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Identification of the inhibitory activity of walnut extract on the E3 ligase Syvn1

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Abstract. Synoviolin (Syvn1), an E3 ubiquitin ligase in endoplasmic reticulum-associated protein degradation, is involved in rheumatoid arthritis, fibrosis, liver cirrhosis and obesity. We previously demonstrated that Syvn1 negatively regulates the function of peroxisome proliferator-activated receptor gamma coactivator-1β (PGC-1β). In addition, treatment with a Syvn1 inhibitor suppressed weight gain in a mouse model of obesity by activating PGC-1β via Syvn1 inhibition. It has been suggested that the Syvn1 inhibitors may have therapeutic benefits in obese patients. The present study tested the inhibitory activity of walnut extract, a natural product, on Syvn1 activity. Walnut extract inhibited the effect of Syvn1 on the cell proliferation of rheumatoid synovial cells and repressed the interaction between PGC-1β and Syvn1 in an *in vitro* binding assay. Polyubiquitination of PGC-1β by Syvn1 was suppressed by walnut extract in a concentration-dependent manner, but walnut extract did not have an inhibitory effect on the autoubiquitination of Syvn1. Treatment with walnut extract in mouse embryonic fibroblasts increased the number of mitochondria, suggesting that exposure to the extract recovered PGC-1β function. These results demonstrated that constituents of walnut extract may serve as lead compounds in drug development efforts aiming to produce drugs to treat patients with obesity and obesity-associated metabolic diseases.

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Abbreviations: Syvn1, synoviolin; PGC-1β, peroxisome proliferator-activated receptor coactivator 1β; RSCs, rheumatoid synovial cells; SPF, specific pathogen-free

Key words: synoviolin, walnut extract, PGC-1β, ubiquitination, E3 ligase

Introduction

Obesity is a global health problem associated with various metabolic disorders, including diabetes, hypertension, cardiovascular disease, and depression (1). There are two major types of medication used to treat obese patients (2). Some anti-obesity drugs, such as appetite suppressants, reduce food intake by regulating the function of the central nervous system, whereas other drugs block absorption of lipids from food in the intestine. In addition, some candidate anti-obesity drugs directly modulate energy metabolism without affecting the central nervous system, such as peroxisome proliferator-activated receptor (PPAR) agonists. However, the undesirable side effects of currently available agonists significantly limit their use.

Natural products have been used for millennia to treat diseases and mitigate the adverse effects of toxic substances (3). Recently, berberine and curcumin, which have antioxidant and anti-inflammatory properties, have been reported to have anti-obesity effects (4-6). Berberine and curcumin ameliorate obesity by increasing energy expenditure. Berberine activates AMP-activated protein kinase (AMPK) (4), a key energy sensor that leads to reduced energy storage and increased energy production. In addition, berberine regulates expression of uncoupling protein 1 (UCP1), which is found in the mitochondria and generates heat in brown adipose tissue (BAT) and white adipose tissue (WAT) in an AMPK- and PPAR gamma coactivator-1 alpha (PGC-1α)-dependent manner (6). These studies suggest that therapeutic chemicals are involved in natural products with antioxidant and anti-inflammatory properties.

Synoviolin (Syvn1), a mammalian homolog of Hrd1p/Der3p, is involved in the development of obesity, rheumatoid arthritis, fibrosis, limb girdle muscular dystrophy, and liver cirrhosis (7-11). Syvn1 was identified in rheumatoid synovial cells (RSCs) as an endoplasmic reticulum (ER)-resident E3 ubiquitin ligase (7) that plays an important role in RSC proliferation (12). LS-102, a Syvn1 inhibitor, repressed proliferation of RSCs in a Syvn1-dependent manner (13). We recently demonstrated that global elimination of *Syvn1* in post-neonatal mice was associated with weight loss and reduced white adipose tissue (14). Adipose tissue from *Syvn1* knockout mice showed significant up-regulation of PGC-1β-target genes, as well as a significant increase in the number of mitochondria, mitochondrial respiration, and basal energy expenditure. Syvn1 interacts with PGC-1β and negatively regulates its function. Therefore, we propose that knockout or inhibition of Syvn1 leads to stabilization of PGC-1β, enhancing energy expenditure. These results suggest that Syvn1 is a therapeutic target for anti-obesity drugs. However, natural products that inhibit Syvn1 activity have not been found.

To identify Syvn1 inhibitors with antioxidant and anti-inflammatory properties in this study, we performed a screening of natural products based on their inhibitory effects on RSC proliferation. We found that walnut extract inhibited Syvn1 activity, indicating that walnut extract could be used to treat patients with obesity.

Materials and methods

Ethical considerations. All human experimental protocols in the present study (no. 2728, 2729, 3758, 3759) were approved by the Ethics Review Committee of Tokyo Medical University (Tokyo, Japan). RA patients received stable doses of methotrexate (6-10 mg/week) before joint replacement surgery. Written informed consent was obtained from all patients prior to the collection of joint tissue samples. 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 (no. S-28038, S-28040).

Mice. 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). Mice had free access to water bottles. Tamoxifen (Tam)-inducible Syvn1 knockout mice (*CAG*-Cre-ER; *Syvn1*$^{flox/flox}$) was generated previously (14). To isolate MEFs, embryos were isolated at E13.5, and the head and internal (including reproductive) organs were removed. The remaining tissue was physically dissociated and incubated in trypsin at 37˚C for 15 min. Cells were resuspended in DMEM and plate the cells in 10 cm tissue culture dishes. On the next day, medium was changed and cells were expanded for two passages before freezing.

Plasmids, antibodies, and walnut extract. The Syvn1 (NM_032431) and PGC-1β (NM_133249) plasmids are described in the literature (7,14,15). The following antibodies were used: Anti-HA (3F10) (Roche Molecular Biochemicals, Indianapolis, IN, USA). Polyclonal antiserum against GST was generated by immunizing rats with purified GST. Anti-PGC-1β antibody has been described previously (14). Walnut extracts were prepared from the branches of walnut trees by the standard ethanol extraction method (16). Briefly, the air-dried walnut branches were milled into fine powder in the blender and the fibrous powder of walnut branches was extracted twice, on each occasion with 60% ethyl alcohol at room temperature for 24 h. The combined ethanol extract was filtered, and the filtrate was concentrated to dryness under reduced pressure in a rotary evaporator. The ethanol extract was freeze-dried. Without any further purification, the plant crude ethanol extract was used in our study. Aliquot portions of the ethanol extract was dissolved in DMSO for use of our experiments.

Cell culture and assessment of cell proliferation. Rheumatoid synovial cells were obtained by standard methods (17). Briefly, the tissue was minced into small pieces and digested with collagenase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany). The single-cell suspension was incubated overnight, and then floating cells were removed, and adherent cells were cultured in dishes. RSCs and MEFs, which were derived from Tam-inducible *Syvn1* knockout mice (*CAG*-Cre-ER; *Syvn1*$^{flox/flox}$), were cultured in Dulbecco's modified Eagle's medium (DMEM) as previously described (14). RSC proliferation was measured with DMSO or walnut extract treatment (1, 3.3, 10, 33.3 μg/ml) for 3 days using the Cell Counting Kit-8 (Dojindo, Tokyo, Japan). MEFs were treated with DMSO or Tamoxifen (2.5 μM) for 2 days, and were then treated with DMSO or walnut extract (50 μg/ml) for 3 days. Electron microscopic analysis was then performed.

GST pull-down assay. The GST pull-down assay was performed as previously described (14,18). Briefly, GST-Syvn1ΔTM and MBP-PGC-1β (1-367) were expressed and purified using glutathione sepharose beads and amylose beads, respectively (GE Healthcare Life Sciences, Little Chalfont, UK). GST-Syvn1ΔTM was incubated with MBP-PGC-1β 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 Na_3VO_4; 5 mM NaF; 1 μg/ml aprotinin; and 1 μg/ml leupeptin) for 4 h at 4˚C. After washing the beads with buffer A, bound proteins were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by western blotting.

In vitro ubiquitination assay. *In vitro* ubiquitination assays were performed as previously described (14). Briefly, GST-PGC-1β (1-367) was incubated with 0.75 μg HA-Ub, 125 ng E1 (Biomol International, Plymouth Meeting, PA, USA), 150 ng UbcH5c, and 150 ng MBP-Syvn1ΔTM in reaction buffer (50 mM Tris-HCl, pH 7.5; 5 mM $MgCl_2$; 0.6 mM DTT; and 2 mM ATP) at 37˚C for 2 h. Glutathione sepharose was added to the solution, after which 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). Ubiquitinated PGC-1β was analyzed by western blotting using anti-PGC-1β antibodies.

Ubiquitination assay. *In vivo* ubiquitination assays were performed as previously described (14). Briefly, 293T cells were transfected with HA-PGC-1β, FLAG Ub, or Syvn1 expression plasmids. Cells were treated with DMSO or walnut extract (50 μg/ml) for 3 days. 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 μg anti-HA antibody conjugated to

Table I. Primers and probes for reverse transcription-quantitative polymerase chain reaction.

Gene	Type	Primer (5'-3')	Probe no.
SYVN1	Forward	ccagtacctcaccgtgctg	16
	Reverse	tctgagctagggatgctggt	
18sRNA	Forward	gcaattattccccatgaacg	48
	Reverse	gggacttaatcaacgcaagc	
MCAD	Forward	tcttgctggaaatgatcaaca	88
	Reverse	gggctctgtcacacagtaagc	
Atp5b	Forward	tgagagaggtcctatcaaaacca	15
	Reverse	cctttatcccagtcaccagaa	
ACTB	Forward	ctaaggccaaccgtgaaaag	64
	Reverse	accagaggcatacagggaca	

SYVN1, synoviolin; MCAD, medium chain acyl-coenzyme A dehydrogenase; Atp5b, mitochondrial ATP synthase β subunit; ACTB, β-actin.

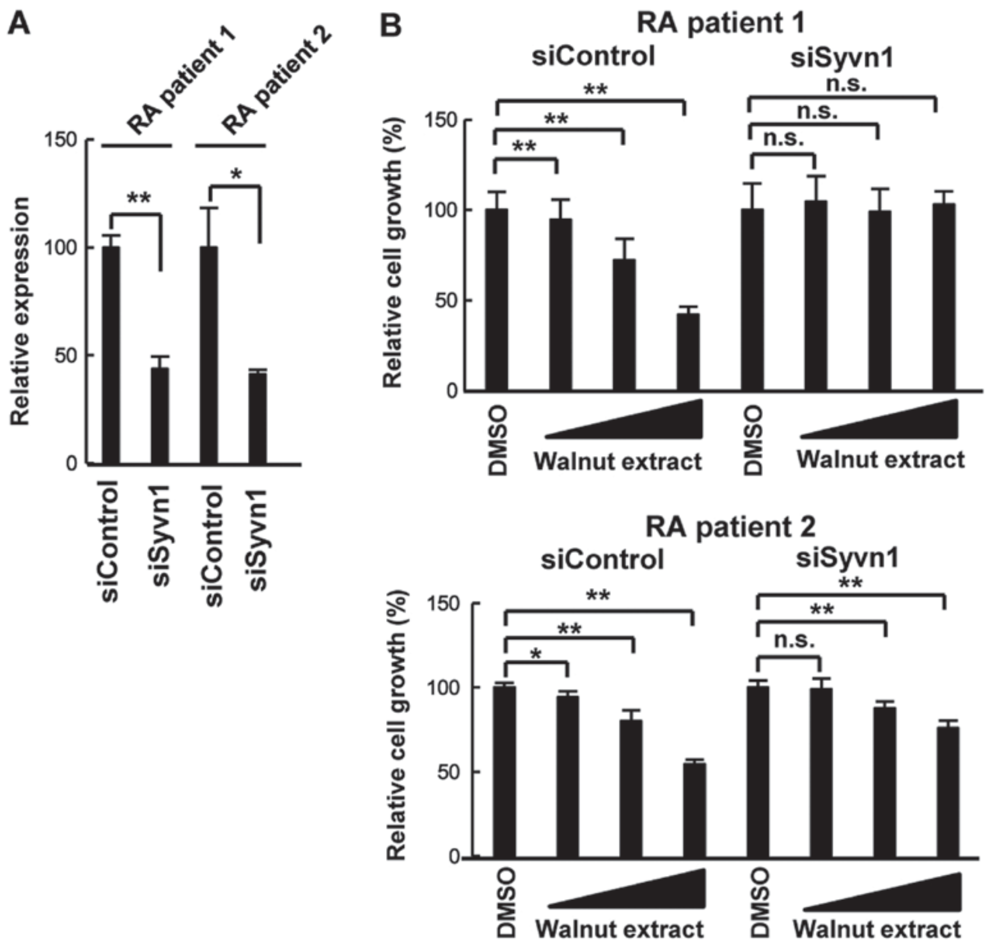

Figure 1. Effect of walnut extract on RSC growth. (A) Effect of Syvn1 knockdown by siRNA. RSCs derived from two patients with RA were transiently transfected with control siRNA (siControl) or siRNA for Syvn1 (siSyvn1). Following 2 days, total RNA were purified and reverse transcription-quantitative polymerase chain reaction was performed. Individual measurements were standardized using 18S RNA, and the average for siControl was set to 100. (B) RSCs were transiently transfected with siControl or siSyvn1. Following 2 days, RSCs were treated with walnut extract (1, 3.3, and 10 μg/ml) for 3 days. Data are presented as the mean ± standard deviation (n=3). *P<0.05 and **P<0.01, as indicated. n.s., not significant; DMSO, dimethyl sulfoxide; RSC, rheumatoid synovial cell; RA, rheumatoid arthritis; si-/siRNA, small interfering RNA; Syvn1, Synoviolin.

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.

MitoTracker staining. For analysis of mitochondria using MitoTracker Red (Molecular Probes, Eugene, OR, USA), MEFs were treated with DMSO or walnut extract (50 μg/ml) for 3 days.

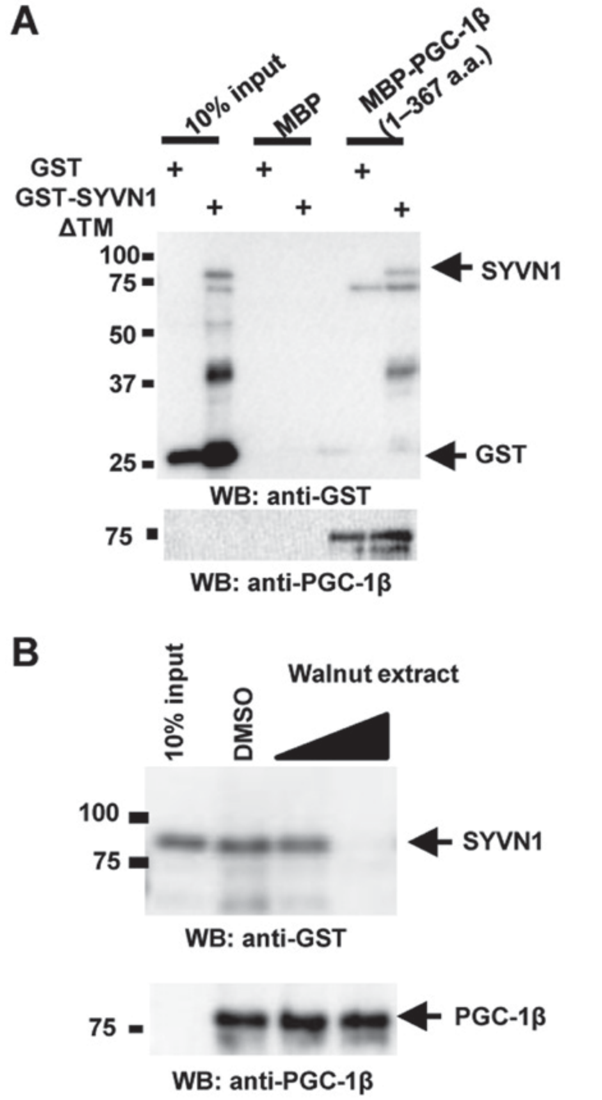

Figure 2. Effect of walnut extract on the interactions between SYVN1 and PGC-1β. (A) An *in vitro* binding assay was performed with GST or GST-SYVN1ΔTM and MBP or MBP-PGC1β (amino acids 1-367). (B) An *in vitro* binding assay was performed using MBP-PGC-1β (amino acids 1-367) and GST-SYVN1ΔTM in the presence of DMSO or walnut extract (0.5 or 5 µg/ml). WB was performed using anti-GST antibodies or anti-PGC1β antibodies. Syvn1, Synoviolin; PGC-1β, PGC-1β, peroxisome proliferator-activated receptor coactivator 1β; GST, glutathione S-transferase; TM, transmembrane domain; MBP, maltose binding protein; DMSO, dimethyl sulfoxide; WB, western blotting.

Mitochondria were stained with the MitoTracker Red probe for 30 min at 37˚C according to the manufacturer's protocol. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). The intensity of staining by mitotracker was measured (n=8).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tam-inducible Syvn1 knockout MEFs were treated with DMSO (WT MEFs) or Tam (Syvn1 knockout MEFs) for 2 days, and then WT MEFs and Syvn1 knockout MEFs were treated with DMSO or walnut extract (50 µg/ml) for 3 days. Total RNA from MEFs treated with DMSO or walnut extract was purified by using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions and reverse transcribed by using ReverTra Ace with random primers (Toyobo, Osaka, Japan). RT-qPCR was performed by using LightCycler 480 Probes Master (Roche Diagnostics, Mannheim, Germany) and the Step One Plus Detection System (Applied Biosystems; Life Technologies Japan, Tokyo, Japan). The thermocycling conditions were as follows: Initial denaturation at 95˚C for 10 min, followed by 45 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 20 sec and extension at 72˚C for 1 sec. Expression levels were determined relative to that of *18sRNA* (RSCs) or *ACTB* (MEFs). Primers and probes used in the present study are shown in Table I. Relative expression was determined using the $2^{-\Delta\Delta Cq}$ method (19).

RNA interference assay. siRNAs for Syvn1 were previously described (14). Transfection with siRNAs (20 µM) was performed by using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol. Total RNA from RSCs was purified 2 days after transfection using ISOGEN (Nippon Gene, Tokyo, Japan) according to the manufacturer's instructions, and reverse transcribed using ReverTra Ace with random primers (Toyobo).

Statistical analysis. All data are expressed as the mean ± standard deviation and were analyzed using Excel Statistics 2012 version 1.00 (Social Survey Research Information Co., Ltd., Tokyo, Japan). Differences between two groups were examined by Student's t-test. One-way analysis of variance with Tukey-Kramer post hoc analysis was used to determine correlations in datasets containing multiple groups. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Screening of natural products for Syvn1 inhibitors. Syvn1 is a crucial factor involved in RSC proliferation (7,13,20). To identify Syvn1 inhibitors in natural products, we tested the effects of natural products on RSC proliferation with or without Syvn1. At first, we performed knockdown experiments with control siRNA (siControl) or siRNA for Syvn1 (siSyvn1). RT-qPCR showed that siSyvn1 induced 60% repression of Syvn1 expression (Fig. 1A). Walnut extract inhibited proliferation in a concentration-dependent manner in two RSC lines treated with siControl (Fig. 1B). Whereas, the inhibitory effect was attenuated in siSyvn1-treated cells (Fig. 1B). In the case of patient 1, walnut extract did not significantly have any effect (n.s.). In the case of patient 2, walnut extract had still inhibited cell growth, however, the strength of the effect was reduced as compared to control siRNA-treated cells.

Regulation of Syvn1-PGC-1β interaction by walnut extract. Syvn1 negatively regulates PGC-1β activity via direct interaction with PGC-1β *in vitro* and *in vivo* (14). To determine whether walnut extract inhibits the interaction of Syvn1 with PGC-1β, we performed *in vitro* binding assays using glutathione S-transferase-tagged Syvn1 lacking the transmembrane domain (GST-Syvn1ΔTM) and maltose binding protein-tagged PGC-1β (amino acids 1-367) (MBP-PGC-1β (1-367)). As previously reported (14), MBP-PGC-1β (1-367) directly bound to GST-Syvn1ΔTM (Fig. 2A). MBP-PGC-1β

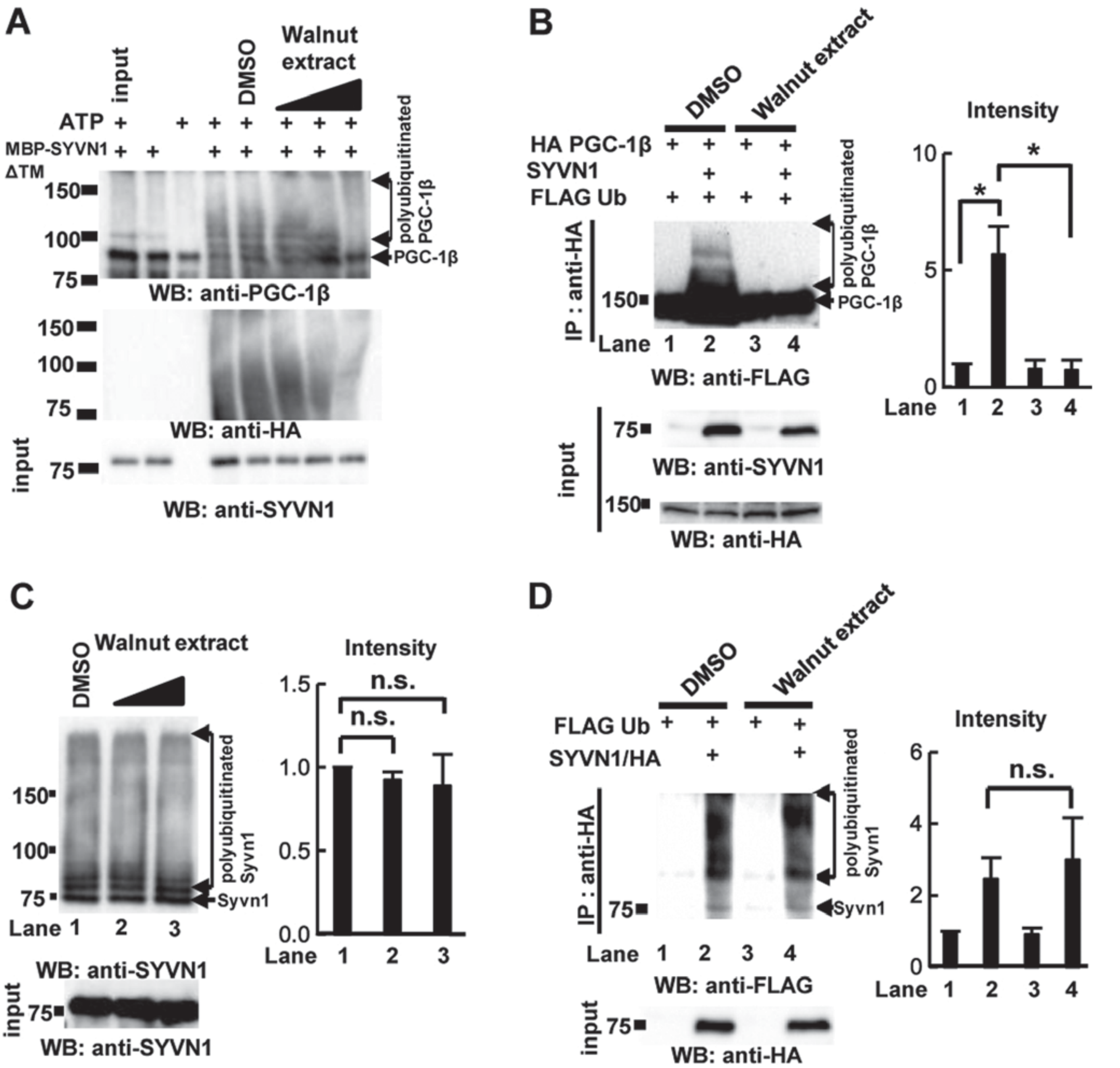

Figure 3. Effect of walnut extract on the polyubiquitination of PGC-1β. (A) *In vitro* ubiquitination assays were performed with MBP-SYVN1ΔTM, GST-PGC-1β (1-367), E1 and E2 enzymes, and HA-Ub in the presence of DMSO or walnut extract (0.5, 5 or 50 μg/ml). WB was performed using anti-PGC-1β antibodies and anti-SYVN1 antibodies. (B) *In vivo* ubiquitination assays were performed. 293T cells were transfected with HA PGC-1β, FLAG Ub and/or SYVN1 expression plasmids and cells were treated with DMSO and walnut extract (50 μg/ml) for 3 days. Whole cell extracts were immunoprecipitated with anti-HA antibody. WB was performed using anti-FLAG and anti-HA antibodies. Quantification of the data is presented. *P<0.05, as indicated. (C) Effect of walnut extract on SYVN1 autoubiquitination. *In vitro* ubiquitination assays were performed with GST-SYVN1ΔTM, E1 and E2 enzymes, and HA-Ub in the presence of DMSO or walnut extract (0.5, 5 or 50 μg/ml). WB was performed using anti-SYVN1 antibodies. Quantification of the data is presented. (D) *In vivo* ubiquitination assays were performed. 293T cells were transfected with FLAG Ub, and SYVN1/HA expression plasmids and cells were treated with DMSO and walnut extract (50 μg/ml) for 3 days. Whole cell extracts were immunoprecipitated with anti-HA antibody. WB was performed using anti-FLAG and anti-HA antibodies. Quantification of the data is presented. The positions of molecular weight standards (in kDa) are indicated to the left of each image. Data are expressed as the mean ± standard deviation (n=3). HA, hemagglutinin; Ub, ubiquitin; DMSO, dimethyl sulfoxide; WB, western blotting; Syvn1, Synoviolin; PGC-1β, PGC-1β, peroxisome proliferator-activated receptor coactivator 1β; ATP, mitochondrial adenosine triphosphate synthase; GST, glutathione S-transferase; TM, transmembrane domain; MBP, maltose binding protein; n.s., not significant.

(1-367) did not bind to GST, and GST-Syvn1ΔTM did not bind to MBP (Fig. 2A). Walnut extract inhibited the interaction of Syvn1 and PGC-1β in a concentration-dependent manner (Fig. 2B).

Inhibition of PGC-1β ubiquitination by walnut extract. Syvn1 ubiquitinates PGC-1β *in vitro* and *in vivo*, negatively regulating PGC-1β abundance (14). To investigate ubiquitination of PGC-1β by Syvn1 in the presence of walnut extract, we performed an *in vitro* assay of ubiquitination with MBP-Syvn1ΔTM and GST-PGC-1β (1-367) in the presence of ATP, hemagglutinin-tagged ubiquitin (HA-Ub), E1, and E2 (UbcH5c) (14). As previously reported (14), Syvn1 induced polyubiquitination of PGC-1β *in vitro*, and polyubiquitination

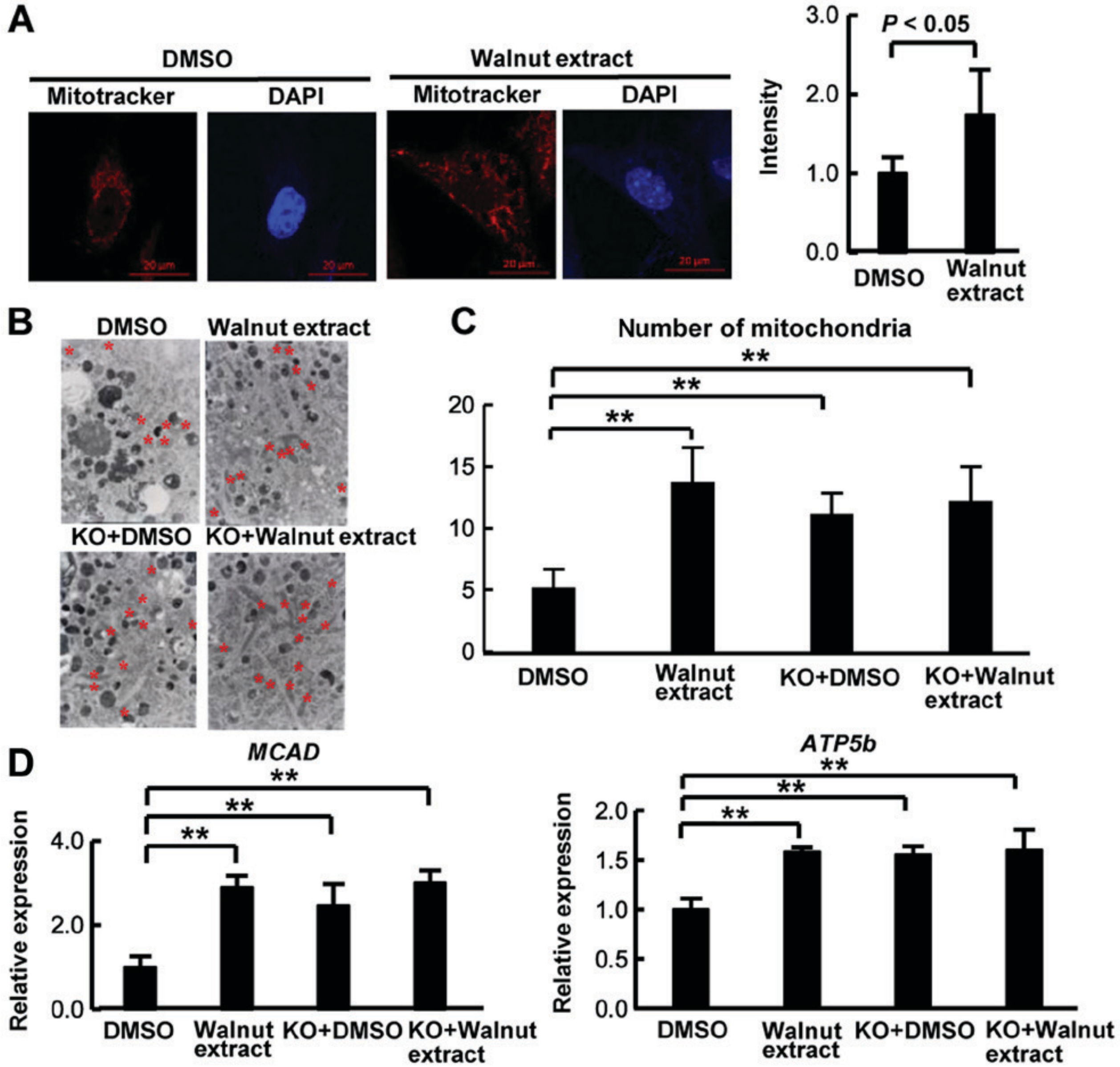

Figure 4. Effect of walnut extract on the number of mitochondria. (A) MEFs were treated with DMSO or walnut extract (50 μg/ml) for 3 days. Cells were immunostained with MitoTracker Red (red), and DAPI (nuclei, blue); scale bars, 20 μm. The intensity of staining was measured by MitoTracker. Data were expressed as the mean ± standard deviation (n=8). (B) Representative electron micrographs of walnut extract-treated cells (50 μg/m) are presented. Increased mitochondrial volume can be observed in the large cytoplasmic areas in MEFs (mitochondria are indicated by red asterisks). Magnification, x10,000. (C) The number of mitochondria in the area (1,000x1,000 pixels) was measured. Data were expressed as the mean ± standard deviation (n=13). (D) Total RNA was isolated from wild-type MEFs and Syvn1 knockout MEFs treated with DMSO or walnut extract, and reverse transcription-quantitative polymerase chain reaction was performed. Individual measurements were standardized using β-actin, and then the average DMSO value was set to 1. Data were expressed as the mean ± standard deviation (n=6). **P<0.01, as indicated. MEFs, mouse embryonic fibroblasts; DMSO, dimethyl sulfoxide; Syvn1, Synoviolin; KO, Syvn1 knockout mice; MCAD, medium chain acyl-coenzyme A dehydrogenase; ATP5b, mitochondrial adenosine triphosphate synthase β subunit.

of PGC-1β was not observed in the absence of ATP or Syvn1. Walnut extract inhibited PGC-1β polyubiquitination (Fig. 3A). To examine the effect of walnut extract *in vivo*, we performed *in vivo* ubiquitination assay. FLAG-tagged Ub and HA-PGC-1β were coexpressed with Syvn1 in HEK 293T cells and cells were treated with DMSO or walnut extract (50 μg/ml) for 3 days. The ubiquitination of PGC-1β was observed in Syvn1-expressing cells (DMSO-treated cells). The treatment with walnut extract decreased the ubiquitination of PGC-1β in Syvn1-expressing cells (Fig. 3B). Walnut extract did not inhibit autoubiquitination of Syvn1 (Fig. 3C). The effect of walnut extract was also examined *in vivo*. FLAG-tagged Ub (FLAG Ub) and Syvn1/HA were coexpressed in HEK 293T cells and cells were treated with DMSO or walnut extract (50 μg/ml) for 3 days. Autoubiquitination of Syvn1 was not inhibited in walnut extract-treated cells (Fig. 3D).

Effects of walnut extract on PGC-1β function. PGC-1β is a coactivator of several transcription factors, including PPARα, and is implicated in various biological processes, including mitochondrial biogenesis (21,22). LS-102 exposure increases the number of mitochondria in cultured cells (14). To investigate the effect of walnut extract on regulation of mitochondria by PGC-1β, we performed mitochondrial staining using

MitoTracker with mouse embryonic fibroblasts (MEFs) (14). MitoTracker staining showed increased mitochondria in MEFs treated with walnut extract compared with MEFs treated with DMSO (Fig. 4A). We used electron microscopy and counted mitochondria. The cells treated with walnut extract had significantly more mitochondria than the cells treated with dimethyl sulfoxide (DMSO) did (Fig. 4B and C). In addition, the number of mitochondria in Syvn1 knockout MEFs treated with DMSO (KO+DMSO) increased compared to that in wildtype MEFs treated with DMSO. However, walnut extract produced no additional effect on the number of mitochondria in the Syvn1 KO MEFs (KO+Walnut extract). Furthermore, the expression of PGC-1β target genes, medium chain acyl-coenzyme A dehydrogenase (MCAD) and mitochondrial ATP synthase β subunit (ATP5b), was also induced in MEFs treated with walnut extract and in Syvn1 KO MEFs treated with DMSO (Fig. 4D). However, walnut extract produced no additional effect on the induction of MCAD and ATP5b in the Syvn1 KO MEFs (Fig. 4D).

Discussion

The development of Syvn1 inhibitors is an active field of study because they have the potential to treat patients with several diseases, including rheumatoid arthritis, fibrosis, liver cirrhosis, and obesity (7-10,13). In a previous study, we demonstrated that the Syvn1 inhibitor, LS102, suppressed weight gain in a mouse model of obesity via inhibition of PGC-1β polyubiquitination by Syvn1 (14). In this study, we showed that walnut extract, a natural product, inhibits Syvn1 activity. Walnut extract inhibited the interaction between Syvn1 and PGC-1β and repressed polyubiquitination of PGC-1β by Syvn1. Taken together, these results suggest that walnut extract has anti-obesity activity.

Selectivity and specificity are important characteristics for targeted drugs. We identified LS-102 as an inhibitor of autoubiquitination of Syvn1 via a high-throughput screening (13) and demonstrated its inhibitory effect on the E3 ligase activity of Syvn1. LS-102 suppressed polyubiquitination of target proteins of Syvn1, including nuclear factor erythroid 2-related factor 2 (NRF2), V247M α-sarcoglycan mutant, and PGC-1β (10,11,13). Interestingly, Syvn1 interacts with NRF2 and V247M α-sarcoglycan mutant through proline-rich domains at the C-terminus, whereas Syvn1 binds to PGC-1β via the Syvn1 unique (SyU) domain (10,11,13,14). Walnut extract did not have an inhibitory effect on autoubiquitination of Syvn1. However, walnut extract decreased polyubiquitination of PGC-1β by inhibiting the interaction of Syvn1 and PGC-1β. Therefore, walnut extract may specifically target the SyU domain of Syvn1. These results indicate that walnut extract might improve obesity by selectively inhibiting the interaction of Syvn1 and PGC-1β.

The mitochondrion is an important organelle involved in cellular energy control that has been reported to be involved in the process of obesity and chronic inflammation (23,24). PGC-1β plays an important role in mitochondrial biogenesis and energy metabolism, including β-oxidation of fatty acids (25). Overexpression of PGC-1β results in increased numbers of mitochondria and increased mitochondrial respiratory function (26). PGC-1β transgenic mice show high energy expenditure and resistance to obesity (27). In addition, PGC-1β attenuated inflammation. PGC-1β diminishes the increase in proinflammatory mediators, such as interleukin-6 (IL-6) and macrophage inflammatory protein 1-alpha (MIP1α), by repressing the activity of nuclear factor-κB (NF-κB) (28). These studies indicate that PGC-1β has anti-obesity and anti-inflammatory properties. In this study, we found that walnut extract inhibited the negative regulation of PGC-1β activity by Syvn1, suggesting that walnut extract activates PGC-1β. Our results suggest that walnut extract may attenuate not only obesity, but also diseases involving chronic inflammation. Further analysis with disease state model will be need to determine whether walnut extract will be helpful in several disease with chronic inflammation. Future studies will be aimed at identifying the bioactive constituents in walnut extract that are responsible for its inhibitory effect on Syvn1.

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Availability of data and materials

All data analyzed in this study are included in this article.

Authors' contributions

HF, SA, KN, NY and TN conceived the project and designed the experiments. HF, SA 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.

Ethics approval and consent to participate

All human experimental protocols in the present study (nos. 2728 and 2729, 3758, 3759) were approved by the Ethics Review Committee of Tokyo Medical University (Tokyo, Japan). Written informed consent was obtained from all of the patients prior to the collection of joint tissue samples.

Patient consent for publication

Consent for publication was obtained from all of the patients.

Competing interests

The authors declare that they have no competing interests.

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