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REPORT

Mitochondrial dysfunction induces *SESN2* gene expression through Activating Transcription Factor 4

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ABSTRACT

We found that inhibitors of mitochondrial respiratory chain complexes III (myxothiazol) and I (piericidin A) in some epithelial carcinoma cell lines induce transcription of the p53-responsive *SESN2* gene that plays an important role in stress response and homeostatic regulation. However, the effect did not depend on p53 because i) there was no induction of p53 after the treatment with piericidin A; ii) after the treatment with myxothiazol the peak of *SESN2* gene upregulation occurred as early as 5h, before the onset of p53 activation (13h); iii) a supplementation with uridine that abolishes the p53 activation in response to myxothiazol did not abrogate the induction of *SESN2* transcripts; iv) in the p53 negative HCT116 p53 -/- cells *SESN2* transcription could be also induced by myxothiazol. In response to the respiratory chain inhibitors we observed an induction of ATF4, the key transcription factor of the integrated stress response (ISR). We found that the induction of *SESN2* transcripts could be prevented by the ISR inhibitory small molecule ISRIB. Also, by inhibiting or overexpressing ATF4 with specific shRNA or ATF4-expressing constructs, respectively, we have confirmed the role of ATF4 in the *SESN2* gene upregulation induced by mitochondrial dysfunction. At a distance of 228 bp upstream from the *SESN2* transcription start site we found a candidate sequence for the ATF4 binding site and confirmed its requirement for the induction of *SESN2* in luciferase reporter experiments. We suggest that the upregulation of *SESN2* by mitochondrial dysfunction provides a homeostatic feedback that attenuates biosynthetic processes during temporal losses of energy supply from mitochondria thereby assisting better adaptation and viability of cells in hostile environments.

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Introduction

Sestrins represent a small family of proteins involved in metabolic regulation and stress response. In mammals sestrins are encoded by 3 genes (*SESN1*, *SESN2* and *SESN3*) presumably sharing similar activities. Sestrins have antioxidant functions and enforce homeostasis, stress resistance and protection against deleterious oxidation-induced DNA modifications and mutations.[1,2] The functions of sestrins are mediated by several different mechanisms. The excess of hydrogen peroxide is eliminated by peroxiredoxins, which are regenerated by a sestrin-induced sulfinic reductase Srx.[1,3] Sesn1 and Sesn2 proteins induce an activity of the transcription factor Nrf2 that controls a set of key antioxidant enzymes, including the Srx, through an activation of the p62-dependent autophagic degradation of Kelch-like ECH-associated protein 1 (Keap1), the inhibitor of Nfr2.[3,4] Besides, the antioxidant effect is achieved through the ability of sestrins to inhibit mTORC1 leading to overall decrease in metabolic activity [5-7] and stimulation of the autophagy.[8,9] Finally sestrins further attenuate metabolism by assisting the LKB1-dependent activation of the AMP-dependent protein kinase.[10] Collectively, the activities of sestrins enforce homeostasis under conditions of changing workloads and facilitate cells' viability and adaptation to different stresses.[11,12]

The expression of sestrins is controlled by a set of transcription factors. In response to genotoxic stresses *SESN1* and *SESN2* are induced by *p*53,[13,14] and *SESN3* – by FOXO[15,16] transcription factors. The induction of *SESN1* and *SESN2* by hypoxia is mediated both by p53 [14] and by the transcription factor HIF1.[17,18] In addition, oxidative stresses induce an Nrf2-mediated stimulation of *SESN2* through the antioxidant response element (ARE) present within the gene.[19] Nutrients availability also affects the expression of sestrins. A deficiency of nutrients, such as low glucose, stimulates SESN2 expression along with some other p53-regulated genes involved in metabolic regulation and cell cycle arrest through the formation of a complex between p53 and PPARγ coactivator–1α (PGC-1α).[20] On the contrary, the excess of saturated fatty acids that result in the endoplasmic reticulum (ER) stress in hepatocytes is accompanied by the SESN2 induction mediated by the transcription factor c/EBP-β.[21] Remarkably, treatment of cells with the anticancer drugs nelfinavir and bortezomib that induce the ER stress and the autophagy were also shown to activate the expression of *SESN2*, along with the known ER stress markers, such as transcription factors ATF3, ATF4 and CHOP. It was found that ATF4 was solely responsible for the induction of SESN2.[22]

ATF4 (Activating transcription factor 4) plays a pivotal role in responses of cells to stresses.[23,24] Different stressful

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conditions induce 4 protein kinases: the PKR-like endoplasmic reticulum kinase (PERK), the general control nonderepressible 2 (GCN2) serine/threonine kinase, protein kinase R (PKR) and heme regulator inhibitor (HRI).[25] These 4 protein kinases induce phosphorylation of the alpha-subunit of translation factor eIF2. This blocks the exchange of eIF2-GDP to eIF2-GTP, thus reducing global translation initiation and subsequent protein synthesis. However, the eIF2α phosphorylation paradoxically enhances the translation of mRNAs containing upstream open reading frames (uORFs), in particular the transcription factor ATF4.[25,26] ATF4 target genes are involved in biosynthesis, folding and assembly of proteins, metabolism, transport of nutrients, protection against oxidative stress and regulation of apoptosis. As ATF4 integrates signals from different kinases upstream of eIF2α,[24] the eIF2α / ATF4 cascade is commonly referred to as the Integrated Stress Response (ISR). Besides the translational regulation in response to stresses, the ATF4 is upregulated at the level of transcription.[27,28] Presumably the combination of transcriptional and translational regulations allows the eIF2-kinase signaling pathway to selectively repress or activate key regulatory genes of the cell.[28]

Mitochondrial dysfunction caused by an inhibition of the respiratory chain induces expression of stress-responsive genes. We have shown recently that the early response to the inhibition of mitochondrial respiratory complex III that enforces cell survival depends on the transcription factor ATF4.[29] However, the sustained inhibition of complex III induces stabilization and activation of the p53 tumor suppressor, due to the shut-off of dihydroorotate dehydrogenase (DHODH), a key enzyme of the *de novo* pyrimidine biosynthesis pathway.[30] We found that the activation of p53 abolishes and reverses the earlier ATF4-mediated pro-survival stress response and induces apoptotic cell death.[29] Upon the p53 activation the expression of many known ATF4-target genes is substantially downregulated, with the exception of *SESN2* whose expression is upregulated not only early (4-5 h) after the addition of complex III inhibitor myxothiazol, but also at later stages (13-17 h) when p53 activity is increased.[29] *SESN2* is known to be a transcriptional target of p53.[14] Here we show that in response to inhibition of mitochondrial respiratory chain the expression of *SESN2* is induced not only by p53, but also by ATF4. We have identified a potential ATF4 binding site and confirmed experimentally its requirement for the induction of *SESN2* in response to ATF4. We conclude that the induction of *SESN2* expression in response to the mitochondrial respiratory chain dysfunction is mediated by ATF4, the key transcription factor of the integrated stress response. Presumably the mechanism provides a homeostatic feedback that attenuates metabolism in response to temporal loss of energy supply from mitochondria and assists in better adaptation and viability of cells in hostile environments.

Results

The induction of SESN2 *expression in response to an inhibition of mitochondrial respiratory chain does not depend on the tumor suppressor p53*

In the previous study[29] we monitored quantitative changes in transcripts following a treatment of HCT116 colon carcinoma cells with the mitochondrial respiration chain complex III inhibitor myxothiazol (1μM). In particular, we found that levels of mRNA for *SESN2* gene are substantially increased (Table 1). As *SESN2* is a transcriptional target of the p53 tumor suppressor[14] we hypothesized that the observed transcriptional activation of SESN2 might depend on p53 that is activated by the inhibition of mitochondrial electron transport chain (ETC) complex III due to a deficiency of the ETC-dependent dihydroorotate dehydrogenase, a mitochondrial membrane enzyme of the de novo pyrimidine biosynthesis pathway.[30,31] To check the hypothesis, the cells were treated with myxothiazol in the presence of uridine that restores pools of uridylic and cytidylic nucleotides, and completely prevents the p53 stabilization and activation.[30] It was found that uridine did not affect the induction of *SESN2* mRNA in response to myxothiazol treatment for 13 h, while completely preventing the p53 activation, which in particular was supported by the lack of induction of transcripts from the p53-reponsive *TP53INP1* (Table 1). We concluded that the induction of *SESN2* expression in this system was not dependent on p53. This was supported by the fact that by 5 h of myxothiazol treatment, before the onset of activation of p53, the expression level of *SESN2* mRNA was higher than that after the 13 h treatment (Table 1).

By RT-qPCR we have confirmed the RNA-seq results. We have shown that a short term inhibition of complex III in HCT116 cells induces *SESN2* mRNA substantially to the same extent as the p53 activation by nutlin-3, an inhibitor of the p53/Mdm2 interaction[32] (Fig. 1A and B). A combined treatment with myxothiazol and nutlin-3 did not show further increase in SESN2 mRNA levels. In HCT116 p53-/- knockout cells the treatment with myxothiazol (but not with nutlin-3) induced *SESN2* mRNA expression (Fig. 1A and B). The respiratory chain complex I inhibitor piericidin A also induced *SESN2* mRNA (Fig. 1C), although no p53 activation was observed (Fig. 1D). In HeLa and RKO human carcinoma cell lines as well as in human embryonic kidney cell line HEK293T the inhibition of mitochondrial respiratory chain also induced *SESN2* mRNA (Figs. 2 and 3), arguing against a cell line specific effect.

As we have previously detected the induction of ATF4 (Table 1) and some of the ATF4-regulated genes after a short-term inhibition of complex III,[29] we hypothesized that ATF4, a key factor of ISR, could be responsible for the induction of *SESN2* upon mitochondrial dysfunction. Therefore, we tested levels of *SESN2* expression after a treatment with the ISR inhibitor ISRIB, as well as in the cells with either inhibited or overexpressed ATF4.

Table 1. Fold change in mRNA levels of HCT116 cells treated with 1μM myxothiazol for 5 h (M5), 13h (M13), 17h (M17) or with 1μM myxothiazol and 1 mM uridine for 13 h (MU13). The data was obtained by RNA-seq;29 FDR <0.05.

	mRNA fold change			
Gene	M 5	M 13	M 17	MU 13
SESN2	5.32	2.71	1.95	2.99
TP53INP1	NC	10.8	28.6	NC
ATF4	2.46	0.43	0.24	1.76

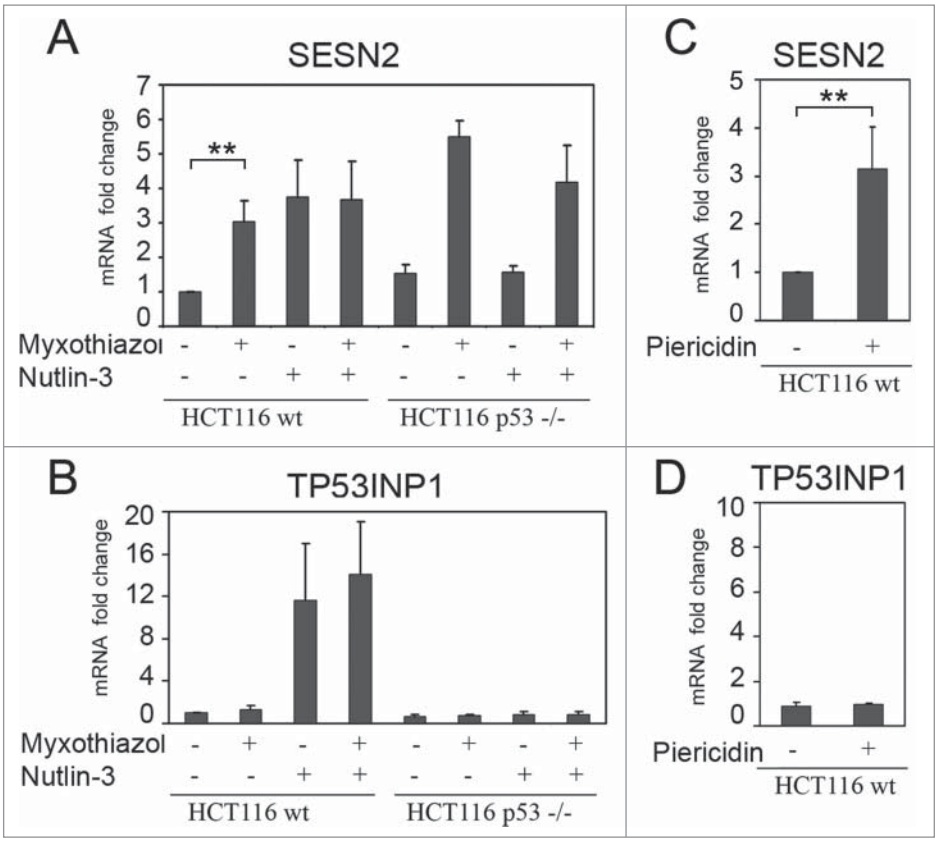

Figure 1. Induction of *SESN2* mRNA in response to the inhibition of the mitochondrial respiratory chain is independent of p53. (A, B) Fold changes of *SESN2* mRNA (A) and of the p53-target gene *TP53INP1* mRNA (B) in wild-type or p53-/- HCT116 cells after myxothiazol (4 h) and/or nutlin-3 (16 h) treatment. (C, D) Fold changes of *SESN2* (C) and *TP53INP1* (D) mRNAs in HCT116 cells treated with piericidin A (4 h). The data was obtained by RT-qPCR, the normalization was to 18S rRNA. The means and standard deviations on the basis of at least 3 independent experiments are presented. Student's t-test was used to analyze statistical significance ($^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P< 0.001$).

Effects of an ISR inhibitor and low and high ATF4 levels on SESN2 gene expression

ISRIB, a small molecule inhibitor of the integrated stress response,[33,34] was shown to counteract negative effects of eIF2α phosphorylation on translation by acting downstream of all eIF2-kinases. Phosphorylation of eIF2α attenuates protein synthesis by inhibiting eIF2B, a guanine nucleotide exchange factor (GEF), which accelerates the exchange of GDP for GTP in the eIF2 complex. ISRIB has been found to stabilize eIF2B, increase its GEF activity and restore protein synthesis even in the presence of the factors that cause cellular stress.[35,36] Besides ISRIB specifically inhibits translation of stress-inducible mRNAs, containing uORFs, including the *ATF4* mRNA. [33]

We found that ISRIB prevented in HeLa cells and reduced significantly in HEK293T and HCT116 cells the induction of *SESN2* mRNA in response to myxothiazol

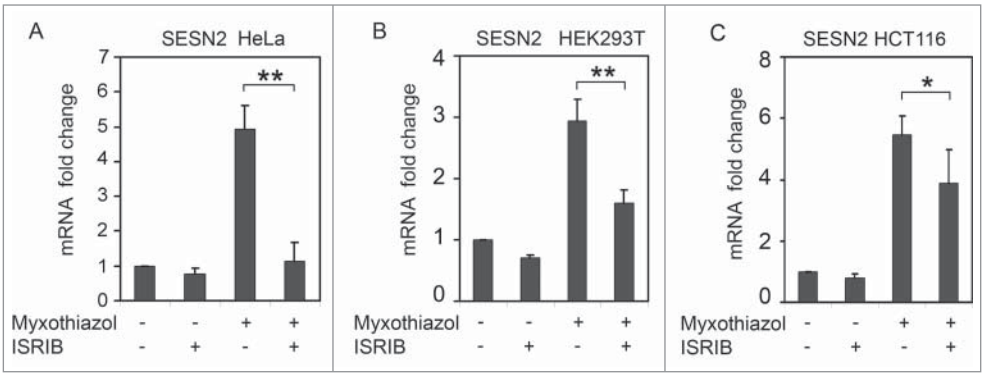

Figure 2. Induction of *SESN2* mRNA in response to the inhibition of mitochondrial respiratory chain depends on ISR. *SESN2* mRNA level in HeLa (A), HEK293T (B) and HCT116 (C) cells after treatment with 1μM myxothiazol, with and without 200 nM ISRIB (4h). The data was obtained by RT-qPCR, normalized to18S rRNA. The means and standard deviations on the basis of at least 3 independent experiments are presented. Student's t-test was used to analyze statistical significance ($^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P< 0.001$).

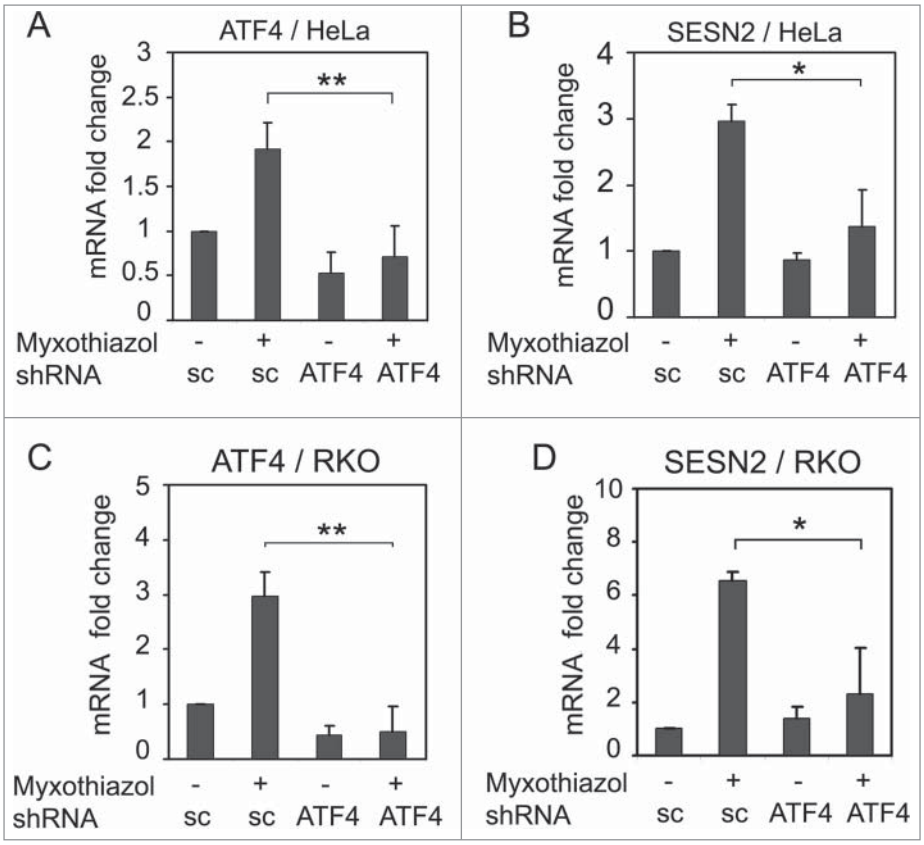

Figure 3. The effect of *ATF4* knockdown on *SESN2* mRNA expression. *ATF4* (A, C) and *SESN2* (B, D) mRNA fold changes in HeLa (A, B) and RKO (C, D) cells expressing *ATF4* shRNA (ATF4) or a scrambled control shRNA (sc). The cells were treated with 1 μM myxothiazol for 4 h where indicated. The data was obtained by RT-qPCR and normalized to 18S rRNA. The means and standard deviations on the basis of 3 independent experiments are presented. Student's t-test was used to analyze statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

treatment (Fig. 2). It confirms a role of ISR in the induction of SESN2 expression in response to mitochondrial dysfunction and suggests an involvement of the transcription factor ATF4.

This conclusion has been directly tested by specific inhibition of *ATF4* expression by RNA interference.[37] Lentiviral constructs expressing shRNA to *ATF4* mRNA were introduced into HeLa or RKO cells with subsequent puromycin selection. Using RT-qPCR, we confirmed that ATF4 mRNA levels were substantially decreased in these cells (Fig. 3A and C). The expression of the ATF4-specific shRNA prevented the induction of *ATF4* and SESN2 mRNAs in response to the short-term treatment with myxothiazol, but did not influence the basal levels of *SESN2* mRNA (Fig. 3). We conclude that the induction of SESN2 gene expression in response to respiratory chain complex III inhibition depends on the ATF4 transcription factor.

In a reciprocal experiment we overexpressed *ATF4* mRNA in HCT116 and HeLa cells by introducing an *ATF4* cDNA expression construct (Fig. 4A and B). The ectopic expression of *ATF4* mRNA in HCT116 cells resulted in a significant (1.8-fold, p-value 0.0023) increase in *SESN2* mRNA levels (Fig. 4C) and even higher induction of *SESN2* (6.5-fold) when ATF4 was overexpressed in HeLa cells (Fig. 4D). The results confirm the ability of ATF4 to stimulate *SESN2* expression.

Molecular mechanism of regulation of SESN2 *expression by ATF4*

To identify DNA elements responsible for the ATF4-mediated regulation, we placed firefly luciferase reporter gene downstream of the promoter and 5'-UTR region of *SESN2* gene. ATF4 regulates transcription of its target genes through the formation of homodimers or heterooligomers with the transcription factors Jun, AP-1 and C/EBP[38,39] that bind to CARE (C/EBP-ATF) responsive elements having the consensus sequence XTTXCATCA (where X = G, A or T).[39] In the region from -625 to -618 bp relative to the *SESN2* translation start codon (from -228 to -221 bp relative to the transcription start site) we found a candidate sequence for the ATF4 binding site TTTTCATCA. To test whether the sequence is important for the ATF4-mediated transcription regulation we amplified by PCR the genome segment comprising 724 bp upstream of the translation start codon of Sesn2 ORF and cloned it into the pGL3-Basic reporter plasmid carrying firefly luciferase gene (Fig. 5A). The reporter construct pGL3-*SESN2* was co-transfected into HCT116 or HeLa cells along with the normalization control plasmid expressing beta-galactosidase driven by a constitutive promoter.

In response to the ectopic expression of ATF4 there was a 2-fold increase in the luciferase activity in HCT116 cells and almost a 6-fold increase in HeLa cells (Fig. 5B and C),

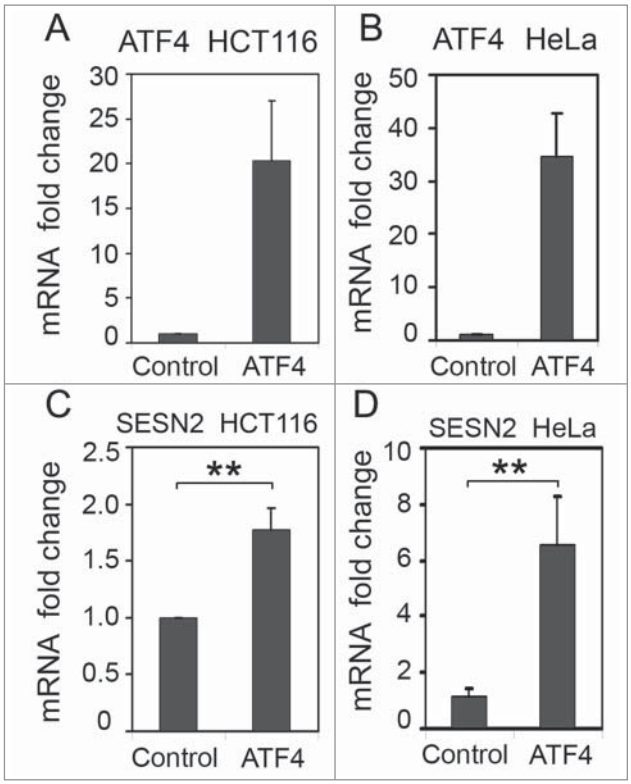

Figure 4. The effect of ATF4 overexpression on *SESN2* mRNA levels. *ATF4* (A, B) and *SESN2* (C, D) mRNA fold changes in HCT116 (A, C) and HeLa (B, D) cells with ectopic expression of *ATF4* mRNA (ATF4) or transfected with a control empty vector (Control). The data was obtained by RT-qPCR and normalized to 18S rRNA. The means and standard deviations on the basis of 3 independent experiments are presented. Student's t-test was used to analyze statistical significance ($^{*}P < 0.05$, $^{**}P<0.01$, $^{***}P< 0.001$).

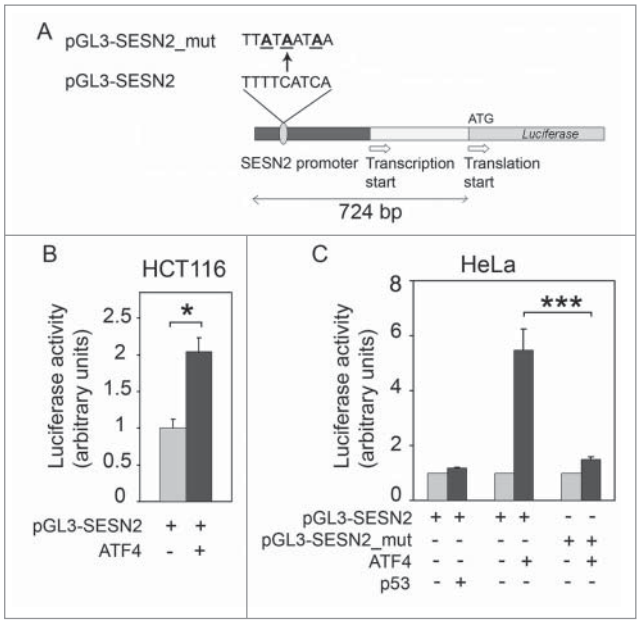

Figure 5. Overexpression of ATF4 (but not p53) stimulates the luciferase reporter controlled by the SESN2 gene promoter region. (A) The reporter constructions. (B) Stimulation of luciferase reporter by ATF4 expression in HCT116 cells compared to the control HCT116 cells transfected with the empty vector. (C) The activity of reporters pGL3-SESN2 or pGL3-SESN2_mut with a mutated putative ATF4 binding site in HeLa cells with ectopic expression of p53 or ATF4 compared to control cells transfected with an empty vector. The effects of ATF4 and p53 expression are presented as relative values in comparison to normalized reporter activities in control cells. The means and standard deviations on the basis of at least 3 independent experiments are presented. Student's t-test was used to analyze statistical significance ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$).

suggesting that the transcription factor ATF4 is capable of stimulating the *SESN2* gene promoter. To test this hypothesis, the putative ATF4 binding site was mutated at the 3 most conserved positions by turning TT**T**C**A**T**C**A into TT**A**T**A**A**T**A**A** in the reporter plasmid pGL3-*SESN2*_mut (Fig. 5A). As expected, the changes prevented the increase in reporter gene activity in response to the ATF4 overexpression (Fig. 5C). We conclude that the regulation of *SESN2* expression by the ATF4 transcription factor is mediated by the TTTTCATCA site located 221-228 bp upstream of the transcription start site. Also, despite the previously identified putative p53-binding site located between the transcription and translation start sites,[40] the ectopic expression of p53 did not result in the increase of the reporter activity (Fig. 5C).

Discussion

In the present study we have demonstrated that in response to the mitochondrial dysfunction caused by the inhibition of mitochondrial respiratory chain there is a substantial induction of *SESN2* gene expression. The induction presumably occurs as a part of the ISR and depends on the ATF4 transcription factor. The inhibition of ISR by ISRIB or the suppression of ATF4 by RNA-interference prevents the induction of *SESN2* mRNA in response to the mitochondrial respiratory chain dysfunction. On the contrary, the ectopic expression of ATF4 is capable of increasing the level of *SESN2* mRNA.

It has been recently reported that the expression of *SESN2* gene is induced by the ER stress.[22,41] The peptidilarginine deiminase (PAM) family inhibitor YW3-56 that is capable of inducing the ER stress, was shown to stimulate the *SESN2* gene expression in an ATF4-dependent manner in triple-negative breast carcinoma cells [41] that is in line with our results. The increased levels of Sesn2 protein were also observed in cells treated with the inducer of the ER stress nelfinavir and proteasome inhibitor bortezomib, and the effect was similar upon the ectopic expression of ATF4 in HeLa cells.[22] However, the molecular mechanism of this regulation has not been elucidated.[22,41] The results of our present experiments with reporter plasmids suggest that the mechanism of *SESN2* regulation includes binding of the ATF4 transcription factor to TTTTCATCA site within 221-228 bp upstream of the transcription start site. ChIP-exo analysis [41] has recently detected ATF4 binding after YW3-56 treatment near the position -138 bp of *SESN2* gene promoter relative to the transcription start site. The data has been confirmed by the conventional ChIP assay. [41] We suppose that the difference might be due to the lack of accuracy of the ChIP method (taking into account that for the ChIP experiments chromatin was fragmented to a range from 0.5 to 1 kb in ref. [41]). It is also worth noting that the DNA sequence TTTGCAGCA located at the position −138 bp of SESN2 gene promoter does not match the canonical ATF4 consensus XTTXCATCA. [39,41]

The ATF4-binding site TTTTCATCA within the *SESN2* gene promoter identified and validated in this study matches

the consensus CARE sequence. We found no absolutely identical sequences among the previously identified ATF4-responsive elements in the promoters of known ATF4-regulated genes.[39] There is only one nucleotide difference between the element in *SESN2* gene and the nutrient-sensing response element 1 (NSRE1) in the ATF4-inducible asparagine synthase gene *ASNS* (**G**TTTCATCA), and the amino acid response element (AARE) in the pseudokinase gene *TRIB3* (TTT**G**CATCA).[39] Both elements are responsible for transcriptional induction of the genes in response to amino acid starvation and ER stress.

Our data does not confirm the presence of functional p53-response elements between *SESN2* transcription and translation start sites.[40] Perhaps the element is located in the region further downstream at position +733 bp or +10 kb, as has been previously suggested.[42,43]

What could be the functional role of *SESN2* gene induction in response to the mitochondrial dysfunction? The induction of *SESN2* gene expression is known to occur in response to oxidative, electrophilic and genotoxic stresses.[3,5,14] Functions of the Sesn2 protein play important roles in the regulation of reactive oxygen species balance and in the control of energy metabolism. An inhibition of the mitochondrial respiratory chain may lead to the formation of reactive oxygen species [44] and a transient energy deficiency. Thus, the induction of *SESN2* gene in response to a mitochondrial dysfunction may have adaptive and pro-survival roles through the ISR, by enforcing the detoxification of reactive oxygen species and aligning metabolic processes with the changing energy supplies. An inhibition of mitochondrial respiratory chain can lead to a shortage of energy required for protein biosynthesis. Being an inhibitor of mTORC1[5] Sesn2 protein attenuates overall biosynthetic activity and contributes to mitigation of the metabolic stress load. Also, during the ER stress a restoration of protein synthesis prior to a complete stress recovery may lead to apoptotic cell death.[45] Therefore, Sesn2 may promote stress resistance and viability of the cell through the inhibition of mTORC1 during energy shortages. Indeed, *SESN2* was recently implicated as a sensor of energy stresses mediated by the reduction of ATP levels in cancer cells treated with the glycolysis inhibitor 2-deoxyglucose. The induction of *SESN2* protein leading to the suppression of mTORC1 was shown to protect cells from energetic stress-induced apoptosis.[46]

The results presented in this study suggest that the ATF4-dependent induction of *SESN2* gene expression in response to mitochondrial dysfunction may contribute to integrated stress response and enforce better adaptation of cells to changing environmental conditions and energy supplies.

Materials and methods

Plasmid constructs

pHM-ATF4

ATF4-coding sequence was amplified by PCR using a total cDNA from the human colon carcinoma cell line RKO as a template and primers Atf4_dir (5′-AGGATCCGCAACATGACCGAAATG-3′) and Atf4_rev (5′-TTGAAGCTTGGTGCGCGCCAGGACCC-3′) containing sites for restriction nucleases BamHI and PstI, respectively. The amplified fragment was cloned into the pUC19 plasmid and sequenced. The sequence-verified insert of ATF4 cDNA was cloned into BamHI and PstI sites of pcDNA4/HisMax/B expression vector (Invitrogen) for a transient expression in mammalian cells.

pGL3-SESN2

To obtain *SESN2* promoter fragment the PCR was performed on a total DNA from the human colon carcinoma cell line HCT116 with primers *SESN2*_dir (5′-GAGAGCTC TTAACCCTAGCCAGTCC-3′) and *SESN2*_rev (5′-TTGAAGCTTGGTGCGCG CCAGGACCC-3′). The PCR product was inserted into the pUC19 vector between SacI and HindIII restriction sites, then sequenced and recloned as a SacI-HindIII fragment into the vector pGL3-Basic (Promega).

pGL3-SESN2mut

Mutations within the putative ATF4-binding site were introduced by the megaprimer PCR. The first PCR was performed with primers SESN2_dir and SESN2_mut_rev (5′-GGGTTGCGTTATTATAAGGGACTTCACTG-3′), the plasmid pUC19 with inserted SESN2 gene promoter was used as a template. After agarose gel purification the PCR product was used as a forward primer in the second PCR with the other primer SESN2_rev. The PCR product was inserted into pUC19 between the sites SacI and HindIII, then sequenced and recloned as a SacI-HindIII fragment into the vector pGL3-Basic.

Cancer cell cultures

Cells were grown in DMEM, containing 10% fetal calf serum (FBS) at 37°C, 5% CO2 to 50-70% confluence. For various treatments we used 1 μM myxothiazol (Sigma-Aldrich Inc.), 1 mM uridine, 2 μM piericidin A (Sigma-Aldrich Inc.), 10 μM nutlin-3 (AdooQ BioScience); treatment conditions are indicated in figure legends. The RNA-seq conditions were previously described in ref. 29, raw sequencing data were deposited in NCBI (Bioproject accession number SRP043021).

Preparation of the lentiviral vector for the expression of ATF4-specific short hairpin RNA (with the sequence sh2 – gatccgGCCAAGCACTTCAAACCTCATCACGTGATGAGGTTTGAAGT GCTTGGCttttg) and its introduction into RKO and HeLa cells was described in ref. 29.

Trancriptional reporters

HCT116 and HeLa cells were grown in 12-well plates to 50-70% confluency and transfected with the TurboFect transfection reagent (Thermo Scientific) according to the manufacturer's protocols. Reporter plasmids pGL3-SESN2 or pGL3-SESN2_mut (0.1 μg), pcDNA4/HisMax/lacZ (Invitrogen), encoding β-galactosidase under control of the constitutive CMV promoter (0.5 μg), and the expression vectors pHM-ATF4 (0.5 μg), encoding the transcription factor ATF4, or pCMV-p53 (0.5 μg), encoding the tumor suppressor p53, were transfected into a single well of the 12-well culture plate. The total plasmid DNA was adjusted to 2 μg by addition of the

"empty" vector pcDNA4/HisMax/B (Invitrogen). Cells were lysed in the Reporter lysis buffer (Luciferase Assay System, Promega) 44 h after transfection, the luciferase and β−galactosidase activities were measured as described previously.[47] Luciferase activity was normalized to β−galactosidase activity of the same lysate. For each measurement, at least 3 biological replicates were used. Mean values with standard deviations are presented.

Real-time PCR

RNA isolation, reverse transcription and real time PCR were performed as previously described.[29] Real-time PCR detection system CFX96 (Bio-Rad) and the following primers were used: ATF4_dir CTTCACCTTCTTACAACCTCTTC, ATF4_rev GTAGTCTGGCTTCCTATCTCC; 18S_dir CGGACAGGATTGACAGATTG, 18S_rev CAGAGTCTCGTTCGTTATCG; SESN2 dir: TTCGGATATGAGGACTTC; SESN2 rev: ATGGTATTGTAGGTGAGG; TP53INP1_dir TCAGCAGAAGAAGAAGAAGAAGAG, TP53INP1_rev AGCAGGAATCA CTTGTATCAGC. Quantification of the target genes was normalized using the reference 18 S rRNA to compensate for inter-PCR variations.

Abbreviations

ATF4	Activating Transcription Factor 4
CARE	C/EBP-ATF responsive element
eIF2α	eukaryotic initiation factor 2, α subunit
ER	endoplasmic reticulum
FDR	false discovery rate
ISR	integrated stress response
ISRIB	inhibitor of the integrated stress response
qPCR	quantitative real-time PCR
RT	reverse transcription
shRNA	small hairpin RNA
TP53INP1	tumor protein p53 inducible nuclear protein 1
uORF	upstream open reading frame
UPR	un-folded protein response
5'-UTR	5'-untranslated region

Disclosure of potential conflicts of interest

The authors declare no conflict of interest.

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