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SUMO-Specific Protease 2 (SENP2) Is an Important Regulator of Fatty Acid Metabolism in Skeletal Muscle

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Small ubiquitin-like modifier (SUMO)-specific proteases (SENPs) that reverse protein modification by SUMO are involved in the control of numerous cellular processes, including transcription, cell division, and cancer development. However, the physiological function of SENPs in energy metabolism remains unclear. Here, we investigated the role of SENP2 in fatty acid metabolism in C2C12 myotubes and in vivo. In C2C12 myotubes, treatment with saturated fatty acids, like palmitate, led to nuclear factor-κB–mediated increase in the expression of SENP2. This increase promoted the recruitment of peroxisome proliferator–activated receptor (PPAR)δ and PPARγ, through desumoylation of PPARs, to the promoters of the genes involved in fatty acid oxidation (FAO), such as carnitine-palmitoyl transferase-1 (CPT1b) and long-chain acyl-CoA synthetase 1 (ACSL1). In addition, SENP2 overexpression substantially increased FAO in C2C12 myotubes. Consistent with the cell culture system, muscle-specific SENP2 overexpression led to a marked increase in the mRNA levels of CPT1b and ACSL1 and thereby in FAO in the skeletal muscle, which ultimately alleviated high-fat diet–induced obesity and insulin resistance. Collectively, these data identify SENP2 as an important regulator of fatty acid metabolism in skeletal muscle and further implicate that muscle SENP2 could be a novel therapeutic target for the treatment of obesity-linked metabolic disorders.

Insulin resistance precedes the development of type 2 diabetes mellitus and is characterized by impaired insulin-dependent glucose metabolism in metabolically active tissues, such as skeletal muscle, liver, and adipose tissues. Among these tissues, skeletal muscle is one of the major sites that expend glucose. There is a negative relationship between muscle triglyceride levels and insulin sensitivity (1–4), and insulin sensitivity is reduced by fatty acid overload in cultured myocytes (5). Several trials to increase fatty acid oxidation (FAO) or to limit fat storage in muscle have been shown to improve obesity-induced insulin resistance (6–9), which can be applied for the treatment of insulin resistance.

Peroxisome proliferator-activated receptors (PPARs) are members of the nuclear receptor superfamily and comprise three isoforms: PPARα, PPARδ, and PPARγ. All are involved in lipid metabolism and glucose homeostasis, although they show different tissue distributions and physiological roles (10). PPARδ is expressed ubiquitously in all tissues, and its agonists facilitate FAO in skeletal muscle via PPARγ coactivator (PGC)1α (11). PPARγ is highly expressed in adipocytes and plays an essential role in adipogenesis (12).

Small ubiquitin-like modifier (SUMO) is conjugated to a variety of proteins and modulates their localization, stability, and interaction with other proteins (13,14). SUMO modification (sumoylation) is involved in the control of various cellular processes, including cell cycle progression, gene expression, and signal transduction (15,16). Sumoylation can be reversed by the action of SUMO-specific cysteine proteases (SENPs). Six mammalian SENP

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family members have been identified, and they have different subcellular locations and substrate specificity (17–20). SENPs also play important roles in the control of various cellular events. Knockout of either *SENP1* or *SENP2* results in embryonic lethality (21–23), suggesting that the regulation of sumoylation is essential for developmental processes. Moreover, disruption of SUMO homeostasis contributes to the development and progression of prostate cancer, in which SENP1 and SENP3 are critically involved (24–26). It is also known that SENP2 desumoylates Mdm2, an ubiquitin E3 ligase of p53, and contributes to the control of p53-mediated processes (27). However, relatively little is known about the role of SENPs in the regulation of energy metabolism. Recent studies have shown that SENP2 represses glycolysis and reprograms glucose metabolism in cancer cells and SENP1 overexpression increases mitochondrial biogenesis in myotubes (28,29).

Several transcription factors involved in metabolic regulation, such as PPARγ and PGC1α, are known to serve as targets for sumoylation (30,31). We have recently shown that SENP2 desumoylates PPARγ and dramatically increases the activity of PPARγ (32). Interestingly, the sumoylation status of PPARγ selectively regulates the expression of some PPARγ target genes in myotubes: desumoylation of PPARγ increases the mRNA level of fatty acid translocase (CD36) but not of adipose differentiation-related protein (ADRP), although both are upregulated by PPARγ agonists. Based on these reports, we hypothesized that SENP2 is involved in metabolic regulation in skeletal muscle. In this study, we investigated the potential role of SENP2 in the regulation of fatty acid metabolism in skeletal muscle by using a cultured cell system and a genetically engineered animal model.

RESEARCH DESIGN AND METHODS

Materials

Expression vectors for PPARs, SUMO-1, SENPs, ubiquitin conjugating enzyme 9 (UBC9), and PPAR response element-thymidine kinase-luciferase (*PPRE-TK-Luc*) reporter vector (p*PPRE-TK-Luc*) were prepared as previously described (32). An expression vector for PGC1α was obtained from Dr. Gang Xu (The Chinese University of Hong Kong, Hong Kong). Reporter vectors for SENP2 were also generated: *(−1980)-Luc, (−868)-Luc*, and *(−157)-Luc* (33). Mutations in the nuclear factor-κB (NF-κB) binding site of *(−157)-Luc* were generated by substituting GGG (−70 to −68 bp) with CTC. Adenovirus containing the human SENP2 expression construct (Ad-SENP2) was prepared as previously described (32). All small interfering (si)RNAs were purchased from Dharmacon except for PPARγ siRNA (Invitrogen). Polyclonal antibody against SENP2 was produced using the peptide representing amino acid 317–335 of mouse SENP2 as an epitope (Abclon, Korea).

Cell Culture and Transfection

C2C12 myoblasts were maintained in DMEM supplemented with 10% FBS (Invitrogen). Differentiation was induced using DMEM containing 2% horse serum (Invitrogen) for 4 days. Transfection of plasmids was performed using Lipofectamin with Plus Reagent (Invitrogen), and siRNAs were transfected using RNAiMAX (Invitrogen).

RNA Preparation and Real-Time PCR

Total RNAs were isolated by using TRIzol (Invitrogen), and real-time PCR was performed using SYBR-master mix (Takara) and ABI 7500 real-time PCR system (Applied Biosystems). Nucleotide sequences of the primers are shown in Supplementary Table 1.

Measurement of Fatty Acid Oxidation

For measurement of FAO, muscle tissues or cells were homogenized in an ice-cold mitochondria isolation buffer (250 mmol/L sucrose, 10 mmol/L Tris-HCl, and 1 mmol/L EDTA). The lysates were incubated for 2 h with 0.2 mmol/L [1-^{14}C]palmitate. $^{14}CO_2$ and ^{14}C-labeled acid-soluble metabolites were quantified using a liquid scintillation counter. Each radioactivity was normalized by protein amount of each lysate.

Electrophoretic-Mobility Shift Assays

The probe was labeled with biotin and incubated with nuclear extracts from C2C12 myotubes treated with palmitate for 24 h in the binding buffer (LightShift Chemiluminescent electrophoretic-mobility shift assay [EMSA] kit). The sequences of the probe and competitors are shown in Supplementary Table 1.

Chromatin Immunoprecipitation Coupled With Quantitative PCR

After cross-linking and DNA fragmentation, nuclear extracts were subjected to immunoprecipitation with antibodies against PPARα, PPARδ, PPARγ, PGC1α, and control IgG. The sequences of primers are listed in Supplementary Table 1.

Microarray

Gene expression of C2C12 cells was profiled using MouseRef-8-v2-BeadChip (Illumina). The array was scanned using a BeadStation 500 System (Illumina).

Animals and Metabolic Analyses

All aspects of animal care and experimentation were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH publ. no. 85-23, revised 1996). The mice were DXA scanned using a LUNAR Prodigy scanner with software version 8.10 (GE Healthcare). The levels of free fatty acid and triglyceride were measured (BioVision). For electron microscopical analysis, thin muscle sections were prepared and examined using a transmission electron microscope (JEM-1400) at 80 Kv.

Statistical Analysis

Statistical analysis was performed using SPSS version 12.0 (SPSS Inc.). Statistical significance was tested using the Mann-Whitney *U* test. A P value <0.05 was considered statistically significant.

RESULTS

Palmitate Elevates the Expression of SENP2 in C2C12 Myotubes

In an attempt to identify the factor(s) that regulates SENP2 expression, C2C12 myotubes were treated with various metabolites, hormones, and cytokines. The expression of SENP2 mRNA was not significantly changed upon treatment with insulin, glucose, interleukin-6, or tumor necrosis factor-α (Supplementary Fig. 1). However, it was markedly increased by treatment with palmitate (Fig. 1*A*), and this increase occurred in dose- and incubation time–dependent fashions, respectively (Fig. 1*B* and *C*). Increase in the SENP2 protein levels by palmitate was also confirmed (Supplementary Fig. 1*B*). On the other hand, unsaturated fatty acids, such as oleate, linoleate, and eicosapentaenoic acid, showed little or no effect on the SENP2 mRNA levels (Fig. 1*A*). In addition, the expression of SENP1, another SENP, was not influenced by any of the tested fatty acids, including palmitate (Supplementary Fig. 1*C*), suggesting that palmitate specifically regulates the expression of SENP2 in myotubes.

SENP2 Expression Is Regulated by Palmitate-Induced NF-κB Activation

Palmitate is known to induce NF-κB activation (34,35). It has also been shown that toll-like receptor 4 (TLR4) and MyD88 are responsible for palmitate-induced NF-κB activation (34,36,37). Treatment with pyrrolidine dithiocarbamate, a selective NF-κB inhibitor, abrogated palmitate-mediated increase in the mRNA level of SENP2 (Fig. 1*D*). Consistently, knockdown of NF-κB by specific siRNAs (confirmed at Supplementary Fig. 2*A*) completely inhibited the effect of palmitate on the SENP2 mRNA level (Fig. 1*E*). Moreover, knockdown of TLR4 or MyD88 (confirmed at Supplementary Fig. 2*B*) led to suppression of palmitate-induced expression of SENP2 mRNA (Fig. 1*F*). These results suggest that the increase in the SENP2 mRNA level is mediated by palmitate-induced NF-κB activation via TLR4 and MyD88.

For determination of whether the promoter of the mouse *SENP2* gene has a *cis*-acting element for NF-κB–mediated regulation, its 5′-flanking region was serially deleted and inserted into a *Luc* reporter vector. Palmitate treatment led to three- to fourfold increase in the Luc activity upon transfection of the constructs having the regions from −1980, −868, and −157 to +93 bp (Fig. 1*G*). Furthermore, a potential NF-κB–binding sequence was found to locate at ~70 bp upstream of the transcription start site (as shown at Fig. 1*H*) and mutations of the sequence abrogated palmitate-induced promoter activity (Fig. 1*G*). To confirm this finding, we performed EMSA by using nuclear extracts of C2C12 myotubes. Palmitate treatment led to a dramatic increase in a binding with a biotin-labeled probe containing the putative NF-κB–binding sequence (Fig. 1*H*, lane 3), and this increased binding could be abolished by treatment with the same but unlabeled probe (lane 4) as well as with the unlabeled consensus NF-κB–binding sequence (lane 6), but not with a oligonucleotide having the mutated sequence (lane 5). Furthermore, the band was supershifted by addition of anti–NF-κB antibody (lane 7). These results suggest that the *SENP2* promoter has a *cis*-acting element for palmitate-induced NF-κB binding.

SENP2 Promotes Palmitate-Mediated Increase in the Expression of Acyl-CoA Synthetase 1, Carnitine-Palmitoyl Transferase 1b, and UCP3

Long-chain acyl-CoA synthetase (ACSL)1 and carnitine-palmitoyl transferase (CPT)1 are known to play a critical role in FAO (38,39). In addition, uncoupling protein (UCP)3 has also been shown to augment FAO (40,41). Henceforth, we referred to ACSL1, CPT1b, and UCP3 as FAO-associated enzymes. Palmitate treatment led to an increase in the mRNA levels of FAO-associated enzymes in a dose-dependent fashion (Fig. 2*A*). Furthermore, knockdown of SENP2 (Supplementary Fig. 3) suppressed the increase in the mRNA levels of all three proteins (Fig. 2*B*). Consistently, palmitate-stimulated FAO was abrogated by SENP2 knockdown (Fig. 2*C*). These results suggest that SENP2 plays a crucial role in the control of FAO under conditions with overloaded saturated fatty acids.

Overexpression of SENP2 Increases FAO Primarily By Upregulating the Expression of FAO-Associated Enzymes

We performed mRNA microarray analysis using C2C12 myotubes that had been infected with adenovirus overexpressing SENP2 (Ad-SENP2). Remarkably, the mRNA levels of the proteins involved in the metabolic process of fatty acids (including ACSL1, CPT1b, and UCP3) as well as of glucose were dramatically upregulated (Fig. 3*A*). Interestingly, however, the mRNA levels of the proteins involved in cell cycle and DNA replication were markedly downregulated by SENP2 overexpression. These results indicated that SENP2 is functionally related to energy metabolism in myotubes.

For further confirmation of this finding, C2C12 myotubes were infected with Ad- SENP1, Ad-SENP2, and its catalytically inactive form (C548S) (Ad-SENP2mt) (Supplementary Fig. 4*A* and *B*). Consistent with the data from microarray analysis, overexpression of SENP2, but not SENP1 or SENP2mt, led to an increase in the mRNA levels of FAO-associated enzymes (Fig. 3*B*). Moreover, the increase in FAO by overexpression of SENP2 was significantly higher than that by overexpression of SENP1 or SENP2mt (Fig. 3*C*). On the other hand, overexpression of SENP2 showed little or no effect on the expression of mRNAs for transcription factors involved in lipid metabolism, such as PPARα, PPARδ, PPARγ, PGC1α, and estrogen-related receptor α (Supplementary Fig. 4*C*). These results suggest that the desumoylating activity of SENP2 is required for the promotion of FAO via upregulation of the expression of FAO-associated enzymes in C2C12 myotubes.

SENP2 Increases FAO Mainly by Promoting PPARδ- and PPARγ-Mediated Expression of FAO-Associated Enzymes

To determine whether SENP2-dependent increase in the expression of FAO-associated enzymes is mediated by

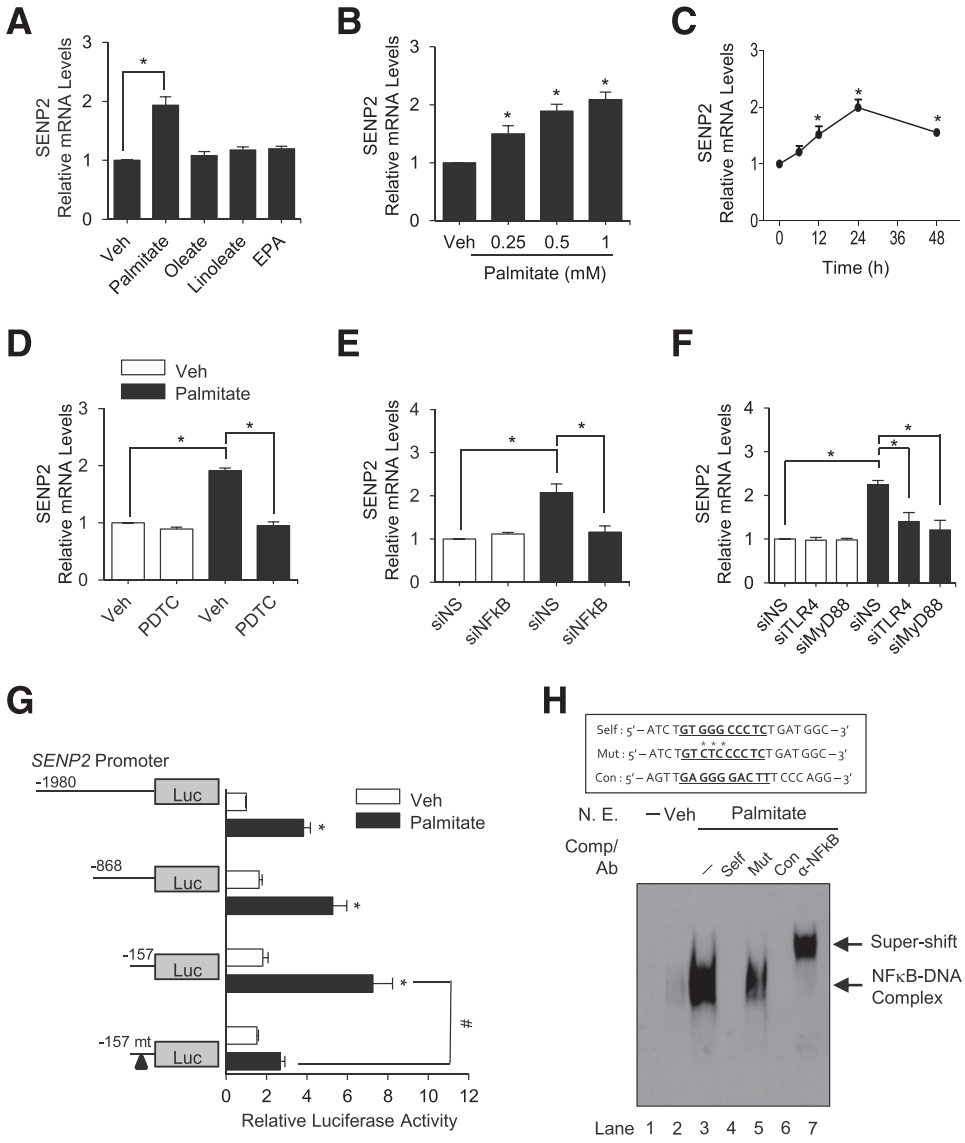

Figure 1—Expression of SENP2 mRNA is regulated by palmitate-induced NF-κB activation. *A*: C2C12 myotubes were treated with various fatty acids (0.5 mmol/L) for 24 h. Free fatty acids (100 mmol/L in EtOH) were diluted in the culture medium containing fatty acid–free BSA in a 1:3 molar ratio. The mRNA levels of SENP2 were then determined by real-time PCR. Each Ct value was subtracted by Ct value of GAPDH (ΔCt) and then subtracted by the ΔCt of each control set (ddCt). Relative mRNA levels were expressed as 2^{-ddCt}. The SENP2 mRNA level of untreated cells (vehicle [Veh]) was expressed as 1.0, and the others were expressed as its relative values. Data are the mean ± SEM of five independent experiments. **P* < 0.05. *B*: C2C12 myotubes were treated with increasing amounts of palmitate for 24 h (*n* = 4). **P* < 0.05 compared with untreated cells (Veh). *C*: C2C12 myotubes were treated with 0.5 mmol/L palmitate for increasing periods and then subjected to assay for SENP2 mRNA levels (*n* = 5). **P* < 0.05 compared with untreated cells. *D*: C2C12 myotubes were treated with palmitate in the absence or presence of 10 μmol/L pyrrolidine dithiocarbamate (*n* = 4). **P* < 0.05. *E*: C2C12 myotubes were transfected with siRNA against NF-κB (50 nmol/L) for 24 h and then treated with palmitate for 24 h (*n* = 3). **P* < 0.05. *F*: C2C12 myotubes transfected with the indicated siRNAs were treated with palmitate (*n* = 4). **P* < 0.05. *G*: C2C12 myoblasts were transfected with *Luc* reporter vectors harboring various deletions of promoter region in the *SENP2* gene. After treatment with palmitate for 24 h, they were subjected to assay for Luc activity (*n* = 4). The arrowhead indicates the mutations shown in *H*. The Luc activity of the cells transfected with *(−1980)-Luc* without palmitate was set to 1, and the other values were expressed relative to that. **P* < 0.05 compared with untreated cells; #*P* < 0.05. *H*: The putative NF-κB–binding sequence in the *SENP2* promoter (−85 bp to −54 bp) is underlined in "Self." The underline in "Con" (consensus) indicates the consensus NF-κB–binding sequence. *Mutated (Mut) bases (upper panel). Nuclear extracts were prepared from C2C12 myotubes that had been incubated without (Veh) and with palmitate for 24 h. They were then subjected to EMSA assay using a biotin-labeled probe (lower panel). For competition assays, unlabeled oligonucleotides (50-fold) (Self, Mut, and Con) were used as competitors (Comp). NF-κB antibody (Ab) was used for supershift. Lane 1 represented the probe only. siNS, nonspecific siRNA.

desumoylation of transcription factors that are involved in lipid metabolism, we first performed knockdown analysis of each of them. Supplementary Fig. 4*D* shows that each siRNA used effectively depletes the mRNA of its target transcription factor (i.e., PPARα, PPARδ, PPARγ, PGC1α, estrogen-related receptor α, and receptor-interacting protein 140). Interestingly, knockdown of PPARδ and PPARγ, but not of the others, dramatically

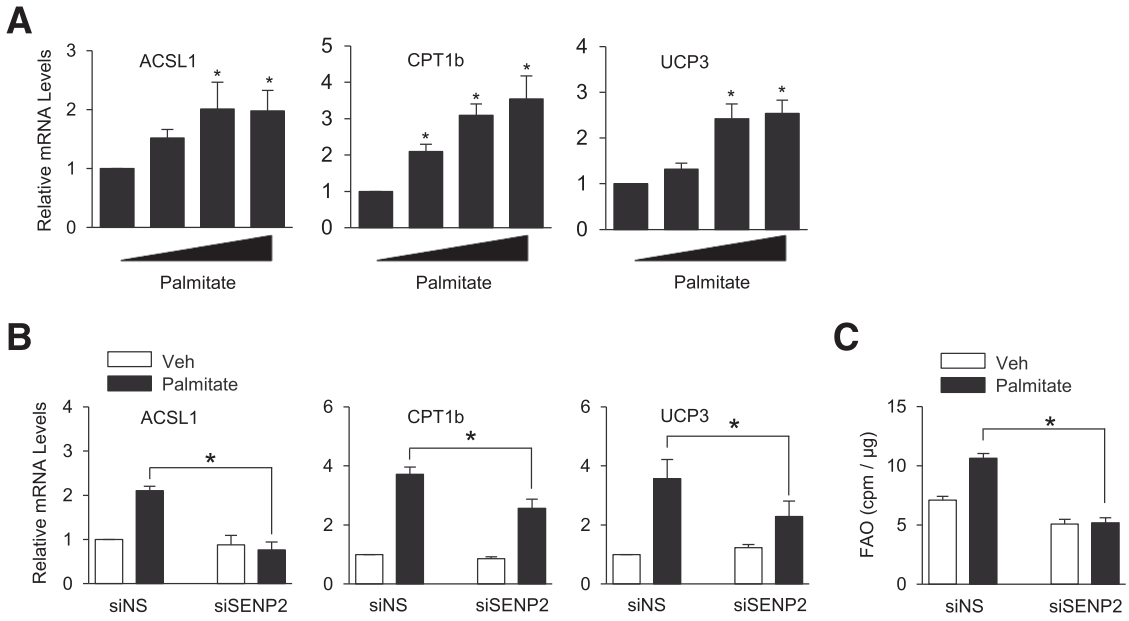

Figure 2—SENP2 is responsible for palmitate-mediated increase of FAO. *A*: Total RNAs were prepared from C2C12 myotubes that had been treated with 0.25, 0.5, or 1 mmol/L palmitate for 24 h. They were then subjected to real-time PCR (n = 4). *P < 0.05 compared with untreated cells. *B* and *C*: Myotubes were transfected with siRNAs against SENP2 (siSENP2). After incubation for 24 h, they were treated with 0.5 mmol/L palmitate (n = 5). The mRNA levels for the indicated proteins (*B*) and FAO (*C*) were then determined. siNS, nonspecific siRNA. *P < 0.05.

suppressed SENP2-mediated increase in the mRNA levels of FAO-associated enzymes and thereby in FAO (Fig. 3*D* and *E*, respectively). On the other hand, the basal FAO level in the absence of SENP2 overexpression was not influenced by knockdown of any of the transcription factors, including PPARδ and PPARγ (Supplementary Fig. 4*E*). These results suggest that SENP2 increases FAO mainly by promoting PPARδ- and PPARγ-mediated expression of FAO-associated enzymes.

For determination of whether SENP2 influences the recruitment of PPARδ and PPARγ to the promoters of their target genes for the control of FAO, chromatin immunoprecipitation–coupled quantitative real-time PCR analysis (ChIP-qPCR) was performed using C2C12 myotubes that had been transfected with Ad-GFP, Ad-SENP2, and Ad-SENPmt. Overexpression of SENP2, but not SENP2mt, markedly promoted the binding of PPARδ and PPARγ to the *CPT1b* promoter region, whereas it showed little or no effect on that of PPARα and PGC1α (Fig. 3*F*). Similar results were obtained using the *ACSL1* promoter region (Fig. 3*G*). These results indicate that SENP2 increases mRNA expression of FAO-associated enzymes by promoting the recruitment of PPARδ and PPARγ to the promoters of the genes.

Next, we tested whether palmitate-induced mRNA level increase of FAO-associated enzymes was also mediated by PPARδ and PPARγ recruitment to their promoters and whether SENP2 was involved in the process. The recruitment of PPARδ and PPARγ to the *CPT1b* or *ACSL1* promoters was increased by palmitate and knockdown of SENP2 suppressed the effect of palmitate (Fig. 3*H* and *I*). These results suggest that SENP2 plays an important role in the palmitate-induced recruitment of PPARδ and PPARγ to the promoters of the FAO-associated enzyme genes.

SENP2 Promotes PPARδ Activity by Desumoylation

We have previously shown that SENP2 desumoylates PPARγ and promotes its activity (32). To determine whether PPARδ also is a target for SENP2, we first examined whether PPARδ is sumoylated. Sequence analysis revealed that PPARδ contains the MK^{104}LE sequence, which is conserved in mouse and human and similar to the consensus sumoylation ΨKXE motif, where Ψ is a hydrophobic amino acid and X is any amino acid (Fig. 4*A*). Therefore, we replaced the Lys^{104} residue with Arg to test whether the mutation (K104R) influences PPARδ sumoylation. Overexpression of SUMO1 and UBC9 (sumoylation E2 enzyme) with PPARδ, but not with its K104R mutant, dramatically increased the appearance of slow-migrating PPARδ bands (Fig. 4*B*). Furthermore, the slow-migrating bands could completely be eliminated by the expression of SENP2 but not by SENP2mt (Fig. 4*C*). These results indicate that PPARδ, like PPARγ, serves as a target of SENP2 and its Lys^{104} residue is a major SUMO acceptor site. The higher–molecular weight bands were also detected with anti-ubiquitin antibody as well as anti-SUMO antibody, and ubiquitination of PPARδ was increased when sumoylation was accompanied (Supplementary Fig. 5*A*). Therefore, it is possible that sumoylation of PPARδ promotes ubiquitination, which generates multiple high–molecular weight bands. We also confirmed desumoylation of endogenous PPARδ by SENP2 overexpression in myotubes (Supplementary Fig. 5*B*).

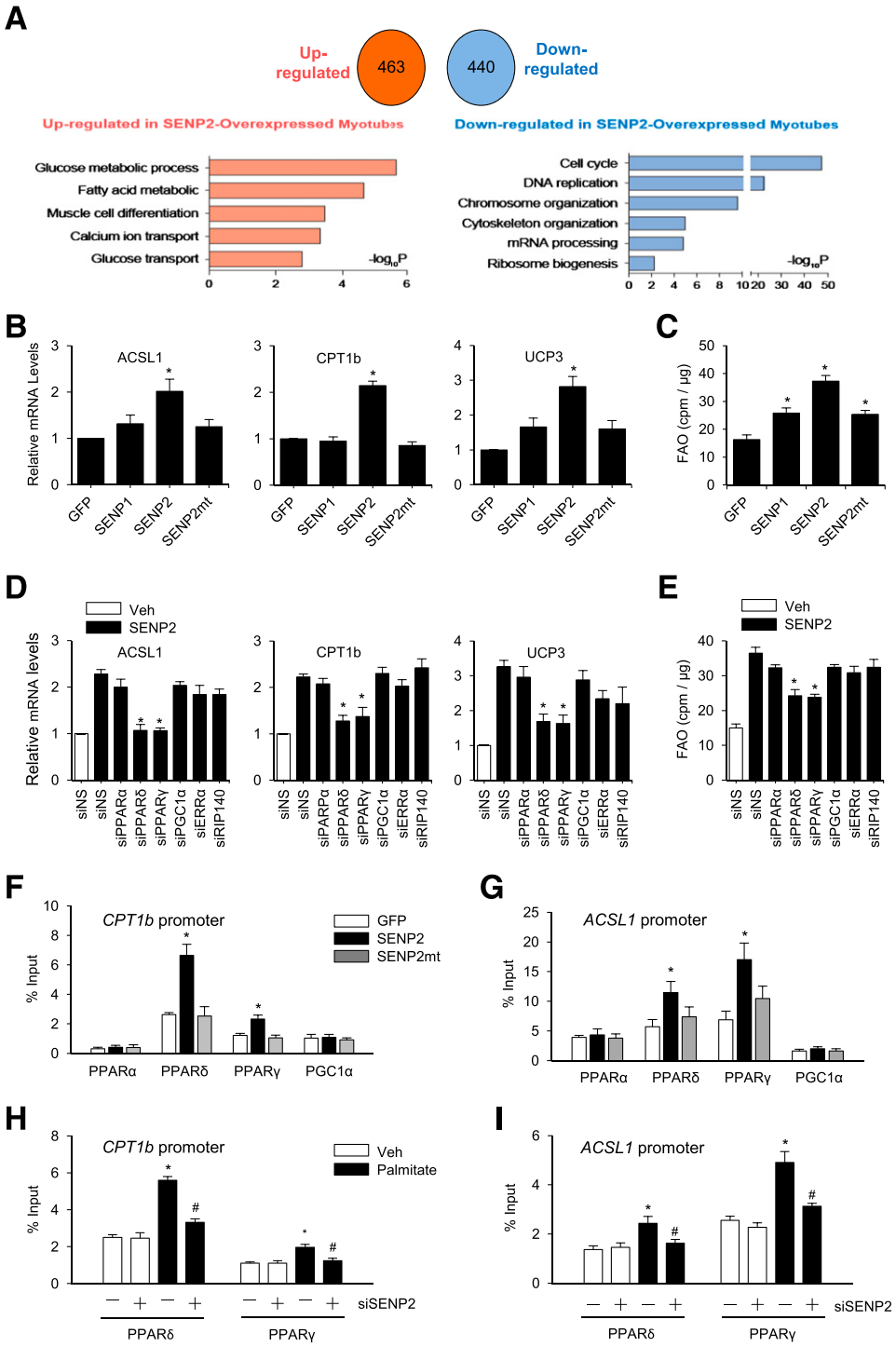

Figure 3—SENP2 increases FAO by promoting PPARδ- and PPARγ-mediated expression of FAO-associated enzymes. *A*: Total RNA was isolated from C2C12 myotubes that had been infected with Ad-GFP or Ad-SENP2 for 3 days and then subjected to microarray. Differentially expressed genes were identified as those with false discovery rates <0.05 and fold change larger than the cutoff of 1.5. *B*: C2C12 myotubes were infected with Ad-GFP, Ad-SENP1, Ad-SENP2, or Ad-SENP2mt for 3 days. Total RNAs were obtained from the cells and then subjected to real-time PCR (n = 4). *C*: Cells prepared as in *B* were subjected to FAO assay (n = 5). *P < 0.05 compared with Ad-GFP infected cells. *D* and *E*: C2C12 myotubes transfected with the indicated siRNAs were infected with Ad-GFP or Ad-SENP2. siNS, nonspecific siRNA. After incubation for 48 h, the mRNA levels of FAO-associated enzymes (n = 4) and FAO (n = 6) were determined. *P < 0.05 compared with cells transfected with nonspecific siRNA and Ad-SENP2. *F* and *G*: C2C12 myotubes infected with Ad-GFP, Ad-SENP2, or Ad-SENP2mt were subjected to ChIP-qPCR analysis with the *CPT1b* promoter region (*F*) and *ACSL1* promoter region (*G*). Data are the means ± SEM (n = 4). *P < 0.05 compared with Ad-GFP–infected cells. *H* and *I*: C2C12 myotubes were treated with siSENP2 for 24 h followed by treatment of palmitate (500 μmol/L) for 24 h and then subjected to ChIP-qPCR (n = 3). Veh, vehicle. *P < 0.05 compared with nonspecific siRNA without palmitate. #P < 0.05 compared with nonspecific siRNA with palmitate.

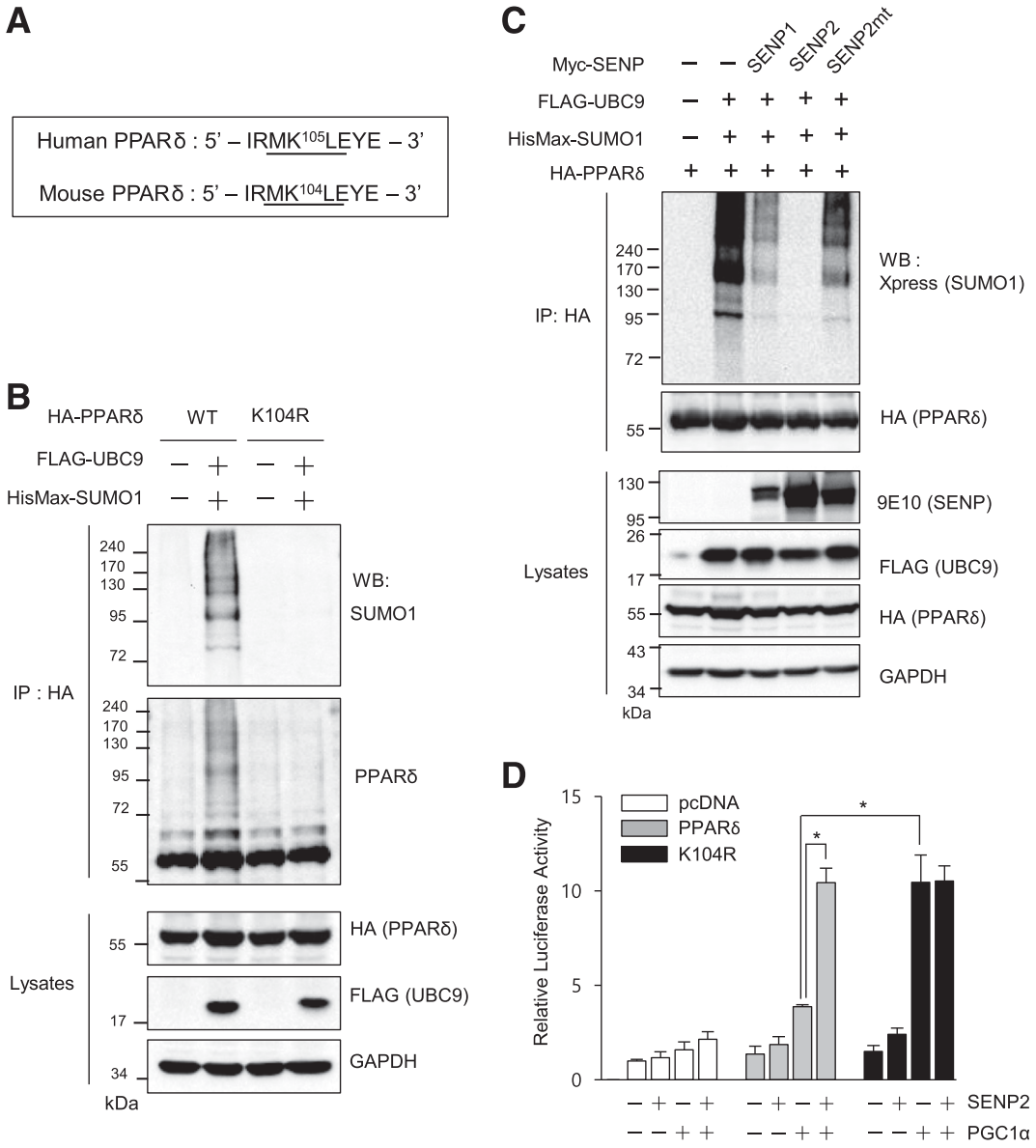

Figure 4—SENP2 promotes the PPARδ activity by desumoylation. *A*: The putative sumoylation sequences of human and mouse PPARδ are shown. *B*: HA-tagged PPARδ and its K104R mutant were expressed in MCF7 cells with HisMax-SUMO1 and FLAG-UBC9. Cell lysates were subjected to immunoprecipitation (IP) with anti-HA antibody followed by Western blotting (WB) with anti-SUMO1 and anti-PPARδ antibodies. Cell lysates were also directly probed with the indicated antibodies. *C*: HA-PPARδ, HisMax-SUMO1, and FLAG-UBC9 were expressed with Myc-tagged SENP1 or SENP2 or its mutant (SENP2mt). Immunoprecipitation was then carried out as in *B*. *D*: COS7 cells were treated with siRNAs (50 nmol/L) against PPARγ to minimize the effect of endogenous PPARγ. The following day, cells were transfected with p*PPRE-TK–Luc* and expression vectors of SENP2, PGC1α, PPARδ, or PPARδ K104R. Luc activity of cells transfected with p*PPRE-TK-Luc* only was expressed as 1.0, and the others were expressed as its relative values ($n = 4$). *$P < 0.05$.

For examination of whether PPARδ sumoylation affects its activity, COS7 cells were transfected with a *PPRE-TK-Luc* reporter vector. Note that coexpression of PGC1α was necessary to detect the PPARδ activity in nonmuscle COS7 cells, which were used instead of C2C12 myotubes having very low transfection efficiency. Overexpression of SENP2 dramatically increased the activity of PPARδ (Fig. 4*D*). Remarkably, the activity of sumoylation-deficient K104R seen without SENP2 was nearly as high as that of wild-type (WT) PPARδ seen with SENP2, indicating that SENP2-mediated desumoylation is required for PPARδ to show its maximal activity. Taken together, these results indicate that SENP2 promotes the activity of PPARδ as well as PPARγ through desumoylation.

SENP2 Overexpression in Muscle Ameliorates High-Fat Diet–Induced Obesity and Insulin Resistance

To determine whether SENP2 is indeed involved in lipid metabolism in vivo, we generated muscle-specific SENP2 transgenic mice (referred to as mSENP2-TG mice) by inserting the *SENP2-FLAG* construct to a β-globin gene

cassette having the *MCK* promoter (Supplementary Fig. 6*A*). The SENP2 mRNA was expressed in all types of muscle tissues of mSENP2-TG mice but not in those of WT mice (Supplementary Fig. 6*B*). It was not expressed in the liver or epididymal fat tissues of both the TG and WT mice (Supplementary Fig. 6*C*). Consistently, the SENP2 protein could be detected in the skeletal muscle of the TG mice but not in that of WT mice (Supplementary Fig. 6*D*). In addition, the total protein level of SENP2 (i.e., FLAG-tagged and endogenous SENP2) was at least three- to fourfold higher in the muscle of mSENP2-TG mice than that of WT mice under both chow- and high-fat diet (HFD)-fed conditions (Supplementary Fig. 6*E*). These results confirmed that the TG mice overexpress SENP2 in muscle.

Remarkably, the body weight and fat mass of mSENP2-TG mice were significantly lower than those of WT mice under HFD-fed conditions, whereas lean mass and bone mineral contents of the TG mice were similar to that of WT mice (Fig. 5*A*–*D*). Analysis by glucose tolerance tests and insulin tolerance tests revealed that mSENP2-TG mice show higher glucose tolerance and insulin sensitivity than WT mice under HFD-fed conditions (Fig. 5*E* and *F*). Moreover, the basal levels of insulin and triglyceride in the serum of HFD-fed TG mice were much lower than those of WT mice (Fig. 5*G* and *H*). On the other hand, little or no difference was detected in the serum cholesterol level under both chow- and HFD-fed conditions (Fig. 5*I*). Moreover, electron microscopic analysis revealed that the gastrocnemius muscle of the TG mice contained much lower fat than that of WT mice under HFD-fed conditions (Fig. 5*J*). On the other hand, the fat level in the liver and fat size of adipose tissues of the TG mice was similar to that of WT mice under both chow- and HFD-fed conditions (Supplementary Fig. 7*A* and *B*). Significantly, insulin-stimulated phosphorylation of insulin receptor substrate 1 and protein kinase B (PKB/Akt) in the gastrocnemius muscle of mSENP2-TG mice was much higher than that of WT mice under both chow- and HFD-fed conditions (Fig. 5*K*), suggesting that SENP2 promotes insulin signaling. Taken together, these results indicate that SENP2 overexpression in muscle alleviates HFD-induced obesity and insulin resistance.

SENP2 Overexpression Increases FAO by Upregulating Expression of FAO-Associated Enzymes in Muscle

Next, we determined the levels of free fatty acids and triglyceride in the gastrocnemius muscle. While the level of free fatty acids in the muscle tissue of the TG mice was similar to that of WT mice (Fig. 6*A*), the triglyceride level in the TG mice was significantly lower than that of WT mice under both chow- and HFD-fed conditions (Fig. 6*B*). In addition, FAO was markedly increased in the muscle of the TG mice under both chow- and HFD-fed conditions compared with that of WT mice (Fig. 6*C*), and ATP contents were also increased in the muscle of HFD-fed TG mice (Supplementary Fig. 7*C*). These results indicate that the muscle tissues of the TG mice show higher FAO, which would reduce fat accumulation.

We next examined whether the increase in FAO is associated with SENP2-mediated changes in the expression of FAO-associated enzymes (i.e., ACSL1, CPT1b, and UCP3). Consistent with the results obtained with cell culture system, the mRNA levels of FAO-associated enzymes were higher in the gastrocnemius muscle of HFD-fed TG mice than in that of WT mice (Fig. 6*D*). In contrast, in the muscle of chow-fed TG mice, only UCP3 mRNA, but not ACSL1 or CPT1b mRNA, levels were increased (Fig. 6*D*). When the mRNA levels of these FAO-associated enzymes of the WT mice muscle were compared under different diet conditions, the levels were similar in chow- and HFD conditions (Fig. 6*D*), and SENP2 mRNA levels of the WT mice were not significantly affected by diet condition, either (Supplementary Fig. 7*D*). In addition, no apparent difference between mSENP2-TG and WT mice muscles was observed in the mRNA levels of transcriptional factors, including PPARα, PPARδ, PPARγ, and PGC1α in both chow- and HFD-fed conditions (Fig. 6*E*). Collectively, these results suggest that SENP2 plays a critical role in the increase of FAO by inducing the expression of FAO-related enzymes and SENP2 overexpression in muscle alleviates HFD-induced obesity and insulin resistance.

DISCUSSION

On the basis of the present findings, we propose a potential mechanism by which SENP2 regulates lipid metabolism in skeletal muscle (Fig. 7). Upon saturated free fatty acid stimulation, TLR4/MyD88 activates NF-κB for expression of SENP2 transcripts, which would increase the cellular level of SENP2 protein. This increase leads to desumoylation of PPARδ and PPARγ and their recruitment to the promoters of the genes encoding FAO-associated enzymes, including ASCL1 and CPT1b. Elevated expression of the enzymes would then promote FAO (the upper panel). In the muscle of mSENP2-TG mice, overexpressed SENP2 increases FAO via the same mechanism described above but without the need of the signal from saturated fatty acids. This increase in FAO then results in fat utilization and suppression of its accumulation, which alleviates HFD-induced obesity and insulin resistance (the lower panel). These findings demonstrate that SENP2 serves as an important regulator of lipid metabolism in muscle.

Several questions may be raised from our data and model. A question is why SENP2-induced FAO is not observed in HFD-fed WT mice. In an in vitro C2C12 myotube culture system, palmitate treatment significantly increases SENP2 expression, which increases FAO. In contrast, SENP2 expression is not substantially increased in the muscle of HFD-fed mice (Supplementary Figs. 6*E* and 7*D*). Similarly, either FAO or the mRNA levels of ACSL1 and CPT1b are not significantly changed by HFD (Fig. 6*C* and *D*). The different results in these models could derive from other metabolic disturbances associated with chronic high-fat feeding or from different experimental systems (in vitro vs. in vivo).

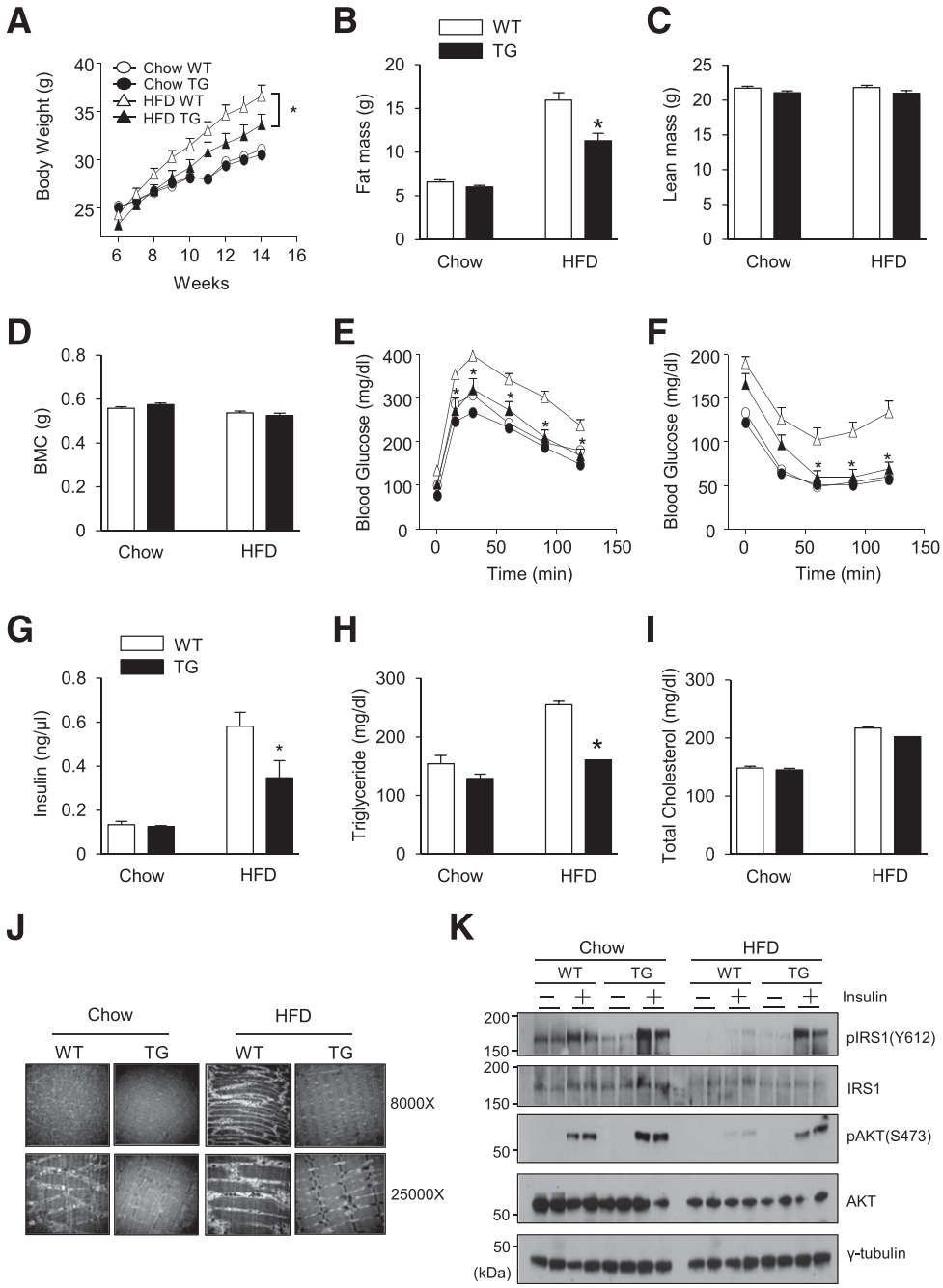

Figure 5— Muscle-specific SENP2 overexpression alleviates HFD-induced obesity and insulin resistance. *A*: WT and mSENP2-TG (TG) mice at 8 weeks were fed with a standard chow diet or HFD, consisting of 60% fat and 6.8% sucrose, for the next 12 weeks. Their body weights were measured every day for 14 weeks (n = 15–20/group). Data are the mean ± SEM. *$P < 0.05$. *B–D*: Body fat mass (*B*), lean mass (*C*), and bone mineral contents (BMC) (*D*) were measured after feeding the mice with chow or HFD for 10 weeks using DXA scanning. *$P < 0.05$. *E* and *F*: Glucose tolerance test (*E*) and insulin tolerance test (*F*) were performed. *$P < 0.05$. *G–I*: The levels of insulin (*G*), triglycerides (*H*), and total cholesterol (*I*) in serum were measured. *$P < 0.05$. Note that the *P* values were in comparison with WT mice fed with HFD (*B* and *E–H*). *J*: Sections of gastrocnemius muscle tissues were obtained and then subjected to electron microscopy. *K*: Mice were treated with insulin for 20 min before sacrifice. Extracts prepared from the gastrocnemius muscle were subjected to immunoblot with respective antibodies.

It is notable that the mRNA levels of UCP3 are higher in the muscle of chow-fed SENP2-TG mice, but unexpectedly the mRNA levels of ACSL1 and CPT1b are not increased (Fig. 6*D*). These are different from the data observed in the myotube culture system. Given that the source of fatty acids available for FAO in the chow-fed condition is limited, it is conceivable that a certain level of fatty acid could be required for inducing ACSL1 and CPT1b gene expression. Additionally, a yet unidentified regulatory system that may suppress ACSL1 or CPT1b expression independently of SENP2 in the context of in vivo milieu is possibly involved in this event.

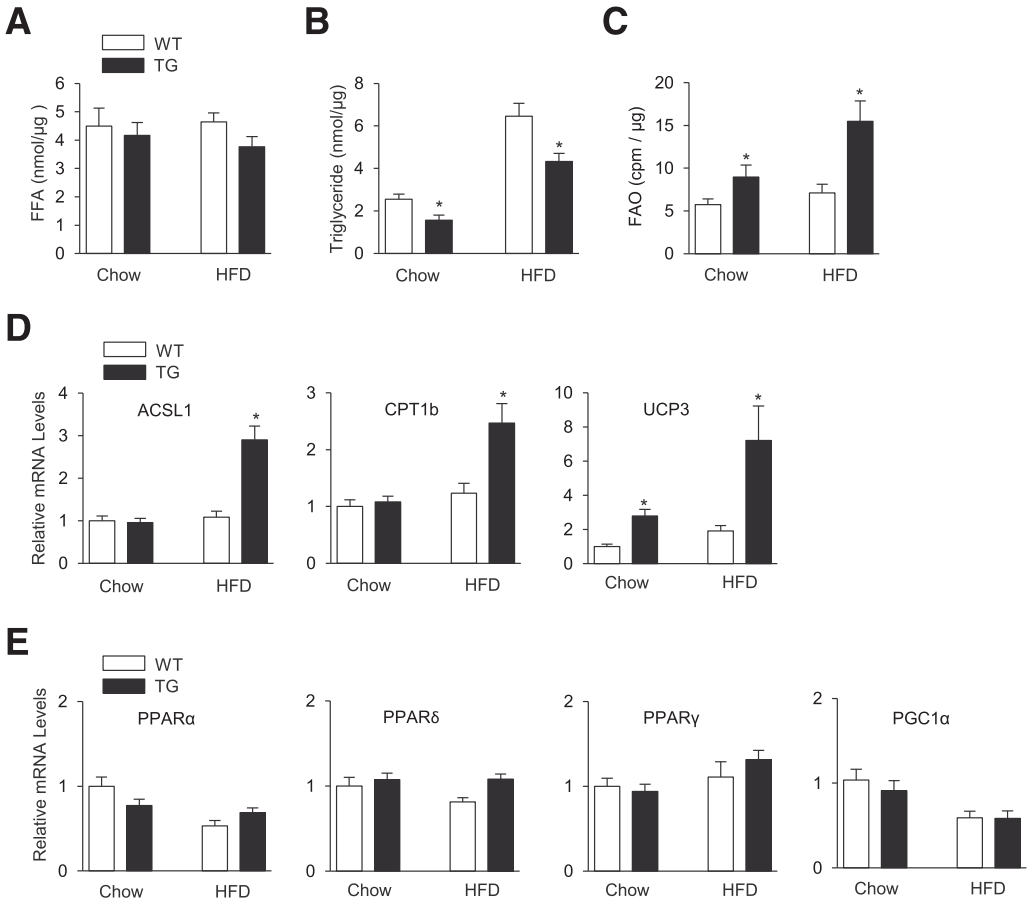

Figure 6—SENP2 overexpression increases FAO by upregulating expression of FAO-associated enzymes in muscle. *A–C*: WT and mSENP2-TG (TG) mice were fed with chow or HFD for 12 weeks. The levels of free fatty acids (FFA) (*A*), triglycerides (*B*), and FAO (*C*) in their gastrocnemius muscle were determined (n = 8/group). *D* and *E*: Total mRNAs were isolated from the muscles of the mice and subjected to real-time PCR (n = 10–14/group) using primers for FAO-associated enzymes (*D*) and PPARs and PGC1α (*E*). The mRNA level seen in chow-fed WT mice muscle was expressed as 1.0, and the others were expressed as its relative values. Data are the mean ± SEM. *$P < 0.05$ compared with WT mice.

Future studies will need to clarify these important issues.

Another question is whether PPARδ and PPARγ are only direct targets of SENP2 to promote FAO in muscle. The effect of palmitate on FAO is completely abolished by SENP2 knockdown, while knockdown of SENP2 partially inhibits palmitate-induced expression of CPT1b and UCP3 (Fig. 2*B* and *C*), although CPT1 is known to be the limiting step of FAO. In addition, knockdown of PPARδ or PPARγ efficiently, but not completely, suppresses SENP2-mediated FAO (Fig. 3*E*). These observations indicate the possibility that SENP2 regulates the function of FAO-associated enzymes through direct modification, at least in part. It is also possible that SENP2 controls the expression or function of another protein(s) that is important for FAO but not tested in this study. Nevertheless, our study clearly shows that SENP2 increases FAO mainly by promoting PPARδ- and PPARγ-mediated expression of FAO-associated enzymes.

While sumoylation of PPARγ was previously reported, to our knowledge, we show for the first time that PPARδ is sumoylated. Of note was the finding that high–molecular weight sumoylated bands of PPARδ were detected (Fig. 4*B* and *C*). However, it is unlikely that PPARδ is sumoylated at multiple sites because its K104R mutation completely abrogated PPARδ sumoylation. The fact that SUMO1 is known to be incapable of forming a polymeric chain and our result showing that ubiquitination of PPARδ is increased by sumoylation suggest that the high–molecular weight bands consist of PPARδ modified by both SUMO1 and ubiquitin. In fact, there are several reports that sumoylation of proteins promotes their ubiquitination (42–44). Further study will determine whether the ubiquitination is linked to proteasome-mediated degradation of PPARδ.

PPARδ is more abundant than PPARγ in muscle, suggesting that the contribution of PPARδ in lipid metabolism in muscle could be greater than that of PPARγ. Interestingly, however, the effect of PPARγ on gene expression of FAO-associated enzymes and in turn on FAO in C2C12 myotubes was nearly similar to that of PPARδ (Fig. 3*D* and *E*). Thus, it appears that PPARγ, in addition

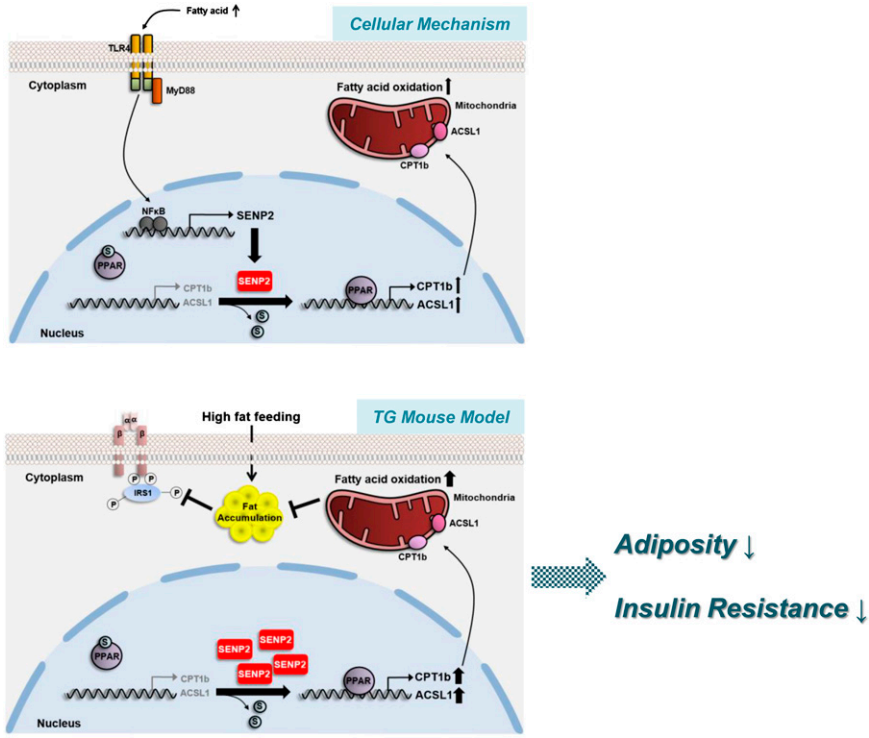

Figure 7—Schematic model shows the role of SENP2 in the control of lipid metabolism in skeletal muscle. Upon sensing by saturated free fatty acids, TLR4/MyD88 activates NF-κB for expression of SENP2 mRNA, which would increase the cellular level of SENP2 protein for desumoylation of PPARδ and PPARγ. These transcription factors are then recruited to the promoters of the genes encoding FAO-associated enzymes, such as ASCL1 and CPT1b, which leads to increase of FAO (the upper panel). Muscle-specific overexpression of SENP2 in TG mice increases the expression of FAO-associated enzymes and thereby increases FAO. This increase would elevate fat utilization and suppress its accumulation, which could alleviate HFD-induced obesity and insulin resistance (lower panel). S, SUMO.

to PPARδ, plays an important role in lipid metabolism in skeletal muscle. This is further supported by experimental evidence showing that muscle-specific deletion of PPARγ results in insulin resistance and promotes adiposity (45,46).

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