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

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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 (Syvn)b/Hrd3/Der3, an ER-resident E3 ubiquitin ligase with known roles in homeostasis maintenance. Syvn1 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)-1β, and Syvn1 mutants showed upregulation of PGC-1β target genes and increase in mitochondrial 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-1β by SYVN1 and prevented weight gain in mice. Thus, SYVN1 is a novel post-translational regulator of PGC-1β and a potential therapeutic target in obesity treatment.

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

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; Leferova & Lazar, 2009); however, there are presently no effective and safe pharmacological options for obesity prevention and treatment. A better understanding
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)-1α, PGC-1β, and PGC-1-related coactivator) (Puigserver et al, 1998; Andersson & Scarpulla, 2001; Lin, 2001; Kressler et al, 2002) play important roles in mitochondrial biogenesis and energy metabolism, including H2O2-based respiration and β-oxidation of fatty acids. Gene knockout studies have suggested functional differences between PGC-1α and PGC-1β, for instance in terms of lethality (Scarpulla, 2008). Interestingly, PGC-1β overexpression results in an increase in mitochondrial number and respiratory function in cultured cells (St-Pierre et al, 2003); furthermore, PGC-1β 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 (Synv1), 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 Synv1 is a key target for inflammatory cytokines such as tumor necrosis factor α (TNFα), interleukin (IL)-1, and IL-17 (Gao et al, 2006; Toh et al, 2006, 2010).

In the present study, conditional Synv1 knockout mice were generated to clarify the role of Synv1 in obesity. The results highlight a novel function for SYVN1 in the control of body weight and mitochondrial biogenesis through negative regulation of PGC-1β.

**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};ob/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-1β**

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 PPARα and β and their
Figure 1. Body weight and WAT in post-neonatal Syvn1 mutant mice.

A Schematic depiction of Syvn1 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;Syvn1 <sup>flox/flox</sup> (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;Syvn1 <sup>flox/flox</sup> mice versus vehicle-treated CAG-Cre-ER;Syvn1 <sup>flox/flox</sup> mice, Tam- or vehicle-treated Syvn1 <sup>flox/flox</sup> 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. *P < 0.05, **P < 0.01.

H–J Fat accumulation in post-neonatal Syvn1 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.
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-SYVN1ΔTM) bound HA-tagged PGC1β but not PPARγ or γ or PGC-1α (Fig 4A). In a GST pull-down assay, PGC-1β 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-1β to aa 195–367 containing an LXXLL motif of middle portion (Supplementary Fig S4C). To verify the interaction in cells, HA-PCG-1β and FLAG-tagged SYVN1 (SYVN1/FLAG) were co-transfected into HEK 293T cells. HA-PCG-1β co-immunoprecipitated with SYVN1/FLAG but not the control FLAG vector (Fig 4D). To further investigate the interaction between SYVN1 and PGC-1β, whole-cell lysates of HEK 293T cells, in which SYVN1 and PGC-1β were expressed, were precipitated with anti-SYVN1 antibody or a control non-immune mouse immunoglobulin (IgG) and probed with an antibody against PGC-1β in an immunoblotting assay. Endogenous PGC-1β 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-1β under normal physiological conditions.

Since SYVN1 is an ER-resident protein and PGC-1β translocates into the nucleus, their subcellular localization was investigated by immunofluorescence staining in transiently transfected HEK 293T cells. HA-PCG-1β 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-PCG-1β with SYVN1R266A, R267A/FLAG, or SYVN1ASyU/FLAG, which do not

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**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, Changes in body weight in Tam-treated mice. CAG-Cre-ER;Syvn1flx/flx, ob/ob (KO; ob/ob) and CAG-Cre-ER;Syvn1flx/flx, db/db (KO; db/db) mice, red line, Syvn1flx/flx, ob/ob, Syvn1flx/flx, ob/ob, and Syvn1flx/flx, db/db; and Syvn1flx/+; 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;Syvn1flx/flx, ob/ob and CAG-Cre-ER;Syvn1flx/flx, db/db mice after Tam administration. Subcutaneous fat is shown by white arrows.

B, C Average daily food intake measured after Tam injection in CAG-Cre-ER;Syvn1flx/flx, ob/ob (D) and CAG-Cre-ER;Syvn1flx/flx, db/db (G) mice.

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

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

PGC-1β is a novel substrate of SYVN1.

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

Since ubiquitinated proteins are degraded by the proteasome, experiments were performed to verify whether the level of PGC-1β protein is regulated by SYVN1. PGC-1β protein level was markedly elevated in the WAT of CAG-Cre;Syvn1<sup>flox/flox</sup> (KO) and Syvn1<sup>flox/flox</sup> mice (Control). The ubiquitination of HA-PGC-1β was measured in mouse embryonic fibroblasts (MEFs) coexpressing HA-PGCB-1β and WT SYVN1 or SYVN1ΔSyU (Supplementary Fig S4G). WT SYVN1 overexpression significantly shortened the half-life of HA-PGC-1β, whereas SYVN1ΔSyU had no effect (Fig 4I). These results indicate that PGC-1β protein expression is negatively regulated by SYVN1 at the post-transcriptional level and strongly suggest that SYVN1 is a major E3 ligase for PGC-1β in cells.

RNA (siRNA), which resulted in a near-complete disappearance of SYVN1 expression in HEK 293 cells: PGC-1β 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-1β degradation, the proteasome inhibitor MG-132 was applied to cultured skin fibroblasts. Similar to Tam treatment, MG-132 upregulated PGC-1β protein levels in vehicle-treated cells by 1.6-fold, but produced no additional effects on Tam-treated skin fibroblasts (Fig 4L). To confirm whether the SYVN1–PGC-1β interaction is critical for SYVN1-mediated PGC-1β degradation, the half-life of PGC-1β was measured in mouse embryonic fibroblasts (MEFs) coexpressing HA-PGC-1β and WT SYVN1 or SYVN1ΔSyU (Supplementary Fig S4G). WT SYVN1 overexpression significantly shortened the half-life of HA-PGC-1β, whereas SYVN1ΔSyU had no effect (Fig 4L). These results indicate that PGC-1β protein expression is negatively regulated by SYVN1 at the post-transcriptional level and strongly suggest that SYVN1 is a major E3 ligase for PGC-1β in cells.

Negative regulation of PGC-1β by SYVN1

PGC-1β functions as a coactivator of several transcription factors including PPARα 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-1β, the activity of PPAR luciferase (PPRE X3-TK-luc) (Kim et al., 1998)—which contains three PPAR binding sites and is
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 PPARα agonist. Syvn1 but not control siRNA transfection significantly enhanced reporter activity under Wy-14643 induction (Fig 5A). Furthermore, the cotransfection of PGC-1β and Syvn1 siRNA activated reporter activity. These results indicate that loss of Syvn1 enhances PPARα-mediated transcription in a PGC-1β-dependent manner. Conversely, Syvn1 overexpression inhibited the coactivator function of PGC-1β (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 Ppard siRNAs (Fig 5D). Furthermore, transcript levels of medium chain acyl-coenzyme A dehydrogenase and mitochondrial ATP synthase β subunit, two known PGC-1β target genes (Rodriguez-Calvo et al., 2006; Shao et al., 2010), were upregulated in Adipoq-Cre;Syvn1lox/lox (Fig 5E and F) and CAG-Cre-ER;Syvn1lox/lox 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;Syvn1lox/lox compared to controls (Fig 5G). To investigate whether the mitochondria in CAG-Cre-ER;Syvn1lox/lox mice were functional, O2 consumption in a single-cell suspension of primary...
mouse adipocytes was measured and was found to be higher in cells from-CAG-Cre-ER;Syvn1^{floxed} than from control mice (Supplementary Fig S5B). Moreover, mitochondrial respiration and activity were higher in-CAG-Cre-ER;Syvn1^{floxed} than in control mice (Fig 5H). Western blotting was performed with anti-SYN1 and anti-PPARγ antibodies. *Non-specific band. Source data are available online for this figure.

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-1β function by SYVN1, as evidenced by reduced ubiquitination of PGC-1β (Fig 6A) and activation of PPARγ-mediated transcription in HEK 293 cells (Fig 6B). In addition, LS-102 induced the expression of PGC-1β 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^{floxed} 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 TNFα 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-TNFα 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-1β was identified as a SYVN1-interacting molecule (Fig 4). PGC-1β, PGC-1α, and
Figure 5. Regulation of PGC-1β 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 ng SYVN1 expression vector was co-transfected (B, C); 16 h later, cells were treated with vehicle or 10 μM 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×. The number of mitochondria in the area (500 pixel × 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^flox/flox (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^flox/flox (right) mice. An increase in mitochondrial volume can be seen as an enlargement in cytoplasmic area (M, mitochondria). Magnification, 10,000×.

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^flox/flox 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 ± 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 PPAREX3-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 Control, 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.
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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-1β 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-1β 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-1β by SYVN1 could be evidenced for the unique role of PGC-1β compared to PGC-1α. Thus, a novel function for SYVN1 in energy metabolism could be exerted via negative regulation of PGC-1β. Recently, knockout mice of Sel-1 suppressor of lin-12-like protein (Sel1L), which is an adapter 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 PPARα—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 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 challenging. In the present study, LS-102—a selective inhibitor of the E3 ligase activity of SYVN1—induced activation of PGC-1β 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-1β 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-1β.

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 (WS-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 Syvn1flkox/+ 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 Syvn1flx/+ and heterozygous CAG-Cre-ER mice (Jackson Laboratories) were crossed to generate double heterozygous CAG-Cre-ERSyvn1flx/+ mice, which were bred with homozygous Syvn1flx/flx mice to produce conditional Syvn1 homozygous (CAG-Cre-ERSyvn1flx/flx) and Syvn1 heterozygous (CAG-Cre-ERSyvn1flx/+ ), homozygous Syvn1flx/flx and heterozygous Syvn1flx/+ mice. Control mice lacking the Cre transgene (Syvn1flx/flx or Syvn1flx/+ ) received Tam injections to eliminate any potential effects of Tam. To generate the CAG-Cre-ERSyvn1flx/flx, ob/ob and CAG-Cre-ERSyvn1flx/prom/d/db genotypes, CAG-Cre-ER: Syvn1flx/flx mice were bred with ob/+ or db/+ mice (Jackson Laboratories), yielding the compound heterozygotes CAG-Cre-ERSyvn1flx/flx, ob/+ and CAG-Cre-ERSyvn1flx/flx, db/+ . These were interbred to obtain CAG-Cre-ERSyvn1flx/flx, ob/ob, Syvn1flx/flx, ob/ob, and Syvn1flx/flx, ob/ob mice or CAG-Cre-ERSyvn1flx/flx, db/+. The genotypes Syvn1flx/flx, ob/ob, Syvn1flx/flx, ob/ob, Syvn1flx/flx, ob/ob, and Syvn1flx/flx, ob/ob were used as control mice. To generate adipose-specific Syvn1 knockout mice, Syvn1flx/flx and Adipoq-Cre mice (Jackson Laboratories) were crossed to generate Adipoq-Cre-Syvn1flx/flx compound heterozygotes, which were mated with Syvn1flx/flx 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, 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 Synt1 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 antisera against PGC-1β was generated by immunizing rabbits with purified GST-PGC-1β (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 µg/ml aprotinin; and 1 µg/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-1β and SYVN1/FLAG expression vectors; 24 h later, cells were lysed in 200 µl 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 µg 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-1β, 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 MgCl2. Immunoprecipitation was carried out with anti-SYVN1 antibody or control IgG, followed by Western blot using anti-FLAG–α-antibody.

In vitro ubiquitination assays

The in vitro ubiquitination assay was performed as previously described (Yamasaki et al., 2007). Briefly, GST-PGC-1β (aa 1–367) was incubated with 0.75 µg HA-Ub, 125 ng E1 (Biomol International, Plymouth Meeting, PA, USA), 150 ng UbH5C, and 150 ng maltose-binding protein-tagged SYVN1-ATM-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-1β was analyzed by Western blotting with anti-HA antibody. For inhibition of ubiquitination by LS102, FLAG-PGC-1β was purified from HEK 293T cells transfected with FLAG-PGC-1β with an anti-FLAG antibody and incubated with 1.5 µg 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-1β 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-1β, Ub/FLAG, SYVN1, or SYVN1 3S expression plasmids; 24 h later, 20 µM MG-132 was added, and cells were lysed in lysis buffer (50 mM HEPES, pH 7.9; 150 mM KCl; 1 mM phenylmethylsulfonyl fluoride; 1% Triton X-100; 10% glycerol; and protease inhibitors). Lysates were mixed with 1 µg 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 (1:2,000) or rabbit anti-FLAG (1:1,000) primary antibody, followed by staining with Alexa Fluor 594 anti-mouse or Alexa Fluor 498 anti-rabbit 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 Synn knock-out mice (Yamasaki et al., 2007) were transfected with 1 µg pcDNA3 Synn/FLAG, empty vector, 1.5 µg pcDNA3 SynnASy/FLAG, and 0.75 µg pcDNA3 HA-PGC-1β; 48 h later, cells were treated with 40 µM 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-1β, 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-PPARα, 25 ng pcDNA3 HA-PGC-1β, 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-PPARα, 75 ng pcDNA3 HA-PGC-1β, 0.1 ng pRL-CMV, and 50 or 100 ng SYVN1 expression vector were transfected. After 12 h, cells were treated with 10 μM 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 (Yamazaki et al., 2007). SiRNAs for mouse Syvn1 and Pparγ1b 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<sup>lox/lox</sup>/lox or Adipoq-Cre;Syvn1<sup>lox/lox</sup> 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<sup>lox/lox</sup> 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.

References


