# Essential role of tuberous sclerosis genes *TSC1* and *TSC2* in NF-κB activation and cell survival

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## Summary

**The TSC1-TSC2 complex has recently been implicated in cell survival responses. We observed that NF-κB signaling is attenuated in *TSC1*- and *TSC2*-deficient MEFs concomitant with reduced survival following DNA damage or TNFα stimulation. Reconstitution of *TSC2* expression in *TSC2*$^{-/-}$ MEFs rescued survival in an NF-κB activity-dependent manner. Furthermore, in *TSC2*$^{-/-}$ MEFs, the rapamycin-mediated inhibition of deregulated mTOR activity restored NF-κB activation and survival. This rapamycin-mediated effect was reversed by inhibition of NF-κB transcriptional activation or by inhibition of ERK1/2 MAP kinase or PI-3K pathways, which lie on signaling cascades that lead to NF-κB activation. These results provide evidence for a crosstalk between the TSC/Rheb/mTOR pathway and the NF-κB induction pathways and indicate that NF-κB functions as an important survival factor that regulates *TSC2*-dependent cell survival.**

## Introduction

Autosomal dominant mutations in the tumor suppressor genes *TSC1* (*Hamartin*) or *TSC2* (*Tuberin*), give rise to hamartomatous lesions or tubers. The common hamartomas are subependymal giant cell astrocytomas (SEGAs) in the brain, rhabdomyomas of the heart, and angiomyolipomas of the kidneys (Kwiatkowski and Manning, 2005). Genetic studies and molecular characterization have placed the TSC1/2 complex in the phosphatidylinositol 3-OH kinase (PI-3K)-mammalian target of rapamycin (mTOR)/S6K1/4E-BP1 signaling pathway, directly downstream of Akt and upstream of mTOR. The C terminus of TSC2 harbors a GTPase-activating protein (GAP) domain that inhibits mTOR by stimulating the GTP hydrolysis activity of a member of the Ras superfamily GTPases, Ras homology enriched in brain (Rheb) (Kwiatkowski and Manning, 2005). Therefore, Rheb•GTP level and mTOR activity are upregulated in *TSC1*- and *TSC2*-deficient cells. Recent studies have shown that lack of *TSC1* or *TSC2* leads to downregulation of Akt and apoptosis under conditions of serum starvation or during exposure to chemotherapeutic agents (Harrington et al., 2004; Shah et al., 2004). The increased sensitivity of *TSC2*$^{-/-}$ mouse embryonic fibroblasts (MEFs) to DNA-damaging reagents has been ascribed to insulin/IGF-1 resistance, as rapamycin treatment restores insulin-stimulated Akt activation and this process correlates with increased chemoresistance in these cells. Since a better understanding of rapamycin-induced chemoresistance mechanisms would be important for identifying targets for therapeutic intervention, we investigated if additional signaling pathways are involved in the TSC1-TSC2 complex-dependent cell survival response.

The nuclear factor-kappa B (NF-κB) transcription factors are important antiapoptotic factors that regulate cell survival (Ghosh and Karin, 2002; Wang et al., 2002). The mammalian NF-κB/Rel family consists of five members (p65/RelA; RelB; c-Rel; p105, which is processed into p50; and p100, which is processed into p52) that bind to cognate DNA sequence as homodimers or heterodimers and elicit transcription of a number of genes. Under basal conditions, the p50/p65 NF-κB complex is sequestered by IκB family proteins. In response to specific stimuli, IκB proteins in complex with NF-κB are phosphorylated at Ser32 and Ser36 by the upstream protein kinase complex containing the IκB kinases α and β (IKK-α/1, IKK-β/2), the scaffold protein NF-κB essential modulator (NEMO)/IKKγ, and ELKS (Ducut

### SIGNIFICANCE

**The triene macrolide rapamycin has been identified as a therapeutic reagent for a wide variety of cancers with defects in the PI-3K-TSC1/2-mTOR signaling pathway. However, acquisition of resistance to chemotherapy remains a major problem in cancer therapy. Since rapamycin-mediated increases in apoptosis resistance in *TSC2*-deficient cells can be reversed by inhibition of NF-κB activation or its upstream signaling pathways, our studies suggest the potential use of NF-κB signaling inhibitors as adjuvants to maximize the efficacy of rapamycin-based therapeutics for *TSC*-derived tumors.**

Sigala et al., 2004). The N-terminally phosphorylated IκB proteins are recognized by SCF-βTrCP, rapidly polyubiquitylated, and subsequently degraded by the 26S proteasome (Ben-Neriah, 2002). The removal of IκB allows the p50/p65 NF-κB complex to bind specific DNA sequences and regulate the expression of its target genes. Recent studies indicate that posttranslational modification of NF-κB is critical for its transcriptional competence (Campbell and Perkins, 2004; Ghosh and Karin, 2002).

A number of signaling events, including phosphorylation and/or ubiquitylation, are important for NF-κB activation (Chen, 2005; Hayden and Ghosh, 2004; Viatour et al., 2005). Activation of NF-κB by the inflammatory cytokine tumor necrosis factor α (TNFα) requires activation of PI-3K and its downstream target, Akt. Akt mediates IKKα phosphorylation at Thr23 (Ozes et al., 1999). NF-κB activation following DNA damage by doxorubicin (adriamycin) and camptothecin also involves a sequential activation of kinase cascades, although the molecular mechanism is independent from cytokine signaling and less well understood. NF-κB is activated by a RIP- and IKK-dependent mechanism following DNA damage, but the functional role of RIP in this response pathway is distinct from the TNFR signaling pathway (Hur et al., 2003). Huang et al. reported that, following DNA damage, ATM phosphorylates NEMO, leading to replacement of inhibitory SUMO with ubiquitin on NEMO. This substitution allows nuclear exit and association with IKK, resulting in IKK activation (Huang et al., 2003). Additionally, UV radiation or doxorubicin-induced DNA damage can induce IKK-independent IκBα degradation (Kato et al., 2003; Tergaonkar et al., 2003). It is well known that ERK1/2 is involved in cell survival signaling (Gardner and Johnson, 1996; Tran et al., 2001; Xia et al., 1995). ERK is activated in response to cisplatin in ovarian cancer cells and may function in the response to DNA damage (Persons et al., 2000; Tang et al., 2002), although its role in mediating apoptosis remains to be clarified (MacKeigan et al., 2000; Wang et al., 2000). However, in this regard, tumors with constitutive ERK activation undergo apoptosis when ERK activity is blocked (Hoshino et al., 2001). ERK1/2 may also serve as an activator of NF-κB responses by stimulating p90Rsk activation (Ghoda et al., 1997; Panta et al., 2004; Ryan et al., 2000; Schouten et al., 1997). Doxorubicin-induced DNA damage has also been shown to activate an ATM-dependent, but p53-independent, MEK/ERK/p90Rsk/IKK signaling pathway that leads to NF-κB activation and cell survival (Panta et al., 2004). Also, MEKK1 and MEKK3 regulate IKKα and IKKβ activation, and a dominant-negative MEKK1 blocks Raf-1-induced NF-κB activation (Baumann et al., 2000; Lee et al., 1998; Yang et al., 2001). NF-κB DNA binding is also abolished by the MEK1 inhibitor PD98059 (Ryan et al., 2000). In this report, we show that one of the key mechanisms of cell survival mediated by the TSC1-TSC2 complex involves the activation of NF-κB. The TSC1-TSC2 complex modulates NF-κB activity by regulating Akt and ERK1/2 signaling cascades that lead to NF-κB activation. These findings may have therapeutic implications for the treatment of TSC-derived tumors.

## Results and discussion

### *TSC1*- and *TSC2*-deficient cells are more sensitive to DNA damage and TNFα-induced cell death and show reduced NF-κB activation

The TSC1-TSC2 complex has recently been implicated in the cell survival response. To investigate TSC1-TSC2 complex-mediated prosurvival signaling, we treated $TSC1^{-/-}$ or $TSC2^{-/-}$ MEFs with reagents known to induce cell death, and by extension, cell survival responses. Compared to MEFs obtained from $TSC2^{+/+}$ $p53^{-/-}$ and $TSC1^{+/+}$ $p53^{+/+}$ littermates, $TSC2^{-/-}$ $p53^{-/-}$ MEFs or $TSC1^{-/-}$ $p53^{+/+}$ MEFs showed increased susceptibility to cell death following treatment with the alkylating agent methyl methanesulfonate (MMS) (0.01% volume/volume) or TNFα (10 ng/ml) plus cycloheximide (5 μg/ml) (Figures 1A and 1B). Cycloheximide alone had no effect (data not shown). Single-blind quantitation of cell viability showed that, in the presence of either MMS or the anthracycline chemotherapeutic compound doxorubicin, there was approximately a 5-fold increase ($p < 0.001$) in cell death in MEFs deficient for *TSC2* (55.12% ± 1.84% for doxorubicin and 56.15% ± 4.24% for MMS) in comparison to $TSC2^{+/+}$ $p53^{-/-}$ (henceforth referred to as wild-type) MEFs (14.16% ± 5.26% and 12.88% ± 4.94%, respectively). TNFα plus cycloheximide treatment also resulted in a similar increase in cell death after 3 hr (70.87% ± 9.11%; Figure 1C). Immunofluorescence staining using phospho-ATM Ser1981 and γ-(phospho)H2AX antibodies confirmed that ATM was activated in response to MMS treatment in both wild-type and $TSC2^{-/-}$ MEFs (Figure 1D). Thus, the DNA damage response checkpoint is functional in the absence of *TSC2.* However, consistent with increased cell death, caspase 3 activation was significantly higher in $TSC2^{-/-}$ MEFs following exposure to MMS and TNFα (Figures S1A and S1B in the Supplemental Data available with this article online). In summary, a deficiency in either of the *TSC* genes, *TSC1* or *TSC2*, both of which are essential for a functional TSC1/2 complex, results in increased cell death following exposure to DNA-damaging reagents or to TNFα. It is interesting to note that the attenuation of prosurvival responses was observed in $TSC2^{-/-}$ MEFs, which are p53 deficient, as well as in the $TSC1^{-/-}$ MEFs, which express wild-type p53, indicating that the increased apoptosis observed in cells lacking functional TSC1/2 complex occurs independently of p53 function.

NF-κB activation is common to and involved in the protection against both TNFα- and DNA damage-induced cell death. Since $TSC2^{-/-}$ MEFs were sensitive to both these apoptosis-inducing stimuli, we tested the status of NF-κB signaling in these cells. In response to MMS or doxorubicin, or alternatively to inflammatory cytokines, such as TNFα, interleukin 1 (IL-1), and the bacterial lipopolysaccharide (LPS), the specific DNA binding activity of p65:p50 NF-κB complexes in nuclear extracts from wild-type MEFs increased, as determined by NF-κB electrophoretic mobility shift assay (EMSA). In contrast, no quantitative increase in active NF-κB-DNA complexes was observed in the $TSC2^{-/-}$ MEFs following these treatments. Control binding of Oct-1 to a specific probe showed no difference in electromobility shift upon induction in either cell line (Figure 1E). Additionally, total p65 and IκBα protein levels, when normalized to actin, were similar between the wild-type and $TSC2^{-/-}$ MEFs (Figure S1C). Taken together, these results indicate that $TSC2^{-/-}$ MEFs are more susceptible to cell death, and decreased cell survival correlates with a defect in NF-κB activation. Therefore, NF-κB activation may play a role in TSC1-TSC2 complex-dependent prosurvival signaling.

### $TSC2^{-/-}$ MEFs show reduced induction of NF-κB-dependent transcripts

NF-κB activation culminates in the transcription of NF-κB target genes. To verify if NF-κB-regulated gene expression is

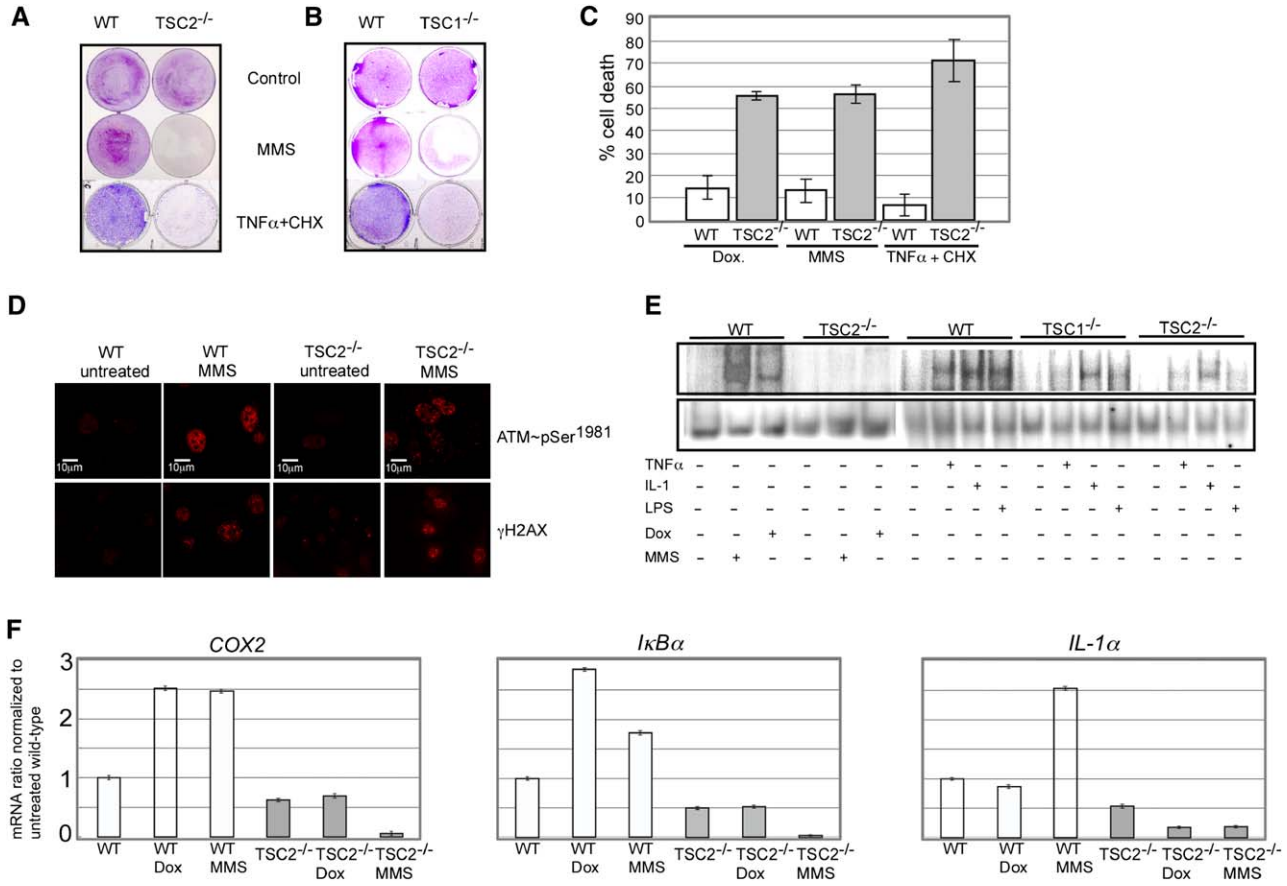


**Figure 1.** Increased DNA damage sensitivity and reduced NF-κB activation in *TSC1*- and *TSC2*-deficient cells

**A and B**: *TSC2*$^{+/+}$ *p53*$^{-/-}$ (*WT*), *TSC2*$^{-/-}$ *p53*$^{-/-}$ littermate-derived MEFs (**A**), or *TSC1*$^{+/+}$ *p53*$^{+/+}$ and *TSC1*$^{-/-}$ *p53*$^{+/+}$ littermate-derived MEFs (**B**) were treated with 0.01% MMS or 10 ng/ml TNFα plus cycloheximide (CHX) (5 μg/ml) for 4 hr or left untreated. Viable cells were fixed and stained with crystal violet.

**C:** *WT* and *TSC2*$^{-/-}$ MEFs were treated with 0.01% MMS for 2 hr, 0.7 μg/ml doxorubicin (Dox) for 6 hr, and 10 ng/ml TNFα plus cycloheximide for 3 hr or treated with cycloheximide alone or DMSO vehicle alone. Percentage of cell death is represented in the bar graph. Bars in graphs represent means ± standard deviations.

**D:** Active ATM and γH2AX foci in wild-type and *TSC2*$^{-/-}$ MEFs were detected by immunofluorescence after MMS treatment for 2 hr.

**E:** Wild-type and *TSC2*$^{-/-}$ MEFs were either untreated (lanes 1, 4, 7, 11, and 15) or treated with 0.7 μg/ml doxorubicin for 2 hr (lanes 3 and 6), 0.01% MMS for 2 hr (lanes 2 and 5), 10 ng/ml TNFα for 20 min (lanes 8, 12, and 16), 10 ng/ml IL-1 for 30 min (lanes 9, 13, and 17) or 10 ng/ml LPS for 30 min (lanes 10, 14, and 18), and NF-κB (top panel) and Oct-1 (bottom panel) DNA binding activity was detected by EMSA assays from wild-type, *TSC1*$^{-/-}$, or *TSC2*$^{-/-}$ MEFs as indicated.

**F:** Real-time PCR analyses of indicated genes from wild-type and *TSC2*$^{-/-}$ MEFs following treatment with MMS for 2 hr or doxorubicin for 6 hr.

compromised in the absence of *TSC2*, we quantitatively analyzed the expression profile of a number of NF-κB-inducible mRNAs by real-time PCR analyses, following exposure of *TSC2*$^{-/-}$ or wild-type MEFs to MMS or doxorubicin. *Cox2*, *IκBα*, and *IL-1α* mRNAs were induced approximately 2- to 2.5-fold after 2 hr of MMS treatment (Figure 1F). *Cox2* and *IκBα* responded similarly to treatment with doxorubicin, showing a 1.5- to 2.5-fold increase in abundance. On the other hand, none of these genes showed any induction following MMS or doxorubicin treatment in *TSC2*$^{-/-}$ MEFs. Similar results were obtained with other NF-κB target genes, such as *iNOS* and *IL-6*, but not with other genes not regulated by NF-κB (data not shown). However, the identities of NF-κB target genes that directly mediate the antiapoptosis response remain unclear. We did not observe inducible differences in antiapoptotic genes, such as *IAP1* or *Bcl-xL*, following MMS stimulation of *TSC2*$^{+/+}$ in our real-time PCR analyses under the conditions that we have used. Nonetheless, consistent with decreased DNA binding of NF-κB complexes, the transcriptional upregulation of some NF-κB-dependent genes was compromised in *TSC2*-deficient MEFs, following exposure to DNA-damaging reagents.

### TSC2 knockdown or Rheb expression in human tumor-derived cell lines results in decreased NF-κB activation, while reconstitution of *TSC2* in *TSC2*$^{-/-}$ MEFs restores NF-κB activation and NF-κB-dependent resistance to DNA damage-induced cell death

Given the possibility that *TSC2*$^{-/-}$ MEFs might have compensatory mutations in other pathways that result in the attenuation of NF-κB activation, we investigated whether acute disruption of TSC2 signaling in other genetic backgrounds has a similar effect on NF-κB activation. Toward this goal, we tested if siRNA-mediated knockdown of TSC2 can recapitulate the attenuation of NF-κB activation in HEK293 cells (Figure 2A). Compared to control siRNA-treated cells, there was a decrease in MMS-induced expression of three NF-κB target genes, *IκBα*, *Cox2*, and *TNFα*, following knockdown of *TSC2* expression as assayed by real-time PCR analyses (Figure 2C). We also transiently

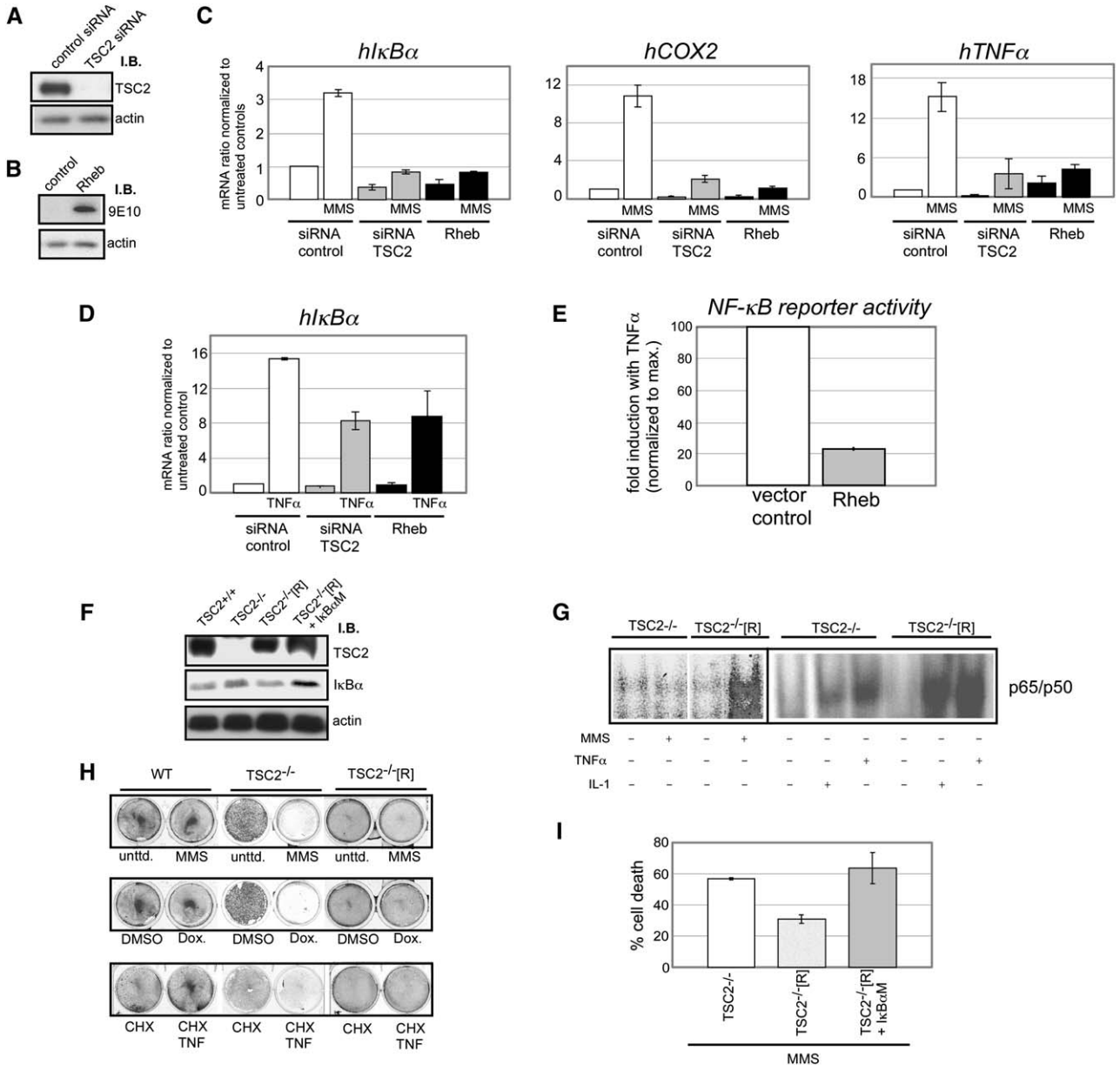


**Figure 2.** siRNA knockdown of TSC2 or Rheb expression in HEK293 cells attenuates NF-κB target gene transcription, while reconstitution of *TSC2* in *TSC2*$^{-/-}$ MEFs restores NF-κB activation and increases resistance to DNA damage-induced cell death in an NF-κB-dependent manner

**A:** TSC2 (top panel) or actin (bottom panel) immunoblot showing knockdown of TSC2 in HEK293 cells transfected with Stealth RNAi (Invitrogen).
**B:** Anti-myc (top panel) and actin (bottom panel) immunoblot showing Rheb expression in HEK293 cells.
**C and D:** Real-time PCR analyses in TSC2 siRNA-transfected, control siRNA-transfected, or Rheb-transfected HEK293 cells following 2 hr MMS treatment (**C**) or 2 hr TNFα treatment (**D**).
**E:** pNF-κBLuc reporter plasmid assay in Rheb-transfected HEK293 cells treated with TNFα for 6 hr.
**F:** Expression of Flag-tagged TSC2 in whole-cell lysates of *TSC2*$^{-/-}$ [R] MEFs.
**G:** *TSC2*$^{-/-}$ and *TSC2*$^{-/-}$ [R] MEFs were treated with MMS for 2 hr (lanes 2 and 4), TNFα for 20 min (lanes 7 and 10), or IL-1 for 30 min (lanes 6 and 9) as described in Figure 1E, and nuclear lysates were analyzed for NF-κB activation by EMSA.
**H:** Wild-type, *TSC2*$^{-/-}$, and *TSC2*$^{-/-}$ [R] MEFs were treated with MMS for 2 hr, doxorubicin for 6 hr, or TNFα plus cycloheximide for 3 hr as described in Figure 1A. Cell viability was assayed by crystal violet staining.
**I:** *TSC2*$^{-/-}$ MEFs were reconstituted with *TSC2* alone (*TSC2*$^{-/-}$ [R]), or sequentially with *TSC2* and *IκBαM* (*TSC2*$^{-/-}$ [R] + *IκBαM*) by infecting them with lentiviral vectors bearing respective expression constructs. Cell viability was assayed after MMS treatment for 2 hr. Bars in graphs represent means ± standard deviations.

overexpressed the TSC2 target Rheb in HEK293 cells (Figure 2B), which leads to increased Rheb•GTP and activation of mTOR, thus phenocopying the loss of *TSC2* (Garami et al., 2003; Im et al., 2002; Inoki et al., 2003). Similar attenuation of NF-κB target gene induction was observed (Figure 2C).

TNFα-induced expression of *IκBα*, as a representative NF-κB target, was also seen to be attenuated in TSC2 siRNA-treated or Rheb-expressing HEK293 cells (Figure 2D). Similarly, overexpression of Rheb repressed induction of an NF-κB:luciferase reporter plasmid by TNFα in HEK293 (Figure 2E) or HeLa cells

(Figure S2A). Thus, the effects of TSC2 on NF-κB activation are mediated by negative regulation of Rheb.

Attenuated induction of NF-κB target genes in response to MMS or TNFα, including *IκBα*, *COX2*, *TNFα*, and *IL-8*, was also observed by real-time PCR analyses using a panel of human tumor-derived cells lines (U2OS, MDA-MB-468, MCF7, and HCT116), following transient siRNA-mediated knockdown of *TSC2* expression (Figures S2B and S2C). Taken together, these results obtained in MEFs and in several different human tumor cell lines strongly suggest that the loss of *TSC2* per se, irrespective of genetic backgrounds, results in defective NF-κB activation.

To demonstrate conclusively that the observed defect in cell survival and NF-κB activation was a consequence of the lack of the *TSC2* gene and not a result of unrelated alterations in the genome of $TSC2^{-/-}$ MEFs, we reintroduced a Flag-tagged *TSC2* gene into these cells using a lentiviral expression vector. Lentiviral vector-mediated expression of *TSC2* restored TSC2 protein to a wild-type level in a pool of $TSC2^{-/-}$ MEFs (Figure 2F). NF-κB EMSA from $TSC2^{-/-}$-reconstituted ($TSC2^{-/-}$ [R]) MEFs showed a restoration of NF-κB activation following MMS, TNFα, or IL-1 treatment (Figure 2G). Reconstitution of the *TSC2* gene was also sufficient to impart protection against MMS, doxorubicin, or TNFα treatment to extents nearly resembling those observed with wild-type MEFs (Figure 2H).

To verify if NF-κB activation directly mediated the observed cell survival, we used a nondegradable mutant of IκBα, IκBαM. This mutant lacks the inducible N-terminal (Ser32 and Ser36) and constitutive phosphorylation sites in the C-terminal PEST domain (Tergaonkar et al., 2003) and therefore functions as a dominant-negative inhibitor of NF-κB activation. Lentivirus-mediated expression of IκBαM in wild-type MEFs significantly reduced cell survival following treatment with MMS or doxorubicin, in comparison to uninfected control cells (Figure S3). While reconstitution of *TSC2* alone ($TSC2^{-/-}$ [R] MEFs) imparted resistance to MMS (percent of cell death = 30.73 ± 2.66), expression of IκBαM in $TSC2^{-/-}$ [R] MEFs reduced cell survival to the levels observed for $TSC2^{-/-}$ MEFs (percent of cell death = 63.47 ± 10.12; Figure 2I). Infection with the IκBαM lentivirus did not affect *TSC2* expression (Figure 2F). The heterogeneity of expression in the TSC2-reconstituted pool contributed to some variability in survival in different experiments. However, IκBMα clearly abrogated the prosurvival effects of the reconstituted TSC2. Our results indicate that *TSC2*-dependent prosurvival signals function, at least in part, by activating the NF-κB pathway.

### ERK1/2 signaling mediates NF-κB activation and cell survival in response to DNA damage, while Akt functions in TNFα-dependent NF-κB activation

A number of protein kinases regulate NF-κB activation either by activating IKK or by modifying p65 after its release from IκB molecules (reviewed in Viatour et al., 2005). Immunoblotting with phospho-IKKαSer180/IKKβSer181-specific antibodies indicated that IKKα/β was activated following 30 min of MMS treatment in wild-type MEFs. Phosphorylation of these sites in the kinase domain correlates with increased IKK kinase activity (Delhase et al., 1999). In contrast, MMS treatment of $TSC2^{-/-}$ MEFs did not elicit sustained IKKα/β activation (Figure 3A, upper panel). Similarly, MMS treatment led to phosphorylation of IκBα at Ser32 in wild-type MEFs, but not $TSC2^{-/-}$ MEFs (Figure 3A, middle panel). Immunoblotting with phospho-p65 Ser536-specific antibodies demonstrated that this IKK-dependent phosphorylation site was phosphorylated in both wild-type MEFs and $TSC2^{-/-}$ MEFs following MMS treatment. While the phosphorylation was robust and sustained in wild-type MEFs, $TSC2^{-/-}$ MEFs failed to sustain the increase in Ser536 phosphorylation (Figure 3B).

Correspondingly, while IKKα/β phosphorylation was also increased in wild-type cells following TNFα treatment, no increase in phosphorylation was observed in $TSC2^{-/-}$ MEFs (Figure 3F). Additionally, the kinetics of Ser536 phosphorylation following TNFα treatment differed between wild-type and $TSC2^{-/-}$ MEFs, with $TSC2^{-/-}$ MEFs showing lower basal levels and a lack of sustained phosphorylation at this site, similar to that observed with DNA damage (Figure 3G). These data suggest that MMS and TNFα treatment fails to activate the IKK complex and maintain p65 transactivation in $TSC2^{-/-}$ MEFs, resulting in the lack of NF-κB activation.

Since deficiency of *TSC1* or *TSC2* leads to defects in PI-3K/Akt signaling, and Akt is a well-known regulator of NF-κB activation (Gustin et al., 2004; Madrid et al., 2000; Mayo et al., 2002; Osawa et al., 2002; Ozes et al., 2001; Reddy et al., 2000; Sizemore et al., 1999, 2002), we investigated if Akt plays a role in NF-κB activation following MMS-induced DNA damage. We failed to detect any significant increase in Ser473 phosphorylation in Akt following the treatment of wild-type, $TSC2^{-/-}$, and $TSC2^{-/-}$ [R] MEFs with MMS (Figure 3C). Consistent with this observation, no increase in Akt kinase activity was detected by in vitro kinase assays following MMS treatment in wild-type cells (data not shown). Furthermore, treatment with an Akt inhibitor, A-443654 (1 μM) (Luo et al., 2005), did not enhance MMS-stimulated cell death in either wild-type or $TSC2^{-/-}$ MEFs, nor did it affect MMS-induced NF-κB-dependent gene expression in wild-type MEFs (data not shown). Therefore, Akt does not appear to be a major regulator of DNA damage-induced NF-κB activation in these cells.

Activation of NF-κB by TNFα requires activation of PI-3K and its downstream target, Akt (Ozes et al., 1999). In our studies, stimulation of wild-type MEFs with TNFα (10 ng/ml) resulted in an increase in Akt activation within 10 min of TNFα stimulation, with maximal activation at 30 min (Figure 3H). Additionally, A-443654 blocked TNFα-induced NF-κB-dependent gene expression (Figure S4D). Consistent with reports that the PI-3K inhibitors LY294002 and wortmannin block TNFα-induced NF-κB activation in many cell types (Gustin et al., 2004), LY294002 prevented TNFα-induced NF-κB activation in wild-type MEFs (Figure S4E). In contrast, $TSC2^{-/-}$ MEFs, which had a lower basal level of Akt Ser473 phosphorylation, did not show any induction following TNFα treatment (Figure 3H). Furthermore, both the activating phosphorylations, Thr308 and Ser473, on Akt were restored in a TNFα-dependent manner following reconstitution of *TSC2* (Figure 3I). It is interesting to note that TNFα treatment of wild-type MEFs induced phosphorylation of TSC2 at the Akt phosphorylation site Thr1462, suggesting that downregulation of *TSC2* may be required for feedback regulation of TNFα signaling and NF-κB downregulation (Figure 3J). We conclude that Akt activation in response to TNFα is defective in $TSC2^{-/-}$ MEFs and that this difference could well account for defective NF-κB-dependent gene expression and protection against TNFα-induced cell death in these cells.

A number of protein kinases have been implicated in regulating NF-κB activity (Viatour et al., 2005). We investigated the activation of candidate kinases following MMS treatment in

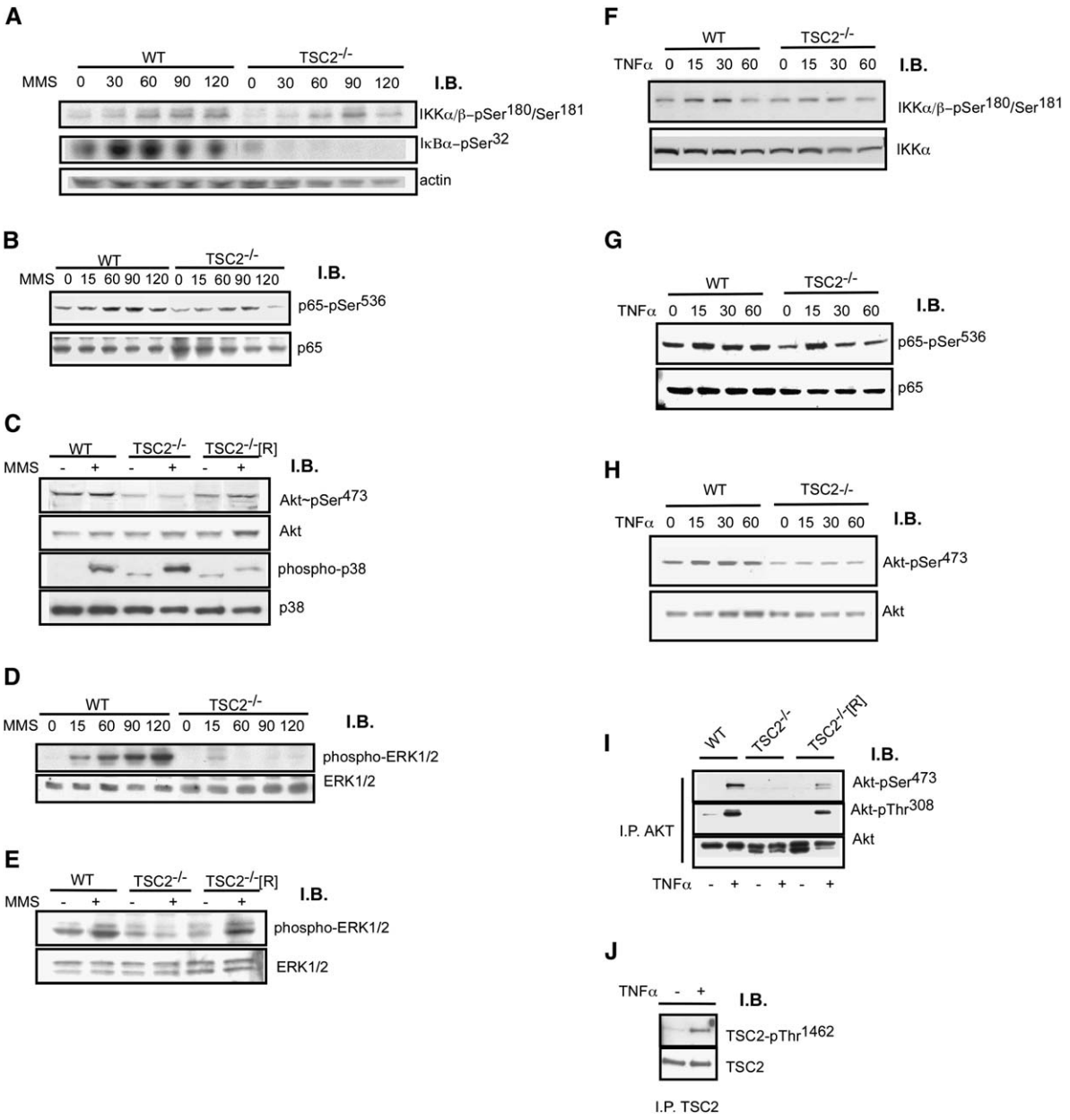


**Figure 3.** ERK1/2 activation in response to DNA damage is suppressed in *TSC2*-deficient MEFs

Wild-type and *TSC2*$^{-/-}$ MEFs were treated with MMS as indicated (**A**, **B**, and **D**) or for 2 hr (**C** and **E**), and whole-cell lysates were analyzed by immunoblotting with phospho-IKKα/β Ser180/181, phospho-IκBα Ser32, and actin antibodies (**A**); phospho-p65 Ser536 and p65 antibodies (**B**); phospho-Akt, Akt, phospho-p38 Thr180/Tyr182, and p38 antibodies (**C**); or phospho-ERK1/2 Thr202/185/Tyr204/187 and ERK1/2 antibodies (**D** and **E**) at the indicated time points. *TSC2*$^{-/-}$ [R] MEFs were included in some experiments (**C** and **E**). Alternatively, wild-type and *TSC2*$^{-/-}$ MEFs were treated with TNFα as indicated (**F**–**H**) or for 20 min (**I** and **J**), and cells were lysed at various time points as indicated. Akt (**I**) or TSC2 (**J**) was immunoprecipitated, or whole-cell lysates (**F**–**H**) were subjected to immunoblotting with phospho-IKKα/β Ser180/181 and IKKα (**F**); phospho-p65 Ser536 and p65 (**G**); phospho-Akt Ser473 and Akt (**H**); phospho-Akt Thr308, phospho-Akt Ser473, and Akt (**I**); or phospho-TSC2 Thr1462 and TSC2 (**J**) antibodies.

wild-type and *TSC2*$^{-/-}$ MEFs. While some kinases, such as Akt and GSK3 (data not shown), did not show any activation following DNA damage, p38 MAP kinase was activated, but to a similar extent in both wild-type and *TSC2*$^{-/-}$ MEFs (Figure 3C). Additionally, the p38 MAP kinase inhibitor SB203580 (10 μM) treatment caused no increase in MMS-induced cell death in *TSC2*$^{-/-}$ MEFs (data not shown). These results indicate that, although p38 MAP kinase may play a role in DNA damage-dependent NF-κB activation, this result cannot account for the differences in cell survival observed between wild-type and *TSC2*$^{-/-}$ MEFs. Investigation of the activation-specific phosphorylation of ERK1/2 indicated that ERK1/2 was induced between 15 and 30 min following MMS exposure and showed sustained activation in wild-type MEFs up to 2 hr (Figure 3D). In contrast, *TSC2*$^{-/-}$ MEFs failed to show sustained activation of ERK1/2 in response to MMS. Reexpression of *TSC2* in *TSC2*$^{-/-}$ [R] MEFs partially restored sustained ERK1/2 activation following treatment with MMS (Figure 3E). Additionally, the MEK1/2 inhibitor U0126, which blocks ERK1/2 activation, inhibited MMS-induced IKKα/β phosphorylation, inhibited NF-κB

activation, and reduced cell survival in wild-type MEFs (Figures S4A, S4B, and S4C). Taken together, our results show a correlation between the difference in ERK1/2 activity in wild-type and *TSC2*$^{-/-}$ MEFs and the differences in NF-κB activation and DNA damage sensitivity in these two cell lines. We did not observe any induction of ERK1/2 activation following TNFα stimulation in either cell type. These results indicate that the TSC1-TSC2 complex modulates NF-κB activation under a variety of different conditions by regulating IKKα/β activation. It is interesting to note that the molecular mechanisms for activation of NF-κB following DNA damage versus TNFα stimulation, although both showing a requirement for *TSC2*, involve different signal transduction pathways: DNA damage activates the ERK1/2 MAP kinase pathway, whereas TNFα signaling involves Akt.

Previous results from this laboratory have shown that rapamycin treatment protects *TSC2*$^{-/-}$ cells from camptothecin- or etoposide-induced cell death (Shah et al., 2004). Therefore, we tested if cell death induced by other classes of DNA-damaging agents, such as MMS, is also blocked by rapamycin treatment. *TSC1*$^{-/-}$ or *TSC2*$^{-/-}$ MEFs were pretreated overnight with 200 nM rapamycin and then subjected to MMS treatment. Rapamycin pretreatment significantly protected both *TSC1*$^{-/-}$ and *TSC2*$^{-/-}$ MEFs from MMS-induced cell death (Figure 4A). Quantitative analyses demonstrated that rapamycin pretreatment reduced MMS-induced cell death in *TSC2*$^{-/-}$ MEFs to 3.62% ± 5.12% (Figure 4B).

To investigate the mechanism of rapamycin-induced protection, we blocked the activation of different signaling components upstream of NF-κB using specific inhibitors and assessed the effect on rapamycin-induced survival of MMS-treated *TSC2*$^{-/-}$ cells. Wild-type MEFs or *TSC2*$^{-/-}$ MEFs that were pretreated overnight with rapamycin were treated with U0126, LY294002, or SB203580. Following treatment with the U0126 (50 μM) or LY294002 (20 μM), but not SB203580 (10 μM), rapamycin-treated *TSC2*$^{-/-}$ MEFs showed dramatically increased sensitivity to MMS-induced DNA damage (percent of cell death with U0126 = 55.87 ± 9.92 and percent of cell death with LY294002 = 51.16 ± 16.50) (Figure 4C). Consonantly, ERK1/2 activation was partially restored in *TSC2*$^{-/-}$ cells in the presence of rapamycin (Figure 4D), and rapamycin treatment restored NF-κB activation (Figure 4E). Similarly, rapamycin treatment restored Akt activation following TNFα treatment in *TSC2*$^{-/-}$ MEFs (Figure 4G), and this correlated with increased NF-κB activation (Figure 4H). The rapamycin-dependent rescue of NF-κB activation following MMS or TNFα treatment was inhibited by U0126 or LY294002, respectively (Figures 4F and 4I).

We also tested the effects of directly interfering with NF-κB activation following rapamycin pretreatment on cell survival, by using a cell-permeant quinazoline compound that acts as a potent inhibitor of NF-κB transcriptional activation (Correa et al., 2005; Tobe et al., 2003). 6-Amino-4-(4-phenoxyphenylethylamino) quinazoline (NAI) (20 μM) treatment, which specifically blocked NF-κB-dependent transcription (Figure S5), effectively reversed the rapamycin-mediated protection from MMS-induced cell death (Figure 4C). Thus, in the absence of *TSC2*, an mTOR-dependent feedback mechanism results in ERK1/2, Akt, and NF-κB downregulation. Rapamycin treatment rescues ERK1/2 and Akt activation, and consequently NF-κB activation. Inhibition of NF-κB activation or upstream signaling overcomes rapamycin-induced chemoresistance in *TSC2*$^{-/-}$ MEFs.

The molecular mechanisms of TSC2-dependent ERK1/2 and Akt activation remain unclear. This may occur through a common or distinct pathway and awaits further investigation. Akt is a target of negative regulation in the absence of TSC2 through an mTOR-S6K1-regulated feedback pathway that inactivates IRS-1 by multiple means, including phosphorylation, protein degradation, and transcriptional downregulation (Harrington et al., 2005; Shah and Hunter, 2005). The absence of TNFα-stimulated Akt activation and its restoration upon rapamycin treatment is consistent with such an mTOR-mediated downregulation of PI-3K and Akt in *TSC2*$^{-/-}$ MEFs. In some, but not all cell types, inhibition of PI-3K leads to suppression of ERK1/2 activity (Chaudhary et al., 2000; Cross et al., 1994; Duckworth and Cantley, 1997; King et al., 1997). Thus, it is conceivable that mTOR-mediated negative regulation of PI-3K in *TSC2*$^{-/-}$ MEFs may also modulate ERK1/2 activation. *TSC2* expression increased ERK1/2 phosphorylation and B-Raf kinase activity in *TSC2*$^{-/-}$ ELT3 cells, whereas siRNA downregulation of *TSC2* resulted in decreased ERK1/2 phosphorylation in HEK293 cells (Karbowniczek et al., 2004). Conversely, Rheb expression decreased ERK1/2 phosphorylation, further supporting a role for *TSC2* in ERK1/2 regulation. However, this regulation appears to be mTOR independent. Interestingly, Finlay et al. (2005) have reported a similar temporal pattern of PDGF-dependent ERK1/2 phosphorylation, yet reduced nuclear translocation of ERK1/2 in *TSC2*$^{-/-}$, in comparison to *TSC2*-reconstituted ELT3 cells. The mechanism of ERK activation in *TSC2*$^{-/-}$ cells, in contrast to *TSC2*$^{+/+}$ cells, was independent of MEK but dependent on reactive oxygen and mTOR. During our studies, we noted that transient activation of ERK1/2 following EGF or TPA stimulation was comparable between wild-type and *TSC2*$^{-/-}$ MEFs, suggesting that the defect in ERK1/2 activation may be signal specific and limited to sustained activation (Figure S6). However, other investigators did not observe any effect of the TSC1-TSC2 complex on ERK1/2 (Garami et al., 2003; Tee et al., 2003). On the basis of the time course required for rapamycin-mediated rescue of Akt or ERK1/2 activation, we cannot rule out complex feedback regulation that may require transcription/translational rather than a direct phosphorylation of substrates by mTOR. Nevertheless, our results establish that there is a crosstalk between the TSC/Rheb/mTOR and the NF-κB pathways that modulates cell survival.

In conclusion, our results demonstrate that the TSC1-TSC2 complex is an important modulator of NF-κB activation. In our model (Figure 5), DNA damage causes activation of ERK1/2 through a pathway whose details remain to be fully elucidated, and this in turn results in activation of IKK and NF-κB. Stimulation of the TNFα receptor elicits Akt activation, and this leads to similar activation of IKK and NF-κB. The molecular details of IKK activation following activation of ERK1/2 or Akt remain unexplored. Nonetheless, with both stimuli, high mTOR activity, as found in *TSC2*$^{-/-}$ cells, blocks coupling to IKK at the level of ERK1/2 and Akt, respectively. Based on the effects of small molecule inhibitors on cell survival, we deduce that resistance of rapamycin-treated *TSC2*$^{-/-}$ cells to DNA damage-induced cell death requires PI3-K activity, ERK activation, and most importantly, downstream NF-κB activation. While other pathways, such as HIF1α or Akt/PKB and ERK-dependent, NF-κB-independent cell survival pathways, such as FOXO or Bim phosphorylation (O.J. Shah and T.H., unpublished data), may also be important, our results demonstrate that

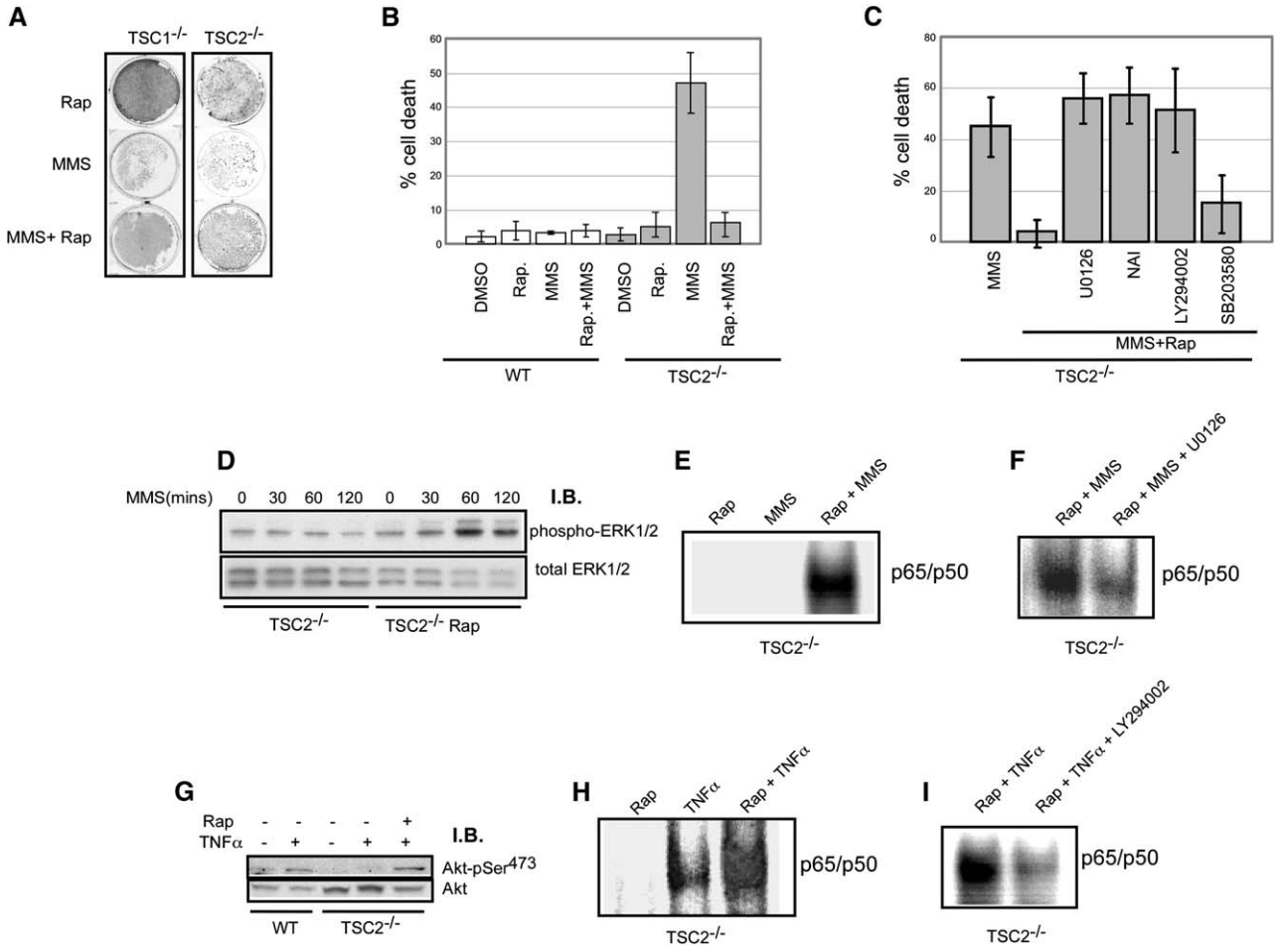


**Figure 4.** Treatment with rapamycin protects against increased DNA damage-sensitive cell death and rescues ERK1/2 signaling and NF-κB activation in *TSC2*$^{-/-}$ MEFs

**A and B:** *TSC1*$^{-/-}$ or *TSC2*$^{-/-}$ MEFs were pretreated overnight with 200 nM rapamycin (Rap) or left untreated. Subsequently, MEFs were exposed to MMS for 2 hr, and cell viability was analyzed. Crystal violet staining (**A**) or bar graph representing survival determined by trypan blue staining (**B**) are shown. Bars in graphs represent means ± standard deviations.

**C:** Cell viability in *TSC2*$^{-/-}$ MEFs treated with MMS for 2 hr in combination with U0126 (50 μM), NF-κB inhibitor (NAI) (20 μM), LY294002 (20 μM), or SB203580 (10 μM) following overnight treatment with 200 nM rapamycin. Cells were pretreated for 30 min with inhibitors alone prior to the application of MMS. Bars in graphs represent means ± standard deviations.

**D and E:** *TSC2*$^{-/-}$ MEFs or *TSC2*$^{-/-}$ MEFs pretreated overnight with 200 nM rapamycin were treated with MMS, and whole-cell lysates were analyzed by immunoblotting with phospho ERK1/2 or ERK1/2 antibodies at various times as indicated (**D**), or NF-κB DNA binding activity in nuclear lysates was analyzed by EMSA (**E**).

**F:** *TSC2*$^{-/-}$ MEFs pretreated overnight with 200 nM rapamycin or *TSC2*$^{-/-}$ MEFs pretreated overnight with 200 nM rapamycin and subsequently with U0126 for 30 min were further treated with MMS or MMS in combination with U0126 for 2 hr, respectively. NF-κB DNA binding activity in nuclear lysates was analyzed by EMSA.

**G and H:** *TSC2*$^{-/-}$ MEFs were left untreated or pretreated overnight with 200 nM rapamycin and subsequently treated with TNFα for 20 min. Lysates were analyzed by immunoblotting with phospho-Akt Ser473 or Akt antibodies (**G**), or NF-κB DNA binding activity in the nuclear lysates was analyzed by EMSA (**H**).

**I:** *TSC2*$^{-/-}$ MEFs pretreated overnight with 200 nM rapamycin or *TSC2*$^{-/-}$ MEFs pretreated overnight with 200 nM rapamycin and subsequently with LY294002 for 30 min were further treated with TNFα or TNFα in combination with LY294002 for 20 min, respectively. NF-κB DNA binding activity in the nuclear lysates was analyzed by EMSA.

NF-κB signaling is a critical component in *TSC2*-mediated cell survival.

Deregulation of the NF-κB pathway is associated with oncogenesis, malignancies, and metastasis (Baldwin, 2001; Huber et al., 2004; Karin et al., 2002). The lack of activation of the NF-κB-dependent cell survival pathway in *TSC2*$^{-/-}$ cells may potentially explain the relatively weak malignancy in *TSC*-derived tumors. NF-κB activation also contributes to the antiapoptotic function of insulin and may contribute to insulin-mediated survival in these cells (Bertrand et al., 1998). Additionally, we demonstrate that NF-κB activation is a critical mechanism that contributes to chemoresistance and cell survival in rapamycin-treated *TSC2*$^{-/-}$ MEFs. Several recent reports have demonstrated that combined therapeutic approaches are required for the treatment of tumors with mutations in the Akt signaling pathway because of redundant proliferation pathways (O'Reilly et al., 2006; Raje et al., 2004; Xing and Orsulic, 2005). The NF-κB pathway is an attractive target for overcoming antiapoptotic responses to promote chemosensitivity in various cancers (Cusack, 2003). Our studies indicate a need for combinatorial targeting of mTOR plus the NF-κB pathway to maximize the benefits of anticancer therapeutics for *TSC*-derived tumors. Although strongly suggestive, it remains to be documented if activation of the NF-κB pathway by rapamycin in human

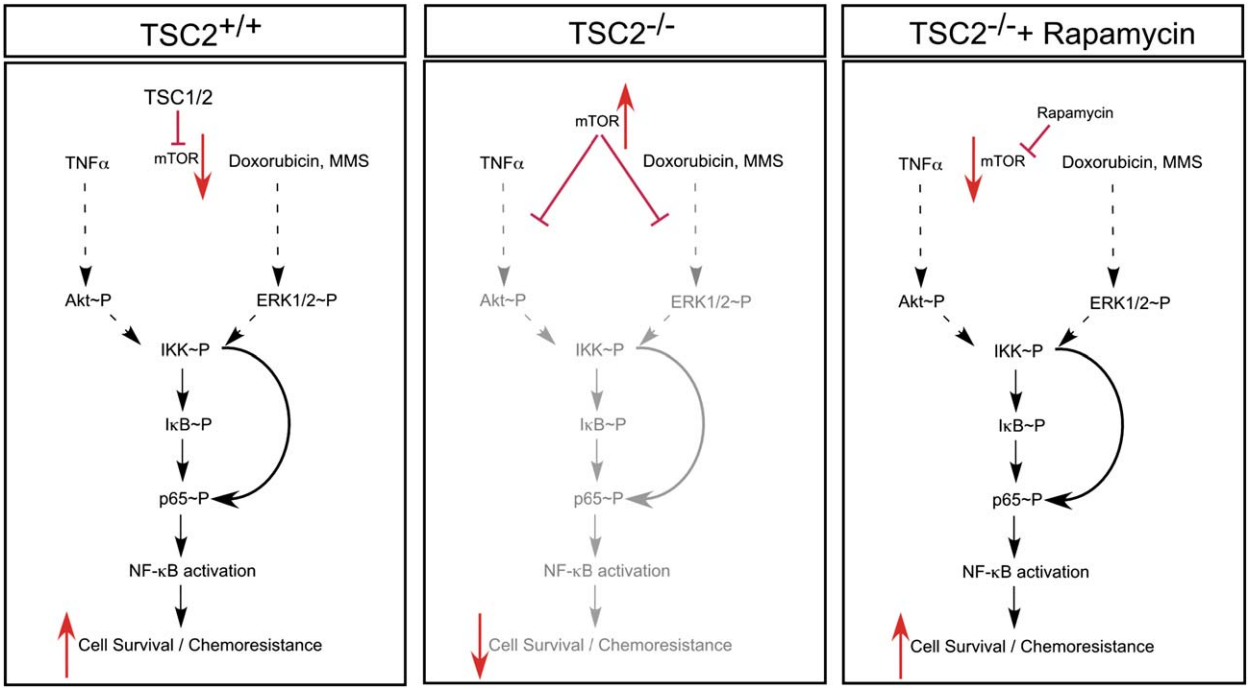


**Figure 5.** Model depicting NFκB activation and cell survival/chemoresistance in wild-type, *TSC2*$^{-/-}$, and *TSC2*$^{-/-}$ MEFs treated with rapamycin following treatment with TNFα and MMS/doxorubicin

The three panels represent pathways leading to NF-κB activation downstream of TNFα and MMS/doxorubicin-induced DNA damage in wild-type, *TSC2*$^{-/-}$, and *TSC2*$^{-/-}$ MEFs treated with rapamycin. Increased mTOR activity in TSC2$^{-/-}$ MEFs (middle panel) results in the downregulation of signaling to Akt and ERK1/2 (red bars), as depicted by the lighter shade of the downstream NF-κB pathway components. This is reversed upon rapamycin-induced inhibition of mTOR.

*TSC*-derived tumors plays a role in the acquisition of chemoresistance. Further studies are required to determine if targeting NF-κB activation and its upstream signaling components, together with rapamycin therapy, may enhance the efficacy of therapeutic regimens in tuberous sclerosis patients.

## Experimental procedures

### Cell culture and reagents

*TSC2*$^{+/+}$ *p53*$^{-/-}$ and *TSC2*$^{-/-}$ *p53*$^{-/-}$ (Kwiatkowski et al., 2002) or *TSC1*$^{+/+}$ *p53*$^{+/+}$ and *TSC1*$^{-/-}$ *p53*$^{+/+}$ MEFs (Zhang et al., 2003), as well as U2OS, MDA-MB-468, MCF7, and HCT116 cells were cultured in DMEM supplemented with 10% FCS (Hyclone), 100 μg/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Gibco). Doxorubicin, MMS, and LPS were purchased from Sigma. TNFα, IL-1, U0126, NF-κB activation inhibitor (NAI) (cat. no. 481406), LY294002, SB203580, and rapamycin were obtained from Calbiochem.

### Antibodies

IKKα, p65 (RelA), IκBα, and TSC2 (tuberin) antibodies were obtained from Santa Cruz Biotechnology. Phospho-IKKα/β Ser180/Ser181, Akt, phospho-Akt Thr308, phospho-Akt Ser 473, phospho-p65 Ser536, ERK1/2, phospho-TSC2 Thr1462, phospho-IκBα Ser 32, γH2AX, and activated caspase 3 Asp175 antibodies were obtained from Cell Signaling Technology. Phospho-ERK1/2 Thr183/Tyr185, Flag-M2 antibodies, and β-actin antibodies were purchased from Sigma. p38α was obtained from R&D Systems. Phospho-p38 Thr180/Tyr182 antibodies were from Zymed. Phospho-ATM Ser1981 antibodies were from Rockland Immunochemicals, Inc.

### Cell survival assay and statistical analyses

MEFs were treated with 0.01% MMS or 0.7 μg/ml doxorubicin or 10 ng/ml TNFα as described or with vehicle alone (DMSO/cycloheximide 5 μg/ml) for the indicated time. Culture dishes were washed three times with phosphate-buffered saline (PBS), and remaining adherent cells were fixed and stained with 0.05% crystal violet solution in 20% ethanol. Alternatively, cell viability was assessed by trypan blue staining. Three representative samples from a uniform suspension of MEFs were diluted 1:10 in 0.4% trypan blue (Sigma). The number of viable cells was determined by counting the trypan blue-excluding cells in a hemocytometer. The percent of cell viability was calculated from the ratio of the number of viable cells (trypan blue-excluding or nonapoptotic cells) divided by the total number of cells. The mean ± standard deviation was calculated from three independent experiments.

### EMSA

Nuclear extracts were prepared, and EMSA were performed as described previously (Tergaonkar et al., 2002).

### Immunofluorescence analysis

Cells grown on coverslips were washed with cold PBS and fixed for 30 min in 4% paraformaldehyde at room temperature. Fixed cells were permeabilized with PBS-1% Triton X-100, blocked with 10% normal goat/horse serum, and incubated with the antibodies mentioned in the text. After extensive washes, the primary antibodies were visualized with Rhodamine red-X (Jackson ImmunoResearch Labs)-labeled fluorescent secondary anti-rabbit Ig antibodies using a Zeiss scanning confocal microscope.

### RNA extraction and reverse transcription

After stimulation, cells were harvested, and total RNA was isolated using the RNeasy mini kit (Qiagen). Reverse transcription was performed according to the manufacturer's instructions with RT Superscript III (Invitrogen Life Technologies).

### Real-time PCR analysis

PCR reactions were performed on a ABI Prism 7700 Sequence Detection System using SYBRGreen PCR master mix (Applied Biosystems, Warrington, UK). Real-time PCR analyses are represented as a ratio of the gene-specific transcripts over 18S rRNA, normalized to untreated wild-type.

### TSC2 knockdown and expression of Rheb

Various cell lines were transfected with pooled validated TSC2 Stealth RNAi 1 and 2 or pooled Stealth RNAi Negative Control LO GC and Stealth RNAi Negative Control Med GC (Invitrogen) at a final concentration of 50 nM using Lipofectamine 2000 according to the manufacturer's suggested protocol. Myc-tagged Rheb in pRK7 (Tee et al., 2003) was transfected using Effectene (Qiagen) or by calcium phosphate method. Cells were assayed 48–72 hr posttransfection.

### NF-κB luciferase reporter assays

HEK293 cells ($2 \times 10^5$) were plated into six-well plates and, after overnight incubation, cotransfected with 0.5 mg of pNF-κBLuc reporter plasmid in combination with 0.2 mg of pCMV-LacZ plasmid and Rheb-expressing plasmid using calcium phosphate. Corresponding empty vector was included in some transfections as control. Two milligrams of total DNA amount was used per transfection. Forty-eight hours posttransfection, cells were treated for 8 hr with TNFα, lysates were prepared, and luciferase activity and β-galactosidase activity were measured. Luminometric and colorimetric reactions were read on the Wallac 1420 plate reader (Perkin Elmer). Luciferase activity was corrected for transfection efficiency using β-galactosidase activity of cotransfected pCMV-LacZ plasmid. Fold reduction after Rheb transfection was normalized to TNFα-induced maximum luciferase activity with control transfection.

### Immunoblots and immunoprecipitations

MEFs were washed with PBS and harvested in RIPA buffer. Whole-cell lysates were resolved by electrophoresis, and proteins were transferred onto PVDF membrane (Immobilon P, Millipore), blocked in TBS containing 0.2% Tween-20 (TBST) and 3% BSA and probed with the indicated antibodies in TBST with 3% BSA.

For immunoprecipitations, total cell lysates were prepared in a buffer containing 50 mM Tris·HCl (pH 8), 170 mM NaCl, 0.5% Nonidet P-40, 50 mM NaF supplemented with complete protease inhibitors (Roche), and phosphatase inhibitors I and II (Sigma) at 4°C. Immunoprecipitations were performed after preclearing the lysates for 1 hr by incubation with protein A-Sepharose (Amersham Biosciences). Precleared supernatants were incubated overnight with primary antibodies at 4°C, and protein A-Sepharose beads were added for the last 2 hr. The immunoprecipitates were washed three times with 10 mM Tris·HCl (pH 8), 250 mM NaCl, 5 mM EDTA, 0.5% Nonidet P-40, and complete protease inhibitors.

### Generation of lentiviral vectors and cell lines

The LV-GFP and LV-TSC2 constructs were derived from p156RRLsin-PPTPGK-eGFP-PRE vector, wherein the transgene is driven by the CMV promoter. The production of lentiviruses was performed as per standard protocols (Ikawa et al., 2002). To generate cell lines, *TSC2*$^{-/-}$ MEFs were infected with either pLV-GFP or pLV-TSC2 lentiviral vectors. Pools of infected cells were used in the assays described.

### Supplemental data

The Supplemental Data include Supplemental Experimental Procedures and six figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/10/3/215/DC1/.

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