

SCIENTIFIC REPORTS



OPEN

Inhibition of ERK1/2 Restores GSK3 β Activity and Protein Synthesis Levels in a Model of Tuberous Sclerosis

Rituraj Pal^{1,2}, Vitaliy V. Bondar², Carolyn J. Adamski², George G. Rodney^{1,4,5} & Marco Sardiello^{2,3}

Tuberous sclerosis (TS) is a multi-organ autosomal dominant disorder that is best characterized by neurodevelopmental deficits and the presence of benign tumors. TS pathology is caused by mutations in tuberous sclerosis complex (TSC) genes and is associated with insulin resistance, decreased glycogen synthase kinase 3 β (GSK3 β) activity, activation of the mammalian target of rapamycin complex 1 (mTORC1), and subsequent increase in protein synthesis. Here, we show that extracellular signal-regulated kinases (ERK1/2) respond to insulin stimulation and integrate insulin signaling to phosphorylate and thus inactivate GSK3 β , resulting in increased protein synthesis that is independent of Akt/mTORC1 activity. Inhibition of ERK1/2 in *Tsc2*^{-/-} cells—a model of TS—rescues GSK3 β activity and protein synthesis levels, thus highlighting ERK1/2 as a potential therapeutic target for the treatment of TS.

Tuberous sclerosis (TS), a hereditary autosomal syndrome caused by defects in either *TSC1* or *TSC2* genes, is characterized by a wide spectrum of clinical manifestations in multiple organs^{1–3}. Molecular pathology of TS includes resistance to insulin signaling, hyper-phosphorylation and thus inactivation of GSK3 β , aberrant activation of mTORC1, and elevated levels of protein synthesis^{4,5}. *TSC1* and *TSC2* form a heterodimeric tumor suppressor complex that negatively regulates the activity of mTORC1, a central hub for the regulation of protein synthesis, cell growth and proliferation^{2,6}. Several upstream signaling pathways that sense growth factors and energy levels converge to *TSC2* and determine its activation status, thus resulting in the modulation of the activity of mTORC1 and thereby of the rate of protein synthesis⁷. Loss of *TSC1* or *TSC2* protein indeed results in constitutively active mTORC1 and elevated levels of protein synthesis⁵.

mTORC1 is a highly conserved regulator of transcription, ribosomal biogenesis, translation and cell growth and is one of the most highly integrated signaling nodes present in all cell types^{8,9}. In response to amino acids, mTORC1 is recruited to lysosomes where it is fully activated by its potent and essential direct activator, Rheb guanosine triphosphatase (Rheb GTPase)¹⁰. Upon insulin stimulation, PI3K activates protein kinase B (Akt/PKB), which then directly phosphorylates *TSC2* resulting in the dissociation of the *TSC1/2* complex from lysosomes where mTORC1 activation takes place². Because *TSC2* acts as a GTPase-activating protein (GAP) specific for Rheb, Akt inhibition of *TSC2* promotes Rheb-GTP-dependent mTORC1 activation¹¹. In cells lacking *TSC2*, mTORC1 and its substrates remain constitutively active and insensitive to insulin availability, which, in turn, results in hyperactivation of the translational machinery.

Glycogen synthase kinase 3 (GSK3) is implicated in multiple biological processes including regulation of protein synthesis, cell proliferation and survival^{12–14} and is found to be phosphorylated and inactivated in multiple cancer types^{15,16}. GSK3 activity is inhibited through phosphorylation of serine 21 in GSK3 α and serine 9 in GSK3 β ¹⁷. Upon insulin stimulation, Akt-mediated phosphorylation of GSK3 β inhibits GSK3 β activity and

¹Department of Molecular Physiology and Biophysics, Baylor College of Medicine, One Baylor Plaza, Houston, TX, 77030, USA. ²Department of Molecular and Human Genetics, Baylor College of Medicine, One Baylor Plaza, Houston, TX, 77030, USA. ³Jan and Dan Duncan Neurological Research Institute, Texas Children's Hospital, Houston, TX, 77030, USA. ⁴Cardiovascular Research Institute, Baylor College of Medicine, One Baylor Plaza, Houston, TX, 77030, USA. ⁵Center for Space Medicine, Baylor College of Medicine Bioscience Research Collaborative, Houston, TX, 77030, USA. Correspondence and requests for materials should be addressed to M.S. (email: sardiell@bcm.edu)

causes its substrate, eukaryotic initiation factor 2B (eIF2B), to become dephosphorylated and thus activated^{17–19}. Insulin-dependent activation of GSK3 β -eIF2B pathway therefore stimulates activation of protein synthesis²⁰. Recent studies have shown that GSK3 phosphorylation is also sensitive to mTORC1 activity^{4,21}. In cells lacking TSC2, where mTORC1 remains constitutively active and resistant to insulin stimulation, GSK3 β is phosphorylated by S6K1, a direct downstream substrate of mTORC1⁴.

Extracellular signal-regulated kinases (ERK1/2) have long been implicated in protein synthesis^{22,23}. The growth factors, EGF and IGF1, stimulate a kinase cascade leading to the activation of ERK1/2 and Ras, which then transmit the upstream signals to TSC1/2 by specific phosphorylation events^{24,25} to activate mTORC1. Previous studies have shown that ERK1/2 integrates IGF1 signal to phosphorylate and inactivate GSK3 β in an Akt-independent manner^{26,27}. Together, these findings suggest that ERK1/2 could possibly play a significant role in insulin regulation of GSK3 β activity and protein synthesis in TS. In this study, we show that insulin activation of ERK1/2 is necessary for insulin-mediated phosphorylation and inactivation of GSK3 β . We demonstrate that insulin-mediated activation of ERK1/2 results in phosphorylation of GSK3 β at S9 independently of Akt/mTORC1 activity in *Tsc2* null mouse embryonic fibroblasts. In addition, we show that inhibition of ERK1/2 rescues GSK3 β activity and restores protein synthesis in *Tsc2*^{-/-} MEFs to normal levels. Together, these findings highlight ERK1/2 as a potential therapeutic target for the treatment of tuberous sclerosis.

Results

Insulin regulation of ERK1/2 is independent of Akt/mTORC1 signaling. TS pathology is characterized by constitutively active mTORC1 that is insensitive to insulin availability. To test the effects of the manipulation of ERK activity in a model of TS, we first investigated the interplay between ERK and insulin regulation of Akt-mTORC1 signaling. Time- and dose-dependency studies have previously established that an optimal activation of the Akt/mTORC1 pathway in human cell lines or mouse embryonic fibroblasts (MEFs) can be obtained by stimulating serum-depleted cells with 1 μ M insulin for 15 min². Using similar conditions, we found that insulin activates ERK1/2 (hereafter referred as ERK) activity in HEK-293 cells (hereafter referred as HEK) (Fig. 1A). Consistent with previous studies², we found that a 15-min period of insulin stimulation resulted in a significant increase in phosphorylation of Akt and the Akt TSC2 target residue S939 (Fig. 1B). Accordingly, robust activation of mTORC1 was observed in these conditions. Pharmacological inhibition of ERK with U0126^{28,29} selectively suppressed the insulin-mediated activation of ERK without altering the phosphorylation levels of Akt, TSC2 or S6K1 (Fig. 1B). As expected, inhibition of Akt by MK2206² or of mTORC1 by rapamycin (Rapa)³⁰ abolished insulin-mediated activation of mTORC1. Insulin stimulates mTORC1 activity by promoting the dissociation of TSC2 from lysosomes *via* Akt-mediated phosphorylation of TSC2². A previous study reported that ERK also phosphorylates TSC2, but at an amino acid residue that is different from the Akt target sites²⁵. To test whether insulin-mediated activation of ERK controls spatial localization of TSC2, we performed confocal microscopy analysis of HEK cells (Fig. 1C) and MEFs (Fig. 1D) using antibodies specific to TSC2 and the lysosomal markers, LAMP1 and LAMP2. Consistent with previous studies, we observed that TSC2 was predominantly localized at lysosomes in serum-starved cells^{2,10}, which was significantly reduced upon insulin stimulation. Notably, inhibition of ERK by U0126 was unable to prevent insulin-induced dissociation of TSC2 from lysosomes (Fig. 1C,D). As a control, inhibition of Akt significantly prevented insulin-induced dissociation of TSC2 from lysosomes. Genetic inhibition of ERK using small interfering RNAs (Fig. 1E) significantly decreased the levels of total and phospho-ERK but, similar to ERK pharmacological inhibition, resulted in no changes in insulin-stimulated mTORC1 activity (Fig. 1F). Together, these results demonstrate that insulin regulation of ERK is independent of Akt/mTORC1 activity and that ERK activation does not alter Akt/mTORC1 signaling.

ERK1/2 regulate insulin-dependent protein synthesis independently of Akt/mTORC1 signaling. TS pathology is associated with constitutively active Akt-mTORC1 signaling pathway that induces elevated levels of protein synthesis. We tested whether ERK regulates protein synthesis in an Akt-mTORC1 independent manner. To test this hypothesis, we used SUNSET, a nonradioactive puromycin end-labeling assay^{31–34}. Immunoblot analysis in HEK cells stimulated with insulin, which activates Akt-mTORC1 signaling, showed a significant increase in protein synthesis compared to starved cells (Fig. 2A). Importantly, the insulin-dependent increase in protein synthesis was significantly diminished by ERK inhibition, similar to control experiments conducted by inhibiting Akt (Fig. 2B) or mTORC1 (Fig. 2C) activities. Together with our findings that insulin-mediated activations of ERK and Akt/mTORC1 are independent of each other, these results indicate that ERK regulates insulin-mediated protein synthesis in an Akt/mTORC1-independent manner.

Insulin regulates ERK1/2 activity in *Tsc2*^{-/-} cells. Based on these results, we hypothesized that ERK activity remains sensitive to insulin in TS. To test this hypothesis, we used *Tsc2*^{-/-} MEFs, where mTORC1 is constitutively active and resistant to insulin. In wild-type MEFs (*Tsc2*^{+/+}), insulin significantly activated ERK, Akt and mTORC1, and neither Akt nor mTORC1 was suppressed by inhibition of ERK (Fig. 3). In these cells, insulin-mediated activation of Akt-mTORC1 was abolished upon Akt inhibition. In *Tsc2*^{-/-} MEFs, the activity of mTORC1 was insensitive to inhibition of either ERK or Akt (Fig. 3), however, insulin stimulation was able to activate ERK. Consistent with earlier observations indicating that mTORC1 shows feedback inhibition of PI3K-Akt pathway in *Tsc2*^{-/-} cells⁴, we also found that Akt activity was decreased in *Tsc2*^{-/-} cells compared to *Tsc2*^{+/+} cells. Interestingly, a similar decrease was also observed for ERK activity, suggesting that mTORC1 could possibly show feedback inhibition of ERK in conditions of insensitivity to insulin such as the absence of TSC2. Collectively, these results indicate that insulin is able to regulate ERK activity in a model of TS.

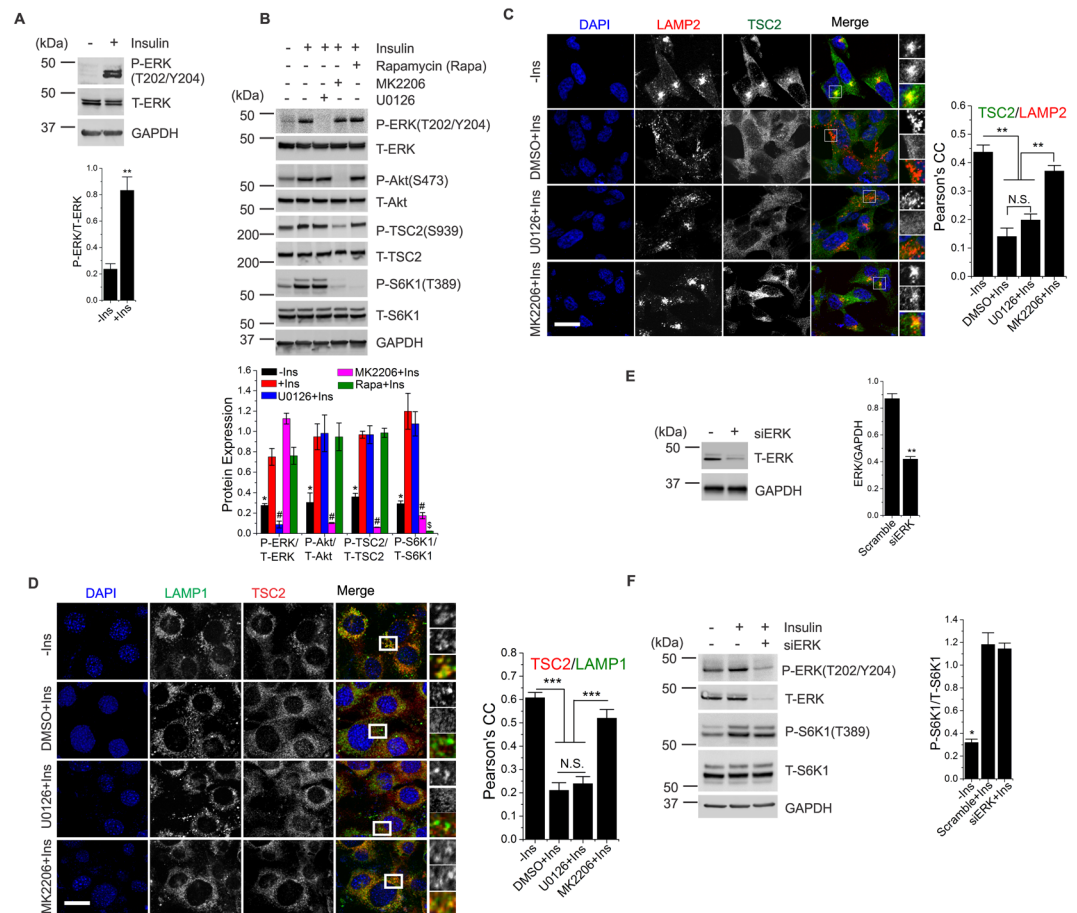


Figure 1. Insulin regulation of ERK and Akt/mTORC1 are independent of each other. **(A)** HEK cells were starved of serum (16 h) prior to insulin stimulation (1 μ M, 15 min). Cell lysates were probed with antibodies as indicated. Quantification of three independent experiments is reported in the bar diagrams. **(B)** HEK cells were starved of serum (16 h) and treated with indicated drugs for 2 h prior to insulin stimulation (1 μ M, 15 min). Lysates were analyzed by immunoblot assay using antibodies as indicated. Quantification of three independent experiments is reported in the bar diagrams. *, # and § indicate significant differences between all conditions in each group. **(C)** HEK cells were treated as in **B** prior to immunofluorescent labeling of endogenous LAMP2 (red) and TSC2 (green). Bar indicates 30 μ m. **(D)** *Tsc2*^{+/+} MEFs were treated as in **B** prior to immunofluorescent labeling of endogenous LAMP1 (green) and TSC2 (red). Bar indicates 20 μ m. Representative cells are shown in **C** and **D**, where yellow or orange pixels indicate co-localization in the merged images. Zoomed images represent the area under the white box. Quantification of three independent experiments and at least 30 cells is reported in the bar diagrams in **C** and **D**. **(E)** HEK cells were transfected with siRNA targeted to *ERK* (or scrambled controls) for 72 h prior to cell lysis. Lysates were probed with antibodies as indicated. **(F)** HEK cells transfected with *siERK* or scramble for 56 h prior to 16 h-serum starvation followed by insulin stimulation (1 μ M, 15 min) where indicated. Lysates were probed with antibodies as indicated. Quantification of three independent experiments is reported in the bar diagrams in **E** and **F**. All quantitative analyses are reported as mean \pm SEM with a significance level of * p < 0.05, ** p < 0.01, *** p < 0.001, # p < 0.05 and § p < 0.01. N.S. indicates not significant. GAPDH was used as a loading control in all immunoblot assays. Cropped blots from full-length gels are displayed in **A**, **B**, **E** and **F**.

ERK1/2 mediates insulin regulation of GSK3 β activity in an Akt/mTORC1-independent manner. GSK3 β is a key regulator that modulates global protein synthesis by regulating components of eukaryotic translation initiation²⁰. Upon insulin stimulation, Akt-mediated phosphorylation and suppression of GSK3 β activity¹⁷ results in dephosphorylation and subsequent activation of the GSK3 β substrate, eukaryotic initiation factor 2B (eIF2B)^{17,18}. Active eIF2B promotes protein synthesis by stimulating translation initiation^{20,35}. Our data indicate that ERK regulates insulin-induced protein synthesis in an mTORC1-independent manner. We then investigated whether ERK regulates protein synthesis in *Tsc2*^{-/-} cells through inactivation of GSK3 β . To test this possibility, we first transfected HEK cells with myc-tagged wild-type GSK3 (Myc-GSK3 β -WT) or constitutively active GSK3 (Myc-GSK3 β -S9A) constructs³⁶. Immunoblot analysis of cells transfected with constitutively active GSK3 β showed a significant decrease in protein synthesis (Fig. 4A). We next examined whether ERK regulates insulin-mediated phosphorylation and inactivation of GSK3 β in an Akt/mTORC1 independent manner. Our results showed that insulin-induced phosphorylation of GSK3 β was significantly reduced upon ERK inhibition by U0126 (Fig. 4B);

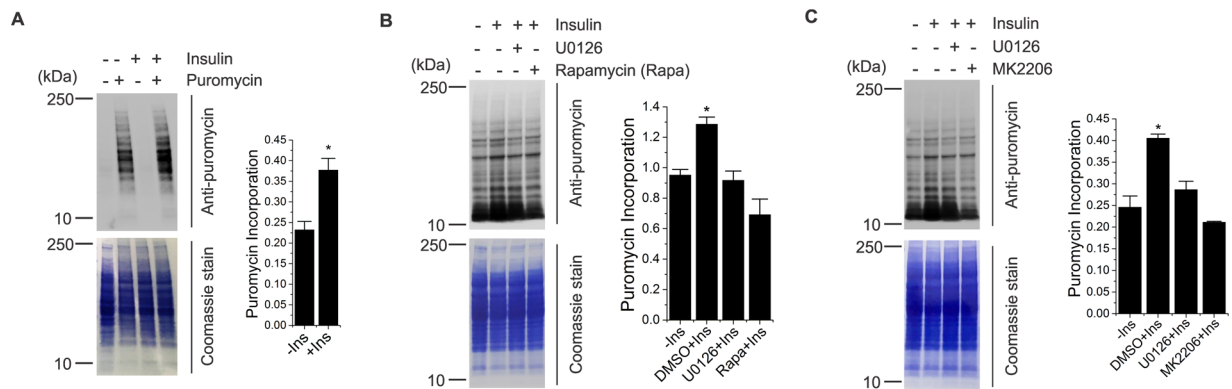


Figure 2. ERK regulates insulin-stimulated protein synthesis. **(A)** HEK cells were starved of serum (16 h) prior to insulin stimulation (1 μ M, 15 min). Cells were then incubated with puromycin for 30 min followed by cell lysis. Lysates were probed with an antibody to puromycin. Blot was stained with coomassie at the end of the immunoblot assay. **(B and C)** HEK cells were starved of serum (16 h) and treated with indicated drugs for 2 h prior to insulin stimulation (1 μ M, 15 min). Cells were then incubated with puromycin for 30 min followed by cell lysis. Lysates were probed with an antibody to puromycin. Blot was stained with coomassie at the end of the immunoblot assay. Quantifications of three independent experiments are reported in the bar diagrams in A, B and C. All quantitative analyses are reported as mean \pm SEM with a significance level of $*p < 0.05$. *Indicates significant differences between all conditions in B and C.

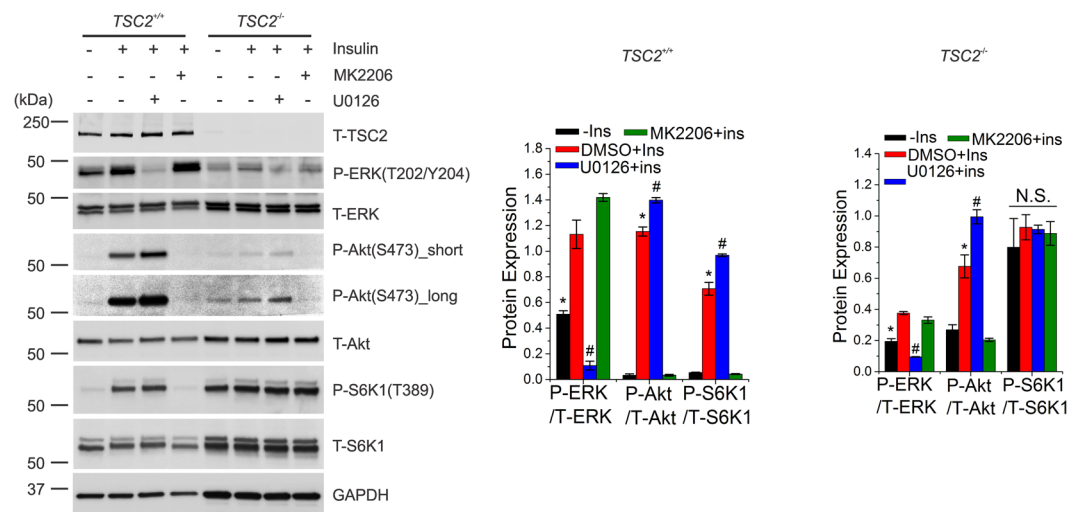


Figure 3. Insulin regulates ERK activity in both *Tsc2*^{+/+} and *Tsc2*^{-/-} cells. *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs were starved of serum (16 h) and treated with the indicated drugs for 2 h prior to insulin stimulation (1 μ M, 15 min). Lysates were analyzed by immunoblot assay using antibodies as indicated. GAPDH was used as a loading control in all immunoblot assays. Quantification of three independent experiments is reported in the bar diagrams. All quantitative analyses are reported as mean \pm SEM with a significance level of $*p < 0.05$ and $\#p < 0.05$. N.S. indicates not significant. * and # indicate significant differences between all conditions in each group. Cropped blots from full-length gels are displayed.

however, no change in Akt activity was observed. As expected, inhibition of Akt also suppressed the phosphorylation of GSK3 β . Notably, rapamycin did not alter the phosphorylation of GSK3 β (Fig. 4B). siRNA-mediated silencing of ERK significantly decreased insulin-mediated phosphorylation of ERK and GSK3 β , but again no obvious change was observed in Akt activity (Fig. 4C). To test that insulin regulation of ERK-GSK3 β signaling is not cell-type specific, we used the MCF7 breast cancer cell line³⁷. We found that insulin stimulation rapidly phosphorylates ERK, Akt, S6K1 and GSK3 β . Insulin-mediated phosphorylation of ERK and GSK3 β was significantly decreased by ERK inhibition; however, no significant changes were observed in Akt or mTORC1 activity (Fig. 4D). We then tested whether PI3K/Akt acts as an upstream regulator of ERK in response to insulin. Immunoblot analysis of HEK cells showed that treatment with an Akt inhibitor (MK2206) or a PI3K inhibitor (LY294002) did not decrease ERK phosphorylation but rather showed a slight, but significant, increase in ERK phosphorylation (Fig. 4E), indicating that Akt and ERK could possibly share a negative feedback loop. Together, these results demonstrate that ERK regulates insulin-mediated GSK3 β activity independent of Akt/mTORC1. We also tested

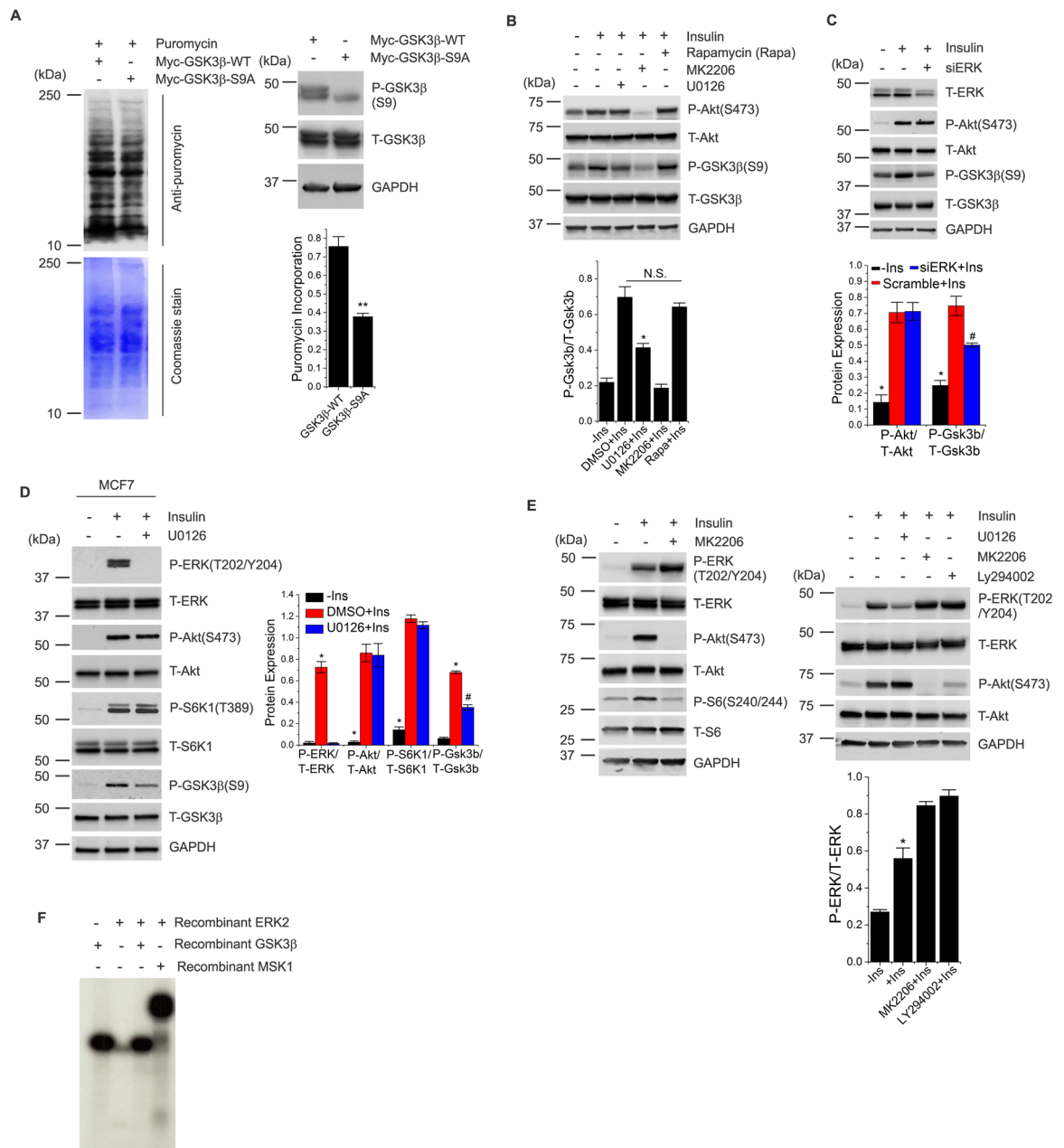


Figure 4. ERK integrates insulin signaling to GSK3 β in an Akt-independent manner. **(A)** HEK cells were transfected with Myc-GSK3 β -WT or Myc-GSK3 β -S9A for 48 h prior to the addition of puromycin in the culture medium. Cell lysates were probed with an antibody to puromycin. The blot was stained with coomassie at the end of the immunoblot assay. Another set of cells were transfected with Myc-GSK3 β -WT or Myc-GSK3 β -S9A for 48 h prior to cell lysis. Lysates were probed with antibodies as indicated. **(B)** HEK cells were starved of serum (16 h) and treated with the indicated drugs for 2 h prior to insulin stimulation (1 μ M, 15 min). Lysates were analyzed by immunoblot assay using antibodies as indicated. Quantification of three independent experiments is reported in the bar diagrams. * indicates significant differences between all conditions in each group. **(C)** HEK cells transfected with siERK or scramble for 56 h prior to 16 h-serum starvation followed by insulin stimulation (1 μ M, 15 min) where indicated. Lysates were probed with antibodies as indicated. Quantification of three independent experiments is reported in the bar diagrams. * and # indicate significant differences between all conditions in each group. **(D)** MCF7 cells were treated as in B prior to the cell lysis. Cell lysates were analyzed with antibodies as indicated. Quantification of three independent experiments is reported in the bar diagrams. * and # indicate significant differences between all conditions in each group. **(E)** HEK cells were starved of serum and treated with the indicated drugs for 2 h prior to insulin stimulation. Lysates were analyzed by immunoblot assay using antibodies as indicated. **(F)** *In vitro* kinase assay (see method for complete details). Quantification of three independent experiments is reported in the bar diagrams. * Indicates significant differences between all conditions in each group. GAPDH was used as a loading control in all immunoblot assays. All quantitative analyses are reported as mean \pm SEM with a significance level of * p < 0.05, ** p < 0.01 and # p < 0.05. N.S. indicates not significant. Cropped blots from full-length gels are displayed in immunoblots.

whether ERK directly phosphorylates GSK3 β by performing *in vitro* kinase assay by using recombinant ERK2 and GSK3 β proteins. We did not observe any obvious changes in GSK3 β phospho-signal in the presence or absence of active ERK, differently from the known ERK substrate, MSK1³⁸, that we used as a positive control (Fig. 4F). These results suggest that ERK does not directly phosphorylate GSK3 β . A co-IP assay performed by pulling down endogenous ERK did not reveal any detectable interaction with GSK3 β (data not shown). Future studies might establish so far unidentified ERK regulatory signals to the GSK3 β that integrates insulin signaling to protein synthesis.

ERK1/2 regulates GSK3 β activity and protein synthesis in *Tsc2*^{-/-} cells. Finally, we tested whether ERK regulates GSK3 β activity and protein synthesis level in *Tsc2*^{-/-} cells. Consistent with previous findings, we found an increased level of phosphorylated and inactivated GSK3 β in *Tsc2*^{-/-} cells compared to *Tsc2*^{+/+} cells, which was further elevated in response to insulin stimulation (Fig. 5A). Similar to HEK and MCF7 cells, we found that insulin-mediated phosphorylation of ERK and GSK3 β was suppressed by ERK inhibition in both *Tsc2*^{+/+} and *Tsc2*^{-/-} cells (Fig. 5A), confirming that ERK regulation of GSK3 β activity in response to insulin stimulation is active in TS. We then investigated whether ERK regulates protein synthesis in *Tsc2*^{-/-} MEFs. As expected, *Tsc2*^{-/-} cells showed a significant increase in the level of protein synthesis compared to *Tsc2*^{+/+} cells (Fig. 5B), which was significantly decreased upon ERK inhibition (Fig. 5C). Immunoblot analysis of *Tsc2*^{-/-} MEFs stimulated with insulin showed a significant increase in protein synthesis compared to starved cells (Fig. 5D). Importantly, the insulin-dependent increase in protein synthesis was significantly diminished by ERK inhibition. We then investigated whether GSK3 β modulates protein synthesis in *Tsc2*^{-/-} MEFs. To test this possibility, we transfected *Tsc2*^{-/-} MEFs with myc-tagged wild-type (Myc-GSK3 β -WT) or constitutively active (Myc-GSK3 β -S9A) GSK3 constructs. Immunoblot analysis of cells transfected with constitutively active GSK3 β showed a significant decrease in protein synthesis in both *Tsc2*^{+/+} and *Tsc2*^{-/-} cells (Fig. 5E). Collectively, these results demonstrate that inhibition of ERK rescues GSK3 β activity and restores the levels of protein synthesis in conditions of absence of TSC2 and hyperactivation of mTORC1 (Fig. 5F).

Discussion

Nutrient availability controls the efficient transition between anabolic and catabolic states in most organisms⁸. Several components of the cell regulate cell growth by coordinating upstream signals from growth factors and sensors of intracellular energy levels and amino acid availability, which are often deregulated in cancer, diabetes and other metabolic disorders^{8,9}. Insulin, a major growth factor, plays a pivotal role in maintaining the metabolic homeostasis in many cell types². Insulin signal funnels through the PI3K/Akt pathway to regulate two major regulators of cell metabolism, mTORC1 and GSK3, which maintain cellular homeostasis through protein and glycogen synthesis, respectively^{17,20}. Tuberous sclerosis complex (TSC) is a rare genetic disorder that is characterized by an increase in protein synthesis and a decrease in GSK3 β activity. In this study, we find that ERK integrates insulin signaling to GSK3 β to regulate global protein synthesis in *Tsc2*^{-/-} cells, a model of tuberous sclerosis³⁹. Inhibition of ERK restores both GSK3 β activity and levels of protein synthesis in *Tsc2*^{-/-} cells, thus identifying ERK as a candidate therapeutic target for the treatment of tuberous sclerosis. In addition, the finding that ERK regulates protein synthesis *via* modulation of GSK3 β activity in an Akt/mTORC1-independent manner provides a new regulatory pathway that could be exploited for combinatorial therapeutic interventions in tuberous sclerosis.

Previous studies have shown that, in absence of TSC2, cells become resistant to insulin stimulation and GSK3 β phosphorylation remains elevated⁴. Lack of TSC2 results in a constitutively active mTORC1, which in turn phosphorylates and inactivates GSK3 β through hyperactivation of the mTORC1 substrate, S6K1⁴. mTORC1 regulation of GSK3 has gained significant interest over the past few years, as this mechanism could have implications in several pathological conditions such as cancer and diabetes, as well as tuberous sclerosis and lymphangiomyomatosis. Our finding that pharmacological inhibition of ERK inhibits GSK3 β activity in *Tsc2*^{-/-} cells suggests that modulation of ERK may be leveraged to regulate GSK3 β activity in multiple pathological conditions characterized by mTORC1 hyperactivation and insulin insensitivity.

Methods

Reagents and materials. DMEM was purchased from HyClone; FBS was from Atlanta Biologicals; glutamine and penicillin-streptomycin (Penn-strep) were purchased from Invitrogen; DMSO, U0126, Rapamycin, LAMP1 (SC-19992) and LAMP2 (SC-18822) antibodies were purchased from Sigma-Aldrich. MK2206 was obtained from Selleckchem. Plasmids Tag5Amyc-GSK3 β WT (Plasmid #16260) and Tag5Amyc-GSK3 β CA (Plasmid #16261) were purchased from Addgene. Anti-GAPDH was purchased from Millipore (MAB374). Anti-TSC2 (for immunoblot) was from Abcam (Ab32554). Anti-puromycin was from Kerfast (EQ0001). LY294002 (9901), siRNA of ERK1/2 (6560), antibodies to P-ERK (9101), T-ERK (9102), P-Akt (4060), T-Akt (9272), P-TSC2 (3615), T-TSC2 (IF, 4308), P-S6K1 (9234 S), T-S6K1 (9202 S), P-GSK3 β (9336 S), T-GSK3 β (12456 S), P-S6 (2215 S), T-S6 (2217 S) were purchased from Cell Signaling.

Cell culture and treatment. HEK-293, MEFs and MCF7 cells were grown in DMEM (1:1) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/mL Penn-strep. For the starvation and insulin stimulation experiments, cells were grown in serum-starved DMEM media for overnight (16 h) prior to stimulation with 1 μ M insulin for 15 min. For the pharmacological inhibitions, 16 h serum-starved cells were treated 2 h with DMSO (as a vehicle) or ERK inhibitor U0126 (10 μ M), or Akt inhibitor MK2206 (10 μ M) or PI3K inhibitor LY294002 (50 μ M) or mTORC1 inhibitor rapamycin (300 nM) prior to stimulation with 1 μ M insulin for 15 min. For knockdown of ERK1/2, cells were transfected with siRNA of ERK1/2 for 56 h prior to 16 h serum starvation, followed by stimulation with 1 μ M insulin for 15 min. Cells were transfected with siRNA of ERK1/2 using the jetPRIMETM siRNA Transfection Reagent (Polyplus transfection).

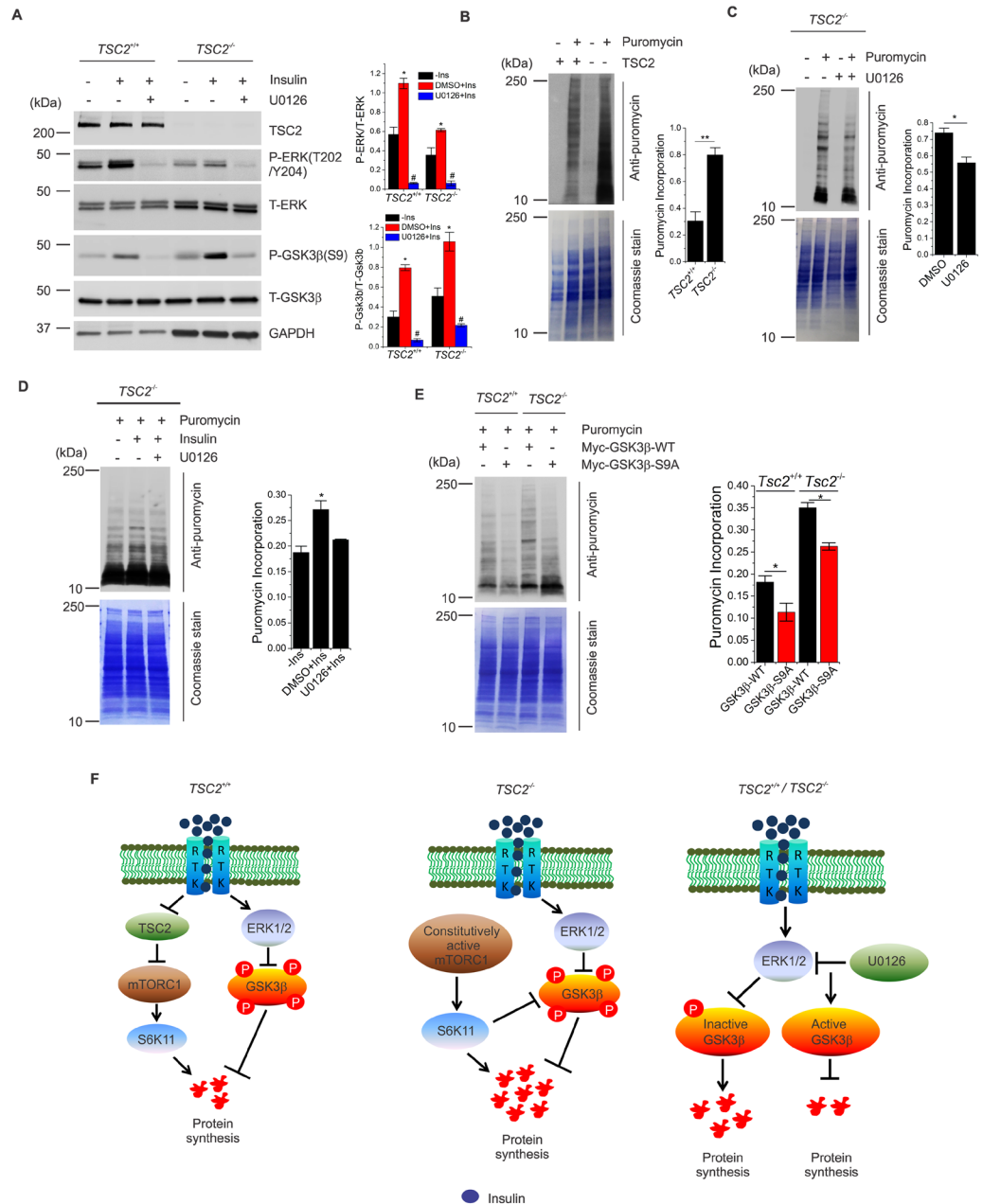


Figure 5. ERK restores GSK3 β activity and protein synthesis levels in *Tsc2*^{-/-} cells. (A) *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs were starved of serum (16 h) and treated with U0126 for 2 h prior to insulin stimulation (1 μ M, 15 min). Lysates were analyzed by immunoblot assay using antibodies as indicated. GAPDH was used as a loading control in all immunoblot assays. Quantification of three independent experiments is reported in the bar diagrams. * and # indicate significant differences between all conditions in each group. (B) *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs were grown 24 h followed by treatment with puromycin for 30 min prior to cell lysis. (C) *Tsc2*^{-/-} MEFs were grown 24 h followed by treatment with U0126 for 24 h prior to the addition of puromycin (30 min) in media. Cell lysates from B and C were probed with an antibody to puromycin. The blots were stained with coomassie at the end of the immunoblot assay. Quantification of three independent experiments is reported in the bar diagrams in B and C. (D) *Tsc2*^{-/-} MEFs were starved of serum (16 h) and treated with U0126 for 2 h prior to insulin stimulation (1 μ M, 15 min). Cells were then incubated with puromycin for 30 min followed by cell lysis. Lysates were probed with an antibody to puromycin. Blot was stained with coomassie at the end of the immunoblot assay. Quantifications of three independent experiments are reported in the bar diagrams. (E) *Tsc2*^{+/+} and *Tsc2*^{-/-} MEFs were transfected with Myc-GSK3 β -WT or Myc-GSK3 β -S9A for 48 h prior to the addition of puromycin in the culture medium. Cell lysates were probed with an antibody to puromycin. The blot was stained with coomassie at the end of the immunoblot assay. Quantitative analyses are reported as mean \pm SEM with a significance level of * p < 0.05 and ** p < 0.01 in B, C, D and E. (F) Model showing that *Tsc2*^{-/-} MEFs have elevated level of protein synthesis compared to *Tsc2*^{+/+} MEFs of protein synthesis. ERK integrates insulin signaling to GSK3 β and protein synthesis in both *Tsc2*^{+/+} and *Tsc2*^{-/-} cells. Inhibition of ERK activates GSK3 β and decreases protein synthesis level. Cropped blots from full-length gels are displayed in A.

Immunoblot assay. Cells were rinsed with ice-cold PBS and harvested and lysed using RIPA buffer (50 mM Tris-HCl, pH 7.4, 1% NP40, 0.5% Na-deoxycholate, 0.1% SDS, 150 mM NaCl, 2 mM EDTA, and 50 mM NaF) including a cocktail of protease (Roche, Basel, Switzerland) and phosphatase (MilliporeSigma, Billerica, MA) inhibitors. Cells lysates were used for protein concentration measurement with the bicinchoninic acid (BCA) protein assay kit (Pierce, Rockford, IL), using BSA as standard. Lysates were then separated via SDS-PAGE followed by transfer to polyvinylidene difluoride (PVDF) membranes. Blots were incubated in blocking buffer (5%, w/v, dried skimmed milk in Tris-buffered saline, pH 7.4, and 0.2% Tween 20, TBST) followed by overnight incubation with appropriate antibodies diluted in fresh blocking buffer. Blots were then washed three times with 1X TBST and then exposed to HRP-conjugated secondary antibodies diluted in blocking buffer for 75 min at room temperature and washed again. Blots were washed three times again prior to detection using appropriate image-developer.

ERK and GSK3 β co-immunoprecipitation analysis. HEK cells were seeded on 10 cm dish and cultured for 24 hours prior to transfection (Lipofectamine 2000, Invitrogen) with 12 μ g of Myc-GSK3 β . After 48 hours, the cells were lysed with NEMT buffer (50 mM Tris pH 7.5, 0.5% NP-40, 150 mM NaCl, and 1 mM EDTA with phosphatase inhibitor, Xpert phosphatase inhibitor cocktail (Gendepot) and Xpert protease cocktail (Gendepot)). The lysates were centrifuged at 13,000 g for 20 min and then the lysate was divided into 2 equal volumes. Cell lysates were immunoprecipitated with either ERK or IgG (as control) and immunoblotted with antibodies to ERK or GSK3 β .

P32 *in vitro* kinase assay. 500 ng of recombinant substrate (GSK3 β , SignalChem; MSK1, SignalChem) was combined with 100 ng of active kinase (ERK2, SignalChem) and then incubated in kinase buffer (25 mM MOPS, 25 mM MgCl₂, 5 mM EGTA, 2 mM EDTA, 0.1 mg/mL BSA, 0.2 mM dithiothreitol (DTT)) with phosphatase inhibitor (Roche) 25 μ M cold ATP (Invitrogen), and 1.2 μ L of 0.250 mCi ³²P ATP (PerkinElmer) for 1 hour at 30 °C. Addition of NuPAGE LDS buffer and sample reducing agent (Invitrogen) followed by boiling for 15 min terminated the kinase reaction. The samples ran on NuPAGE 4–12% Bis-Tris Gel (Invitrogen) followed by exposure to x-ray film (GE) for 1 hour.

Confocal microscopy assay. Cells were grown on glass coverslips in 24-well plates overnight prior to treatment. After the end of the treatment, cells were rinsed with PBS and fixed with 4% paraformaldehyde (in PBS) at room temperature (RT) for 15 min. Cells were then rinsed five times with PBS prior to permeabilization with 0.1% Triton X-100 in PBS for 5 min. Cells were then rinsed five times with PBS and blocked with blocking reagent (0.1% saponin, 10% goat serum in PBS) for 90 min at RT. After blocking, cells were washed twice with PBS, followed by incubation with primary antibody in blocking reagent for overnight at 4 °C. Cells were then washed five times with PBS and incubated with labeled secondary antibodies for 1 h at RT in the dark. Cells were then washed five times with PBS and coverslips were then mounted with vectashield containing DAPI (H-1200) prior to microscopy. Images were acquired through 63x oil immersion objectives with either a Zeiss Axiotome fluorescence microscope with Apotome feature engaged or a Zeiss 710 confocal laser microscope (Zeiss, Oberkochen, Germany). Representative images are shown in all figures at the same exposure and magnification and in merged color images; colocalization is indicated by yellow and orange regions. For quantitative analyses, thresholded Pearson's correlation coefficients were analyzed using raw images of split red and green channels. For each condition, images of at least 30–50 cells were analyzed.

Measurement of Protein synthesis by SUNSET- puromycin end-labeling assay. After the end of the treatment, cells were incubated with puromycin (1 μ M) for 30 min prior to cell lysis. Cell lysates were then subjected to immunoblot analysis. Anti-puromycin antibody was used to detect the level of puromycin incorporation. Protein synthesis was determined by measuring total lane signal from 250–10 kDa and subtracting unlabelled protein control. The signal from total protein loading was detected by coomassie staining as a control. Puromycin signal was normalized with the coomassie signal from each lane. Signals were quantified using ImageJ.

Statistical analyses. All quantitative analyses are reported as mean \pm SEM, unless otherwise specified. Statistical differences between groups were determined using ANOVA with Tukey's post-hoc test. All statistical analyses were performed in Origin Pro (OriginLab Corporation, Northampton, MA) with a significance level of * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, # $p < 0.05$ and \$ $p < 0.01$.

References

1. Auerbach, B. D., Osterweil, E. K. & Bear, M. F. Mutations causing syndromic autism define an axis of synaptic pathophysiology. *Nature* **480**, 63–68, doi:10.1038/nature10658 (2011).
2. Menon, S. *et al.* Spatial Control of the TSC Complex Integrates Insulin and Nutrient Regulation of mTORC1 at the Lysosome. *Cell* **156**, 771–785, doi:10.1016/j.cell.2013.11.049 (2014).
3. Zhang, J. *et al.* A TSC signaling node at the peroxisome regulates mTORC1 and autophagy in response to ROS. *Nature cell biology* **15**, 1186–1196, doi:10.1038/ncb2822 (2013).
4. Zhang, H. H., Lipovsky, A. I., Dibble, C. C., Sahin, M. & Manning, B. D. S6K1 regulates GSK3 under conditions of mTOR-dependent feedback inhibition of Akt. *Molecular cell* **24**, 185–197, doi:10.1016/j.molcel.2006.09.019 (2006).
5. Zhang, Y. *et al.* Coordinated regulation of protein synthesis and degradation by mTORC1. *Nature* **513**, 440–443, doi:10.1038/nature13492 (2014).
6. Demetriades, C., Doumpas, N. & Teleman, A. A. Regulation of TORC1 in response to amino acid starvation via lysosomal recruitment of TSC2. *Cell* **156**, 786–799, doi:10.1016/j.cell.2014.01.024 (2014).
7. Astrinidis, A. A. Tuberous sclerosis complex: linking growth and energy signaling pathways with human disease. *Oncogene* **24**, 7475–7481, doi:10.1038/sj.onc.1209090.
8. Howell, J. J. & Manning, B. D. mTOR couples cellular nutrient sensing to organismal metabolic homeostasis. *Trends in endocrinology and metabolism: TEM* **22**, 94–102, doi:10.1016/j.tem.2010.12.003 (2011).

9. Laplante, M. & Sabatini, D. M. mTOR signaling in growth control and disease. *Cell* **149**, 274–293, doi:10.1016/j.cell.2012.03.017 (2012).
10. Sancak, Y. *et al.* The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. *Science (New York, N.Y.)* **320**, 1496–1501, doi:10.1126/science.1157535 (2008).
11. Li, Y., Corradetti, M. N., Inoki, K. & Guan, K.-L. TSC2: filling the GAP in the mTOR signaling pathway. *Trends in Biochemical Sciences* **29**, 32–38, doi:10.1016/j.tibs.2003.11.007 (2004).
12. Duronio, R. J. & Xiong, Y. Signaling Pathways that Control Cell Proliferation. *Cold Spring Harbor Perspectives in Biology* **5**, a008904, doi:10.1101/cshperspect.a008904 (2013).
13. Shin, S., Wolgamott, L., Yu, Y., Blenis, J. & Yoon, S.-O. Glycogen synthase kinase (GSK)-3 promotes p70 ribosomal protein S6 kinase (p70S6K) activity and cell proliferation. *Proceedings of the National Academy of Sciences* **108**, E1204–E1213 (2011).
14. Huo, X. *et al.* GSK3 Protein Positively Regulates Type I Insulin-like Growth Factor Receptor through Forkhead Transcription Factors FOXO1/3/4. *The Journal of Biological Chemistry* **289**, 24759–24770, doi:10.1074/jbc.M114.580738 (2014).
15. Ma, C. *et al.* The Role of Glycogen Synthase Kinase 3 β in the Transformation of Epidermal Cells. *Cancer Research* **67**, 7756 (2007).
16. Sugden, P. H., Fuller, S. J., Weiss, S. C. & Clerk, A. Glycogen synthase kinase 3 (GSK3) in the heart: a point of integration in hypertrophic signalling and a therapeutic target? A critical analysis. *British Journal of Pharmacology* **153**, S137–S153, doi:10.1038/sj.bjp.0707659 (2008).
17. Cohen, P. & Frame, S. The renaissance of GSK3. *Nat Rev Mol Cell Biol* **2**, 769–776 (2001).
18. Wang, X. *et al.* Eukaryotic initiation factor 2B: identification of multiple phosphorylation sites in the ϵ -subunit and their functions *in vivo*. *The EMBO Journal* **20**, 4349–4359, doi:10.1093/emboj/20.16.4349 (2001).
19. Welsh, G. I., Miller, C. M., Loughlin, A. J. & Price, N. T. Regulation of eukaryotic initiation factor eIF2B: glycogen synthase kinase-3 phosphorylates a conserved serine which undergoes dephosphorylation in response to insulin. *FEBS Letters* **421**, 125–130, doi:10.1016/S0014-5793(97)01548-2 (1998).
20. Proud, C. G. Regulation of protein synthesis by insulin. *Biochemical Society Transactions* **34**, 213 (2006).
21. Porstmann, T. *et al.* SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth. *Cell Metabolism* **8**, 224–236, doi:10.1016/j.cmet.2008.07.007 (2008).
22. Salto, R., Vilchez, J. D., Cabrera, E., Guinovart, J. J. & Girón, M. D. Activation of ERK by sodium tungstate induces protein synthesis and prevents protein degradation in rat L6 myotubes. *FEBS Letters* **588**, 2246–2254, doi:10.1016/j.febslet.2014.05.004 (2014).
23. Servant, M. J., Giasson, E. & Meloche, S. Inhibition of Growth Factor-induced Protein Synthesis by a Selective MEK Inhibitor in Aortic Smooth Muscle Cells. *Journal of Biological Chemistry* **271**, 16047–16052 (1996).
24. Dibble, C. C. & Manning, B. D. Signal integration by mTORC1 coordinates nutrient input with biosynthetic output. *Nat Cell Biol* **15**, 555–564, doi:10.1038/ncb2763 (2013).
25. Ma, L., Chen, Z., Erdjument-Bromage, H., Tempst, P. & Pandolfi, P. P. Phosphorylation and Functional Inactivation of TSC2 by Erk: Implications for Tuberous Sclerosis and Cancer Pathogenesis. *Cell* **121**, 179–193 (2005).
26. Ding, Q. *et al.* Erk Associates with and Primes GSK-3 β for Its Inactivation Resulting in Upregulation of β -Catenin. *Molecular Cell* **19**, 159–170, doi:10.1016/j.molcel.2005.06.009 (2005).
27. Fang, X. *et al.* Phosphorylation and inactivation of glycogen synthase kinase 3 by protein kinase A. *Proceedings of the National Academy of Sciences* **97**, 11960–11965 (2000).
28. Lou, T.-F. *et al.* Extracellular Regulating Kinase (ERK) Inhibition by U0126: A Novel Mechanism for γ -Globin Gene Activation. *Blood* **104**, 3590 (2015).
29. Han, M.-Y., Kosako, H., Watanabe, T. & Hattori, S. Extracellular Signal-Regulated Kinase/Mitogen-Activated Protein Kinase Regulates Actin Organization and Cell Motility by Phosphorylating the Actin Cross-Linking Protein EPLIN. *Molecular and Cellular Biology* **27**, 8190–8204, doi:10.1128/MCB.00661-07 (2007).
30. Weichhart, T., Hengstschlager, M. & Linke, M. Regulation of innate immune cell function by mTOR. *Nat Rev Immunol* **15**, 599–614, doi:10.1038/nri3901 (2015).
31. Schmidt, E. K., Clavarino, G., Ceppi, M. & Pierre, P. SUNSET, a nonradioactive method to monitor protein synthesis. *Nat Meth* **6**, 275–277, http://www.nature.com/nmeth/journal/v6/n4/supinfo/nmeth.1314_S1.html (2009).
32. Dieck, S. T. *et al.* Direct visualization of identified and newly synthesized proteins *in situ*. *Nature methods* **12**, 411–414, doi:10.1038/nmeth.3319 (2015).
33. Bhattacharya, A. *et al.* Genetic Removal of p70 S6 Kinase 1 Corrects Molecular, Synaptic, and Behavioral Phenotypes in Fragile X Syndrome Mice. *Neuron* **76**, 325–337, doi:10.1016/j.neuron.2012.07.022 (2012).
34. Santini, E. *et al.* Exaggerated Translation Causes Synaptic and Behavioral Aberrations Associated with Autism. *Nature* **493**, 411–415, doi:10.1038/nature11782 (2013).
35. Sonenberg, N. & Hinnebusch, A. G. Regulation of Translation Initiation in Eukaryotes: Mechanisms and Biological Targets. *Cell* **136**, 731–745, doi:10.1016/j.cell.2009.01.042 (2009).
36. Zhou, B. P. *et al.* Dual regulation of Snail by GSK-3[β]-mediated phosphorylation in control of epithelial-mesenchymal transition. *Nat Cell Biol* **6**, 931–940, http://www.nature.com/ncb/journal/v6/n10/supinfo/ncb1173_S1.html (2004).
37. Liang, X. H. *et al.* Induction of autophagy and inhibition of tumorigenesis by beclin 1. *Nature* **402**, 672–676 (1999).
38. McCoy, C. E. *et al.* Simon C. MSK1 activity is controlled by multiple phosphorylation sites. *Biochemical Journal* **387**, 507–517, doi:10.1042/BJ20041501 (2005).
39. Uhlmann, E. J. E. J. Heterozygosity for the tuberous sclerosis complex (TSC) gene products results in increased astrocyte numbers and decreased p27-Kip1 expression in TSC2 $^{-/-}$ cells. *Oncogene* **21**, 4050–4059, doi:10.1038/sj.onc.1205435.

Acknowledgements

We thank Vera P. Krymskaya for the TSC2 mutant MEFs. We would also like to thank Mien-Chie Hung for the generous gift of plasmids Tag5Amyc-GSK3 β WT and Tag5Amyc-GSK3 β CA to Addgene. This work was supported by NIH grant NS079618 to M.S. and from The National Institute of Arthritis and Musculoskeletal and Skin Diseases of the National Institutes of Health under Award Number AR061370 to G.G.R. The authors declare that they have no conflicts of interest with the contents of this article. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author Contributions

R.P. conceived the project, designed the experiments and analyzed the data under the supervision of M.S. and G.G.R. R.P., V.V.B. and C.J.A. carried out the experiments. R.P. wrote the manuscript with the help of M.S. and G.G.R. All authors read and approved the final manuscript.

Additional Information

Competing Interests: The authors declare that they have no competing interests.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2017