The TSC1 and TSC2 tumor suppressors are required for proper ER stress response and protect cells from ER stress-induced apoptosis

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Tuberous sclerosis complex (TSC)1 and TSC2 are tumor suppressors that inhibit cell growth and mutation of either gene causes benign tumors in multiple tissues. The TSC1 and TSC2 gene products form a functional complex that has GTPase-activating protein (GAP) activity toward Ras homolog enriched in brain (Rheb) to inhibit mammalian target of rapamycin complex 1 (mTORC1), which is constitutively activated in TSC mutant tumors. We found that cells with mutation in either TSC1 or TSC2 are hypersensitive to endoplasmic reticulum (ER) stress and undergo apoptosis. Although the TSC mutant cells show elevated eIF2 α phosphorylation, an early ER stress response marker, at both basal and induced conditions, induction of other ER stress response markers, including ATF4, ATF6 and C/EBP homologous protein (CHOP), is severely compromised. The defects in ER stress response are restored by raptor knockdown but not by rapamycin treatment in the TSC mutant cells, indicating that a rapamycin-insensitive mTORC function is responsible for the defects in ER stress response. Consistently, activation of Rheb sensitizes cells to ER stress. Our data show an important role of TSC1/TSC2 and Rheb in unfolded protein response and cell survival. We speculate that an important physiological function of the TSC1/2 tumor suppressors is to protect cells from harmful conditions. These observations indicate a potential therapeutic application of using ER stress agents to selectively kill TSC1 or TSC2 mutant cells for TSC treatment.

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Tuberous sclerosis complex (TSC) is an autosomal dominant disease characterized by the growth of benign tumors in multiple vital organs such as brain, which results in severe neurological disorders.¹ Mutations in either TSC1 or TSC2 lead to constitutive activation of mTORC1 (the mammalian target of rapamycin complex 1) and downstream signaling cascades, resulting in the development of TSC. The TSC1 and TSC2 proteins interact with each other to form a physical and functional complex that regulates cell growth by inhibiting the Ras homolog enriched in brain (Rheb) and the mammalian target of rapamycin (mTOR) pathway.^{2–7} mTOR protein functions in two different complexes called mTOR complexes 1 and 2 (mTORC1 and mTORC2). A linear TSC1/2-Rheb-mTORC1 pathway has been revealed, in which Rheb is an essential molecule mediating the effect of TSC1/2 to control mTORC1 activity.^{8–10} In contrast, Rheb does not directly stimulate mTORC2 activity.

Excessive expression of unfolded or misfolded proteins, nutrient deprivation and calcium homeostasis perturbation cause a variety of endoplasmic reticulum (ER) stress conditions that can be sensed by intracellular signaling pathways collectively called the unfolded protein response

(UPR) or ER stress.^{11,12} Initiation of this signaling cascade is primarily achieved by the activation of three ER stress sensors. The type I transmembrane endonuclease/protein kinase inositol-requiring enzyme 1 (IRE1), PKR-like ER kinase (PERK) and a type II transmembrane protein called activating transcription factor 6 (ATF6)^{11,12} collectively sense unfolded proteins in ER and elicit cellular responses, including general inhibition of protein synthesis, selective protein translation and induction of stress responsive genes. UPR is believed to be a mechanism to protect cells from unfavorable conditions. However, uncontrolled UPR can lead to apoptosis. Activation of PERK leads to phosphorylation of eukaryotic translation initiation factor 2 α (eIF2 α) and therefore, results in reduced protein synthesis by inhibiting translation initiation.^{13,14} The phosphorylated eIF2 α also selectively translates some proteins, such as the ATF4 transcription factor that in turn induces expression of stress responsive genes. The nuclease of IRE1 specifically cleaves the mRNA of a transcription factor called X-box binding protein-1 (XBP-1); it removes 26 bp from the XBP-1 mRNA precursor to produce a mature form of XBP-1 mRNA, therefore stimulating XBP-1 protein expression.^{12,15,16} ER stress also induces site-specific

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Abbreviations: GAP, GTPase-activating protein; TSC, Tuberous sclerosis complex; mTOR, mammalian target of rapamycin; UPR, unfolded protein response; CHOP, C/EBP homologous protein

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proteolysis of ATF6, and the cleaved cytoplasmic domain of ATF6 moves into the nucleus to act as a transcription factor to increase the transcription of ER chaperones.^{17,18} Furthermore, ER stress promotes proteasome-dependent protein degradation. Failure in relief of ER stress-induced protein overload could result in cell death mediated by massive induction of proapoptotic transcription factor C/EBP homologous protein (CHOP) and activation of caspase cascade.¹⁹

In this study, we investigated ER stress response in TSC mutant cells. We found that the TSC1^{-/-} or TSC2^{-/-} cells are sensitive to ER stress-induced apoptosis. We observed that loss of either TSC1 or TSC2 results in a truncated ER stress response. The TSC mutant cells show elevated eIF2 α phosphorylation but activation of transcription factors such as ATF4, ATF6 and CHOP are significantly reduced. As a consequence, the TSC mutant cells are much more sensitive to ER stress-induced apoptosis. The hypersensitivity to ER stress is not affected by rapamycin treatment, but suppressed by raptor knockdown, and can be mimicked by Rheb activation. These results led to the conclusion that TSC1 and TSC2 protect cells from ER stress and suggest a possibility of using ER stress agents for TSC treatment.

Results

TSC mutant cells are sensitive to ER stress-induced apoptosis. Previously we showed that the TSC mutant cells are sensitive to glucose starvation and undergo apoptosis during prolonged glucose deprivation.²⁰ We conclude that this effect is due to the defects of TSC mutant cells in energy starvation response. However, glucose starvation could also cause ER stress.²¹ We therefore examined the effect of ER stress on TSC mutant cells. Thapsigargin is an ER calcium ATPase inhibitor and induces ER stress. We found that the TSC1^{-/-} MEF cells but not the control TSC1^{+/+} cells

were very sensitive to thapsigargin treatment and showed a massive cell death during 18 h of treatment (Figure 1a). Tunicamycin blocks protein glycosylation and is also a commonly used ER stress inducer. Tunicamycin treatment also induced a robust cell death in the TSC1^{-/-} but not in the control cells (Figure 1a). Knowing TSC1^{-/-} cells are sensitive to thapsigargin and tunicamycin, we examined the sensitivity to MG132, a proteasome inhibitor that also induces UPR. As expected, MG132-induced cell death in TSC1^{-/-} but not the control cells (Figure 1a). To determine whether the high sensitivity to ER stress is unique to TSC1 inactivation, similar experiments were performed in the TSC2^{-/-} LEF cells and controls that re-express TSC2 (labeled as TSC2^{+/+} for convenience). Massive cell death was observed in the TSC2^{-/-} cells in response to thapsigargin, tunicamycin or MG132 treatment while the TSC2^{+/+} cells were not killed by similar treatments (Figure 1b). Together, our data show that mutation in TSC1 or TSC2 sensitizes cells to ER stress inducers and the TSC mutant cells initiate apoptosis under ER stress conditions. The above results show that TSC1 and TSC2 normally protect cells from ER stress.

Uncontrolled ER stress is known to induce apoptosis.¹² To test whether the cell death induced by ER stress in the TSC cells was due to apoptosis, we determined caspase activation. Site-specific cleavage of procaspase to smaller mature caspase is a hallmark of caspase activation. We found that MG132 treatment induced a dramatic increase in active (cleaved) caspase 12, 9 and 3 in TSC1^{-/-} cells (Figure 2a). In contrast, no caspase activation was observed in the control TSC1^{+/+} cells under similar treatment conditions. These data support that MG132-induced apoptosis in TSC1^{-/-} cells. We also tested the effect of thapsigargin and tunicamycin. Our data showed that both ER stress inducers caused activation of caspase 12, 9 and 3 although the effect of tunicamycin is less dramatic than thapsigargin

Figure 1 TSC-deficient cells are sensitive to ER stress-induced cell death. TSC1^{+/+} or ^{-/-} MEFs (a) and TSC2^{+/+} or ^{-/-} LEFs (b) were incubated with thapsigargin (TG, 1 μ M), tunicamycin (Tm, 2 μ g/ml), or MG 132 (MG, 10 μ M) for 18 h, and cell death was observed. All results in this paper are the representatives of 2–3 independent experiments

Figure 2 ER stress induces caspase activation in TSC-deficient cells. TSC1 +/+ or -/- MEFs (a) and TSC2 +/+ or -/- LEFs (b) were incubated with MG 132 (MG), thapsigargin (TG) or tunicamycin (Tm) for the indicated times. Cell lysates were analyzed by western blotting using antibodies specific to caspase 12, cleaved caspase 9 or cleaved caspase 3. Actin levels were measured as loading controls

(Figures 2a and b). Consistent with cell death observed in Figure 1, ER stress-induced caspase activation in TSC2-/- cells as determined by the appearance of cleaved mature caspase 9 and 3, whereas ER stress did not induce caspase activation in the control TSC2 +/+ cells (Figure 2b). Caspase 12 has been implicated in ER stress-induced apoptosis to function upstream of caspase 9, which is in the intrinsic cell death pathway.^{22,23} Caspase 3 functions downstream of caspase 9 and is the effector caspase involved in the execution of cell death.^{24,25} Our data establish that the ER stress-induced cell death in TSC mutant cells correlates with the activation of caspase 12, 9 and 3. ER stress is also known to induce autophagy.²⁶ We also tested whether deletion of TSC affects the ER stress-mediated induction of autophagy, and we observed that autophagy was induced in both TSC1 -/- and wild-type MEFs by ER stress (data not shown).

Elevated basal and stress-induced eIF2 α phosphorylation in TSC mutant cells. Phosphorylation of eIF2 α is one of the best-characterized markers for ER stress. A key physiological response to ER stress is to suppress protein synthesis, which is mediated in large part by the phosphorylation-dependent inhibition of eIF2 α . TSC1-/- cells showed a significant level of basal eIF2 α phosphorylation even under no treatment (Figure 3), an observation consistent with previous report that the TSC

mutant cells are under constant ER stress because of overload of elevated protein synthesis caused by the uncontrolled mTORC1 activation.^{27,28} We also found that not only was the eIF2 α phosphorylation increased by MG132 but also at significantly higher levels (Figures 3a and b). Both thapsigargin and tunicamycin caused a stronger eIF2 α phosphorylation in the TSC1-/- cells than the control cells as determined by western blotting (Figure 3a). Similar observations were made in the TSC2-/- cells (Figure 3b). Our data indicate that the TSC mutant cells show an elevated basal UPR and a stronger eIF2 α phosphorylation induced by ER stress.

TSC mutant cells show a truncated ER stress response. ER stress response protects cells to cope with unfavorable stress environment. Then, it is perplexing why the TSC mutant cells are sensitive to ER stress-induced apoptosis on one hand while these cells also show stronger eIF2 α phosphorylation on the other hand. Besides eIF2 α phosphorylation by PERK, activation of the three ER stress sensors also induces expression of many ER stress proteins. We examined several ER stress markers, including ATF4, ATF6, XBP-1 and CHOP, which have critical roles in proper ER stress response. ER stress also activates two other ER stress sensors, ATF6 and IRE1. IRE1 activation results in expression of XBP-1 while eIF2 α phosphorylation induces

Figure 3 ER stress-induced eIF2 α phosphorylation is increased in TSC-deficient cells. TSC1 +/+ or -/- MEFs (a) and TSC2 +/+ or -/- LEFs (b) were treated with MG 132 (MG), thapsigargin (TG) or tunicamycin (Tm), and cell lysates were prepared at the indicated time points. Phosphorylation of eIF2 α was measured by western blotting using anti-phospho-eIF2 α (p-eIF2 α) antibody. Loading of cell lysates was analyzed by reprobing the membrane with anti-eIF2 α and anti-actin antibodies

the selective translation of ATF4. Furthermore, both ATF4 and ATF6, two transcription factors, contribute to the expression of CHOP. Multiple ER stress responses collectively protect cells from ER stress. TSC1 +/+ or -/- cells were treated with MG132 and nuclear fractions were collected. Western blotting with ATF4-specific antibody showed that MG132 robustly increased ATF4 protein levels in the TSC1 +/+ cells (Figures 4a and b). Surprisingly, the induction of ATF4 was significantly blunted in TSC1 -/- cells. Next, we examined the effect of MG132 on CHOP expression. Similarly, CHOP induction was significantly compromised in the TSC1 -/- cells compared with the TSC1 +/+ control cells. Western blots were also performed for ATF6 and XBP-1. We observed that the MG132-induced XBP-1 expression was decreased in the TSC1 -/- cells. In contrast, the induction of ATF6 was comparable in both TSC1 +/+ and TSC1 -/- cells. Similar experiments were performed in TSC2 -/- cells and identical results were observed (Figure 4a). Therefore, our data indicate that the induction of ER stress response, including ATF4, CHOP and XBP-1, is compromised in TSC mutant cells.

We further analyzed the ER stress response induced by thapsigargin and tunicamycin. As expected, both compounds induced expression of ATF4, CHOP and ATF6 in the control cells although the induction was less robust than the MG132 treatment (Figures 4a and b). Importantly, the induction of these three ER stress response genes was abolished in the TSC1 -/- cells. We did not detect XBP-1 expression by thapsigargin or tunicamycin in our western blotting. This could be due to a combination of a low sensitivity of the antibody and a weaker ER stress response induced by the two compounds. The induction of ATF4, CHOP, ATF6

and XBP-1 were also examined in TSC2 -/- cells and a blunted ER stress response was observed (Figure 4a), suggesting that the defective ER stress response is common to both TSC1 -/- and TSC2 -/- cells. Our results suggest a critical role of TSC1 and TSC2 for a full range ER stress response. Moreover, the truncated ER stress response may contribute to the high ER stress-induced apoptosis in the TSC mutant cells.

Reconstitution of TSC2 blocks while knockdown of TSC1 increases ER stress-induced apoptosis.

To rule out the possibility that the hypersensitivity of TSC mutant cells to ER stress is due to acquired mutations other than TSC1 or TSC2, we next re-expressed TSC2 in TSC2 -/- LEF cells and transiently knocked down TSC1 in HeLa cells (Figures 5a and e). The ER stress response was measured by western blotting for active ATF6 induction, and apoptosis was analyzed by detecting the level of cleaved caspase 9 by western blotting and by flow cytometry analysis of annexin V/propidium iodide stained cells. As shown in Figure 5, TSC2-rescued cells restored ER stress response (Figure 5b) and concomitantly underwent less apoptosis compared with control TSC2 -/- cells (Figures 5c and d). Although knockdown of TSC1 in HeLa cells decreased the level of active ATF6 induced by thapsigargin and tunicamycin (Figure 5f), and the ER stress-induced apoptosis was increased (Figures 5g and h). These results indicate that the truncated ER stress response and higher sensitivity to apoptosis in TSC mutant cells are indeed caused by the mutation of TSC1 or TSC2, and not only in MEF but also in other cell types, such as HeLa cells.

Figure 4 TSC-deficient cells show defective ER stress response. **(a)** TSC1 $+/+$ or $-/-$ MEFs and TSC2 $+/+$ or $-/-$ LEFs were incubated with MG 132 (MG), thapsigargin (TG) or tunicamycin (Tm), and nuclear extracts (NE) or whole cell lysates (WCL) were prepared at the indicated time points. Induction of ATF4, CHOP, ATF6 and spliced form of XBP-1 were examined by western blotting using specific antibodies. Equal protein loading was determined using anti-U1SnRNP 70 (for NE) or anti-actin (for WCL) antibodies. **(b)** Induction of ATF4 and CHOP by ER stress in TSC1 $+/+$ or $-/-$ MEFs. Cells were incubated with MG 132 (MG), thapsigargin (TG) or tunicamycin (Tm) for 4 h, and total RNA was prepared. Quantitative RT-PCR was performed using specific primers and the fold induction was normalized with actin mRNA levels

Rapamycin does not protect TSC mutant cells from ER stress-induced apoptosis. Mutation in either TSC1 or TSC2 results in constitutive activation of Rheb and mTORC1. We first analyzed the role of mTORC1 by using rapamycin, which is a potent and specific inhibitor of mTORC1. Glucose starvation is known to induce apoptosis in TSC mutant cells.²⁵ The glucose starvation-induced apoptosis could be effectively blocked by rapamycin (Figure 6a), consistent with a critical role of high mTORC1 activation in energy starvation-induced apoptosis. To test the role of mTORC1, rapamycin was added together with MG132, thapsigargin or tunicamycin to treat TSC1 $-/-$ cells. We were surprised to find that rapamycin had little effect in protecting TSC1 $-/-$ cells from apoptosis induced by

the ER stress (Figure 6a). Western blotting for active caspase 3 supported the morphological cell death data (Figure 6b). Rapamycin completely blocked the appearance of active caspase 3 in response to glucose starvation in TSC1 $-/-$ cells. In contrast, rapamycin did not block caspase 3 activation caused by MG132, thapsigargin or tunicamycin. In fact, rapamycin even slightly enhanced the appearance of active caspase 3 in the TSC1 $-/-$ cells in response to thapsigargin and tunicamycin (Figure 6b). Parallel experiments were performed with TSC2 $-/-$ cells and we also found that rapamycin did not protect ER stress-induced apoptosis (Figures 6a and b). Quantification of apoptosis by FACS analysis further supports this conclusion (Figure 6c). The above observations indicate that a rapamycin-insensitive downstream signaling pathway sensitizes TSC mutant cells to ER stress.

Higher apoptosis induced by ER stress in TSC mutant cells is Rheb and raptor-dependent. We next tested the effect of the Rheb GTPase, which is a direct target of TSC1/TSC2. We stably overexpressed calmodulin-binding protein (CBP) epitope-tagged wild type or constitutively active Rheb (S16H) in HeLa cells (Figure 7a). We were unable to generate HeLa cells that stably overexpress dominant-negative Rheb (S20N) because we could not detect the expression of dominant-negative Rheb after hygromycin B selection. Active Rheb overexpression or Rheb knockdown MEFs were also generated as confirmed by western blotting with Rheb-specific antibody (Figure 7d). The induction of apoptosis by different ER stress inducers in these cells was examined. Overexpression or knockdown of Rheb did not induce apoptosis under normal culture conditions (Figures 7b–d). However, expression of wild type and active Rheb-induced activation of caspase 9 and caspase 3 when cells were treated with MG132, thapsigargin or tunicamycin, supporting a role of Rheb in ER stress (Figures 7b and d), and active Rheb showed a more dramatic effect as shown by higher level of cleaved caspase 9 and higher proportion of cell death measured by FACS analysis (Figures 7b and c). In contrast, knockdown of Rheb did not sensitize cells to thapsigargin- or tunicamycin-induced apoptosis (Figure 7d). It is worth noting that Rheb knockdown did sensitize cells to MG132 (Figure 7d). Given the fact that MG132 causes global accumulation of ubiquitinated protein, the effect of MG132 could be broader than ER stress. Together, our data indicate that high Rheb activity sensitizes cells to ER stress, supporting a notion that the high Rheb activity in the TSC mutants is responsible for the ER stress-induced apoptosis.

Although the ER stress-induced apoptosis in TSC mutant cells is rapamycin-insensitive, it is still possible that mTORC1 is involved. We further examined whether raptor is required for ER stress hypersensitivity in the TSC mutant cells. We performed raptor knockdown in TSC1 $-/-$ MEFs, and raptor protein knockdown and decrease in S6K phosphorylation was confirmed (Figure 8a). We tested ER stress response and apoptosis in raptor knockdown and control TSC1 $-/-$ MEFs when treated with medium, thapsigargin or tunicamycin. Surprisingly, when raptor was knockdown, the TSC mutant cells restored ER stress response as shown by similar ATF6 activation as in the TSC1 $+/+$ MEFs (Figures 4a and 8b) and

simultaneously, TSC mutant cells were protected from apoptosis induced by thapsigargin and tunicamycin (Figures 8c and d). Taken together, our data suggest a raptor-dependent rapamycin-insensitive function of mTORC is responsible for hypersensitivity to ER stress in TSC mutant cells.

Discussion

The TSC1/TSC2 tumor suppressor complex functions as GTPase-activating protein (GAP) toward Rheb, which is a potent and direct activator of mTORC1. In TSC mutant cells, mTORC1 is constitutively active, therefore stimulates translation and promotes cell growth. The TSC-Rheb-mTOR pathway integrates a wide range of intracellular signals to coordinate cell growth with cellular environment. Under unfavorable conditions, activation of TSC1/TSC2 would inhibit unwanted cell growth, and thus protect cells from harmful conditions. In this report, we showed that TSC1 and TSC2 are required for a full range ER stress response and function to protect cells from ER stress-induced apoptosis. Interestingly, this function of TSC1/TSC2 is mediated by Rheb and raptor,

but in a manner insensitive to rapamycin inhibition. Different from conventional kinase inhibitor, rapamycin forms a complex with FKBP12 then binds to the FRB domain in mTOR to inhibit the ability of mTORC1 to phosphorylate downstream substrates, such as S6K1. Therefore, rapamycin does not directly inhibit the intrinsic mTOR kinase activity. Interestingly, Choo *et al.*²⁹ showed that a rapamycin-resistant mTORC1 activity is required for 4EBP1 phosphorylation under some conditions, showing that not all functions of mTORC1 are inhibited by rapamycin. Our study indicates that a rapamycin-insensitive mTORC1 activity may be responsible for the hypersensitivity to ER stress in the TSC mutant cells.

A potential function of TSC in ER stress response has been suggested. Ozcan *et al.*²⁸ showed that TSC mutant cells have higher basal eIF2 α phosphorylation and higher apoptosis in response to ER stress. These investigators concluded that the elevated ER stress response contributes to cell death in the TSC mutant cells. However, besides eIF2 α phosphorylation other ER stress response markers were not examined. Our study showed that the TSC mutant cells show a defective ER stress response, as indicated by the blunted induction of ATF4, ATF6 and CHOP although higher level of eIF2 α

Figure 5 TSC1 and TSC2 functions are required for cell survival in response to ER stress. (a–d) Reconstitution of TSC2 blocks the ER stress-induced apoptosis. (a) Overexpression of TSC2 was confirmed by western blotting using anti-TSC2 antibody. (b–d) Cells were treated with medium (none), thapsigargin or tunicamycin. Cell lysates were prepared after 4 or 24 h to detect the ER stress marker ATF6 (b) or induction of cleaved caspase-3 (c) by western blotting. Cells were also harvested after 24 h to measure the induction of apoptosis by FACS analysis (d). (e–h) ER stress-induced apoptosis in TSC1 knockdown HeLa cells. (e) HeLa cells were infected with shRNA targeting nothing (control) or TSC1, and knockdown of TSC1 was confirmed by western blotting using anti-TSC1 antibody. (f–h) Cells were treated with medium (none), thapsigargin or tunicamycin, and cell lysates were obtained after 6 or 24 h to detect ATF6 (f) or cleaved caspase-9 (g) by western blotting. Induction of apoptosis was measured by FACS analysis after 24 h (h). Numbers in each quadrant indicate the average percentage of population

Figure 5 Continued

phosphorylation occurred. We suggest that the incomplete ER stress response in TSC mutant cells fails to protect cells from unfolded protein stress and contributes to the high level of apoptosis under ER stress.

CHOP has been implicated in ER stress-induced apoptosis. Surprisingly, we observed that CHOP induction is abolished in TSC mutant cells in response to thapsigargin and tunicamycin, yet the TSC cells show a massive apoptosis. These data indicate that CHOP induction is not required for apoptosis induced by ER stress, at least in the TSC mutant cells. Furthermore, our data suggest that a normal function of CHOP induction (a moderate induction) by ER stress may protect rather than kill cells. Only when expressed at a very high level, CHOP may contribute to apoptosis. This function of CHOP is similar to p53, of which a moderate induction protects cells by stopping cell cycle while a massive p53 induction promotes apoptosis.^{19,30} The cell-protecting function of TSC1 or TSC2 is likely to be cell type independent. For example, the TSC1 mutant neurons also show hypersensitivity to ER stress.³¹

We found that TSC mutant cells showed a truncated ER stress response. The TSC1^{-/-} or TSC2^{-/-} cells show elevated basal and stress-induced eIF2 α phosphorylation, which is catalyzed by PERK. However, there are three ER stress sensors, IRE1, ATF6 and PERK. In TSC mutant cells, the induction of ATF4, ATF6 and CHOP are severely compromised in response to ER stress. XBP-1 induction by MG132 treatment is also diminished. These observations indicate that the TSC mutant cells showed a partial ER stress response as indicated by the elevated eIF2 α phosphorylation

but decreased induction of other ER stress markers. High eIF2 α phosphorylation is implicated to promote translation of selective mRNAs, such as ATF4. However, ATF4 is not induced in TSC mutant cells although eIF2 α phosphorylation is high, indicating a possibly uncoupling between early ER stress response and the late ER stress events in the TSC mutant cells. MG132 inhibits proteasome-mediated degradation and causes a general UPR, including ER stress response. The TSC mutant cells similarly show hypersensitivity to MG132. We suggest that the incomplete ER stress response in the TSC mutant cells contributes to the high sensitivity to ER stress.

Hyperactivation of mTORC1 has been shown to contribute, to much of the abnormality in the TSC mutant cells. As a potent inhibitor of mTORC1, rapamycin has been implicated as a potential drug for TSC treatment.³² Interestingly, rapamycin does not protect TSC mutant cells from ER stress-induced apoptosis. In contrast, rapamycin potently protects TSC mutant cells from glucose starvation-induced apoptosis. However, the high sensitivity to ER stress in TSC mutant cells is Rheb- and raptor-dependent, indicating a rapamycin-insensitive activity of mTORC1 in ER stress-induced cell death. Recent studies have indicated that rapamycin may inhibit some but not all mTORC1 functions.^{29,33} The rapamycin-FKBP12 complex may interfere with phosphorylation of some but not all mTORC1 substrates. Thus, it will be interesting to examine how raptor has a role in response to ER stress and what new substrates of mTORC1 are involved. A role of Rheb in ER stress response is clearly supported by the observation that increased Rheb activity

sensitizes cells to ER stress and Rheb knockdown abolished the apoptosis induction by thapsigargin and tunicamycin. Future studies to elucidate the mechanism of Rheb- and mTORC1-dependent function downstream of TSC1/TSC2 in cellular regulation would provide new insights into the mechanisms of TSC1 and TSC2 in normal cell growth regulation, stress response and tumorigenesis.

Our study further supports the critical function of the TSC1 and TSC2 tumor suppressor genes in coordinating cell growth with extracellular and intracellular signals. A key physiological

function of TSC1 and TSC2 is to provide a protection mechanism for cells in response to unfavorable conditions. As the TSC mutant cells are highly sensitive to ER stress, ER stress agents, which can selectively kill TSC cells, could be used for TSC treatment. Velcade is a proteasome inhibitor approved by FDA as a cancer drug. We speculate that Velcade may also have a beneficial effect for TSC. Consistently, we have observed that injection of Velcade induces apoptosis in TSC1^{-/-} liver cells in a TSC1 tissue-specific knockout mouse model (data not shown). Rapamycin mainly

Figure 6 Rapamycin does not protect TSC mutant cells from ER stress-induced apoptosis. (a) TSC1^{-/-} MEFs or TSC2^{-/-} LEFs were incubated with or without rapamycin (10 nM) for 1 h, and then treated with culture medium, MG 132 (MG), thapsigargin (TG) or tunicamycin (Tm), or incubated with glucose-free medium (-glucose) as indicated. After 18 h, cell death was observed (a) and cell lysates were prepared to analyze the induction of cell death by using anti-cleaved caspase-3 antibody (b). Protein loading was measured by reprobing the membrane using anti-actin antibody. Cells were harvested after 24 h to measure the induction of cell death by flow cytometry (c)

Figure 6 Continued

provides cytostatic effect to inhibit TSC tumor growth. Given the fact that rapamycin does not suppress ER stress-induced apoptosis in TSC mutant cells, a combination of rapamycin (to inhibit TSC cell growth) and Velcade (to induce TSC cell apoptosis) could be considered for TSC treatment. Moreover, our studies also suggest that Velcade may be more effective for cancers that have low TSC1 or TSC2 activity, such as those with activated PI3K-AKT that inhibits TSC2.

Materials and Methods

Reagents and antibodies. Proteasome inhibitor MG132, ER stress inducers thapsigargin and tunicamycin, and α -tubulin antibody were purchased from Sigma (St Louis, MO, USA). Rapamycin was obtained from Calbiochem (San Diego, CA, USA). Caspase-12, cleaved caspase-3 (Asp175), cleaved caspase-9 (Asp353), phospho-eIF2 α , eIF2 α , raptor, TSC1 and phospho-p70 S6 Kinase (Thr389) antibodies were purchased from Cell Signaling (Beverly, MA, USA). Antibodies to ATF4, CHOP, XBP-1, actin, U1 SnRNP 50, GAPDH, raptor and TSC2 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-ATF6 antibody was purchased from IMGEX (San Diego, CA, USA), and anti-Rheb antibody was from ProSci (Poway, CA, USA). CBP epitope tag antibody was purchased from Millipore (Billerica, MA, USA). The plasmids containing short hairpin RNAs (shRNAs) targeting the messenger RNA for mouse Raptor (pLKO mouse

shRNA1 raptor) and scrambled control shRNA plasmid were obtained from Addgene, Inc. (Cambridge, MA, USA).

Lentiviral overexpression and shRNA plasmids. Constitutively active Rheb (RhebL64) was amplified by PCR method and cloned into lentiviral overexpression vector pCDH1-puro. Rheb shRNA was generated by cloning the Rheb targeting oligonucleotides into pLKO.1 lentiviral plasmid. The sequences of the oligonucleotides are as follows: Rheb sense, 5'-CCGGTATGGAAAGGGTGATCAGTTACTCGAGTA ACTGATCACCCTTTTCATATTTTCATACCTTCCCACTAGTCAATGAGCTCATTGACTAGTGGGAAAGGTATAAAAAGTTAA-3'; Rheb antisense, 5'-AATTGAAAAATATGGAAAGGGTGATCAGTTACTCGAGTAAGTATCACCCTTTCCATA-3'. Lentiviral plasmids were propagated in and purified from Stbl2 competent cells (Invitrogen, Carlsbad, CA, USA) and co-transfected with the lentiviral packaging plasmids psPAX2 and pMD2.G into HEK293T cells for virus production. TSC1 shRNA sequence has been described.³⁴ The TSC1 targeting oligonucleotides were cloned into the *AgeI* and *EcoRI* sites of pLKO.1 lentiviral vector. The sequences of the oligonucleotides are as follows: TSC1 shRNA sense: 5'-CCGGGGGAGGTCAACGAGCTCTATTAAGCTTTAATAGAGCTCGTTGACCTCCCTTTTTC-3'; TSC1 shRNA antisense: 5'-AATTGAAAAAGGGAGGTCAACGAGCTCTATTAAGCTTTAATAGAGCTCGTTGACCTCCCTTTTC-3'. The lentiviral plasmids were co-transfected with psPAX2 and pMD2.G into HEK293 cells for virus production. The lentiviruses encoding mouse raptor shRNAs or scramble shRNA were used to infect TSC1^{-/-} MEF cells. HeLa cells were infected with lentiviruses encoding TSC1 shRNA or scramble shRNA. Stable shRNA-expressing pools of TSC1^{-/-} MEF or HeLa cells were selected with puromycin.

Cell culture. The TSC1^{+/+} and TSC1^{-/-} MEF, and TSC2^{+/+} and TSC2^{-/-} LEF cells were maintained in DMEM supplemented with 10% FBS. To generate Rheb-overexpressing or knockdown cell lines, cells were infected with lentivirus for 2 days and selected in 5 μ g/ml puromycin in culture medium.

Generation of cell lines. pPGS-TSC2 retroviral plasmid, pPGS vector, pQCXIH-Rheb wild type, pQCXIH-Rheb S16H or pQCXIH retroviral vector was transfected into HEK293P cells for retroviruses production. The retroviruses encoding TSC2 or empty pPGS vector were used to infect TSC2^{-/-} LEF cells. HeLa cells were infected with retroviruses encoding Rheb wild type, S16H or pQCXIH vector. At 48 h after infection, cells were selected with G418 or hygromycin B.

Preparation of cell lysates and nuclear extracts. To prepare cell lysates, cells were washed twice with ice-cold PBS and then lysed with lysis buffer (20 mM Tris, pH 8.0, 137 mM NaCl, 1% Nonidet P40, 10% glycerol, 1 mM Na₃VO₄, 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN, USA). Cell lysates were prepared by centrifugation for 10 min at 4 °C, and total protein concentration was determined by Bradford method.

Nuclear extracts were prepared as follows; cells were washed twice with ice-cold PBS, and pelleted by centrifugation at 1.500 $\times$ g for 10 min. The pellet was resuspended in 160 μ l of buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride) and cells were allowed to swell on ice for 15 min, after which 40 μ l of a 2.5% of Nonidet P-40 was added and the tube was vigorously vortexed for 10 s. The homogenate was centrifuged at 12 000 g for 5 min. The nuclear pellet was resuspended in 40 μ l of buffer C (20 mM HEPES, pH 7.9, 0.4 M NaCl, 1 mM EDTA, 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride) and the tube was vigorously vortexed for 20 min and centrifuged at 12 000 $\times$ g for 5 min at 4 °C and the supernatant was frozen in aliquots at -70 °C. Protein concentration was determined by Bradford method.

Western blot analysis. Cell lysates or nuclear extracts were resolved by SDS-PAGE and western blot analysis using specific antibodies and SuperSignal West Femto system (Thermo Scientific, Rockford, IL, USA) for detection.

RNA isolation and real-time reverse transcription PCR. Total RNA was isolated using Trizol reagent (Invitrogen) from wild-type or knockout cells and cDNA was synthesized using SuperScript III reverse transcriptase (Invitrogen). Induction of ATF4 and CHOP was quantitated by real-time PCR using a TaqMan gene expression system with Sybr Green (Applied Biosystems, Foster City, CA, USA). The primer sequences are as follows: ATF4 sense, 5'-CCTAGCTTGGCTGACAGAGG-3'; ATF4 antisense, 5'-CTGCTCC TTCTCCTTCATGC-3'; CHOP sense,

Figure 7 High Rheb activity sensitizes cells to ER stress-induced apoptosis. **(a)** HeLa cells were infected with control, wild type or constitutively active mutant (S16H) Rheb-encoding lentiviruses, and the overexpression was confirmed by western blotting using anti-CBP-tag antibody. **(b, c)** Cells were treated with medium (none), thapsigargin or tunicamycin. After 24 h, cell lysates were prepared to detect the induction of cleaved caspase-9 by western blot analysis **(b)**, and cells were harvested to measure the induction of cell death by FACS analysis **(c)**. **(d)** Wild-type MEFs were infected with lentiviral vectors that express none (N), constitutively active Rheb (Act) or shRNA targeting Rheb (KD). After 48 h, cells were treated with medium (none), MG 132 (MG), thapsigargin (TG) or tunicamycin (Tm) for 18 h, and cell lysates were prepared to analyze the induction of cleaved forms of caspase 9 or caspase 3 by western blotting using specific antibodies. Overexpression or knocking down of Rheb was measured by anti-Rheb antibody and protein loading was measured by using anti-actin antibody

5'-TGAAACCTCATGGGTTCTCC-3'; CHOP antisense, 5'-GTGTCATCCAACGTG GTCA-3'; actin sense, 5'-TACAGCTT CACCACCACAGC-3'; actin antisense, 5'-AAGGAAGG CTGGAAGAGC-3'. All values were normalized to the level of actin mRNA and fold expression was calculated according to the $\Delta\Delta C_T$ method: $\Delta\Delta C_T = \Delta C_{T\text{sample}} - \Delta C_{T\text{actin}}$.

Flow cytometry analysis of apoptosis. Cells were treated with culture medium, thapsigargin or tunicamycin for 24 h, and harvested and washed with PBS. Cells were stained with annexin V-FITC and PI for 15 min (Apoptosis Detection Kit, eBioscience, San Diego, CA, USA), and subjected to FACS analysis.

Figure 8 Raptor knockdown in TSC1^{-/-} MEFs restores ER stress response and protects the cells from ER stress-induced apoptosis. TSC1^{-/-} MEF cells were infected with shRNA-encoding lentiviruses targeting nothing (control) or raptor, and the knockdown of raptor was confirmed by western blot analysis. (b–d) Cells were treated with medium (none), thapsigargin or tunicamycin. Cell lysates were prepared after 4- or 24-h treatments and used to detect ATF6 (b) or cleaved caspase-3 (c) by western blotting. Induction of cell death was measured by FACS analysis after 24 h (d)

Conflict of interest

The authors declare no conflict of interest.

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