Under these conditions, we observed enhanced phosphorylation of Akt at Ser-473 (Fig. 1, B and D). These data suggest that rapamycin did not inhibit mTORC2 activity in TSC2^{-/-} MEFs and in 293 cells.

To test the involvement of mTOR in more detail, we examined whether kinase-dead mTOR, which acts as a dominant negative kinase, affects expression of PTEN in TSC2-deficient MEFs. The results show that expression of kinase-dead mTOR in TSC2-null MEFs inhibited PTEN mRNA and protein expression and attenuated TORC1-mediated phosphorylation of S6 kinase (Fig. 1, E and F) similarly to the effects obtained with rapamycin above. However, unlike the results obtained with rapamycin, kinase-dead mTOR inhibited the TORC2-mediated basal phosphorylation of Akt at Ser-473 (Fig. 1F). These data are consistent with the mutation in the kinase-dead construct affecting the kinase activity of mTOR irrespective of whether it is a constituent of TORC1 or TORC2. On the other hand, we observed increased phosphorylation of Akt at Thr-308 (Fig. 1F). This is probably due to an increase in PIP₃ levels associated with decreased PTEN (Fig. 1F, top panel), which would stimulate the PDK-1 activity responsible for Akt Thr-308 phosphorylation in TSC2^{-/-} MEFs (Fig. 1F).

To investigate further the physiological relevance of PTEN regulation by mTOR, we examined its effect in growing human 293 cells. Expression of dominant negative mTOR kinase in these cells inhibited PTEN mRNA (Fig. 1G) and protein abundance concomitantly with reduced phosphorylation of S6 kinase, which was used as a surrogate to determine TORC1 activation (Fig. 1H). Because kinase-dead mTOR would block TORC2 activity, phosphorylation of Akt at Ser-473 was inhibited in 293 cells (Fig. 1H). In contrast, phosphorylation of Akt at Thr-308 was increased by dominant negative mTOR (Fig. 1H). Together these data indicate that similarly to TSC2^{-/-} MEFs dominant negative mTOR blocks both TORC1 and TORC2 activity to regulate phosphorylation of Akt at Thr-308 and Ser-473 in a reciprocal manner.

To confirm our results demonstrating a role of TORC1 in the regulation of PTEN expression, we next used a constitutively active mutant of mTOR (supplemental Fig. S2) in human 293 cells (43). Expression of CA mTOR in 293 cells increased the expression of PTEN mRNA and protein concomitantly with increased phosphorylation of S6 kinase (Fig. 2, A and B). This constitutively active mutant of mTOR has been shown to dis-

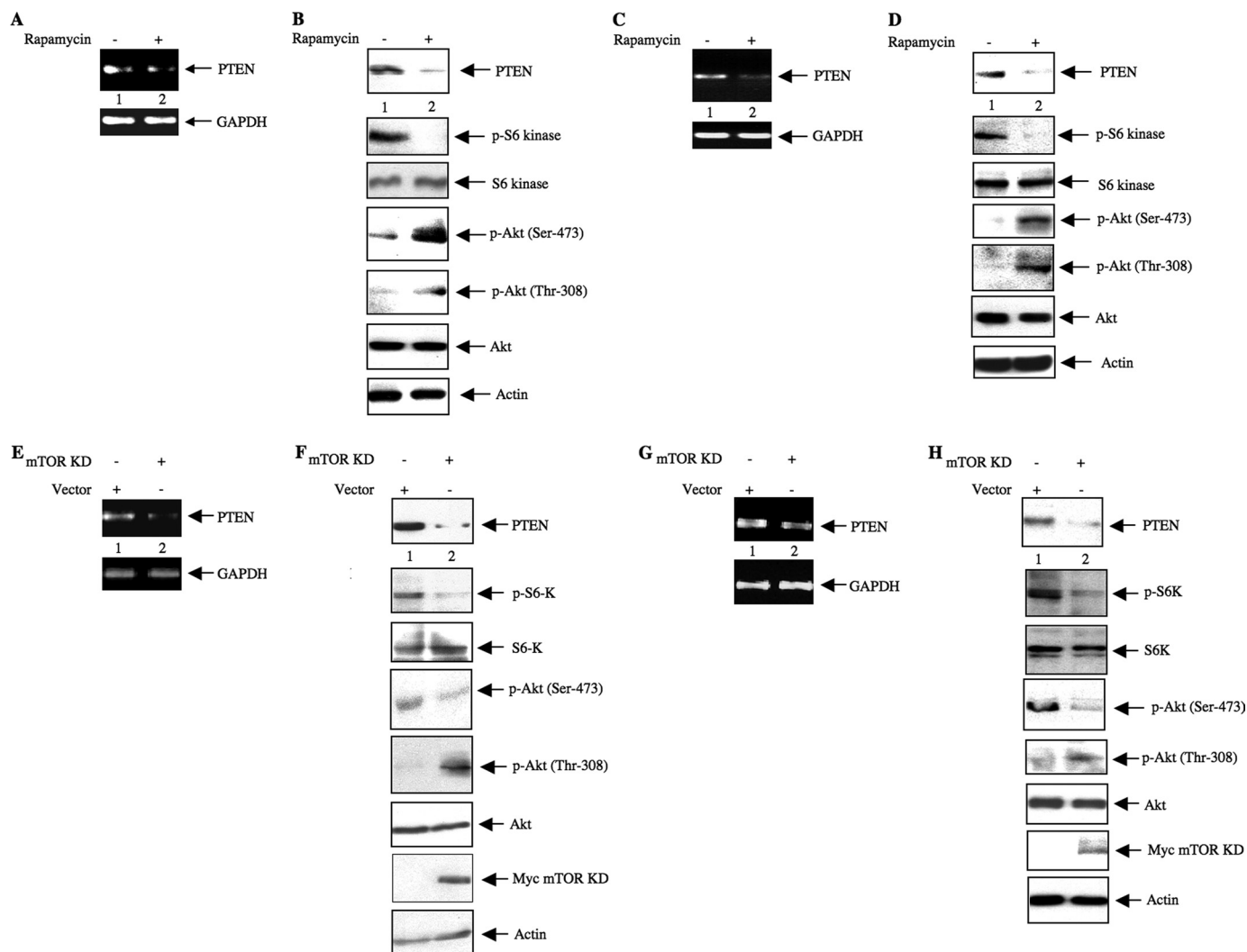


FIGURE 1. mTOR regulates expression of PTEN and phosphorylation of Akt. *A–D*, rapamycin lowers PTEN expression to modulate Akt phosphorylation. *A* and *B*, TSC2^{-/-} MEFs were grown to near confluence and serum-starved for 24 h prior to incubation with 25 nM rapamycin for 24 h. *A*, total RNA was used in semiquantitative RT-PCR to detect PTEN and GAPDH mRNAs as indicated. *B*, the cell lysates were immunoblotted with the indicated antibodies as described under “Experimental Procedures.” *C* and *D*, 293 cells were grown to confluence in the presence of 10% serum prior to incubation with 25 nM rapamycin for 24 h. *C*, total RNA was analyzed for the expression of PTEN and GAPDH mRNAs by semiquantitative RT-PCR. *D*, cleared cell lysates were immunoblotted with the indicated antibodies. *E–H*, dominant negative mTOR inhibits PTEN expression to affect phosphorylation of Akt. *E* and *F*, TSC2^{-/-} MEFs in a 12-well culture dish were transfected with 500 ng of Myc-tagged mTOR KD or vector plasmid as described under “Experimental Procedures.” Twenty-four hours post-transfection, the cells were incubated with serum-free medium for 24 h. *E*, total RNA was analyzed for PTEN and GAPDH mRNAs by semiquantitative RT-PCR. *F*, the cleared cell lysates were immunoblotted with the indicated antibodies. *G* and *H*, 293 cells were transfected with Myc-mTOR KD and grown for 48 h in 10% serum. *G*, total RNAs were analyzed for PTEN and GAPDH mRNAs by semiquantitative RT-PCR. *H*, the cleared cell lysates were immunoblotted with the indicated antibodies. *p-Akt*, phospho-Akt; *S6K*, S6 kinase; *p-S6K*, phospho-S6 kinase.

play only TORC1 activity (43). As a result, phosphorylation of Akt at Ser-473 was significantly inhibited in constitutively active mTOR-expressing cells (Fig. 2*B*). Furthermore, Akt phosphorylation at Thr-308 was also inhibited in the presence of constitutively active mTOR (Fig. 2*B*). This is probably because increased PTEN would decrease the PIP₃ level, which would in turn lead to diminished PDK-1 activity. Together our results suggest that increased expression of PTEN due to active TORC1 reduces phosphorylation of Akt.

mTOR Regulates Transcription of PTEN—The above findings indicate that TORC1 regulates PTEN mRNA expression. To gain more insight into the mechanism, we investigated the role of TORC1 in the transcription of PTEN. We used a reporter plasmid in which the firefly luciferase gene is driven by the PTEN promoter (PTEN-Luc) (41, 47). Transient transfection

of this reporter plasmid leads to significant luciferase activity in TSC2^{-/-} MEFs (Fig. 3*A*) (41). Incubation of the reporter-transfected cells with rapamycin significantly inhibited the luciferase activity, indicating a reduction in transcription of PTEN (Fig. 3*A*). Similarly, expression of a kinase-dead mutant of mTOR prevented the activity of the reporter in TSC2^{-/-} MEFs (Fig. 3*B*). To confirm these results, we transiently transfected PTEN-Luc into human 293 cells. Treatment of these cells with rapamycin attenuated the reporter activity (Fig. 3*C*). Furthermore, cotransfection of dominant negative mTOR with the reporter plasmid markedly inhibited the luciferase activity (Fig. 3*D*). Finally, we determined whether constitutively active mTOR increases this activity. Cotransfection of a constitutively active mutant of mTOR with PTEN-Luc in 293 cells resulted in a significant up-regulation of luciferase activity (Fig. 3*E*). These

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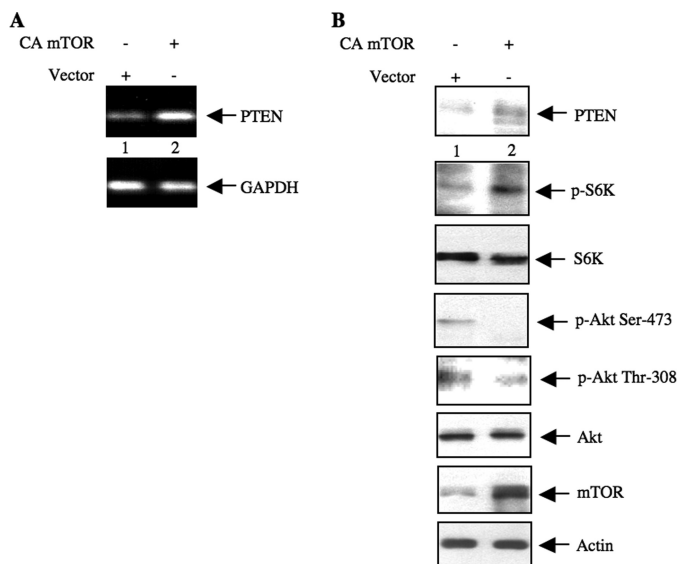


FIGURE 2. CA mTOR increases PTEN to regulate phosphorylation of Akt in 293 cells. 293 cells were transfected with CA mTOR or vector plasmids. Twenty-four hours post-transfection, the cells were incubated in serum-free medium for 24 h prior to harvest. *A*, total RNA was analyzed for expression of PTEN and GAPDH mRNAs by semiquantitative RT-PCR. *B*, cleared cell lysates were immunoblotted with the indicated antibodies. *p*-Akt, phospho-Akt; *S6K*, S6 kinase; *p*-*S6K*, phospho-S6 kinase.

results suggest that TORC1 regulates PTEN promoter activity to increase PTEN expression.

TORC1 and TORC2 Regulate PTEN Expression via Transcription—Use of rapamycin, kinase-dead mTOR, and constitutively active mTOR mutants conclusively pointed to a significant role of the kinase in the regulation of PTEN expression. To further dissect the molecular mechanisms, we first examined the contribution of TORC1 by inhibiting raptor, an exclusive component of the TORC1 kinase (31). Knockdown of raptor using a pool of siRNA in *TSC2*^{-/-} MEFs decreased the level of PTEN (Fig. 4A). As expected, raptor knockdown reduced the TORC1 activity as judged by the phosphorylation of S6 kinase in these cells (Fig. 4A). Reduced levels of PTEN due to decreased TORC1 activity were associated with increased phosphorylation of Akt at the Ser-473 and Thr-308 (Fig. 4A). These findings suggest a specific requirement of TORC1 activity in the up-regulation of PTEN in *TSC2* deficiency. We also examined the effect of down-regulation of raptor in growing human 293 cells. Knockdown of raptor using two independent shRNA vectors, shRaptor 1 and shRaptor 2, decreased the basal PTEN levels concomitantly with reduced phosphorylation of S6 kinase (Fig. 4, B and C). The reduction in PTEN resulted in an increase in the phosphorylation of Akt at Ser-473 and Thr-308 (Fig. 4, B and C). To confirm this observation, we also used an siRNA pool to down-regulate raptor in 293 cells. As expected, expression of PTEN was inhibited along with decreased phosphorylation of S6 kinase and increased phosphorylation of Akt (supplemental Fig. S3A).

We have demonstrated that mTOR regulates the expression of PTEN mRNA by a transcriptional mechanism (Fig. 3). To examine whether TORC1 specifically regulates the transcription of PTEN, we cotransfected PTEN-Luc reporter plasmid with an siRNA pool against raptor in *TSC2*^{-/-} MEFs. Knock-

down of raptor in *TSC2*^{-/-} MEFs significantly inhibited the luciferase activity (Fig. 4D). Similarly, cotransfection of PTEN-Luc with shRaptor 1 or shRaptor 2 attenuated reporter activity in 293 cells (Fig. 4, E and F). Identical results were obtained when we used a pool of siRNA targeting raptor in 293 cells (supplemental Fig. S3B).

Next, we investigated whether TORC2 plays any role in PTEN expression. 293 cells grown in serum were used. We down-regulated rictor, a component of TORC2 necessary for its kinase activity (48), in growing 293 cells using two independent shRNA vectors, shRictor 1 and shRictor 2. Knockdown of rictor with both rictor shRNAs inhibited PTEN protein expression (Fig. 5, A and B). As expected, the phosphorylation of Akt at Ser-473 was decreased as it is the substrate of TORC2 (Fig. 5, A and B). Interestingly, a decrease in phosphorylation of Akt at Thr-308 was evident in response to rictor down-regulation (Fig. 5, A and B). Identical results were obtained using an siRNA pool to down-regulate rictor in 293 cells (supplemental Fig. S4A). To further confirm the role of TORC2, we used two shRNA plasmids targeting mSin1, an exclusive component of TORC2. Two isoforms of this protein, mSin1.1 and mSin1.2, are predominantly present in the TORC2 complex (33). Down-regulation of both mSin1 complexes with two independent shRNAs inhibited expression of PTEN (Fig. 5, C and D). Phosphorylation of Akt was also blocked (Fig. 5, C and D). Finally, to elucidate the role of TORC2 in PTEN transcription, 293 cells were cotransfected with PTEN-Luc reporter and two independent shRNA vectors, shRictor 1 and shRictor 2. Knockdown of rictor with both shRNAs significantly blocked the reporter activity (Fig. 5, E and F). Similar results were obtained using an siRNA pool targeting rictor in these cells to inhibit rictor expression (supplemental Fig. S4B). When shRNAs against mSin1 were used to down-regulate TORC2, identical results were obtained (Fig. 5, G and H). These results demonstrate that both TORC1 and TORC2 contribute to the expression of PTEN protein via a transcriptional mechanism.

TORC2 Contributes to Hif1 α Expression by Translational Mechanism—We have recently shown that a Hif1 α -responsive DNA element present in the PTEN promoter increases its transcription in *TSC2*-null MEFs and in 293 cells (41). Previously, rapamycin was shown to inhibit mTOR activity and Hif1 α -mediated transcription in *TSC2*-null MEFs (49). We directly tested whether TORC1 regulates Hif1 α protein in *TSC2*-deficient MEFs. siRNA pool-mediated down-regulation of raptor in these cells decreased Hif1 α protein levels (Fig. 6A). Similarly, inhibition of raptor expression using two independent shRNA vectors, shRaptor 1 and shRaptor 2, also blocked Hif1 α expression in 293 cells (Fig. 6, B and C). Identical results were obtained when we used an siRNA pool to down-regulate raptor in these cells (supplemental Fig. S5). These results conclusively demonstrate that TORC1 regulates Hif1 α protein expression.

We have shown above that along with TORC1 TORC2 also contributes to the expression of PTEN (Figs. 4 and 5). Because PTEN is a Hif1 α target gene (41), we tested the involvement of TORC2 in Hif1 α expression using rictor siRNA. A pooled siRNA-directed down-regulation of rictor in *TSC2*-null MEFs decreased Hif1 α protein levels (Fig. 6D). siRNA-directed down-regulation of mSin1 in these cells also suppressed Hif1 α expres-

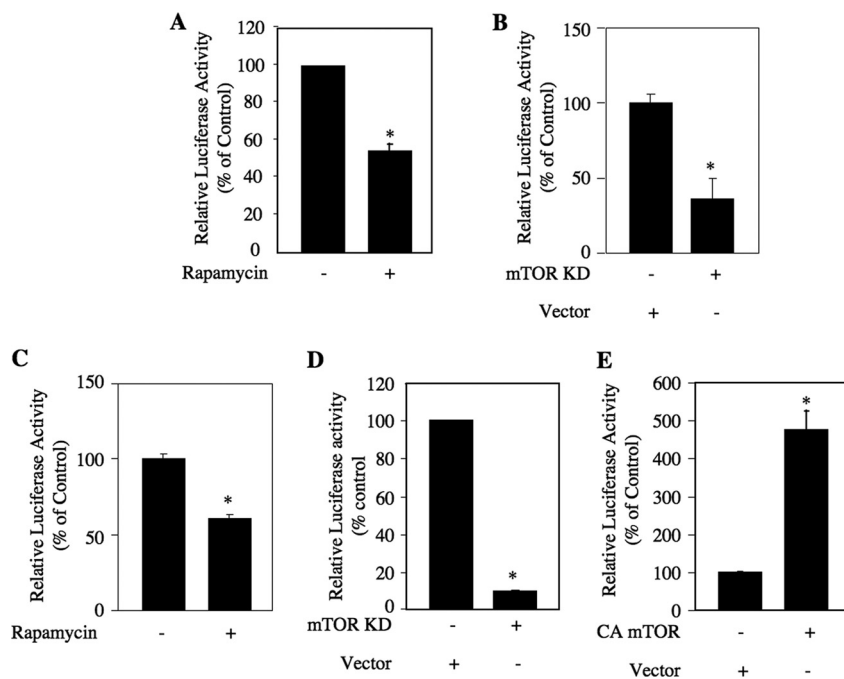


FIGURE 3. mTOR regulates transcription of PTEN in TSC2^{-/-} MEFs and 293 cells. *A* and *B*, TSC2^{-/-} MEFs were transfected with PTEN-Luc along with mTOR KD as indicated. Twenty-four hours post-transfection, the cells were serum-starved for 24 h and treated with rapamycin for 24 h before harvesting (*A*). Luciferase activity was determined in the cell lysates as described under "Experimental Procedures" (41, 44, 45, 65). *C–E*, 293 cells were transfected with PTEN-Luc along with mTOR KD or CA mTOR as indicated followed by incubation in the presence of serum for 48 h (*C* and *D*). The cells in *C* were incubated with rapamycin for 24 h before harvesting. For *E*, 24 h post-transfection, the cells were incubated in serum-free medium for 24 h. The cell lysates were used for luciferase activity assay as described (41, 44, 45, 65). For *A–D*, mean \pm S.E. of 12 measurements is shown. For *A*, *B*, and *D*, * represents $p = 0.001$ versus control. For *C*, * represents $p = 0.002$ versus control. For *E*, mean \pm S.E. of six measurements is shown. *, $p = 0.0007$ versus control.

sion (Fig. 6E). Analogous to these observations, down-regulation of either rictor or mSin1 using two independent shRNA vectors for each protein reduced Hif1 α protein abundance in 293 cells (Figs. 6, F–I). Expression of Hif1 α protein has been ascribed to increased translation of its mRNA due to the presence of a TOP in the 5'-UTR (50, 51). In fact, in TSC2-null MEFs, Hif1 α protein expression is sensitive to rapamycin, indicating a role for TORC1 (supplemental Fig. S6) (49, 50). To test directly the translational mechanism, we used a Hif1 α 5'-UTR-driven reporter plasmid along with a raptor expression vector (42). Overexpression of raptor increased Hif1 α 5'-UTR-mediated reporter activity (Fig. 6J). Interestingly, expression of rictor and both mSin1.1 and mSin1.2 also increased the Hif1 α 5'-UTR-driven luciferase activity (Fig. 6, K–M).

The above results suggest a role of TORC2 in increasing translation from the Hif1 α 5'-UTR. The rate-limiting step in protein synthesis involves phosphorylation of the translation repressor 4EBP-1 by TORC1 (52). We tested the role of TORC2 in phosphorylation of 4EBP-1 using rictor and mSin1 shRNAs. Down-regulation of either rictor or mSin1 inhibited the phosphorylation of 4EBP-1 at Thr-37/46 and Ser-65 (Fig. 7, A–D). Phosphorylation induces dissociation of 4EBP-1 from the eIF4E·4EBP-1 complex to release eIF4E, allowing the eIF4E to associate with eIF4G to form the initiation complex to commence mRNA translation (52). We tested the role of TORC2 in this process using shRNAs targeting rictor and mSin1 in 293 cells. Coimmunoprecipitation experiments revealed that down-regulation of rictor as well as mSin1 using two independent shRNAs for each protein increased the association of eIF4E with 4EBP-1 (Fig. 8, A–D). Reciprocally, immunoprecipitation

tation of eIF4E from rictor shRNA- or mSin1 shRNA-down-regulated samples followed by immunoblotting with 4EBP-1 showed increased association of 4EBP-1 with eIF4E (supplemental Fig. S7, A–D).

Initiation of translation occurs due to binding of the eIF4F complex composed of eIF4E, eIF4G, and eIF4A to the cap of the mRNA. Therefore, we tested the involvement of TORC2 in formation of the eIF4E·eIF4G complex. eIF4E immunoprecipitates from shRictor- or shmSin1-transfected cells using two independent shRNAs for each protein were immunoblotted with eIF4G. Down-regulation of rictor or mSin1 suppressed the formation of the eIF4E·eIF4G complex (Fig. 8, E–H). Reciprocal immunoprecipitation-immunoblotting of the same samples showed similar results (supplemental Fig. S7, E and H). Together these results provide evidence that TORC2 regulates the initiation phase of mRNA translation and support our observation of the increased Hif1 α translation described above.

TORC2 Regulates Hif1 α -dependent PTEN Expression—We have shown previously that expression of PTEN is regulated by Hif1 α (41). Also, we have demonstrated that TORC2 regulates the expression of Hif1 α protein (Fig. 6, F–I). We examined the hypothesis that TORC2-regulated Hif1 α contributes to the expression of PTEN. 293 cells were transfected with either shRictor or shmSin1 plasmids along with the Hif1 α expression vector. As expected, down-regulation of rictor or mSin1 and expression of Hif1 α decreased and increased the levels of PTEN protein, respectively (Fig. 9, A–D). Phosphorylation of Akt at Ser-473 was inhibited due to inhibition of TORC2 and increased PTEN expression. Interestingly, expression of Hif1 α prevented the shRictor- or shmSin1-induced inhibition of

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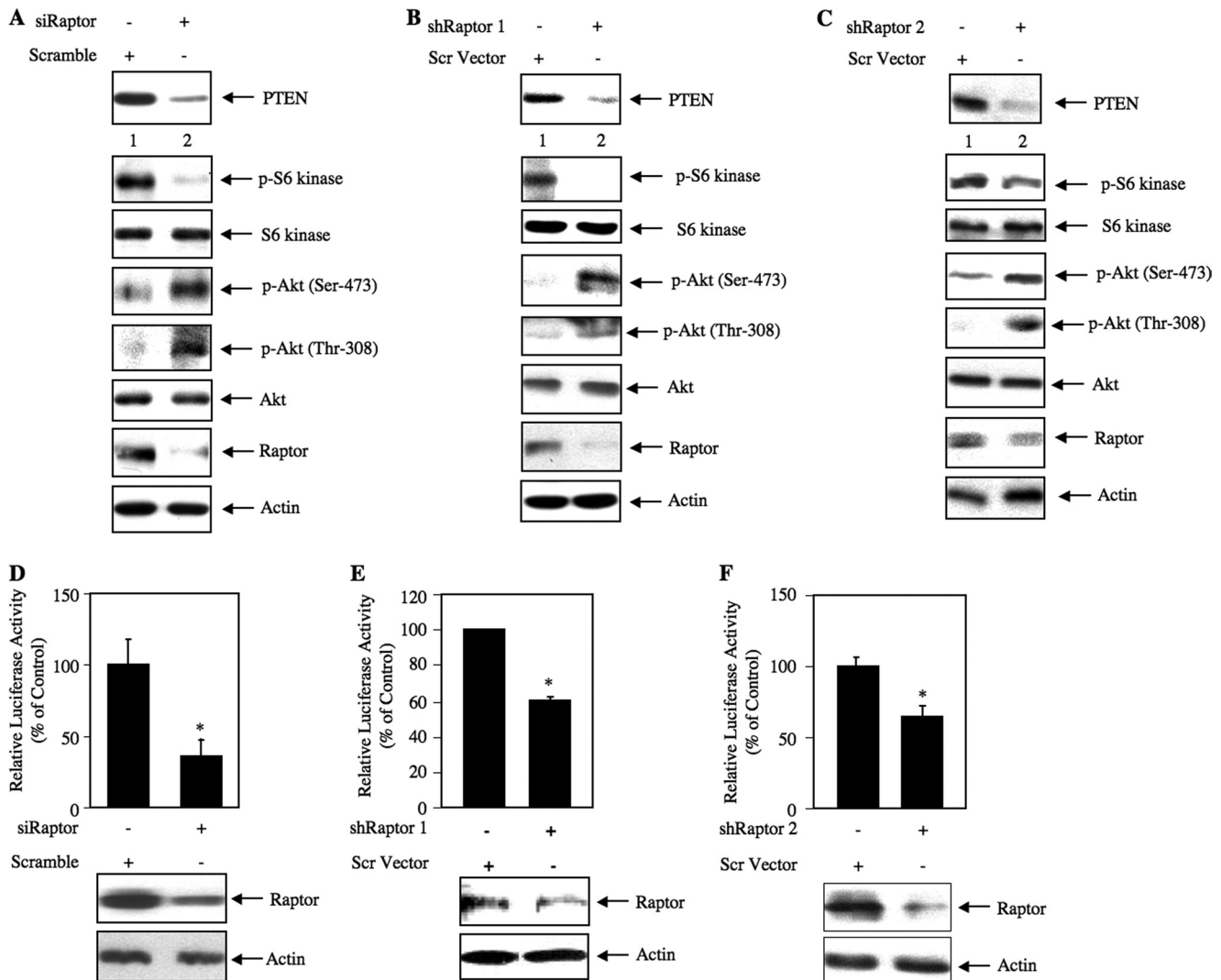


FIGURE 4. Down-regulation of raptor prevents expression of PTEN and increases Akt phosphorylation in $TSC2^{-/-}$ MEFs and 293 cells. *A*, $TSC2^{-/-}$ MEFs were transfected with siRNA pool (20 nM) targeting raptor or scrambled RNA as described under "Experimental Procedures" (41, 44, 45, 65). Twenty-four hours post-transfection, the cells were serum-starved for 24 h. The cell lysates were immunoblotted with the indicated antibodies. *B* and *C*, 293 cells were transfected with two independent shRNA expression plasmids, shRaptor 1 and shRaptor 2, targeting two different regions in the raptor gene (500 ng) or scrambled (*Scr*) vector as described under "Experimental Procedures" (41, 44, 45, 65). Forty-eight hours post-transfection, the cell lysates were immunoblotted with the indicated antibodies. *D–F*, $TSC2^{-/-}$ MEFs (*D*) or 293 cells (*E* and *F*) were transfected with PTEN-Luc along with either an siRNA pool (20 nM) against raptor (*D*) or plasmid expressing shRaptor 1 (*E*) or shRaptor 2 (*F*) (250 ng). Twenty-four hours post-transfection, the $TSC2^{-/-}$ MEFs were serum-starved for 24 h (*D*). In the case of 293 cells (*E* and *F*), the cells were harvested at 48 h post-transfection. The cell lysates were assayed for luciferase activity as described (41, 44, 45, 65). Mean \pm S.E. of 12 measurements is shown for *D* and *E*. For *F*, mean \pm S.E. of six measurements is shown. For *D*, * represents $p = 0.002$ versus control. For *E*, * represents $p = 0.0008$ versus control. For *F*, * represents $p = 0.02$ versus control. The expression of raptor is shown at the bottom of histograms for representative wells. *p*-Akt, phospho-Akt; *p*-S6 kinase, phospho-S6 kinase.

PTEN expression, which resulted in decreased Akt (Ser-473) phosphorylation (Fig. 9, *A–D*, compare lanes 4 with lanes 2). These results indicate that TORC2-mediated expression of Hif1 α regulates PTEN levels.

Next, we tested the involvement of the TORC2-Hif1 α axis in the transcription of PTEN. 293 cells were transiently transfected with PTEN-Luc reporter and shRictor or shmSin1 in the presence of the Hif1 α expression vector. As expected, expression of shRictor or shmSin1 inhibited luciferase activity (Fig. 9, *E–H*). Overexpression of Hif1 α prevented the shRictor- or shmSin1-induced inhibition of reporter activity (Fig. 9, *E–H*). These results conclusively demonstrate that TORC2 contributes to the increased expression of Hif1 α to enhance PTEN expression.

DISCUSSION

This study establishes an important contribution of mTOR activity to the up-regulation of PTEN in $TSC2$ deficiency (Fig. 10). Our results provide the first evidence that both TORC1 and TORC2 regulate the expression of PTEN to control Akt activation in a reciprocal manner. Finally, we demonstrate the involvement of TORC2 in regulating the Hif1 α transcription factor, which results in the increased transcription of PTEN (Fig. 10).

Tumors from TSC patients display increased phosphorylation of S6 kinase and 4EBP-1, which is mimicked by cultured cells lacking $TSC2$, indicating a constitutive activation of TORC1 (25, 49, 53). Elevated TORC1 activity has been recognized as the principal driver of cancer cell proliferation in cul-

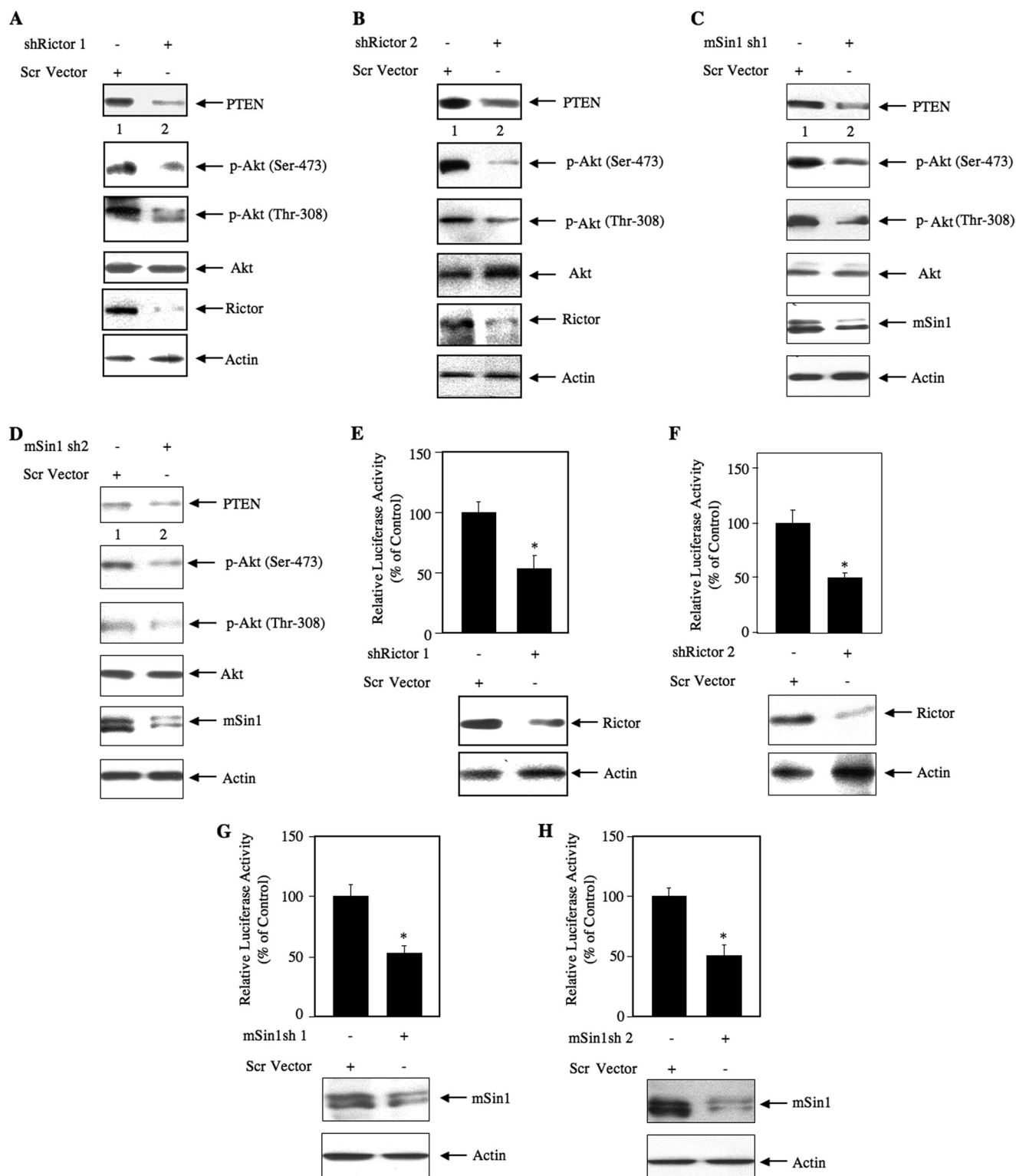


FIGURE 5. Down-regulation of rictor and mSin1 prevents expression of PTEN and decreases Akt phosphorylation in 293 cells. *A* and *B*, 293 cells were transfected with two independent shRNA expression plasmids, shRictor 1 and shRictor 2, targeting two different regions in the rictor gene (500 ng) or scrambled (*Scr*) vector. *C* and *D*, 293 cells were transfected with two independent shRNA expression plasmids, mSin1 sh1 and mSin1 sh2, targeting two different regions in the mSin1 mRNA (500 ng) or scrambled vector. Forty-eight hours post-transfection, the cell lysates were immunoblotted with the indicated antibodies. *E* and *F*, 293 cells were cotransfected with PTEN-Luc along with shRictor 1 (*E*), shRictor 2 (*F*), mSin1 sh1 (*G*), or mSin1 sh2 (*H*) expression vector. The cells were harvested at 48 h post-transfection. The cell lysates were assayed for luciferase activity as described (41, 44, 45, 65). For *E*, mean \pm S.E. of 12 measurements is shown. *, $p = 0.002$ versus control. For *F*, mean \pm S.E. of six measurements is shown. *, $p = 0.03$ versus control. For *G*, mean \pm S.E. of six measurements is shown. *, $p = 0.04$ versus control. For *H*, mean \pm S.E. of six measurements is shown. *, $p = 0.002$ versus control. The expression of rictor and mSin1 is shown at the bottom of the histogram. *p*-Akt, phospho-Akt.

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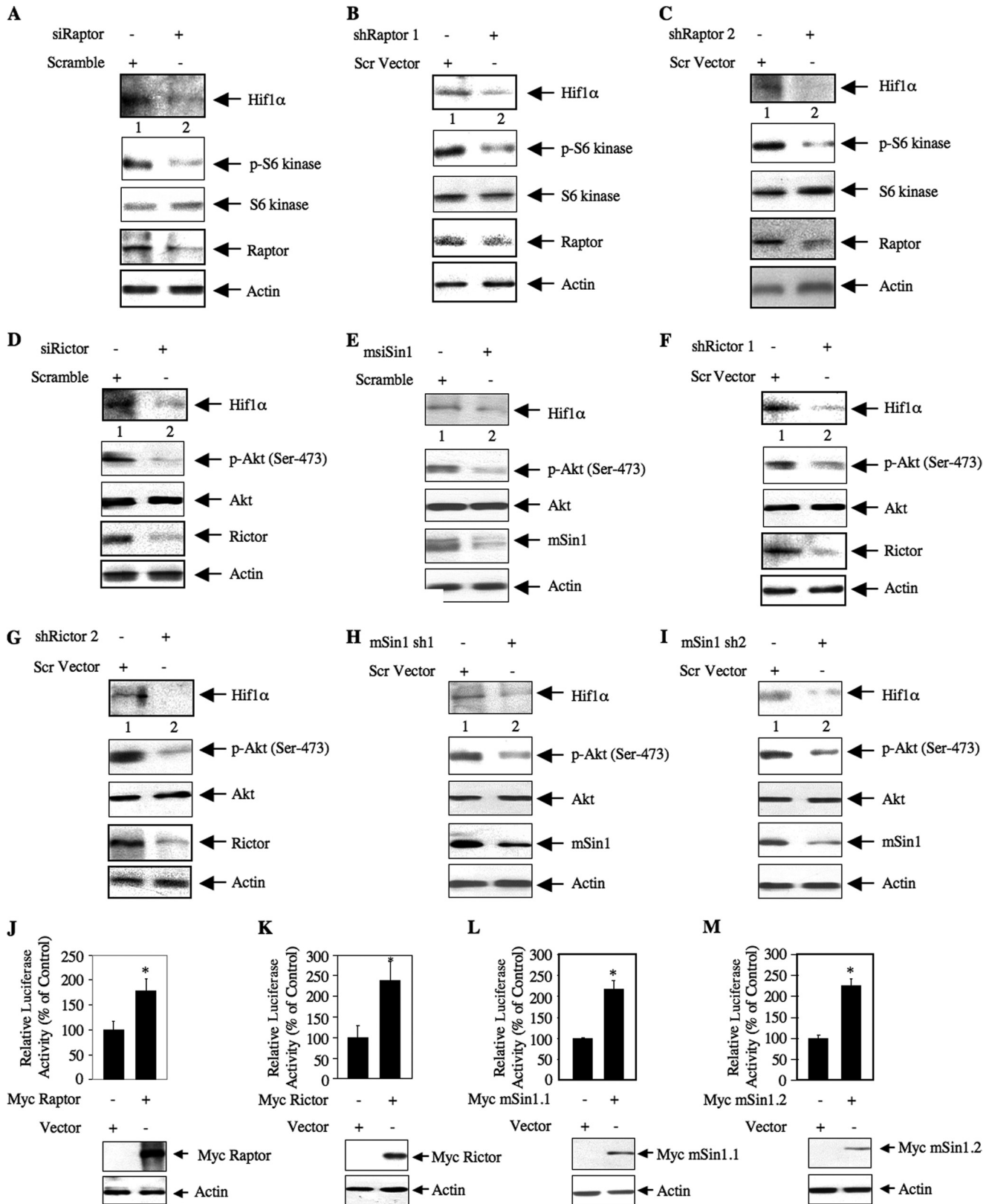


FIGURE 6. TORC1 and TORC2 regulate Hif1 α expression in TSC2^{-/-} MEFs and 293 cells. *A, D, and E*, TSC2^{-/-} MEFs were transfected with siRNAs (20 nM) targeting raptor, rictor, mSin1, or scrambled RNA as described under "Experimental Procedures." Twenty-four hours post-transfection, the cells were serum-starved for 24 h. The cell lysates were immunoblotted with the indicated antibodies. *B, C, F, G, H, and I*, 293 cells were transfected with 500 ng of two independent shRNA expression plasmids, shRaptor 1 (*B*) and shRaptor 2 (*C*), shRictor 1 (*F*) and shRictor 2 (*G*), or mSin1 sh1 (*H*) and mSin1 sh2 (*I*), or scrambled (*Scr*) vector as described under "Experimental Procedures." Forty-eight hours post-transfection, the cell lysates were immunoblotted with the indicated antibodies. *J–M*, Hif1 α 5' TOP-Lux reporter plasmid (42) was cotransfected with either Myc-tagged raptor (*J*), Myc-tagged rictor (*K*), Myc-tagged mSin1.1 (*L*), or Myc-tagged mSin1.2 (*M*) in 293 cells. Luciferase activity was determined in the cell lysates. Mean \pm S.E. of six measurements is shown. For *J*, * represents $p = 0.005$ versus control. For *K*, * represents $p = 0.03$ versus control. For *L*, * represents $p = 0.005$ versus control. For *M*, * represents $p = 0.001$ versus control. *p-Akt*, phospho-Akt; *p-S6 kinase*, phospho-S6 kinase.

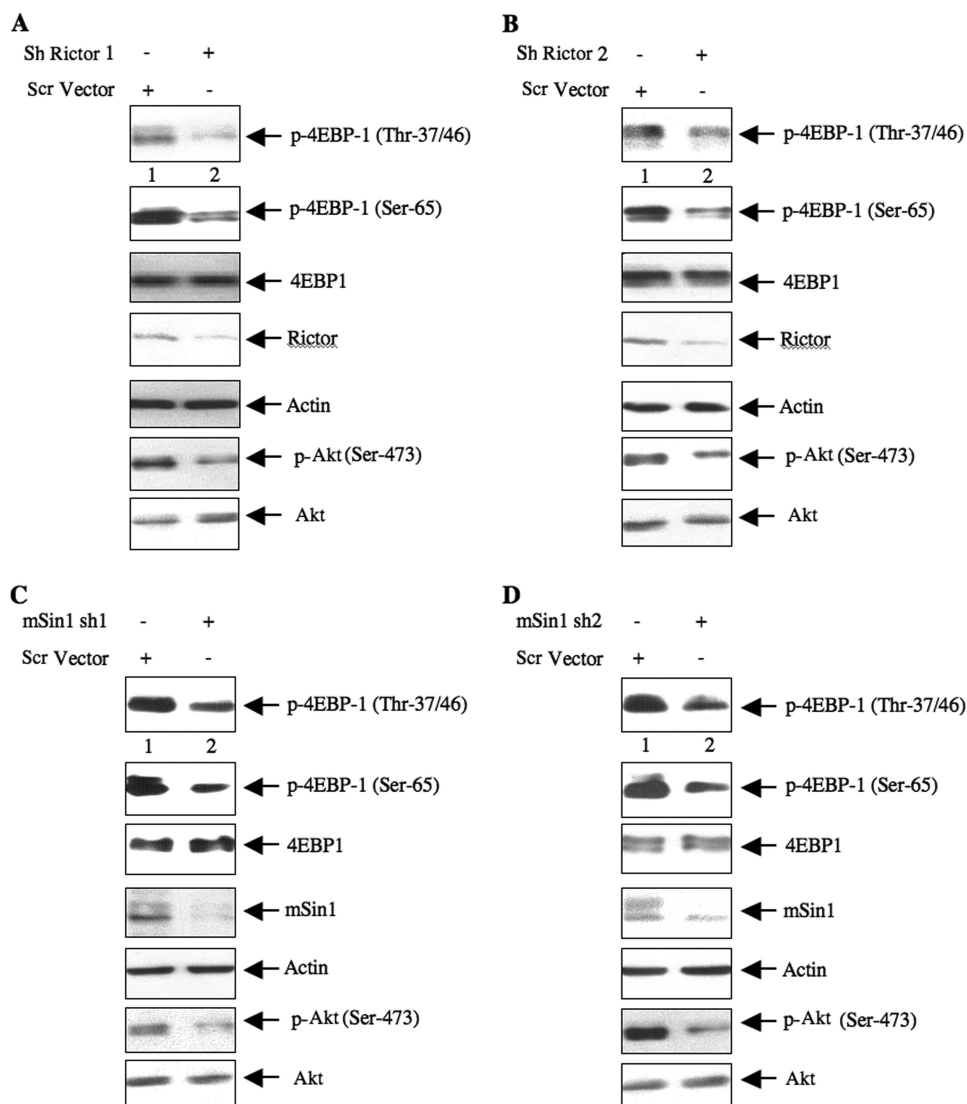


FIGURE 7. Down-regulation of rictor or mSin1 decreases phosphorylation of 4EBP-1. 293 cells were transfected with two independent shRNA plasmids targeting either rictor (A and B) or mSin1 (C and D) or with scrambled (Scr) vector as indicated. The cell lysates were immunoblotted with phospho-4EBP-1 (p-4EBP-1) (Thr-37/46), phospho-4EBP-1 (Ser-65), and 4EBP-1 antibodies. Immunoblotting of the same samples with antibodies against rictor, mSin1, phospho-Akt (p-Akt) (Ser-473), Akt, and actin is also shown.

ture and in animal models (34, 54). Enhanced inactivating TSC2 phosphorylation has been detected in human cancers with elevated mTOR signaling (55). However, recent clinical trials using different mTOR inhibitors showed rather modest antitumor activity only in a minority of patients (56, 57). These disappointing results may be partially explained by the release of the feedback inhibition of the PI 3-kinase pathway similar to that observed in TSC2-null cells involving IRS-1. The increased TORC1-mediated S6 kinase activity reduces the levels of IRS-1, leading to inhibition of PI 3-kinase-dependent Akt phosphorylation (37, 53). Indeed, in TSC2-deficient cells, rapamycin blocks S6 kinase activity and relieves the negative regulation of IRS-1, leading to the restoration of Akt phosphorylation in response to insulin/IGF-1. A recent report demonstrated increased expression and hence tyrosine phosphorylation of multiple receptor tyrosine kinases including members of the human epidermal growth factor receptor family and insulin and IGF-1 receptors in response to chronic Akt inhibition (58).

However, reduced Akt activity in TSC2 deficiency is not sufficient to increase insulin/IGF-1 signaling (37). TSC2 deficiency causes a loss of Akt phosphorylation in response to multiple growth factors such as EGF and PDGF, which do not use IRS-1 to signal (19, 39, 40, 59). These results indicate that multiple feedback mechanisms exist to regulate Akt phosphorylation.

Recently, we have reported that in TSC2 deficiency the level of PTEN is significantly elevated, and this prevents Akt phosphorylation (41). In the present study, we show that treatment of TSC2-null MEFs with rapamycin diminishes PTEN protein with a concomitant increase in Akt phosphorylation at Thr-308 (Fig. 1B). Phosphorylation at this site is mediated by PDK-1 downstream of PIP₃ (12). This observation was also confirmed in growing human 293 cells (Fig. 1D). Together our results show that PTEN gene expression is downstream of TORC1. Note that the incubation of cells with rapamycin was for 24 h, which has been shown to down-regulate TORC2 activity in many cancer cells (29). However, under this incubation condi-

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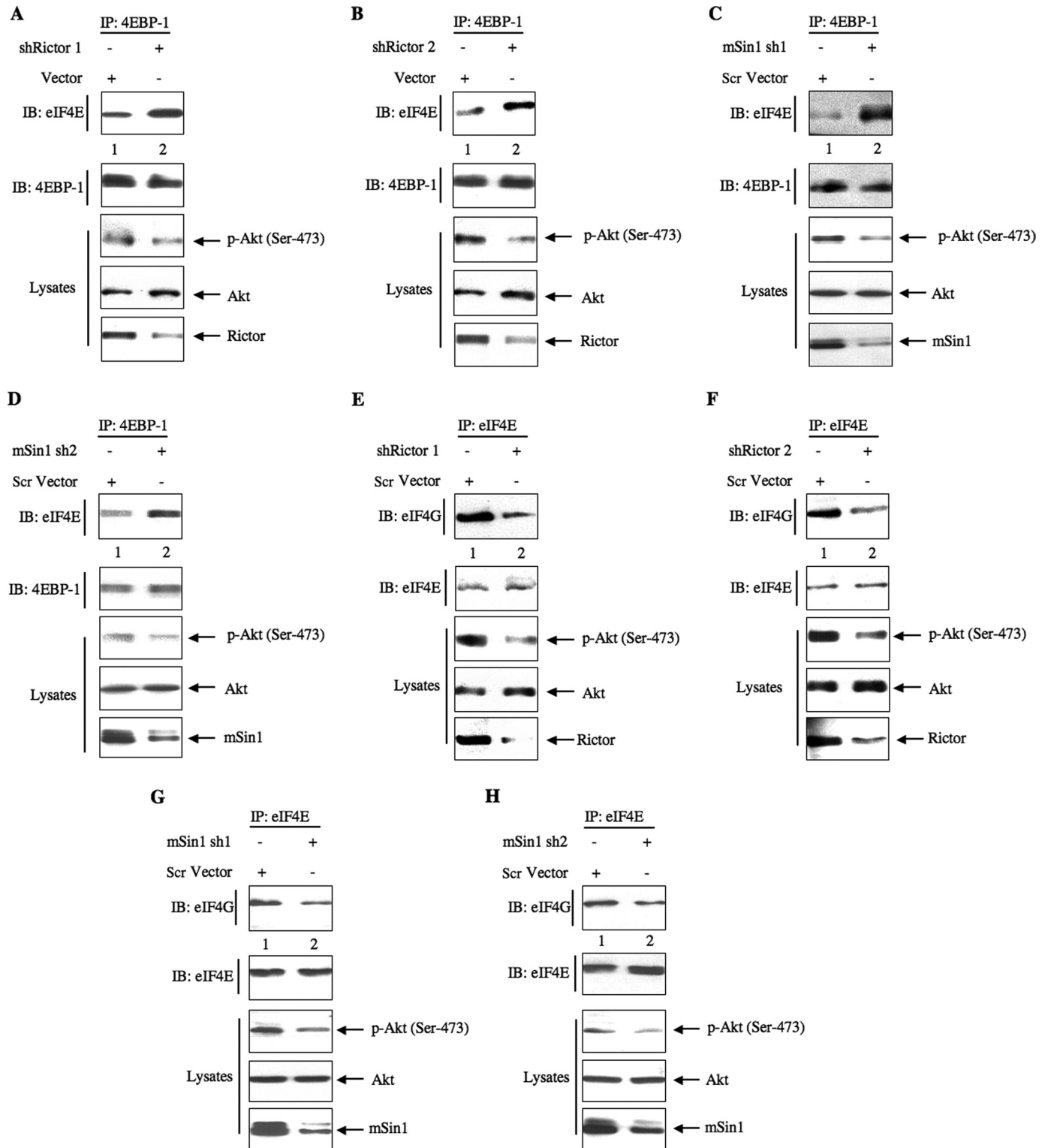


FIGURE 8. TORC2 regulates dissociation of eIF4E-4EBP-1 complex and formation of eIF4E-eIF4G complex. 293 cells were transfected with either shRictor 1 (A and E), shRictor 2 (B and F), mSin1 sh1 (C and G), or mSin1 sh2 (D and H) expression vectors. Forty-eight hours post-transfection, the cells were lysed, and the extracts were immunoprecipitated with 4EBP-1 followed by immunoblotting (IB) with eIF4E and 4EBP-1 antibodies (A–D). E–H, the same cell lysates were immunoprecipitated (IP) with eIF4E followed by immunoblotting with eIF4G and eIF4E antibodies. Phosphorylation of Akt at Ser-473 to demonstrate TORC2 activity in rictor-down-regulated cells and expression of rictor and mSin1 in the cell lysates are shown in the bottom panels. Scr, scrambled; p-Akt, phospho-Akt.

tion, rapamycin acted as a classical TORC1 inhibitor, blocking the phosphorylation of S6 kinase in *TSC2*^{-/-} MEFs and in 293 cells (Fig. 1, B and D). Moreover, rapamycin increased the

hydrophobic site phosphorylation of Akt at Ser-473 concomitantly with PTEN down-regulation (Fig. 1, B and D). It has been shown recently that PIP₃ regulates the activation of TORC2

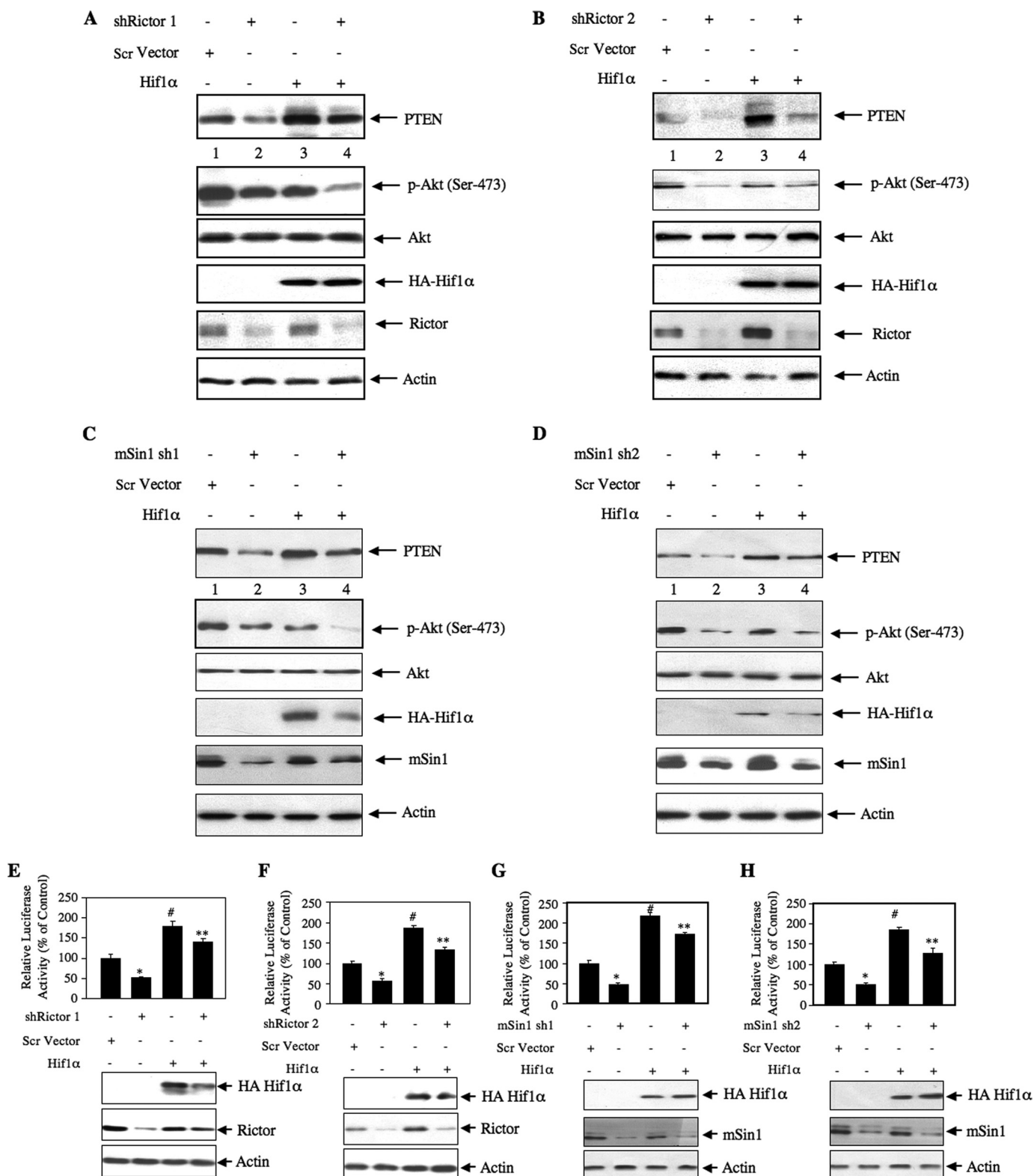


FIGURE 9. TORC2 regulates Hif1α-driven PTEN expression. A–D, 293 cells were transfected with HA-tagged Hif1α along with either shRictor 1 (A), shRictor 2 (B), mSin1 sh1 (C), or mSin1 sh2 (D). Forty-eight hours post-transfection, the cells were lysed, and the lysates were immunoblotted with the indicated antibodies. E and F, TORC2 regulates Hif1α-dependent PTEN transcription. 293 cells were cotransfected with PTEN-Luc and HA-Hif1α along with shRictor 1 (E), shRictor 2 (F), mSin1 sh1 (G), or mSin1 sh2 (H). Forty-eight hours post-transfection, luciferase activity was determined in the cell lysates. Mean ± S.E. of six determinations is shown. In E, * represents $p < 0.01$ versus control, # represents $p < 0.001$ versus control, and ** represents $p < 0.001$ versus shRictor 1 alone. In F, * represents $p < 0.001$ versus control, # represents $p < 0.001$ versus control, and ** represents $p < 0.001$ versus shRictor 2 alone. In G, * represents $p < 0.001$ versus control, # represents $p < 0.001$ versus control, and ** represents $p < 0.001$ versus mSin1 sh1 alone. In H, * represents $p < 0.01$ versus control, # represents $p < 0.001$ versus control, and ** represents $p < 0.001$ versus mSin1 sh2 alone. Scr, scrambled; p-Akt, phospho-Akt.

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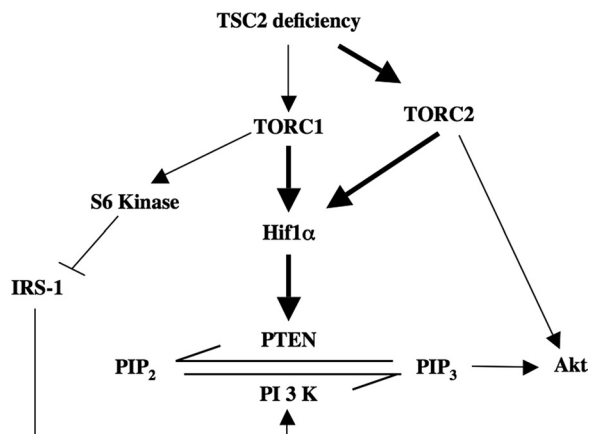


FIGURE 10. **Schema summarizing our results.** The solid arrows indicate the results described in our study. PIP₂, phosphatidylinositol 4,5-bisphosphate.

(60). Our data indicate that due to down-regulation of PTEN the resulting increase in PIP₃ enhances the TORC2 activity to augment the phosphorylation of Akt at Ser-473. Furthermore, rapamycin does not inhibit TORC2 activity.

The results obtained with rapamycin were further strengthened with the use of a kinase-dead mutant of mTOR that mimicked the action of rapamycin on the expression of PTEN and on TORC1 activity in TSC2-deficient MEFs and in 293 cells (Fig. 1, E–H). Because mTOR is the common kinase subunit for both TORC1 and TORC2, kinase-dead mTOR is expected to block the activity of both these kinase complexes. Indeed, we observed inhibition of TORC1 and TORC2 activity in both TSC2^{-/-} MEFs and in 293 cells expressing dominant negative mTOR (Fig. 1, F and H). The phosphorylation of Akt at Thr-308 by PDK-1 is under the direct regulation of PTEN (12). However, this phosphorylation was reported to be regulated by phosphorylation of Ser-473, and the loss of components of TORC2 also diminished the phosphorylation of both these sites in Akt (35, 61, 62). In contrast to these results, we demonstrate increased phosphorylation of Thr-308 in the presence of reduced phosphorylation of Akt at Ser-473 (Fig. 1, F and H). These results support the idea that down-regulation of PTEN by dominant negative mTOR increases the phosphorylation of Akt at Thr-308. Furthermore, inhibition of TORC1 also relieves the negative feedback loop, resulting in increased phosphorylation of Akt at Thr-308.

In contrast to our data obtained with rapamycin and kinase-dead mTOR, as expected, we found increased expression of PTEN when we used a constitutively active mutant of mTOR (Fig. 2, A and B). This mutant increased phosphorylation of S6 kinase due to enhanced TORC1 activity, resulting in the inhibition of Akt phosphorylation (Fig. 2B). Unlike the kinase-dead mutant, constitutively active mTOR did not affect TORC2 activity as we observed no increase in Akt phosphorylation at Ser-473. Rather, the phosphorylation was inhibited in the presence of constitutively active mTOR (Fig. 2B), indicating that this mutant affects only TORC1 activity in cells. These results are consistent with the studies in which Ohne *et al.* (43) established that this constitutively active mutant exclusively up-regulated TORC1 activity in the absence of any effect on TORC2.

Thus, our data point to a specific involvement of TORC1 in the up-regulation of PTEN, which contributes to the decreased

phosphorylation of Akt in TSC2 deficiency. This conclusion is further supported by our results with raptor down-regulation in TSC2-null MEFs and in 293 cells, which show reduced PTEN protein levels in association with increased phosphorylation of Akt at both Thr-308 and Ser-473 (Fig. 4, A–C). The increase in phosphorylation of both these sites may result from attenuation of PTEN, which increases PIP₃ levels to increase PDK-1 and TORC2 activity (12, 60). Another possibility is that due to raptor down-regulation more mTOR kinase may be available for assembly into TORC2 in the absence of TORC1 formation. This will result in increased phosphorylation of Akt at Ser-473 by TORC2.

We described the effect of dominant negative mTOR kinase on the expression of PTEN in 293 cells (Fig. 1H). However, this mutant affects activity of both TORC1 and TORC2 as evident by the reduced phosphorylation of S6 kinase and Akt Ser-473, respectively (Fig. 1, F and H). Consequently, TORC2 along with TORC1 may be involved in the regulation of PTEN expression. Down-regulation of rictor or mSin1 to specifically block TORC2 activity indeed decreased the level of PTEN in 293 cells (Fig. 5, A–D). Interestingly, a decreased level of PTEN was not sufficient to increase the phosphorylation of Akt at Thr-308. This could be due to the fact that rictor or mSin1 down-regulation, which results in the loss of TORC2 formation, leaves more mTOR kinase available to form TORC1. We tested this hypothesis. mTOR immunoprecipitates from rictor- or mSin1-down-regulated 293 cells were immunoblotted with raptor antibody. Independent down-regulation of each of these TORC2 components increased the formation of mTOR-raptor complex, indicating enhanced TORC1 formation (supplemental Fig. S8, A–D). Consequently, down-regulation of rictor or mSin1 increased TORC1 activity as judged by phosphorylation of S6 kinase (supplemental Fig. S9, A–D). Increased TORC1 activity is known to diminish phosphorylation of Akt at Thr-308 due to the presence of a negative feedback loop involving IRS-1 (37, 38, 53). Therefore, this phenomenon may explain our observation of diminished phosphorylation of Akt at Thr-308 under the condition of reduced rictor or mSin1 expression (Fig. 5, A–D).

Our study shows that the effect of TORC1 and TORC2 on up-regulation of PTEN appears to be due to an increased level of its mRNA (Fig. 1, E and G). Multiple transcription factors have been reported to regulate the transcription of PTEN including NFκB, p53, and Egr1 (47, 63, 64). We identified a Hif1α recognition element in the PTEN promoter and showed that this transcription factor regulates the transcription of PTEN in TSC2-null MEFs (41). Previously, it was shown that an increased Hif1α protein level in TSC2-null MEFs is due to the presence of constitutively active TORC1 and enhanced translation of 5' TOP-containing Hif1α mRNA (49, 50). Now we show that both rapamycin and dominant negative mTOR as well as down-regulation of raptor block the PTEN promoter activity in both TSC2^{-/-} MEFs and in 293 cells (Figs. 3 and 4). These results raise the possibility that TORC1-sensitive Hif1α regulates the expression of PTEN. In fact, treatment of the TSC2-null MEFs with rapamycin and siRNA-mediated down-regulation of raptor decreased Hif1α protein levels (Fig. 6A and supplemental Fig. S6), suggesting a role of TORC1 in PTEN

expression (41). Furthermore, in the present study, we demonstrate a contribution of TORC2 to the expression of Hif1 α in TSC2-deficient MEFs and in 293 cells (Fig. 6, D–I). We provide evidence that TORC2 regulates the TOP-mediated translation of Hif1 α due to its effect on mRNA translation initiation (Fig. 6, K–M). Indeed, TORC2 increased the phosphorylation of 4EBP-1 and the formation of the eIF4E·eIF4G complex (Figs. 7 and 8). Moreover, we present direct evidence that TORC2-mediated up-regulation of Hif1 α increases the protein levels of PTEN due to enhanced transcription (Fig. 9). These data also suggest that TORC1 and TORC2 share a common regulatory pathway to control PTEN expression.

Our results demonstrate a reciprocal relationship between TORC1 and TORC2 in regulating the phosphorylation of Akt as evident by the down-regulation of raptor and rictor (Figs. 4, A–C, and 5, A–D). Furthermore, our data show that increased expression of PTEN in TSC2-deficient cells may contribute to a reduced malignant potential of TSC tumors in a cell-autonomous manner. TSC2 phosphorylation and inactivation are evident due to increased growth factor signaling in many cancers. Our results showing rapamycin-mediated down-regulation of PTEN leading to an increase in Akt phosphorylation represent a novel mechanism that may explain why different TORC1 inhibitors are not very effective in cancer clinical trials (56, 57). Finally, in patients with TSC, the efficacy of TORC1 inhibitors may depend upon the PTEN status of their tumors.

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