Molecular Cancer Research

mTORC2 Balances AKT Activation and eIF2α Serine 51 Phosphorylation to Promote Survival under Stress

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Abstract

The mTOR nucleates two complexes, namely mTOR complex 1 and 2 (mTORC1 and mTORC2), which are implicated in cell growth, survival, metabolism, and cancer. Phosphorylation of the α-subunit of translation initiation factor eIF2 at serine 51 (eIF2αS51P) is a key event of mRNA translation initiation and a master regulator of cell fate during cellular stress. Recent studies have implicated mTOR signaling in the stress response, but its connection to eIF2αS51P has remained unclear. Herein, we report that genetic as well as catalytic inhibition of mTORC2 induces eIF2αS51P. On the other hand, the allosteric inhibitor rapamycin induces eIF2αS51P through pathways that are independent of mTORC1 inactivation. Increased eIF2αS51P by impaired mTORC2 depends on the inactivation of AKT, which primes the activation of the endoplasmic reticulum (ER)-resident kinase PERK/PEK. The biologic function of eIF2αS51P was characterized in tuberous sclerosis complex (TSC)-mutant cells, which are defective in mTORC2 and AKT activity. TSC-mutant cells exhibit increased PERK activity, which is downregulated by the reconstitution of the cells with an activated form of *AKT1*. Also, TSC-mutant cells are increasingly susceptible to ER stress, which is reversed by *AKT1* reconstitution. The susceptibility of TSC-mutant cells to ER stress is further enhanced by the pharmacologic inhibition of PERK or genetic inactivation of eIF2αS51P. Thus, the PERK/eIF2αS51P arm is an important compensatory prosurvival mechanism, which substitutes for the loss of AKT under ER stress.

Implications: A novel mechanistic link between mTOR function and protein synthesis is identified in TSC-null tumor cells under stress and reveals potential for the development of antitumor treatments with stress-inducing chemotherapeutics. *Mol Cancer Res; 13(10); 1377–88. ©2015 AACR.*

Introduction

The mTOR is a serine/threonine kinase involved in various cellular processes ranging from energy metabolism and inflammation to protein translation, apoptosis and autophagy (1, 2). mTOR is found in two distinct multiprotein complexes termed mTOR complex 1 (mTORC1) and mTORC2, which are associated with the development of several human diseases, including cancer, type II diabetes, obesity, and neurodegeneration (1–3). In addition to sharing mTOR, mLST8, DEPTOR, and Tti1/Tel2, each complex consists of different components, namely RAPTOR and PRAS40 for mTORC1, or RICTOR, mSIN1, and PROTOR1/2 for mTORC2 (1–3). mTOR activity is intricately linked to the PI3K signaling pathway, which regulates key cellular functions such as cell growth, survival, and mobility (1–3). Receptor tyrosine kinases (RTK) signal through PI3K to activate phosphoinositide-dependent protein kinase-1 (PDK1), which in turn phosphorylates the AGC family kinase AKT or protein kinase B (PKB) at threonine (T) 308 (4). AKT requires a second phosphorylation at serine (S) 473 to become activated, a modification that is mediated by mTORC2 (5). AKT indirectly activates mTORC1 through an inhibitory phosphorylation of the tuberous sclerosis complex (TSC) tumor suppressor, which acts as GTPase-activating protein (GAP) for RHEB (Ras homolog enriched in brain; refs. 6, 7). Inactivating mutations in either *TSC1* or *TSC2*, which encode for hamartin and tuberin, respectively, cause TSC disorder (6, 7). TSC is an autosomal dominant disease characterized by hamartomatous lesions or tubers in multiple vital organs, including the skin, kidneys, lungs, and brain, resulting in neurologic disorders such as severe epilepsy, mental retardation, and autism (8). Disruption of TSC leads to constitutive activation of mTORC1, which in turn stimulates protein synthesis through the phosphorylation of several proteins, including the ribosomal S6 kinase 1 (S6K-1)

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Note: Supplementary data for this article are available at Molecular Cancer Research Online (http://mcr.aacrjournals.org/).

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and eIF4E-binding protein 1 (4EBP1; refs. 9, 10). TSC deficiency is associated with impaired mTORC2 activity, which is caused by a feedback mechanism initiated by hyperactivated mTORC1, leading to the inactivation of insulin receptor substrate 1 (IRS1) and inhibition of insulin or insulin growth factor 1 (IGF1) signaling to PI3K (11, 12). mTORC2 activation requires binding to TSC, which can also account for impaired mTORC2 activity in TSC-mutant cells (6, 13).

Cells respond to various forms of environmental stress by blocking general protein synthesis via the phosphorylation of the α subunit of the translation initiation factor eIF2 at S51 (herein referred to as eIF2αS51P; ref. 10). In mammalian cells, induction of eIF2αS51P is mediated by a family of four kinases, each of which responds to distinct forms of stress (14). The family includes the heme-regulated inhibitor (HRI), which becomes activated by heme deficiency and controls globin synthesis in erythroid cells; the general control nonderepressible-2 (GCN2), which is activated by uncharged t-RNA caused by amino acid deficiency; the RNA-dependent protein kinase PKR, an IFN-inducible protein that becomes activated by binding to double-stranded (ds)RNA; and the PKR-like endoplasmic reticulum (ER)–resident protein kinase PERK, whose activity is induced by the accumulation of misfolded proteins in the ER (14). Despite the general inhibition of mRNA translation, induction of eIF2αS51P can also lead to de-repression of translation of specific mRNAs like those encoding for GCN4 in yeast or activating transcription factor 4 (ATF4) in mammalian cells to increase the expression of genes encoding proteins that alleviate cells from stress (15, 16).

Aberrant eIF2αS51P is observed in several pathophysiologic conditions, including neurodegeneration, obesity, diabetes, as well as cancer (17–21). To date, several studies have demonstrated that activation of the PERK–eIF2αS51P arm conveys prosurvival effects in response to various forms of stress associated with tumor progression and protect tumor cells from death caused by treatments with chemotherapeutic drugs (14, 22). The PERK–eIF2αS51P arm is a key branch of the unfolded protein response (UPR), which is elicited by imbalances between the load of proteins entering the ER and ER's ability to process the client proteins (17). UPR coordinates expression of chaperons, enzymes, and other ER components, whose primary role is to facilitate adaptation to oncogenic stress or stress in the tumor microenvironment (e.g., hypoxic stress; ref. 17).

mTOR is intimately involved in mRNA translation inasmuch as mTORC1 activation leads to stimulation of cap-dependent mRNA translation whereas mTORC2 activation depends on binding to actively translating polyribosomes (23, 24). However, the role of mTOR in eIF2αS51P and translational control in mammalian cells under stress conditions is not well understood. For example, eIF2αS51P was shown to be induced by either the inhibition or the activation of mTORC1 in response to rapamycin or TSC inactivation, respectively, rendering the interpretation of the findings difficult (25, 26). We have investigated the regulation of eIF2αS51P in mouse and human cells rendered defective in mTORC1 and/or mTORC2 activity by genetic or pharmacological means. We observed that mTORC2 plays a major role in the inhibition of PERK and eIF2αS51P through its ability to activate AKT. We also found that the functional interplay between mTORC2 and eIF2αS51P has an important role in the biology of TSC-mutant cells and their response to stress. Specifically, we show that the inherited impairment of the mTORC2–AKT axis in TSC-mutant cells is responsible for the induction of eIF2αS51P, which functions in place of AKT to promote survival in response to chemotherapeutic drugs causing ER stress.

Materials and Methods

Cell culture and treatments

Cells were maintained at 37°C, 5% CO_2 in DMEM (Wisent) supplemented with 10% FBS (Wisent) and antibiotics (100 U/mL penicillin–streptomycin; Wisent). Primary mouse embryonic fibroblasts (MEF) were isolated from $mTOR^{flx/flx}$ mice (27) whereas immortalized MEFs with tamoxifen-inducible knockout of RAPTOR or RICTOR were generated as described previously (28). Reconstitution of $TSC2^{-/-}$ MEFs with human TSC2 was previously described (29). The origin of 621-101 cells was described elsewhere (30). Cells were treated with rapamycin (LC Laboratories), KU0063794 (Bethyl Laboratories), Torin-2 (Tocris Bioscience), Bortezomib (LC Laboratories), or thapsigargin (Sigma) at concentrations indicated in the Fig. 3 for Rapamycin, KU0063794, and Torin-2, and Fig. 6 for Thapsigargin and Bortezomib. Treatments with GSK2656157 (Vibrant Pharma) were performed as previously described (31).

Transfections and transductions

The origin and preparation of viruses expressing small hairpin (sh)RNAs against human mTOR, RICTOR or RAPTOR were previously described (5). Primary $mTOR^{flx/flx}$ MEFs were immortalized by infection with pBABE-retroviruses expressing the simian virus 40 (SV40) large T antigen (Addgene) and selection with 2 μg/mL puromycin (Sigma). Immortalized $mTOR^{flx/flx}$ MEFs were subsequently transduced with Cre-expressing pBABE retroviruses or with insert-less pBABE-retroviruses and selected with 200 μg/mL hygromycin (BioShop Canada). To generate $TSC2^{-/-}$ MEFS deficient in eIF2αS51P, cells were infected with pBABE retroviruses expressing an HA-tagged form of eIF2α S51A and selected with 200 μg/mL zeocin (Invitrogen). $TSC2^{-/-}$ as well as $TSC2^{-/-}$ +TSC2 cells were transduced with either pBABE-puro-myr-Flag-AKT1 (Addgene) followed by selection with 2 μg/mL puromycin. Transient transfections of Cos-1 cells were performed with the Lipofectamine Plus Reagent (Invitrogen) according to the manufacturer's instructions.

Generation of antibodies specific for phosphorylated PERK

Rabbit antiserum for mouse PERK phosphorylated at T779 was produced by immunizing animals against a chemically synthesized phosphopeptide (PYVRSRERUISSSIVFEDSGC where U represents phosphothreonine) of mouse PERK conjugated with keyhole limpet hemocyanin (KLH; Creative Biolabs). Production of antibodies in the serum from various bleeds was quantified by ELISA. Serum with the highest titer was pooled and purified by negative preadsorption against the nonphosphorylated form of the peptide. The final product was generated by affinity chromatography using the phosphorylated peptide. The specificity of the antibody for phosphorylated PERK T799 was tested experimentally as shown in Supplementary Fig. S1A.

The human PERK T982 (LLY-71) rabbit monoclonal phosphoantibody was developed at Epitomics as a contracted project by immunizing rabbits with a synthetic peptide (Cys-PAYARH-pT-GQVGTK) derived from sequence surrounding Thr-982 of human PERK conjugated to keyhole limpet

hemocyanin. Splenocytes were isolated from the animal with the strongest differential titer to the nonphosphorylated version of the immunizing peptide and fused for hybridoma development. Clone supernatants were screened against cell lysates expressing wild-type, K622M kinase dead mutant, or T982A-mutant human PERK to confirm activity and specificity

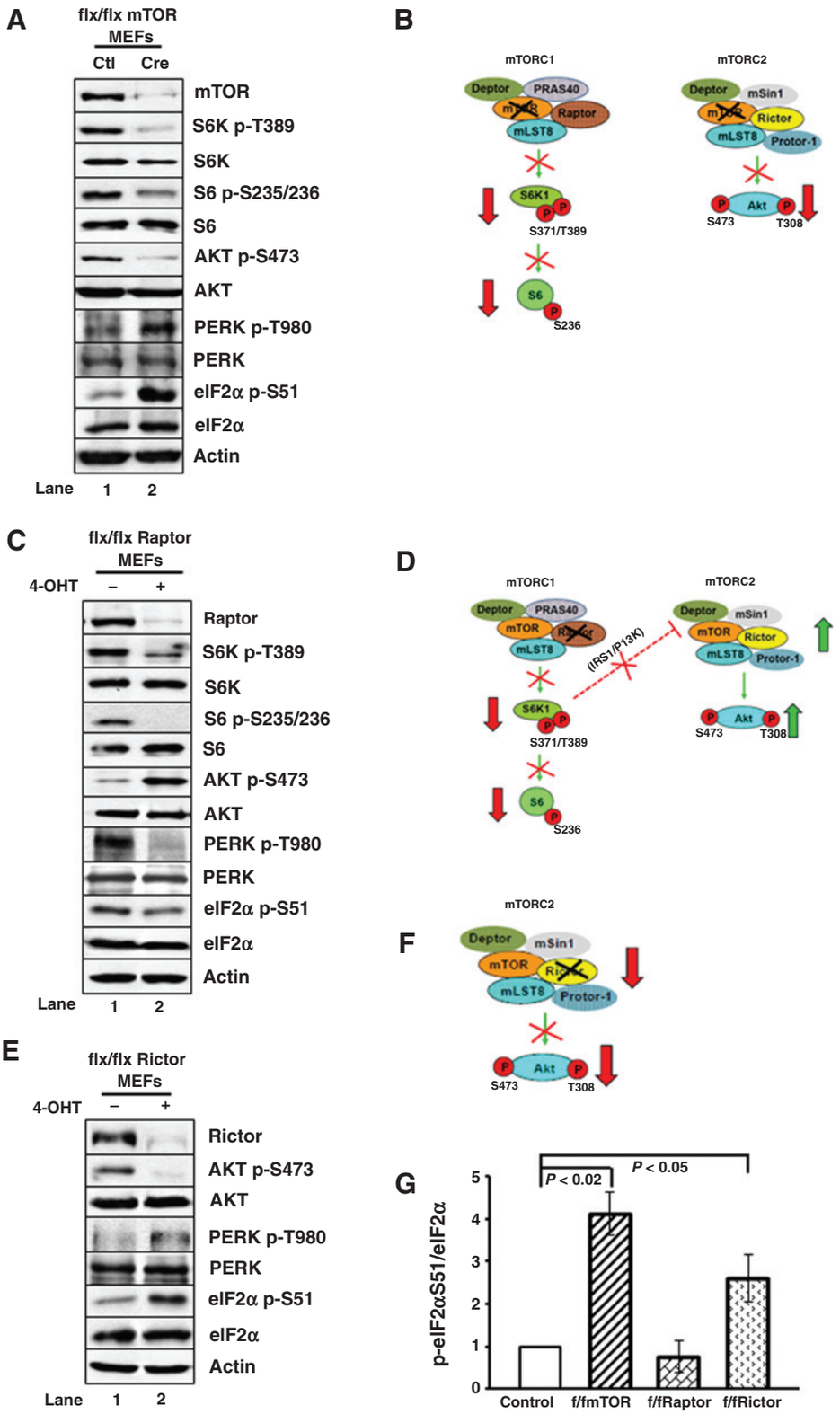

Figure 1.
Genetic inactivation of mTORC2 induces the PERK–eIF2αS51P pathway in mouse cells. A, immortalized mTOR$^{flx/flx}$ MEFs were infected with either insert-less (lane 1) or Cre-expressing pBABE retroviruses (lane 2) and maintained under selection with hygromycin for 72 hours. C and E, immortalized RAPTOR$^{flx/flx}$ (B) or RICTOR$^{flx/flx}$ MEFs (E) expressing Cre-ERT2 were left untreated (lane 1) or treated with 1 μmol/L tamoxifen for 72 hours (lane 2). A, C, and E, protein extracts (50 μg) were subjected to immunoblot analyses for the indicated proteins. B, D, and F, schematic representation of the pathways affected by mTOR complex disruption. G, the graph indicates the ratio of eIF2αS51P to total eIF2α in mouse extracts subjected to analyses in A, C, and E. Each bar represents the mean of three independent experiments and the error bars indicate the SE.

(Supplementary Fig. S1B). Clone LLY-71-1-6 was chosen for large scale IgG production.

Protein extraction and immunoblotting

Protein extraction and immunoblot analyses were performed as described previously (32). The antibodies were obtained from Cell Signaling Technology unless otherwise indicated. The antibodies used were as follows: rabbit monoclonal against phosphorylated eIF2α at S51 (Novus Biologicals), mouse monoclonal against eIF2α, rabbit monoclonal against phosphorylated AKT at S473, rabbit polyclonal against AKT, rabbit monoclonal against PERK phosphorylated at T980, mouse monoclonal against PERK (32), rabbit monoclonal against S6K phosphorylated at T389, rabbit polyclonal against S6K, rabbit polyclonal against S6 phosphorylated at S235/236, rabbit monoclonal against RAPTOR, rabbit polyclonal against RICTOR, rabbit polyclonal against mTOR, rabbit monoclonal against Tuberin (Santa Cruz Biotechnology), mouse monoclonal against S6, rabbit polyclonal against ATF4 (Proteintech) and mouse monoclonal antibody to actin (ICN Biomedicals Inc.). All antibodies were used at a final concentration of 0.1 to 1 μg/mL. Anti-mouse or anti-rabbit IgG antibodies conjugated to horseradish peroxidase (HRP; Mandel Scientific) were used as secondary antibodies (1 × 1,000 dilution) and proteins were visualized with the enhanced chemiluminescence (ECL; Thermo Scientific) detection system according to the manufacturer's instructions. Quantification of bands was performed by densitometric analysis within the linear range of exposure using Image J Software (NIH).

Flow-cytometry analysis

Analysis of cell death by flow cytometry was performed as previously described (32). Cells were analyzed on a BD FACScalibur using the FlowJo software (Tree Star Inc.).

Statistical analysis

Error bars represent SE as indicated, and significance in differences between arrays of data tested was determined using the two-tailed Student *t* test (Microsoft Excel).

Results

Disruption of mTORC2 results in the activation of the PERK–eIF2αS51P arm

We addressed mTOR function in the regulation of eIF2αS51P in immortalized MEF containing a conditional floxed/floxed allele of mTOR ($mTOR^{flx/flx}$) in which mTOR was disrupted by expression of Cre recombinase (Fig. 1A). Downregulation of mTOR was accompanied by a substantial decrease in AKT S473 phosphorylation and ribosomal S6 kinase 1 (S6K1) T389 and S6 235/236 phosphorylation owing to the inactivation of mTORC2 and mTORC1, respectively (Fig. 1A). Also, mTOR inactivation was associated with a substantial increase in PERK T980 autophosphorylation, which is a marker of its activation (33), and increased eIF2αS51P (Fig. 1A). To assess the role of mTORC1 and mTORC2 in this process, we used immortalized $RAPTOR^{flx/flx}$ or $RICTOR^{flx/flx}$ MEFs, which were engineered to express a chimeric protein consisting of Cre and the ligand-binding domain of the estrogen receptor (ERT2; ref. 28). Treatment of cells with 4-hydroxytamoxifen (4-OHT) induced Cre activity in the nucleus and impaired the expression of either RAPTOR (Fig. 1C) or RICTOR (Fig. 1E). Conditional inactivation of RAPTOR resulted in the inhibition of S6K T389 and S6 S235/236 phosphorylation as a result of mTORC1 inactivation (Fig. 1C). Moreover, RAPTOR inactivation increased AKT S473 phosphorylation due to increased mTORC2 activity caused by the inhibition of the negative feedback effect of mTORC1 on IRS1 and PI3K signaling as previously reported (11, 12). Downregulation of RAPTOR decreased PERK T980 autophosphorylation, which was

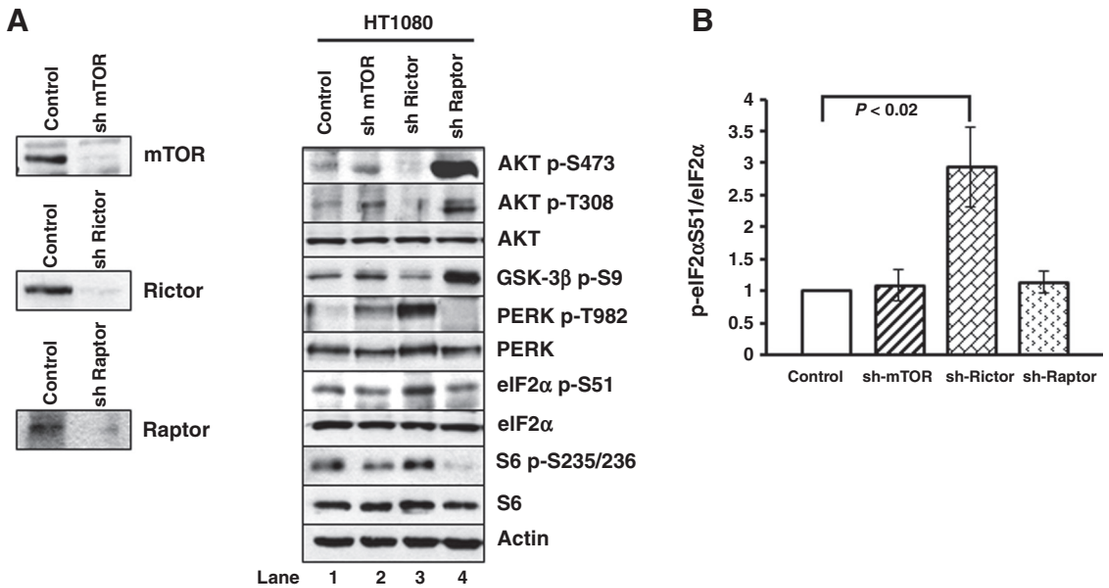

Figure 2.
Genetic disruption of mTORC2 induces the PERK–eIF2αS51P pathway in human cells. A, human fibrosarcoma HT1080 cells were infected with pLKO lentiviruses expressing shRNAs against mTOR, RICTOR, or RAPTOR as well as insert-less pLKO retroviruses used as control. Protein extracts (50 μg) were subjected to immunoblot analyses for the indicated proteins. B, the graph shows the ratio of eIF2αS51P to total eIF2α in human cell extracts subjected to immunoblot analyses shown in A. Each bar represents the mean of three independent experiments and the error bars indicate the SE.

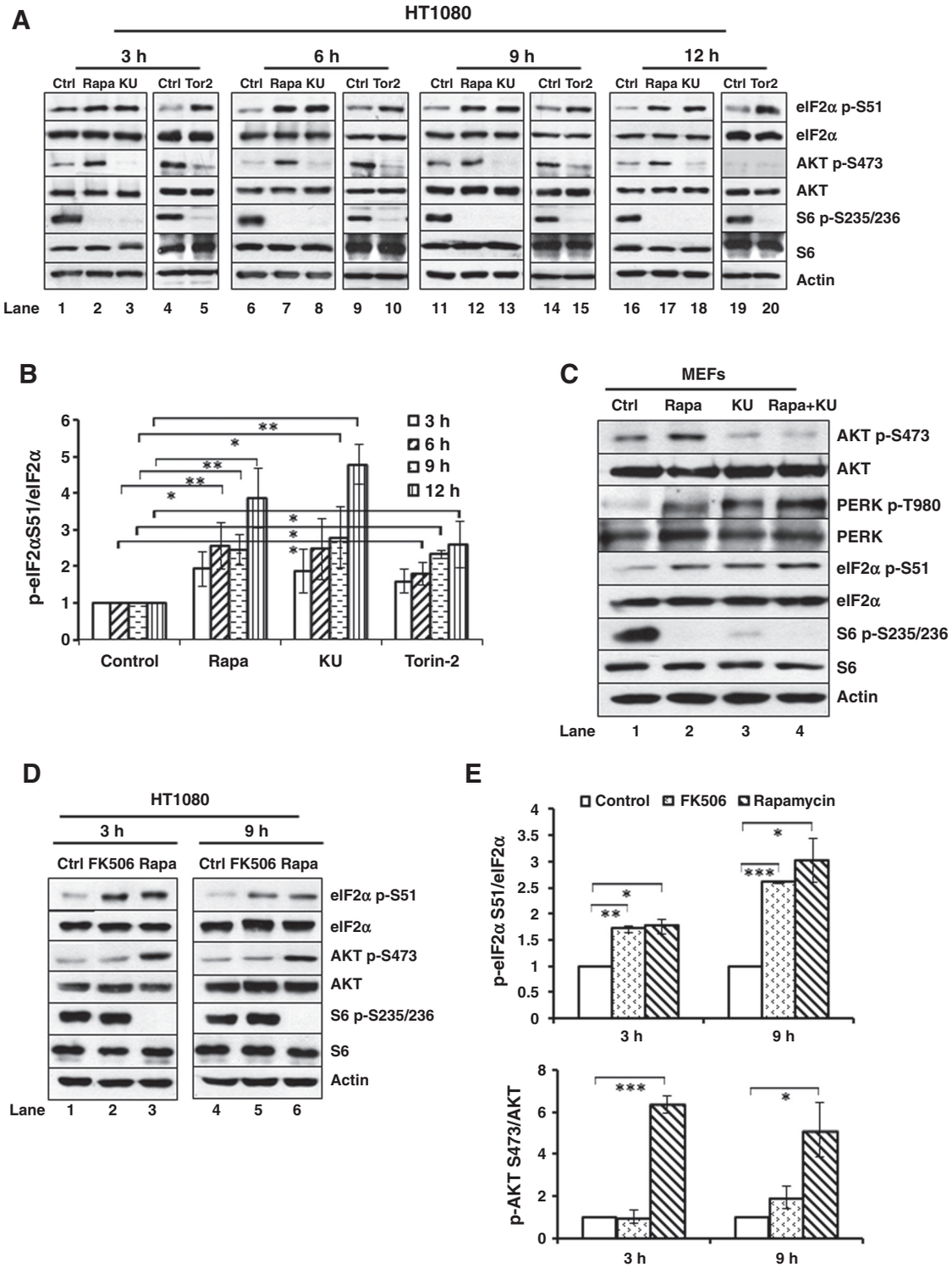

Figure 3.
Pharmacologic inhibition of mTOR induces eIF2αS51P. A, HT1080 were left untreated (lanes 1, 4, 6, 9, 11, 14, 16, and 19) or treated with 20 nmol/L rapamycin (Rapa, lanes 2, 7, 12, and 17), 1 μmol/L KU0063794 (KU, lanes 3, 8, 13, and 18) or 20 nmol/L Torin-2 (Tor2, lanes 5, 10, 15, and 20) for the indicated time points (3, 6, 9, or 12 hours). C, immortalized MEFs were left untreated (lane 1) or treated with 20 nmol/L rapamycin (Rapa), 1 μmol/L KU0063794 (KU), or 20 nmol/L rapamycin and 1 μmol/L KU0063794 for 6 hours. D, HT1080 cells were treated with 10 μmol/L FK506 (lanes 2 and 5) or 20 nmol/L rapamycin (lanes 3 and 6) for 3 or 9 hours. A, C, and D, protein extracts (50 μg) were used for Western blotting with antibodies against the indicated proteins. B, the ratio of eIF2αS51P to total eIF2α quantified from experiments in A is shown in the graphs. E, the graphs indicate the ratio of eIF2αS51P to total eIF2α as well as AKT phosphorylated at S473 to total AKT from the data shown in D. B and E, each bar represents the mean of three independent experiments and the error bars indicate the SE; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

associated with a modest reduction of eIF2αS51P (Fig. 1C). On the other hand, RICTOR downregulation caused a substantial reduction of AKT phosphorylation at S473, which was in line with previous work demonstrating the AKT S473 phosphorylation by mTORC2 (Fig. 1E; ref. 5). Furthermore, RICTOR downregulation led to increased PERK activation by autophosphorylation at T980 and induction of eIF2αS51P (Fig. 1E). Data analyses from several experiments showed that downregulation of either mTOR or RICTOR resulted in a substantial induction of eIF2αS51P consistent with an inhibitory effect of mTORC2 on eIF2αS51P (Fig. 1G).

mTOR function in eIF2αS51P was further addressed in human fibrosarcoma HT1080 cells, which were rendered deficient in mTOR, RAPTOR, or RICTOR by the shRNA approach (5). We found that downregulation of either mTOR or RAPTOR was associated with increased AKT phosphorylation at S473 and T308, as well as increased phosphorylation of the AKT substrate glycogen synthase kinase 3β (GSK-3β) at S9 compared with control cells (Fig. 2A). However, AKT phosphorylation was higher in RAPTOR than in mTOR-deficient cells most likely because AKT activation by impaired mTORC1 from RAPTOR downregulation counterbalances the inactivation of AKT by mTORC2 disruption from mTOR downregulation (Fig. 2A, compare lane 2 with lane 4). On the other hand, RICTOR downregulation resulted in the inhibition of AKT S473 phosphorylation owing to mTORC2 disruption (Fig. 2A, lane 3). Decreased AKT S473 phosphorylation in RICTOR-deficient cells was associated with increased PERK T982 autophosphorylation and eIF2αS51P consistent with an inhibitory effect of mTORC2 on the PERK–eIF2αS51P arm (Fig. 2A, lane 3). Data analyses from different experiments verified that mTORC2 disruption by RICTOR downregulation resulted in a substantial increase of eIF2αS51P in human cells (Fig. 2B), which was in agreement with the effects of mTORC2 disruption on eIF2αS51P in mouse cells (Fig. 1).

Pharmacologic inhibition of mTOR induces the PERK–eIF2αS51P arm

Next, we examined the effects of pharmacologic inhibition of mTOR on eIF2αS51P in human and mouse cells. Cells were treated with the allosteric inhibitor of mTORC1 rapamycin, as well as the catalytic mTOR inhibitors KU0063794 and Torin-2 (34, 35). Treatment of HT1080 cells and immortalized MEFs with each inhibitor caused an increase in eIF2αS51P in a time-dependent manner (Fig. 3A–C). Increased eIF2αS51P was associated with a decrease in AKT phosphorylation in either human cells or MEFs treated with the catalytic inhibitors of mTOR as opposed to the same cells treated with rapamycin, which caused an increase in AKT phosphorylation (Fig. 3A and C). These data indicated that genetic inactivation of mTORC1 and rapamycin elicit different effects on eIF2αS51P and suggested that rapamycin increases eIF2αS51P independently of its inhibitory effect on mTORC1. This notion was supported by data showing that treatment of

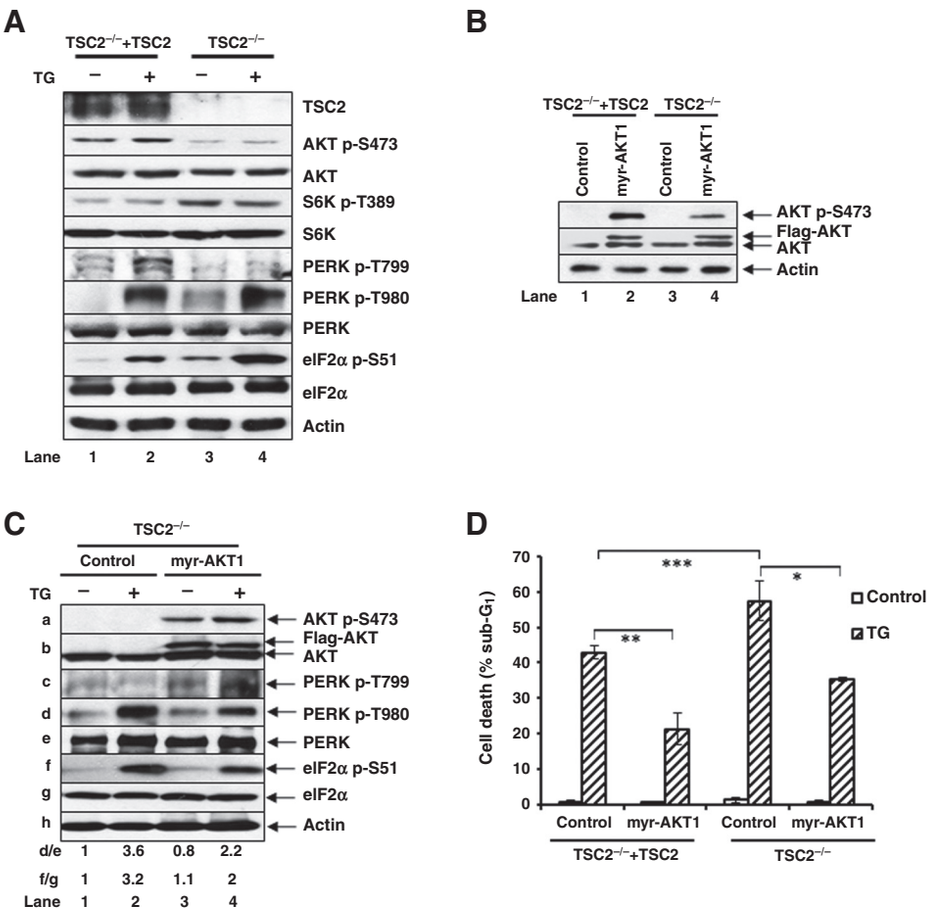

Figure 4.
AKT inactivation in TSC-mutant cells increases death by ER stress. A, $TSC2^{-/-}$ MEFs reconstituted with TSC2 ($TSC2^{-/-}$+TSC2) as well as $TSC2^{-/-}$ MEFs were treated with 1 μmol/L thapsigargin (TG) for 2 hours. B, TSC2-proficient ($TSC2^{-/-}$+TSC2) and deficient ($TSC2^{-/-}$) MEFs were infected with pBABE retroviruses expressing a Flag-tagged myristoylated (myr) form of wild-type *AKT1*. C, TSC-deficient cells either lacking (control) or expressing myr-Flag-*AKT1* were treated with 1 μmol/L thapsigargin (TG) for 2 hours. A to C, protein extracts (50 μg) were immunoblotted for the indicated proteins. Quantification of eIF2αS51P to eIF2α for each lane is shown in C. D, TSC-proficient and -deficient cells lacking (control) or expressing myr-*AKT1* were treated with 1 μmol/L TG 48 hours. The percentage of cell death was determined by measuring the population of cells in sub-G_1 by propidiun iodide staining and FACS analysis. The histograms represent the mean of three independent experiments and the error bars indicate the SE; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

HT1080 cells with FK506, a compound that is structurally related to rapamycin, resulted in the induction of eIF2αS51P (Fig. 3D). Contrary to rapamycin, however, FK506 had no effect on mTORC1 activity as indicated by the lack of an effect on either S6 or AKT phosphorylation (Fig. 3D and E). These data showed that the catalytic inhibitors of mTOR increase eIF2αS51P through mTORC2 inactivation as opposed to rapamycin, which increases eIF2αS51P independently of mTORC1 inhibition.

Disruption of mTORC2 induces eIF2αS51P as a result of AKT inactivation

The physiologic relevance of the regulation of eIF2αS51P by mTORC2 was investigated in TSC2-deficient cells, which are impaired for mTORC2 and AKT activity (29, 36). We used immortalized TSC2$^{-/-}$ MEFs and their isogenic controls consisting of TSC2$^{-/-}$ MEFs reconstituted with TSC2 (herein referred to as TSC-deficient or proficient cells, respectively; ref. 29). TSC-deficient cells displayed decreased AKT S473 phosphorylation and increased S6K1 T389 phosphorylation due to decreased mTORC2 activity and hyperactivated mTORC1, respectively (Fig. 4A; refs. 29, 36). In addition, TSC-deficient cells exhibited elevated levels of PERK activity as a result of T980 autophosphorylation and eIF2αS51P, which were further enhanced by treatment with the ER stressor thapsigargin (Fig. 4A). Previous work by our group demonstrated a negative effect of AKT on PERK activity by phosphorylation at T799 (32). We noticed that ER stress increased PERK T799 phosphorylation in TSC-proficient compared with TSC-deficient cells, which was in line with the higher AKT activity in the former than latter cells (Fig. 4A). These data suggested that hyperactivation of the PERK–eIF2αS51P arm in TSC-deficient cells is mediated, at least in part, by decreased AKT activity, which in turn relieves the negative regulation of PERK by T799 phosphorylation (Fig. 4A).

To better address the role of AKT in the regulation of PERK, TSC-proficient and deficient cells were infected with retroviruses bearing a Flag-tagged form of myristoylated *AKT1* (myr-*AKT1*; Fig. 4B). Although myr-AKT1 was equally expressed in TSC-proficient and deficient cells, myr-AKT1 phosphorylation at S473 was higher in the former than latter cells, indicating that loss of mTORC2 is the major determinant of decreased AKT S473 phosphorylation in TSC-mutant cells (Fig. 4B). Reconstitution of TSC-deficient cells with myr-*AKT1* resulted in the downregulation of eIF2αS51P in response to ER stress, which was associated with a reduction of T980 and induction of T799 phosphorylation of PERK (Fig. 4C). The data showed that AKT inactivation is responsible for the induction of the PERK–eIF2αS51P arm in TSC-deficient cells subjected to ER stress. Concerning the biologic implications of the findings, myr-AKT1 increased the survival of TSC-deficient cells under ER stress, suggesting that loss of AKT plays an important role in the increased susceptibility of these cells to death in response to ER stress (Fig. 4D).

Upregulation of the PERK–eIF2αS51P arm increases the survival of TSC-mutant cells under ER stress

To determine the role of PERK in TSC-deficient cells, PERK activity was impaired by the treatment with GSK2656157, which is an ATP-competitive inhibitor of the kinase (31, 37). Treatment with GSK2656157 resulted in a substantial inhibition of PERK T980 phosphorylation and eIF2αS51P, which was stronger in TSC-deficient than proficient cells subjected to ER stress (Fig. 5A). Also, pharmacologic inhibition of PERK increased cell death

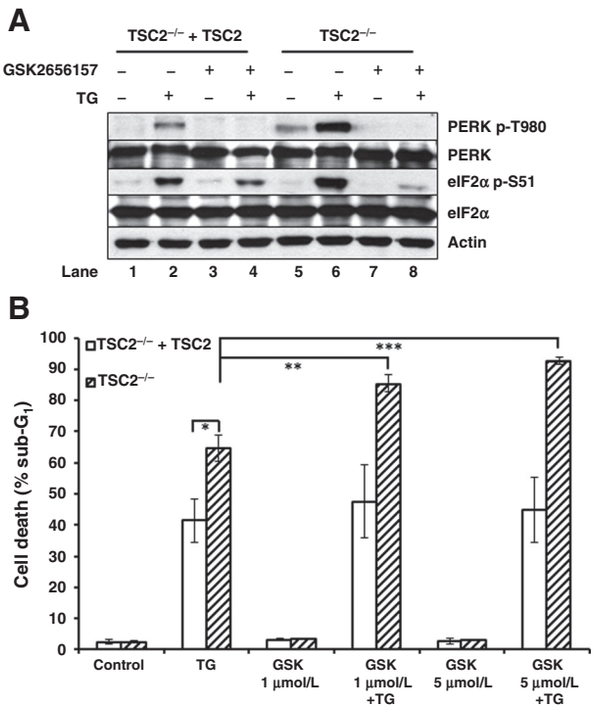

Figure 5.
PERK exhibits a prosurvival role in TSC-mutant cells under ER stress. A, TSC2$^{-/-}$ MEFs reconstituted with TSC2 (TSC2$^{-/-}$+TSC2) as well as TSC2$^{-/-}$ MEFs were pretreated with 1 μmol/L PERK inhibitor GSK2656157 for 20 hours followed by 1 μmol/L thapsigargin (TG) and 2 hours. Protein extracts (50 μg) were immunoblotted for the indicated proteins. B, the same cells were treated with 1 μmol/L thapsigargin (TG) and either 1 μmol/L or 5 μmol/L GSK2656157 for 48 hours. The percentage of cell death was determined by measuring the population of cells in sub-G_1 by propidiun iodide staining and FACS analysis. The histograms represent the mean of three independent experiments and the error bars indicate the SE; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

caused by ER stress, which was more evident in TSC-deficient than proficient cells (Fig. 5B). These data provided evidence for a prosurvival role of PERK in TSC-mutant cells under ER stress.

To examine the implications of eIF2αS51P in the prosurvival effects of PERK, eIF2αS51P was impaired by the stable expression of a hemagglutinin (HA)-tagged form of the nonphosphorylatable human eIF2αS51A, which exhibits dominant negative effects (38). HA-eIF2αS51A expression was evident by its delayed electrophoretic mobility in the polyacrylamide gels caused by the HA tag as well as by its ability to block endogenous eIF2αS51P in cells subjected to ER stress (Fig. 6A). It should be noted that the different expression levels of endogenous eIF2α and HA-eIF2αS51A were due to a higher specificity of the antibodies for mouse than human eIF2α (Fig. 6A). ER stress resulted in a higher PERK activation by T980 autophosphorylation in TSC-deficient than proficient cells (Fig. 6A). Interestingly, ATF4 expression, which is under the translational control of eIF2αS51P (39), was substantially increased in TSC-proficient but not deficient cells under ER stress. This is in agreement with a previous study showing an incomplete UPR response of TSC-mutant cells in response to ER stress, which is associated with impaired ATF4 expression (40). Inhibition of eIF2αS51P did not cause an effect on either AKT or S6K phosphorylation, suggesting that neither mTORC2 nor mTORC1 is under the control of eIF2αS51P in TSC-

deficient cells (Fig. 6A). However, inhibition of eIF2αS51P substantially increased the susceptibility of TSC-deficient cells to death in response to ER stress consistent with a prosurvival function of eIF2αS51P in this process (Fig. 6B).

We further investigated the role of eIF2αS51P in human lymphangioleiomyomatosis (LAM) cells (621-101 cells), which are deficient in TSC2 (30). 621-101 cells were rendered impaired for eIF2αS51P by the expression of HA-eIF2αS51A and subsequent downregulation of endogenous eIF2α by an shRNA targeting the 3′ untranslated region (UTR; ref. 41). This approach produced cells that were substantially deficient in eIF2αS51P as was evident by the lack of phosphorylation in the absence or presence of thapsigargin treatment (Fig. 6C). We observed that impaired eIF2αS51P increased the susceptibility of 621-101 cells to death by treatment with either thapsigargin or the proteasome inhibitor bortezomib, which is a potent inducer of ER stress (Fig. 6D and E; refs. 42, 43). Collectively, the data demonstrated that eIF2αS51P plays a prosurvival role in TSC-deficient mouse and human cells exposed to pharmacologic inducers of ER stress.

Induction of eIF2αS51P in TSC-mutant cells under ER stress is independent of mTORC1

Increased protein synthesis by hyperactivated mTORC1 was thought to induce UPR (26), which contributes to induction of eIF2αS51P in TSC-mutant cells. To determine the possible

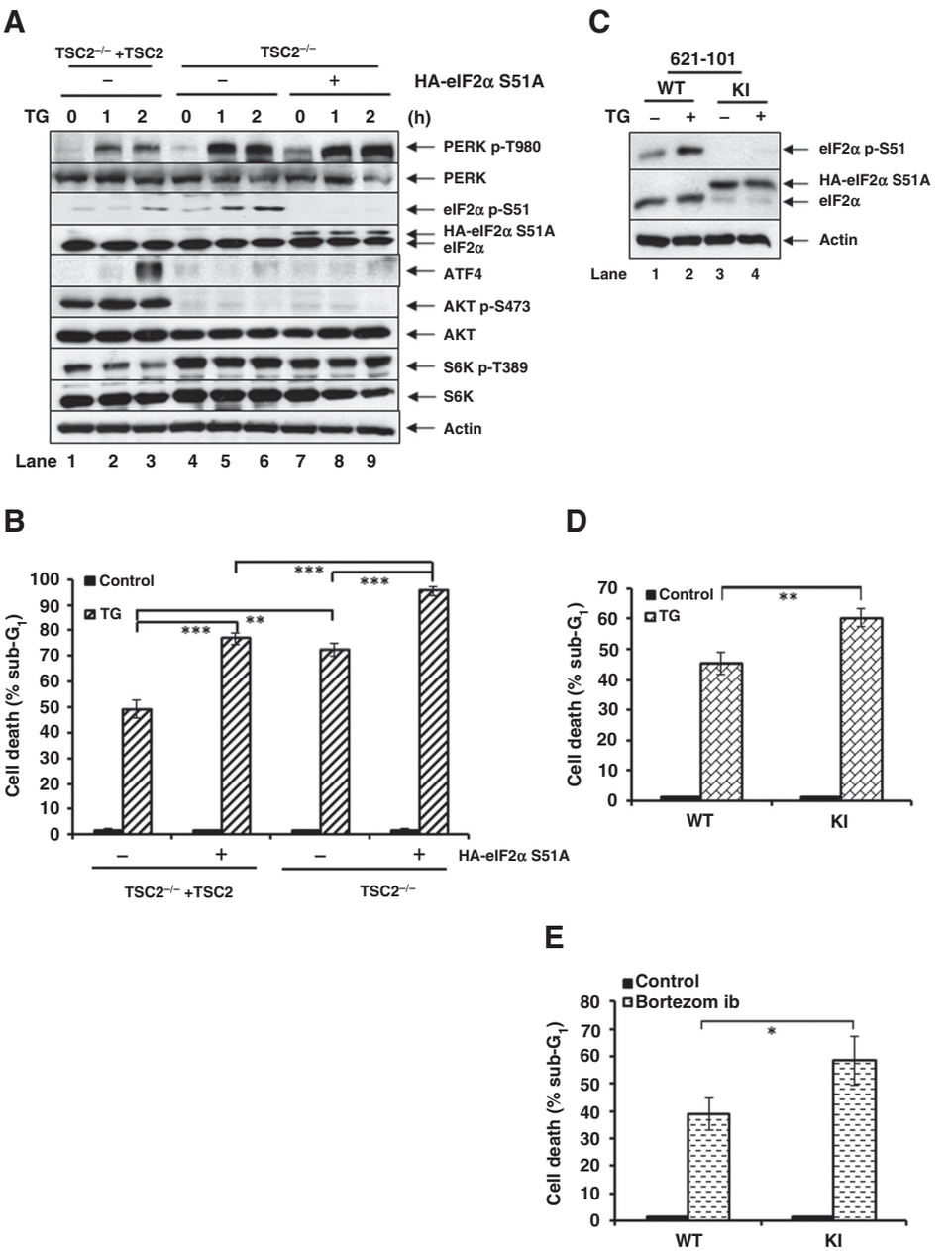

Figure 6.
Induction of eIF2αS51P protects TSC mutant cells from death by ER stress. A, TSC-proficient ($TSC2^{-/-}$+TSC2) and TSC-deficient ($TSC2^{-/-}$) cells infected with pBABE insert-less retroviruses as well as TSC-deficient ($TSC2^{-/-}$) cells infected with pBABE retroviruses expressing HA-eIF2αS51A were subjected to 1 μmol/L thapsigargin (TG) treatment for the indicated time points and protein extracts (50 μg) were immunoblotted for the indicated proteins. B, cells described in A, including TSC-proficient cells expressing HA-eIF2αS51A were subjected to 1 μmol/L TG treatment and were analyzed 48 hours later for the induction of death by FACS analysis of sub-G_1 population. C, 621-101 LAM cells were engineered to be deficient in eIF2αS51P by expressing HA-eIF2αS51A under conditions of downregulation of endogenous eIF2α by shRNA [knock-in (KI) cells]. eIF2αS51P in control wild-type (WT) as well as KI cells was assessed by immunoblotting after treatment with 1 μmol/L TG for 2 hours. D and E, WT and KI 621-101 cells were subjected to treatments with either 1 μmol/L TG (D) or 100 nmol/L bortezomib (E) for 48 hours followed by FACS analysis of dead cells (sub-G_1 population). B, D, and E, the histograms represent the mean of three independent experiments and the error bars indicate the SE; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

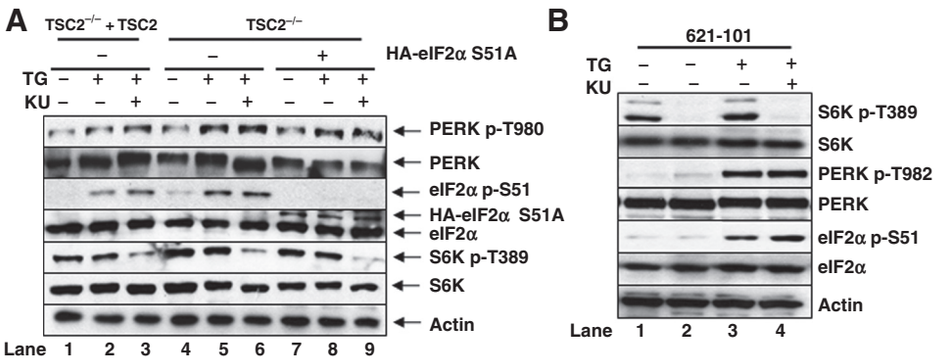

Figure 7.
Induction of PERK activity and eIF2αS51P in TSC-mutant cells by ER stress is not mediated by mTORC1. A and B, TSC-proficient cells or deficient MEFs (A) as well as human 621-101 LAM cells (B) were pretreated with 1 μmol/L KU0063794 (KU) for 4 hours in the absence or presence of 1 μmol/L of thapsigargin (TG) for an additional 2 hours. Protein extracts (50 μg) were immunoblotted for the indicated proteins.

implication of mTORC1 in this process, we examined the regulation of PERK activity and eIF2αS51P in TSC-deficient cells under conditions of catalytic inhibition of mTOR. Considering that TSC-mutant cells do not contain functional mTORC2, we reasoned that catalytic inhibition of mTOR impairs the activity of mTORC1 only and is a suitable way to bypass the side effects of mTORC1 inhibition on eIF2αS51P by rapamycin (Fig. 3). We observed that mTORC1 inhibition with KU0063794 had no significant effect on PERK T980/982 autophosphorylation in either TSC-deficient MEFs or LAM cells in response to thapsigargin treatment (Fig. 7A and B). In addition, KU006379 treatment did not significantly affect eIF2αS51P in TSC-mutant MEFs or human cells after thapsigargin treatment (Fig. 7A and B). These results argued against a role of mTORC1 in the regulation of the PERK–eIF2αS51P arm in TSC-mutant cells under ER stress.

Discussion

Our work establishes a role of mTOR signaling in the regulation of eIF2αS51P in proliferating as well as stressed cells. Genetic disruption of mTORC1 had no significant effects on eIF2αS51P in proliferating cells as opposed to genetic inactivation of mTORC2, which resulted in PERK activation and induction of eIF2αS51P (Figs. 1 and 2). Previous studies by our group and others established a link between AKT inactivation and PERK activation

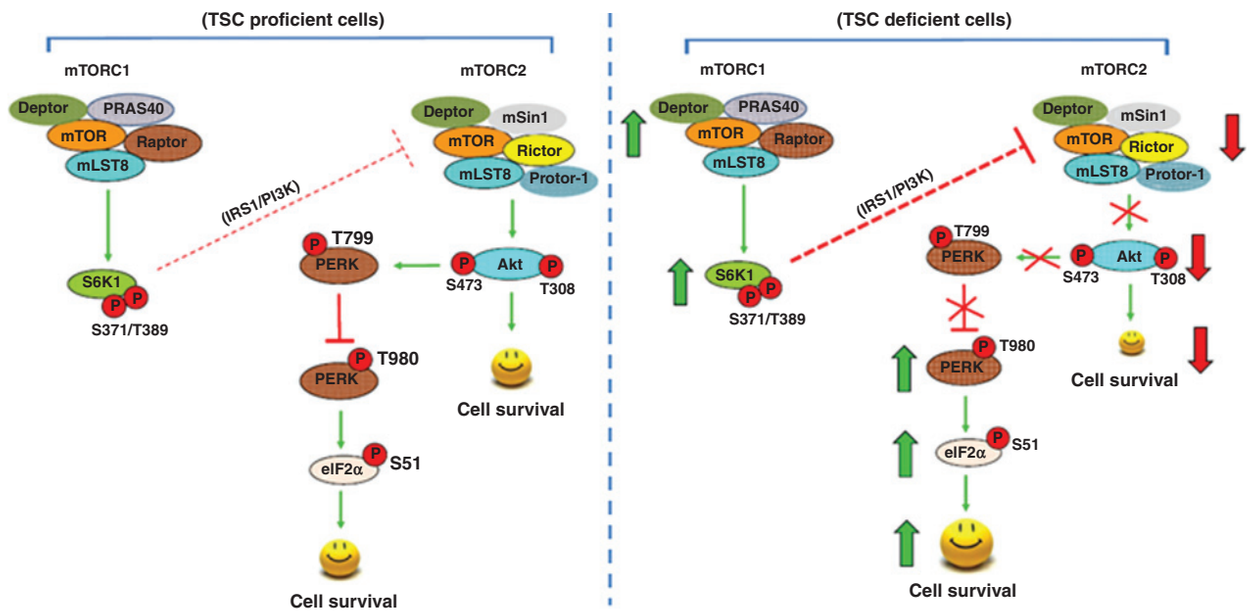

Figure 8.
Schematic model of biologic function of the PERK–eIF2αS51P pathway in TSC-mutant cells. Left, in TSC-proficient cells, AKT activation is balanced by a negative feedback loop initiated by mTORC1 leading to inhibition of IRS1 and PI3K. Activated AKT also mediates a feedback loop resulting in T799 phosphorylation and inactivation of PERK as a means to balance the cytostatic effects of eIF2αS51P. When TSC-proficient cells are exposed to ER stress, both AKT and PERK become hyperactivated and contribute to the survival of stressed cells. Right, in TSC-mutant cells, mTORC1 hyperactivation results in the upregulation of the negative feedback effect on IRS1 and PI3K leading to inhibition of mTORC2. Another mechanism that causes mTORC2 inactivation involves the loss of its interaction with TSC. Low mTORC2 activity accounts for AKT inactivation, which in turn results in decreased PERK T799 phosphorylation and increased PERK activation by T980 autophosphorylation. Loss of AKT activity disarms a major prosurvival pathway, which renders stressed cells highly susceptible to ER stress. Under these conditions, hyperactivation of PERK as a result of AKT inactivation leads to increased eIF2αS51P to help cells survive under stress and balance the loss of the prosurvival function of AKT. Inhibition of either PERK or eIF2αS51P renders TSC-deficient cells highly susceptible to death under ER stress and this may represent an effective strategy for the treatment of TSC tumors.

(32, 44), which can account, at least in part, for the activation of PERK by mTORC2 disruption. On the other hand, genetic disruption of mTORC1 by RAPTOR downregulation exhibited a modest inhibitory effect on eIF2αS51P in MEFs but not in HT1080 cells despite the substantial induction of AKT phosphorylation (Figs. 1 and 2). Also, mTOR downregulation in human cells caused a modest increase in PERK phosphorylation without a detectable effect on eIF2αS51P as opposed to mTOR downregulation in MEFs, which resulted in PERK activation and eIF2αS51P (Figs. 1 and 2). These different responses of mouse and human cells to mTOR signaling disruption may be caused by differences in the stoichiometry of mTORC1 and mTORC2. That is, mTORC2 formation may be higher in MEFs than in human HT1080 cells, resulting in a higher induction of eIF2αS51P by the downregulation of either mTOR or RICTOR in the former than in latter cells, respectively.

Our findings may have important implications in therapies targeting mTOR signaling. Specifically, we observed that inhibition of mTOR with a new generation of drugs that act as ATP-competitive inhibitors resulted in a substantial induction of the PERK–eIF2αS51P pathway (Fig. 3). On the basis of genetic evidence that inactivation of mTORC2 rather than mTORC1 is involved in PERK activation, we conclude that induction of eIF2αS51P by the catalytic inhibitors of mTOR is due to mTORC2 inactivation. On the other hand, unlike the genetic inactivation of mTORC1, which did not increase eIF2αS51P, mTORC1 inhibition by rapamycin resulted in the induction of eIF2αS51P (Fig. 3). The best-characterized function of rapamycin is the allosteric inhibition of mTORC1 in complex with FKBP12, which impairs the phosphorylation of some but not all of mTORC1 substrates (35). However, recent studies revealed new functions of the rapamycin–FKBP12 complex such as the regulation of Ras trafficking or TGFβ1/small mother against decapentaplegic (Smad) signaling, which are independent of mTORC1 inhibition (45, 46). The ability of rapamycin to act through mTORC1-independent mechanisms was strongly implied by our data showing that FK506 increased eIF2αS51P independently of mTORC1 signaling (Fig. 3). Both FK506 and rapamycin bind to and inhibit the peptidyl-prolyl *cis-trans* isomerase activity of the FK506/rapamycin–binding protein FKBP12 (47). FKBP12 exerts multiple cellular functions (48), and therefore, it is reasonable to speculate that some of these mechanisms account for the induction of eIF2αS51P by rapamycin in mTORC1-independent fashion.

Our data demonstrate a role of eIF2αS51P in the regulation of mTOR signaling by TSC (Fig. 7). TSC deficiency is functionally linked to mTORC2 inactivation as well as mTORC1 hyperactivation, both of which are thought to be major players of TSC disease (8, 13). Previous studies proposed that induction of UPR is an important pathologic feature of TSC disease that contributes to critical functional abnormalities in insulin/IGF1 action and cell survival (26, 40, 49). These studies also suggested that mTORC1 hyperactivation by TSC deficiency increases the sensitivity of cells to ER stress–mediated death revealing an unusual property of mTOR to promote cell death under certain circumstances (26, 40, 49). However, TSC-deficient cells were found to launch an incomplete UPR as was indicated by the impaired expression of ATF4, ATF6, and CCAAT/enhancer-binding protein homologous protein (CHOP) despite the upregulation of the PERK–eIF2αS51P arm (40). The incomplete UPR was thought to deprive TSC-deficient cells from survival mechanisms in response to ER stress and increase their susceptibility to death through the selective activation of the IRE1–JNK pathway (40, 49). Our work demonstrates that AKT inactivation represents a major mechanism that induces the PERK–eIF2αS51P arm in TSC-deficient cells by ER stress. Nevertheless, additional mechanisms cannot be excluded as for example the ability of Rheb GTPase to physically interact with and activate PERK (50). We also demonstrate that an important property of mTORC2 inactivation is the activation of PERK and induction of eIF2αS51P, which represents a prosurvival mechanism used by TSC-deficient cells to counteract the deleterious effects of ER stress (Fig. 8). Previous work provided evidence that mTORC1 hyperactivation in response to ER stress does not impact on the PERK–eIF2αS51P arm (49). This is consistent with our interpretation that it is the inactivation of mTORC2 rather than mTORC1 hyperactivation that leads to the induction of the PERK–eIF2αS51P arm to promote survival of TSC-mutant cells under ER stress (Fig. 8).

Increased susceptibility of TSC-deficient cells to ER stress was thought to have therapeutic implications for the treatment of TSC disease (26, 40). Because ER stress inducing drugs like thapsigargin or tunicamycin are toxic to normal cells, killing of TSC-mutant cells through the induction of ER stress requires drugs that selectively induce apoptosis in tumor cells at low doses (26). Interestingly, proteasome inhibitors like MG132 or bortezomib are strong inducers of ER stress in many tumors, including TSC-mutant tumors (51, 52). Our data show that LAM cells are increasingly susceptible to death by bortezomib, a process that is antagonized by eIF2αS51P (Fig. 7). Rapamycin and other rapalogs inhibit TSC tumor growth through the induction of cytostatic effects, which are reversed after discontinuation of the treatment as shown in mouse models of TSC disease and in patients with renal AML (53, 54). Our data suggest alternative approaches of TSC tumor treatment, which may involve the utilization of ER stress–inducing drugs under conditions of impaired PERK or eIF2αS51P.

Disclosure of Potential Conflicts of Interest

Z. Mounir is a scientist and has provided expert testimony for Genentech. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C. Tenkerian, J. Krishnamoorthy, A. Khoutorsky, A.S. Kristof

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Tenkerian, Z. Mounir, A.S. Kristof, S. Wang

Writing, review, and/or revision of the manuscript: C. Tenkerian, Z. Mounir, U. Kazimierczak, A.S. Kristof, A.E. Koromilas

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): K.A. Staschke, A.E. Koromilas

Study supervision: A.E. Koromilas

Other (provided custom phospho-PERK rabbit monoclonal antibody for studies): K.A. Staschke

Other (intellectual contribution to the development of the project): M. Hatzoglou

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