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# Sestrin2 inhibits uncoupling protein 1 expression through suppressing reactive oxygen species

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**Uncoupling protein 1 (Ucp1), which is localized in the mitochondrial inner membrane of mammalian brown adipose tissue (BAT), generates heat by uncoupling oxidative phosphorylation. Upon cold exposure or nutritional abundance, sympathetic neurons stimulate BAT to express *Ucp1* to induce energy dissipation and thermogenesis. Accordingly, increased *Ucp1* expression reduces obesity in mice and is correlated with leanness in humans. Despite this significance, there is currently a limited understanding of how *Ucp1* expression is physiologically regulated at the molecular level. Here, we describe the involvement of Sestrin2 and reactive oxygen species (ROS) in regulation of *Ucp1* expression. Transgenic overexpression of Sestrin2 in adipose tissues inhibited both basal and cold-induced *Ucp1* expression in interscapular BAT, culminating in decreased thermogenesis and increased fat accumulation. Endogenous Sestrin2 is also important for suppressing *Ucp1* expression because BAT from *Sestrin2*$^{-/-}$ mice exhibited a highly elevated level of *Ucp1* expression. The redox-inactive mutant of Sestrin2 was incapable of regulating *Ucp1* expression, suggesting that Sestrin2 inhibits *Ucp1* expression primarily through reducing ROS accumulation. Consistently, ROS-suppressing antioxidant chemicals, such as butylated hydroxyanisole and *N*-acetylcysteine, inhibited cold- or cAMP-induced *Ucp1* expression as well. p38 MAPK, a signaling mediator required for cAMP-induced *Ucp1* expression, was inhibited by either Sestrin2 overexpression or antioxidant treatments. Taken together, these results suggest that Sestrin2 and antioxidants inhibit *Ucp1* expression through suppressing ROS-mediated p38 MAPK activation, implying a critical role of ROS in proper BAT metabolism.**

aging | mouse | homeostasis | β-adrenergic signaling

Although reactive oxygen species (ROS) are normal products of cellular metabolism, excessive accumulation of ROS resulting from nutritional imbalance and/or environmental stresses can provoke oxidative damage of diverse cellular macromolecules, such as DNA, RNA, and proteins (1). Accumulation of ROS has been associated with diverse degenerative diseases, such as cancer, neurodegeneration, and obesity-associated metabolic syndrome (2–4). To minimize detrimental consequences of ROS accumulation, cells are equipped with various antioxidant proteins, including superoxide dismutases, catalases, peroxiredoxins, and sestrins (5–7). Several ROS-scavenging chemicals or dietary supplements, such as butylated hydroxyanisole (BHA), *N*-acetylcysteine (NAC), and antioxidant vitamins, can assist with eliminating excessive amounts of ROS (8–10) and were once considered to be potential inhibitors of degenerative diseases associated with aging and obesity (11–13). However, most animal and human clinical studies failed to demonstrate the benefits of dietary antioxidants in restoring metabolic homeostasis or in promoting health and lifespan (13, 14).

Uncoupling protein 1 (Ucp1) is an anion-carrier protein located in the inner membrane of the mitochondria. By dissipating the proton gradient across the mitochondrial inner membrane, Ucp1 uncouples substrate oxidation from ATP synthesis, ultimately reducing ATP production and generating heat (15). Ucp1-mediated mitochondrial uncoupling also suppresses ROS production during respiration (16). *Ucp1* expression is induced upon exposure to cold temperature or nutritional overload, and this induction is important for protection of organisms against cold and obesity (17). Despite the significance of Ucp1 in energy metabolism, it is poorly understood how *Ucp1* expression is regulated, other than the fact that cAMP and p38 MAPK signaling pathways (18–20) are necessary for *Ucp1* induction upon cold stimuli. It also has not yet been explored whether subcellular ROS can regulate *Ucp1* expression and subsequent heat generation.

Sestrins are a family of stress-inducible proteins that regulate metabolic homeostasis (21). Sestrins have two independent biological activities largely divided into regulating AMP-activated protein kinase (AMPK)-mammalian target of rapamycin complex 1 (mTORC1) signaling and suppressing ROS accumulation (22). Loss of endogenous sestrins can provoke a variety of metabolic pathologies, including insulin resistance, fat accumulation, mitochondrial dysfunction, and oxidative damage (23, 24). Given the known roles of endogenous sestrins in reducing oxidative stress, fat accumulation, and insulin resistance, we thought that overexpression of sestrins may protect animals from developing obesity or obesity-associated metabolic pathologies. Among the three mammalian sestrins (Sestrin1–3), the metabolism-regulating

## Significance

Antioxidant therapy was once considered useful for treating metabolic syndrome because excessive accumulation of reactive oxygen species (ROS) was identified as an inducer of diverse metabolic pathologies. However, the effectiveness of dietary antioxidants in treating obesity-associated diseases had been largely controversial in numerous animal and human clinical studies, some of which actually show adverse effects upon antioxidant consumption. Here, we show that Sestrin2 and other antioxidants can interfere with *uncoupling protein 1* (*Ucp1*) expression through suppression of ROS-mediated p38 MAPK activation. Ucp1, a protein responsible for heat generation and energy dissipation, is known to suppress diverse metabolic pathologies associated with obesity and aging. Thus, our results explain why some antioxidant therapies were not successful in treating obesity-associated diseases and extending health and lifespan in mammals.

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The authors declare no conflict of interest.

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functions of Sestrin2 have been the most rigorously characterized in metabolic organs, such as the liver and adipose tissue (AT) (23, 25). Thus, to examine the effects of Sestrin2 overexpression, we generated tetracyclin-regulated promoter-*Sestrin2* (*tet-Sesn2*) transgenic mice that can express Sestrin2 in a tissue-specific manner when crossed with tissue-specific tetracycline activator (tTA) strains.

Using *tet-Sesn2* and AT-specific peroxisome proliferator-activated receptor γ (Pparγ)-tTA (*Pparγ-tTA*) strains, we generated *Pparγ-tTA*/*tet-Sesn2* mice that express Sestrin2 specifically in AT. Given the ROS- and mTORC1-suppressing functions of Sestrin2 (21), we expected that the *Pparγ-tTA*/*tet-Sesn2* (PG-Sn2) strain would exhibit decreased fat accumulation and improved metabolic homeostasis compared with the control strain. However, we found that Sestrin2 overexpression unexpectedly increased fat accumulation, which is associated with dramatic suppression of *Ucp1* expression in brown adipose tissue (BAT). The redox-regulating function of Sestrin2, rather than its mTORC1-inhibiting function, was responsible for its *Ucp1* regulation. We also discovered that administration of chemical antioxidants, such as BHA or NAC, could inhibit cAMP (in vitro)- or cold (in vivo)-induced *Ucp1* expression. These results reveal a critical role of ROS in basal and cold-induced expression of *Ucp1* in BAT.

## Results

**Generation of Transgenic Mice That Express Sestrin2 in AT.** To investigate the metabolic consequences of Sestrin2 overexpression, we engineered mice to express Sestrin2 specifically in AT by crossing *tet-Sesn2* and *Pparγ-tTA* strains. PG-Sn2 offspring that inherited both transgenes have AT-specific Sestrin2 expression because *Pparγ-tTA* drives the expression of tTA only in AT (26), and tTA is required for transcriptional activation of the *tet-Sesn2* transgene (Fig. 1*A*). Indeed, PG-Sn2 mice had prominent overexpression of transgenic Sestrin2 in all AT, including BAT, epididymal white adipose tissue (eWAT), and s.c. white adipose tissue (sWAT) (Fig. 1*B*). Transgenic Sestrin2 expression was not detected in other tissues, such as the liver or skeletal muscle (Fig. 1*B*), confirming the strict tissue specificity. Offspring that inherited only the *Pparγ-tTA* (PG) transgene were used as control mice.

**AT-Specific Transgenic Sestrin2 Increases Body and Tissue Weights.** Because Sestrin2 is a potent suppressor of mTORC1 and mTORC1 promotes adipogenesis, we originally hypothesized that Sestrin2 overexpression in AT would provoke typical consequences of mTORC1 inhibition, such as decreased AT volume (27). However, we found that PG-Sn2 mice actually exhibited a slight increase in body weight (Fig. 1*C*), which was associated with an increase in liver (Fig. 1*D*) and eWAT (Fig. 1*E*) weights but was unrelated to sWAT (Fig. S1*A*) weight. These differences in body and tissue weights were gradually diminished after induction of obesity via a high-fat diet (HFD) (Fig. S2 *A*–*D*).

**Transgenic Sestrin2 Induces Whitening of BAT.** We examined the AT histology of control and PG-Sn2 mice. Interestingly, Sestrin2 overexpression dramatically increased the lipid droplet size of BAT in mice kept on both a low-fat diet (LFD) and the HFD (Fig. 1 *F* and *H*). In contrast, the effect of Sestrin2 on lipid droplet size was negligible in eWAT (Fig. 1 *G* and *H*) and sWAT (Fig. S1 *B* and *C*). Sestrin2 overexpression also did not alter the level of free fatty acids in serum (Fig. S1*D*). Because the energy-dissipating function of BAT is important for balancing nutrient intake and energy expenditure (28), it is likely that Sestrin2 affected the organism-level energy balance by disrupting BAT homeostasis.

**Transgenic Sestrin2 Regulates Both AMPK-mTORC1 and Redox Signaling in AT.** We examined if downstream signaling pathways of Sestrin2, especially redox signaling and AMPK-mTORC1 signaling (21), were regulated by transgenic Sestrin2 in AT. As expected, Sestrin2 overexpression strongly reduced ROS levels in BAT (Fig. S3 *A*–*D*), although it had only marginal effects in eWAT (Fig. S3 *E*–*H*). In addition, both BAT and eWAT of PG-Sn2 mice exhibited AMPK activation, mTORC1 inhibition, and AKT activation (Fig. S4). Consistent with the known metabolic activities of AMPK (29), Sestrin2-induced AMPK activation was associated with reduced lipogenic gene expression (Fig. S5*A*) and increased mitochondrial contents (Fig. S5 *B* and *C*) in BAT. However, these indications do not provide an explanation of how Sestrin2-expressing BAT accumulates more lipid droplets than control BAT (Fig. 1 *F* and *H*). It is possible that the ROS-controlling function of Sestrin2, rather than the

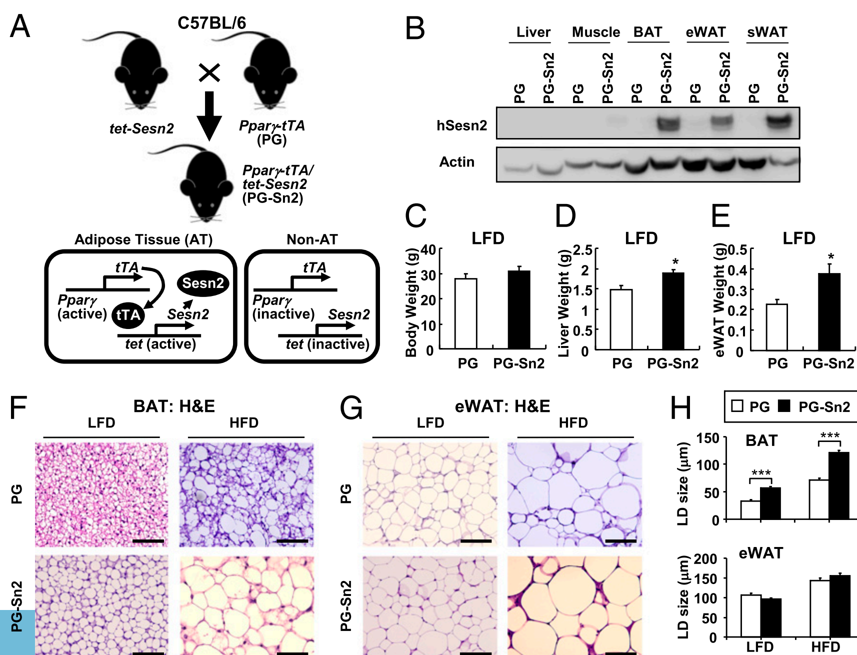


**Fig. 1.** Transgenic Sestrin2 expression increases fat accumulation in BAT. (*A*) Sestrin2 was overexpressed in AT of PG-Sn2 mice that were obtained from a genetic cross between *Pparγ-tTA* (PG) mice and *tet-Sesn2* mice. (*B*) Transgenic expression of human Sestrin2 in the indicated tissues was determined by immunoblotting. Total body (*C*), liver (*D*), and eWAT (*E*) weights were measured from 3-mo-old PG and PG-Sn2 mice kept on the LFD ($n$ = 3). (*F* and *G*) PG and PG-Sn2 mice were kept on the LFD for 3 mo and then on the HFD for 3 additional months ($n \geq 3$). BAT (*F*) and eWAT (*G*) from the indicated mice were analyzed by H&E staining. (*H*) Quantification of lipid droplet (LD) size of indicated tissues. Data are presented as mean ± SEM. $*P < 0.05$; $***P < 0.001$. (Scale bars, 200 μm.)



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AMPK-mTORC1–controlling function, was more significantly involved in regulation of BAT metabolism in PG-Sn2 mice.

**Sestrin2 Overexpression Does Not Alter 3T3-L1 Adipocyte Differentiation.** To determine whether Sestrin2 overexpression affects differentiation of adipocytes, we infected 3T3-L1 preadipocytes with Sestrin2-overexpressing lentiviruses and examined the level of differentiation. As predicted from the fact that 3T3-L1 cells differentiate into WAT-like tissues (30), Sestrin2 overexpression did not significantly alter adipocyte differentiation (Fig. S6 *A* and *B*). This result is also consistent with the finding that AT-specific transgenic Sestrin2 expression does not alter the differentiation and morphology of eWAT (Fig. 1*G*) or sWAT (Fig. S1*B*).

**Sestrin2 Overexpression Suppresses *Ucp1* Expression in Brown Adipocytes.** We then specifically examined the differentiation of brown adipocytes by isolating primary preadipocytes from interscapular BAT of WT mice and subjecting them to in vitro differentiation. As in 3T3-L1 cells, Sestrin2 overexpression did not interfere with differentiation of the primary brown preadipocytes as measured by lipid droplet accumulation (Fig. S6 *C* and *D*) or by expression of the general adipocyte marker (Pparγ) expression (Fig. 2*A*). However, expression of *Ucp1*, which is observed only in fully differentiated brown adipocytes, was dramatically reduced by Sestrin2 overexpression at both protein (Fig. 2*A*) and mRNA (Fig. 2*B*) levels. Interestingly, $Sestrin2^{C125S}$, a mutant Sestrin2 that is still capable of regulating AMPK-mTORC1 signaling (31) but incapable of suppressing ROS (7) (Fig. 2 *C* and *D* and Fig. S6 *E* and *F*), was unable to down-regulate *Ucp1* expression (Fig. 2 *A* and *B*). These data indicate that Sestrin2's antioxidant role, rather than its AMPK-mTORC1–controlling role, mediates *Ucp1* suppression in brown adipocytes.

***Ucp1* Expression, but Not *Ucp2* Expression, Is Reduced in BAT of PG-Sn2 Mice.** Ucp1 is essential for energy dissipation and thermogenesis in BAT (15), and loss of *Ucp1* can lead to complete whitening of BAT (32). Furthermore, increased lipid droplet size in BAT is observed in many animal models with reduced expression of *Ucp1* (32–35). Therefore, Sestrin2-mediated inhibition of *Ucp1* expression may provide a basis for the BAT-whitening phenotype observed in PG-Sn2 mice (Fig. 1 *F* and *H*). Supporting this idea, PG-Sn2 mice showed a dramatic reduction in mRNA (Fig. 2*E*) and protein (Fig. 2 *F* and *G*) expression levels of *Ucp1* in BAT. In contrast, expression of *Ucp2*, which does not mediate energy dissipation or thermogenesis in BAT (15), was unaltered by transgenic Sestrin2 overexpression (Fig. 2*E*).

**Transgenic Sestrin2 Inhibits Cold-Induced *Ucp1* Expression.** *Ucp1* expression becomes induced in BAT upon exposure to cold temperature. Low temperature increases the sympathetic neuronal activity that stimulates BAT to accumulate a large amount of cAMP, which then results in activation of p38 MAPK (18–20). Activated p38 MAPK subsequently phosphorylates several transcription factors that induce *Ucp1* (20, 36). As expected, *Ucp1* expression was up-regulated in BAT of control mice upon cold exposure and resultant p38 MAPK activation (Fig. 2 *E*–*G*). However, PG-Sn2 mice were defective in these processes; both p38 MAPK activation and *Ucp1* induction were strongly suppressed (Fig. 2 *E*–*G*). Also, in cultured brown adipocytes, Sestrin2 overexpression strongly inhibited isoproterenol (ISO, a β-adrenergic receptor agonist)-induced or forskolin (FSK, an adenylate cyclase activator that induces cAMP accumulation)-induced expression of *Ucp1* (Fig. 2 *H* and *I*), demonstrating that Sestrin2 can antagonize the action of the cold-induced cAMP signaling that promotes *Ucp1* expression.

**Transgenic Sestrin2 Interferes with Cold-Induced Thermogenesis.** Ucp1-mediated nonshivering thermogenesis in BAT can expend up to 20% of cold-induced oxygen consumption ($VO_2$) in mice initially kept at room temperature (37). The remaining $VO_2$ is attributed to other processes, such as shivering. We put control and PG-Sn2 mice in metabolic cages to examine their metabolic responses to cold exposure. Cold-induced elevation of $VO_2$ was significantly impaired in PG-Sn2 mice (Fig. 3 *A*–*C*) with no

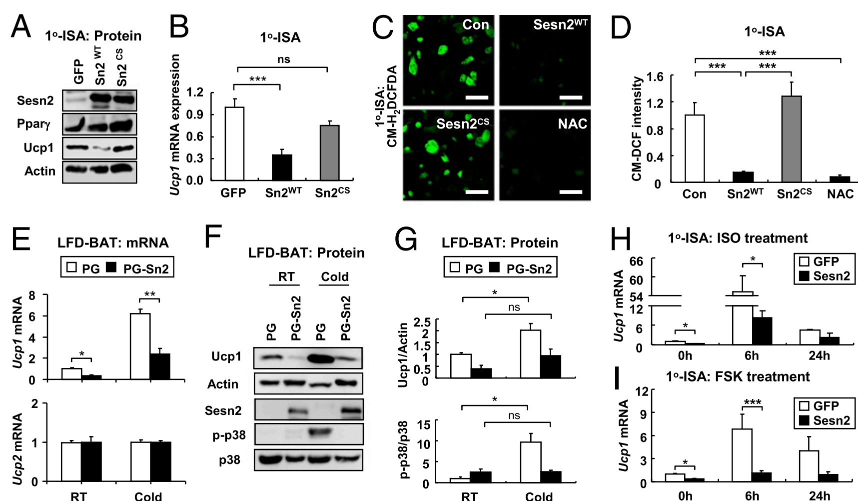


**Fig. 2.** Sestrin2 inhibits *Ucp1* expression by suppressing ROS. (*A*–*D*) Primary preadipocytes isolated from the interscapular depot (1°-ISA) were transduced with lentiviruses expressing GFP, luciferase (Con, *C* and *D*), WT Sestrin2 ($Sn2^{WT}$), or redox-inactive Sestrin2 mutant ($Sn2^{CS}$) and were differentiated into brown adipocytes ($n$ = 3). At day 8 of differentiation, protein expression was analyzed by immunoblotting (*A*), mRNA expression was examined by quantitative RT-PCR (*B*), and intracellular ROS were visualized by chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (CM-$H_2$DCFDA) staining (*C*). (*D*) Fluorescence intensities of CM-DCF, the oxidized form of 2′,7′-dichlorodihydrofluorescein (CM-$H_2$DCF), were quantified. NAC (10 μM) was administered to cells for 1 h before analysis. (*E*–*G*) Three-month-old PG and PG-Sn2 mice fed the LFD ($n$ = 3) were kept at 22 °C [room temperature (RT)] or 4 °C (cold) for 24 h. (*E*) From indicated BAT, relative mRNA expression was examined through quantitative RT-PCR. Protein phosphorylation and expression were examined through immunoblotting (*F*) and quantified by densitometry (*G*). (*H* and *I*) At day 8 of differentiation, 1°-ISA cells transduced with GFP- or Sestrin2-expressing lentiviruses were stimulated with ISO (1 μM) or FSK (10 μM) for the indicated number of hours. mRNA expression was examined through quantitative RT-PCR. Data are presented as mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant. (Scale bars, 100 μm.)

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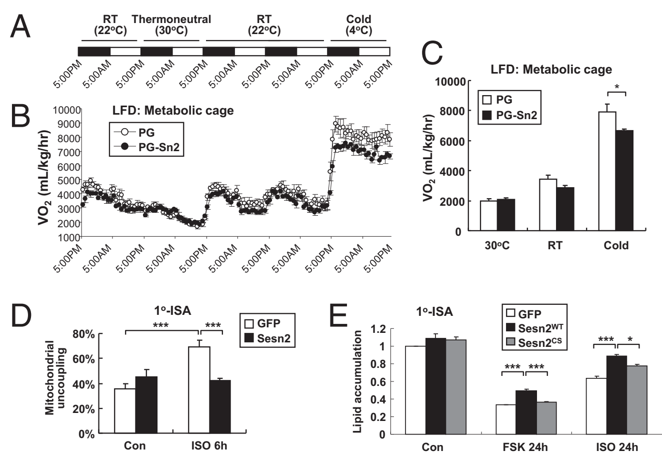


**Fig. 3.** Effect of Sestrin2 on energy metabolism of BAT. (*A*) Four- to 6-mo-old PG and PG-Sn2 mice kept on the LFD (age-matched, $n = 4$) at RT (22 °C) were put into metabolic cages with dark/light cycle and temperature controls. (*B*) $VO_2$ was monitored at RT for 1 d, at thermoneutrality (30 °C) for 1 d, at RT again for 2 d, and in the cold (4 °C) condition for 1 d. $VO_2$ was normalized to lean body mass. (*C*) Averaged daytime $VO_2$ is presented as a bar graph. (*D* and *E*) At day 8 of differentiation, 1°-ISA cells transduced with GFP- or Sestrin2-expressing lentiviruses were stimulated with ISO (1 μM) or FSK (10 μM) for the indicated number of hours. (*D*) Mitochondrial uncoupling was expressed as the percentage of basal mitochondrial respiration. (*E*) Lipid accumulation was measured by Oil Red O staining and expressed as relative Oil Red O intensities. Data are presented as mean ± SEM. $*P < 0.05$; $***P < 0.001$.

changes in food consumption or physical activity (Fig. S7 *A*–*C*), an observation consistent with suppressed *Ucp1* expression. $VO_2$ at a thermoneutral temperature (30 °C), at which Ucp1-mediated thermogenesis is not activated (37), did not differ between control and PG-Sn2 groups (Fig. 3 *A*–*C*). These data suggest that Sestrin2 overexpression and subsequent inhibition of *Ucp1* expression interfere with cold-induced thermogenesis.

**Sestrin2 Inhibits ISO-Induced Mitochondrial Uncoupling of Brown Adipocytes.** We further examined the effect of Sestrin2 on the mitochondrial metabolism of primary brown adipocytes. We found that ISO-induced elevation of mitochondrial uncoupling in brown adipocytes was diminished by Sestrin2 overexpression (Fig. 3*D*), consistent with decreased *Ucp1* expression (Fig. 2*H*). We also found that Sestrin2-overexpressing brown adipocytes retained more fat than the control group after treatment with ISO or FSK (Fig. 3*E*). These data indicate that Sestrin2 controls mitochondrial respiration and lipid metabolism through regulation of *Ucp1* expression.

**Chemical Antioxidants Interfere with *Ucp1* Expression.** Because Sestrin2$^{C125S}$ mutant mice, which cannot suppress ROS, was unable to regulate *Ucp1* expression (Fig. 2 *A*–*D*) or lipid metabolism (Fig. 3*E*), ROS may be the target of Sestrin2 that is required for cold- or cAMP-induced *Ucp1* expression. To test for such involvement of ROS, we treated cultured brown adipocytes with the chemical antioxidants BHA and NAC. The p38 MAPK inhibitor SB203580, which was previously reported to inhibit *Ucp1* expression (20), was used as a positive control. BHA, NAC, and SB203580 all strongly suppressed both basal and FSK-induced *Ucp1* expression in cultured brown adipocytes (Fig. 4*A*). Also, BHA and NAC strongly inhibited p38 MAPK activation and *Ucp1* expression in mouse BAT upon cold exposure (Fig. 4 *B*–*D*). Because ROS are known activators of p38 MAPK (38), these results collectively indicate that ROS-dependent activation of p38 MAPK is essential for *Ucp1* expression in BAT.

**Endogenous Sestrin2 Regulates *Ucp1* Expression.** Finally, we questioned the impact of endogenous Sestrin2 on *Ucp1* expression and BAT metabolism. Therefore, we analyzed BAT phenotypes of *Sesn2*$^{-/-}$ mice. BAT isolated from *Sesn2*$^{-/-}$ mice showed a slight increase in fat accumulation compared with WT mice (Fig. 5 *A* and *C*), whereas eWAT tissue morphology was not affected by Sestrin2 loss (Fig. 5 *B* and *C*). This adipose tissue phenotype of *Sesn2*$^{-/-}$ mice was quite unexpected because Sestrin2 overexpression also increased fat accumulation in BAT (Fig. 1 *F* and *H*). To comprehend the underlying mechanism, we performed gene expression analyses in WT and *Sesn2*$^{-/-}$ BAT. Interestingly, expression of *Ucp1* was significantly up-regulated in *Sesn2*$^{-/-}$ BAT (Fig. 5*D*), whereas *Ucp2* expression was reduced (Fig. 5*E*). On the other hand, the mitochondrial biogenesis markers *Nrf1* and *Tfam* were dramatically down-regulated upon Sestrin2 loss (Fig. 5*F*), and *Sesn2*$^{-/-}$ BAT contained many fewer mitochondria compared with WT BAT (Fig. 5*G*). These results are consistent with our observation that transgenic Sestrin2 overexpression increased mitochondrial contents (Fig. S5*C*) but reduced *Ucp1* expression (Fig. 2 *E*–*G*). Thus, even though *Ucp1* expression is up-regulated by Sestrin2 deficiency, BAT of *Sesn2*$^{-/-}$ mice accumulated more fat due to decreased mitochondrial content. Conversely, although Sestrin2 overexpression increased mitochondrial content, decreased *Ucp1* expression prevented BAT from burning lipids. Therefore, either overexpression or

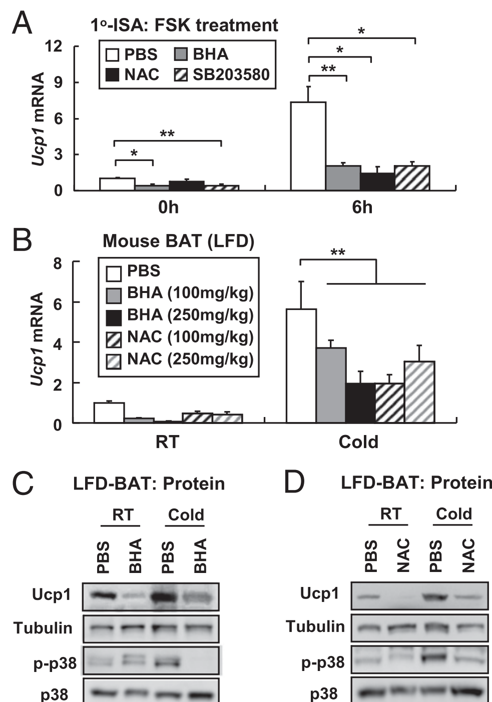


**Fig. 4.** Antioxidants suppress *Ucp1* expression. (*A*) The 1°-ISA cells were differentiated into brown adipocytes. At day 8 of differentiation, cells were treated with BHA (10 μM), NAC (10 μM), or SB203580 (10 μM) as indicated. At 30 min after these treatments, cells were stimulated with FSK (10 μM) for the indicated period. Relative *Ucp1* mRNA expression was analyzed through quantitative RT-PCR ($n = 3$). (*B*–*D*) Three-month-old C57BL/6 mice kept on the LFD ($n = 3$) were administered the indicated antioxidants i.p. on a daily basis for 3 d. On the final day of injection, the mice were kept at 22 °C (RT) or 4 °C (cold) for 24 h. From BAT of the indicated mice, mRNA expression was analyzed through quantitative RT-PCR (*B*, $n = 3$), and protein phosphorylation and expression were examined through immunoblotting (*C*, BHA, 100 $mg \cdot kg^{-1} \cdot d^{-1}$; *D*, NAC, 250 $mg \cdot kg^{-1} \cdot d^{-1}$). Data are presented as mean ± SEM. $*P < 0.05$; $**P < 0.01$.



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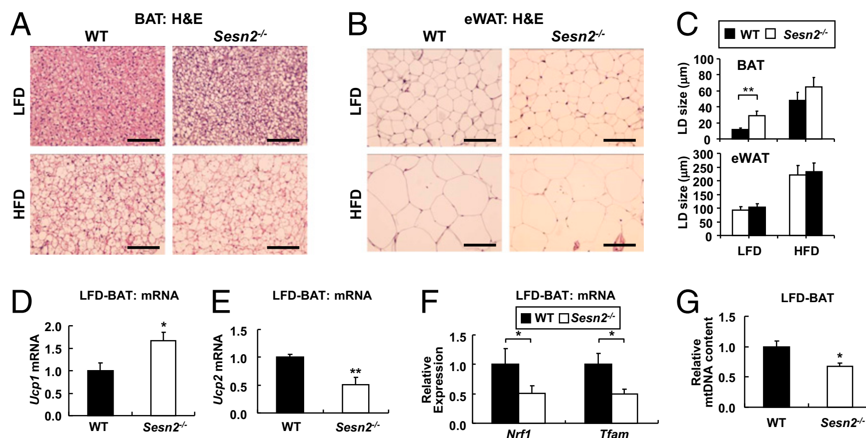


**Fig. 5.** Increased *Ucp1* expression in *Sesn2*$^{-/-}$ mice. WT and *Sesn2*$^{-/-}$ mice were kept on the LFD for 3 mo and then on the HFD for an 3 additional months ($n = 4$). (*A* and *B*) BAT and eWAT from indicated mice were analyzed by H&E staining. (*C*) Quantification of LD size of indicated tissues. Relative mRNA expression of *Ucp1* (*D*), *Ucp2* (*E*), and mitochondrial biogenesis markers (*F*) was determined by quantitative RT-PCR. (*G*) Ratio of mtDNA to nuclear DNA was determined by quantitative PCR of nuclear and mitochondrial genes. Data are presented as mean ± SEM. *$P < 0.05$; **$P < 0.01$. (Scale bars, 200 μm.)

deficiency of Sestrin2 can be detrimental for the energy homeostasis in mouse BAT.

## Discussion

The sestrin family proteins were originally characterized as critical antioxidant proteins that contribute to the recycling of peroxiredoxins (7). Although sestrins are not stand-alone oxidoreductases (39), they can regulate antioxidant defense by promoting activities of other oxidoreductases, such as sulfiredoxin (25). Independent of this redox-regulating activity, sestrins can inhibit mTORC1 by activating AMPK (31). The Cys125 residue of Sestrin2, which is conserved throughout the sestrin-family proteins, is important for Sestrin2's antioxidant activity (7) but negligible for its AMPK-mTORC1–regulating activity (24, 31). According to genetic studies in mice and *Drosophila*, AMPK-mTORC1–regulating activity was suggested to be more physiologically important for control of metabolic homeostasis than its redox-regulating activity (23, 24).

Because endogenous sestrins play essential roles in metabolic homeostasis, we investigated whether we could use their beneficial activities against age- or obesity-associated metabolic pathologies. Thus, we attempted to express Sestrin2 in AT, where the pathogenetic roles of ROS and mTORC1 have been well characterized in the context of obesity (27, 40) and endogenous Sestrin2 expression is relatively low (23). Although transgenic Sestrin2 overexpression suppressed both ROS and mTORC1 as expected, it resulted in unexpected consequences for BAT metabolism; BAT had significantly down-regulated its *Ucp1* expression and thermogenic capacity and accumulated large amounts of lipid droplets. We found that the effect of *Ucp1* loss outweighed all of the beneficial effects of Sestrin2-dependent AMPK-mTORC1 regulation, including decreased lipogenesis and increased mitochondrial contents. Therefore, transgenic overexpression of Sestrin2 in AT was paradoxically revealed to be detrimental to metabolic homeostasis.

We then investigated the molecular mechanism underlying this phenomenon. Interestingly, Sestrin2's antioxidant activity, rather than its more recognized AMPK-mTORC1–controlling activity, was required for its *Ucp1* down-regulating activity. Sestrin2-mediated suppression of ROS resulted in a dramatic reduction in p38 MAPK activation, consistent with the known relationships among ROS, p38 MAPK signaling, and *Ucp1* expression (18–20, 38). Inhibition of ROS by chemical antioxidants was sufficient to inhibit both p38 MAPK activation and *Ucp1* expression, demonstrating that an adequate level of ROS in BAT is essential for metabolic functionality.

Recent studies have demonstrated that BAT is not the only *Ucp1*-expressing tissue in mammals. Brown-like adipocytes, also known as beige fat cells (41), can conditionally express *Ucp1* when stimulated by environmental or physiological cues, such as cold exposure. Unlike BAT, which has relatively well-defined anatomical locations, beige fat cells are dispersed throughout WAT. Nevertheless, the mechanism of cold-induced *Ucp1* expression is shared between BAT and beige fat cells (17), and Ucp1 in beige fat cells has its full thermogenic capacity (42). Therefore, ROS may be essential for inducible *Ucp1* expression and Ucp1-mediated thermogenesis in beige fat cells as well. In the future, it would be interesting to investigate the effect of dietary antioxidants on *Ucp1* expression in human beige fat cells and on homeostatic regulation of energy metabolism.

Antioxidants were once thought to be beneficial for treating age- and obesity-associated metabolic derangements because excessive ROS accumulation was considered a cause of diverse pathologies, such as chronic inflammation, fibrotic damage, and insulin resistance (2, 43, 44). However, many animal and human clinical studies failed to show benefits of antioxidants in treating age- or obesity-associated diseases (12, 14) and revealed several harmful side effects instead (45–48). It is plausible that those antioxidant therapies may have interfered with some ROS-dependent physiological processes that are important for metabolic homeostasis. Our current study suggests that the role of ROS in promoting *Ucp1* expression (Fig. S8) may be one such process that can override the beneficial effects of antioxidants.

## Materials and Methods

Detailed methods for all experiments are available in *SI Materials and Methods*.

**Mice and Diets.** The *tet-Sesn2* mice were generated by pronuclear injection of a linearized pMCStet95 plasmid that contains a tetracycline promoter-conjugated human Sestrin2 (Hi-95) cDNA. The *tet-Sesn2* mice were backcrossed into a C57BL/6 background for more than eight generations. *Pparγ-tTA* mice in a C57BL/6 background were obtained from the Jackson Laboratory (no. 8227) and crossed with *tet-Sesn2* mice. Mice were maintained in filter-topped cages and were given free access to an autoclaved regular chow LFD or the HFD (S3282; Bio-serv; a detailed nutritional profile is provided in Table S1) and water at the University of Michigan (UM) according to the National Institutes of Health and institutional guidelines. All animal studies were overseen by the University Committee on Use and Care of Animals at the UM.

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**Primary Cell Culture.** Interscapular BAT was dissected from 6-wk-old C57BL/6 male mice, and stromal vascular fractions of BAT were grown and differentiated as detailed in *SI Materials and Methods*.

**Lentiviruses.** Lentiviral plasmids for overexpressing WT Sestrin2 or redox-inactive Sestrin2 mutant were previously described (7, 23). Lentiviruses were generated and amplified in the Vector Core facility at the UM.

**Analyses of RNA and Proteins.** Quantitative RT-PCR and immunoblotting were performed to examine the level of RNA and protein expression (49), respectively, as detailed in *SI Materials and Methods*.

**Histology.** Tissues were fixed in 10% (vol/vol) buffered formalin, embedded in paraffin, and stained with H&E. ROS measurements were performed with chloromethyl 2′,7′-dichlorodihydrofluorescein diacetate (Invitrogen) or dihydroethidium (Invitrogen), as detailed in *SI Materials and Methods*.

**Metabolic Analyses.** Mitochondrial uncoupling of primary brown adipocytes was measured using the Seahorse XF24 analyzer at the UM Metabolomics Core. The $VO_2$ rate of live mice was measured using the Comprehensive Laboratory Monitoring System (CLAMS; Columbus Instruments) at the UM Animal Phenotyping Core. $VO_2$ was normalized to lean body mass, as measured by an NMR analyzer (Minispec LF90II; Bruker Optics). Details of metabolic analyses are described in *SI Materials and Methods*.

**Statistical Analysis.** Data are presented as mean ± SEM. The statistical significance of differences between two groups was calculated by a two-tailed Student *t* test. *P* values equal to or above 0.05 were considered not statistically significant.

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