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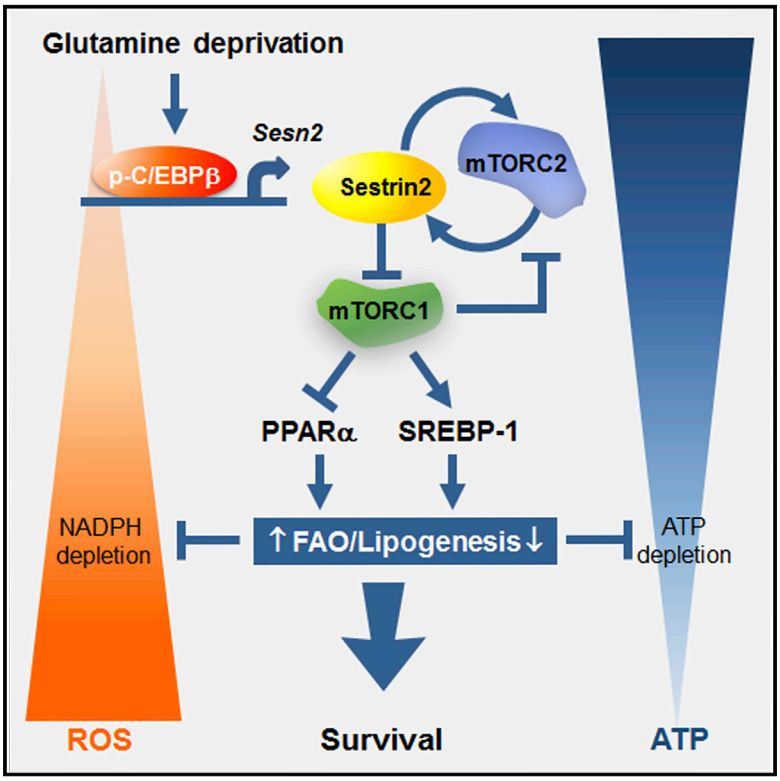
A Positive Feedback Loop between Sestrin2 and mTORC2 Is Required for the Survival of Glutamine-Depleted Lung Cancer Cells

Graphical Abstract



Highlights

- Induction of Sestrin2 is critical for glutamine-depleted lung cancer cell survival
- Glutamine depletion creates a positive feedback loop between Sestrin2 and mTORC2
- Differential regulation of mTORC1 and 2 by Sestrin2 maintains ATP and redox balance
- Co-targeting SLC1A5 and Sestrin2 is a promising therapeutic strategy for lung cancer

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In Brief

Byun et al. find that a positive feedback loop between Sestrin2 and mTORC2 is necessary to suppress mTORC1 activity in glutamine-depleted lung cancer cells. Differential regulation of mTORC1 and mTORC2 by Sestrin2 prevents ATP depletion and maintains redox balance, enabling lung cancer cells to survive under glutamine-depleted conditions.

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A Positive Feedback Loop between Sestrin2 and mTORC2 Is Required for the Survival of Glutamine-Depleted Lung Cancer Cells

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SUMMARY

Proper regulation of mTORC1 and mTORC2 upon nutrient starvation is critical for cancer cell survival. Upregulation of Sestrin2 in response to glutamine deprivation rescues cell death by suppressing mTORC1. However, the contribution of mTORC2 to Sestrin2-mediated mTORC1 suppression remains unclear. Here, we report that both Sestrin2 and mTORC2 are upregulated in glutamine-depleted lung cancer cells. Moreover, glutamine depletion caused Sestrin2 to associate with mTORC2, which was required for the increase in Sestrin2 protein stability and the reduction in mTORC1 activity. Ultimately, differential regulation of mTORC1 and 2 by Sestrin2 reprogramed lipid metabolism and enabled glutamine-depleted lung cancer cells to survive by maintaining energy and redox balance. Importantly, combined inhibition of glutamine utilization and Sestrin2 induced lung cancer cell death both in vitro and in vivo. This study shows that differential Sestrin2-mediated regulation of mTORC1 and mTORC2 is necessary for the survival of glutamine-depleted lung cancer cells.

INTRODUCTION

Recent advances in the study of tumor metabolism suggest that reprogramming altered nutrient metabolism in tumors is a promising anticancer strategy (Zhao et al., 2013). Proliferating cancer cells show high utilization of glutamine for growth; for this reason, targeting the tumor-specific characteristics of glutamine metabolism has attracted attention as a potential therapeutic strategy for cancers (Mohamed et al., 2014). However, this approach turned out to be infeasible, because cancer cells adapt to nutrient stress (Reid et al., 2013). Thus, understanding the mechanism by which cancer cells reprogram their metabolism to survive under conditions of glutamine depletion is critical if we are to develop effective cancer therapies. Proliferating cancer cells must maintain ATP levels and prevent excessive accumulation of reactive oxygen species (ROS) if they are to survive nutrient starvation (Jeon et al., 2012). The mechanistic target of rapamycin complex (mTORC) senses nutrient and energy levels and integrates diverse environmental signals to control cellular growth and homeostasis within an organism (Laplante and Sabatini, 2012). mTORC1 regulates cell growth and proliferation, which are tightly controlled by multiple signals such as growth factor stimulation, amino acid withdrawal, and hypoxia (Bhaskar and Hay, 2007). Although most tumor cells exhibit upregulated mTORC1 activity during rapid growth (Zoncu et al., 2011), inhibiting mTORC1 protects proliferating cells from energetic stress-induced death by balancing metabolic demand with nutrient supply under nutrient-starved conditions (Choo et al., 2010). In contrast to mTORC1, the role of mTORC2 in metabolic adaptation of cancer cells to energy starvation is unclear.

Mounting evidence shows that Sestrin2 plays a pivotal role in cellular energy homeostasis in response to a variety of environmental stresses, including genotoxic, oxidative, and nutrient stress (Lee et al., 2013). Sestrin2 promotes activation of AMP-dependent protein kinase (AMPK), thereby suppressing mTORC1 signaling and transmitting stress signals to restore organismal homeostasis (Budanov and Karin, 2008; Lee et al., 2010). On the other hand, recent studies show that upon nutrient removal, Sestrin2 directly suppresses mTORC1 signaling in an AMPK-independent manner, thereby participating in the reprogramming of cellular metabolism (Peng et al., 2014). Under conditions of amino acid deprivation, Sestrin2 is induced by GCN2 and ATF4, which then maintains mTORC1 suppression by blocking its lysosomal localization (Ye et al., 2015). Furthermore, the latest molecular functional studies demonstrate that Sestrin2 directly senses leucine, leading to mTORC1 activation; leucine deprivation

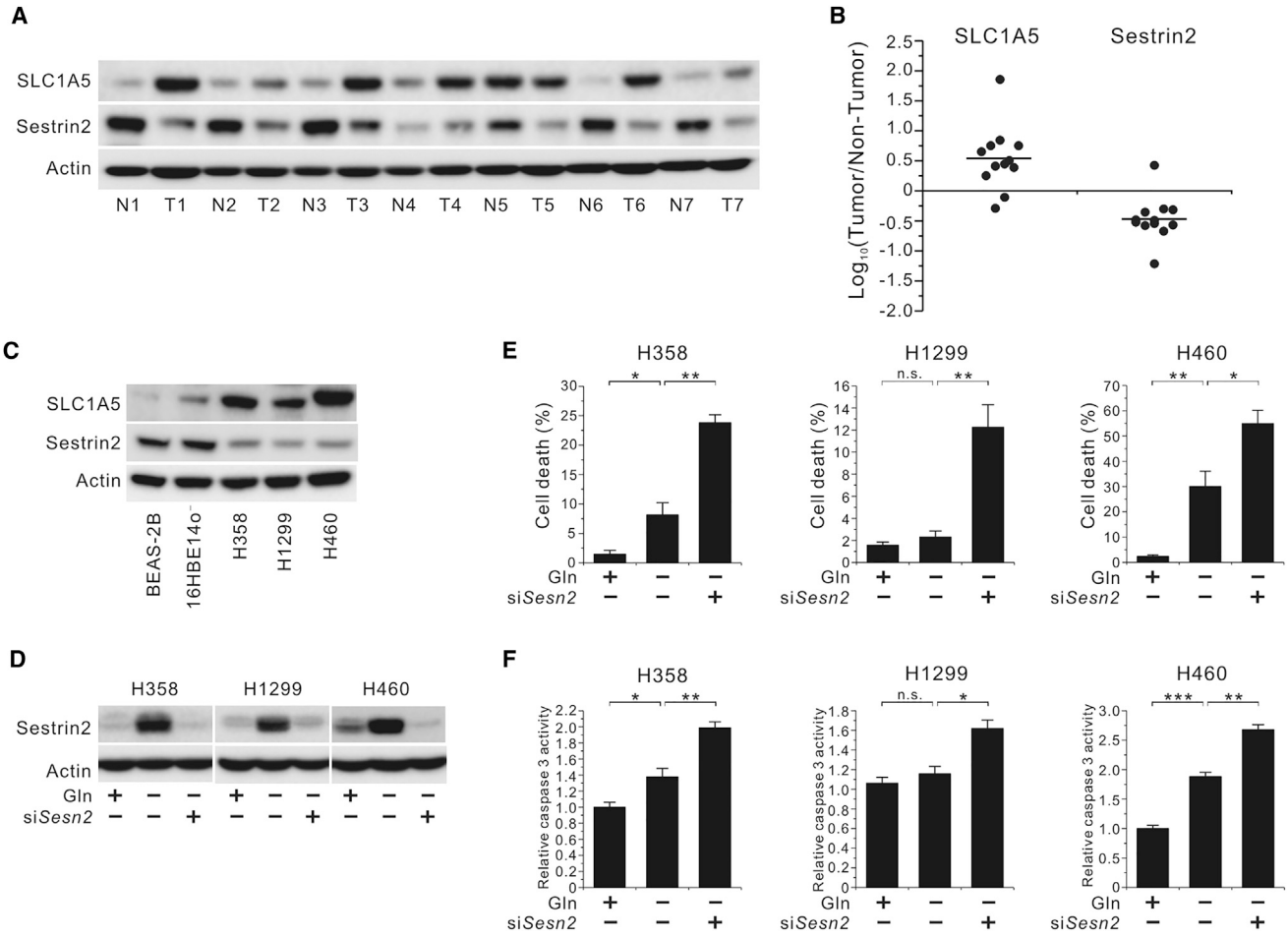

Figure 1. Induction of Sestrin2 upon Glutamine Depletion Reduces NSCLC Cell Death

(A) Levels of SLC1A5 and Sestrin2 protein in human NSCLC tissue (T) and adjacent non-tumor tissue (N) samples from 7 out of 12 patients.

(B) SLC1A5 and Sestrin2 protein levels were normalized to that of actin and plotted as the log10 ratio of T/N tissues (n = 12 patients).

(C) Levels of SLC1A5 and Sestrin2 protein in human bronchial epithelial (BEAS-2B and 16HBE14o-) and human NSCLC (H358, H1299, and H460) cells.

(D) NSCLC cells were transfected with scrambled siRNA or siSesn2 for 24 hr, followed by glutamine deprivation for 24 hr.

(E and F) NSCLC cells were transfected with a scrambled siRNA or siSesn2 for 24 hr followed by glutamine deprivation for 72 hr. (E) Cell death was measured by trypan blue exclusion. (F) Caspase-3 activity.

Data are expressed as the mean ± SEM of three independent experiments and normalized against values measured in complete medium. n.s., not significant; *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S1.

triggers the interaction between Sestrin2 and GATOR2, which inhibits mTORC1 signaling (Saxton et al., 2016; Wolfson et al., 2016). As mentioned above, the role of Sestrin2 and the mTORC1 pathway in the maintenance of cancer cell viability under nutrient-starved conditions is well known; however, the interplay between Sestrin2 and mTORC2 in response to nutrient starvation, which contributes to Sestrin2-mediated suppression of mTORC1 to promote cancer cell survival, remains unclear.

Therefore, to identify the mechanism by which Sestrin2 mediates metabolic adaptation to ensure non-small cell lung cancer (NSCLC) cell survival under glutamine-depleted conditions, we examined whether Sestrin2 differentially co-ordinates the activities of mTORC1 and mTORC2, and whether mTORC2 plays a role in Sestrin2-mediated mTORC1 suppression. We also investigated whether co-targeting glutamine utilization mechanisms and Sestrin2 has potential as a therapeutic strategy for NSCLC.

RESULTS

Induction of Sestrin2 Is Required for NSCLC Survival upon Glutamine Depletion

We first measured the levels of SLC1A5, a high-affinity transporter of glutamine, and Sestrin2 in 12 NSCLC tissues and compared them with levels in matched adjacent normal lung tissues. Tumor tissue expressed higher levels of SLC1A5 protein but lower levels of Sestrin2 protein than adjacent normal tissue (Figures 1A, 1B, and S1A). Consistent with this, human NSCLC cell lines (H358, H1299, and H460 cells) showed higher expression of SCL1A5 but lower expression of Sestrin2 than lung

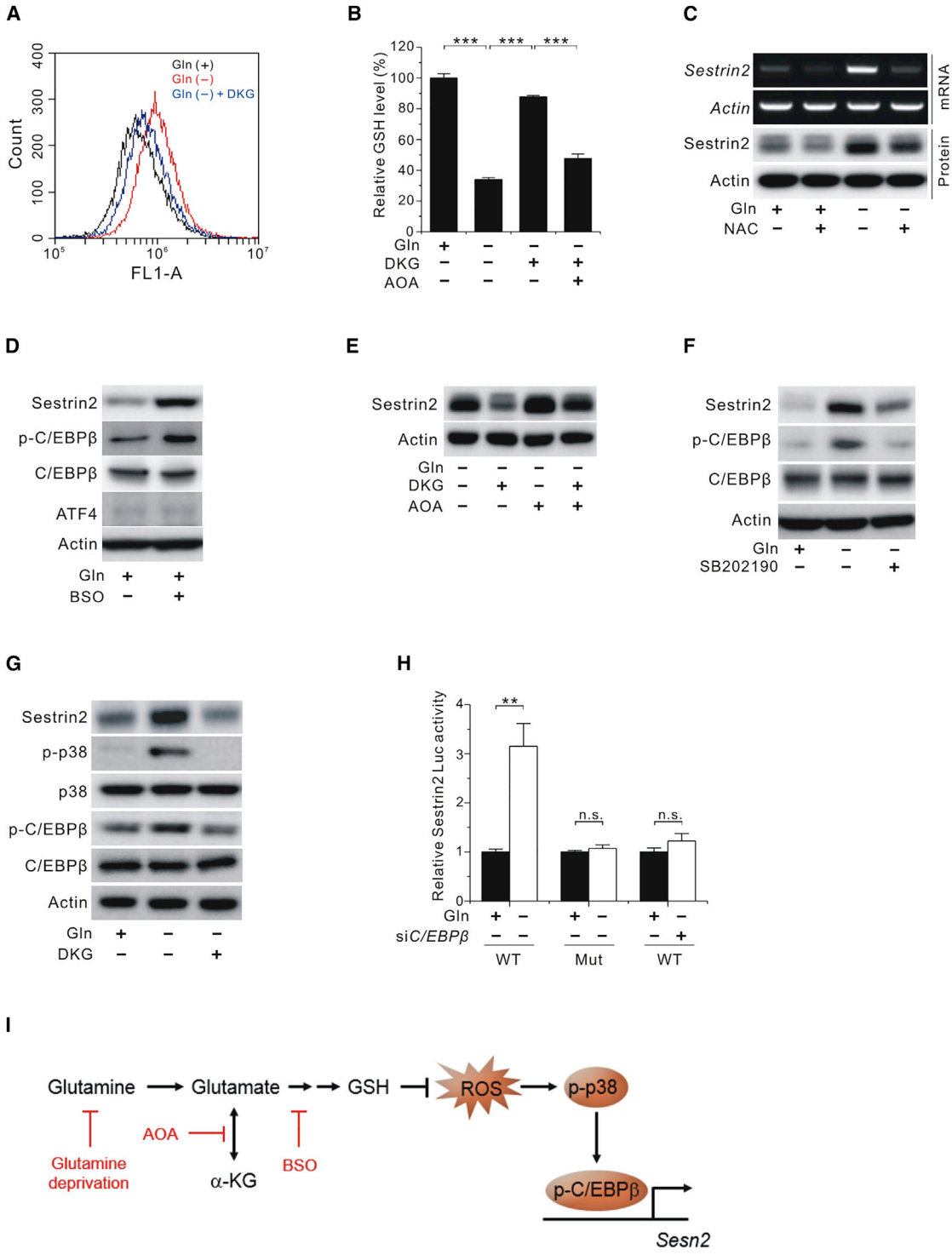

Figure 2. Sestrin2 Is Upregulated via a ROS-p38-C/EBPβ-Dependent Pathway under Conditions of Glutamine Deprivation

(A) Flow cytometric analysis of intracellular ROS levels after 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) staining of H358 cells after glutamine deprivation in the presence/absence of DKG (4 mM) for 24 hr.

(B) Relative changes in GSH levels in glutamine-depleted H358 cells supplemented with DKG (4 mM) with/without AOA (0.5 mM) for 24 hr. Data are expressed as the mean ± SEM of three independent experiments.

(C) H358 cells were treated with NAC (4 mM) in the presence/absence of glutamine for 24 hr. Sestrin2 mRNA and protein level was measured by RT-PCR and western blot analysis, respectively.

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bronchial epithelial cell lines (BEAS-2B and 16HBE14o-) (Figure 1C). As expected, the levels of Sestrin2 protein in NSCLC cells increased markedly upon glutamine deprivation (Figure 1D) (Ye et al., 2015). By contrast, levels of Sestrin1 and Sestrin3 in NSCLC cells were not significantly altered upon glutamine deprivation (Figure S1B). To determine whether induction of Sestrin2 contributes to cell survival under conditions of glutamine depletion, we next checked whether small interfering RNA (siRNA)-mediated knockdown of Sestrin2 (siSesn2) increased cell death and caspase-3 activity in glutamine-deprived NSCLC cells. The results showed that siSesn2 increased both glutamine-deprivation-induced cell death and caspase-3 activity (Figures 1E and 1F), indicating that induction of Sestrin2 in response to glutamine deprivation prevents NSCLC cell death.

Sestrin2 Is Upregulated via a ROS-p38 MAPK-C/EBPβ-Dependent Pathway under Glutamine Deprivation

Given that glutamine supports redox homeostasis by contributing to glutathione (GSH) synthesis, we asked whether impaired GSH synthesis is implicated in Sestrin2 induction in glutamine-deprived NSCLC cells. As expected, ROS levels increased, whereas GSH levels decreased, in NSCLC cells subjected to glutamine deprivation; ROS levels correlated positively, but GSH levels correlated negatively, with Sestrin2 mRNA and protein levels (Figures 2A–2C). We noticed that treatment with the antioxidant N-acetylcysteine (NAC) prevented glutamine-deprivation-induced expression of Sestrin2 mRNA and protein (Figure 2C), whereas treatment with the GSH inhibitor buthionine sulfoximine (BSO) increased Sestrin2 protein levels, even in the presence of glutamine (Figure 2D). To further explore whether Sestrin2 induction upon glutamine deprivation is caused by reduced GSH synthesis due to impaired supply of precursors derived from glutamate, we treated glutamine-deprived NSCLC cells with dimethyl-2-ketoglutarate (DKG), which is metabolized to glutamate and is an initial substrate for the GSH synthetic pathway. Supplementation of glutamine-deficient NSCLC cells with DKG led to recovery of GSH and Sestrin2 levels to those observed in the presence of glutamine. This DKG-induced recovery of GSH and Sestrin2 levels was abolished by treatment with the transaminase inhibitor aminooxyacetic acid (AOA), which inhibits conversion of DKG to glutamate (Figures 2B, 2E, and S2A). These results indicate that glutamine-depletion-induced inhibition of GSH synthesis and subsequent ROS generation play a role in inducing Sestrin2.

Next, we examined the signaling pathway that drives ROS-mediated induction of Sestrin2. In glutamine-deprived NSCLC cells, the level of phospho-CCAAT/enhancer binding protein β (p-C/EBPβ) increased along with that of Sestrin2, and both were decreased by treatment with the p38 inhibitor SB202190 (Figure 2F). In line with this, we found that supplementation with DKG prevented glutamine-deprivation-induced increases in p-p38 mitogen-activated protein kinase (MAPK) and p-C/EBPβ levels (Figures 2G and S2B). Similar to the induction of Sestrin2 by inhibition of GSH, levels of p-C/EBPβ also increased upon treatment with BSO in the presence of glutamine (Figure 2D). Mutagenesis analysis of the Sestrin2 promoter revealed that cells transfected with a mutated putative C/EBPβ-binding site showed impaired glutamine-deprivation-induced activation of the Sestrin2 promoter, which did occur in cells transfected with the wild-type Sestrin2 promoter (Figure 2H). C/EBPβ-mediated Sestrin2 promoter activation upon glutamine deprivation was further confirmed by transfection of siRNA-C/EBPβ (Figure 2H). Given that ATF4 mediates Sestrin2 transcription in amino-acid-depleted cells (Ye et al., 2015), we investigated whether ATF4 also mediates GSH depletion and ROS-mediated induction of Sestrin2 under glutamine deprivation. As observed in other cancer cell lines (Ye et al., 2010), ATF4 protein levels were also increased in glutamine-depleted NSCLC cells (Figure S2C). Unlike the levels of Sestrin2 and C/EBPβ, levels of ATF4 protein were not increased by BSO treatment (Figure 2D). Taken together, these data demonstrate that glutamine/GSH-depletion-induced ROS generation promotes transcriptional activation of Sestrin2 via the p38 MAPK-C/EBPβ pathway (Figure 2I).

Sestrin2 Increases mTORC2 Activity in Glutamine-Depleted NSCLC Cells

mTORC1 inhibition and concomitant activation of mTORC2 are critical for cancer cell survival under nutrient-starved conditions (Hung et al., 2012). Thus, we investigated whether Sestrin2 affects the activity of mTORC1 and mTORC2 under glutamine-deprived conditions. Glutamine deprivation or treatment with a specific SLC1A5 inhibitor (L-γ-glutamyl-p-nitroanilide [GPNA]) reduced mTORC1 activity but increased mTORC2 activity, as measured by phosphorylation of S6K (p-S6K) (T389) and phosphorylation of AKT (p-AKT) (S473), respectively (Figures 3A, S3A, and S3B). Interestingly, knockdown of Sestrin2 by siSesn2 decreased not only glutamine-deprivation-induced suppression of p-S6K (T389) but also glutamine-deprivation-induced activation of p-AKT (S473) (Figure 3A). Activation of p-AKT was further confirmed by increased phosphorylation of downstream molecules, including p-TSC2 (T1462) and p-CREB (S133) (Figure S3C). We confirmed that the Sestrin2-mediated increase in p-AKT (S473) occurs in an mTORC2-dependent manner from the results showing that Ku-0063794 abolished the upregulation of p-AKT (S473) following Sestrin2 overexpression (Figure S3D).

(D) The level of Sestrin2, phosphorylated C/EBPβ, and ATF4 protein in H358 cells treated with BSO (100 μM in complete medium) for 24 hr.
(E) Western blot analysis showing the effects of DKG (4 mM) and AOA (0.5 mM) on Sestrin2 protein levels in H358 cells glutamine depleted for 24 hr.
(F) Western blot analysis of the effect of a p38 MAPK inhibitor (SB202190; 10 μM) on the levels of Sestrin2 protein and phosphorylated C/EBPβ in H358 cells in the absence of glutamine for 24 hr.
(G) Western blot analysis showing the effect of DKG (4 mM) on Sestrin2, phosphorylated p38, and C/EBPβ in H358 cells glutamine depleted for 24 hr.
(H) Sesn2 promoter luciferase activity. H358 cells were co-transfected with C/EBPβ-binding-site-intact (WT) or C/EBPβ-binding-site-mutated (Mut) Sestrin2 promoter constructs along with scramble siRNA or siC/EBPβ for 36 hr, and luciferase activity was measured after glutamine deprivation for 24 hr. Data are expressed as the mean ± SEM of three independent experiments.
(I) Schematic showing Sestrin2 induction under conditions of glutamine deprivation.
n.s., not significant; $^{**}p < 0.01$ and $^{***}p < 0.001$. See also Figure S2.

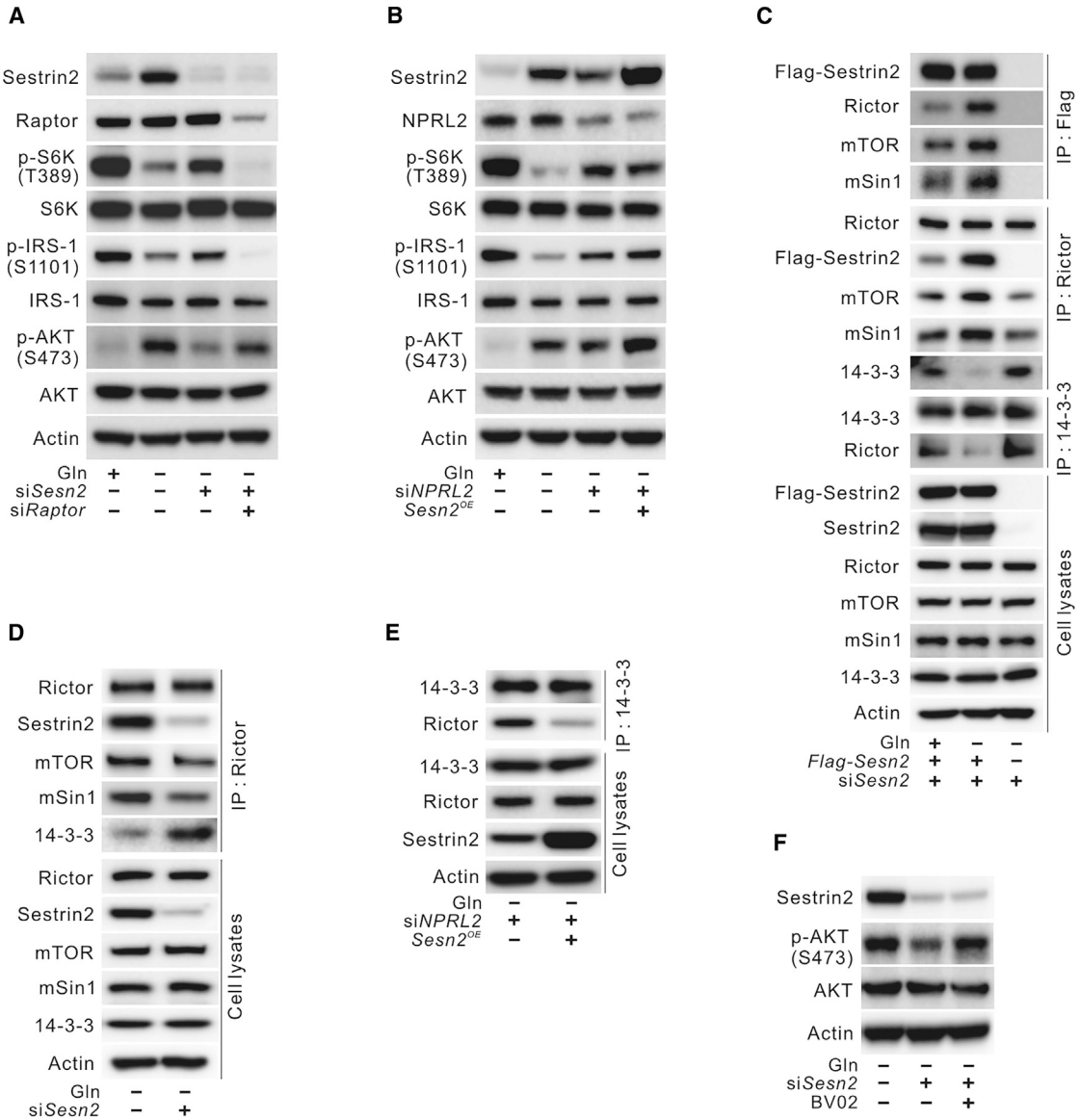


Figure 3. Sestrin2 Increases mTORC2 Activity in Glutamine-Depleted NSCLC Cells

(A and B) Levels of phosphorylated S6K (T389), IRS-1 (S1101), and AKT (S473). (A) H358 cells were transfected with scrambled siRNA, siSesn2, or siSesn2 plus siRaptor for 24 hr, followed by glutamine deprivation for 24 hr. (B) H358 cells were transfected with scrambled siRNA, siNPRL2, or siNPRL2 plus a Sestrin2-expression vector for 24 hr, followed by glutamine deprivation for 24 hr.

(C) Immunoprecipitation showing the effect of glutamine deprivation on the interaction between Rictor and Flag-tagged Sestrin2, or the interaction between Rictor and 14-3-3. H358 cells were transfected with siSesn2, a Flag-Sesn2 expression vector, or an empty vector for 24 hr followed by glutamine depletion for 24 hr.

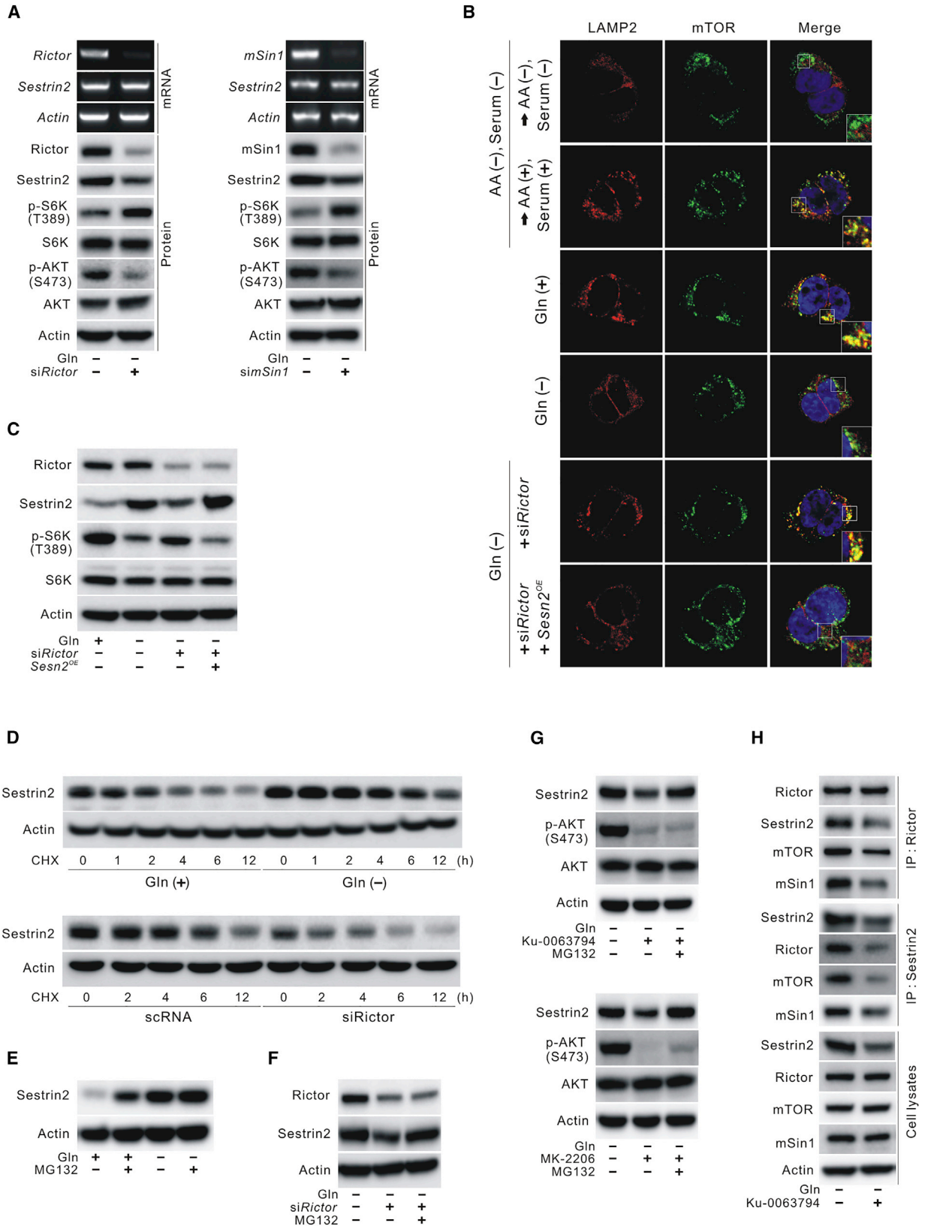
(D) Effect of knocking down Sestrin2 on the interaction between Rictor and 14-3-3. H358 cells were transfected with scrambled siRNA or siSesn2 for 24 hr and then subjected to glutamine depletion for 24 hr.

(E) Effects of Sestrin2 overexpression on the interaction between Rictor and 14-3-3. H358 cells were transfected with siNPRL2, or siNPRL2 plus a Sestrin2-expression vector for 24 hr, followed by glutamine deprivation for 24 hr.

(F) Effects of 14-3-3 inhibition on p-AKT (S473) levels in Sestrin2-knockdown cells. H358 cells were transfected with scramble siRNA or siSesn2 for 24 hr, followed by treatment with BV02 (5 μM) in the absence of glutamine for 24 hr. Levels of p-AKT (S473) were measured by western blotting.

See also Figure S3.

Given the inhibitory effect of mTORC1 on mTORC2 activity, it was necessary to evaluate whether Sestrin2-induced inhibition of mTORC1 plays a role in activating mTORC2. Thus, we examined whether inhibiting mTORC1 using a siRNA targeting Raptor (siRaptor) increases p-AKT (S473) levels in Sestrin2-knockdown cells. Although siRaptor led to a significant reduction in IRS-1 phosphorylation (S1101), it had only a small effect on suppressed p-AKT (S473) levels when Sestrin2 was silenced



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(Figure 3A). To verify the involvement of mTORC1-independent effects of Sestrin2 on mTORC2 activation, we examined the effects of Sestrin2 overexpression on p-AKT (S473) in mTORC1-activated cells. mTORC1 activation was achieved by knockdown of NPRL2, a key component t of GATOR1(Parmigiani et al., 2014). Although siNPRL2-mediated mTORC1 activation resulted in significant recovery of glutamine-deprivation-induced p-IRS-1 (S1101) suppression, there was only a minimal reduction in glutamine-deprivation-induced upregulation of p-AKT (S473) levels. Moreover, overexpression of Sestrin2 caused a significant increase in p-AKT (S473) levels, despite the increase in p-S6K (T389) and p-IRS-1 (S1101) levels upon NPRL2 knockdown (Figure 3B). Additionally, we further confirmed the importance of the mTORC1-independent mechanism for glutamine-deprivation-induced mTORC2 activation by comparing the effects of glutamine deprivation on p-AKT (S473) levels between rapamycin-treated and untreated cells. Although rapamycin treatment increased p-AKT (S473) levels in the presence of glutamine, the increase was much less than that observed after glutamine deprivation (Figure S3E). Furthermore, glutamine deprivation in rapamycin-treated cells produced a further increase in p-AKT (S473) levels, which was similar to the fold increase in p-Akt (S473) levels induced by glutamine deprivation in rapamycin-untreated cells (Figure S3E).

We next sought to explain the mechanism by which Sestrin2 increases mTORC2 activity under glutamine deprivation by investigating a potential interaction between Sestrin2 and mTORC2 components. To prevent glutamine-deprivation-induced changes in Sestrin2 protein levels influencing the interaction between Sestrin2 and mTORC2 components, we silenced endogenous Sestrin2 using a specific siRNA; cells then overexpressed equal amounts of FLAG-tagged Sestrin2. Immunoprecipitation revealed that Sestrin2 physically associated with mTORC2 components, including Rictor, mTOR, and mSin1, in the presence of glutamine and that this interaction was markedly enhanced by glutamine deprivation (Figure 3C). Interestingly, we observed that binding of 14-3-3 to Rictor was markedly reduced by glutamine deprivation (Figure 3C). Accordingly, we asked whether Sestrin2 affects the binding of 14-3-3 to Rictor, as this interaction inhibits mTORC2 activity (Dibble et al., 2009; Jeon et al., 2013). Indeed, knockdown of Sestrin2 in glutamine-depleted NSCLC cells led to a significant increase in binding of Rictor to 14-3-3 and a concomitant decrease in binding of Rictor to mTOR and mSin1 (Figure 3D). By contrast, Sestrin2 overexpression reduced binding of 14-3-3 to Rictor in mTORC1-activated NSCLC cells induced with siNPRL2 (Figure 3E), which is consistent with previous results showing mTORC1-independent upregulation of p-AKT (S473) by Sestrin2. Moreover, we observed that BV02, an inhibitor of 14-3-3, rescued siSesn2-induced suppression of AKT (S473) phosphorylation (Figure 3F), supporting the finding that Sestrin2 increases mTORC2 activity by, at least in part, disrupting the interaction between 14-3-3 and Rictor.

An Increase in Sestrin2 Protein Stability by mTORC2 Is Required for the Suppression of mTORC1 Activity

We further examined whether mTORC2 also has a role in glutamine-deprivation-induced increase in Sestrin2 protein levels. As shown in Figure 4A, downregulation of mTORC2 by siRictor or si-mSin1 decreased Sestrin2 protein levels. To our surprise, downregulation of mTORC2 by siRictor or si-mSin1 increased p-S6K (T389) levels in glutamine-deprived NSCLC cells (Figure 4A), which is contrary to the previous known function of mTORC2 in positive regulation of mTORC1 activity (Huang and Manning, 2009). These results raised the possibility that the effects of mTORC2 on mTORC1 activity under glutamine starvation are different from those under normal nutrient conditions. Therefore, we next examined whether Sestrin2 and mTORC2 in glutamine-deprived NSCLC cells inhibit mTORC1 activity by measuring lysosomal recruitment of mTORC1. Indeed, downregulation of mTORC2 recovered glutamine-deprivation-induced disruption of mTORC1 lysosomal localization, as evidenced by co-localization with the lysosomal membrane protein LAMP2 (Figure 4B). Moreover, this recruitment of mTORC1 to lysosomes was prevented when Sestrin2 was overexpressed (Figure 4B). Additionally, we observed that overexpression of Sestrin2 prevented the siRictor-induced increase in p-S6K (T389) (Figure 4C), indicating that mTORC2 inhibits mTORC1 activity in a Sestrin2-dependent manner and supporting the results from immunofluorescence analysis.

Figure 4. An Increase in Sestrin2 Protein Stability by mTORC2 Is Required to Suppress mTORC1 Activity

(A) H358 cells were transfected with scrambled siRNA, siRictor, or si-mSin1 for 24 hr, followed by glutamine deprivation for 24 hr. Levels of Sestrin2 mRNA and protein and phosphorylated S6K (T389) and AKT (S473) were measured by RT-PCR and western blot analysis.

(B and C) H358 cells were transfected with scrambled siRNA, siRictor, or siRictor plus a Sestrin2-expressing vector for 24 hr, followed by glutamine deprivation for 24 hr. (B) Immunofluorescence analysis of lysosomal recruitment of mTOR (red) and LAMP2 (green). As a negative and positive control for mTORC1 localization, H358 cells were subjected to glutamine-, arginine-, leucine-, and fetal bovine serum (FBS)-free media for 2 hr (negative control) and then maintained in media supplemented with glutamine (300 mg/L), arginine (200 mg/L), leucine (50 mg/L), and 10% FBS for 20 min (positive control). (C) Levels of Sestrin2 protein and phosphorylated S6K (T389).

(D) Stability of the Sestrin2 protein. (Upper) H358 cells were maintained in the presence or absence of glutamine for 24 hr and then treated with cycloheximide (CHX, 0.5 mM) for the indicated times. (Lower) H358 cells were transfected with scrambled siRNA or siRictor for 24 hr, followed by glutamine deprivation for 24 hr and treatment with cycloheximide (CHX, 0.5 mM) for the indicated times.

(E and F) Effects of MG132 on Sestrin2 protein levels. (E) H358 cells were maintained in the presence or absence of glutamine for 24 hr and then treated with or without MG132 (4 μM) for 4 hr. (F) H358 cells were transfected with scramble siRNA or siRictor for 24 hr, followed by glutamine depletion for 24 hr, and then treated with MG132 (4 μM) for 4 hr.

(G) Effects of mTOR or AKT inhibition on Sestrin2 protein levels. H358 cells were maintained in the absence of glutamine for 24 hr, followed by treatment with Ku-0063794 (1 μM) or MK-2206 (1 μM) in the presence or absence of MG132 (4 μM) for 4 hr. Levels of Sestrin2 and phosphorylated AKT (S473) were then measured.

(H) Effect of inhibition of mTORC2 activity on the interaction between Sestrin2 and mTORC2. H358 cells were incubated in the absence of glutamine for 24 hr, followed by treatment of Ku-0063794 (1 μM) for 4 hr.

See also Figure S4.

Because siRictor or si-mSin1-mediated inhibition of mTORC2 activity reduced the amount of Sestrin2 protein in glutamine-deprived NSCLC cells without affecting Sestrin2 mRNA expression (Figures 4A and S4A), we examined whether mTORC2 affects the stability of Sestrin2 protein under glutamine-depleted conditions. Glutamine-deprived NSCLC cells maintained their levels of Sestrin2 following treatment with a protein synthesis inhibitor (cycloheximide) when compared with NSCLC cells cultured in complete medium (Figure 4D, top). Sestrin2 protein stability upon glutamine deprivation was further confirmed by treating the cells with the proteasome inhibitor MG132. Consistently, MG132 markedly increased Sestrin2 protein levels in the presence of glutamine while it increased weakly Sestrin2 protein levels in glutamine-depleted cells (Figure 4E). Notably, after glutamine deprivation, cells treated with Rictor siRNA showed a more rapid disappearance of Sestrin2 protein than cells treated with control siRNA (Figure 4D, bottom). In addition, MG132 resulted in the recovery of siRictor or si-mSin1-induced suppression of Sestrin2 protein levels in the absence of glutamine, while overexpression of Rictor or mSin1 increased the levels of Sestrin2 protein and p-AKT (S473) in the presence of glutamine (Figures 4F, S4B, and S4C). We next investigated whether the catalytic activity of mTORC2 is necessary for the increase in Sestrin2 protein stability in glutamine-depleted conditions. As shown in Figure 4G, inhibition of mTORC2 activity by Ku-0063794 or inhibition of AKT activity by MK-2206 decreased Sestrin2 protein levels in glutamine-depleted cells, and these levels were almost fully restored by MG132 treatment. Moreover, Ku-0063794 treatment reduced not only the assembly of mTORC2 components, including Rictor, mTOR, and mSin1, but also the interaction between Sestrin2 and mTORC2 components (Figure 4H). In agreement with the results shown in Figure 4A, we found that Sestrin2 mRNA levels were not altered by Ku-0063794 or MK-2206 (Figure S4D). Taken together, these data demonstrate that mTORC2 activation is required for Sestrin2 protein stability and the increase in Sestrin2 protein levels in glutamine-depleted NSCLC cells. Finally, to verify that Sestrin2 expression upon glutamine starvation corresponds kinetically with mTORC1 inactivation and mTORC2 activation, we measured Sestrin2, p-S6K (T389), and p-AKT (S473) levels at various time points after glutamine deprivation. The results showed that the Sestrin2 level increased 4 hr after glutamine deprivation and that this was accompanied by an increase in p-AKT (S473). p-S6K (T389) levels were slightly decreased at 1 hr after glutamine deprivation whereas p-S6K (T389) levels were markedly decreased at 4 hr after glutamine deprivation, corresponding to the time when Sestrin2 and p-AKT (S473) levels started to increase (Figure S4E). Additionally, mTORC2-dependent regulation of Sestrin2 and mTORC1 activity upon glutamine deprivation was observed in colon cancer (HCT116) and HCC (Hep3B) cells (Figure S4F), suggesting that the results of this study may be applicable to other cancer cell types.

Differential Regulation of mTORC1 and mTORC2 by Sestrin2 Helps to Maintain Energy and Redox Balance in Glutamine-Deprived NSCLC Cells

Regulation of catabolic and anabolic pathways that prevent ATP depletion and ROS overproduction is critical for cancer cells to survive nutrient stress (Carracedo et al., 2013). Thus, we next investigated whether differential regulation of mTORC1 and mTORC2 activities by Sestrin2 affects the survival of glutamine-deprived NSCLC cells by modulating intracellular ATP and ROS levels. As shown in Figure 5A, siSesn2 further increased glutamine-deprivation-induced NSCLC cell death, which was rescued by rapamycin. Likewise, activation of mTORC1 by siNPRL2, or inhibition of mTORC2 by siRictor, significantly increased glutamine-depletion-induced NSCLC cell death, which was rescued by rapamycin treatment or Sestrin2 overexpression (Figures 5B and 5C). Collectively, these data indicate that Sestrin2-mediated mTORC1 suppression and mTORC2 activation support the survival of glutamine-deprived NSCLC cells.

Considering the critical role of ATP and NADPH for cell survival under nutrient-starved conditions (Choo et al., 2010; Jeon et al., 2012), we next examined whether Sestrin2-induced NSCLC cell survival is attributable to metabolic flexibility, which means that the cells can utilize other nutrients and preserve ATP and NADPH levels. Because glucose uptake did not increase after glutamine deprivation (data not shown), we next examined whether Sestrin2 modifies lipid metabolism in glutamine-deprived NSCLC cells. Indeed, we found that knockdown of Sestrin2 increased glutamine-deprivation-induced suppression of the mature forms of SREBP-1, FASN, and ATP-citrate lyase protein, whereas rapamycin reversed these changes (Figures 5D and S5). Expression of mRNAs encoding these genes was consistent with the results of western blot analysis (Figure 5E). By contrast, siSesn2 reduced CPT1 protein and mRNA levels and PPARα activity under conditions of glutamine deprivation; however, levels recovered upon rapamycin treatment (Figures 5D–5F). In addition, XF analysis showed that the reduced oxygen consumption rate (OCR) observed after treatment with the CPT1 inhibitor etomoxir was more attenuated in Sestrin2-knockdown cells than in scrambled RNA (scRNA)-treated cells, supporting the notion that Sestrin2 promotes fatty acid oxidation (FAO) under glutamine-depleted conditions (Figures 5G). Furthermore, treatment with C75 (an inhibitor of fatty acid synthase) or WY-14643 (an activator of PPARα) rescued siSesn2-induced NSCLC cell death upon glutamine deprivation (Figures 5H and 5I). Taken together, these results demonstrate that Sestrin2-mediated mTORC1 suppression reprograms fatty acid metabolism and eventually limits glutamine-depleted NSCLC cell death.

Next, we examined whether induction of Sestrin2 maintains intracellular ATP and NADPH levels under glutamine-depleted conditions. Indeed, Sestrin2-knockdown cells showed reduced intracellular ATP and NADPH levels, which were rescued by rapamycin (Figures 6A and 6B). Similarly, the reduction of intracellular ATP and NADPH levels in Rictor-knockdown cells was partially but significantly compensated for by overexpression of Sestrin2 (Figures 6C and 6D). Glutamine-deprivation-induced increases in intracellular ROS levels were further increased in Sestrin2-knockdown NSCLC cells but returned to levels observed in glutamine-deprived cells upon rapamycin treatment (Figure 6E). Given that compensatory activation of alternative redox systems is critical for cell survival in the absence of glutamine, we investigated whether Sestrin2-preserved NADPH contributes to compensatory increases in thioredoxin

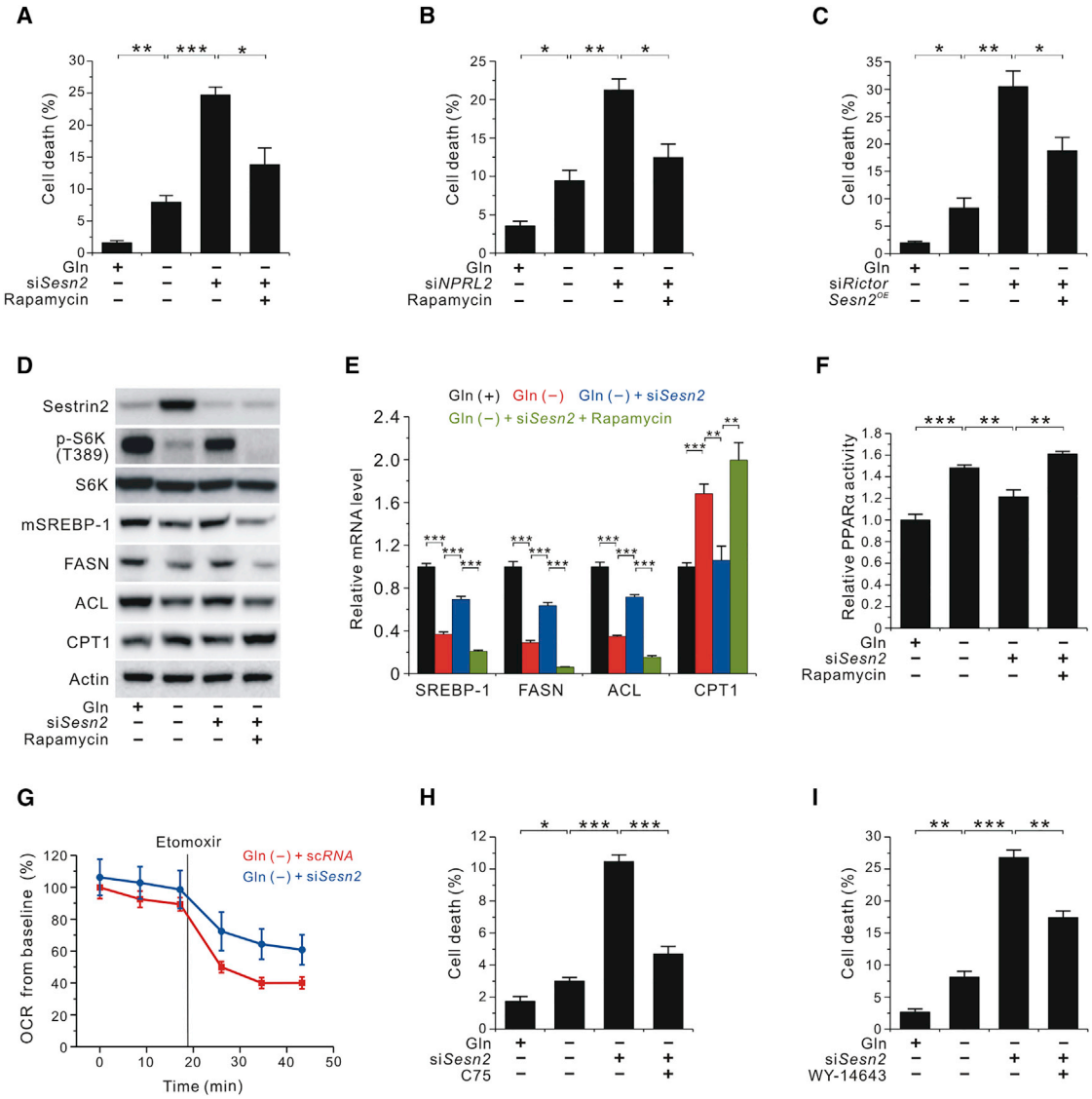

Figure 5. Differential Sestrin2-Mediated Regulation of mTORC1 and 2 Is Required for NSCLC Cell Survival upon Glutamine Deprivation

(A–C) Cell death was measured by trypan blue exclusion. Data are expressed as the mean ± SEM of three independent experiments. (A) H358 cells were transfected with scrambled siRNA or siSesn2 for 24 hr, followed by glutamine deprivation with/without rapamycin (50 nM) for 72 hr. (B) H358 cells were transfected with scrambled siRNA or siNPRL2 for 24 hr, followed by glutamine deprivation with or without rapamycin (50 nM) for 72 hr. (C) H358 cells were transfected with scrambled siRNA, siRictor, or siRictor plus a Sestrin2-expressing vector for 24 hr, followed by glutamine deprivation for 72 hr.

(D–F) H358 cells were transfected with scrambled siRNA or siSesn2 for 24 hr, followed by glutamine deprivation with or without rapamycin (50 nM) for 24 hr. Protein levels (D), mRNA levels (E), and PPARα activity (F) were measured. Data are expressed as the mean ± SEM of three independent experiments and normalized against the values measured in complete medium.

(G) The oxygen consumption rate (OCR) in response to etomoxir treatment (starting from the black line, 50 μM). H358 cells were transfected with scramble siRNA or siSesn2 for 24 hr, followed by glutamine deprivation for 24 hr.

(H and I) H358 cells were transfected with scrambled siRNA or siSesn2 for 24 hr, followed by glutamine deprivation with or without C75 (10 μg/mL) for 36 hr (H) or WY-14643 (25 μM) for 72 hr (I). Cell death was measured by trypan blue exclusion.

Data are expressed as the mean ± SEM of three independent experiments. *p < 0.05, **p < 0.01, and ***p < 0.001. See also Figure S5.

activity, another NADPH-dependent antioxidant system in glutamine-deprived cells. As shown in Figure 6F, glutamine deprivation maintained the reduced form of thioredoxin, which was diminished by siSesn2. Rapamycin partially prevented the siSesn2-induced decrease in reduced thioredoxin. In addition, treatment of glutamine-deprived cells with the thioredoxin reductase inhibitor auranofin (AUR) led to a marked increase in NSCLC cell death; however, AUR had a minimal effect in the presence of glutamine (Figure 6G). These results suggest that an alternative thioredoxin system is required to buffer excessive ROS and rescue NSCLC cell death when glutamine is depleted. Thus, mTORC2 and Sestrin2 cooperate

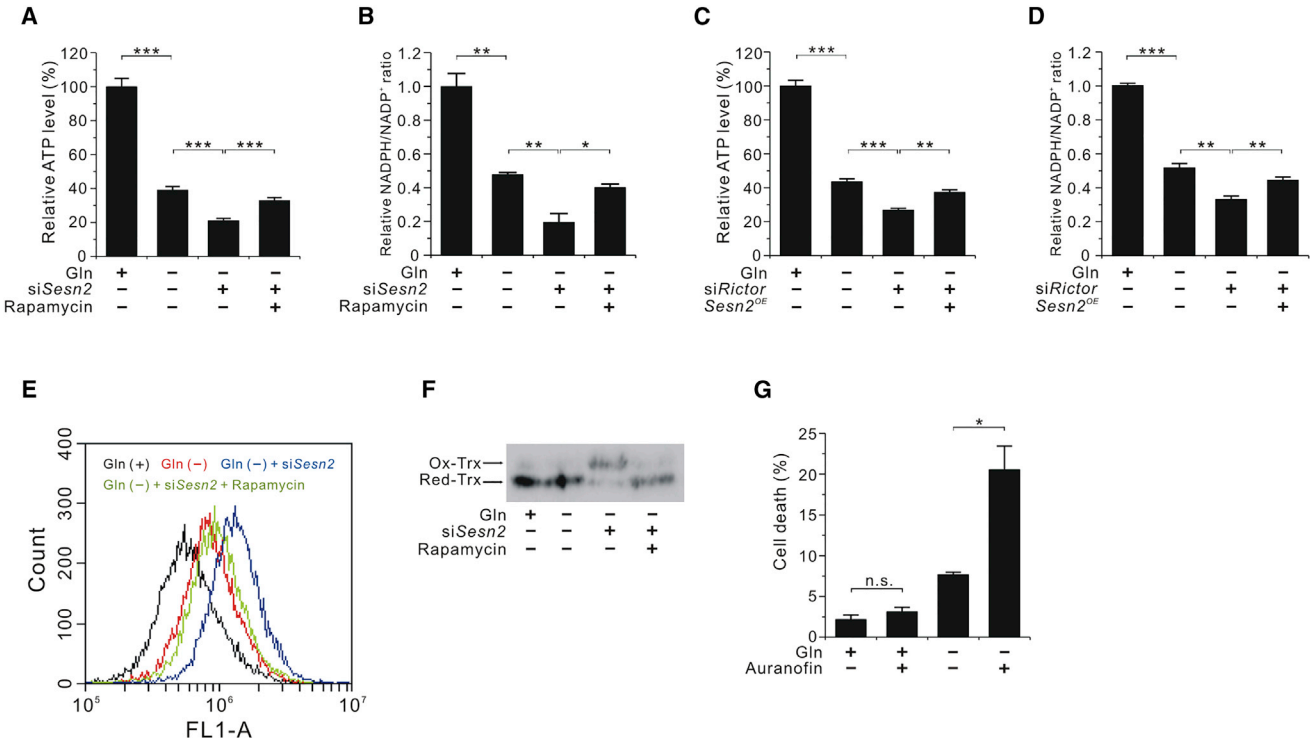

Figure 6. Sestrin2-Mediated Modulation of mTORC1 and mTORC2 Contributes to Energy and Redox Homeostasis in Glutamine-Deprived NSCLC Cells

(A and B) H358 cells were transfected with scrambled siRNA or siSesn2 for 24 hr, followed by glutamine deprivation with or without rapamycin (50 nM) for 24 hr. (A) Measurement of relative ATP levels and (B) the NADPH/$NADP^+$ ratio.

(C and D) H358 cells were transfected with scrambled siRNA, siRictor, or siRictor plus a Sestrin2-expressing vector for 24 hr, followed by glutamine deprivation for 24 hr. (C) Measurement of relative ATP levels and (D) NADPH/$NADP^+$ ratios. Data are expressed as the mean ± SEM of three independent experiments and normalized against the values measured in complete medium.

(E and F) Intracellular ROS levels (E) and levels of reduced thioredoxin (F) in H358 cells.

(G) H358 cells were treated with auranofin (100 nM) in the absence or presence of glutamine for 72 hr, and cell death was measured by trypan blue exclusion. Data are expressed as the mean ± SEM of three independent experiments. n.s., not significant; *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$.

to inhibit mTORC1, thereby preserving ATP and maintaining the redox balance to ensure survival of glutamine-deprived NSCLC cells.

Inhibition of Sestrin2 Induces GPNA-Treated NSCLC Cell Death and Further Augments GPNA-Induced Inhibition of Tumor Growth

Finally, we used nude mice bearing Sestrin2-knockdown H358 (shSesn2-H358) xenografts to examine whether co-targeting glutamine utilization and Sestrin2 induces NSCLC cell death in vivo. Stable knockdown of Sestrin2 and cooperative induction of cell death with shSesn2 and GPNA were confirmed in vitro (Figures 7A and 7B). Although GPNA treatment led to a marked reduction in tumor mass in mice bearing control-H358 tumors compared with control mice, it did not completely inhibit tumor growth (Figure 7C). However, GPNA treatment of mice bearing shSesn2-H358 cell tumors (shSesn2-mice) completely suppressed the increase in tumor volume; indeed, the tumor volume after treatment was less than that of the original tumor (Figure 7C). The reduction in tumor volume induced by shSesn2 was significantly reversed by rapamycin (mean tumor volume: shCtrl, 228.23 mm^3; shCtrl + GPNA, 108.99 mm^3; shSesn2 + GPNA, 11.09 mm^3; shSesn2 + GPNA$^+$rapamycin, 66.27 mm^3) (Figure 7C). We found that glutamine levels in tumor tissues were markedly decreased by GPNA (Figure 7D). Consistent with the in vitro results, tumor tissues from shCtrl + GPNA mice showed higher levels of Sestrin2 protein and p-AKT (S473) but lower levels of p-S6K (T389) than tumor tissues from shCtrl mice (Figure 7E). However, this effect of GPNA was not so evident in tumor tissues from shSesn2 mice; however, the effect was partially restored by rapamycin treatment (Figure 7E). Importantly, GPNA-induced increases in caspase-3 activity and in the number of TUNEL-positive cells were markedly higher in tumor tissues from shSesn2 mice than in those from shCtrl mice (Figures 7F–7H). These data suggest that shSesn2-induced cell death contributes to the reduced tumor volume in GPNA-treated mice via mTORC1 activation and mTORC2 inhibition. The body weights of the control and treated mice were measured to assess non-specific toxicity caused by GPNA, rapamycin, or loss of Sestrin2. The average body weight of control and treated mice did not differ significantly (data not shown).

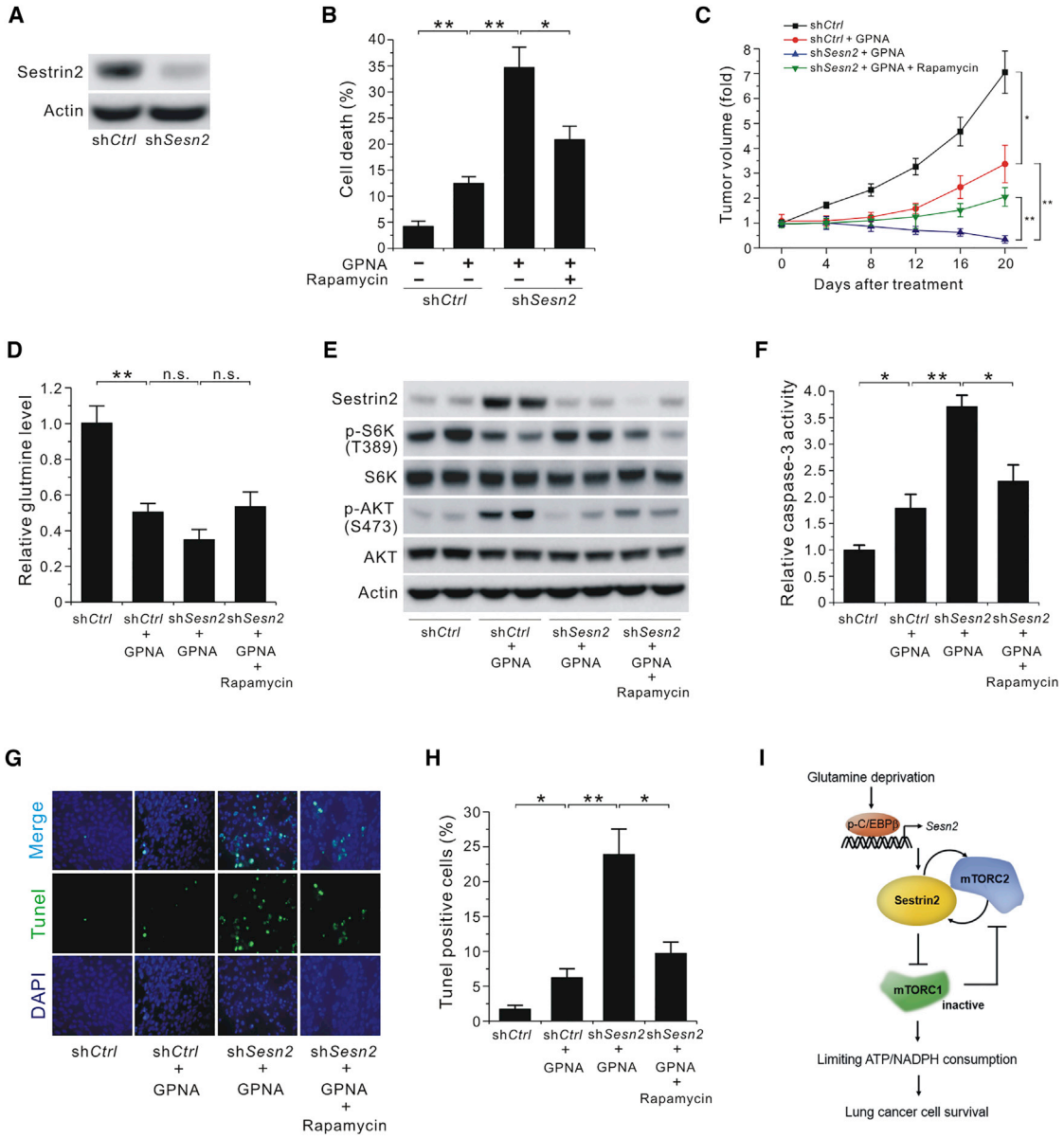

Figure 7. Inhibition of Sestrin2 Induces GPNA-Treated NSCLC Cell Death and Further Augments GPNA-Induced Inhibition of Tumor Growth

(A) Western blot showing shSesn2-mediated knockdown of Sestrin2 in H358 cells (shSesn2-H358).

(B) shSesn2-H358 or control cells were treated with GPNA (1 mM) with/without rapamycin (50 nM) for 96 hr. Cell death was measured by trypan blue exclusion.

(C–H) Effects of Sestrin2 inhibition and GPNA treatment on tumor growth in vivo. (C) Growth curve. Data are expressed as the mean ± SEM (n = 9 mice per group). Relative glutamine levels (D), levels of the indicated proteins (E), and caspase-3 activity (F) in tumors derived from xenografted shCtrl or shSesn2-H358 cells and treated with GPNA (20 mg/kg) or GPNA plus rapamycin (2.5 mg/kg). TUNEL assay (G) and quantification of TUNEL assay results (H).

(I) Schematic showing Sestrin2-mediated survival pathways in glutamine-deprived NSCLC cells.

Data are expressed as the mean ± SEM. n.s., not significant; *$p < 0.05$ and **$p < 0.01$.

DISCUSSION

The present study showed that despite a marked reduction in NSCLC cell growth under glutamine-deprived conditions, inhibiting glutamine utilization alone has little effect on NSCLC cell death. Differential regulation of mTORC1 and mTORC2 is a key aspect of cancer cell survival under nutrient-limited conditions (Choo et al., 2010). In addition, a recent study showed that Sestrin2-mediated inhibition of mTORC1 has an important role in restoring cellular and organismal homeostasis under conditions of amino acid deprivation (Ye et al., 2015). However, little is known about how the interplay between Sestrin2 and mTORC2 governs cancer cell survival under glutamine-depleted conditions. The data presented herein show that induction of

Sestrin2 during glutamine deprivation increases mTORC2 activity by promoting mTORC2 assembly and inhibiting binding of 14-3-3 to Rictor. Given that mTORC1 negatively regulates mTORC2 activity (Huang and Manning, 2009; Moloughney et al., 2016), glutamine-depletion-induced or Sestrin2-mediated activation of mTORC2 activity could be due to a less active mTORC1-mediated negative feedback loop. Our data, therefore, provide evidence that Sestrin2 increases mTORC2 activity through its ability to interact with mTORC2, in addition to its ability to downregulate the mTORC1-mediated negative feedback loop. Interestingly, unlike the previous finding that mTORC2 has a positive regulatory effect on mTORC1 (Huang and Manning, 2009), we showed that under glutamine-depleted conditions, mTORC2 signaling inhibits mTORC1 activity. This contradictory finding can be explained by the increase in the association between Sestrin2 and mTORC2 and the subsequent increase in Sestrin2 abundance under glutamine-depleted conditions. Here, we demonstrated that a positive feedback interaction between Sestrin2 and mTORC2 allows sustained suppression of mTORC1 activity in glutamine-depleted NSCLC cells. Taken together, the present study shows that Sestrin2-mediated differential regulation of mTORC1 and mTORC2 activity plays a pivotal role in the survival of glutamine-depleted NSCLC cells.

Under conditions of nutrient starvation, inhibition of mTORC1 halts anabolic fatty acid synthesis (FAS) for cell growth but stimulates catabolic FAO for survival (DeBerardinis et al., 2008; Levine and Yuan, 2005). mTORC1 drives lipid synthesis by promoting SREBP-1 activation (Laplante and Sabatini, 2012). By contrast, mTORC1 inhibition promotes FAO through PPARα activation (Sengupta et al., 2010). Here, we found that Sestrin2-mediated suppression of mTORC1 reduced the expression of lipogenic genes, including SREBP-1, FASN, and ACL, but increased FAO by increasing CPT1 expression and PPARα activity. Given that ATP and NADPH are generated via FAO but consumed for FAS (Carracedo et al., 2013), Sestrin2-mediated suppression of mTORC1 and the subsequent reprogramming of fatty acid metabolism explain how ATP and NADPH levels are maintained under conditions of glutamine deprivation. In addition, an alternative NADPH-dependent antioxidant pathway, the thioredoxin redox system, can compensate for GSH depletion and enable the survival of glutamine-depleted cancer cells (Harris et al., 2015). Indeed, we observed that in glutamine-depleted NSCLC cells, Sestrin2 maintains thioredoxin in its reduced form. Combined with the finding that inhibiting thioredoxin reductase markedly increases NSCLC cell death, these results highlight that Sestrin2-mediated mTORC1 inhibition impedes cell death under conditions of glutamine-deprivation-induced metabolic stress by preventing ATP depletion and excessive ROS accumulation.

Several mechanisms are thought to be responsible for regulating Sestrin2 expression (Lee et al., 2013). Sestrin2 expression is induced by p53 in response to various stresses, including oxidative stress and DNA damage (Budanov and Karin, 2008), whereas its transcription is increased by hypoxia in a p53-independent manner (Budanov et al., 2002). We showed that Sestrin2 expression increases in p53-null H358 and H1299 cells upon glutamine-depletion-induced ROS generation, indicating that upregulated Sestrin2 expression is p53 independent. A recent study reported that Sestrin2 expression is induced in a C/EBPβ-dependent manner upon oxidative stress (Papadia et al., 2008). Furthermore, p38 MAPK directly phosphorylates and modulates the activity of C/EBPβ, which is stimulated by production of ROS (Wagner and Nebreda, 2009). In view of glutamine's role in maintaining GSH and attenuating ROS levels, glutamine-deprivation-induced GSH depletion results in ROS-mediated activation of the p38 MAPK-C/EBPβ pathway, which in turn induces expression of Sestrin2. In addition to C/EBPβ, ATF4 also mediates Sestrin2 transcription under amino acid deprivation via the activation of GCN2 (Ye et al., 2015). In this study, we found that ATF4 was induced in glutamine-depleted NSCLC cells, but its level was unaffected by the GSH synthesis inhibitor, indicating that GSH depletion and ROS-mediated induction of Sestrin2 is mediated via the C/EBPβ pathway and is less likely to be mediated by ATF4. However, we consider that ATF4 cooperates with C/EBPβ for the induction of Sestrin2 expression in glutamine-depleted cells, because these two transcription factors are known to function on promoter lesion via heterodimer formation (Podust et al., 2001). Taken together, our data reveal that ROS-induced Sestrin2 expression in glutamine-depleted NSCLC cells eventually limits excessive ROS accumulation via the alternative thioredoxin redox system.

In agreement with a previous report demonstrating increased glutamine utilization in NSCLC (Mohamed et al., 2014), we showed that SLC1A5 levels were higher in human NSCLC tissues than in adjacent non-tumor tissues, suggesting that inhibiting SLC1A5 may be a therapeutic strategy for NSCLC. However, we found that a SLC1A5 inhibitor did not induce significant cell death despite a marked reduction in tumor mass. When these findings are combined with the results identifying Sestrin2 as a metabolic gatekeeper for glutamine-depleted NSCLC cell survival, we propose that co-targeting of SLC1A5 and Sestrin2 may be an effective NSCLC treatment. Indeed, our results show that inhibiting both SLC1A5 and Sestrin2 synergistically reduces the tumor burden via profound induction of NSCLC cell death, underscoring the importance of co-targeting SLC1A5 and Sestrin2. In addition to nutrient stress, our data also suggest that targeting Sestrin2 activity may overcome the metabolic adaptation necessary for NSCLC cell survival through modulation of mTORC1 and mTORC2 in response to various stresses. Further studies are needed to examine the potential roles of Sestrin2 in therapeutic responses to conventional chemotherapy.

In summary, the present study shows that restricting glutamine reduces GSH synthesis, which in turn induces Sestrin2 expression via the ROS-p38 MAPK-C/EBP pathway. Under glutamine-depleted conditions, differential regulation of mTORC1 and mTORC2 by Sestrin2 leads to an increase in FAO but a decrease in lipid synthesis. This metabolic reprogramming enables NSCLC cells to survive under glutamine deprivation by preventing intracellular ATP depletion and excessive ROS accumulation (Figure 7I), indicating that targeting glutamine utilization alone is not sufficient to eradicate NSCLC cells. Therefore, co-targeting SCL1A5 and the interaction between Sestrin2 and mTORC2 components may overcome this adaptive mechanism in glutamine-depleted NSCLC cells and would thus be a promising therapeutic strategy for advanced NSCLC. However, before this strategy can be actualized, a future study will be required to

identify the physical interaction sites between Sestrin2 and mTORC2 components.

EXPERIMENTAL PROCEDURES

Patients and Specimens

Protein levels in tumor and adjacent non-tumor tissues taken from 12 patients with NSCLC who underwent surgical resection at the Keimyung University Dongsan Hospital in Daegu, Korea between 2013 and 2015 were determined by western blot analysis (see below). Patients who had received prior chemotherapy were excluded. The study was approved by the institutional review board (IRB) of Keimyung University Dongsan Hospital (IRB no. 2015-07-034). Written informed consent was obtained from each patient.

siRNA Knockdown and Transfection of Expression Vectors

Cells were transiently transfected with control siRNA, siSesn2, siRictor, si-mSin1 (all from Bioneer), control siRNA, siC/EBPβ, siRaptor, siNPRL2 (from Santa Cruz Biotechnology), a Sesn2-expressing vector (Korea Human Gene Bank, Medical Genomics Research Center [KRIBB]), a FLAG-Sesn2 expressing vector (a gift from Dr. Jun Hee Lee, University of Michigan), a myc-Rictor-expressing vector from Dr. David Sabatini (Addgene plasmid 11367), an mSin1-HA-expressing vector from Dr. Jie Chen and Taekjip Ha (Addgene 73388), or a control vector using Lipofectamine 2000 reagent (Invitrogen).

Animal Experiments

Scrambled or shSesn2-expressing H358 cells (6×10^6) were injected subcutaneously into 7-week-old male nude mice. After confirming a palpable xenografted tumor, mice received a daily intraperitoneal (i.p.) injection of vehicle, GPNA, or rapamycin for 20 days. Tumor volume (calculated as length × width^2 × 0.5 mm^3) and body weight were measured every 4 days. For the TUNEL assay, tumor sections were stained using the In Situ Cell Death Detection Kit, Fluorescein (Roche Applied Science). The last dose of drug was administered 4 hr before the experimental endpoint. All animal procedures were approved by the Institutional Animal Care and Use Committee of Kyungpook National University.

Statistical Analyses

Statistical analyses were performed using an unpaired t test or the Mann-Whitney test. Data are presented as the mean ± SEM. $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and five figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2017.06.066.

AUTHOR CONTRIBUTIONS

J.-K.B. performed and conceived the experiments and analyzed and interpreted the data. Y.-K.C. conceived the experiments, analyzed and interpreted the data, and drafted and wrote the manuscript. M.-K.K. and S.-Y.L. obtained lung tissues from patients with NSCLC and interpreted the clinical data. J.-H.K. and H.-J.J. performed experiments and analyzed and interpreted the data. I.H. and J.Y.J. evaluated immunohistochemical staining and scored the tissue microarray. Y.M.L. and I.-K.L. analyzed and interpreted the data and edited the manuscript. K.-G.P. designed the experiments, analyzed and interpreted the data, wrote and edited the manuscript, and supervised the project. All authors approved the final version of the manuscript.

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