The E3 ligase synoviolin controls body weight and mitochondrial biogenesis through negative regulation of PGC-1 β

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Abstract

Obesity is a major global public health problem, and understanding its pathogenesis is critical for identifying a cure. In this study, a gene knockout strategy was used in post-neonatal mice to delete *synoviolin* (*Syn*)1/*Hrd1*/*Der3*, an ER-resident E3 ubiquitin ligase with known roles in homeostasis maintenance. *Syn*1 deficiency resulted in weight loss and lower accumulation of white adipose tissue in otherwise wild-type animals as well as in genetically obese (*ob/ob* and *db/db*) and adipose tissue-specific knockout mice as compared to control animals. SYVN1 interacted with and ubiquitinated the thermogenic coactivator peroxisome proliferator-activated receptor coactivator (PGC)-1 β , and *Syn*1 mutants showed upregulation of PGC-1 β target genes and increase in mitochondrion number, respiration, and basal energy expenditure in adipose tissue relative to control animals. Moreover, the selective SYVN1 inhibitor LS-102 abolished the negative regulation of PGC-1 β by SYVN1 and prevented weight gain in mice. Thus, SYVN1 is a novel post-translational regulator of PGC-1 β and a potential therapeutic target in obesity treatment.

Keywords endoplasmic reticulum; mitochondria; obesity; PGC-1 β ; synoviolin

Subject Categories Metabolism; Post-translational Modifications, Proteolysis & Proteomics

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Introduction

Obesity is characterized by the excessive accumulation of adipose tissue, as well as increased risk of diabetes, hypertension, cardiovascular diseases, and depression, and is an enormous economic and social burden (Wickelgren, 1998). White adipose tissue (WAT) functions as an energy reservoir and as an endocrine organ, and various inflammatory mediators and cytokines are overexpressed in the WAT of obese individuals, which leads to chronic inflammation (Hotamisligil, 2006). Metabolism in adipocytes has been extensively studied (Rosen & MacDougald, 2006; Lefterova & Lazar, 2009); however, there are presently no effective and safe pharmacological options for obesity prevention and treatment. A better understanding

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of the underlying mechanisms is therefore necessary to develop suitable treatments for obesity and associated metabolic diseases.

The mitochondrion is a key organelle in cellular energy control and has been implicated in obesity (Bournat & Brown, 2010). Peroxisome proliferator-activated receptors (PPARs) (Viana Abranches *et al*, 2011) and their coactivators (PPAR coactivator (PGC)-1 α , PGC-1 β , and PGC-1-related coactivator) (Puigserver *et al*, 1998; Andersson & Scarpulla, 2001; Lin, 2001; Kressler *et al*, 2002) play important roles in mitochondrion biogenesis and energy metabolism, including H₂O₂-based respiration and β -oxidation of fatty acids. Gene knockout studies have suggested functional differences between PGC-1 α and PGC-1 β , for instance in terms of lethality (Scarpulla, 2008). Interestingly, PGC-1 β overexpression results in an increase in mitochondrion number and respiratory function in cultured cells (St-Pierre *et al*, 2003); furthermore, PGC-1 β transgenic mice show high energy expenditure and resistance to obesity (Kamei *et al*, 2003). In addition to the mitochondrion, the endoplasmic reticulum (ER) is also thought to play an important role in obesity (Cnop *et al*, 2012). For example, mutations or deficiency in genes that function in the unfolded protein response produces insulin resistance in a mouse model (Cnop *et al*, 2012; Wang & Kaufman, 2012). Nonetheless, the role of the ER-associated protein degradation (ERAD) pathway in obesity remains obscure.

Synoviolin (*Syvn1*), a mammalian homolog of *Hrd1p/Der3p*, is an E3 ubiquitin (Ub) ligase that was identified from the cDNA of rheumatoid synovial cells (Amano *et al*, 2003) and plays important roles in the ERAD pathway (Yamasaki *et al*, 2005). In a series of studies, we have demonstrated the importance of SYVN1 expression in arthritis (Amano *et al*, 2003) and fibrosis (Hasegawa *et al*, 2010). Inflammation plays a critical role in regulating metabolic status, and *Syvn1* is a key target for inflammatory cytokines such as tumor necrosis factor α (TNF α), interleukin (IL)-1, and IL-17 (Gao *et al*, 2006; Toh *et al*, 2006, 2010).

In the present study, conditional *Syvn1* knockout mice were generated to clarify the role of *Syvn1* in obesity. The results highlight a novel function for SYVN1 in the control of body weight and mitochondrial biogenesis through negative regulation of PGC-1 β .

Results

Generation of *Syvn1*-deficient mice

To study the post-neonatal function of SYVN1, tamoxifen (Tam)-inducible *Syvn1* knockout mice were generated that carry homozygous floxed-*Syvn1* alleles and a Cre-estrogen receptor (ER) transgene (Hayashi & McMahon, 2002) (Fig 1A). Efficient recombination was confirmed in *Syvn1* conditional knockout (CAG-Cre-ER; *Syvn1*^{flox/flox}) and control (*Syvn1*^{flox/flox} and *Syvn1*^{flox/+}) mice following Tam administration by PCR (genome), real-time PCR (mRNA), and Western blotting (protein) (Fig 1B–D). A significant reduction in body weight was observed in CAG-Cre-ER;*Syvn1*^{flox/flox} mice as early as 1 week after Tam administration until it was approximately 80% that of control mice (Fig 1E and F). A slight decrease in weight was also observed in *Syvn1* heterozygous CAG-Cre-ER;*Syvn1*^{flox/+} mice (Supplementary Fig S1). Three experiments were used to determine whether the weight loss was due to low

food intake, absorption-related malnutrition, or both. First, the daily food intake was measured; however, differences were almost not found in terms of food consumption between CAG-Cre-ER;*Syvn1*^{flox/flox} and control mice at day 1 and 7, and higher food consumption was observed in CAG-Cre-ER;*Syvn1*^{flox/flox} mice than in control mice at day 14 (Fig 1G). Second, macroscopic and microscopic examinations showed comparable amounts of food residue in the gut and no histological abnormalities in the intestine of CAG-Cre-ER;*Syvn1*^{flox/flox} mice compared to controls. Third, serum biochemical tests showed no significant differences in the levels of several biomarkers of nutrition and liver and kidney function between the two groups (Supplementary Table S1). However, a detailed anatomical analysis showed a marked reduction of WAT in CAG-Cre-ER;*Syvn1*^{flox/flox} mice relative to controls (Fig 1H–J).

Loss of *Syvn1* in *ob/ob* mice and *db/db* mice causes body weight loss

Two well-established mouse models of obesity (*ob/ob* and *db/db*) in which the leptin signal is constitutively inactivated (Tartaglia *et al*, 1995) were used to determine whether *Syvn1* deficiency is associated with a reduction in body weight at the level of the central nervous system under conditions of constitutive food intake. The expression level of SYVN1 was higher in *ob/ob* and *db/db* than in *ob/+* and *db/+* mice (Fig 2A). Moreover, Tam administration resulted in a significant loss of body weight in CAG-Cre-ER;*Syvn1*^{flox/flox};*ob/ob* and CAG-Cre-ER;*Syvn1*^{flox/flox};*db/db* compound mutants (Fig 2B and C). An anatomical dissection revealed a reduction in fat mass in CAG-Cre-ER;*Syvn1*^{flox/flox};*ob/ob* and CAG-Cre-ER;*Syvn1*^{flox/flox};*db/db* mice compared to *Syvn1*^{flox/flox};*ob/ob* and *Syvn1*^{flox/flox};*db/db* mice, respectively (Fig 2D and E, and Supplementary Fig S2). No differences in food intake were noted across groups (Fig 2F and G). Taken together, these results indicate that SYVN1 directly controls body weight at the level of peripheral energy expenditure, and not at the level of the central nervous system.

WAT is the source of body weight loss resulting from *Syvn1* deletion

To directly test the effect of *Syvn1* knockout on peripheral energy expenditure in WAT, adipose-specific *Syvn1* knockout mice were generated by crossing *Syvn1*^{flox/flox} mice with the *adiponectin* (*Adipoq*)-Cre line (Herman *et al*, 2012; Kleiner *et al*, 2012) to obtain *Adipoq*-Cre;*Syvn1*^{flox/flox} mice. Cre-mediated *Syvn1* deletion in WAT was confirmed by PCR (Fig 3A) and Western blotting (Fig 3B). The body weight of *Adipoq*-Cre;*Syvn1*^{flox/flox} mice was approximately 90% that of controls (Fig 3C and D), an effect that was associated with a reduction in WAT (Fig 3E and F). These results indicate that SYVN1 directly targets WAT to control body weight.

SYVN1 directly interacts with PGC-1 β

To understand the molecular mechanism, we first used microarray analysis to establish the expression of genes in WAT. Genes involved in β oxidation and mitochondria biogenesis (Supplementary Fig S3) were increased in CAG-Cre-ER;*Syvn1*^{flox/flox} mice compared with *Syvn1*^{flox/flox} mice. Several studies have indicated that thermogenic nuclear receptors PPAR α and β and their

Figure 1. Body weight and WAT in post-neonatal *Syun1* mutant mice.

A Schematic depiction of *Syun1* gene targeting strategy. Homologous recombination resulted in exons 2–12 being flanked by loxP sites; deletion was achieved by Tam-induced Cre recombinase-mediated excision.

B PCR products amplified from genomic DNA isolated from tails on day 7 after Tam administration.

C Real-time PCR analysis of adipocyte mRNA from control (Control) and CAG-Cre-ER;*Syun1*^{flox/flox} (KO) mice on day 7 after Tam administration.

D Western blotting of spleen proteins on day 7 after Tam administration.

E, F Changes in body weight. **P* < 0.05, ***P* < 0.01 for Tam-treated CAG-Cre-ER;*Syun1*^{flox/flox} mice versus vehicle-treated CAG-Cre-ER;*Syun1*^{flox/flox} mice, Tam- or vehicle-treated *Syun1*^{flox/flox} mice, Tam- or vehicle-treated C57BL/6J mice (analysis of variance with Tukey–Kramer *post hoc* analysis).

G Average daily food intake measured after Tam injection.

H–J Fat accumulation in post-neonatal *Syun1* KO mice on 7 day after Tam administration. Subcutaneous adipose (H), epididymal adipose (I), and mesentery adipose (J) tissues are shown (Control, *n* = 8; KO, *n* = 3). Mesentery fat is shown by black arrows.

Data information: All data are expressed as mean ± SD. Data were analyzed with the Student's *t*-test. **P* < 0.05, ***P* < 0.01.

Source data are available online for this figure.

Figure 2. Changes in body weight and WAT in post-neonatal *Syvn1* knockout (KO) and genetically obese (*ob/ob* and *db/db*) mice.

Compound mutants of *Syvn1* and *ob/ob* or *db/db* were generated as described in Materials and Methods.

A SYVN1 expression in the WAT of *ob/+*, *ob/ob*, *db/+*, and *db/db* mice.

B, C Changes in body weight in Tam-treated mice. CAG-Cre-ER;*Syvn1*^{flox/flox};*ob/ob* (KO;*ob/ob*) and CAG-Cre-ER;*Syvn1*^{flox/flox};*db/db* (KO;*db/db*) mice, red line; *Syvn1*^{flox/flox};*ob/ob*, *Syvn1*^{flox/+};*ob/ob*, and *Syvn1*^{+/+};*ob/ob* (Control;*ob/ob*) and *Syvn1*^{flox/flox};*db/db*, *Syvn1*^{flox/+};*db/db*, and *Syvn1*^{+/+};*db/db* (Control;*db/db*) mice, blue line.

D, E Subcutaneous fat accumulation in CAG-Cre-ER;*Syvn1*^{flox/flox};*ob/ob* and CAG-Cre-ER;*Syvn1*^{flox/flox};*db/db* mice after post-neonatal *Syvn1* knockout. Histological analysis of adipose tissue from Control;*ob/ob* and Control;*db/db* mice (left), and CAG-Cre-ER;*Syvn1*^{flox/flox};*ob/ob* and CAG-Cre-ER;*Syvn1*^{flox/flox};*db/db* mice (right) after Tam administration. Subcutaneous fat is shown by white arrows.

F, G Average daily food intake measured after Tam injection in CAG-Cre-ER;*Syvn1*^{flox/flox};*ob/ob* (D) and CAG-Cre-ER;*Syvn1*^{flox/flox};*db/db* (G) mice.

Data information: Data were analyzed by the Student's *t*-test and are expressed as mean $\pm$ SD. **P* < 0.05, ***P* < 0.01.

Source data are available online for this figure.

coactivators of the PGC-1 family transcriptionally regulate peripheral energy expenditure via β oxidation and mitochondria biogenesis (Lowell & Spiegelman, 2000; Scarpulla, 2006); therefore, the interaction of SYVN1 with these factors was tested *in vitro*. Glutathione-S-transferase (GST)-tagged SYVN1 lacking the transmembrane domain (GST-SYVN1 Δ TM) bound HA-tagged PGC1 β but not PPAR α or γ or PGC-1 α (Fig 4A). In a GST pull-down assay, PGC-1 β bound the SyU domain (amino acids, aa 236–270) of SYVN1 (Fig 4B), which is highly conserved from *Caenorhabditis elegans* to humans, but not in yeast SYVN1 orthologs (Supplementary Fig S4A). In addition, an R266A/R267A double mutation in the SyU domain decreased this interaction (Fig 4C), but had no effect on the E3 ligase activity of SYVN1 (Supplementary Fig S4B). The GST pull-down assay mapped the SYVN1-binding domain of PGC-1 β to aa 195–367 containing an LXXLL motif of middle portion (Supplementary Fig S4C). To verify the interaction in cellulo, HA-PGC-1 β and FLAG-tagged SYVN1 (SYVN1/FLAG) were co-transfected into HEK 293T cells. HA-PGC-1 β co-immunoprecipitated with SYVN1/FLAG

but not the control FLAG vector (Fig 4D). To further investigate the interaction between SYVN1 and PGC-1 β , whole-cell lysates of HEK 293 cells, in which SYVN1 and PGC-1 β were expressed, were precipitated with anti-SYVN1 antibody or a control non-immune mouse immunoglobulin (IgG) and probed with an antibody against PGC-1 β in an immunoblotting assay. Endogenous PGC-1 β was detected in the precipitate with anti-SYVN1 but not with IgG (Fig 4E). These results clearly indicate that SYVN1 interacts *in vivo* with PGC-1 β under normal physiological conditions.

Since SYVN1 is an ER-resident protein and PGC-1 β translocates into the nucleus, their subcellular localization was investigated by immunofluorescence staining in transiently transfected HEK 293T cells. HA-PGC-1 β was mainly detected in the nucleus (Fig 4F), as previously reported (Kelly *et al*, 2009). However, when coexpressed with SYVN1/FLAG, the two proteins predominantly colocalized in the perinuclear region, but were not observed in the nucleus (Fig 4F). In contrast, the coexpression of HA-PGC-1 β with SYVN1R266A, R267A/FLAG, or SYVN1 Δ SyU/FLAG, which do not

Figure 3. Effect of adipose tissue-specific *Syvn1* deletion on body weight and fat mass.

A PCR products amplified from genomic DNA isolated from WAT, liver, tail, and muscle of *Adipoq-Cre;Syvn1^{flox/flox}* (KO) and *Syvn1^{flox/flox}* mice (Control).
B Western blotting of WAT proteins 15 weeks after birth.
C, D Changes in body weight after weaning. *Adipoq-Cre;Syvn1^{flox/flox}* mice (KO), red line; control (*Syvn1^{flox/flox}* and *Syvn1^{flox/+}*) mice, blue line. Data were analyzed with the Student's *t*-test and represent the mean $\pm$ SD. * $P < 0.05$. (D) Control and KO mice 15 weeks after birth.
E, F Reduction in subcutaneous fat (E) and epididymal adipose tissue (F) in KO mice 15 weeks after birth. Data were analyzed with the Student's *t*-test and represent the mean $\pm$ SD (Control, $n = 10$; KO, $n = 8$). * $P < 0.05$, ** $P < 0.01$.

Source data are available online for this figure.

interact with PGC-1 β (Fig 4B and C), resulted in the nuclear localization of HA-PGC-1 β (Fig 4F, Supplementary Fig S4D). These results indicate that SYVN1 traps PGC-1 β in the perinuclear region.

PGC-1 β is a novel substrate of SYVN1

An *in vitro* assay was carried out to determine whether PGC-1 β is a substrate of SYVN1, which is an E3 Ub ligase (Amano *et al*, 2003). Polyubiquitinated PGC-1 β was detected in the presence of ATP, HA-Ub, E1, E2, and SYVN1 (Fig 4G). The ubiquitination of PGC-1 β was also examined *in vivo*. FLAG-tagged Ub and HA-PGC-1 β were coexpressed with wild-type (WT) or mutant SYVN1 (3S)—which lacks Ub ligase activity (Amano *et al*, 2003)—in HEK 293T cells. Ubiquitinated HA-PGC-1 β was observed in WT SYVN1- but not SYVN1 3S-expressing cells (Fig 4H). Collectively, these results suggest that PGC-1 β is a SYVN1 substrate.

Since ubiquitinated proteins are degraded by the proteasome, experiments were performed to verify whether the level of PGC-1 β protein is regulated by SYVN1. PGC-1 β protein level was markedly elevated in the WAT of *CAG-Cre-ER;Syvn1^{flox/flox}* (Fig 4I) and *Adipoq-Cre;Syvn1^{flox/flox}* (Supplementary Fig S4E) mice; however, the transcript level of *Pparg1b* (encoding PGC-1 β) was unaltered (Supplementary Fig S4F). PGC-1 β protein level was 1.4-fold higher in Tam-treated skin fibroblasts from *CAG-Cre-ER;Syvn1^{flox/flox}* mice than in vehicle-treated cells (Fig 4J). A similar observation was made by knocking down *Syvn1* expression using small interfering

RNA (siRNA), which resulted in a near-complete disappearance of SYVN1 expression in HEK 293 cells: PGC-1 β protein expression in *Syvn1* siRNA-treated cells was 2.5-fold higher than in controls (Fig 4K). Finally, to investigate the role of SYVN1 in PGC-1 β degradation, the proteasome inhibitor MG-132 was applied to cultured skin fibroblasts. Similar to Tam treatment, MG-132 upregulated PGC-1 β protein levels in vehicle-treated cells by 1.6-fold, but produced no additional effects on Tam-treated skin fibroblasts (Fig 4J). To confirm whether the SYVN1–PGC-1 β interaction is critical for SYVN1-mediated PGC-1 β degradation, the half-life of PGC-1 β was measured in mouse embryonic fibroblasts (MEFs) coexpressing HA-PGC-1 β and WT SYVN1 or SYVN1 Δ SyU (Supplementary Fig S4G). WT SYVN1 overexpression significantly shortened the half-life of HA-PGC-1 β , whereas SYVN1 Δ SyU had no effect (Fig 4L). These results indicate that PGC-1 β protein expression is negatively regulated by SYVN1 at the post-transcriptional level and strongly suggest that SYVN1 is a major E3 ligase for PGC-1 β in cells.

Negative regulation of PGC-1 β by SYVN1

PGC-1 β functions as a coactivator of several transcription factors including PPAR α and is implicated in various biological processes such as mitochondrial biogenesis, β oxidation, and body weight control (Scarpulla, 2008). To investigate the role of SYVN1 in the regulation of the coactivator and mitochondrial biogenesis functions of PGC-1 β , the activity of PPAR luciferase (PPRE X3-TK-luc) (Kim *et al*, 1998)—which contains three PPAR binding sites and is

Figure 4.

specifically regulated by PPAR and its coactivators—was measured. As previously reported (Lin *et al*, 2003), reporter activity was induced by treatment with Wy-14643, a PPAR α agonist. *Syvn1* but not control siRNA transfection significantly enhanced reporter activity under Wy-14643 induction (Fig 5A). Furthermore, the co-transfection of PGC-1 β and *Syvn1* siRNA activated reporter activity. These results indicate that loss of *Syvn1* enhances PPAR α -mediated transcription in a PGC-1 β -dependent manner. Conversely, SYVN1 overexpression inhibited the coactivator function of PGC-1 β (Fig 5B), but this effect was decreased for the SYVN1R266A, R267A mutant (Fig 5C).

The role of SYVN1 in the regulation of mitochondrial biogenesis was also examined. *Syvn1* siRNA-treated cells contained large

numbers of mitochondria compared to those treated with control siRNA, and the increase was not observed in cells that were co-transfected with both *Syvn1* and *Ppargc1b* siRNAs (Fig 5D). Furthermore, transcript levels of *medium chain acyl-coenzyme A dehydrogenase* and *mitochondrial ATP synthase β subunit*, two known PGC-1 β target genes (Rodriguez-Calvo *et al*, 2006; Shao *et al*, 2010), were upregulated in *Adipoq*-Cre;*Syvn1*^{fllox/fllox} (Fig 5E and F) and *CAG*-Cre-ER;*Syvn1*^{fllox/fllox} compared to control mice (Supplementary Fig S5A). An electron microscopic analysis revealed increases in both the number and size of mitochondria in *CAG*-Cre-ER;*Syvn1*^{fllox/fllox} compared to controls (Fig 5G). To investigate whether the mitochondria in *CAG*-Cre-ER;*Syvn1*^{fllox/fllox} mice were functional, O₂ consumption in a single-cell suspension of primary

Figure 4. PGC-1 β is a substrate of SYVN1.

- A GST and GST-tagged SYVN1 lacking the transmembrane domain (GST-SYVN1 Δ TM) were incubated with HEK 293T whole-cell extracts expressing HA-PPAR α , HA-PPAR γ , HA-PGC-1 α , or HA-PGC-1 β .
- B, C Interaction of SYVN1 with PGC-1 β . (B) Schematic representation of SYVN1. TM, transmembrane domain; SyU, SYVN1 unique domain. (B, C) An *in vitro* binding assay was performed with HA-PGC-1 β and GST or GST-tagged SYVN1 deletion mutants.
- D, E SYVN1 interacts with PGC-1 β *in vivo*. (D) Whole-cell extracts from HEK 293T cells transfected with HA-PGC-1 β and/or SYVN1/FLAG expression plasmids were immunoprecipitated with anti-FLAG antibody. *Immunoprecipitated IgG heavy chain. (E) Whole-cell extracts from HEK 293 cells were immunoprecipitated with anti-SYVN1 antibody. Western blotting was performed using anti-PGC-1 β antibody. *Non-specific band.
- F HEK 293T cells were transfected with HA-PGC-1 β (green) and/or SYVN1/FLAG, SYVN1 R266A, R267A/FLAG (red) expression plasmids. After 24 h, cells were immunolabeled with anti-HA and anti-FLAG antibodies.
- G *In vitro* ubiquitination assay was performed with maltose-binding protein (MBP)-tagged SYVN1 Δ TM-His, GST-PGC-1 β (aa 1–367), E1 and E2 enzymes, and HA-Ub. Lower panel shows the loading control.
- H Whole-cell extracts from HEK 293T cells transfected with HA-PGC-1 β , Ub/FLAG, and WT or mutant SYVN1 (SYVN1 WT or 3S, respectively) expression plasmids were immunoprecipitated with anti-HA antibody. Lower panels show the loading controls.
- I Cell extracts from adipose tissues were obtained from control and CAG-Cre-ER; *Syvn1*^{fllox/fllox} mice 7 days after Tam administration. Western blotting was performed using an anti-PGC-1 β antibody. Representative data from three sets of control and CAG-Cre-ER; *Syvn1*^{fllox/fllox} mice are shown.
- J Skin fibroblasts from CAG-Cre-ER; *Syvn1*^{fllox/fllox} mice were cultured, and 48 h after Tam or vehicle treatment and addition of 10 μ M MG-132 for 2 h, cell lysates were collected for Western blotting.
- K HEK 293 cells were transiently transfected with control or *Syvn1* siRNA. After 48 h, cells were incubated with 10 μ M MG-132 for 2 h. Western blotting was performed with anti-SYVN1 and anti-PGC-1 β antibodies.
- L Measurement of PGC-1 β half-life. Mouse embryonic fibroblasts derived from *Syvn1* knockout mice were transfected with HA-PGC-1 β and SYVN1/FLAG or SYVN1 Δ SyU/FLAG; 48 h later, cells were treated with 40 μ M cycloheximide for the indicated times, and immunoblotting was performed with anti-PGC-1 β antibody. Intensity of bands was measured (right panel). Each experiment was performed at least three times.

Source data are available online for this figure.

mouse adipocytes was measured and was found to be higher in cells from CAG-Cre-ER; *Syvn1*^{fllox/fllox} than from control mice (Supplementary Fig S5B). Moreover, mitochondrial respiration and activity were higher in CAG-Cre-ER; *Syvn1*^{fllox/fllox} than in control mice (Fig 5H). The high respiration rate was also observed in *Syvn1* knockout pre-adipocyte cells compared to *Syvn1* WT cells, and the high respiration was not observed by treatment of *Syvn1* knockout cells with *Ppargc1b* siRNAs (Supplementary Fig S5C). These results indicated that the respiratory phenotype of *Syvn1* knockout is PGC1b dependent. Finally, the basal energy expenditure was increased in CAG-Cre-ER; *Syvn1*^{fllox/fllox} relative to control mice (Fig 5I). These results indicate that *Syvn1* deletion enhances mitochondrial activity *in vivo* and strongly suggests that PGC-1 β is a functional target of SYVN1.

Selective SYVN1 inhibition attenuates weight gain

We previously demonstrated that LS-102 selectively inhibits the E3 Ub ligase activity of SYVN1 (Yagishita *et al*, 2012) and suppresses rheumatoid arthritis (Yagishita *et al*, 2012), liver cirrhosis (Wu *et al*, 2014), and sarcoglycanopathy (Bianchini *et al*, 2014) in a mouse model. Here, it was used to examine the effects of SYVN1 inhibition on obesity. LS-102 suppressed the regulation of PGC-1 β function by SYVN1, as evidenced by reduced ubiquitination of PGC-1 β (Fig 6A) and activation of PPAR α -mediated transcription in HEK 293 cells (Fig 6B). In addition, LS-102 induced the expression of PGC-1 β in WT MEFs, but not SYVN1 KO MEFs (Supplementary Fig S6). Next, C57BL/6J mice were treated with the vehicle control dimethylsulfoxide (DMSO) or LS-102 and their body weight was monitored over a 2-month treatment period (Fig 6C). Mice treated with LS-102 showed no weight gain as a result of normal food intake, while food intake itself was unaffected (Fig 6D); analysis by dissection revealed a reduction in WAT fat mass of the epididymis and fewer lipid droplets in these mice compared to controls (Fig 6E and F). Similar to the WAT of CAG-Cre-ER; *Syvn1*^{fllox/fllox} mice, C57BL/6J mice treated with LS-102 had greater numbers of mitochondria (Fig 6G). The effect of LS-102 on obesity was

examined in *db/db* mice. The body weight of LS-102-treated animals gradually decreased to approximately 85% of that of DMSO-treated mice (Fig 6H and I), but there was no effect on food intake (Fig 6J). LS102 also significantly decreased blood glucose in *db/db* mice compared to controls (Fig 6K). These findings indicate that the inhibition of SYVN1 can suppress weight gain in a mouse model of obesity.

Discussion

Gene knockout technology is a useful method for assessing gene function and often yields unexpected results, such as the reductions in body weight and WAT accumulation observed in post-neonatal *Syvn1* knockout mice in the present study (Fig 1). This weight loss was also observed in crosses with two genetically obese mouse lines, *ob/ob* and *db/db*, as well as in adipose tissue-specific *Syvn1* knockout mice (Figs 2 and 3). Taken together, these results indicate that the loss of *Syvn1* causes peripheral activation of energy expenditure in WAT, eventually leading to weight loss.

Obesity is a risk factor for other chronic diseases such as cardiovascular disorders and diabetes (Wickelgren, 1998). Recently, the relationship between obesity and chronic inflammation—especially the contributions of adipokines such as TNF α and IL-1 that link obesity to rheumatic diseases (Abella *et al*, 2014)—has been closely examined (Johnson *et al*, 2012). For instance, recent studies have shown that obesity impairs the efficacy of anti-TNF α therapy in rheumatoid arthritis (RA) patients (Gonzalez-Gay & Gonzalez-Juanatey, 2012). We have identified SYVN1 as a causative factor for RA, and many studies have confirmed that SYVN1 is an important target of cytokines (Yamasaki *et al*, 2005; Gao *et al*, 2006; Toh *et al*, 2010). Our study is the first demonstration that SYVN1 is a key for understanding the common feature among obesity, chronic inflammatory, and RA.

The thermogenic transcriptional coactivator PGC-1 β was identified as a SYVN1-interacting molecule (Fig 4). PGC-1 β , PGC-1 α , and

Figure 5. Regulation of PGC-1 β function by SYVN1.

A–C HEK 293 cells (1×10^5 cells) were transiently co-transfected with a reporter plasmid containing PPAR binding sites (PPRE X3-TK-luc) and expression constructs or siRNAs (A). For WT and mutant SYVN1 overexpression, 50 or 100 μ g SYVN1 expression vector was co-transfected (B, C); 16 h later, cells were treated with vehicle or 10 μ M Wy-14643 for 6 h. Each experiment was performed at least three times.

D Representative electron micrographs of siRNA-treated cells. An increase in mitochondrial volume can be seen as the large cytoplasmic area in 3T3 L1 cells (a mitochondrion is shown by the white arrow). Magnification, 5,000 $\times$. The number of mitochondria in the area (500 pixel $\times$ 500 pixel) was measured (right panel). Each experiment was performed at least three times.

E Total RNA was isolated from adipose tissue 15 weeks after birth, and mRNA expression was measured by real-time PCR relative to 18S rRNA level, with the average for control mice set to 1 ($n = 3$).

F Cell extracts from adipose tissue were obtained from control and *Adipoq*-Cre;*Syvn1*^{fllox/fllox} (KO) mice 15 weeks after birth. Western blotting was performed using anti-medium chain acyl-coenzyme A dehydrogenase (MCAD) antibody.

G Representative electron micrographs of adipose tissue of control (left) and *CAG*-Cre-ER;*Syvn1*^{fllox/fllox} (right) mice. An increase in mitochondrial volume can be seen as an enlargement in cytoplasmic area (M, mitochondria). Magnification, 10,000 $\times$.

H Mitochondrial respiration was measured in WAT 7 days after Tam administration (the value for control mice was set to 100%) ($n = 3$).

I Basal energy expenditure of control and *CAG*-Cre-ER;*Syvn1*^{fllox/fllox} mice 7 days after Tam administration.

Data information: Data were analyzed with the Student's *t*-test (A–E, I) or Mann–Whitney *U*-test (H) and represent the mean $\pm$ SD. * $P < 0.05$, ** $P < 0.01$.

Source data are available online for this figure.

Figure 6. Effect of SYVN1 inhibition on diet-induced weight gain or genetic obesity.

- A** HEK 293 cells were transfected with FLAG-PGC-1β plasmid and immunoprecipitated with anti-FLAG antibody. An *in vitro* ubiquitination assay was performed with GST-SYVN1ΔTM, immunoprecipitated FLAG-PGC-1β, E1 and E2 enzymes, and HA-Ub in the presence or absence of LS-102. Each experiment was performed at least three times.
- B** HEK 293 cells were transiently transfected with PPARE3-TK-luc and PPARα expression plasmid; 16 h later, cells were treated with vehicle or 10 μM Wy-14643 for 6 h. ** $P < 0.01$. Each experiment was performed at least three times.
- C, D** Body weight (C) and average food intake (D) were measured daily for C57BL/6J mice treated once a day with DMSO or 50 mg/kg LS-102. * $P < 0.05$ for C57BL/6J LS-102-treated versus C57BL/6J DMSO-treated mice.
- E** Epididymal adipose tissue of C57BL/6J mice treated with DMSO (left) or LS-102 (right) on day 57 ($n = 4$).
- F** Hematoxylin and eosin staining of epididymal adipose tissue of C57BL/6J mice treated with DMSO (left) or LS-102 (right) (lipid droplets are shown by black arrows). Magnification, 400×.
- G** Representative electron micrographs of adipose tissue from DMSO- (left) and LS-102-treated (right) mice on day 57. An increase in mitochondrial volume can be seen as enlarged cytoplasmic areas (a mitochondrion is shown by the white arrow). Magnification, 10,000×. The number of mitochondria in the area (2,000 pixel × 2,000 pixel) was measured (right panel).
- H–J** Obese *db/db* mice were treated daily with DMSO or LS-102, and body weight (H, I) and average daily food intake (J) were measured (Control, $n = 3$; LS-102, $n = 8$).
- K** Blood glucose was measured on days 13 and 27 (Control, $n = 3$; LS-102, $n = 8$).

Data information: All data were analyzed with the Student's *t*-test and are shown as mean ± SD. * $P < 0.05$, ** $P < 0.01$.

Source data are available online for this figure.

PRC regulate mitochondrial function in the control of energy expenditure (Lin, 2001; Kamei *et al*, 2003; Liu & Lin, 2011). Although these coactivators share functional and structural identities the primary structure of PGC-1 β has several unique features—including an middle LXXLL motif (Lin, 2001)—, and knockout studies have suggested functional differences among these proteins (Scarpulla, 2008). In the present study, the aa 195–367 region of PGC-1 β encompassing the middle LXXLL motif was found to interact with and serve as a target of negative regulation by SYVN1 (Fig 4). This novel regulatory mechanism was also confirmed *in vivo* in global and tissue-specific *Syvn1* knockout mice (Figs 4 and 5). The selective regulation of PGC-1 β by SYVN1 could be evidenced for the unique role of PGC-1 β compared to PGC-1 α . Thus, a novel function for SYVN1 in energy metabolism could be exerted via negative regulation of PGC-1 β . Recently, knockout mice of Sel-1 suppressor of lin-12-like protein (Sel1L), which is an adaptor protein for SYVN1 and functions in the ERAD pathway, were shown to be resistant to diet-induced obesity (Sha *et al*, 2014). Therefore, these studies point to a new direction for research on obesity that includes a role for the ERAD pathway in energy control.

Nuclear receptors are transcription factors involved in various metabolic processes and are therefore attractive targets for therapeutic interventions. Several studies on PPARs—the main regulators of lipid, glucose, and energy metabolism—have been published in the past two decades and have led to the development of PPAR-related drugs. Although many PPAR agonists are clinically efficacious, their use is associated with many side effects (Swanson *et al*, 2013). The PPAR coactivators PGC-1 α and β are also possible drug targets, and adequate modulation or activation of PGC-1 expression has great potential in the treatment of diseases associated with mitochondrial dysfunction and dysregulation of oxidative metabolism (Liu & Lin, 2011). Although targeting a coactivator in new drug development is attractive, the pharmacological upregulation of nuclear coactivators is challenging. In the present study, LS-102—a selective inhibitor of the E3 ligase activity of SYVN1—induced activation of PGC-1 β and prevented weight gain and WAT accumulation in mice (Fig 6). In addition, SYVN1 was more highly expressed in obese (*ob/ob*) mice than in their non-obese (*ob/+*) counterparts (Fig 2). These results provide novel insight into the mechanistic basis of obesity, since the pharmacological inhibition of SYVN1 enhances energy expenditure by preventing PGC-1 β degradation. In addition, LS-102 treatment improved blood glucose before stimulating weight loss (Fig 6). Although further studies are needed to clarify the role of SYVN1 in glucose metabolism, SYVN1 is an important drug candidate for obesity and associated metabolic diseases. In conclusion, this study demonstrated a novel role for SYVN1 in the control of energy metabolism via negative regulation of PGC-1 β .

Materials and Methods

Mice

All procedures involving animals were performed in accordance with institutional and national guidelines for animal experimentation and were approved by the Institutional Animal Care and Use Committee of Tokyo Medical University (#S-24021). Mice were kept in SPF under conditions (20–26°C temperature; 40–65% humidity) on a

12-h light/12-h dark cycle. F-1 Foods (5.1% fat, 21.3% protein) were purchased from Funabashi farm (Chiba, Japan). All mice used in the study were of the C57BL/6J background. Conditional *Syvn1*^{loxP/loxP} mice were generated through homologous recombination in embryonic stem (ES) cells (Fig 1). The mouse *Syvn1* locus was cloned from a BAC clone, and the targeting construct was linearized and transfected into ES cells by electroporation. Recombinant ES cell clones expressing the neomycin gene were selected in medium supplemented with G418 and injected into C57BL/6 mouse-derived blastocysts using standard procedures. The neomycin selection cassette was flanked by Frt recombination sites and excised *in vivo* by crossing with the general FLP deleter strain (Jackson Laboratories, West Grove, PA, USA). Floxed heterozygous *Syvn1*^{lox/+} and heterozygous CAG-Cre-ER mice (Jackson Laboratories) were crossed to generate double heterozygous CAG-Cre-ER;*Syvn1*^{lox/+} mice, which were bred with homozygous *Syvn1*^{lox/lox} mice to produce conditional *Syvn1* homozygous (CAG-Cre-ER;*Syvn1*^{lox/lox}) and *Syvn1* heterozygous (CAG-Cre-ER;*Syvn1*^{lox/+}), homozygous *Syvn1*^{lox/lox}, and heterozygous *Syvn1*^{lox/+} mice. Control mice lacking the Cre transgene (*Syvn1*^{lox/lox} or *Syvn1*^{lox/+}) received Tam injections to eliminate any potential effects of Tam. To generate the CAG-Cre-ER;*Syvn1*^{lox/lox}, *ob/ob* and CAG-Cre-ER;*Syvn1*^{lox/lox};*db/db* genotypes, CAG-Cre-ER;*Syvn1*^{lox/lox} mice were bred with *ob/+* or *db/+* mice (Jackson Laboratories), yielding the compound heterozygotes CAG-Cre-ER;*Syvn1*^{lox/+};*ob/+* and CAG-Cre-ER;*Syvn1*^{lox/+};*db/+*. These were interbred to obtain CAG-Cre-ER;*Syvn1*^{lox/lox};*ob/ob*, *Syvn1*^{lox/lox};*ob/ob*, and *Syvn1*^{lox/+};*ob/ob* mice or CAG-Cre-ER;*Syvn1*^{lox/lox};*db/db*, *Syvn1*^{lox/lox};*db/db*, and *Syvn1*^{lox/+};*db/db* mice. The genotypes *Syvn1*^{lox/lox};*ob/ob*, *Syvn1*^{lox/+};*ob/ob*, *Syvn1*^{+/+};*ob/ob*, *Syvn1*^{lox/lox};*db/db*, *Syvn1*^{lox/+};*db/db*, and *Syvn1*^{+/+};*db/db* were used as control mice. To generate adipose-specific *Syvn1* knockout mice, *Syvn1*^{lox/lox} and *Adipoq*-Cre mice (Jackson Laboratories) were crossed to generate *Adipoq*-Cre;*Syvn1*^{lox/+} compound heterozygotes, which were mated with *Syvn1*^{lox/lox} mice. For blinding and randomization, genotyping and measurement of body weight were separately performed by different person, respectively. In addition, treatment with LS-102 and measurement of body weight were also performed by different person, respectively. We randomly allocated mice and estimated sample size as the number was more than 3 at least. We excluded mice with sickness or injury from experiment before starting the experiment.

Tamoxifen, LS-102 administration

Tamoxifen stock solution was prepared using 100 mg tamoxifen suspended in corn oil (both from Sigma, St. Louis, MO, USA). Starting from 7 to 8 weeks after birth, mice were injected intraperitoneally with 125 mg/kg tamoxifen per day for five consecutive days. LS-102 solution (50 mg/kg) or DMSO was injected intraperitoneally once a day.

Plasmids and antibodies

Coding sequences of full-length *Ppara*, *Pparg*, *Ppargc1a*, and *Ppargc1b* were PCR-amplified from mouse 3T3-L1 cDNA. Fragments of *Ppargc1b* deletion mutants were obtained by PCR amplification, and these along with full-length *Ppargc1b* were inserted into the pcDNA3 HA plasmid (Invitrogen, Carlsbad, CA, USA) for GST

pull-down and transient transfection assays. Plasmid sequences were confirmed by sequencing. PPRE X3-TK-luc was purchased from Addgene (Cambridge, MA, USA). SYVN1 plasmids were previously described (Amano *et al*, 2003; Yamasaki *et al*, 2007), and *Syvn1* point mutants were obtained by PCR amplification. The following antibodies were used: anti-FLAG (M2), anti-tubulin (both from Sigma), and anti-HA (12CA5 and 3F10; Roche, Indianapolis, IN, USA). The anti-SYVN1 rabbit polyclonal antibody that was used was previously reported (Yamasaki *et al*, 2007). Polyclonal antiserum against PGC-1 β was generated by immunizing rabbits with purified GST-PGC-1 β (1–367 aa).

GST pull-down assay

GST fusion proteins were expressed and purified using glutathione sepharose beads (GE Healthcare, Little Chalfont, UK) (Aratani *et al*, 2001; Fujita *et al*, 2003). Cell extracts were incubated with each GST fusion protein bound to resin in 1 ml buffer A [20 mM Tris–HCl, pH 8.0; 100 mM NaCl; 1 mM ethylenediaminetetraacetic acid (EDTA); 1 mM dithiothreitol (DTT); 0.1% Nonidet P-40 (NP-40); 5% glycerol; 1 mM Na₃VO₄; 5 mM NaF; 1 μ g/ml aprotinin; and 1 μ g/ml leupeptin] for 4 h at 4°C. After washing with buffer A, bound proteins were fractionated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) followed by Western blotting.

Immunoprecipitation assays

HEK 293T cells were transfected with HA-PGC-1 β and SYVN1/FLAG expression vectors; 24 h later, cells were lysed in 200 μ l lysis buffer (20 mM Tris–HCl, pH 8.0; 100 mM NaCl; 1 mM EDTA; 1 mM DTT; 1% NP-40; 5% glycerol; and protease inhibitors). Lysates were diluted in binding buffer (of the same composition as lysis buffer, except that it contained 0.1% NP-40) and mixed with 2 μ g anti-FLAG (M2) antibody conjugated to protein G-sepharose beads. After a 4-h incubation at 4°C, beads were washed three times with binding buffer. Bound proteins were fractionated by SDS–PAGE and analyzed by Western blotting. To detect the interaction between endogenous SYVN1 and PGC-1 β , HEK 293 cells were lysed in a solution composed of 100 mM Tris–HCl, 80 mM NaCl, 1 mM EDTA, 5 mM ethylene glycol tetraacetic acid, 5% glycerol, 2% (w/v) digitonin, 0.1% Brij 35, protease inhibitor cocktail, and 20 mM MG132. Immunoprecipitation was carried out with anti-SYVN1 antibody or control IgG, followed by Western blot using anti-PGC-1 β antibody.

In vitro ubiquitination assays

The *in vitro* ubiquitination assay was performed as previously described (Yamasaki *et al*, 2007). Briefly, GST-PGC-1 β (aa 1–367) was incubated with 0.75 μ g HA-Ub, 125 ng E1 (Biomol International, Plymouth Meeting, PA, USA), 150 ng UbCh5C, and 150 ng maltose-binding protein-tagged SYVN1 Δ TM–His in reaction buffer (50 mM Tris–HCl, pH 7.5; 5 mM MgCl₂; 0.6 mM DTT; and 2 mM ATP) at 37°C for 2 h. Glutathione sepharose was then added, and the mixture was washed with GST wash buffer (50 mM Tris–HCl, pH 7.5; 0.5 M NaCl; 1% Triton X-100; 1 mM EDTA; 1 mM DTT; and protease inhibitors). The ubiquitinated PGC-1 β was analyzed by

Western blotting with anti-HA antibody. For inhibition of ubiquitination by LS102, FLAG-PGC-1 β was purified from HEK 293T cells transfected with FLAG-PGC-1 β with an anti-FLAG antibody and incubated with 1.5 μ g HA-Ub, 250 ng E1, 300 ng UBE2G2, and 150 ng GST-SYVN1 (aa 233–338) in reaction buffer (50 mM Tris–HCl, pH 7.5; 5 mM MgCl₂; 0.6 mM DTT; and 2 mM ATP) at 37°C for 2 h. The ubiquitinated PGC-1 β was re-immunoprecipitated with anti-FLAG antibody and analyzed by Western blotting with anti-HA antibody.

In vivo ubiquitination assay

HEK 293T cells were transfected with HA-PGC-1 β , Ub/FLAG, SYVN1, or SYVN1 3S expression plasmids; 24 h later, 20 μ M MG-132 was added, and cells were lysed in lysis buffer (50 mM HEPES, pH 7.9; 150 mM KCl; 1 mM phenylmethanesulfonyl fluoride; 1% Triton X-100; 10% glycerol; and protease inhibitors). Lysates were mixed with 1 μ g anti-HA antibody conjugated to protein G-sepharose beads. After a 4-h incubation at 4°C, beads were washed three times with lysis buffer. Bound proteins were fractionated by SDS–PAGE and analyzed by immunoblotting.

Cells cultures and transient transfection assay

HEK 293, HEK 293T, and 3T3-L1 cell lines; MEF; and skin fibroblasts were cultured in Dulbecco's modified Eagle's medium as previously described (Yamasaki *et al*, 2007). Transient transfection was performed with Lipofectamine 2000 or LTX according to the manufacturer's protocol (Invitrogen). Cells were lysed with cell lysis buffer (Promega, Madison, WI, USA) 24 h after transfection, and luciferase activity was measured. To ensure equal amounts of DNA, empty plasmids were added to each transfection.

Immunocytochemistry

The detailed procedure has been described in detail elsewhere (Fujita *et al*, 2003, 2005). Briefly, cells were transfected with plasmids and fixed 24 h later with 3.7% formaldehyde, permeabilized with 0.2% Triton X-100, and blocked with 2% bovine serum albumin. Cells were incubated with rat anti-HA (1:1,000) or rabbit anti-FLAG (1:1,000) primary antibody, followed by staining with Alexa Fluor 594 anti-mouse or Alexa Fluor 498 anti-rat secondary antibody (1:1,000; Molecular Probes, Eugene, OR, USA).

Measurement of protein half-life

The assay was performed using a previously described method with some modifications (Yamasaki *et al*, 2007; Bernasconi *et al*, 2010). MEFs derived from *Syvn* knockout mice (Yamasaki *et al*, 2007) were transfected with 1 μ g pcDNA3 *Syvn*/FLAG, empty vector, 1.5 μ g pcDNA3 *Syvn* Δ SyU/FLAG, and 0.75 μ g pcDNA3 HA-PGC-1 β ; 48 h later, cells were treated with 40 μ M cycloheximide for various times, then lysed with buffer (10 mM Tris–HCl, pH 8.0; 150 mM NaCl; 1 mM EDTA; 1% NP-40; 1 mM DTT; and protease inhibitors), and analyzed by immunoblotting with antibodies against PGC-1 β , SYVN1, or α -tubulin (loading control). Each experiment was performed at least three times.

Luciferase assay

The assay was performed as previously described (Chakravarti *et al*, 1996; Nakajima *et al*, 1996). HEK 293 cells were transiently transfected with 12.5 ng PPRE X3-TK-luc reporter plasmid, 25 ng pcDNA3 HA-PPAR α , 25 ng pcDNA3 HA-PGC-1 β , 0.1 ng pRL-CMV, and 15 nM *Syvn1* siRNA. For SYVN1 overexpression, 12.5 ng PPRE X3-TK-luc reporter plasmid, 25 ng pcDNA3 HA-PPAR α , 75 ng pcDNA3 HA-PGC-1 β , 0.1 ng pRL-CMV, and 50 or 100 ng SYVN1 expression vector were transfected. After 12 h, cells were treated with 10 μ M WY-14643 for 6 h and lysed with cell lysis buffer, followed by measurement of luciferase activity. Each experiment was performed at least three times.

RNA interference and real-time PCR

SiRNAs for human *SYVN1* have been previously described (Yamasaki *et al*, 2007). SiRNAs for mouse *Syvn1* and *Pparg1b* were purchased from Ambion Inc. (Austin, TX, USA). SiRNA transfection was performed with Lipofectamine 2000. Total RNA from adipose tissue was purified 7 days after Tam injection using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions and reverse-transcribed using ReverTra Ace with random primers (Toyobo, Osaka, Japan). Real-time PCR was performed using LightCycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany). Expression levels were determined relative to that of 18s rRNA. Primers and probes used in this study are shown in Supplementary Table S2.

Mitochondrial respiration

WAT was collected from control and CAG-Cre-ER;*Syvn1*^{flox/flox} or *Adipoq*-Cre;*Syvn1*^{flox/flox} mice. The detailed procedure for measurement of mitochondrial respiration is described elsewhere (Sjovall *et al*, 2013). Pre-adipocyte cells were obtained from CAG-Cre-ER;*Syvn1*^{flox/flox} mice. White adipose tissues were minced and incubated with collagenase solution for 45 min at 37°C. Then, cell suspension was filtered twice. Cells were cultured in DMEM/F12. Cells were treated with DMSO or tamoxifen, and transient transfection of siRNA was performed 5 days before experiment.

Measurement of basal metabolism

Oxygen consumption and carbon dioxide production were measured using an Oxymax Equal Flow System (Columbus Instruments, Columbus, OH, USA) during the rest period after a 4-h fast 7 days after Tam administration. In addition, motor activity (number of movements) was measured using the DAS system (Neuroscience, Inc, Tokyo, Japan).

Statistical analysis

One-way analysis of variance was used to determine correlations among individual genotypes of mice and body weight at each time point. The non-paired Student's *t*-test was used to analyze mean differences between control and LS-102-treated mice in luciferase activity, tissue weight, basal metabolism, and body weight change at each time point. The Mann–Whitney *U*-test was used in the

mitochondrial respiration assay. A *P* value < 0.05 was considered statistically significant.

Supplementary information for this article is available online: <http://emboj.embopress.org>

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Author contributions

HF, NY, SA, and TN conceived the project and designed the experiments. HF, NY, SA, KS, HK, TS-F, IH, SM, NH, and TN performed the experiments and analyzed the data. HF and TN wrote the manuscript. All authors discussed the results and commented on the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

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