EXTRA VIEW

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Sestrin2 facilitates death receptor-induced apoptosis in lung adenocarcinoma cells through regulation of XIAP degradation

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Apoptosis plays a critical physiological role in controlling cell number and eliminating damaged, non-functional and transformed cells. Cancerous cells as well as some types of normal cells are often resistant to cell death induced by pro-inflammatory cytokines through death receptors. This potentially allows cancer cells to evade the control from the immune system and to proceed toward a more malignant stage, although the mechanisms of this evasion are not well established. We have recently identified the stress-responsive Sestrin2 protein as a critical regulator of cell viability under stress conditions. Sestrin2 is a member of a small family of antioxidant proteins and inhibitors of mechanistic Target of Rapamycin Complex 1 (mTORC1) kinase. Down-regulation of Sestrin1/2 leads to genetic instability and accelerates the growth of lung adenocarcinoma xenografts. Here we addressed the potential role of Sestrin2 in regulation of cell death induced by TNFR1 and related Fas and TRAIL receptors in lung adenocarcinoma cells. We found that Sestrin2 silencing strongly inhibits cytokine-induced cell death through a mechanism independent of ROS and mTORC1 regulation. We determined that the X-linked inhibitor of apoptosis protein (XIAP) plays a critical role in the control of cytokine-induced cell death by Sestrin2. Thus our study defines a new, previously unrecognized role of Sestrin2 in the regulation of apoptosis.

Keywords: Sesn2, XIAP, death receptors, caspases, apoptosis

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Introduction

Carcinogenesis is a process often opposed by a stress and accompanied by acute inflammation, which may cause elimination of cancer cells through induction of apoptosis; however, sustained inflammation is considered to be a promoter of carcinogenesis.[1] Many cancer cells acquire resistance to cell death through downregulation of proapoptotic proteins and up-regulation of cell death inhibitors.[2] The stress-responsive Sestrin2 (Sesn2) gene belongs to an evolutionary-conserved Sestrin gene family found in most eukaryotes.[3-5] Sestrins support cell viability under oxidative and metabolic stress but sensitize cells to DNA-damage.[3,6,7] The variability of the Sestrins-mediated responses is associated with several activities of Sestrins such as suppression of reactive oxygen species and inhibition of mechanistic Target of Rapamycin Complex 1 (mTORC1) kinase.[6,8,9] The effects of mTORC1 on cell viability can be mediated by regulation of protein synthesis through phosphorylation p70S6K and 4EBP1 proteins or autophagosomal-lysosomal proteolysis via phosphorylation of ULK1 and ATG13 proteins.[10-12] Sesn2 might have tumor suppressive function as it is a target of tumor suppressor p53,[3] and is inactivated in the majority of human tumors.[13] Deficiency of Sesn2 can facilitate transformation and stimulation of growth of lung adenocarcinoma xenografts,[8,14,15] although the precise role of Sesn2 in suppression of carcinogenesis is yet to be established.

The immune system provides an additional level of protection from carcinogenesis by eliminating malignant cells through activation of death receptors (DR) such as Fas, TRAILR1/2 and, possibly, TNFR1. DR belong to the Tumor Necrosis Factor Receptor (TNFR) superfamily of type-I transmembrane proteins

containing N-terminal cysteine-rich extracellular domain, transmembrane domain and C-terminus containing 80 amino-acid length peptide called death domain (DD).[16,17] After interaction with cognate ligands, DR undergo conformational changes, leading to their oligomerization and recruitment of effector proteins transducing signals from the receptor.[18] For example, activated TNFR1 recruits TRADD (TNFR1-associated Death Domain) and RIP1 (receptor interacting protein kinase 1) followed recruitment of FADD (Fas Associated Death Domain) protein via their DD. FADD in turn interacts with pro-caspase 8/10 death effector domain (DED), forming a complex called DISC, where procaspase 8/10 is cleaved and activated which triggers the activation of executive caspases 3, 6 and 7.[19-21] Activated caspases also cleave Bid protein, a proapoptotic Bcl2 family member, which translocates to mitochondria and stimulates apoptosome formation and activation of caspase 9, 3, 6 and 7 amplifying the apoptotic cascade.[22]

TNFR1 also recruits TRAF2 (TNFR-associated factor 2), cIAP1 and cIAP2 (cellular inhibitors of apoptosis 1 and 2) proteins in a TRADD-dependent manner. RIP1 is ubiquitinated by cIAP1/2 following recruitment and activation of TAK and IKK kinases. IKK phosphorylates and stimulates proteosomal degradation of IκBα (inhibitor of κBα) and IκBα-related proteins, which work as inhibitors of NF-κB transcription factor. Once activated, NF-κB translocates to the nucleus and activates the expression of antiapoptotic genes such as cFLIP, cIAP1/2, XIAP, Bcl2, BclXL. For example, cFLIP is a close homolog of caspase 8 lacking its protease activity. When tethered to DISC, cFLIP competes with caspase 8 and inhibits caspase activation.[17,23] The IAP family proteins, such as XIAP, cIAP1 and cIAP2, are other critical apoptotic inhibitors. They contain several N-terminal BIR domains and a C-terminal RING domain. While BIR domains may interact with and inhibit the activation of caspases directly, RING domains possess an E3 ubiquitin ligase activity. Despite their structural similarity, the different IAP members inhibit cell death through different although overlapping mechanisms. cIAP1/2 are mostly involved in ubiquitination of TRAF2 followed by NF-κB activation. In contrast, XIAP directly binds caspases 9, 3 and 7 and inhibits their proteolythic activity. The activities of IAPs are also regulated by direct interaction with their natural inhibitor Smac/Diablo which is released from mitochondria after induction of cell death.[24] Moreover, IAPs can also be regulated on the level of protein stability. Besides activation of caspases and NF-kB, TNFR1 also stimulates the members of the mitogen-activated protein kinase (MAPK) family, JNK, p38 and ERK, which modulate the cell death response.[18]

DISC complex also mediates cell death triggered by the other members of the TNFR family: Fas and TRAILR1/2. However, activation of Fas or TRAILR1/2 leads to direct recruitment of FADD and caspase 8 to the receptors causing robust induction of cell death.[17,23] Nevertheless, under certain conditions, Fas and TRAILR1/2 can tether TRADD via FADD recruitment, stimulating complex formation with RIP1, TRAF2 and cIAP1/2 proteins and activation of the pro-survival pathway.[23]

Here we demonstrate that Sesn2 supports DR-induced cell death in lung adenocarcinoma cells by a mechanism independent on ROS and mTORC1. Sesn2 silencing leads to accumulation of IAP family members such as cIAP1/2 and XIAP. We also show that XIAP is responsible for regulation of DR-induced cell death by Sesn2 which controls XIAP stability through regulation of its lysosomal degradation.

Results

Sesn2 silencing suppresses DR-induced apoptosis

Sesn2 is an important modulator of cell death.[3,6, 25] To study whether Sesn2 regulates DR-induced cell death in human adenocarcinoma cells, we silenced Sesn2 in H460 and A549 cells with shSesn2 lentiviral vector and treated cells with TNFα in the presence of translation inhibitor cycloheximide (CHX) or, alternatively, transcription inhibitor actinomycin D (ActD). Both CHX and ActD are inhibitors of the pro-survival pathway induced by TNFα which interferes with cell death by TNFα *in vitro*. Either TNFα+CHX or TNFα+ActD treatment strongly activated cell death in control shLuc-expressing cells as determined by accumulation of apoptotic cells in sub-G1 phase, and Sesn2 silencing significantly inhibited TNFα-induced cell death (**Fig. 1A, C**, S2A). To exclude the possibility of off-target effects of shSesn2, we treated H460 cells expressing alternative shSesn2–2 with TNFα+CHX and obtained similar results (**Fig. S1A, B**). Interestingly, Sesn2 silencing did not affect the caspase-dependent cell death induced by staurosporin (**Fig. S2B**), indicating that Sesn2 is a specific regulator of DR-induced cell death.

To study whether Sesn2 is important for apoptosis, we analyzed TNFα-induced activation of caspases examining cleaved caspases 8, 3, 7, and 9 by immunoblotting. We also examined cleavage of PARP and Bid mediated by caspases.[22] We observed strong activation of the caspases by TNFα+CHX treatment in control cells although these effects were compromised in Sesn2-silenced cells (**Fig. 1B, D**), which indicates that Sesn2 is a major regulator of apoptosis. To distinguish Sesn2 effects on apoptosis from other types of cell death, we utilized Annexin V-PI staining, that allows the discrimination between necrosis and apoptosis. We found that most cells were AnnexinV+ (early apoptotic) or AnnexinV+PI+ (late apoptotic) but not AnnexinV-PI+, indicating that apoptosis was the major mechanism of cell death (**Fig. S1C, D**). Accordingly, we did not observe any inhibitory effects of Sesn2 silencing on necroptosis induced by TNFα+CHX treatment in the presence of a pan-caspase inhibitor Z-VAD-FMK[26] (**Fig. S2C**).

Activated Fas and TRAILR1/2 also induce cell death via DISC formation and caspase activation. To determine the role of Sesn2 in Fas and TRAIL -induced cell death, we treated H460 cells with FasL, an activatory anti-Fas antibodies (Ab) or TRAIL and observed that all of these treatments activated cell death in a Sesn2-dependent manner (**Fig. 1E–G**).

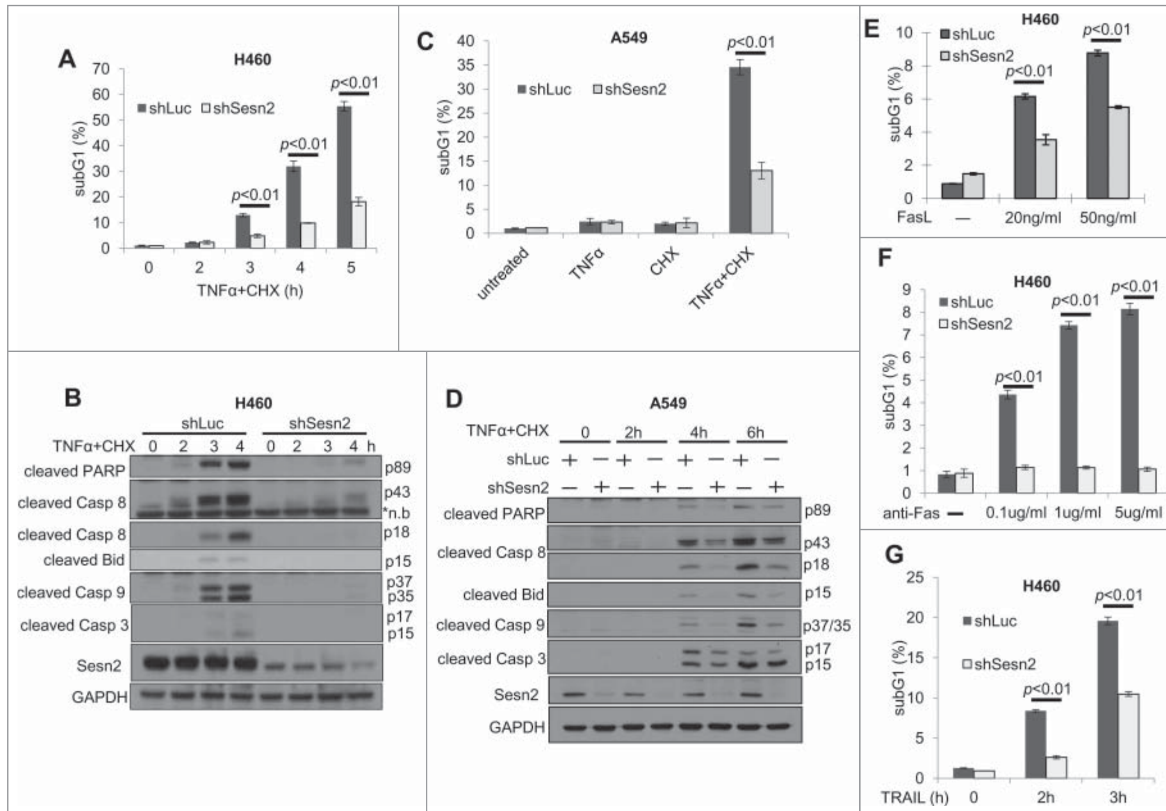

Figure 1. Sesn2 supports TNFα, Fas and TRAIL induced cell death. (**A–C**) Sesn2 silencing compromises TNFα+cycloheximide (CHX)-induced cell death in lung adenocarcinoma cells. (**A**) Sesn2-silenced or control (shLuc) H460 cells were treated with TNFα (10 ng/ml)+CHX (10 ug/ml) for the indicated time intervals and number of cells with sub-G1 DNA content stained with propidium iodide (PI) was assessed by flow cytometry. (**B**) Cells were treated as in (**A**) for 3 hrs and expression of full-size and cleaved forms of the corresponding proteins were analyzed by immunoblotting. (**C**) Sesn2-silenced and control A549 cells were treated with TNFα+CHX for 4h and analyzed as in (**A**). (**D**) Cells were treated with TNFα+CHX for different time intervals and analyzed as in (**B**). (**E, F**) Sesn2 silencing inhibits Fas-induced cell death. (**E**) Sesn2-silenced or control H460 cells were treated with FasL for 24 hrs and sub-G1 content was analyzed by flow cytometry as in (**A**). (**F**) Sesn2-silenced or control H460 cells were treated with activatory anti-Fas antibody and analyzed 30 hrs later as in (**A**). (**G**) Sesn2 silencing suppresses TRAIL-induced cell death. Sesn2-silenced or control H460 cells were treated with TRAIL for indicated time intervals and analyzed as in (**A**).

Sesn2 does not affect expression of NF-κB-regulated genes or mediators of TNFα-induced cell death

Although TNFα induces the apoptotic cascade, it also activates the pro-survival signaling pathways triggering NF-κB-dependent transcriptional activation of anti-apoptotic genes.[27,28] To determine whether Sesn2 modulates expression of NF-κB-regulated genes, we analyzed expression of several NF-kB targets in untreated and TNFα-treated cells by quantitative real-time PCR (qPCR). Although many of these genes such as IKBα, IL6, cIAP1, COX2 and A20 were activated by TNFα treatment, we did not observe a difference in their mRNA levels between Sesn2-silenced and control cells. Moreover we did not observe activation of other reported potential NF-κB targets such as BAX, Bcl-XL, BFL-1/A1, BIM and cIAP2 in our experimental conditions and the expression of these genes was not affected by Sesn2 silencing (**Fig. 2A, Fig. S3**).

As reported earlier, Sesn2 is involved in regulation of protein synthesis or degradation.[8,29,30] Considering the possible impact of Sesn2 on the expression of proteins transducing signaling from TNFα toward caspases, we compared the levels of TNFR1, RIP1 and TRADD proteins in control and Sesn2-silenced cells by immunoblotting, and observed no difference in the expression levels of these proteins between these 2 cell lines (**Fig. 2B, C**). We also measured levels of cFLIPs, the components of DISC and inhibitors of cell death, and did not observe any difference in the expression of long $cFLIP_L$ and short $cFLIP_S$ isoforms between the 2 cell lines. Also cFLIP was degraded with a similar rate in response to TNFα+CHX treatment in both cell lines (**Fig. 2D**).[16]

Sesn2 silencing does not affect activation of MAPK and AKT kinases by TNFα

MAPKs are involved in positive and negative regulation of DR-induced cell death.[31,32] To determine whether Sesn2 plays a role in activation of JNK, p38 and MEK by TNFα, we examined their phosphorylation by immunoblotting. As shown in **Figure 3A**, Sesn2 did not affect the magnitude of JNK, p38 or ERK activation by TNFα, nor the duration of JNK activation. In addition, treatment with the JNK inhibitor SP600125 suppressed TNFα-induced apoptosis in both control and Sesn2-

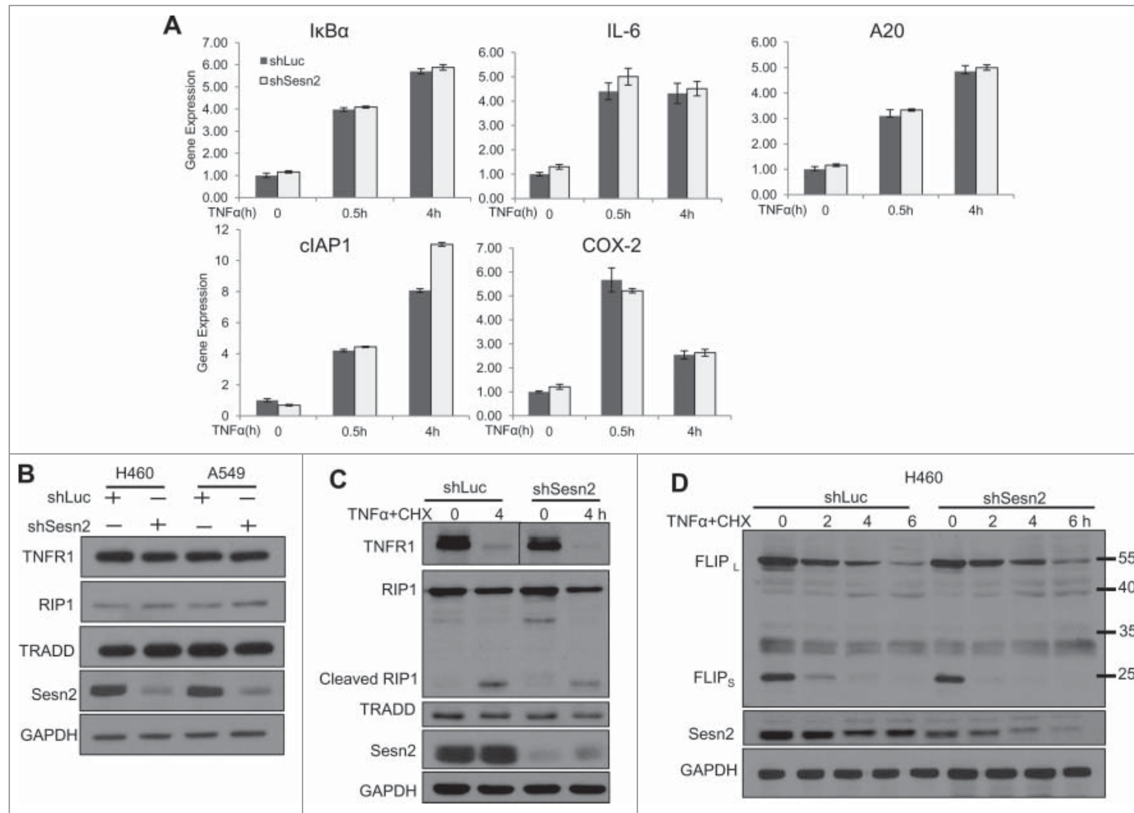

Figure 2. NFκB activity and the expression of critical regulators of TNFR1-induced cell death are not altered in Sesn2-silenced cells (**A**) Sesn2-silenced and control cells have similar levels of expression of NF-κB regulated genes under normal conditions and after TNFα treatment. (**B**) Sesn2-silenced or control H460 cells were treated with TNFα and the expression of the corresponding NF-κB-inducible genes was analyzed by qPCR. Sesn2 silencing does not affect the expression of the mediators of TNFα signaling TNFR1, RIP1 and TRADD in H460 and A549 cells. Sesn2-silenced or control cells were analyzed by immunoblotting with the indicated antibody. (**C**) Sesn2 silencing does not affect the expression of TNFR1, TRADD and RIP1 in untreated and TNFα+CHX treated cells. Sesn2-silenced or control cells were treated with TNFα+CHX and the expression of the indicated proteins was analyzed by immunoblotting. (**D**) Sesn2 silencing does not affect $cFLIP_L$ and $cFLIP_S$ expression in response to TNFα+CHX treatment. Sesn2-silenced or control cells were treated with TNFα+CHX for different time intervals and the expression of FLIP and control GAPDH proteins were analyzed by immunoblotting.

silenced cells in a similar fashion, preserving the ratio in cell death between control and Sesn2-silenced cells (**Fig. 3B**). We also analyzed phosphorylation of AKT, another regulator of apoptosis and a potential target of Sesn2,[7,33] and did not observe any effect of Sesn2 silencing on AKT phosphorylation in untreated or TNFα-treated cells (**Fig. 3A**). These data indicate that Sesn2 does not play any significant role in regulation of MAPK or AKT signaling in response to TNFα treatment.

Sesn2 regulates TNFα-induced cell death in the ROS- and mTORC1-independent manner

We have previously shown that Sestrins suppress ROS accumulation and inhibit mTORC1 activity.[6,8,34] Since elevated ROS might support cell death through activation of JNK,[32] we analyzed ROS levels in Sesn2-silenced and control cells treated with TNFα. As expected,[6,14] Sesn2 knockdown increased ROS levels in untreated cells (**Fig. 4A**). Treatment with TNFα slightly increased ROS levels and co-incubation with a ROS scavenger N-acetylcysteine (NAC) decreased ROS accumulation in both control and Sesn2-silenced cells (**Fig. 4A**). To study whether Sesn2 silencing can affect cell death via a ROS-dependent mechanism, we induced cell death by TNFα+CHX in the presence or absence of NAC and found that NAC treatment suppressed cell death in a similar manner in both control and Sesn2-silenced cells, preserving the difference in the levels of cell death induced by TNFα between these 2 cell lines (**Fig. 4B**).

In parallel, we also analyzed the potential impact of Sesn2 on mTORC1 regulation by TNFα. While we observed the activation of mTORC1 in response to TNFα treatment (depicted by increased p70S6K and S6 phosphorylation), there was no difference in p70S6K, S6 and 4EBP1 phosphorylation between Sesn2-silenced and control cells (**Fig. 4C**). Moreover, treatment with mTORC1 inhibitor rapamycin strongly suppressed phosphorylation of mTORC1 targets (**Fig. 4C**), but had no significant impact on the TNFα-induced cell death in both Sesn2-silenced and control cells (**Fig. 4D**). Thus, we concluded that control of ROS or mTORC1 is dispensable

for the regulation of TNFα-indu-ced cell death by Sesn2.

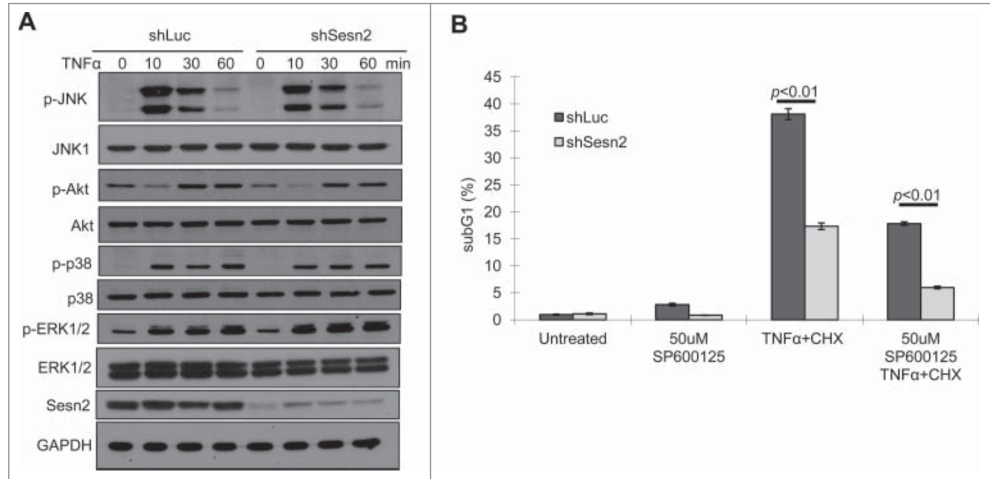

Figure 3. Sesn2 supports TNFα+CHX-induced cell death not via a MAPK-dependent mechanism. (**A**) Sesn2 is not involved in the regulation of JNK, p38, ERK and AKT phosphorylation. Sesn2-silenced or control H460 cells were treated with TNFα for different time interval and phosphorylation and expression of corresponding proteins were analyzed by immunoblotting. (**B**) JNK inhibition has similar effect on cell death in control and Sesn2-silenced cells. Sesn2-silenced or control H460 cells were treated with TNFα+CHX in the presence or absence of JNK inhibitor SP600125 for 4 hrs and the sub-G1 population of apoptotic cells was analyzed by flow cytometry.

IAP proteins accumulate in Sesn2-silenced cells and XIAP mediates the effects of Sesn2 on TNFα-induced apoptosis

The IAP family members are major regulators of DR-induced cell death.[35] To study whether Sesn2 can regulate expression of IAP proteins we analyzed their levels by immunoblotting and found that cIAP1/2 and XIAP were accumulated in the Sesn2-silenced cells as compared to control (**Fig. 5A**). Within the IAP family, XIAP is the most potent regulator of cytokine-induced cell death due to its direct strong inhibitory effect on caspases.[24,36] Therefore, we reasoned that XIAP might be responsible for regulation of DR-induced apoptosis by Sesn2. To study whether Sesn2 controls XIAP mRNA levels we analyzed XIAP mRNA levels in Sesn2-silenced and control cells by qPCR, but did not observe any difference between these 2 cell lines (**Fig. 5B**). Thus the regulation of XIAP expression by Sesn2 is mediated by post-transcriptional mechanisms such as protein synthesis and/or degradation. To study whether XIAP is responsible for regulation of TNFα-induced cell death by Sesn2, we silenced XIAP by shRNA lentivirus (**Fig. 5C**) and treated cells with TNFα+CHX. Strikingly, XIAP knockdown in shSesn2-silenced H460 cells restored the levels of cell death observed in control cells and eliminated the difference in the levels of cell death between control and Sesn2-silenced cells (**Fig. 5D**), indicating that XIAP plays a major role in regulation of TNFα-induced cell death by Sesn2.

Sesn2 controls XIAP through regulation of protein stability

Protein synthesis and/or degradation are the major mechanisms of post-transcriptional control of protein expression. To study the impact of Sesn2 on these processes we pulse-labeled control and Sesn2-silenced cells with ^{35}S methionine-cysteine for 1 hr, removed the radioactivity from the medium and monitored the levels of newly-synthesized XIAP protein at different time intervals after replacement with non-radioactive medium by SDS-PAGE electrophoresis and autoradiography. This allowed us to analyze the contribution of Sesn2 to XIAP synthesis comparing the XIAP levels immediately after ^{35}S labeling, as well as the half-life of the XIAP protein in control and Sesn2-silenced cells. We did not observe any significant difference in the levels of freshly-synthesized Sesn2 protein immediately after pulse chase labeling, suggesting that Sesn2 does not considerably contribute to XIAP protein synthesis (**Fig. 6A**). However, we found striking differences in XIAP protein levels at different time points after replacement with non-radioactive medium. While the half-life of XIAP was about 5 hrs in control cells, it increased 2-fold in Sesn2-silenced cells. Thus Sesn2 knockdown causes XIAP protein stabilization by suppressing its degradation (**Fig. 6A and B**).

Sesn2 can regulate XIAP protein degradation through the lysosomal pathway

The majority of intracellular proteins are degraded through the proteosomal and/or lysosomal pathways. To establish the mechanism responsible for the decreased XIAP degradation in shSesn2 cells, we examined how inhibition of either of these pathways affects XIAP degradation in control or Sesn2-silenced cells. We treated cells with either proteosomal inhibitor MG132 or lysosomal inhibitor chloroquine (CQ) and measured XIAP protein levels at different time points after treatment. Both treatments led to accumulation of XIAP protein in control cells indicating that XIAP can be degraded through both mechanisms (**Fig. 7A and B**). Nevertheless we observed a conspicuous difference in XIAP accumulation in Sesn2-silenced cells treated with either MG132 or CQ. Although the XIAP level was originally higher in Sesn2-silenced cells as compared to the control, XIAP continued to accumulate in response to MG132 treatment with similar dynamic as in the control cells (**Fig. 7A**), indicating that Sesn2 is not likely to be affecting the rate of XIAP proteosomal degradation. In contrast, although CQ treatment led to accumulation of XIAP in control cells, no additional accumulation of XIAP was observed in Sesn2-silenced cells (**Fig. 7B**). These data suggest that XIAP lysosomal degradation was already impaired in the Sesn2-silenced cells and inhibition of the lysosomal pathway had no additional

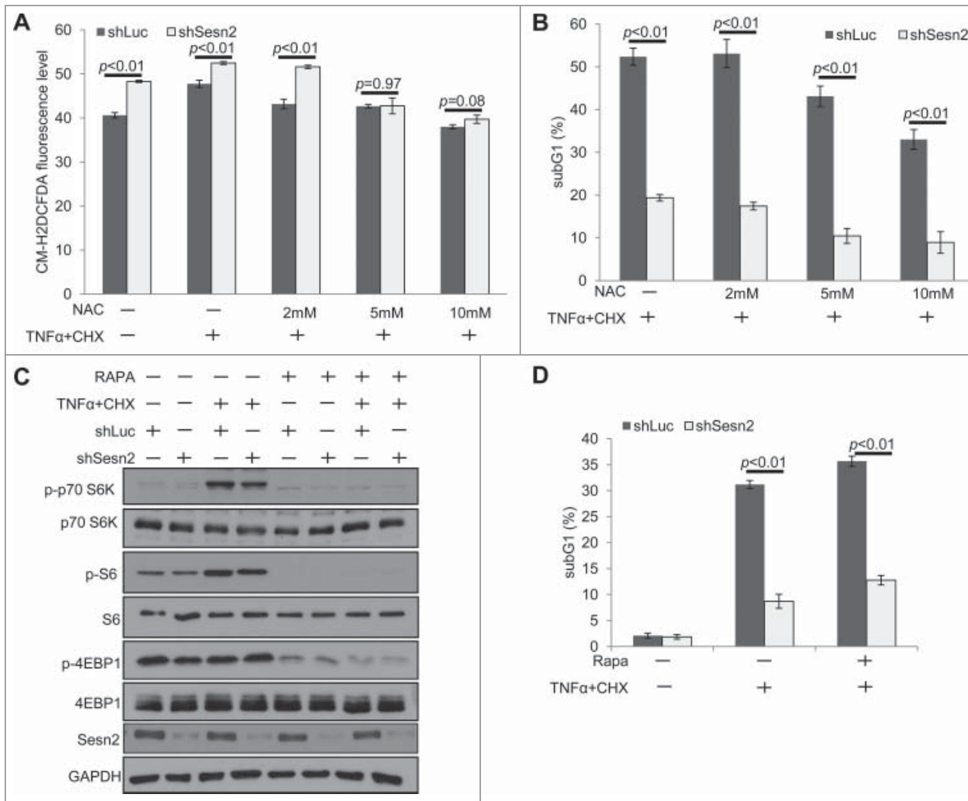

Figure 4. The stimulatory effect of Sesn2 on TNFα+CHX-induced cell death is not mediated by ROS or mTORC1 regulation. (**A**) Sesn2-silenced cells have increased ROS levels as compared to control cells which can be reversed by NAC treatment. Sesn2-silenced or control H460 cells were treated with TNFα+CHX for 4 hrs in the presence or absence of different concentrations of NAC. Cells were incubated with DCFDA and analyzed by flow cytometry. (**B**) NAC treatment slightly inhibits cell death in both Sesn2-silenced and control cells, but does not eliminate the difference in cell death between these 2 cell lines. Cells were treated as in (**A**) and sub-G1 DNA content was analyzed by flow cytometry. (**C**) Sesn2 silencing does not affect mTORC1 activation by TNFα+CHX treatment. Sesn2-silenced or control cells were treated with TNFα+CHX in the presence or absence of rapamycin and phosphorylation and expression of the indicated proteins were analyzed by immunoblotting. (**D**) mTORC1 inhibition by rapamycin does not affect the magnitude of TNFα+CHX-induced cell death in Sesn2-silenced and control cells. Sesn2-silenced or control cells were treated with TNFα+CHX in the presence or absence of rapamycin (10 nM) and cell death was determined as in **B**.

effect on XIAP protein levels in Sesn2-silenced cells.

Total autophagosomal-lysosomal protein degradation can be monitored by p62/SQSTM1 degradation or accumulation of the high-mobility LC3-II form. We measured p62 and LC3-II levels by immunoblotting and did not observe any difference between control and Sesn2-silenced cells, although p62 protein levels were decreased in both cell lines in response to TNFα+CHX (**Fig. S4**). Therefore, although total autophagy can be stimulated by TNFα treatment, it does not depend on Sesn2. It was also reported previously that Sesn2 can be involved in specific degradation of Keap1 via direct interaction with p62 and Keap1.[30] Anticipating potential similarity in the mechanisms of degradation of Keap1 and XIAP, we analyzed whether Sesn2 can interact with p62 and XIAP and whether Sesn2 can affect interaction between p62 and XIAP. We did not observe any interaction between Sesn2 and either p62 or XIAP in H460 cells (data not shown). Although we detected an interaction between p62 and XIAP, such interaction was not affected by the Sesn2 status (**Fig. S5**).

Discussion

Tumor suppressive mechanisms rely on apoptosis for elimination of pre-malignant and malignant cells.[37] The immune system is responsible for surveillance of transformed cells and their elimination through activation of DRs.[17,18] Cancer cells, in turn, develop resistance to apoptosis, through disregulation of expression of antiapoptotic and proapoptotic proteins.[38] Here, we determined that down-regulation of Sesn2, a major controller of cell viability under different stress conditions,[3,6] is a new potential strategy of evasion from DR-induced cell death for adenocarcinoma lung cancer cells. The importance of Sesn2 in modulating cell death prompted us to study its potential impact on regulation of DR-induced cell death. As we demonstrated here, Sesn2 is important for cell death induced by TNFα, Fas and TRAIL in human lung adenocarcinoma H460 and A549 cells. While we observed an inhibition of caspase activation by Sesn2 silencing, it played no role in the control of NF-κB-regulated transcription or regulation of the expression of cell death mediators such as TNFR1, TRADD and RIP1. Sesn2 also had no impact on the activation of JNK, p38 or ERK kinases, ruling out their role in Sesn2-dependent processes. This is a new and previously uncharacterized function of Sesn2, which is not relevant to inhibition of ROS accumulation or mTORC1 activity.

Examining different regulators of DR-induced cell death, we found that Sesn2 silencing caused accumulation of the IAP family members XIAP, cIAP1 and cIAP2 which all can contribute to suppression of cell death by Sesn2 silencing. cIAP1/2 inhibit cell death mostly through ubiquitination and degradation of RIP1 and RIP1-dependent activation of NF-κB, so we compared RIP1 expression and NF-κB activation between Sesn2-silenced and control cells, and were not able to assign

the regulation of any of these proteins by Sesn2 to its effects on cell death.[24,39] In contrast, XIAP directly binds caspases and inhibits their proteolytic activity and we demonstrated the clear effect of Sesn2 on activation of caspases in response to DR activation. Although we could not completely waive away the role of cIAP1/2 in regulation of DR-induced apoptosis by Sesn2, in the following experiments we focused on the XIAP protein as the most prominent potential mediator of the effects of Sesn2 on cell death (**Fig. 7C**). Accordingly, we found that XIAP knockdown in Sesn2-silenced cells restored the levels of cell death to those observed in control cells, indicating that XIAP is the major contributor to the suppression of DR-induced cell death by Sesn2 downregulation. Although a direct inhibitory effect of XIAP was demonstrated on caspases 3, 7 and 9, these caspases can amplify activation of caspase 8 via a positive feedback loop,[40,41] explaining the inhibition of caspase 8 activation in the Sesn2-silenced cells.

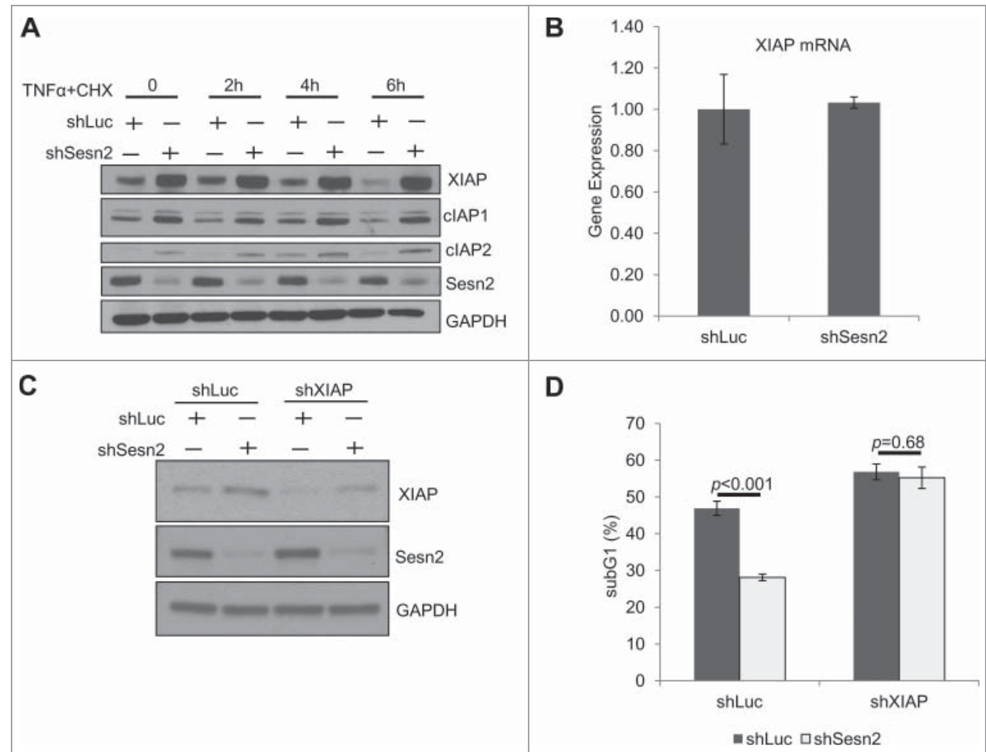

Figure 5. Sesn2 supports TNFα+CHX-induced cell death through regulation of XIAP. (**A**) Sesn2-silencing causes accumulation of IAP proteins. (**A**) XIAP, cIAP1 and cIAP2 proteins are accumulated in Sesn2-silenced cells as compared to the control. The levels of the indicated proteins in untreated and TNFα+CHX treated cells were determined by immunoblotting. (**B**) Sesn2 silencing does not affect XIAP mRNA-expression. RNA from Sesn2-silenced or control H460 cells was isolated with Trizol reagent, converted to cDNA and analyzed by qPCR. (**C, D**) XIAP knockdown restore the levels of TNFα+CHX-induced cell death in Sesn2-silenced H460 cells. (**C**) Analysis of XIAP protein expression in XIAP-silenced H460 cells. Sesn2-silenced or control H460 cells were infected with lentiviral vector expressing shXIAP and the proteins were analyzed by immunoblotting. (**D**) Analysis of the effect of XIAP knockdown on TNFα+CHX-induced cell death. Cells were generated as in **C** and cell death was determined by the analysis of sub-G1 population by flow cytometry.

To study the potential mechanism of XIAP regulation by Sesn2, we analyzed its stability by pulse-chase labeling and observed that XIAP half-life was extended 2-fold in Sesn2-silenced cells. Moreover, we found that Sesn2 regulates XIAP levels through control of lysosomal degradation. The potential role of Sesn2 in regulation of the autophagy-lysosomal pathway via mTORC1 inhibition was previously reported,[8,42,43] however, we did not see any effects of Sesn2 silencing on the mTORC1 activation by TNFα. The autophagic rate can be monitored by degradation of p62 protein, which works as a cargo for delivery of many proteins to autophagosomes, and by conversion of LC3-I into the processed LC3-II form. We did not observe any difference in the p62 levels between Sesn2-silenced or control TNFα+CHX treated or untreated cells, although TNFα+CHX treatment caused down-regulation of p62 in both cell lines (**Fig. S4**). Similarly, we did not see any effect of Sesn2 silencing on the expression of LC3-I and LC3-II forms, arguing against a significant role of Sesn2 in regulation of general autophagy in our experimental system.

Nevertheless, Sesn2 can be involved in the specific lysosomal degradation of some proteins, such as PDGFRβ[44,45] and Keap1.[30] As demonstrated, Sesn2 regulates Keap1 degradation through direct interaction with Keap1 and p62, directing Keap1 to the autophagosomal-lysosomal pathway. Speculating that a similar mechanism for XIAP degradation might be involved, we analyzed whether Sesn2 interacts with either XIAP or p62, but did not observe any noticeable interaction between Sesn2 and any of these proteins. Moreover, although we were able to co-immunoprecipitate XIAP and p62, Sesn2 silencing did not affect interaction between these 2 proteins (**Fig. S5**). Thus, although p62 can take part in the XIAP lysosomal degradation, we were not able to assign any role of Sesn2 in this process. Although the mechanism of XIAP degradation by Sesn2 is yet to be characterized, we speculate that Sesn2 can label and target XIAP and other IAP members to lysosomes via several potential mechanisms. One of them might involve interaction with and activation of ULK1,[46] which plays an important role in early stages of autophagosome biogenesis and could direct some proteins into autophagosomes. Another mechanism might involve the recently characterized GATOR complex as the major Sestrin interactor.[9,47] GATOR proteins can be associated with lysosomes[48] and could mediate the effects of Sesn2 in the recognition and specific

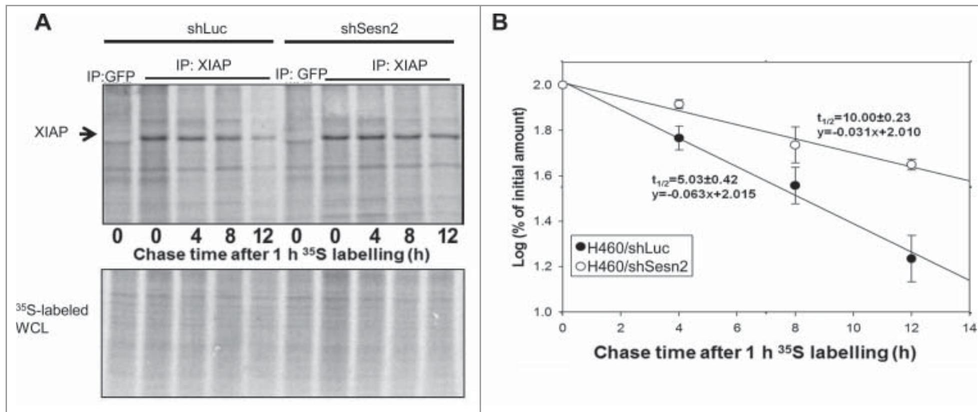

Figure 6. Sesn2 controls XIAP expression via regulation of protein stability. (**A, B**) XIAP protein is stabilized in Sesn2-silenced cells. (**A**) ^{35}S pulse-chase labeling experiment demonstrated significant effect of Sesn2 knockdown on the stability of XIAP protein. Sesn2-silenced or control H460 cells were incubated with ^{35}S protein labeling mix for 1 hr followed by incubation with non-radioactive medium for different time intervals. At each time-point the cells were lyzed and XIAP protein was immunoprecipitated with anti-XIAP antibody (anti-GFP antibodies were used as a negative control) and analyzed by SDS-PAGE followed by autoradiography, On the lower panel, the whole cell lysates (WCL) were analyzed by SDS-PAGE electrophoresis. (**B**) Quantification of the radioactively-labeled proteins in Sesn2-silenced and control cells. Cells were treated as in **A** and the amount of labeled Sesn2 protein and its half-life were analyzed on a phosphoimager with ImageQuant software.

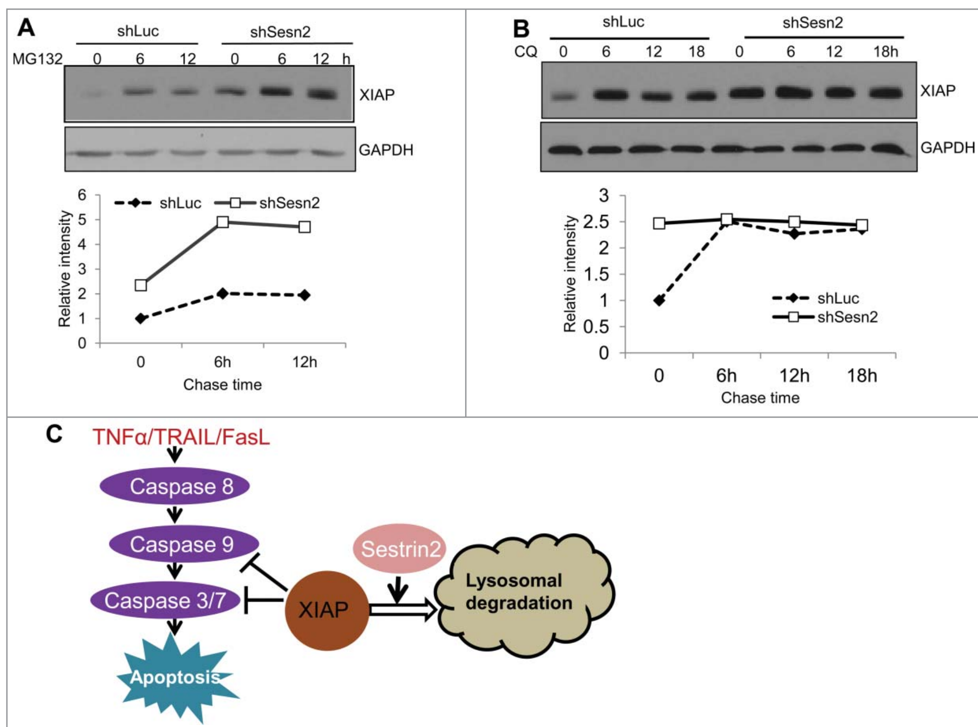

Figure 7. Sesn2 controls XIAP stability through regulation of lysosomal degradation. (**A**) Inhibition of proteosomal synthesis causes XIAP accumulation in both Sesn2-silenced and control H460 cells with similar rates. Sesn2-silenced or control H460 cells were treated with proteosomal inhibitor MG132 (20 μM) for different time intervals and XIAP and control GAPDH protein levels were analyzed by immunoblotting. (**B**) Inhibition of lysosomal synthesis does not cause additional XIAP protein accumulation in Sesn2-silenced cells. Sesn2-silenced or control H460 cells were treated with lysosome inhibitor chloroquine (CQ) (50 μM) for the indicated time intervals and XIAP and GAPDH protein levels were examined by immunoblotting. (**C**) The scheme illustrating regulation of DR-induced cell death by Sesn2. Sesn2 stimulates degradation of XIAP via lysosomal pathway, as result supporting DR-induced cell death.

degradation of particular proteins. Other members of the Sestrin family might be also involved in regulation of DR-induced cell death via control of IAP proteins. Although the dramatic effects of Sesn2 inactivation on DR-induced cell death in lung adenocarcinoma cells can be explained by high relative expression of Sesn2 as compared to the other Sestrins members or, alternatively, its higher specificity for XIAP degradation. Nevertheless we anticipate that Sesn1/3 can play a role in the regulation of DR-induced cell death under some experimental conditions, especially in the cell types where these proteins are predominantly expressed.

Tumors are always associated with inflammation and infiltration of immune cells, which play an ambivalent role in carcinogenesis. Despite the potential role of these factors in eliminating malignant cells via activation of cell death, they can also contribute to tumor progression via activation of pathways supporting cell proliferation, angiogenesis and metabolic re-programming. Thus cancer cells that are capable of inhibition of pro-apoptotic machinery induced by DR, and still maintain intact pro-survival pathways, might be selected. One of the most vivid examples is the inactivation of tumor suppressor p53, the important positive regulator of DR-induced cell death, found in the majority of human cancers.[13] Another example is the accumulation of the IAP proteins. XIAP and other IAP family members are up-regulated in advanced lung and some other human cancers,[24,39, 49,50] although the mechanisms of accumulation of IAP proteins are yet to be characterized. Here we demonstrated that Sesn2 silencing is involved in accumulation of XIAP and other IAP members. Sesn2 and Sesn1 can be downregulated in human

cancers due to inactivation of its master regulator p53 found mutated in majority of human cancers,[14,34] or due to some other yet to be characterized mechanisms. Accordingly, loss of heterozygosity in Sesn2 locus 1p34 or in Sesn1 locus 6q21 is found in many human cancers[5,51] arguing for a potential tumor suppressive function of Sestrins. The ability of Sesn2 to support DR-induced cell death along with its antioxidant and mTORC1-suppressing functions, make this protein a primary candidate as a suppressor of lung carcinogenesis.

Materials and Methods

Cell culture, transfection, infection and treatment

Human lung adenocarcinoma H460 and A549 cells and human embryonic kidney HEK 293T cells were cultured in high-glucose DMEM supplemented with 10% FBS and penicillin/streptomycin. All transfections and infections were performed as described previously.[8] Human recombinant TNFα, TRAIL and FasL were from R&D System and reconstituted in PBS with 0.2% BSA. N-Acetyl-L-cysteine (NAC), chloroquine (CQ), MG132, cycloheximide, actinomycin D and rapamycin were from Sigma Aldrich; SP600125 and anisomycin were from Cell Signaling Inc.; Z-VAD-FMK was from Enzo Lifescience For ROS examination cells were incubated with DCFDA (Life Science) for 45 min followed by flow cytometry analysis.

Cell lysis, immunoprecipitation and immunoblot analyses

For immunoblot analysis cells were lysed in RIPA-SDS buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS, and protease/phosphatase inhibitors (Roche)). For immunoprecipitation experiments cells were lysed in NP40 buffer (50mM Tris pH 7.4, 150 mM NaCl, 0.5% NP40, 0.1 mM EDTA, and protease/phosphatase inhibitors). The lysates were incubated with the mix of indicated antibodies and protein A:G-sepharose beads for 3 hrs, then washed 4 times with lysis buffer. The proteins were separated by SDS-PAGE and analyzed by immunoblotting; Quantification was performed by Image J software. The antibodies used were: Sesn2 from Proteintech; FLIP from Enzo Lifescience; PARP, GAPDH, p53, p62/SQSTM1 from Santa Cruz; all others were from Cell Signaling.

Constructs

pLSLPw-shLuc was previously described.[8] The sequence for shSesn2 is 5′-GAAGACCCTACTTTCGGAT-3′, sh Sesn2–2: 5′-GAGATGGAGAGCCG-CTTT-3′, shXIAP: 5′-CCAGAATGGT-CAGTACAAA-3′. The primers used for qPCR were: A20: 5′-CTGCCCAG-GAATGCTACAGATAC-3′ and 5′-GTGGAACAGCTCGGATTTCAG-3′; COX2: 5′-CACCCATGTCAAAACC-GAGG-3′ and 5′-CCGGTGTTGAG-CAGTTTTCTC-3′; IκBα: 5′-GATCC-GCCAGGTGAAGGG-3′ and 5′-GCAATTTCTGGCTGGTTGG-3′; IL6: 5′-AATTCGGTACATCCTCGAC-GG-3′ and 5′-GGTTGTTTTCTGCC-AGT-GCC-3′; XIAP: 5′-AGCCAAGGGGAA-TGAAGTGA-3′ and 5′-GGGGA-AGG-GCATTTGAAGAA-3′; GAPDH: 5′-CATGGGTGTGAACCATGAGA-3′ and 5′-CAGTGATGGCATGGACTG-TG-3′.

Analysis of cell death

Cell death was evaluated by analysis of sub-G1 population and Annexin V-propidium iodide (PI) staining. For sub-G1 analysis, cells were fixed with 70% ice-cold ethanol and kept at −20°C overnight. After washing with PBS, cells were incubated with 100 μg/ml RNase A and 40 μg/ml PI at room temperature for 30 min in the dark. Samples were acquired with a BD FACS Calibur and analyzed with CellQuest Pro software. For Annexin V-PI staining, cells were re-suspended in Annexin V Binding Buffer and stained with anti-Annexin V FITC antibody and PI as per manufacturer's recommendations (BD Biosciences).

Quantitative Real-Time PCR

Total RNA was extracted with Trizol reagent (Invitrogen). Purified RNA (1 ug) was converted into cDNA according to the Tetro cDNA synthesis kit (Bioline). qPCR was performed with the iTaq universal SYBR green supermix (Bio-rad). Data were analyzed by the $2^{-\Delta\Delta CT}$ method for relative quantification. Experimental Ct values were normalized to GAPDH levels and relative mRNA expression was calculated versus a control sample.

Pulse-chase protein labeling and examination of protein stability

Cells (1×10^6 per 6 cm well) were incubated with 200μCi Sprotein labeling mix (PerkinElmer), containing ^{35}S-labeled methionine and cysteine for 60 min, washed and replaced with normal unlabeled medium contained 3 mM methionine and 1mM cysteine. Cells were harvested and lysed in a buffer containing 50 mM Tris (pH 7.5), 0.5 mM EDTA, 1% SDS, and 1 mM DTT, and sonicated for 10 minutes. The normalized lysates were diluted 1:9 with immunoprecipitation buffer (50 mM Tris 7.5, 300 mM NaCl, 1% NP40, and 1 mM DTT), and immunoprecipitated with equal amount (1.2 μg) of either anti-XIAP (Santa Cruz sc-55552) or control anti-GFP (Santa Cruz sc-9996) monoclonal antibodies followed by SDS-PAGE. The gels were fixed, dried, and the signal intensities were acquired using ImageQuant software.

Statistical analysis

Differences between samples were analyzed by Student's t-test, or one-way Anova according to data distribution. Analyses were performed using GraphPad Prism (GraphPad Software Inc.). P values <0.05 were considered significant. Results are presented as mean ± standard deviation of at least 3 independent experiments.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Supplemental Material

Supplemental data for this article can be accessed on the publisher's website.

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