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p53 Target Genes Sestrin1 and Sestrin2 Connect Genotoxic Stress and mTOR Signaling

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SUMMARY

The tumor suppressor p53 is activated upon genotoxic and oxidative stress and in turn inhibits cell proliferation and growth through induction of specific target genes. Cell growth is positively regulated by mTOR, whose activity is inhibited by the TSC1:TSC2 complex. Although genotoxic stress has been suggested to inhibit mTOR via p53-mediated activation of mTOR inhibitors, the precise mechanism of this link was unknown. We now demonstrate that the products of two p53 target genes, Sestrin1 and Sestrin2, activate the AMP-responsive protein kinase (AMPK) and target it to phosphorylate TSC2 and stimulate its GAP activity, thereby inhibiting mTOR. Correspondingly, Sestrin2-deficient mice fail to inhibit mTOR signaling upon genotoxic challenge. Sestrin1 and Sestrin2 therefore provide an important link between genotoxic stress, p53 and the mTOR signaling pathway.

INTRODUCTION

The major tumor suppressor p53 is a stress-activated transcription factor that can either inhibit cell proliferation or induce apoptotic cell death (Levine, 1997). The different functions of p53 are mediated through numerous target genes, such as $p21^{Waf1}$, which serves as a cell-cycle inhibitor (Levine, 1997). In addition to cell proliferation, p53 also inhibits cell growth, a function that is important for preventing the wasteful buildup of proteins and other cellular building blocks during periods of stress (Vousden and Lane, 2007). Cell growth is regulated by the target of rapamycin (TOR), an evolutionary conserved protein (280 kDa) that belongs to the phosphatidylinositol kinase-related kinase (PIKK) subfamily (Wullschleger et al., 2006). The mammalian TOR protein (mTOR) forms two distinct signaling complexes, called mTORC1 and mTORC2. The mTORC1 complex, which, in addition to mTOR, consists of raptor, PRAS40, and mLST8, is responsible for control of cell growth and protein synthesis (Guertin and Sabatini, 2007; Yang and Guan, 2007). The mTORC2 complex, consisting of mTOR, rictor, Sin1, PROTOR, and mLST8, controls the actin cytoskeleton and cell spreading (Astrinidis et al., 2002; Sarbassov et al., 2004; Yang and Guan, 2007). Rapamycin, an inhibitor of cell growth, rapidly inhibits mTORC1 through interaction with FKBP12, but after a while, it may also inhibit mTORC2 (Sarbassov et al., 2006). mTORC1 activity is positively regulated by growth factors through the insulin/insulin-like growth factor (IGF1)-phosphoinositide-3′ kinase (PI3K)-AKT pathway, the Wnt-GSK3 pathway, and the ERK-RSK kinase cascade (Wullschleger et al., 2006; Yang and Guan, 2007). mTORC1 activity is inhibited by adverse environmental and metabolic conditions, including nutrient limitation, hypoxia, and DNA damage (Corradetti and Guan, 2006; Inoki et al., 2003a).

The positive and negative control of mTORC1 activity is exerted through the TSC1:TSC2 complex, whose TSC2 subunit serves as a GAP for the small GTPase Rheb, which activates mTOR (Corradetti and Guan, 2006; Hay and Sonenberg, 2004; Wullschleger et al., 2006). TSC2 activity is regulated by several kinases, including AKT, ERK, RSK, and AMP-activated protein kinase (AMPK) (Corradetti and Guan, 2006; Kwiatkowski and Manning, 2005). Genotoxic stress was suggested to inhibit mTORC1 activity through p53-dependent upregulation of negative regulators such as PTEN, TSC2, and AMPKβ1 (Feng et al., 2007). p53 also increases the phosphorylation of the AMPKα subunit, leading to AMPK activation (Feng et al., 2005). However, the precise physiological mechanism by which p53 activates AMPK and inhibits mTOR is not established.

Two recently described p53 target genes genes are Sestrin1 (Sesn1)/PA26 and Sestrin2(Sesn2)/Hi95 (Budanov et al., 2002; Peeters et al., 2003; Velasco-Miguel et al., 1999). Previous studies have shown that Sesn1 and Sesn2, whose expression is induced upon DNA damage and oxidative stress, may have a cytoprotective function based on regeneration of overoxidized peroxiredoxins (Budanov et al., 2004). We now demonstrate that Sesn1 and Sesn2 are negative regulators of mTOR signaling and that they execute this redox-independent function through activation of AMPK and TSC2 phosphorylation. We also provide in vivo evidence for the functional importance of Sesn2 in inhibition of mTOR signaling in mice treated with a p53-activating alkylating agent. Hence, Sesn1 and Sesn2 link genotoxic stress, p53, and mTOR signaling.

RESULTS

Sestrins Inhibit mTOR-Dependent S6K and 4E-BP1 Phosphorylation

To examine whether Sestrins regulate mTOR, we used p70S6K phosphorylation (Burnett et al., 1998; Corradetti and Guan, 2006) as a readout. p70S6K activation can in turn be determined by phosphorylation of ribosomal S6 protein at S235/236 (Dufner and Thomas, 1999; Pende et al., 2004). We cotransfected HEK293 cells with HA-tagged p70S6K together with Sesn1-Flag (F), Sesn2F, both TSC1F and TSC2F, or GFP expression vectors. HA-p70S6K was immunoprecipitated, and its phosphorylation was examined with phospho-specific antibodies. Ectopic expression of either Sesn1F or Sesn2F dramatically inhibited T389 p70S6K phosphorylation, as did TSC1F:TSC2F overexpression (Figure 1A). Although Sesn1F was expressed less efficiently than Sesn2F, its inhibitory effect was pronounced (Figure 1A). However, when expressed at similar levels, no substantial differences in the inhibitory activity of the two Sestrins were observed (Figure S1A available online). To examine regulation of endogenous mTOR and p70S6K, we generated MCF7-tet OFF cells with doxycycline-regulated Sesn2F. To reduce possible toxicity due to Sesn2 overexpression, we infected cells with low-titer virus and selected cells on puromycin as described (Budanov et al., 2002). After 24 hr of doxycycline removal, Sesn2F was expressed, and considerable inhibition of p70S6K and S6 phosphorylation was observed (Figure 1B). This inhibitory effect of Sesn2 was seen as early as 6 hr after doxycycline withdrawal, when its expression was barely detectible (Figure S1B). Another important target of mTOR is 4E-BP1, which is phosphorylated on many sites (Hay and Sonenberg, 2004). We examined 4E-BP1 phosphorylation using anti-phospho-S65 antibodies, as well as changes in its electrophoretic mobility (Guan et al., 2007). Sesn2 induction decreased the abundance of the hyperphosphorylated γ form of 4E-BP1 and increased the abundance of the hypophosphorylated α form (Figure 1B). Similar results were seen upon infection of cells with lentiviral vectors expressing Sesn1 or Sesn2 (Figure S1C).

In an attempt to localize the protein domain responsible for inhibition of mTOR, we expressed Sesn2 deletion mutants in H1299 cells. Deletion of either the N- or C-terminal portions completely blocked the ability of Sesn2 to inhibit mTOR signaling (Figure S1D). mTOR is redox sensitive (Dames et al., 2005; Sarbassov and Sabatini, 2005), and the Sestrins were reported to be involved in redox-mediated reactivation of peroxiredoxins (Budanov et al., 2004). However, a redox-inactive Sesn2(C125S) mutant (Budanov et al., 2004) was as effective as WT Sesn2 in inhibition of p70S6K and S6 phosphorylation (Figure 1C). Expression of peroxiredoxin 1 (Prx1) had no effect on p70S6K and S6 phosphorylation.

Nonphosphorylated 4E-BP1 binds and sequesters eIF-4E, thereby inhibiting formation of the eIF-4F complex involved in initiation of Cap-dependent translation of a subset of mRNAs that include cyclin D1 and c-Myc (Thomas, 2006; Averous and Proud, 2006; Mamane et al., 2004). Phosphorylated 4E-BP1 cannot interact with eIF-4E and does not inhibit eIF-4F formation (Richter and Sonenberg, 2005). To determine whether Sesn1 and Sesn2 modulate the interaction between 4E-BP1 and eIF-4E, we

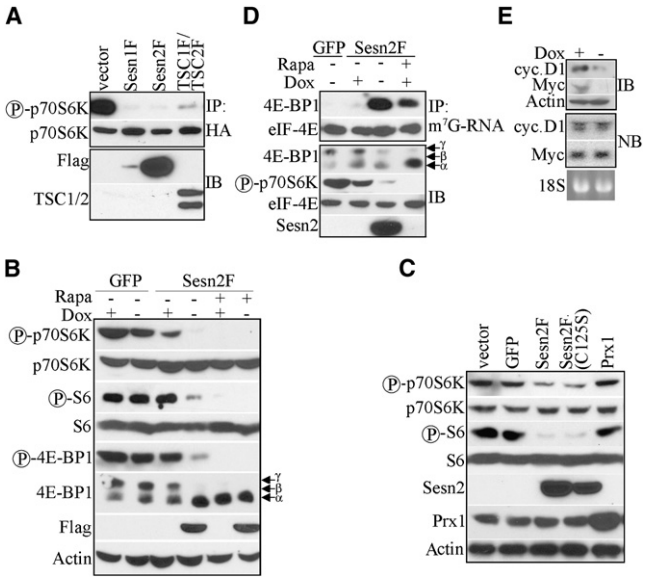

Figure 1. Sesn1 and Sesn2 Inhibit mTOR Signaling

(A) Ectopic Sesn1, Sesn2, or TSC1:TSC2 expression decreases p70S6K phosphorylation. HEK293 cells were cotransfected with HA-p70S6K together with Sesn1F, Sesn2F, TSC1F plus TSC2F, or GFP expression vectors. After 48 hr, HA-p70S6K was immunoprecipitated (IP), and its phosphorylation was examined by immunoblotting. Sesn1 and Sesn2 and TSC1:TSC2 expression in total lysates was examined by immunoblotting (IB).

(B) Induction of Sesn2 inhibits mTOR signaling. Sesn2F was induced in MCF7-tet OFF Sesn2F cells by doxycycline (Dox) removal. Control cultures were left with doxycycline. After 22 hr, rapamycin (Rapa; 20 ng/ml) was added to some of the cultures. Cell lysates were prepared 2 hr later, and expression and phosphorylation of the indicated mTOR pathway components was examined by immunoblotting.

(C) Sesn2 redox activity is not required for inhibition of mTOR signaling. H1299 cells were infected with WT or mutant Sesn2F-lentiviruses as well as GFP- and Prx1-lentiviruses and analyzed for phosphorylation and expression of the indicated proteins as above.

(D) Sesn2 increases association of 4E-BP1 and eIF-4E with m^7G-RNA Cap structures. Sesn2 was induced as in B and cell lysates were incubated with m^7G-Sepharose beads for 6 hr, and bead-bound proteins were examined by immunoblotting (upper panel). Expression levels and phosphorylation state of the indicated proteins in the total lysates were analyzed as in (B) (lower panel).

(E) Sesn2 induction inhibits cyclin D1 and c-Myc protein expression but does not affect their mRNAs. Sesn2F was induced by doxycycline withdrawal, and expression of cyclin D1 and c-Myc was examined 24 hr later by immunoblotting (IB). RNA from the same cells was analyzed by northern blotting (NB) with cyclin D1 or c-Myc probes. 18S RNA was used as a loading control.

isolated Cap-binding complexes with 7-methyl-guanosine (m^7G)-RNA- Sepharose beads from control and doxycycline-deprived MCF7-tet OFF Sesn2F cells. Expression of Sesn2F induced 4E-BP1:eIF-4E complex formation as effectively as rapamycin treatment (Figure 1D). Sesn2F induction inhibited expression of both cyclin D1 and c-Myc but did not affect their mRNA amounts (Figure 1E).

TSC2 Is Required for Sestrin-Mediated Inhibition of mTOR Signaling

To elucidate how the Sestrins inhibit mTOR, we coexpressed either Sesn2 or GFP with HA-p70S6K, and the transfected cells

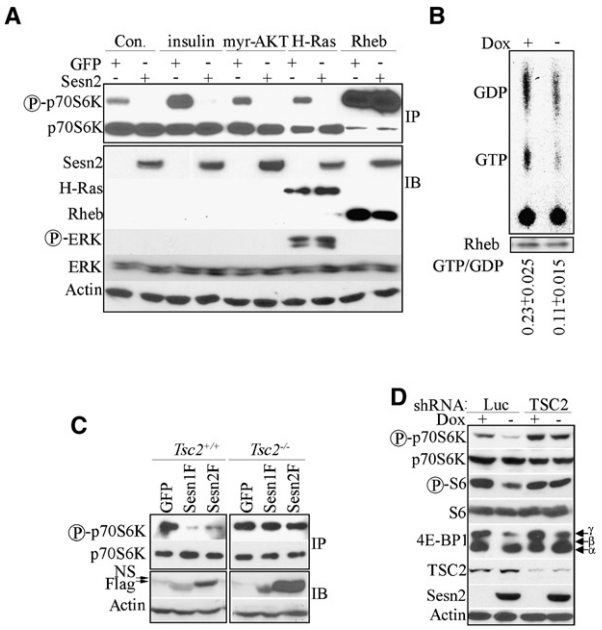

Figure 2. Sestrins Inhibit mTOR Signaling Upstream to the TSC1:TSC2 Complex

(A) Rheb abrogates Sesn2-induced inhibition of p70S6K phosphorylation. HEK293 cells were cotransfected with HA-p70S6K and either Sesn2 or GFP plasmids together with either myristoylated (myr)-AKT, H-Ras or Rheb constructs or were treated without (Con.) or with insulin for 1 hr as indicated. HA-p70S6K was immunoprecipitated (IP) with anti-HA antibody, and its phosphorylation was examined by immunoblotting. Total cell lysates were immunoblotted (IB) with the indicated antibodies.

(B) Sesn2 expression decreases Rheb GTP loading. Sesn2 was induced in MCF7-tet OFF Sesn2F cells for 24 hr, and cells were metabolically labeled with ^{32}P for 4 hr. Rheb was immunoprecipitated, and guanine nucleotides were extracted, separated by thin-layer chromatography, autoradiographed, and quantified by QuantityOne software. Rheb recovery was examined by immunoblotting and used to normalize the calculated GTP/GDP ratio. The values shown are averages ± SD of three experiments.

(C) TSC2 is required for downregulation of p70S6K phosphorylation by Sesn1 and Sesn2. *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ fibroblasts were cotransfected with HA-p70S6K together with Sesn1, Sesn2, or GFP expression vectors. HA-p70S6K was immunoprecipitated (IP), and its phosphorylation was examined by immunoblotting. Sesn1 and Sesn2 expression in total lysates was examined by immunoblotting (IB). NS, nonspecific band.

(D) Inhibition of mTOR signaling by Sesn2 requires TSC2. MCF7-tet OFF Sesn2F cells were infected with shTSC2- or shLuc-lentiviruses. After 48 hr, Sesn2F was induced by doxycycline removal, and protein phosphorylation and expression was examined 24 hr later.

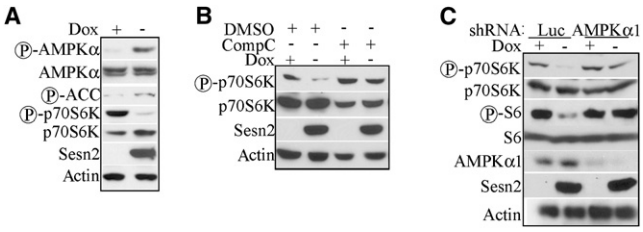

Figure 3. Sestrins Inhibit p70S6K Phosphorylation via an AMPK-Dependent Mechanism

(A) Sesn2 expression enhances AMPK phosphorylation. Sesn2F was induced in MCF7-tet OFF Sesn2F cells, and phosphorylation of the indicated proteins was examined 24 hr later.

(B) Compound C abrogates Sestrin-induced inhibition of p70S6K phosphorylation. Sesn2F was induced in MCF7-tet OFF Sesn2F cells in the presence of compound C (CompC) or vehicle control (DMSO), and protein phosphorylation was examined 24 hr later.

(C) Knockdown of AMPKα1 abrogates Sesn2 inhibition of p70S6K and S6 phosphorylation. MCF7-tet OFF Sesn2F cells were infected with shAMPKα1- or shLuc-lentiviruses. Protein phosphorylation and expression were analyzed by immunoblotting 72 hr after infection and 24 hr after doxycycline removal.

were treated with insulin to activate the PI3K-AKT-mTOR pathway. We also cotransfected cells with constructs expressing constitutively active H-Ras, myristoylated AKT (myr-AKT), or Rheb, which upon overexpression is no longer inhibited by endogenous TSC1:TSC2. Sesn2 inhibited p70S6K phosphorylation in all cases except when coexpressed with Rheb (Figure 2A), suggesting that Sesn2 affects mTOR signaling upstream of Rheb (Figure 2A).

To determine whether Sesn2 modulates Rheb GTP loading, we induced its expression in MCF7-tet OFF cells that were labeled with ^{32}P and immunoprecipitated endogenous Rheb. Inhibition of p70S6K phosphorylation by Sesn2F in these cells was confirmed by immunoblot analysis (Figure S2A). Rheb-associated guanine nucleotides were extracted and separated by thin-layer chromatography. Sesn2F expression induced a 2-fold decrease in the relative amount of Rheb-associated GTP (Figure 2B). These results suggest that Sesn2 negatively regulates Rheb GTP loading.

Rheb GTP loading is controlled by the TSC1:TSC2 complex (Garami et al., 2003; Inoki et al., 2003b; Tee et al., 2003). To assess the role of this complex in inhibition of mTOR by Sesn1 and Sesn2, we used *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ cells. Whereas Sesn1 and Sesn2 inhibited p70S6K phosphorylation in WT cells, they failed to do so in TSC2-deficient cells (Figure 2C). To confirm the critical role of TSC1:TSC2 in the response to Sesn1 and Sesn2 in human cancer cell lines, we knocked down TSC2 in both H1299 and MCF7-tet OFF Sesn2F cells. Downregulation of TSC2 compromised Sestrin-induced inhibition of p70S6K, S6, and 4E-BP1 phosphorylation (Figure 2D, Figure S2B).

AMPK Is Important for Sestrin-Induced mTOR Inhibition

To study whether AMPK is involved in negative regulation of mTOR by Sesn1 and Sesn2, we examined phosphorylation of AMPK and its target acetyl-CoA carboxylase (ACC) (Motoshima et al., 2006). Expression of Sesn1 or Sesn2 strongly enhanced AMPK phosphorylation and slightly increased ACC phosphorylation (Figure 3A, Figure S3A). In comparison to Sesn1 and Sesn2 or campotecin treatment, activation of AMPK in response to 2-deoxyglucose (2-DG) resulted in much stronger ACC phosphorylation (Figure S3B). We considered whether the Sestrins activate AMPK through induction of metabolic stress by lowering of cellular ATP. We measured cellular ATP after Sesn2F induction and found no difference from control cells (data not shown). To further examine the role of AMPK in Sestrin-modulated mTOR inhibition, we used compound C, an AMPK inhibitor (Zhou et al., 2001). Compound C prevented inhibition of p70S6K phosphorylation by Sesn2F (Figure 3B). To rule out nonspecific effects of compound C, we knocked down AMPKα1, the major form of

AMPK in MCF7 cells (data not shown), and found that this also prevented inhibition of p70S6K and S6 phosphorylation (Figure 3C). Similar results were obtained with another shRNA to AMPKα1 (data not shown).

Sestrins Interact with TSC1, TSC2, and AMPK

To determine whether the Sestrins can interact with TSC1, TSC2, or AMPK, we expressed TSC1-Myc, TSC2-HA, and AMPKα2-Myc together with Sesn2F in HEK293 cells. Immunoprecipitation of Sesn2F resulted in coprecipitation of TSC1-Myc, TSC2-HA, and AMPKα2–Myc (Figure 4A). None of these proteins was precipitated by the Flag antibody if they were coexpressed with GFP instead of Sesn2F. Reciprocal immunoprecipitation experiments confirmed the interaction of Sesn2 with TSC1, TSC2, and AMPKα2 (Figure S4A). Similar results were obtained with Sesn1 and AMPKα1 (Figures S4B and S4C). We also performed immunoprecipitation experiments with Sesn2 mutants lacking the N-terminal (ΔN), C-terminal (ΔC), or middle segments (ΔM) of the protein and found that the N-terminal segment was important for interaction with AMPKα2. However, all three mutants retained interaction with the TSC1:TSC2 complex (Figure S4D).

To study whether a complex containing Sesn2, TSC1, TSC2, and AMPK can form under more physiological conditions, we immunoprecipitated lysates of MCF7-tet OFF Sesn2F or MCF7-tet OFF GFP cells with the Flag antibody. The MCF7-tet OFF Sesn2F cells were kept on low concentration of doxycycline to achieve Sesn2F amounts similar to those seen in the same cells after camptothecin treatment (Figure 4B). Under these conditions, endogenous TSC1, TSC2, AMPKα1, and its phosphorylated form were coprecipitated along with Sesn2F (Figure 4B). To obtain further biochemical evidence for a complex containing TSC1, TSC2, AMPKα1, and Sesn2, we used gel filtration chromatography. TSC1, TSC2, AMPKα1, and Sesn2 coeluted in several high molecular weight fractions ranging from 0.4 to 1.1 × 10^6 Da (Figure 4C). Only a portion of the total Sesn2 or AMPKα1 pool coeluted with the TSC1:TSC2 complex. The AMPK-activating kinase LKB1 eluted as a low molecular weight protein and was not present in the high molecular weight fractions (Figure 4C). Because immunoprecipitation of endogenous Sesn2 with the available antibody from a variety of cell lines failed to detect the Sesn2:AMPK:TSC1:TSC2 complex, probably because of low antibody affinity or low Sesn2 amounts, we repeated the gel filtration experiment using extracts of livers isolated from mice injected with the hepatocarcinogen diethylnitrosamine (DEN; see below). As described above, a portion of Sesn2 eluted in fractions ranging from 0.3 to 1.15 × 10^6 Da (data not shown). We pooled these fractions and immunoprecipitated them with the Sesn2 antibody. This resulted in coimmunoprecipitation of AMPKα1, AMPKα2, TSC1, and TSC2 (Figure 4D).

Sestrins Activate AMPK and Enhance TSC2 Phosphorylation

To determine whether the Sestrins stimulate AMPK kinase activity, we coexpressed Flag-tagged AMPKα1 with either GFP, WT Sesn2, or the Sesn2-ΔN deletion mutant, which cannot interact with AMPK. The tagged AMPKα1 subunit was immunoprecipitated, and the resulting immunecomplex was assayed for its ability to phosphorylate the TSC1:TSC2 complex isolated from transiently transfected HEK293 cells. Coexpression of WT Sesn2 strongly enhanced the ability of AMPK to phosphorylate TSC2 and undergo autophosphorylation (Figure 5A). No TSC1 phosphorylation was detected. The truncated Sesn2 protein had only a marginal effect on AMPK kinase activity. We also examined the ability of Sesn2 to induce TSC1 or TSC2 phosphorylation in live cells by metabolic labeling. Expression of Sesn2 in HEK293 cells resulted in a substantial increase in TSC2 phosphorylation (Figure 5B). No TSC1 phosphorylation could be

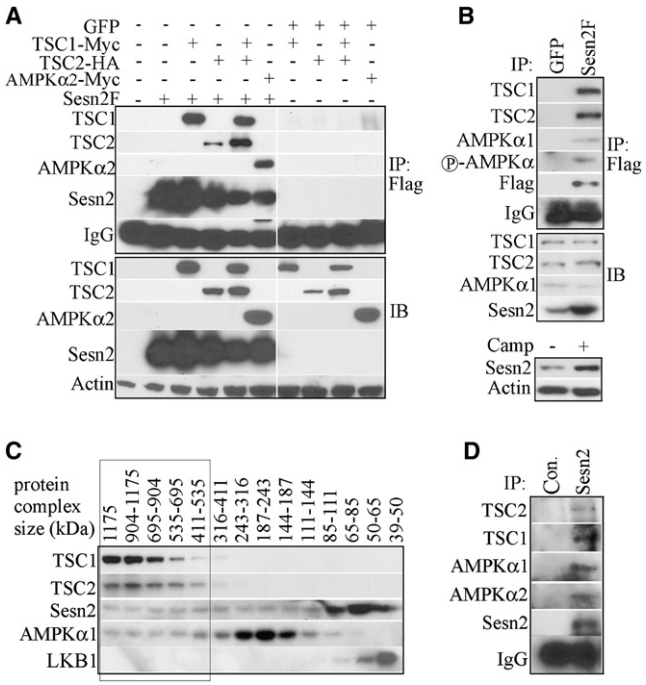

Figure 4. Sestrins Interact with TSC1:TSC2 and AMPK

(A) Coimmunoprecipitation of TSC1, TSC2, and AMPKα2 with Sesn2. HEK293 cells were cotransfected with Sesn2F or GFP expression vectors along with tagged TSC1, TSC2, TSC1 plus TSC2, or AMPKα2 plasmids as indicated. Sesn2F was immunoprecipitated with anti-Flag, and presence of the indicated proteins in the immunecomplexes (IP) and total lysates (IB) was examined by immunoblotting.

(B) Coimmunoprecipitation of endogenous TSC1, TSC2, and AMPKα1 with Sesn2. Sesn2F or GFP were induced in MCF7-tet OFF Sesn2F or GFP cells by incubation in low doxycycline concentration (0.01 μg/ml). After 24 hr, the cells were lysed. Sesn2F was immunoprecipitated with anti-Flag, and presence of the indicated proteins in the immunoprecipitates (IP) and original lysates (IB) was examined. For comparison, MCF7 cells were incubated with camptothecin (Camp) for 12 hr to induce Sesn2 expression that was examined by immunoblotting of the same amount of cell lysates as above.

(C) Sesn2 coelutes with TSC1, TSC2, and AMPKα in high molecular weight fractions. Extracts of H1299 cells were separated on a Superdex 200 gel filtration column and analyzed by immunoblotting. The elution positions (in kDa) of molecular weight standards are indicated at the top.

(D) Coimmunoprecipitation of TSC1, TSC2, and AMPKα with endogenous Sesn2. Two-month-old WT male mice were injected with DEN (100 mg/kg). After 24 hr, the liver was removed, homogenized, and filtered. The lysate was separated by gel filtration as above, and the high molecular weight fractions were combined and immunoprecipitated with anti-Sesn2 or control anti-rabbit (Con.) IgG. Presence of the indicated proteins in the immunepreciptates was analyzed by immunoblotting.

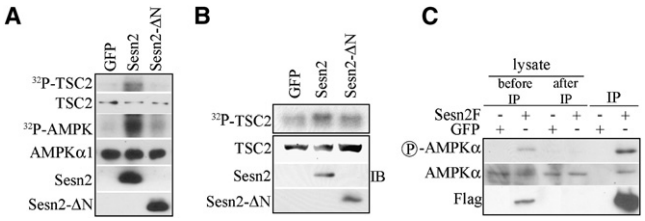

Figure 5. Sesn2 Activates AMPK and Induces TSC2 Phosphorylation

(A) WT but not N-terminally truncated Sesn2 activates AMPK. HEK293 cells were cotransfected with AMPKα1F expression vector along with GFP, Sesn2F, and Sesn2F-ΔN expression vectors. After 48 hr, the cells were lysed, and AMPKα1F immune complexes were isolated and assayed for their ability to phosphorylate a TSC1:TSC2 complex isolated by immunoprecipitation from lysates of transiently transfected HEK293 cells. The amounts of AMPKα1F, Sesn2, and TSC2 in the kinase reaction were examined by immunoblotting (IB).

(B) Sesn2 enhances TSC2 phosphorylation. HEK293 cells were cotransfected with TSC1Myc and TSC2F expression vectors along with WT Sesn2F, N-terminally truncated Sesn2F, or GFP expression vectors. After 48 hr, cells were metabolically labeled with ^{32}P-orthophosphate, and the TSC1:TSC2 complex was immunoprecipitated 4 hr later, gel separated, and autoradiographed. TSC2 and Sesn2 expression were examined by immunoblotting (IB).

(C) Sesn2-associated AMPKα is more extensively phosphorylated than AMPK that is not associated with Sesn2. Sesn2F or GFP were induced by doxycycline withdrawl in MCF7-tet cells. After 24 hr, cells were lysed, and the lysates were subjected to immunoprecipitation with anti-Flag antibody. The supernatant of the immunoprecipitation reaction, the immune complexes, and the total unfractionated lysate were examined for AMPKα phosphorylation and content by immunoblotting.

detected, and, as above, the truncated Sesn2-ΔN protein had little stimulatory effect on TSC2 phosphorylation. We also compared the phosphorylation of Sesn2-associated and Sesn2-unassociated AMPKα1. As shown in Figure 5C, AMPKα1 that was coprecipitated with Sesn2 was more intensely phosphorylated at residue T172 within its activation loop than AMPKα1 that was not coprecipitated with Sesn2. Collectively, these results suggest that the Sestrins activate AMPK through direct interaction and stimulate its kinase activity toward TSC2, which is present in a complex with AMPK and Sesn1 and Sesn2.

Sestrins Play a Physiologically Important Role in mTOR Regulation

To study the role of endogenous Sestrins in mTOR regulation, we knocked down Sesn1 or Sesn2 using shRNA in H1299 cells (Budanov et al., 2004). Silencing of Sesn1 had a marginal effect in this cell line, whereas the knockdown of Sesn2 increased phosphorylation of both p70S6K and S6 (Figure S6A). Two different Sesn1 and Sesn2 shRNAs exerted similar effects (data not shown). Because we suspected that Sesn1 does not significantly contribute to mTOR regulation in the p53-negative H1299 cells, we examined its function in p53-positive 3T3 fibroblasts. Silencing of Sesn1 with two different lentiviral-delivered shRNAs increased p70S6K and S6 phosphorylation, indicating mTOR activation (Figure 6A). Furthermore, Sesn1 knockdown accelerated the proliferation of these cells (Figure 6B).

p53 activation can downregulate mTOR signaling (Feng et al., 2005; Horton et al., 2002). To examine the role of Sesn1 and Sesn2 in this response, we silenced their expression in p53-negative H1299 cells and reinfected the silenced cells with GFP- or

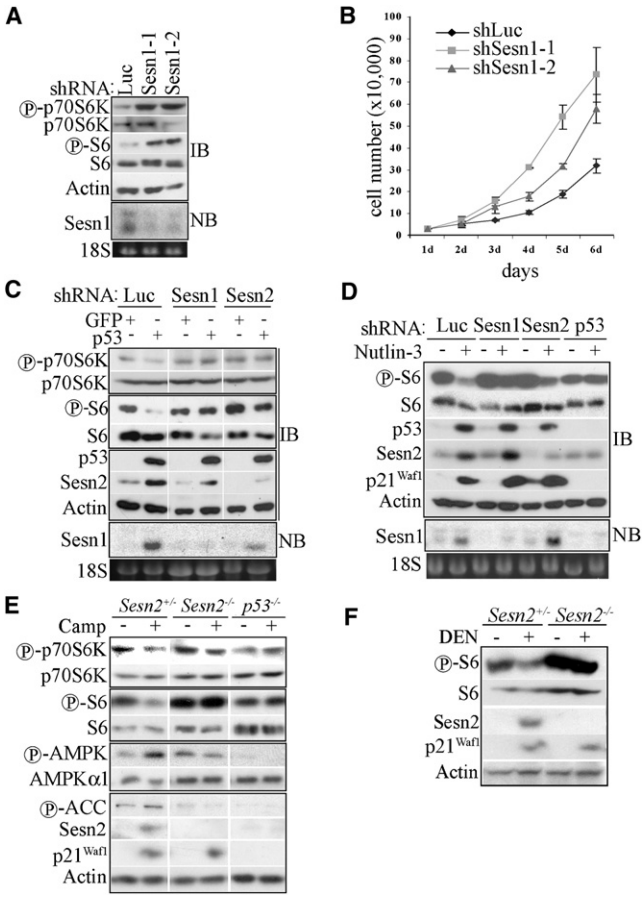

Figure 6. Sesn1 and Sesn2 Are Required for Negative Regulation of mTOR under Basal and Stressed Conditions

(A) Sesn1 negatively regulates mTOR signaling in mouse fibroblasts. 3T3 cells were infected with shLuciferase (shLuc)-, shSesn1-1-, or shSesn1-2-lentiviruses, selected with puromycin, and analyzed 48 hr later for protein phosphorylation (IB) and Sesn1 mRNA expression by northern blotting (NB). 18S RNA was used to control for RNA loading.

(B) Cells (30,000) from (A) were plated onto 6-well plates and counted every 24 hr for 6 days.

(C) Sestrins are required for p53-induced mTOR inhibition. H1299 cells were infected with shLuciferase (shLuc)-, shSesn1-, or shSesn2- lentiviruses and 48 hr later were infected with p53- or GFP-lentiviral vectors. After an additional 48 hr, the cells were lysed and analyzed by immunoblotting (IB) for p70S6K and S6 phosphorylation and p53 and Sesn2 expression. Sesn1 expression was examined by northern blotting (NB).

(D) Sesn1 and Sesn2 are required for downregulation of S6 phosphorylation in Nutlin-3-treated cells. U2OS cells were infected with shLuciferase (shLuc)-, shSesn1-, shSesn2-, or shp53-lentiviruses. After 48 hr, the cells were incubated or not with Nutlin-3 (6 μM) for 12 hr as indicated to induce p53 accumulation. Expression of p53, Sesn2, and $p21^{Waf1}$ and S6 phosphorylation were examined by immunoblotting (IB). Sesn1 mRNA was analyzed by northern blotting (NB).

(E) Sesn2 and p53 are required for inhibition of mTOR signaling during camptothecin-induced genotoxic stress. 3T3 cells of the indicated genotypes were treated with camptothecin (Camp, 20 μM) for 12 hr, and protein expression and phosphorylation were analyzed by immunoblotting.

(F) Sesn2 is required for DEN-induced inhibition of S6 phosphorylation in mouse liver. Two-month-old *Sesn2*$^{+/-}$ and *Sesn2*$^{-/-}$ male mice were injected with DEN (100 mg/kg). After 24 hr, protein expression and phosphorylation in liver homogenates were analyzed as above.

p53-lentiviral vectors. As expected (Sablina et al., 2005), p53 induced expression of both Sesn1 and Sesn2 (Figure 6C). Importantly, the ability of p53 to inhibit mTOR signaling, as measured by p70S6K and S6 phosphorylation, was attenuated by silencing of either Sesn1 or Sesn2 (Figure 6C). A similar abrogation of p53-induced inhibition of S6 phosphorylation was observed upon knockdown of either TSC2 or AMPKα1 (Figure S6B). To further examine the role of Sesn1 and Sesn2 in p53-induced mTOR inhibition, we treated U2OS osteosarcoma cells, which contain amplified MDM2, a negative regulator of p53 (Florenes et al., 1994), with a specific inhibitor of the MDM2-p53 interaction, Nutlin-3 (Vassilev et al., 2004). Nutlin-3 induced p53 accumulation and expression of $p21^{Waf1}$, Sesn1, and Sesn2 and downregulation of S6 phosphorylation (Figure 6D). Knockdown of Sesn1 and to a lesser extent Sesn2 compromised the inhibition of S6 phosphorylation but had no effect on accumulation of p53 and induction of $p21^{Waf1}$, both of which were abolished by p53 silencing, which also prevented the inhibition of S6 phosphorylation.

Genotoxic stress can inhibit mTOR signaling through activation of p53 (Feng et al., 2005). To study the role of Sestrins in this response in vivo, we generated *Sesn2* knockout mice using gene trap ES cells from Bay Genomics. The Sesn2-deficient ES cells contain a pGT0Lxf expression vector with a strong splice acceptor site integrated into the third intron of the mouse *Sesn2* gene, which results in expression of a truncated mRNA and no Sesn2 protein (Figure S6C). Mice generated from these cells were indeed Sesn2 null (Figures 6E and 6F). We treated $Sesn2^{+/-}$, $Sesn2^{-/-}$, and $p53^{-/-}$ mouse embryo fibroblasts (MEFs) immortalized by the 3T3 procedure with camptothecin, a topoisomerase I inhibitor, to activate p53. Camptothecin treatment inhibited p70S6K and S6 phosphorylation while enhancing AMPK and ACC phosphorylation in $Sesn2^{+/-}$ cells but not in $Sesn2^{-/-}$ or $p53^{-/-}$ cells (Figure 6E). Silencing of Sesn1 in $Sesn2^{+/-}$ cells also compromised the inhibitory effect of camptothecin on mTOR (Figure S6D). Sesn2, however, had no effect on activation of AMPK by AICAR (Figure S6E).

We treated 2-month-old $Sesn2^{+/-}$ and $Sesn2^{-/-}$ mice with DEN, which, upon metabolic activation in zone 3 hepatocytes, becomes a potent alkylating agent (Buhler et al., 1992; Verna et al., 1996) that causes p53 activation and induction of p53 target genes, such as *Sesn1*, *Sesn2*, and $p21^{Waf1}$ (Figure S6F). DEN also led to inhibition of S6 phosphorylation (Figure S6D), an effect that was not seen in $Sesn2^{-/-}$ mice (Figure 6G). Immunohistochemistry revealed that inhibition of S6 phosphorylation was restricted to zone 3 hepatocytes, the main site of DEN metabolic activation (Figure S6G). This effect was much weaker in $Sesn2^{-/-}$ mice. Using hepatocyte-specific p53 knockout mice created by crossing $p53^{F/F}$ mice (Jonkers et al., 2001) with *Alb-Cre* mice (Postic et al., 1999), we confirmed that induction of Sesn2 and $p21^{Waf1}$ by DEN was p53 dependent (Figure S6H).

The Growth-Inhibitory Activity of Sestrins Is Mediated, in Part, through Inhibition of mTOR Signaling

mTOR is a critical regulator of cell growth and proliferation (Fingar et al., 2004; Wullschleger et al., 2006). To examine whether Sestrins negatively regulate cell growth and proliferation, we expressed Sesn1 and Sesn2 in HEK293 cells and measured cell size and cell-cycle distribution. Either Sesn1 or Sesn2 decreased cell size (similar to the effect of rapamycin), especially in G1 cells whose fraction was increased upon Sesn1 and Sesn2 expression (Figures 7A and 7B). To examine whether p53 regulates cell size in a Sestrin-dependent manner, we infected H1299 cells with shSesn2- or shLuc-lentiviruses and reinfected the cells with either p53- or GFP-lentiviruses. p53 expression decreased cell size, but this effect was abrogated in Sesn2-silenced cells (Figure S7). We also found that Sesn2 truncation mutants that were incapable of mTOR inhibition and AMPK activation (Figure S8A) failed to inhibit clonogenic growth of HEK293 cells, which was readily inhibited by WT Sesn1 or Sesn2 (Figure S8B). The redox-defective Sesn2(C125S) mutant also inhibited clonogenic growth, but not as effectively as WT Sesn2, suggesting that some of the growth-inhibitory activity of Sesn2 may be redox dependent and mTOR independent. To examine whether the Sestrin effect on cell size and proliferation is TSC2 dependent, we infected $Tsc2^{+/+}$ and $Tsc2^{-/-}$ cells with a Sesn2- or GFP-lentiviruses and measured cell size and clonogenic growth. Sesn2 expression decreased cell size and inhibited clonogenic growth of $Tsc2^{+/+}$ but not $Tsc2^{-/-}$ cells (Figures 7C and 7D).

Ectopic Sesn2 expression inhibits cell growth and proliferation (Budanov et al., 2002). To determine the role of mTOR in this effect, we cotransfected MCF7 cells with Sesn2 or GFP expression vectors together with Rheb or H-Ras constructs. Sesn2 expression inhibited clonogenic growth by about 50%, and coexpression of Rheb, but not H-Ras, attenuated this effect (Figure 7E). In fact, H-Ras slightly potentiated the growth-inhibitory activity of Sesn2.

DISCUSSION

The mTOR signaling pathway is a central regulator of cell growth and survival (Corradetti and Guan, 2006; Fingar and Blenis, 2004; Guertin and Sabatini, 2005; Hay and Sonenberg, 2004; Soliman, 2005; Thomas, 2006; Wullschleger et al., 2006). It is therefore not surprising that adverse environmental conditions negatively regulate cell growth by inhibiting mTOR (Corradetti and Guan, 2006). In addition to nutrient limitation, mTOR activity is negatively regulated by genotoxic stress and hypoxia, conditions that activate tumor suppressor p53. The ability of p53 to inhibit mTOR signaling is in line with its function as a negative regulator of cell growth and proliferation (Levine et al., 2006). The results described above strongly suggest that the ability of p53 to inhibit mTOR signaling depends on two of its target genes: *Sesn1* and *Sesn2*.

The Sestrins belong to a small and evolutionary conserved family composed of three members in mammals, of which Sesn1 and Sesn2 are stress inducible and p53 regulated (Budanov et al., 2002; Velasco-Miguel et al., 1999). The ability of Sesn1 and Sesn2 to inhibit cell growth and proliferation was attributed to their redox activity (Budanov et al., 2004). The present work, however, demonstrates that Sesn1 and Sesn2 are potent inhibitors of mTOR signaling, acting in a manner that does not depend on their redox activity, which only makes a partial contribution to their growth-inhibitory activity. Sesn1 and Sesn2 inhibit mTORC1 activity toward p70S6K and 4E-BP1 in a variety of human and mouse cell lines, as well as in mouse liver.

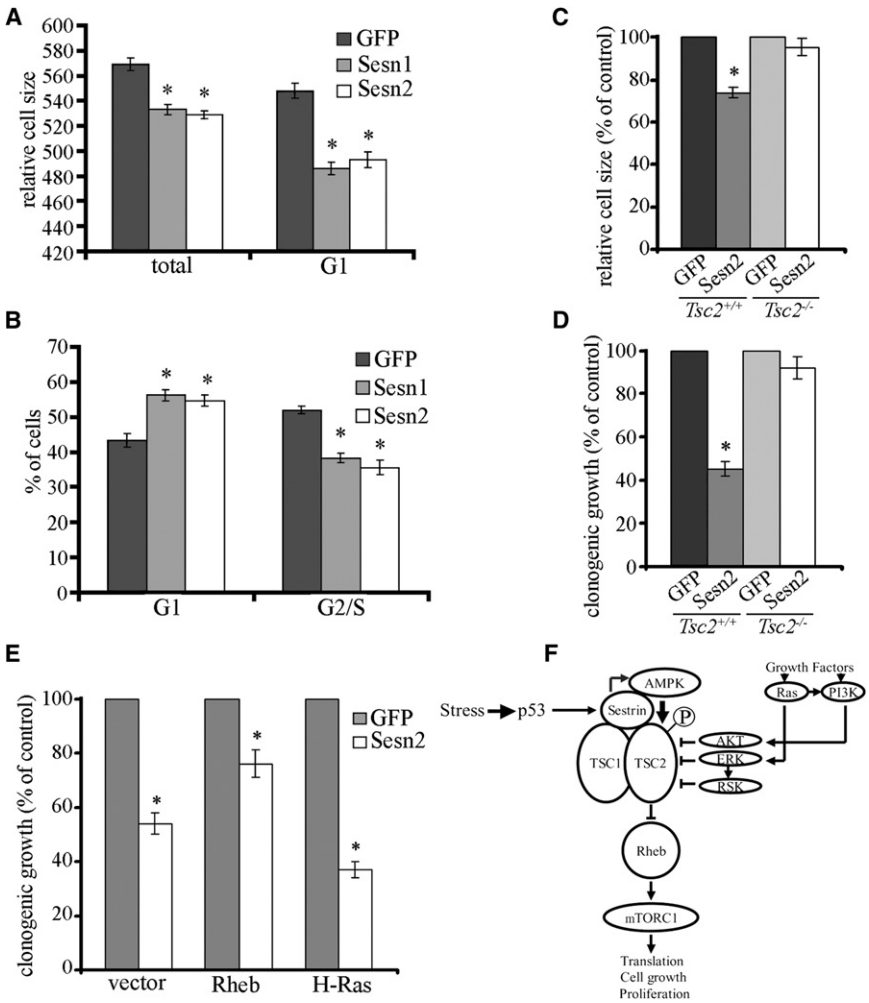

Figure 7. Sestrins Inhibit Cell Growth and Proliferation via mTOR

(A and B) Sesn1 and Sesn2 expression decreases cell size and inhibits cell proliferation at G1. HEK293 cells were infected with Sesn1-, Sesn2-, or GFP-lentiviruses. After 48 hr, cell size (A) and cell-cycle distribution (B) were analyzed by flow cytometry.

(C and D) Sesn2 expression decreases cell size and inhibits clonogenic growth in $Tsc2^{+/+}$ but not $Tsc2^{-/-}$ cells. $Tsc2^{+/+}$ and $Tsc2^{-/-}$ cells were infected with Sesn2- or GFP-lentiviruses. After 48 hr, cell size (C) was analyzed by flow cytometry. Colony formation (D) was analyzed 14 days after infection.

(E) Sesn2 inhibits clonogenic growth upstream to Rheb. MCF7 cells were transfected with Sesn2 or GFP expression vectors along with Rheb, H-Ras, or an empty expression vector. The cells were selected in puromycin-containing medium and colony formation was quantitated after 3 weeks.

(F) A model explaining our results. Sesn1 and Sesn2 are induced upon stress-triggered p53 activation. Sesn1 and Sesn2 dimers interact with TSC1:TSC2 and AMPKα and activate AMPK by induced proximity-dependent autophosphorylation. This results in TSC2 phosphorylation and stimulation of its GAP activity, leading to inhibition of mTOR signaling, which is positively regulated by growth factors via Ras and PI3K.

Results in (A)–(E) are averages ± SE. * indicates $p \leq 0.05$ by Student's t test.

Notably, the ability of the hepatocarcinogen DEN to inhibit S6 phosphorylation is restricted to zone 3 hepatocytes, which are the main site in which it undergoes metabolic activation to become a potent alkylating agent (Buhler et al., 1992), and this inhibitory activity is Sesn2 dependent. By inhibiting 4E-BP1 phosphorylation, Sesn2 enhances its interaction with eIF-4E and inhibits expression of growth regulatory proteins, such as cyclin D1 and c-Myc, whose translation is eIF-4E dependent and sensitive to 4E-BP1 phosphorylation (Averous et al., 2007; Averous and Proud, 2006; Mamane et al., 2004).

The Sestrins impact mTORC1 activity through the TSC1:TSC2 complex. Being a GAP for Rheb, the direct activator of mTORC1, the TSC1:TSC2 complex is a central regulator of mTOR signaling (Corradetti and Guan, 2006; Kwiatkowski and Manning, 2005). Sesn2 expression decreases Rheb GTP loading, and the ability of both Sesn1 and Sesn2 to inhibit mTOR signaling is TSC2 dependent. One way to regulate TSC1:TSC2 GAP activity is through TSC2 phosphorylation, but other modes of regulation may also exist (Corradetti and Guan, 2006). Although the Sestrins have no effect on ERK and its target RSK or GSK3β, which can serve as TSC2 kinases, they stimulate the activity of AMPK, a major TSC2 kinase (Corradetti and Guan, 2006). Furthermore, Sestrin expression enhanced TSC2 phosphorylation in live cells, and this effect required the N terminus of Sesn2, which mediates AMPKα binding. Sesn2 did not stimulate TSC1 phosphorylation, and Sesn2-activated AMPK did not phosphorylate TSC1.

Importantly, the mTOR inhibitory activity of Sesn1 and Sesn2 depends on AMPKα, whose phosphorylation at the activation loop was enhanced upon Sestrin expression. Inhibition of AMPK with compound C as well as shRNA silencing of AMPKα1 attenuated the ability of Sesn2 to inhibit mTOR signaling. Coimmunoprecipitation and gel filtration analyses revealed an interaction between Sesn2 and AMPKα, suggesting that Sestrins are engaged in formation of a large protein complex containing AMPK and TSC1:TSC2. We propose that Sesn1 and Sesn2 induction in response to genotoxic stress results in binding of Sestrins, most likely as dimers (data not shown), to AMPK and TSC1:TSC2, as well as autoactivation of AMPK through a mechanism based on induced proximity. In addition to activation of AMPK, the Sestrins recruit it to phosphorylate TSC2 (Figure 7F). Phosphorylation of TSC2 correlates with enhancement of its GAP activity that leads to inhibition of Rheb and mTOR.

Importantly, we obtained ample and clear evidence that Sesn1 and Sesn2 are critical mediators of p53's ability to inhibit mTOR signaling. Using shRNA-mediated silencing, we found that both Sesn1 and Sesn2 participate in mTOR inhibition upon p53 activation in human cancer cells. Furthermore, disruption of the *Sesn2* gene in mice attenuated the inhibition of p70S6K activity by the DNA-damaging agents: camptothecin in fibroblasts and DEN in hepatocytes. In both cases, inhibition of p70S6K was p53 mediated, but unlike the p53 deficiency, the absence of Sesn2 has no effect on induction of $p21^{Waf1}$, another p53 target gene. Thus, Sesn2 (and presumably Sesn1) seems to mediate only one aspect of p53 signaling—inhibition of mTOR. Correspondingly, the growth-inhibitory activity of Sesn2 is not as strong as that of p53, which has additional targets with antiproliferative activity, such as $p21^{Waf1}$.

p53 deficiency and activation of mTOR signaling are hallmarks of human cancer (Levine, 1997; Sabatini, 2006; Thomas, 2006). Several mechanisms account for mTOR activation in cancer, including activation of Ras, PI3K, and AKT and inactivation of tumor suppressors that negatively regulate these molecules: PTEN, TSC1, TSC2, and LKB1 (Corradetti and Guan, 2006; Sabatini, 2006; Thomas, 2006). Although p53 can induce expression of several negative regulators of mTOR, including PTEN, TSC2, AMPKβ1, and IGF-BP3, in a cell type-dependent manner (Feng et al., 2007), our results demonstrate that p53-mediated inhibition of mTOR depends mainly on Sesn1 and Sesn2 in mouse fibroblasts and certain human cancer cell lines and on Sesn2 in mouse liver.

Inhibition of mTOR suppresses cell growth and proliferation (Fingar et al., 2004; Wullschleger et al., 2006). Sesn2 was known to inhibit cell proliferation (Budanov et al., 2002), but its mechanism of action was heretofore unknown. Our results strongly suggest that Sesn1 and Sesn2 exert their growth-inhibitory effect via mTOR and may cooperate with other antiproliferative p53 targets, such as $p21^{Waf1}$. Interestingly, the *SESN1* (6q21) and *SESN2* (1p35) loci are frequently deleted in a variety of human cancers (Velasco-Miguel et al., 1999; Ragnarsson et al., 1999; Schwab et al., 1996), suggesting that they harbor one or more tumor suppressors. We found that Sesn2 deficiency renders murine fibroblasts more susceptible to oncogenic transformation, and this effect may depend on mTOR inhibition (Figure S9). Hence, *SESN1* and *SESN2* may indeed be important components of the tumor-suppressor network activated by p53.

In summary, although more remains to be learned about Sestrin biology and mechanism of action, our results establish these proteins as critical links between p53 and mTOR that enable p53 to inhibit cell growth.

EXPERIMENTAL PROCEDURES

Mice

$Sesn2^{-/-}$ mice on a mixed C57BL/129 background were generated from ES cells obtained from Bay Genomics. These cells were created by a random gene trap approach and contain a pGT0Lxf construct within exon 3 of the *Sesn2* gene with a strong splice acceptor site expressing a β-gal-Neo fusion protein that disrupts the *Sesn2* ORF. $p53^{\Delta hep}$ mice were generated by crossing of $p53^{F/F}$ mice on a mixed FVB/129 (Jonkers et al., 2001) with *Alb-Cre* mice (Postic et al., 1999) on a C57BL6 background for at least five generations. Efficient and specific deletion of p53 in hepatocytes was confirmed by PCR analysis. All mice were maintained in filter topped cages on autoclaved food and water at University of California, San Diego (UCSD) according to National Institutes of Health guidelines.

Cell Culture, Transfection, Infection, and Treatment

$Tsc2^{+/+}$ and $Tsc2^{-/-}$ 3T3 fibroblasts were a gift from D. Kwiatkowsky. MCF7-tet OFF cells were from Clontech. $Sesn2^{+/-}$ and $Sesn2^{-/-}$ 3T3 fibroblasts were derived via the 3T3 protocol from MEFs. All cells were cultured in high-glucose DMEM supplemented with 10% FCS, L-glutamine, penicillin, and streptomycin. For the MCF7-tet OFF system, doxycycline was added at 0.5 μg/ml. Lipofectamine-Plus (Invitrogen) was used as a transfection reagent. Infection with retroviral vectors was described (Budanov et al., 2004; Budanov et al., 2002). For rapamycin treatment, cells were usually incubated with 20 ng/ml rapamycin. For camptothecin treatment, cells were incubated 12 hr after plating with camptothecin (20 μM) for 12 hr more. For Nutlin-3 treatment, cells were incubated with 6 μM of Nutlin-3. For insulin and IGF1 treatment, cells were kept in serum-free DMEM for 12 hr and incubated with the growth factors for 1 hr. For colony formation assays, cells were transfected with the different plasmids as described (Budanov et al., 2002), cultured for 14–21 days under selective pressure, fixed, and stained with methylene blue.

Metabolic Labeling

HEK293 cells were transfected with TSC1-Myc plus TSC2-Flag along with GFP, WT, or N-terminally trancated Sesn2 expression vectors. After 48 hr, the growth medium was replaced with phosphate-free DMEM supplemented with dialysed serum and 1 mCi of orthophosphate. After 4 hr, cells were lysed, and the TSC1:TSC2 complex was immunoprecipitated with anti-Flag antibody.

AMPK Kinase Assay

HEK293 cells were cotransfected with AMPKα1-Flag along with GFP, WT or N-terminally truncated Sesn2 expression vectors. After 48 hr, the cells were lysed, and AMPKα1F was immunoprecipitated with anti-Flag agarose (Sigma). Kinase activity was examined in AMPK reaction buffer (20 mM HEPES-NaOH [pH 7.5], 0.4 mM dithiotriethol, 18 mM $MgCl_2$, 125 μM unlabeled ATP, 6 μM β-glycerophosphate, 1 mM EGTA, and 0.25 mM Na_3VO_4) containing 5 μCi [γ-^{32}P]ATP.

Cell Lysis, Immunoprecipitation, and Immunoblot Analyses

For ordinary immunoblot analysis, cells were lysed in RIPA-SDS buffer (Budanov et al., 2002). For immunoprecipitation analysis, cells or livers were lysed in NP40 buffer (20 mM Tris-HCl, 5% glycerol, 138 mM NaCl, 2.7 mM KCl, 1% NP40, 20 mM NaF, 18 μM pepstatin, 5 mM EDTA, 1 mM Na_3VO_4, 20 μM leupeptin, 2.5 mM Na pyrophosphate, and 1 mM β-glycerophosphate). Cell lysates were incubated with the indicated antibodies for 1 hr, and incubation with a 1:1 mixture of protein A- and protein G-Sepharose beads followed. For Cap-binding assay, cell lysates were incubated with m^7G-RNA-Sepharose beads (Amersham) for 6 hr. After centrifugation, the beads were washed four times in lysis buffer. Total and immunoprecipitated proteins were resolved by SDS-PAGE, transferred onto polyvinylidene fluoride membranes, and probed with the relevant antibodies (Budanov et al., 2004).

Analysis of Rheb GTP/GDP Content

Cells were metabolically labeled with ^{32}P-orthophosphate and immunoprecipitated with anti-Rheb antibody (Santa Cruz, CA), and guanine nucleotides were extracted and resolved by thin-layer chromatography (Garami et al., 2003).

Gel Filtration Analysis

Gel Filtration analysis was performed on a Superdex 200 column (Amersham Pharmacia Biotech) with a fast-flow liquid chromatography system. The column buffer was PBS. The column was calibrated with protein markers ranging from 29 to 2000 kDa (Sigma).

SUPPLEMENTAL DATA

Supplemental Data include eight figures, Supplemental Experimental Procedures, and Supplemental References and can be found with this article online at http://www.cell.com/cgi/content/full/134/3/451/DC1/.

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