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# Sestrins Activate Nrf2 by Promoting p62-Dependent Autophagic Degradation of Keap1 and Prevent Oxidative Liver Damage

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## SUMMARY

**Sestrins (Sesns) protect cells from oxidative stress. The mechanism underlying the antioxidant effect of Sesns has remained unknown, however. The Nrf2-Keap1 pathway provides cellular defense against oxidative stress by controlling the expression of antioxidant enzymes. We now show that Sesn1 and Sesn2 interact with the Nrf2 suppressor Keap1, the autophagy substrate p62, and the ubiquitin ligase Rbx1 and that the antioxidant function of Sesns is mediated through activation of Nrf2 in a manner reliant on p62-dependent autophagic degradation of Keap1. Sesn2 was upregulated in the liver of mice subjected to fasting or subsequent refeeding with a high-carbohydrate, fat-free diet, whereas only refeeding promoted Keap1 degradation and Nrf2 activation, because only refeeding induced p62 expression. Ablation of Sesn2 blocked Keap1 degradation and Nrf2 activation induced by refeeding and thereby increased the susceptibility of the liver to oxidative damage resulting from the acute stimulation of lipogenesis associated with refeeding.**

## INTRODUCTION

The products of two p53 target genes, Sestrin1 (Sesn1) and Sesn2, were implicated in defense against reactive oxygen species (ROS); their antioxidant function was proposed to be attributable to their sulfinic acid reductase activity (Budanov et al., 2004), with such activity being necessary to maintain peroxiredoxins (Prxs) in an active state. Prxs constitute a family of peroxidases in which cysteine serves as the primary site of oxidation during the reduction of peroxides. Certain Prxs are inactivated via substrate-mediated hyperoxidation of the active-site cysteine to sulfinic acid (Cys-$SO_2H$). The hyperoxidized, inactivated form of Prx is reactivated via a reaction catalyzed by sulfiredoxin (Srx) (Biteau et al., 2003; Rhee and Woo, 2011). It was proposed that Sesns also catalyze this cysteine-sulfinic acid reduction reaction and thereby inhibit ROS accumulation by maintaining Prx activity. Sesns and Srx, however, show no similarity in amino acid sequence. Several lines of evidence subsequently refuted the reductase function of Sesns (Woo et al., 2009).

Although the underlying mechanism has remained unknown, it is clear that Sesns protect cells against oxidative stress (Lee et al., 2010; Papadia et al., 2008). While studying the protective role of Sesn2 in mouse liver, we found that Sesn2 is necessary for Srx induction in the liver of mice subjected to refeeding with a high-carbohydrate, fat-free diet after food deprivation. The Srx gene is a target of the transcription factor Nrf2 (nuclear factor erythroid 2-related factor 2) (Bae et al., 2009), which mediates the induction of many cytoprotective genes in response to oxidative or electrophilic stress (Itoh et al., 2010; Kensler et al., 2007). Kelch-like ECH-associated protein 1 (Keap1) is a repressor of Nrf2 activity and plays a key role in its regulation (Itoh et al., 2010). We looked for a link between Sesn2 and the Nrf2 pathway and found that Sesn2 promotes the autophagic degradation of Keap1 and thereby upregulates Nrf2 signaling, leading to the induction of genes for antioxidant enzymes including Srx. We have therefore identified a role for Sesn2 as a positive regulator of the Nrf2 pathway, with this role likely accounting for the antioxidant function of Sesns. We also show that Sesn2-dependent activation of Nrf2 is important for the protection of mouse liver from oxidative injury caused by the acute lipogenic stimulus that results from refeeding after food deprivation.

## RESULTS

### Sesn2 Induces Keap1 Downregulation and Nrf2 Activation

To investigate whether Sesn2 might regulate the Nrf2-Keap1 pathway, we transfected cultured cells with expression vectors for FLAG epitope-tagged Sesn2 (F-Sesn2) and HA-tagged Keap1 (H-Keap1). Forced expression of F-Sesn2 reduced the abundance of H-Keap1 in a concentration-dependent manner in HEK293 cells (Figure 1A) as well as in HCT116 and HeLa cells (Figures S1A and S1B), whereas the amount of Keap1 mRNA remained unaffected (Figure 1B). Similar experiments showed that F-Sesn1 also reduced the abundance of H-Keap1 (Figure S1C). Sesns have been shown to possess a conserved, redox-sensitive cysteine residue that corresponds to C125 of Sesn2. However, expression of a Sesn2 mutant (C125S) in which this cysteine residue has been replaced with serine still reduced the abundance of H-Keap1 (Figure S1D).

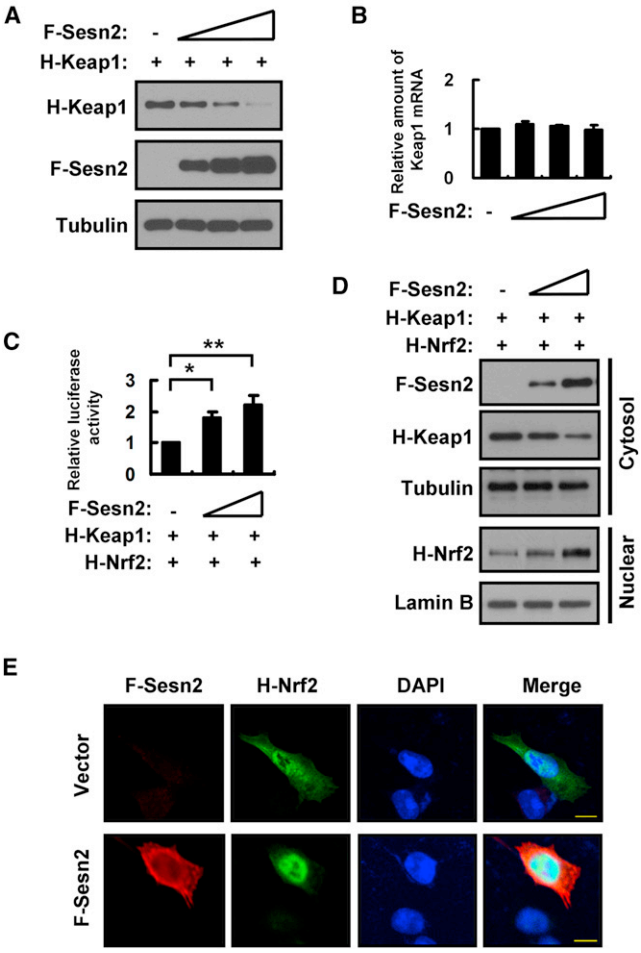


**Figure 1. Sesn2 Induces Downregulation of Keap1 and Activation of Nrf2**

(A) HEK293 cells transfected with an expression vector for H-Keap1 and various amounts (1×, 2×, 4×) of a vector for F-Sesn2 were lysed and subjected to immunoblot analysis with antibodies to the HA or FLAG tags or to α-tubulin (loading control).

(B) HEK293 cells expressing various amounts (1×, 2×, 4×) of F-Sesn2 were assayed for Keap1 mRNA by real-time PCR and RT-PCR analysis. Data are means ± SD from three independent experiments.

(C) HEK293 cells transfected with vectors for H-Nrf2, H-Keap1, and F-Sesn2 (1×, 2×) as well as with the luciferase reporter plasmid pNqo1-ARE and pRL-TK (internal control) were lysed and assayed for luciferase activity. Data are means ± SD from three independent experiments. *$p < 0.05$, **$p < 0.02$.

(D) Cytosolic and nuclear fractions of HEK293 cells transfected with vectors for H-Keap1, H-Nrf2, and F-Sesn2 (1×, 2×) were subjected to immunoblot analysis with antibodies to FLAG, HA, α-tubulin, and lamin B.

(E) HEK293 cells expressing F-Sesn2 (or transfected with the empty vector) and HA-Nrf2 were subjected to immunofluorescence analysis with antibodies to FLAG and HA. Nuclei were also stained with DAPI. Scale bars, 20 μm.

Keap1 is a repressor of Nrf2 activation. Forced expression of F-Sesn2 thus upregulated the transactivation activity of Nrf2 as revealed by a luciferase reporter assay (Figure 1C). This Sesn2-induced increase in Nrf2 activity was accompanied by an increase in the amount of Nrf2 in the nucleus as detected by both subcellular fractionation (Figure 1D) and immunofluorescence staining (Figure 1E).

We examined the sulfinic acid reductase activity and ability to downregulate Keap1 abundance of both Sesn2 and Srx in HEK293 cells expressing various amounts of F-Sesn2 or F-Srx together with H-Keap1. Forced expression of F-Srx resulted in the complete reduction of sulfinic PrxI, sulfinic PrxII, and partial reduction of sulfinic PrxIII in $H_2O_2$-treated cells but had no effect on H-Keap1 abundance in $H_2O_2$-treated or control cells (Figure S1E). Conversely, forced expression of F-Sesn2 induced a decrease in the abundance of H-Keap1 in both $H_2O_2$-treated and control cells but had no effect on that of the sulfinic form of Prxs (Figure S1F). These results thus suggested that Sesn2 is not a sulfinic acid reductase and Sesn2 and Srx do not share a biochemical function.

## Sesn2-Induced Keap1 Degradation Is Mediated by p62-Dependent Autophagy and Promoted by Rbx1

Several pathways that regulate the Keap1-Nrf2 interaction have been identified, one of which involves p62, an autophagy substrate that competes with Nrf2 for binding to Keap1 (Komatsu et al., 2010; Lau et al., 2010). We first examined whether the downregulation of Keap1 abundance by Sesn2 is mediated by degradation of Keap1. HEK293 cells expressing F-Sesn2 and H-Keap1 were exposed to the proteasome inhibitor MG132 or to the autophagy inhibitors chloroquine or 3-methyladenine. Immunoblot analysis showed that the Sesn2-induced decrease in the abundance of Keap1 was slightly enhanced by MG132 (Figure S2A), whereas it was attenuated by chloroquine and 3-methyladenine (Figures S2B and S2C), suggesting that the downregulation of Keap1 by Sesn2 is likely mediated via autophagic degradation. To determine whether Sesn2 stimulates autophagy, we transfected HEK293 cells with vectors for F-Sesn2 and H-Keap1 and examined the lipidation of LC3, a characteristic of autophagy that converts the LC3-I isoform to the electrophoretically more mobile LC3-II isoform. We found that the amount of LC3-II was increased by forced expression of Sesn2 (Figure 2A) and that F-Sesn2 changed the distribution of GFP-tagged LC3 from a diffuse pattern to a cytoplasmic punctate pattern (Figure 2B), the latter of which represents decoration of the membranes of autophagosomes by lipidated LC3.

The role of autophagy was further studied using autophagy-defective, ATG7-deficient (*Atg7*$^{-/-}$), or p62-deficient (*p62*$^{-/-}$) MEFs after transfecting them with vectors for F-Sesn2 and H-Keap1. The lack of ATG7 (Figures 2C and 2D) or p62 (Figures 2E and 2F) blocked Keap1 degradation induced by Sesn2, suggesting that p62-dependent autophagy is the major pathway for such degradation. Moreover, forced expression of p62 enhanced the Sesn2-induced degradation of Keap1 in a concentration-dependent manner (Figure S2D).

Consistent with the previous observation that Keap1 degradation was induced by overexpression of Rbx1 (Zhang et al., 2005), we found that expression of Myc epitope-tagged Rbx1 (M-Rbx1) in *p62*$^{+/+}$ MEFs destabilized H-Keap1 in a concentration-dependent manner (Figures 2G and 2H). Moreover, this effect of Rbx1 was not apparent in *p62*$^{-/-}$ MEFs (Figures 2G and 2H). The Rbx1-induced degradation of Keap1 was also significantly attenuated in *Sesn2*$^{-/-}$ MEFs compared with that in *Sesn2*$^{+/+}$ MEFs (Figures 2I and 2J). The degradation of H-Keap1 induced by M-Rbx1 in HEK293 cells was enhanced by the presence of F-Sesn2 (Figure S2E). In line with the fact

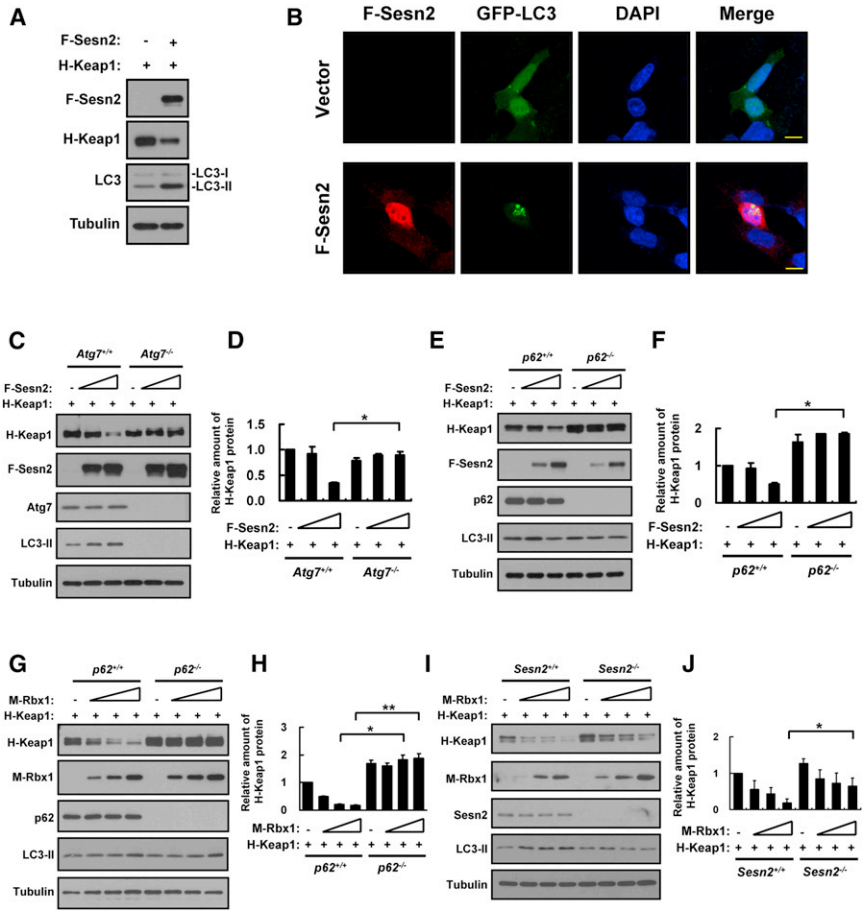


**Figure 2. Sesn2 Induces Keap1 Degradation via p62-Dependent Autophagy**

(A) HEK293 cells transfected with vectors for F-Sesn2 and H-Keap1 were lysed and subjected to immunoblot analysis with antibodies to FLAG, HA, and LC3.

(B) HEK293 cells expressing F-Sesn2 (or transfected with the empty vector) and GFP-LC3 were subjected to immunofluorescence analysis with antibodies to FLAG and GFP. Nuclei were also stained with DAPI. Scale bars, 20 μm.

(C) $Atg7^{+/+}$ or $Atg7^{-/-}$ MEFs transfected with vectors for H-Keap1 and F-Sesn2 (1×, 2×) were lysed and subjected to immunoblot analysis with antibodies to HA, FLAG, ATG7, and LC3.

(D) Densitometric analysis of H-Keap1 in immunoblots similar to that in (C). Data are means ± SD from three independent experiments. *$p < 0.05$.

(E) MEFs ($p62^{+/+}$ or $p62^{-/-}$) expressing H-Keap1 and F-Sesn2 (1×, 2×) were lysed and subjected to immunoblot analysis with antibodies to HA, FLAG, p62, and LC3.

(F) Densitometric analysis of H-Keap1 in immunoblots similar to that in (E). Data are means ± SD from three independent experiments. *$p < 0.05$.

(G) MEFs ($p62^{+/+}$ or $p62^{-/-}$) expressing H-Keap1 and M-Rbx1 (1×, 2×, 3×) were lysed and subjected to immunoblot analysis with antibodies to HA, Myc, p62, and LC3.

(H) Densitometric analysis of H-Keap1 in immunoblots similar to that in (G). Data are means ± SD from three independent experiments. *$p < 0.03$, **$p < 0.01$.

(I) $Sesn2^{+/+}$ or $Sesn2^{-/-}$ MEFs transfected with vectors for H-Keap1 and M-Rbx1 (1×, 2×, 3×) were lysed and subjected to immunoblot analysis with antibodies to HA, Myc, Sesn2, and LC3.

(J) Densitometric analysis of H-Keap1 in immunoblots similar to that in (I). Data are means ± SD from three independent experiments. *$p < 0.05$.

that Rbx1 interacts with Keap1 indirectly through Cul3 (Zhang et al., 2005), M-Rbx1-induced H-Keap1 degradation was significantly attenuated in Cul3-depleted HEK293 cells (Figure S2F).

Keap1 is steadily degraded through p62-mediated autophagy under normal (unstressed) conditions (Taguchi et al., 2012). Given that p62 interacts directly with Keap1 and LC3 through its Keap1 interacting region (KIR) and LC3 interacting region (LIR) domains (Pankiv et al., 2007; Komatsu et al., 2010), respectively, the formation of an LC3-p62-Keap1 ternary complex on the autophagosome membrane is likely responsible for autophagic degradation of Keap1. It is known that Keap1 is ubiquitinated by Cul3-Rbx1 and undergoes proteasome-independent degradation (Zhang et al., 2005). It is also known that p62 binds ubiquitinated proteins through its ubiquitin-associated (UBA) domain and directs them to the autophagic machinery. We tested the role of the p62 UBA domain in Sesn2-induced Keap1 degradation using HEK293 cells transfected with vectors for F-Sesn2, H-Keap1, and p62 wild-type or UBA deletion mutant. p62's lack of the UBA domain did not block Keap1 degradation induced by F-Sesn2 (Figure S2G). In the absence of F-Sesn2, however, the steady-state level of H-Keap1 in wild-type p62-expressing cells was lower than in UBA deletion mutant-expressing cells (Figure S2G). Similar experiments with M-Rbx1-expressing cells indicated that the ubiquitin-UBA interaction became unnecessary when M-Rbx1 levels were elevated (Figure S2H). These results suggest that the interaction between the ubiquitin moiety of Keap1 and the p62 UBA domain contributes to the formation of an LC3-p62-Keap1 ternary complex when the concentrations of Sesn2 or Rbx1 are low but that this interaction is not required in the presence of elevated levels of Sesn2 or Rbx1, probably because Sesn2 or Rbx1 can strengthen the otherwise weak association between Keap1 and p62.

To test whether Sesn2-mediated Keap1 degradation mediates Nrf2 activation, we transfected $Keap1^{+/+}$ or $Keap1^{-/-}$ MEFs with vectors for F-Sesn2, p62, and H-Nrf2 and evaluated the expression levels of three Nrf2 target gens: Srx, NAD(P)H dehydrogenase quinone 1 (NQO1), and glutathione S-transferase A1 (GSTA1). The lack of Keap1 completely blocked the Sesn2-induced increase of the three Nrf2-target genes (Figure S2I), suggesting that Sesn2 confers its antioxidant function by facilitating the degradation of Keap1.

## Sesn2 Interacts Specifically with p62, Rbx1, and Keap1

To explore whether Sesn2 interacts with p62, Rbx1, or Keap1, we transfected HEK293 cells with expression vectors for p62,

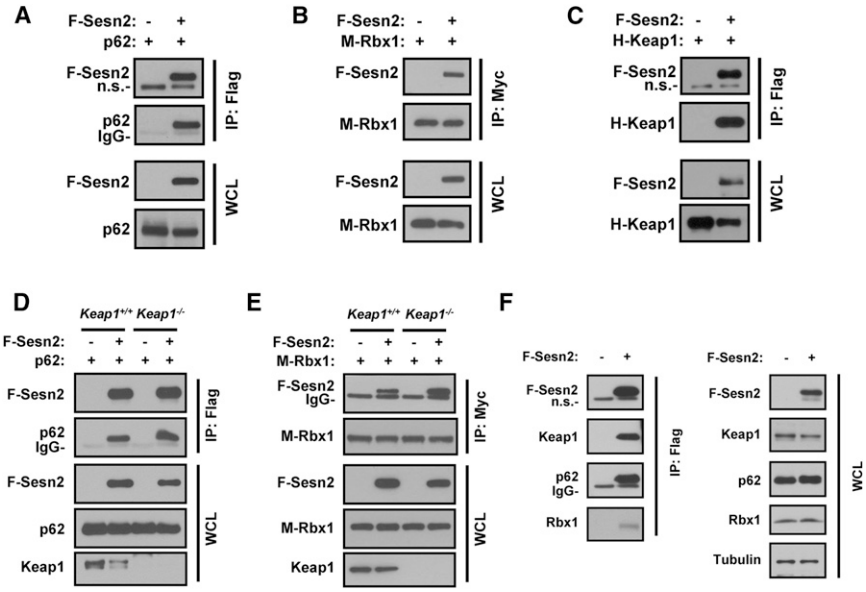


**Figure 3. Sesn2 Interacts Specifically with p62, Rbx1, and Keap1**

(A–C) Lysates of HEK293 cells transfected with vectors for F-Sesn2 and either p62 (A), M-Rbx1(B), or H-Keap1 (C) were subjected to immunoprecipitation with antibodies to FLAG (A and C) or to Myc (B), and the resulting immunoprecipitates (IPs) as well as the whole-cell lysates (WCL) were subjected to immunoblot analysis with antibodies to the indicated proteins. IgG, immunoglobulin G; n.s., nonspecific band.

(D and E) Lysates of *Keap1*$^{+/+}$ or *Keap1*$^{-/-}$ MEFs transfected with vectors for F-Sesn2 and either p62 (D) or M-Rbx1 (E) were subjected to immunoprecipitation and immunoblot analysis as in (A) and (B), respectively.

(F) Lysates of HEK293 cells transfected with a vector for F-Sesn2 (or the corresponding empty vector) were subjected to immunoprecipitation with antibodies to FLAG, and the resulting precipitates as well as the whole-cell lysates were subjected to immunoblot analysis with antibodies to the indicated proteins.

M-Rbx1, or H-Keap1 together with a vector for F-Sesn2 and then subjected cell lysates to coimmunoprecipitation analysis. We found that p62 was efficiently coprecipitated with F-Sesn2 (Figure 3A), F-Sesn2 with M-Rbx1 (Figure 3B), and H-Keap1 with F-Sesn2 (Figure 3C). To determine whether the association of F-Sesn2 with p62 or M-Rbx1 was indirect and mediated through Keap1, we examined their interactions in *Keap1*$^{+/+}$ or *Keap1*$^{-/-}$ MEFs. We detected p62 in F-Sesn2 immunoprecipitates (Figure 3D) as well as F-Sesn2 in M-Rbx1 immunoprecipitates (Figure 3E) prepared from either *Keap1*$^{+/+}$ or *Keap1*$^{-/-}$ MEFs, suggesting that the interactions between Sesn2 and both p62 and Rbx1 are not mediated through Keap1. Endogenous Keap1, p62, and Rbx1 were also coimmunoprecipitated with F-Sesn2 (Figure 3F). The C125S mutant of F-Sesn2 associated with ectopic p62, M-Rbx1, or H-Keap1 to a similar extent to the wild-type protein (Figures S3A–S3C). A similar coimmunoprecipitation analysis indicated that Sesn1 also interacts with p62, Rbx1, and Keap1 (Figures S3D–S3F).

To localize the regions of p62, Rbx1, and Keap1 required for their interaction with Sesn2, coimmunoprecipitation analyses were performed with HEK293 cells transfected with expression vectors for various deletion mutants of M-p62, H-Rbx1, or Keap1 together with a vector for F-Sesn2. Deletion of the Phox and Bem1p (PB1) domain of M-p62 or the N-terminal residues (24–41) of H-Rbx1 completely disrupted the binding of F-Sesn2 to M-p62 and H-Rbx1, respectively, whereas deletion of other regions in M-p62 and H-Rbx1 did not alter their association with F-Sesn2 (Figures S3G and S3H), suggesting that p62 and Rbx1 bind Sesn2 via their PB1 domain and N-terminal residues 24–41, respectively. Keap1 consists of five domains: an N-terminal region, a broad-complex, tramtrack, and bric-á-brac (BTB) domain, an intervening region (IVR), a Kelch repeat domain, and a C-terminal region. All three Keap1 deletion mutants (ΔBTB, ΔIVR, and ΔKelch) interacted with F-Sesn2, although with much weaker affinities than wild-type Keap1 (Figure S3I). Thus, Keap1 may interact with Sesn2 through all three domains, or indirectly through Cul3, which binds to the BTB and IVR domains of Keap1, and p62, which binds to the Kelch domain of Keap1.

## Regulation of Sesn2 Expression, Keap1 Degradation, and Nrf2 Activation in Mouse Liver by Fasting-Refeeding

Sesns are major downstream targets of p53, which is activated by stress due to energy deprivation (Sen et al., 2011). The expression of Sesns is also induced by persistent activation of target of rapamycin (TOR) signaling in the setting of overnutrition (Laplante and Sabatini, 2012).

To explore the role of Sesn2 in the physiological setting, under conditions in which p53 or mTORC1 signaling pathways are activated, we subjected mice to metabolic challenge by overnight fasting followed by refeeding with a high-carbohydrate, fat-free diet. The activity of mTORC1 in the liver, as monitored by immunoblot analysis of phosphorylated ribosomal protein S6, was decreased by overnight fasting; increased markedly at 3, 6, and, 16 hr after the onset of refeeding; and decreased again after refeeding for 36 hr (Figure 4A). In contrast, the hepatic abundance of Sesn2 was increased after overnight fasting (Figure 4B), with the amount of Sesn2 mRNA increased 4-fold relative to the livers of nonfasted mice (Figure 4C). The amounts of Sesn2 protein and mRNA had decreased to nonfasted levels by 3 hr after the onset of refeeding but then increased gradually until 16 hr, achieving levels slightly lower than those apparent after fasting, before decreasing again by 36 hr (Figures 4B and 4C). The effect of fasting on Sesn2 expression in the liver was measured in more detail in mice fasted for 3, 6, 9, or 16 hr. The amounts of Sesn2 protein (Figure 4I) and mRNA (Figure 4J) increased gradually with time, with the abundance of Sesn2 mRNA increasing 5-fold by 16 hr, relative to that in nonfasted controls. These results show that Sesn2 expression is increased by both fasting and refeeding.

In *Drosophila*, Sesn expression is induced through ROS-mediated activation of the transcription factor FOXO (Lee et al., 2010). Recent studies have also shown that the Sesn2 gene is a target of the transcription factor C/EBP, which is

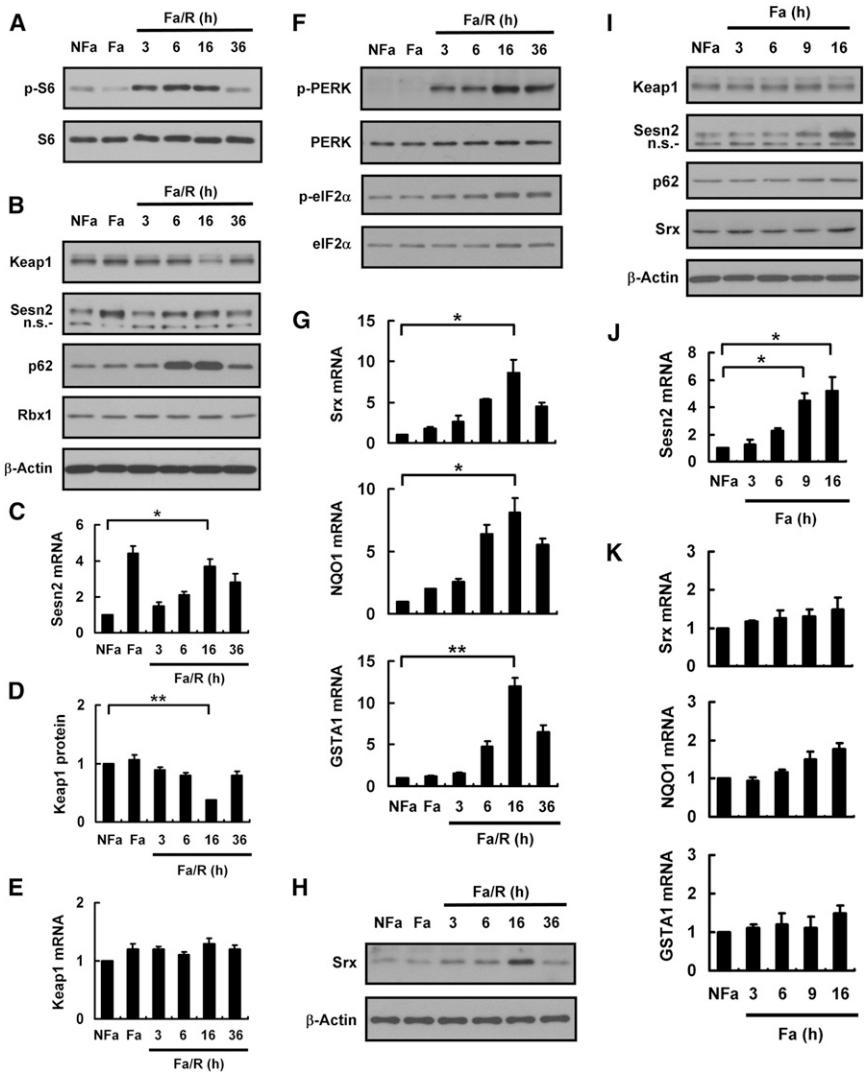


**Figure 4. Effects of Fasting and Refeeding on the Expression of Sesn2 and p62, on Keap1 Degradation, and on Nrf2 Activation in Mouse Liver**

(A–H) Mice were maintained in a nonfasted state (NFa), fasted overnight (Fa), or fasted overnight and then refed a high-carbohydrate, fat-free diet for the indicated times (Fa/R). The livers from three mice of each group were pooled and homogenized, and the homogenates (20 μg of protein) were subjected to immunoblot analysis with antibodies to phosphorylated (p-) or total forms of S6 (A), with those to Keap1, Sesn2, p62, Rbx1, or the loading control β-actin (B), with those to phosphorylated or total forms of PERK or eIF2α (F), or with those to Srx or β-actin (H). Densitometric analysis of Keap1 bands in immunoblots similar to that in (B) is shown in (D); data are means ± SD from three independent experiments and are expressed relative to the value for the nonfasted state (**$p < 0.03$). Total RNA isolated from liver homogenates was also subjected to quantitative RT-PCR analysis of mRNAs for Sesn2 (C) and Keap1 (E) or for Srx, NQO1, and GSTA1 (G); data are expressed relative to the corresponding value for nonfasted mice and are means ± SD for three mice of each group (*$p < 0.05$ and **$p < 0.03$).

(I–K) Mice were maintained in a nonfasted state or deprived of food for the indicated times. The livers from three mice of each group were pooled and homogenized, and the homogenates (20 μg of protein) were subjected to immunoblot analysis with antibodies to Keap1, Sesn2, p62, and Srx (I). Total RNA isolated from liver homogenates was also subjected to quantitative RT-PCR analysis of mRNAs for Sesn2 (J) or for Srx, NQO1, and GSTA1 (K). The mRNA data are expressed relative to the corresponding value for nonfasted mice and are means ± SD for three mice of each group. *$p < 0.03$.

activated downstream of ER stress (Ozcan et al., 2008; Papadia et al., 2008). We found that refeeding in mice induced hepatic ER stress, as indicated by increased phosphorylation of double-stranded RNA-activated protein kinase-like ER kinase (PERK) and eukaryotic initiation factor 2α (eIF2α) (Figure 4F), and that constitutive mTORC1 activation as a result of TSC2 loss led to Sesn2 induction (Figure S4A), suggesting that refeeding might induce Sesn2 expression in mouse liver through activation of mTORC1 and downstream signaling mediated by ROS and ER stress.

Deprivation of food for 16 hr resulted in downregulation of lipogenesis in the liver as monitored by RT-PCR and real-time PCR analyses of the mRNAs for three lipogenic proteins: sterol regulatory element-binding protein 1c (SREBP1c), fatty acid synthase (FAS), and stearoyl-coenzyme A desaturase 1 (SCD1) (Horton et al., 1998) (Figure S4B). Refeeding elicited an acute lipogenic response in the liver as revealed by marked increases in the expression of these three lipogenic genes, which reached a maximum at 16 hr before decreasing again by 36 hr (Figure S4B). This acute lipogenic response, mediated by mTORC1 activation (Li et al., 2010), has been shown to result in fatty acid accumulation and enhanced ROS production in hepatocytes (Browning and Horton, 2004). In turn, ROS production damages DNA and proteins, inducing inflammatory responses, liver fibrosis, and cell death, all of which are often associated with nonalcoholic steatohepatitis (Browning and Horton, 2004). On hematoxylin and eosin (H&E) staining, this type of liver injury manifests as an increase in the number of ballooning degenerated hepatocytes, characterized by cell swelling with rarefaction of the cytoplasm, around the central vein area (zone 3) (Kleiner et al., 2005). In order to test whether mice undergo similar injury in response to fasting-refeeding, we performed a time series of wild-type mice subjected to fasting for 16 hr followed by refeeding for various times. H&E staining of liver sections showed that fasting-refeeding elicited ballooning degeneration (Figure S4C). The refeeding-induced liver injury was also apparent from higher levels of apoptosis measured by the TUNEL assay (Figures S4D and S4E) and from elevated serum levels of ALT (Figure S4F). The liver injury resulting from the acute lipogenic stimulation of

fasting-refeeding, as assessed by all of these parameters, reached a maximum at 16 hr before decreasing again by 36 hr.

The expression of Nrf2-dependent antioxidant genes Srx and NQO1 and GSTA1 mRNAs were increased slightly after an overnight fast, and they increased gradually and markedly (maximum of ~10-, ~8-, and ~12-fold, respectively) during refeeding for 16 hr before declining again at 36 hr (Figure 4G). The abundance of Srx protein exhibited a pattern similar to that of Srx mRNA (Figure 4H). Srx, NQO1, and GSTA1 mRNAs were increased <2-fold during fasting for 16 hr (Figure 4K). We examined whether Keap1 degradation might contribute to the induction of these Nrf2 target genes. Immunoblot analysis revealed that the abundance of Keap1 in the liver was not affected by overnight fasting (Figures 4B, 4D, and 4I). However, the amount of Keap1 decreased gradually during refeeding, reaching its nadir (~40% of the level in nonfasted mice) after 16 hr, before increasing again at 36 hr (Figures 4B and 4D). In contrast, the amount of Keap1 mRNA was not affected by fasting or refeeding (Figure 4E). These results suggest that the refeeding-induced downregulation of Keap1 protein is likely due to protein degradation. The abundance of p62 in mouse liver was not affected by overnight fasting, but it was increased substantially at 6 and 16 hr after the onset of refeeding before declining at 36 hr (Figures 4B and 4I). In contrast, the amount of Rbx1 was not affected by fasting or refeeding (Figure 4B).

In summary, the abundance of Sesn2 in mouse liver was increased to similar extents by fasting for 16 hr and by refeeding for 16 hr, that of p62 was increased by refeeding for 16 hr, but not by overnight fasting, and that of Rbx1 was not affected by fasting or refeeding, whereas the levels of Keap1 degradation and expression of Nrf2 target genes were not increased substantially by fasting for 16 hr but were markedly increased after refeeding for 16 hr. These observations, together with our results obtained with $p62^{+/+}$ or $p62^{-/-}$ MEFs showing that p62 is essential for Sesn2-induced Keap1 degradation (Figures 2E and 2F), suggest that concurrent increases in the concentrations of Sesn2 and p62 are critical for Keap1 degradation.

## Sesn2 Is Required for Keap1 Degradation and Nrf2 Activation Induced by Refeeding

To further examine the role of endogenous Sesn2 in Nrf2 regulation, we subjected $Sesn2^{+/+}$ or $Sesn2^{-/-}$ mice to fasting for 16 hr followed by refeeding for 16 hr. As shown above (Figures 4G and 4K), the abundance of mRNAs for Nrf2 target genes in the liver of $Sesn2^{+/+}$ mice was increased <2-fold by fasting but was increased markedly (~15-fold for Srx, ~8-fold for NQO1, and ~25-fold for GSTA1 relative to the values for nonfasted controls) by refeeding (Figure 5A). The mRNA levels for the Nrf2 target genes were not affected by fasting in $Sesn2^{-/-}$ mice. Furthermore, the marked upregulation of the expression of these genes by refeeding apparent in $Sesn2^{+/+}$ mice was virtually abolished in $Sesn2^{--}$ mice (Figure 5A). Immunoblot analysis showed that refeeding induced a pronounced increase in the hepatic abundance of Srx protein in $Sesn2^{+/+}$ mice but a much smaller increase in $Sesn2^{-/-}$ mice (Figure 5B). These results suggested that ablation of Sesn2 resulted in substantial attenuation of the Nrf2 activation associated with refeeding. As shown in Figures 4B and 4D, the amount of Keap1 protein in the liver was reduced by 50% in refed $Sesn2^{+/+}$ mice (Figures 5C and 5D). In contrast, refeeding had only a marginal effect on Keap1 abundance in $Sesn2^{-/-}$ mice (Figures 5C and 5D). The amount of Keap1 mRNA was not affected by fasting or refeeding in either $Sesn2^{+/+}$ or $Sesn2^{-/-}$ mice (Figure 5E).

The amount of p62 was low in the liver of nonfasted $Sesn2^{+/+}$ or $Sesn2^{-/-}$ mice, and it was not affected by fasting in mice of either genotype (Figure 5C). The hepatic abundance of p62 mRNA was also not affected by fasting in these animals (Figure 5F); it was increased by a factor of ~2.5 in $Sesn2^{+/+}$ mice but only slightly in $Sesn2^{-/-}$ mice after refeeding (Figure 5F), suggesting that Sesn2 ablation downregulated p62 gene transcription. Of note, although the p62 gene is a target of Nrf2 (Jain et al., 2010), the extent of the increase in the amount of p62 mRNA induced by refeeding in $Sesn2^{+/+}$ mice (Figure 5F) was much smaller than that for other Nrf2 targets (Figure 5A). In contrast to its mRNA, the amount of p62 protein was markedly increased in both $Sesn2^{+/+}$ and $Sesn2^{-/-}$ mice (Figure 5C). Thus, despite the fact that the abundance of p62 mRNA in refed $Sesn2^{-/-}$ mice was much lower than that in refed $Sesn2^{+/+}$ mice, refeeding increased p62 protein levels to a similar extent in both types of mice. One possible explanation for this finding might be that the autophagic substrate p62 is degraded much more slowly in the absence of Sesn2, as seen with Keap1.

The activity of mTORC1 in the liver, as reflected by S6 phosphorylation, was markedly reduced after 3 hr of fasting in $Sesn2^{+/+}$ mice, whereas such an effect was not observed in $Sesn2^{-/-}$ mice (Figures 5G and 5H), suggesting that Sesn2 is required for the fasting-induced inhibition of mTORC1. However, the activity of mTORC1 remained inhibited at the same level after 3, 6, or 9 hr of fasting before recovering to the level of nonfasted control mice at 16 hr (Figures 5G and 5H), whereas the amount of Sesn2 increased gradually with time during fasting for 3, 6, 9, or 16 hr (Figure 5G). These observations suggest that the basal level of Sesn2 may be sufficient for the fasting-induced inhibition of mTORC1 activity and that induction of Sesn2 is not a signal to mTORC1.

We evaluated the role of Sesn2 in the formation of autophagosomes by measuring the autophagic marker LC3-II. The amount of LC3-II formed during fasting for 3 and 6 hr in the livers of $Sesn2^{+/+}$ mice was lower than in the livers of $Sesn2^{-/-}$ mice (Figures S5A and S5B). Although refeeding markedly reduced the amount of LC3-II in the livers of both $Sesn2^{+/+}$ and $Sesn2^{-/-}$ mice, LC3-II levels were still noted to be higher in the livers of refed $Sesn2^{+/+}$ mice compared to refed $Sesn2^{-/-}$ mice (Figures S5C and S5D). These results support the notion that Sesn2 together with p62 can activate selective autophagy for degradation of Keap1 even when mTORC1 is activated (Figure 4A) and macroautophagy is subsequently suppressed.

## Sesn2-Induced Nrf2 Activation Protects the Liver from Oxidative Damage Due to Acute Lipogenic Stimulation

Refeeding after fasting elicited oxidative liver damage (Figures S4C–S4F). We evaluated the role of Sesn2 in modulating this liver injury by subjecting $Sesn2^{+/+}$ and $Sesn2^{-/-}$ mice to the fasting-refeeding protocol. The ablation of Sesn2 resulted in a greater increase in liver damage as measured by H&E staining (Figure 6A), serum ALT levels (Figure 6B), and TUNEL assay (Figures 6C and 6D). To determine whether the exaggerated liver injury observed in fasted-refed $Sesn2^{-/-}$ mice was related to the

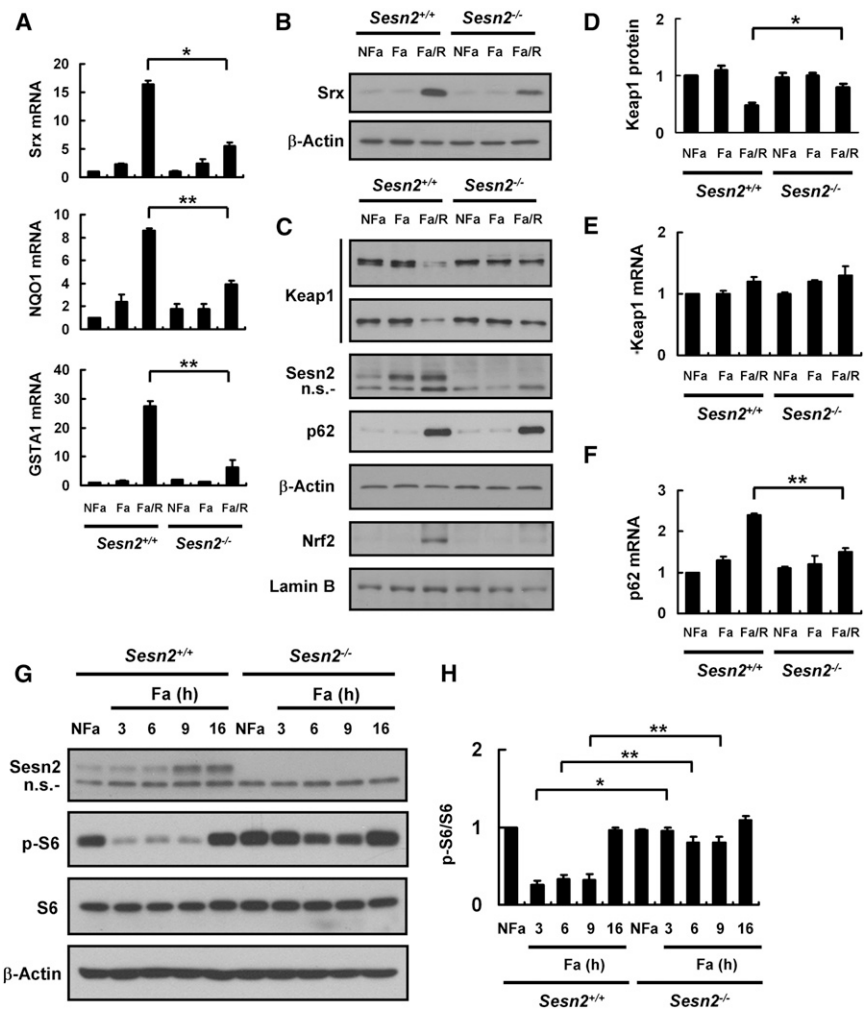


**Figure 5. Effects of Sesn2 Ablation on Keap1 Degradation, Expression of Nrf2 Target Genes, and mTORC1 Activity in the Liver during Fasting or Refeeding in Mice**

(A–F) *Sesn2*$^{+/+}$ or *Sesn2*$^{-/-}$ mice were maintained in a nonfasted state (NFa) or deprived of food for 16 hr without (Fa) or with (Fa/R) refeeding a high-carbohydrate, fat-free diet for 16 hr. The livers from three mice of each group were pooled and homogenized, and the homogenates (20 μg of protein) or nuclear fraction (40 μg of protein) was subjected to immunoblot analysis with antibodies to the indicated proteins (B and C). Two representative Keap1 blots are shown. The intensity of Keap1 bands in immunoblots similar to that in (C) was determined by densitometry (D); data are expressed relative to the corresponding value for nonfasted mice and are means ± SD from three independent experiments (*$p < 0.05$). Total RNA isolated from liver homogenates was also subjected to quantitative RT-PCR analysis of Srx, NQO1, and GSTA1 mRNAs (A) as well as to that of Keap1 (E) and p62 (F) mRNAs. Data are expressed relative to the corresponding value for nonfasted mice and are means ± SD for three mice of each group. *$p < 0.05$ and **$p < 0.03$ (A); **$p < 0.02$ (F).

(G) *Sesn2*$^{+/+}$ or *Sesn2*$^{-/-}$ mice were maintained in a nonfasted state or fasted for the indicated times. The livers from three mice of each group were pooled and homogenized, and the homogenates (20 μg of protein) were subjected to immunoblot analysis with antibodies to Sesn2 and to phosphorylated or total forms of S6.

(H) The intensity of p-S6 bands normalized by that of S6 bands in immunoblots similar to that in (G) was determined by densitometry. Data are means ± SD from three independent experiments. *$p < 0.05$, **$p < 0.03$.

failure to induce Nrf2 activation, we examined the role of Nrf2 in protection against oxidative liver injury by subjecting *Nrf2*$^{+/+}$ or *Nrf2*$^{-/-}$ mice to the fasting-refeeding protocol. As expected, refeeding induced expression of Srx, NQO1, and GSTA1 genes in the liver of *Nrf2*$^{+/+}$ mice, but not in that of *Nrf2*$^{-/-}$ mice (Figures S6A and S6B). Nrf2 deficiency rendered the liver more susceptible to fasting-refeeding-induced injury, as demonstrated by H&E staining (Figure S6C), serum ALT levels (Figure S6D), and TUNEL assay (Figures S6E and S6F). These results suggest that Sesn2-dependent Nrf2 activation is critical for protection of the liver from refeeding-induced injury.

To test whether Nrf2 overexpression could rescue the damaging effect of Sesn2 ablation, we used tail vein injections in *Sesn2*$^{-/-}$ mice to transduce their livers with adenovirus expressing either Nrf2 (Ad-Nrf2) or GFP (Ad-GFP) and subjected mice to refeeding. The activity of Ad-Nrf2 was verified in Hepa1c1c7 mouse hepatoma cells (Figures S7A and S7B). Injection of Ad-Nrf2, but not Ad-GFP, increased the induction of Nrf2 target genes (Figures 7A and 7B) as well as the amount of Nrf2 in the nucleus (Figure 7C) in the livers of both nonfasted and fasted mice. Importantly, overexpression of Nrf2 attenuated the extent of refeeding-induced liver damage in *Sesn2*$^{-/-}$ mice as revealed by less ballooning degeneration (Figure S7C), reduced serum ALT levels (Figure 7D), and reduced apoptotic cell death (Figures 7E and 7F) compared to Ad-GFP-treated mice. These data confirm that Sesn2 protects the liver from refeeding-induced oxidative damage through Nrf2 activation.

## DISCUSSION

The activity of Nrf2 is negatively regulated by Keap1, a cysteine-rich protein that acts as a substrate adaptor for the ubiquitination of Nrf2 by the Cul3-Rbx1 E3 ubiquitin ligase complex. Keap1 thereby targets Nrf2 for proteasomal degradation and maintains the transcription factor at a low level under nonstress conditions (Itoh et al., 2010). Keap1 binds through its Kelch domain to the N-terminal Neh2 domain of Nrf2, and the BTB domain of Keap1 recruits Cul3. A hinge-and-latch model was proposed to explain this adaptor and repressor function of Keap1 (McMahon et al., 2006). In this model, the Kelch domains of a Keap1 homodimer bind to one Nrf2 molecule through a low-affinity DLG motif (latch) or a high-affinity ETGE motif (hinge) located in the Neh2 domain of Nrf2. Under nonstress conditions, the Keap1 homodimer binds both the DLG and ETGE motifs of Nrf2 and thereby presents Nrf2 in the correct orientation for ubiquitination. On exposure to oxidants or electrophiles, Keap1 is modified at

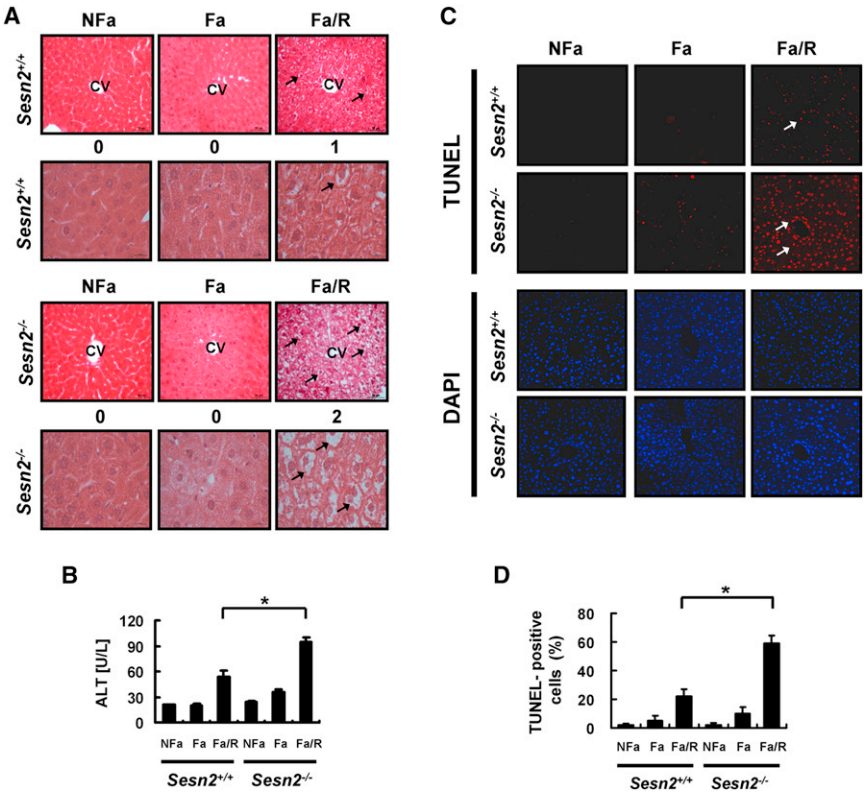


**Figure 6. Sesn2 Protects the Liver from the Acute Lipogenic Stimulus Elicited by Refeeding after Food Deprivation**

(A) *Sesn2*$^{+/+}$ or *Sesn2*$^{-/-}$ mice were maintained in a nonfasted state (NFa), deprived of food for 16 hr (Fa), or fasted for 16 hr and then refed a high-carbohydrate, fat-free diet for 16 hr (Fa/R). Liver sections prepared from the mice were subjected to H&E staining. The extent of ballooning degeneration (arrows) is indicated below each upper set of images, which are shown at higher magnification in the lower panels. CV, central vein. Original magnification, ~100 (upper panels) and ~400 (lower panels).

(B) The serum level of alanine aminotransferase (ALT) was measured from mice treated as in (A). Data are means ± SD for three mice of each group. *$p < 0.05$.

(C) TUNEL analysis of liver sections from mice treated as in (A). Original magnification, ~100.

(D) Quantitation of TUNEL analysis in the liver of mice treated as in (A). Data are means ± SD for three mice of each group. *$p < 0.05$.

one or more residues (Itoh et al., 2010), which is thought to induce a conformational change that disrupts its interaction with the low-affinity DLG motif of Nrf2 and perturbs its ability to direct Nrf2 ubiquitination. The net effect of such Keap1 modification is thus that Nrf2 is no longer degraded in the cytosol and is able to translocate to the nucleus and induce transcription of its target genes.

In the present study, we have found that forced expression of Sesn2 induces degradation of Keap1 and upregulation of Nrf2 activity and that the Sesn2-induced degradation of Keap1 is mediated mostly by autophagy rather than by the proteasome. Furthermore, Sesn2-induced Keap1 degradation was abolished in the absence of p62 and was promoted by forced expression of Rbx1. Although the role of Sesn was not known, activation of the Nrf2 pathway via Keap1 degradation and the role of Rbx1 and p62 in Keap1 degradation were described previously (Tanigawa et al., 2007; Zhang et al., 2005; Taguchi et al., 2012). Degradation of Keap1 results in stabilization and a consequent increase in the transactivation activity of Nrf2. However, degradation of Keap1 is not necessary for Nrf2 activation. Disruption of the interaction between Nrf2 and Keap1 is sufficient to trigger activation of the Nrf2 pathway as exemplified in the case of Nrf2 activation by the cyclin-dependent kinase inhibitor $p21^{Cip/WAF1}$, which competes with Keap1 for binding to the DLG motif of Nrf2 and thereby protects Nrf2 from ubiquitination by opening the DLG latch (Chen et al., 2009). p62 was identified as another protein that activates Nrf2 by disrupting the Keap1-Nrf2 interaction in autophagy-deficient cells (Komatsu et al., 2010; Lau et al., 2010). Impairment of autophagy results in the accumulation of p62, a specific substrate for this process. It was found that p62 binds to the Kelch domain of Keap1 via a motif designated the Keap1-interacting region (KIR), which resembles the ETGE motif of Nrf2, and that it thereby competes with Nrf2 for binding to Keap1. p62 is a multifaceted adaptor protein that performs diverse biological functions by interacting with many other proteins and promoting protein aggregation (Moscat and Diaz-Meco, 2011). Furthermore, through its ability to interact with LC3, p62 regulates autophagic removal of protein aggregates and damaged intracellular organelles (Pankiv et al., 2007). Although proteins that bind tightly to p62 are known to be degraded together with the autophagy substrate (Fan et al., 2010), it has not been clear until our present study whether Keap1 is also degraded by binding to p62. Keap1 degradation was observed in Keap1-overexpressing cells (Jain et al., 2010), but it was not apparent in control cells even when p62 degradation was induced (Fan et al., 2010). These observations suggest that the interaction between p62 and Keap1 is not strong enough to render Keap1 susceptible to autophagic degradation unless the equilibrium favoring dissociation is opposed by a substantial increase in Keap1 concentration.

We have found that Sesn2 as well as Sesn1 interact with p62, Keap1, and Rbx1, likely accounting for our observation that Keap1 degradation was induced by forced expression of Sesn2 or Sesn1. Sesn isoforms thus appear to serve as scaffold proteins that strengthen the otherwise weak association of Keap1 with p62. Both the Sesn2- and Rbx1-induced degradation of Keap1 depends entirely on p62.

To study the role of Sesn2 in a physiological setting, we subjected mice to fasting and refeeding. The abundance of Sesn2 in the liver was increased by both food deprivation and refeeding, but these increases were likely mediated by distinct mechanisms. The amount of p62 was increased by refeeding, but not by fasting. Moreover, Sesn2 appears to serve different functions in the different metabolic states: it suppressed

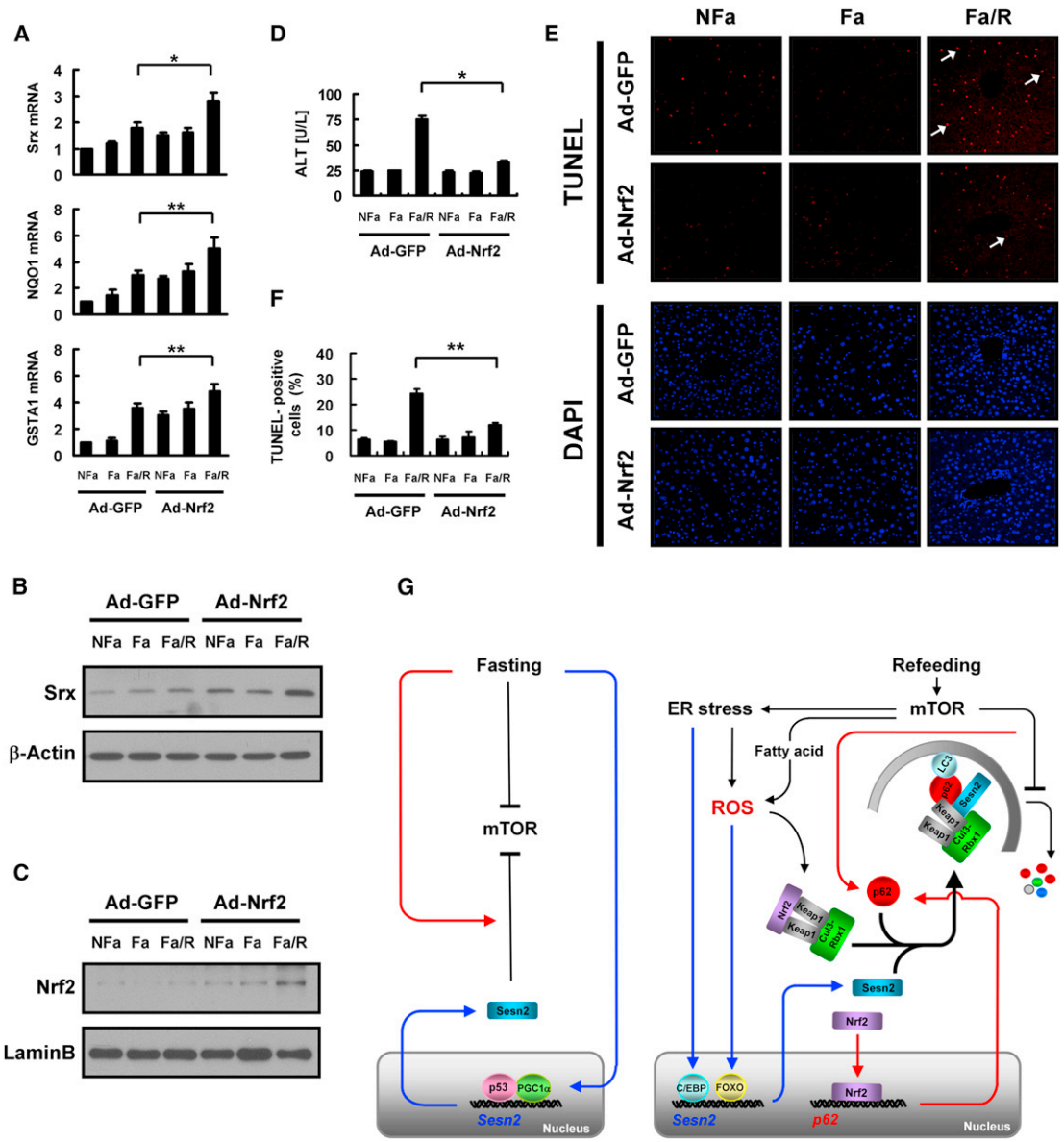


**Figure 7. Ectopic Expression of Nrf2 Protects the Liver in *Sesn2*$^{-/-}$ Mice from the Acute Lipogenic Stimulus Elicited by Refeeding after Food Deprivation**

(A) *Sesn2*$^{-/-}$ mice that had been injected with GFP (Ad-GFP) or Nrf2 (Ad-Nrf2) adenovirus were maintained in a nonfasted state (NFa), deprived of food for 16 hr (Fa), or fasted for 16 hr and then refed with a high-carbohydrate, fat-free diet for 16 hr (Fa/R). Total RNA isolated from the liver of these mice was subjected to quantitative RT-PCR analysis of Srx, NQO1, and GSTA1 mRNAs. Data are expressed relative to the corresponding value for nonfasted mice and are means ± SD for three mice of each group. *$p < 0.03$, **$p < 0.05$.

(B) Immunoblot analysis of Srx in liver homogenates of mice treated as in (A).

(C) Immunoblot analysis of Nrf2 in nuclear fraction of livers of mice treated as in (A).

(D) Serum levels of alanine aminotransferase (ALT) were measured in mice treated as in (A). Data are presented as means ± SD for three mice of each group. *$p < 0.05$.

(E) TUNEL analysis of the liver from mice treated as in (A).

(F) Quantitation of TUNEL analysis in the liver of mice treated as in (A). Data are presented as means ± SD for three mice of each group. **$p < 0.05$.

(G) Model for the function of Sesn2 in the liver of mice subjected to fasting and refeeding. Left panel: fasting induces inhibition of mTORC1 activity in a manner dependent on the basal level of Sesn2 (red arrow), and it induces Sesn2 expression by increasing the transactivation activity of p53 and PGC1α (blue arrow). Right panel: refeeding with a high-carbohydrate, fat-free diet increases mTORC1 activity, which in turn results in fatty acid accumulation and ER stress, both of which lead to ROS accumulation. Sesn2 expression is induced (blue arrows) by the action of the transcription factors C/EBP (activated by ER stress) and FOXO (activated by ROS). Activated mTORC1 inhibits autophagy and thereby allows p62 accumulation (upper red arrow). Accumulated p62 and Sesn2 together sequester Keap1 and the Cul3-Rbx1 complex to autophagosomes and thereby promote Keap1 degradation (black arrow). The degradation of Keap1 results in Nrf2 activation. ROS also induce Nrf2 activation through modification of Keap1. Activation of Nrf2 increases p62 gene transcription (lower red arrows). See Discussion for details.

mTORC1 activity in the fasting condition, whereas it cooperated with p62 to promote the autophagic degradation of Keap1 during refeeding. On the basis of our findings as well as those of previous studies, we propose a model for the mechanisms underlying regulation of the abundance and function of Sesn2 during fasting and refeeding (Figure 7G).

Starvation upregulates p53 activity and downregulates mTORC1 activity through several mechanisms, which are intimately connected to coordinate the cellular response to metabolic changes (Vousden and Ryan, 2009; Laplante and Sabatini, 2012). We found that mTORC1 activity, as reflected by S6 phosphorylation, was markedly decreased in the liver at 3, 6, and 9 hr after food deprivation but had recovered to the level apparent in nonfasted mice by 16 hr after the onset of fasting. These changes are consistent with the recent observations that mTORC1 signaling is inhibited during the initiation of autophagy associated with food withdrawal, but that the restoration of cellular levels of amino acids resulting from autophagic catabolism eventually reactivates mTORC1 despite ongoing food deprivation (Yu et al., 2010). The inhibition of mTORC1 activity and the activation of autophagy apparent in *Sesn2*$^{+/+}$ mice fasted for 3, 6, or 9 hr were severely dampened in *Sesn2*$^{-/-}$ mice, suggesting that Sesn2 is necessary for mTORC1 inhibition and subsequent autophagy activation. Genotoxic stress inhibits mTORC1 signaling through activation of p53; Sesn2 is essential for this effect of p53 (Budanov and Karin, 2008). Sesn1 and Sesn2 form a complex with AMPK, TSC1, and TSC2 and thereby induce AMPK activation and TSC2 phosphorylation, thus accounting for the p53-dependent inhibition of mTORC1 activity in response to DNA damage (Budanov and Karin, 2008). We found that the basal level of Sesn2 is sufficient to downregulate the AMPK-TSC1/TSC2-mTORC1 pathway and that further accumulation of Sesn2 does not enhance this effect.

Sesn2 induction in the liver of fasted mice is likely the result of an increase in the transactivation activity of p53 that occurs downstream of the energy sensor AMPK and the transcriptional coactivator PGC1$\alpha$ in the energy-starved cells (Sen et al., 2011) (Figure 7G). Whereas the hepatic abundance of Sesn2 in mice deprived of food for 16 hr was slightly higher than that in those allowed to refeed for 16 hr, the upregulation of Sesn2 was accompanied by Keap1 degradation in the latter condition, but not in the former, likely because the amount of p62 was increased by refeeding but not by fasting. Fasting activates macroautophagy through multiple mechanisms involving p53, AMPK, and mTORC1 in order to provide additional energy sources (Vousden and Ryan, 2009). Although the abundance of Sesn2 in the liver was increased and Sesn2 can upregulate autophagic catabolism by inhibiting mTORC1 during starvation, Keap1 degradation was not observed in the liver of fasted mice. This finding suggests that Keap1 is not degraded by macroautophagy but by highly selective autophagy, probably because Keap1 degradation requires the presence of both p62 and Sesns or excess amount of Sesns. Consistent with the absence of Keap1 degradation and apparent lack of ROS production in the liver of fasted mice, Nrf2 activation was minimal. Under the starvation condition, p62 was also not likely degraded, rendering unnecessary its resupply through transcriptional activation of its gene, as indicated by the observation that the level of p62 mRNA was not affected by fasting.

Feeding mice with a high-carbohydrate, fat-free diet after fasting provokes an acute lipogenic response with accumulation of fatty acids in the liver. Fatty acid metabolism in hepatocytes results in the production of ROS (Browning and Horton, 2004). Thus, mTORC1 activation and subsequent fatty acid accumulation in the liver of refed mice may trigger the ROS-FOXO pathway to induce Sesn2 expression as reported in *Drosophila* (Lee et al., 2010). We found that refeeding induced ER stress in mouse liver, suggesting that the induction of Sesn2 expression by refeeding is also likely mediated via sequential activation of mTORC1, ER stress, and C/EBP in addition to the ROS-FOXO pathway (Ozcan et al., 2008; Hotamisligil, 2010).

The increased level of p62 apparent in the liver of refed mice is also likely due to upregulation of mTORC1 activity. Activated mTORC1 attenuates autophagy, resulting in the accumulation of p62 (Laplante and Sabatini, 2012). On the other hand, the accumulated p62 together with Sesn2 induced via FOXO and C/EBP signaling would be expected to sequester Keap1 at autophagosomes and thereby to promote its degradation, resulting in Nrf2 activation. At the same time, the p62 gene is a target of Nrf2 (Jain et al., 2010), with p62 accumulation and Nrf2 activation thus constituting a positive feedback loop (Figure 7G). The key role of Sesn2 in the refeeding-induced activation of Nrf2 is evident from our observation that the induction of Nrf2 target genes by refeeding was markedly attenuated in *Sesn2*$^{-/-}$ mice. The fact that such induction was not completely abolished in the absence of Sesn2 is probably due to the presence of other Sesn isoforms or to Nrf2 activation via Keap1 cysteine oxidation by ROS produced from fatty acid metabolism.

The amount of p62 mRNA was significantly increased by refeeding in the liver of *Sesn2*$^{+/+}$ mice, but not in that of *Sesn2*$^{-/-}$ mice. This finding can be explained by the fact that the p62 gene is a target of Nrf2 and that refeeding markedly activates the Nrf2 pathway in *Sesn2*$^{+/+}$ mice, but not in *Sesn2*$^{-/-}$ mice. Despite the differential effect of refeeding on the level of p62 mRNA in *Sesn2*$^{+/+}$ and *Sesn2*$^{-/-}$ mice, the amount of p62 protein was increased to a similar marked extent by refeeding in mice of both genotypes. Although autophagic catabolism is inhibited by refeeding, autophagy occurs constitutively at low levels, and autophagic degradation of certain proteins is even upregulated under nutrient-rich conditions (Mizushima and Komatsu, 2011), as exemplified in the present study by the autophagic degradation of Keap1. Given that Keap1 degradation occurs in association with that of the autophagic substrate p62, increased synthesis of p62 mRNA might be necessary to compensate for the loss of p62 in the liver of refed *Sesn2*$^{+/+}$ mice. In the liver of refed *Sesn2*$^{-/-}$ mice, p62 mRNA was not upregulated, yet the abundance of p62 was highly increased and similar to that in *Sesn2*$^{+/+}$ mice, probably because Keap1 degradation (which is accompanied by p62 degradation) is blocked by the absence of Sesn2, whereas p62 synthesis is maintained. The fact that refeeding-induced Keap1 degradation occurred in *Sesn2*$^{+/+}$ mice, but not in *Sesn2*$^{-/-}$ mice despite the similar p62 protein levels in these animals, further supports the notion that the accumulation of p62 is not sufficient and that the scaffolding function of Sesn proteins is essential for this effect.

Mice fed a high-carbohydrate diet accumulate fatty acids in the liver, resulting in the generation of large amounts of ROS. To protect against these ROS, hepatocytes produce antioxidant

enzymes such as Srx through activation of the Nrf2 signaling pathway. Our data in *Sesn2*$^{-/-}$ and *Nrf2*$^{-/-}$ mice refed a high-carbohydrate diet demonstrate that the failure to induce such antioxidant enzymes results in severe liver injury, as manifested by an increase in ballooning degeneration, serum ALT levels, and apoptotic cell death. Further, the extent of this liver injury in *Sesn2*$^{-/-}$ mice was attenuated by ectopic expression of Nrf2. Thus, our observations suggest that Sesn2-dependent Nrf2 activation is essential for protection of the liver from injury inflicted by an acute lipogenic stimulus. Further, our data show that the ROS-suppressor function of Sesn proteins is attributable to their capacity to promote the p62-dependent autophagic degradation of Keap1 and consequent Nrf2 activation.

## EXPERIMENTAL PROCEDURES

### Cell Culture and Biochemical Analyses

HEK293 cells, HeLa cells, HCT116 cells, Hepa1c1c7 cells, and MEFs were maintained under 5% $CO_2$ at 37° in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, and streptomycin. Transfection of cells with expression vectors (see Supplemental Experimental Procedures) was performed with the Lipofectamine Plus reagent (Invitrogen). For adenoviral transduction, Hepa1c1c7 cells were transduced in serum-deprived medium with Ad-GFP or Ad-Nrf2 at different multiplicities of infection (moi) for 24 or 48 hr (Shih et al., 2005). For siRNA experiment, HEK293 cells were transfected with control siRNA or siRNA against Cul3 for 24 hr. Immunoprecipitation and immunoblot analysis are described in the Supplemental Experimental Procedures.

### Animal Treatments

Male C57BL/6J mice were obtained from the Jackson Laboratory (Bar Harbor), maintained on a 12 hr-light, 12 hr-dark cycle, and allowed free access to food and water before experiments. Breeding pairs of Nrf2 knockout (*Nrf2*$^{-/-}$) mice were obtained from RIKEN BioResource Center (Tsukuba, Japan). Sesn2 knockout (*Sesn2*$^{-/-}$) mice were generated as described (Woo et al., 2009). Ad-GFP and Ad-Nrf2 were a generous gift from T. Murphy. Recombinant adenoviruses ($2 \times 10^9$ pfu) were delivered by tail vein injection to mice. For refeeding experiments, male mice (8 weeks old) were maintained in a nonfasted condition with normal chow, were deprived of food for various times, or were refed with a high-carbohydrate, fat-free diet (Harlan Teklad Diets) after fasting (Horton et al., 1998). Serum alanine aminotransferase (ALT) levels were measured by a 7020 Clinical Analyzer (Hitachi). All animal experiments were approved by the Animal Care and Use Committee of Ewha Womans University.

### Statistical Analysis

Quantitative data are presented as means ± SD and were compared between two groups with the use of the two-tailed Student's t test. A p value of $< 0.05$ was considered statistically significant.

### Other Experimental Procedures

Chemical reagents, antibodies, expression vectors, immunofluorescence analysis, luciferase reporter assays, quantitative RT-PCR analysis, histological analysis, and TUNEL assay are described in Supplemental Experimental Procedures.

## SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2012.12.002.

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## REFERENCES

Bae, S.H., Woo, H.A., Sung, S.H., Lee, H.E., Lee, S.K., Kil, I.S., and Rhee, S.G. (2009). Induction of sulfiredoxin via an Nrf2-dependent pathway and hyperoxidation of peroxiredoxin III in the lungs of mice exposed to hyperoxia. Antioxid. Redox Signal. *11*, 937–948.

Biteau, B., Labarre, J., and Toledano, M.B. (2003). ATP-dependent reduction of cysteine-sulphinic acid by S. cerevisiae sulphiredoxin. Nature *425*, 980–984.

Browning, J.D., and Horton, J.D. (2004). Molecular mediators of hepatic steatosis and liver injury. J. Clin. Invest. *114*, 147–152.

Budanov, A.V., and Karin, M. (2008). p53 target genes sestrin1 and sestrin2 connect genotoxic stress and mTOR signaling. Cell *134*, 451–460.

Budanov, A.V., Sablina, A.A., Feinstein, E., Koonin, E.V., and Chumakov, P.M. (2004). Regeneration of peroxiredoxins by p53-regulated sestrins, homologs of bacterial AhpD. Science *304*, 596–600.

Chen, W., Sun, Z., Wang, X.J., Jiang, T., Huang, Z., Fang, D., and Zhang, D.D. (2009). Direct interaction between Nrf2 and p21(Cip1/WAF1) upregulates the Nrf2-mediated antioxidant response. Mol. Cell *34*, 663–673.

Fan, W., Tang, Z., Chen, D., Moughon, D., Ding, X., Chen, S., Zhu, M., and Zhong, Q. (2010). Keap1 facilitates p62-mediated ubiquitin aggregate clearance via autophagy. Autophagy *6*, 614–621.

Horton, J.D., Bashmakov, Y., Shimomura, I., and Shimano, H. (1998). Regulation of sterol regulatory element binding proteins in livers of fasted and refed mice. Proc. Natl. Acad. Sci. USA *95*, 5987–5992.

Hotamisligil, G.S. (2010). Endoplasmic reticulum stress and the inflammatory basis of metabolic disease. Cell *140*, 900–917.

Itoh, K., Mimura, J., and Yamamoto, M. (2010). Discovery of the negative regulator of Nrf2, Keap1: a historical overview. Antioxid. Redox Signal. *13*, 1665–1678.

Jain, A., Lamark, T., Sjottem, E., Larsen, K.B., Awuh, J.A., Overvatn, A., McMahon, M., Hayes, J.D., and Johansen, T. (2010). p62/SQSTM1 is a target gene for transcription factor NRF2 and creates a positive feedback loop by inducing antioxidant response. J. Biol. Chem. *285*, 22576–22591.

Kensler, T.W., Wakabayashi, N., and Biswal, S. (2007). Cell survival responses to environmental stresses via the Keap1-Nrf2-ARE pathway. Annu. Rev. Pharmacol. Toxicol. *47*, 89–116.

Kleiner, D.E., Brunt, E.M., Van Natta, M., Behling, C., Contos, M.J., Cummings, O.W., Ferrell, L.D., Liu, Y.C., Torbenson, M.S., Unalp-Arida, A., et al.; Nonalcoholic Steatohepatitis Clinical Research Network. (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology *41*, 1313–1321.

Komatsu, M., Kurokawa, H., Waguri, S., Taguchi, K., Kobayashi, A., Ichimura, Y., Sou, Y.S., Ueno, I., Sakamoto, A., Tong, K.I., et al. (2010). The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. Nat. Cell Biol. *12*, 213–223.

Laplante, M., and Sabatini, D.M. (2012). mTOR signaling in growth control and disease. Cell *149*, 274–293.

Lau, A., Wang, X.J., Zhao, F., Villeneuve, N.F., Wu, T., Jiang, T., Sun, Z., White, E., and Zhang, D.D. (2010). A noncanonical mechanism of Nrf2 activation by autophagy deficiency: direct interaction between Keap1 and p62. Mol. Cell. Biol. *30*, 3275–3285.

Lee, J.H., Budanov, A.V., Park, E.J., Birse, R., Kim, T.E., Perkins, G.A., Ocorr, K., Ellisman, M.H., Bodmer, R., Bier, E., and Karin, M. (2010). Sestrin as a feedback inhibitor of TOR that prevents age-related pathologies. Science *327*, 1223–1228.

Li, S., Brown, M.S., and Goldstein, J.L. (2010). Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis. Proc. Natl. Acad. Sci. USA *107*, 3441–3446.

McMahon, M., Thomas, N., Itoh, K., Yamamoto, M., and Hayes, J.D. (2006). Dimerization of substrate adaptors can facilitate cullin-mediated ubiquitylation of proteins by a "tethering" mechanism: a two-site interaction model for the Nrf2-Keap1 complex. J. Biol. Chem. *281*, 24756–24768.

Mizushima, N., and Komatsu, M. (2011). Autophagy: renovation of cells and tissues. Cell *147*, 728–741.

Moscat, J., and Diaz-Meco, M.T. (2011). Feedback on fat: p62-mTORC1-autophagy connections. Cell *147*, 724–727.

Ozcan, U., Ozcan, L., Yilmaz, E., Düvel, K., Sahin, M., Manning, B.D., and Hotamisligil, G.S. (2008). Loss of the tuberous sclerosis complex tumor suppressors triggers the unfolded protein response to regulate insulin signaling and apoptosis. Mol. Cell *29*, 541–551.

Pankiv, S., Clausen, T.H., Lamark, T., Brech, A., Bruun, J.A., Outzen, H., Øvervatn, A., Bjørkøy, G., and Johansen, T. (2007). p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. J. Biol. Chem. *282*, 24131–24145.

Papadia, S., Soriano, F.X., Léveillé, F., Martel, M.A., Dakin, K.A., Hansen, H.H., Kaindl, A., Sifringer, M., Fowler, J., Stefovska, V., et al. (2008). Synaptic NMDA receptor activity boosts intrinsic antioxidant defenses. Nat. Neurosci. *11*, 476–487.

Rhee, S.G., and Woo, H.A. (2011). Multiple functions of peroxiredoxins: peroxidases, sensors and regulators of the intracellular messenger $H_2O_2$, and protein chaperones. Antioxid. Redox Signal. *15*, 781–794.

Sen, N., Satija, Y.K., and Das, S. (2011). PGC-1$\alpha$, a key modulator of p53, promotes cell survival upon metabolic stress. Mol. Cell *44*, 621–634.

Shih, A.Y., Imbeault, S., Barakauskas, V., Erb, H., Jiang, L., Li, P., and Murphy, T.H. (2005). Induction of the Nrf2-driven antioxidant response confers neuroprotection during mitochondrial stress *in vivo*. J. Biol. Chem. *280*, 22925–22936.

Taguchi, K., Fujikawa, N., Komatsu, M., Ishii, T., Unno, M., Akaike, T., Motohashi, H., and Yamamoto, M. (2012). Keap1 degradation by autophagy for the maintenance of redox homeostasis. Proc. Natl. Acad. Sci. USA *109*, 13561–13566.

Tanigawa, S., Fujii, M., and Hou, D.X. (2007). Action of Nrf2 and Keap1 in ARE-mediated NQO1 expression by quercetin. Free Radic. Biol. Med. *42*, 1690–1703.

Vousden, K.H., and Ryan, K.M. (2009). p53 and metabolism. Nat. Rev. Cancer *9*, 691–700.

Woo, H.A., Bae, S.H., Park, S., and Rhee, S.G. (2009). Sestrin 2 is not a reductase for cysteine sulfinic acid of peroxiredoxins. Antioxid. Redox Signal. *11*, 739–745.

Yu, L., McPhee, C.K., Zheng, L., Mardones, G.A., Rong, Y., Peng, J., Mi, N., Zhao, Y., Liu, Z., Wan, F., et al. (2010). Termination of autophagy and reformation of lysosomes regulated by mTOR. Nature *465*, 942–946.

Zhang, D.D., Lo, S.C., Sun, Z., Habib, G.M., Lieberman, M.W., and Hannink, M. (2005). Ubiquitination of Keap1, a BTB-Kelch substrate adaptor protein for Cul3, targets Keap1 for degradation by a proteasome-independent pathway. J. Biol. Chem. *280*, 30091–30099.