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Inappropriate Activation of the TSC/Rheb/mTOR/S6K Cassette Induces IRS1/2 Depletion, Insulin Resistance, and Cell Survival Deficiencies

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Summary

Tuberous sclerosis is a largely benign tumor syndrome derived from the acquisition of somatic lesions in genes encoding the tumor suppressor products, TSC1 or TSC2. Loss of function of the TSC1-TSC2 complex, which acts as a Rheb GAP, yields constitutive, unrestrained signaling from the cell growth machinery comprised of Rheb, mTOR, and S6K. We demonstrate herein that constitutive activation of the Rheb/mTOR/S6K cassette, whether by genetic deletion of TSC1 or TSC2 or by ectopic expression of Rheb, is sufficient to induce insulin resistance. This is the result of downregulation of the insulin receptor substrates, IRS1 and IRS2, which become limiting for signal transmission from the insulin receptor to PI3K. Downstream of PI3K, the survival kinase, Akt, is completely refractory to activation by IRS-dependent growth factor pathways such as insulin or IGF-I in TSC1- or TSC2-deficient cells but not to activation by IRS-independent pathways such as those utilized by PDGF. The antiapoptotic program induced by IGF-I but not PDGF is severely compromised in TSC2 null cells. Our results suggest that inappropriate activation of the Rheb/mTOR/S6K pathway imposes a negative feedback program to attenuate IRS-dependent processes such as cell survival.

Results and Discussion

The turnover of IRS1 and IRS2 (*i*nsulin *r*eceptor *s*ubstrates *1* and *2*) is a physiological mechanism to attenuate insulin signaling during prolonged exposure to insulin. This process is mTOR (*m*ammalian *t*arget *o*f *r*apamycin)-dependent, as rapamycin prevents IRS1 and/or IRS2 turnover in multiple cell types treated chronically with insulin [1–3]. We therefore assayed the steady-state abundance of IRS1 and IRS2 in TSC1 and TSC2 (*t*uberous *s*clerosis *c*omplex *1* and *2*) null MEFs (*m*ouse *e*mbryo *f*ibroblasts), which exhibit consititutively elevated mTOR signaling. IRS1, and to lesser extent IRS2, levels were reduced in TSC1- and TSC2-deficient MEFs (Figure 1A), suggesting that IRS1/2 expression is promoted by the TSC1-TSC2 complex. Concordantly, the insulin-induced phosphorylation of Ser473, the Akt hydrophobic motif, is undetectable in TSC1 and TSC2 null MEFs, although the expression of Akt is unaltered (Figure 1A). IRS1/2 expression and phosphorylation of Ser473 are concomitantly rescued to wild-type levels in TSC-deficient cells after a 24 hr treatment with the mTOR inhibitor, rapamycin. The insulin-stimulated in vitro kinase activity of Akt is also similar to wild-type levels in TSC null cells treated with rapamycin (Figure 1B).

If in TSC1 and TSC2 null cells, IRS depletion underlies the Akt activation deficiency, then growth factor pathways dependent upon IRS1/2 should display subnormal Akt activation similar to the insulin pathway, whereas IRS-independent pathways should remain relatively unaffected. Indeed, Akt activation in response to IGF-I (*i*nsulin-like *g*rowth *f*actor-*I*), a pathway in which IRS proteins are obligatory, is also undetectable in TSC-deficient cells (Figures 1C and S1A in the Supplemental Data available with this article online). In contrast, PDGF (*p*latelet-*d*erived *g*rowth *f*actor) treatment, which activates PI3K (*p*hosphatidyl*i*nositol *3*-*k*inase) independently of IRS1/2, induced Akt activation (Figure 1C). However, the extent of Akt activation was reduced in TSC1 and TSC2 null cells compared to wild-type cells (Figure S1B), presumably due to reduced expression of the PDGFR in these cells [4]. In TSC-deficient cells, rapamycin treatment for 24 hr increased Akt activation in response to both insulin and IGF-I to similar extents, whereas rapamycin did not enhance the ability of PDGF to activate Akt (Figure 1C).

In the TSC-deficient state, IRS depletion becomes critical only if IRS1 and IRS2 are reduced to limiting levels, effectively uncoupling the activated InsR from PI3K. Analysis of immunoprecipitates from TSC1 and TSC2 null cells reveals a drastic reduction in p85-associated IRS1, IRS2, and InsRβ compared to wild-type immunoprecipitates (Figure 1D). Moreover, tyrosine phosphorylation within the 150–180 kDa size range is conspicuously absent, reflecting the absence of IRS1 and IRS2 in these complexes (Figure 1D). Tyrosine phosphorylation of InsRβ is normal in TSC-deficient cells (data not shown).

To eliminate the possibility that the lack of IRS1 and IRS2 could arise from random genetic alterations frequently observed as MEFs are propagated in culture, we infected TSC2 null MEFs with wild-type, human TSC2-expressing retrovirus and selected two clonal lines stably expressing TSC2 at levels similar to wild-type MEFs. TSC2 reconstitution of TSC2 null cells restored endogenous levels of both IRS1 and IRS2 in addition to the magnitude and kinetics of Akt activation by insulin (Figure 1E). Furthermore, the basal and insulin-stimulated activation profiles of four effectors of mTOR, S6K1, rpS6, eIF4G, and 4EBP1 were similar between wild-type cells and TSC2 null cells reconstituted with TSC2. These effectors showed constitutively high basal phosphorylation in TSC2 null cells as expected if mTOR signaling was unrestrained. Thus, reintroduction of TSC2 into the TSC2-deficient background returns the cell to a biochemical state indistinguishable from wild-type.

Extended insulin exposure has been shown to induce proteasomal destruction of IRS proteins [1, 5]. To determine whether or not the rate of turnover of IRS1/2 is increased as a consequence of TSC deficiency, we mea-

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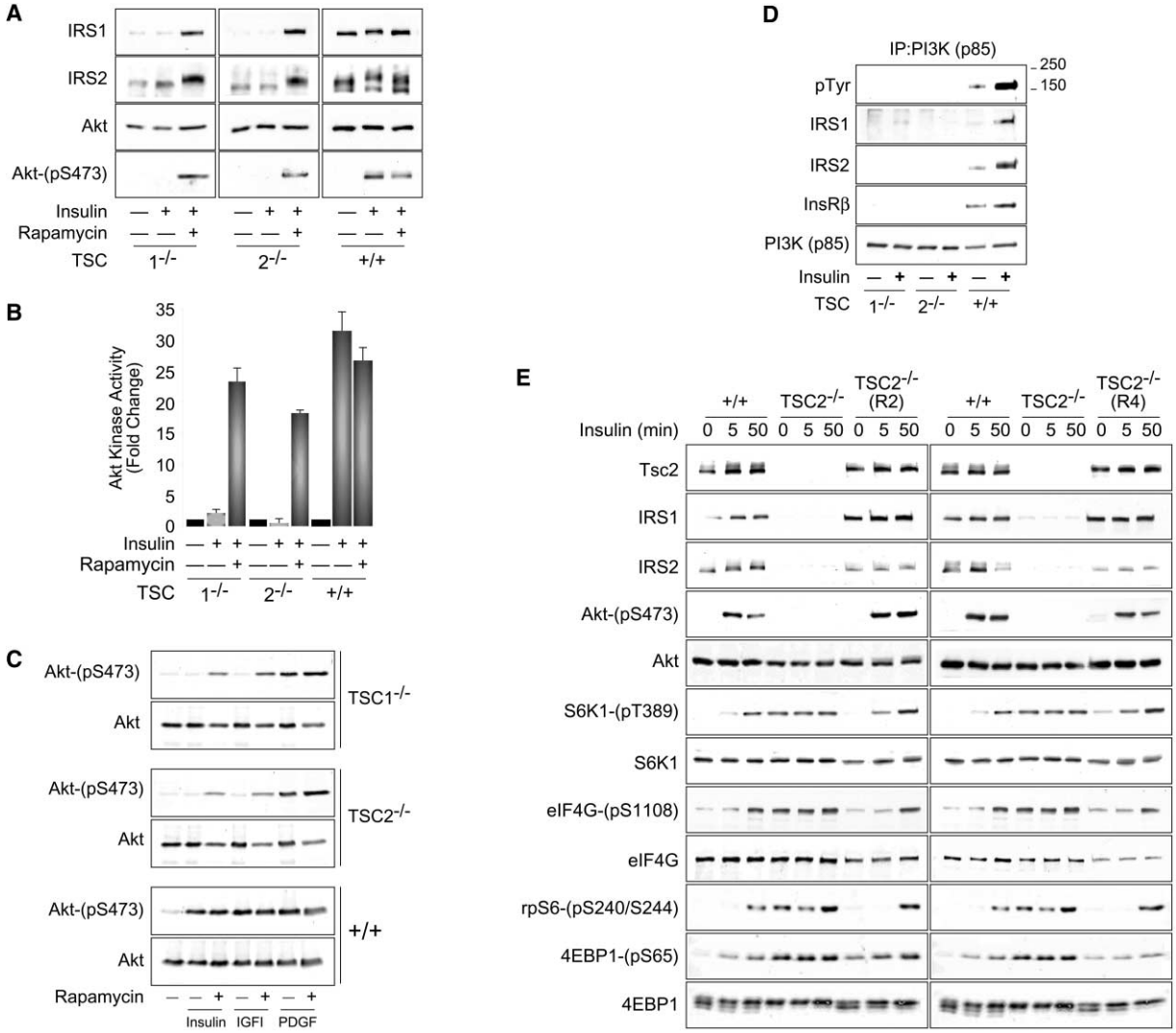


Figure 1. Genetic TSC Deficiency Causes Insulin Resistance

(A) MEFs were cultured in DMEM/0.4% FCS (fetal calf serum) in the presence or absence of 100 nM rapamycin for 24 hr and then stimulated with 200 nM insulin for 5 min. Immunoblot analysis of whole-cell lysates are shown.

(B) MEFs were treated as in (A) followed by immunoprecipitation of Akt. The activity of Akt was determined by in vitro kinase assay using the Akt substrate peptide, RPRAATF. The data presented are means ± standard error for three determinations and are representative of three independent trials.

(C) MEFs were cultured in DMEM/0.4% FCS in the presence or absence of 100 nM rapamycin for 24 hr prior to stimulation with 200 nM insulin, 100 ng/ml IGF-I, or 100 ng/ml PDGF for 5 min. Immunoblot analysis of cell extracts is shown.

(D) Cells were cultured in DMEM/0.4% FCS overnight prior to stimulation with 200 nM insulin for 5 min. The regulatory subunit (p85) of PI3K was immunoprecipitated and immune complexes analyzed by immunoblotting with the indicated antibodies.

(E) TSC2 null MEFs were infected with TSC2-encoding retrovirus, and infected cells were selected with puromycin. Two clonal lines stably reexpressing TSC2 at levels similar to wild-type MEFs were isolated and designated TSC2$^{-/-}$ (R2) and TSC2$^{-/-}$ (R4). Cells were cultured in DMEM/0.4% FCS overnight, then stimulated with 200 nM insulin for the indicated times. Immunoblot analysis of whole-cell extracts is shown.

sured the decay rate of IRS proteins in the absence of protein synthesis and asked whether IRS1 and IRS2 protein could be stabilized by inhibition of mTOR. Cells were pretreated with rapamycin for 24 hr and then cultured in the presence of the protein synthesis inhibitor, cycloheximide, for a subsequent 24 hr period over which IRS1/2 protein decay was monitored. The slow decay rate of IRS1 and IRS2 was unaffected by rapamycin treatment in wild-type cells (Figures 2C and 2F). In contrast, rapamycin reduced the decay rates of IRS1/2 in TSC null cells, indicating that mTOR drives IRS protein turnover in these cells. The IRS1 and IRS2 proteins were inherently less stable in the absence of TSC1 or TSC2 (Figure 2). IRS1 and IRS2 mRNA levels were also reduced in TSC-deficient cells (Figures S2A and S2B), an effect not due, however, to altered mRNA stability (Figures S2C and S2D). Moreover, IRS1/2 mRNA is restored to near-wild-type levels following 24 hr treatment with mTOR inhibitors (Figure S2B). Thus, in the absence of TSC1/2, IRS downregulation is the cumulative effect

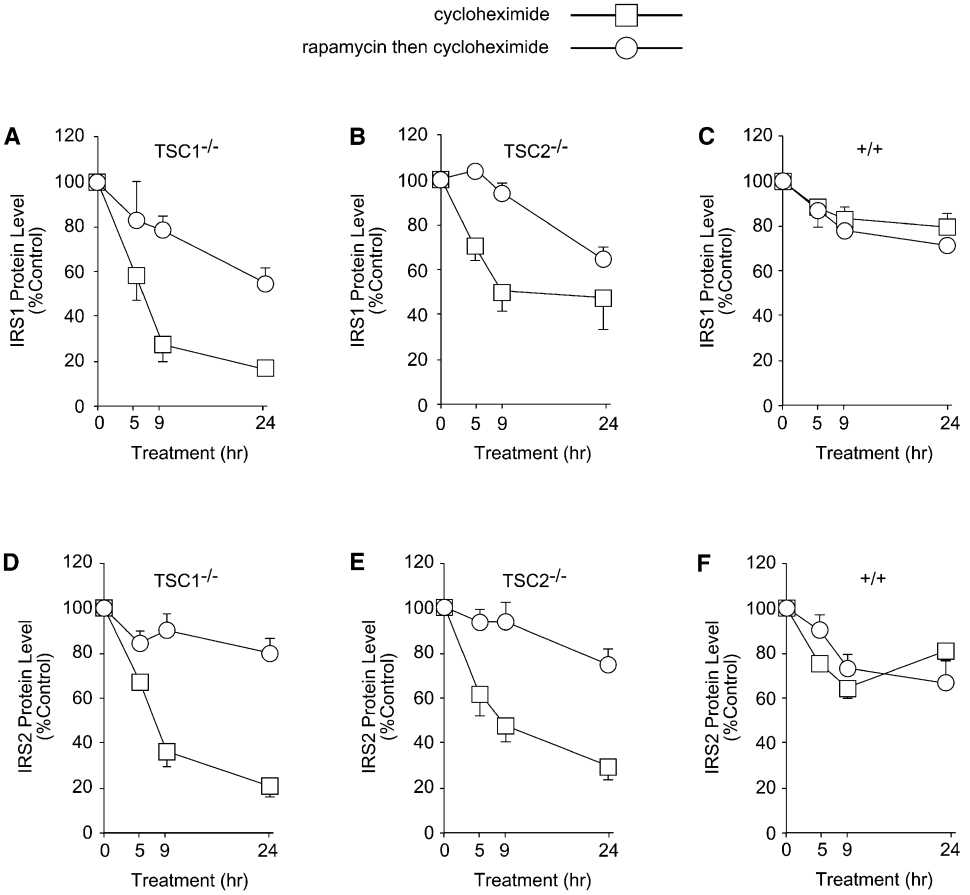


Figure 2. The Degradation of IRS1 and IRS2 Protein Is Accelerated in TSC-Deficient Cells

Cells were cultured in DMEM/10% FCS in the presence or absence of 100 nM rapamycin for 24 hr, then treated with 1 μM cycloheximide for an additional 24 hr. The rate of decay of IRS1 (A–C) or IRS2 (D–F) was monitored by immunoblot analysis and quantified using an Odyssey infrared detection system (Licor). The data are the means ± standard error of three independent experiments.

of reduced mRNA transcription and accelerated protein degradation.

Perturbation of the TSC1-TSC2 complex, either by genetic ablation or by the acquisition of specific disruptive mutations, inhibits the GAP activity of TSC2 toward Rheb (*R*as *h*omolog *e*nriched in *b*rain) [6, 7]. Consequently, Rheb accumulates in the active, GTP bound form and, thus, signals constitutively and unabatedly to mTOR and S6K (ribosomal protein *S6 k*inase). Moreover, owing to relatively limiting intracellular Rheb GAP activity, overexpression of wild-type Rheb leads to a marked increase in GTP loading and, hence, activation of exogenous Rheb [8]. Therefore, cells expressing ectopic Rheb in many ways phenocopy TSC deficiency. Because depletion of IRS levels is a response common to deficiency of either TSC1 or TSC2, we tested whether expression of Rheb was also sufficient to reduce IRS levels. As indicated by GFP expression, HEK293 cells are transfected at 80–90% efficiency under the conditions used in this study (data not shown). Ectopically expressed Rheb reduced the expression of endogenous IRS1 and IRS2 in a dose-dependent manner 72 hr posttransfection (Figure 3A), suggesting that the biological response of constitutive signaling through the mTOR pathway observed in TSC1 and TSC2 null MEFs is recapitulated in HEK293 cells overexpressing wild-type Rheb.

Similar to TSC1 and TSC2 null cells, the activation of Akt by insulin in HEK293 cells expressing Rheb is severely compromised. When Myc-tagged Rheb and HA-tagged Akt are coexpressed, the insulin-induced phosphorylation of Akt on Ser473 and the T loop site, Thr308, is reduced (Figure 3B). In light of the functional conservation between cell types and due to the ease and efficiency with which HEK293 cells can be transfected, this system was suited to dissect the genetic interactions linking Rheb activation to the apparent insulin resistance. Expression of Myc-tagged Rheb with HA-tagged Akt resulted in the complete abrogation of insulin-induced Akt kinase activity detected in HA immunoprecipitates (Figure 3C). Furthermore, whereas the Rheb effector domain mutant, Ile39Lys-Rheb, shows compromised inhibition of Akt, a Rheb mutant with 2- to 3-fold higher activity than wild-type, Gln64Leu-Rheb [7], inhibits Akt as well as wild-type Rheb (Figure 3C). Akt phosphorylation of Thr308 and Ser473 paralleled kinase activity measurements in these experiments (Figure 3C). The effects of wild-type and Gln64Leu-Rheb on phosphorylation of

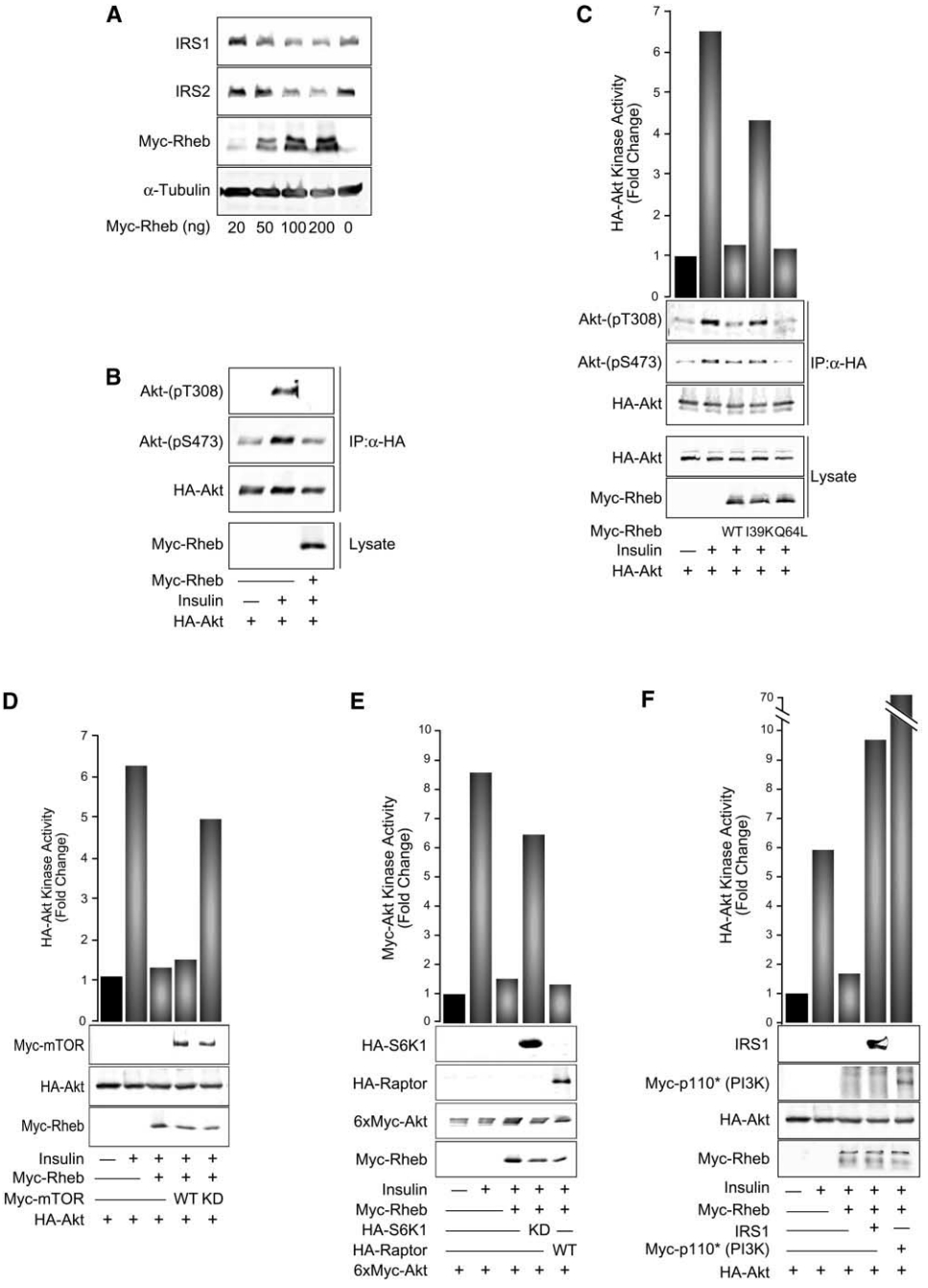


Figure 3. Rheb Reduces IRS1/2 Expression and Represses Akt Activation through mTOR and S6K1

(A) HEK293 cells were transfected with incremental doses of plasmid encoding Myc-Rheb as indicated. 72 hr post-transfection, the expression of endogenous IRS1/2 was monitored by immunoblot analysis of whole-cell extracts.

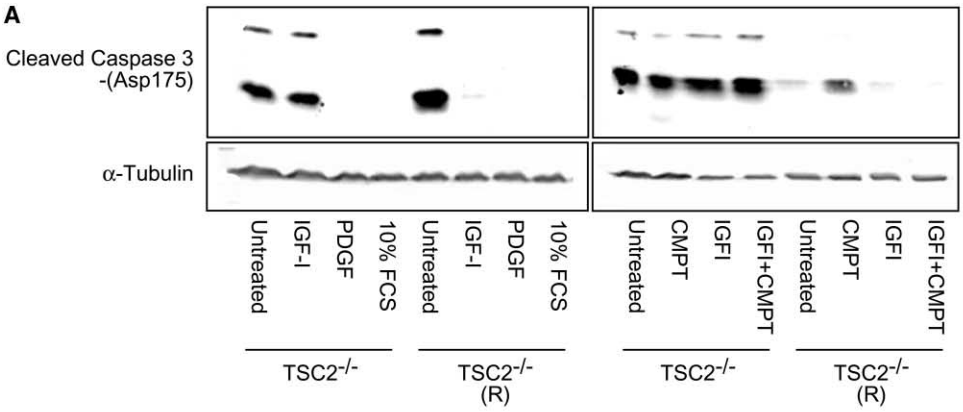
(B) HEK293 cells were transfected with HA-Akt in the presence or absence of Myc-Rheb. 24 hr post-transfection cells were switched to serum-free DMEM and cultured for an additional 48 hr prior to stimulation with 200 nM insulin for 5 min. HA-Akt was immunoprecipitated with anti-HA (12CA5) antibody from whole-cell lysates and subjected to immunoblotting.

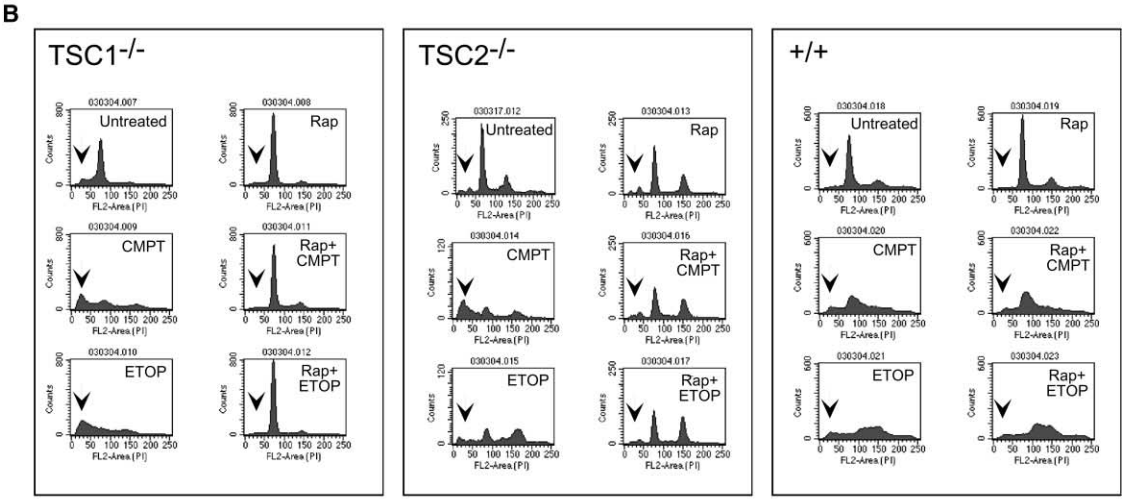
(C) Cells were cultured and transfected as in (B) with either of the following Myc-Rheb constructs: wild-type (WT), Ile39Lys (I39K), or Gln64Leu (Q64L). Akt activity was determined by immunoprecipitation of HA-Akt followed by in vitro kinase assay. Data are the means of duplicate measurements and are representative of three independent trials. Representative immunoblots are shown.

(D) HEK293 cells were cotransfected with Myc-Rheb, HA-Akt with or without wild-type, or kinase-deficient (KD) mTOR. Akt activity was determined in HA immunoprecipitates. Data are the means of duplicate measurements and are representative of three independent trials. Representative immunoblots are shown.

(E) 6×Myc-Akt was coexpressed with Myc-Rheb, kinase-deficient HA-S6K1, or wild-type HA-Raptor. Akt activity was determined following Myc immunoprecipitation. Data are the means of duplicate measurements and are representative of three independent trials. Representative immunoblot analysis of this experiment is displayed.

(F) HA-Akt and Myc-Rheb were transfected with IRS1 or activated, IRS-independent PI3K (Myc-p110*). HA-Akt was immunoprecipitated and assayed for Akt kinase activity. Representative immunoblot analysis of the experiment is presented.





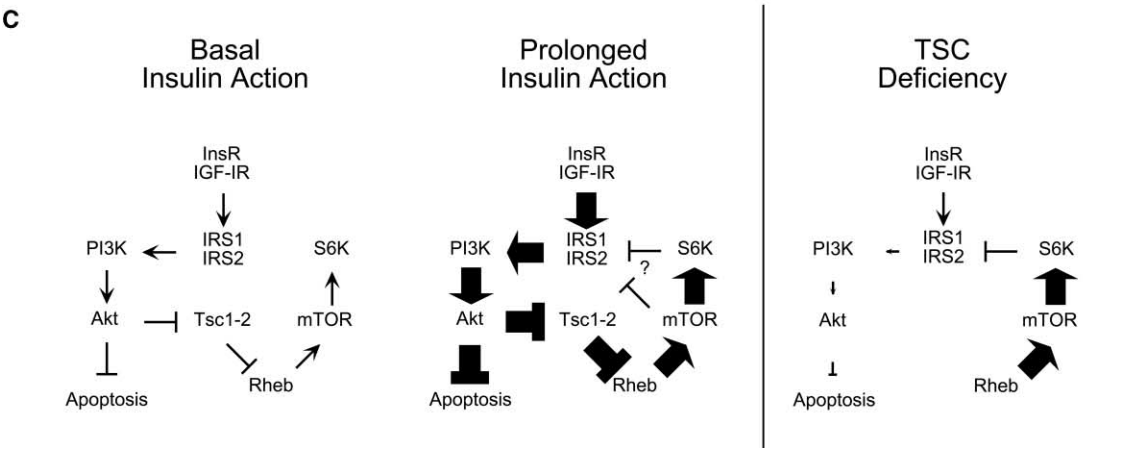


Figure 4. TSC-Deficient Cells Exhibit Compromised Cell Survival

(A) TSC2$^{-/-}$ MEFs or a pooled population of TSC2$^{-/-}$–(R2) and –(R4) (designated TSC2$^{-/-}$[R]) were first placed in serum-free medium to eliminate all growth factors from the medium. Then the indicated growth factors or 10% FCS was added for 6 hr. Immunoblot analysis was carried out on whole-cell lysates (left panel). Alternatively, cells were cultured in medium containing 0.4% FCS overnight, then stimulated for 4 hr with 100 ng/ml IGF-I. Cells were then cultured in the presence of 5 μg/ml camptothecin (CMPT) for an additional 24 hr. Immunoblot analysis is shown (right panel).

(B) Cells were cultured in the presence or absence of 100 nM rapamycin for 24 hr prior to the addition of CMPT or etoposide (ETOP) for an additional 24 hr. Cells were fixed with 70% ethanol, stained with propidium iodide, and subjected to FACS to resolve sub-G1 (apoptotic) populations (designated by arrows).

coexpressed S6K1 and endogenous 4EBP1 are equivalent to or greater than, respectively, insulin-stimulated cells expressing vector alone (Figure S3 and [7]). In contrast, Ile39Lys-Rheb caused little increase in phosphorylation of these mTOR substrates (Figure S3).

The failure of Rheb to inhibit Akt in HEK293 cells expressing catalytically dead mTOR but not wild-type mTOR demonstrates that mTOR participates in this process downstream of Rheb (Figure 3D). Expression of the mTOR binding partner, Raptor, has been shown to inhibit the kinase activity of mTOR and modestly reduce S6K1 phosphorylation [9]. Nevertheless, expression of Raptor failed to influence Rheb inhibition of Akt (Figure 3E). Because insulin activates S6K1 independently of the mTOR-Raptor interaction [9], Raptor's modulation of mTOR may be restricted to nutrient regulation and therefore may not affect the ability of Rheb to repress insulin-induced Akt activity. Kinase-deficient S6K1, however, mitigated the Rheb-induced Akt inhibition to an extent similar to loss-of-function Rheb and kinase-dead mTOR (Figure 3E). Wild-type S6K1 does not affect the ability of Rheb to inhibit Akt (data not shown). Collectively, these data indicate that the intact Rheb/mTOR/S6K pathway is required for Rheb to inhibit Akt.

Constitutive signaling through Rheb, either secondary to genetic TSC deficiency or by overexpression of Rheb itself, mimics the insulin-resistant condition evoked by chronic hyperinsulinemia through constitutive feedback regulation of IRS1/2. Coexpression of either IRS1 or an IRS-independent, active allele of the catalytic subunit of PI3K (p110*) dramatically enhances the activation of Akt in cells overexpressing Rheb (Figure 3F). Thus, by forcing the expression of IRS1 or PI3K ectopically, the negative feedback regulation of IRS signaling is bypassed, as is the dependence of insulin on endogenous IRS1/2 to signal Akt activation.

Because IRS1/2 and Akt support IGF-I-mediated cell survival, we assayed the comparative ability of growth factors to engage survival responses in $TSC2^{-/-}$ and $TSC2^{-/-}$ MEFs reconstituted with TSC2 ($TSC2^{-/-}$[R]). The absence of growth factors induced the cleaved, active form of Caspase 3, a marker of apoptosis in both cell lines (Figure 4A, left panel). In $TSC2^{-/-}$ but not $TSC2^{-/-}$(R) MEFs, however, the addition of IGF-I failed to prevent Caspase 3 activation, whereas PDGF or 10% FCS was effective in either cell line. IGF-I also failed to protect $TSC2^{-/-}$ but not $TSC2^{-/-}$ (R) cells from camptothecin (CMPT)-induced caspase 3 activation (Figure 4A, right panel). Chemosensitivity to both CMPT and etoposide (ETOP) was apparent in logarithmically growing cultures of $TSC1^{-/-}$ and $TSC2^{-/-}$ cells as both chemotherapeutic agents increased the sub-G1 population as shown by FACS (Figure 4B). Furthermore, rapamycin protected TSC1 and TSC2 null cells but not wild-type cells from CMPT- and ETOP-induced cell death. This observation is consistent with TSC deficiency fostering a "dependence" on mTOR signaling, rendering cells exquisitely sensitive to rapamycin. Both the $TSC2^{-/-}$ and wild-type cells used are p53-deficient, whereas the $TSC1^{-/-}$ cells are not (Figure S4). Thus, the sensitivity to CMPT and ETOP can be dissociated from loss of p53 and is specific to TSC deficiency.

Our findings demonstrate that in TSC-deficient cells, mTOR signaling constitutively engages a negative feedback mechanism to regulate IRS1/2 expression, which in normal cells, is engaged by insulin or IGF-I hypersufficiency (Figure 4C). In *Drosophila* larvae, deletion of *d*TSC1 inhibits Akt activity, which can be rescued by codeletion of *d*S6K [10]. Ser473 phosphorylation of Akt is also repressed in $TSC2^{-/-}$ MEFs stimulated with insulin [11]. Our findings are consistent with S6K1 (and perhaps other S6Ks) providing an essential function in IRS1/2 depletion downstream of TSC1-2, Rheb, and mTOR. Consistent with this postulation, S6Ks can also inhibit the ability of IRS1 to signal to PI3K, in part, through direct phosphorylation [12].

We have observed that stable 70–80% depletion of TSC2 by siRNA in HEK293 cells only marginally affects S6K or Akt activation or IRS1/2 abundance (data not shown), indicating that little TSC2 is necessary to restrain mTOR signaling in some cell contexts. Therefore, TSC1 or TSC2 haploinsufficiency, as is manifest in individuals with tuberous sclerosis, may not give rise to generalized diabetes. Rather, insulin resistance may be restricted to specific lesions having undergone loss of heterozygosity at either TSC locus. We suggest that the intrinsic insulin/IGF-I resistance of these lesions could be exploited with tailored interventions, such as chemotherapy, to manage the disorder.

Supplemental Data

Supplemental Data including Experimental Procedures and four additional figures are available at http://www.current-biology.com/cgi/content/full/14/18/1650/DC1/.

Acknowledgments

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(C) Model of regulation of IRS signaling by the TSC1-TSC2 complex. Under basal conditions, activation of the InsR/IGF-IR signals efficiently through PI3K to Akt owing to the abundance of IRS1 and IRS2. As a result, the antiapoptotic and the cell growth machineries are sufficiently engaged. In wild-type cells, prolonged insulin action results in degradation of IRS molecules. This serves to uncouple InsR/IGF-IR activation from PI3K, rendering affected cells refractory to subsequent stimulation by growth factors that require the IRS system. Because this state of insulin resistance is reversed upon mTOR inhibition, mTOR or some distal component (e.g., S6K1) mediates this response. TSC deficiency is characterized by constitutive, unrestrained signaling through the Rheb/mTOR/S6K cassette resulting in the sustained depletion of IRS molecules. Not only are TSC-deficient cells refractory to insulin stimulation, but they also display IGF-I-mediated cell survival deficiencies consistent with insulin resistance. Thus, inactivation of TSC1 or TSC2 mimics the cellular response to prolonged insulin action observed in wild-type cells.

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Note Added in Proof

In the IEP (immediate early publication) version of this paper published online August 12, 2004, reference [12] was cited as personal communication but had been published online July 12, 2004.