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ORIGINAL ARTICLE

F-box only protein 9 is an E3 ubiquitin ligase of PPAR γ

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Peroxisome proliferator-activated receptor gamma (PPAR γ) is a critical regulator of carbohydrate and lipid metabolism, adipocyte differentiation and inflammatory response. Post-translational modification of PPAR γ and its degradation involve several pathways, including the ubiquitin-proteasome system. Here, we identified F-box only protein 9 (FBXO9) as an E3 ubiquitin ligase of PPAR γ . We screened interacting partners of PPAR γ using immunoprecipitation and mass spectrometric analysis and identified FBXO9 as an E3 ubiquitin ligase of PPAR γ . FBXO9 directly interacted with PPAR γ through the activation function-1 domain and ligand-binding domain. FBXO9 decreased the protein stability of PPAR γ through induction of ubiquitination. We found that the F-box motif of FBXO9 was required for its ubiquitination function. The activity of PPAR γ was significantly decreased by FBXO9 overexpression. Furthermore, FBXO9 overexpression in 3T3-L1 adipocytes resulted in decreased levels of endogenous PPAR γ and suppression of adipogenesis. These results suggest that FBXO9 is an important enzyme that regulates the stability and activity of PPAR γ through ubiquitination.

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INTRODUCTION

Peroxisome proliferator-activated receptor gamma (PPARy) is a member of the nuclear hormone receptor superfamily and is a master regulator of adipocyte differentiation.¹ There are two isoforms of PPARy: PPARy1 and PPARy2. Thirty extra amino acids are added to the N-terminal of PPARy2, but the other sequences are identical in both isoforms. While PPARy1 is expressed in a number of tissues, PPARy2 is only observed in adipocytes.² PPARy activity is upregulated by binding of its ligands, such as thiazolidinediones (TZDs).² TZDs increase fat accumulation and improve insulin sensitivity via activation of PPARy. The activity of PPARy is also regulated by modulating the protein level of PPARy. PPARy (+/-) heterozygous mice, which have lower levels of PPARy than wild-type mice, are protected from high-fat diet-induced weight gain and insulin resistance.3-5 These findings suggest that obesity and insulin resistance can be ameliorated by controlling the abundance of PPARy proteins in adipose tissue, independent of TZD activation.

Several reports have demonstrated that PPARy is degraded by the ubiquitin-proteasome system.^{6,7} The ligand-binding domain (LBD) of PPARy contains major ubiquitination sites, and both the N-terminal activation function-1 (AF-1) domain and the LBD are important for recognition by the proteasome.8 Interestingly, PPARy ubiquitination and degradation are increased by its specific ligands, TZDs, in adipocytes.9,10 The reduction in PPARy protein levels by TZDs is not observed in other cell types, such as myotubes or liver cells. A report has demonstrated that Drosophila seven-in-absentia homolog 2 (Siah2) is an E3 ubiquitin ligase that regulates PPARy ubiquitination in adipocytes, and its expression is increased by TZDs.¹¹ Recently, other E3 ligases, such as makorin ring finger protein 1 (MKRN1) and neural precursor cell-expressed developmentally downregulated 4 (NEDD4-1)¹², were reported to be involved in PPARy ubiquitination and proteasomedependent degradation.¹³

F-box proteins are substrate-recruiting subunits of the Skp1-Cullin-F-box (SCF) ubiquitin ligase complex.¹⁴ Little is

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known about the regulation and functional role of F-box only protein 9 (FBXO9), a member of the F-box proteins. Expression of FBXO9 was increased in vascular smooth muscle cells under high-glucose culture conditions and in streptozotocin (STZ)-induced diabetic rat vessels.¹⁵ FBXO9 mediated ubiquitination of telomere maintenance 2 (Tel2) and Tel2 interacting protein 1 (Tti1), integral components of mTORC1, in response to growth factor withdrawal, which inactivates mTORC1 and leads to cell survival in myeloma.¹⁶

In this study, we analyzed PPAR γ -interacting proteins using co-immunoprecipitation in the presence of MG132, a specific proteasome inhibitor, and mass spectrometric analysis to find a novel E3 ubiquitin ligase specific to PPAR γ . FBXO9 was shown to interact with PPAR γ and to facilitate ubiquitination and subsequent degradation of PPAR γ , suggesting that FBXO9 regulates PPAR γ at the protein level as an E3 ubiquitin ligase.

MATERIALS AND METHODS

Antibodies and chemicals

Antibodies against FBXO9, PPAR γ , ubiquitin and HA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies against γ -tubulin and FLAG were purchased from Sigma-Aldrich (St Louis, MO, USA). Cycloheximide, 3-isobutyl-1-methylxanthine (IBMX), dexamethasone, insulin and dimethyl sulfoxide (DMSO) were also purchased from Sigma-Aldrich. MG132 was purchased from Calbiochem (Boston, MA, USA).

Cell culture

COS7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS) in a 5% CO₂ incubator. 3T3-L1 preadipocytes were maintained in DMEM containing 10% calf serum in a 5% CO₂ incubator. Adipocyte differentiation was induced by incubation in DMEM containing 10% FBS, 0.5 μ M 3-isobutyl-1-methylxanthine (IBMX), 0.25 μ M dexamethasone and 5 μ g ml⁻¹ insulin for 2 days. Cells were incubated in DMEM with 10% FBS and 1 μ g ml⁻¹ insulin for the following 2 days and further cultured in DMEM with 10% FBS for four additional days.

Construction of plasmids and adenoviruses

The mouse FBXO9 (FLAG-FBXO9) expression vector was constructed by subcloning the corresponding cDNAs into the pFLAG-CMV vector using the HindIII and NotI sites. The F-box deletion mutant of mouse FBXO9 (FLAG-FBXO9 Δ F) was generated by subcloning as follows. The region encoding amino acids 1-178 of mouse FBXO9 was ligated into pFLAG-CMV using the HindIII and NotI sites, and the region encoding amino acids 230-437 of mouse FBXO9 was ligated using the NotI and BglII sites. The preparation of the HA-tagged mouse PPARy2 expression vector was previously described.17 The His-ubiquitin expression vector was kindly provided by CH Chung (Seoul National University, Seoul, South Korea). Adenoviruses encoding FLAG-tagged mouse FBXO9 (Ad-FLAG-FBXO9) were prepared following a previously reported method using the GFP co-expression adenovirus system. Briefly, FLAG-tagged mouse FBXO9 was inserted into pAdTrack-CMV. Ad-FLAG-FBXO9 was generated by homologous recombination between pAdTrack-CMV-Flag-FBXO9 and pAdEasy and by adenovirus packaging in HEK-293. The deletion mutants of mouse PPARy2 were cloned into HA-tagged pcDNA3.1 as follows. The cDNAs encoding the AF1 domain from amino acids 1 to 138, the DNA-binding domain and the hinge domain from amino acids 139 to

279, and ligand-binding domain (LBD/AF2) from amino acids 279 to 505 of mouse PPAR γ 2 were generated using PCR. The cDNAs were then ligated into HA-tagged pcDNA3.1 using the *Kpn*I and *Xba*I sites.

Glutathione-S-transferase (GST) pull-down assay

Glutathione Sepharose 4B (20 µl) (GE Healthcare, Pittsburgh, PA, USA), GST-FBXO9 (1 µg) (Antibodies, Atlanta, GA, USA) and PPARγ LBD (2 µg) (Cayman Chemical, Ann Arbor, MI, USA) were mixed and incubated in 20 mM Tris-HCl, pH 7.4, 10 mM Na₄P₂O₇, 100 mM NaF, 2 mM Na₃VO₄, 1% NP-40 buffer supplemented with protease inhibitors (10 µg µl⁻¹ aprotinin, 10 µg µl⁻¹ leupeptin and 1 mM phenylmethylsulfonyl fluoride) overnight at 4 °C. The precipitates were washed four times and eluted using Laemmli sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% sodium dodecyl sulfate, 25% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue) (Sigma, St Louis, MI, USA). The precipitates were blotted with either anti-PPARγ antibody or anti-FBXO9 antibody.

Immunoprecipitation and western blot analysis

To evaluate the interaction between PPARy and FBXO9, cell lysates (500 µg) were prepared with lysis buffer containing 20 mM Tris-HCl (pH 7.4), 1% NP-40, 5 mm EDTA, 2 mm Na₃VO₄, 100 mm NaF, 10 mM Na₄P₂O₇, 1 mM phenylmethylsulfonyl fluoride, 7 μ g ml⁻¹ aprotinin and $7 \mu g m l^{-1}$ leupeptin. To assess the FBXO9-dependent ubiquitination of PPARy, COS7 cells were transfected with pHA-PPARy, pHis-Ub and pFLAG-FBXO9 for 12 h. MG132 (10 µM) was added for 4 h before harvesting. Cell lysates (500 µg) were prepared in denaturing condition using lysis buffer containing 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% Na-deoxycholate, 0.1% sodium dodecyl sulfate, 10 mm Na₄P₂O₇, 2 mM Na₃VO₄, 100 mM NaF, 7 μg ml⁻¹ aprotinin, 7 µg ml⁻¹ leupeptin and 1 mM phenylmethylsulfonyl fluoride. Cell lysates were used for immunoprecipitation with anti-HA antibodycoupled agarose beads (Roche, Basel, Switzerland) for 4 h at 4 °C. The precipitates were washed five times and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then blotted with specific antibodies. All blots were developed using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Boston, MA, USA).

Transient transfection and reporter assay

COS7 cells were seeded 1 day before transfection and transfected with expression vectors using LipofectAMINE Plus reagent (Invitrogen, Carlsbad, CA, USA). Reporter activity was detected using a luciferase assay system kit (Promega, Fitchburg, WI, USA) and Lumat LB9507 (Berthold Technologies, Bad Wildbad, Germany). Luciferase activity was normalized to β -galactosidase activity.

Real-time PCR

Total RNA was isolated using TRIzol (Invitrogen). cDNAs were synthesized by reverse transcription with 1 μ g of total RNA. Real-time PCR was performed using the SYBR master mix (Takara, Otsu, Shiga, Japan) with specific primers for each gene and an ABI 7500 Real-time PCR system (Applied Biosystems, Carlsbad, CA, USA). Primer sets are as follows: mouse PPAR γ forward primer, 5'-TCAGGGCTGCCACTTTCG-3'; reverse primer, 5'-GTAATC AGCAACCATTGGGTCA-3'; mouse 18 S rRNA forward primer, 5'-C GCGGTTCTATTTTGTTGGT-3'; reverse primer, 5'-AGTCGGCA TCGTTTATGGTC-3'. 18S rRNA was used as an endogenous control for normalization.

Staining of lipid droplets

Cells were fixed with 3.5% paraformaldehyde for 10 min and washed with phosphate-buffered saline three times. Cells were stained using oil red O solution (1.5% oil red O in 60% isopropanol) for 1 h and washed with phosphate-buffered saline three times. Cells were mounted with 50% glycerol and observed.

Mass spectrometry analysis

To isolate interacting proteins of PPAR γ , Plat-E cells were overexpressed with FLAG-PPAR γ 2 or pcDNA, as a negative control, for 20 h. Cells were treated with MG132 (10 μ M) for 6 h prior to harvesting. Cell lysates were prepared with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100 and protease inhibitors. A total of 8 mg of cell lysates was subjected to immunoprecipitation with an anti-FLAG M2 affinity gel (Sigma-Aldrich, 30 μ l) for 1.5 h at 4 °C. Beads were washed three times using wash buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.2% TritonX-100 followed by two washes using wash buffer without TritonX-100. The eluate for interacting proteins of PPAR γ was prepared by a competitive elution using 3X FLAG elution buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl and 3X FLAG



Figure 1 FBXO9 interacts with PPAR_Y. (a) COS7 cells were seeded in a 60-mm dish. The next day, HA-PPAR_Y2 (1 µg) and FLAG-FBXO9 (1 µg) were co-transfected for 20 h. Whole-cell lysates were subjected to immunoprecipitation with anti-HA antibody-conjugated beads and blotted with anti-FLAG or anti-PPAR_Y antibody. As a loading control, γ -tubulin was detected using anti- γ -tubulin antibodies. (b) COS7 cells were co-transfected with HA-PPAR_Y (100 ng) and FLAG-FBXO9 (100 ng, 300 ng) or an empty vector (CMV) as a negative control. The protein levels of PPAR_Y, FBXO9 or γ -tubulin were detected using anti-HA, anti-FLAG or anti- γ -tubulin antibodies, respectively.

peptides (225 ng μ l⁻¹ final concentration; Sigma-Aldrich), and mass spectrometry analysis was then performed.

Statistics

Statistical analyses were performed using SPSS Statistics 20 (SPSS Inc., Chicago, IL, USA). Data are expressed as the mean \pm s.e., and the difference between the means was analyzed using the Mann–Whitney *U*-test. A *P*-value less than 0.05 indicated a statistically significant difference.

RESULTS

FBXO9 interacts with PPAR γ and regulates PPAR γ protein levels

Plat-E cells were transfected with FLAG-PPARy, and MG132 was added 6 h before harvesting. Cell lysates were immunoprecipitated with FLAG antibody and then the precipitated proteins were subjected to mass spectrometry analysis to identify PPARy-interacting proteins. Forty proteins were identified (Supplementary Table 1), and we chose an E3 ubiquitin ligase, FBXO9, to further determine whether it is specific to PPARy. Interaction between PPARy and FBXO9 was confirmed by transfection of expression vectors for HA-PPARy and FLAG-FBXO9 into COS7 cells and immunoprecipitation with an anti-HA antibody. FLAG-FBXO9 was coimmunoprecipitated with HA-PPARy, demonstrating a physical interaction between FBXO9 and PPARy (Figure 1a). When FBXO9 was co-expressed, the protein level of PPARy was decreased (Figure 1a). This finding was more apparent with increasing amounts of FBXO9: higher expression levels of FBXO9 led to lower levels of PPARy (Figure 1b). Next, we identified the PPARy domain that interacts with FBXO9 using deletion mutants of PPARy: HA-PPARy AF1, HA-PPARy DNA-binding domain and HA-PPARy LBD (Figures 2a and b). HA-PPARy AF1 and HA-PPARy LBD interacted with FBXO9, but HA-PPARy DNA-binding domain did not. In addition, we performed a GST pull-down assay using the recombinant proteins GST-FBXO9 and PPARy LBD. PPARy LBD was co-precipitated with GST-FBXO9, confirming the direct interaction between these proteins (Figure 2c). To summarize, FBXO9 interacts with PPARy and may regulate PPARy protein levels.

FBXO9 regulates PPAR γ stability by facilitating PPAR γ ubiquitination

To determine whether FBXO9 overexpression affects the stability of PPAR γ protein, COS7 cells were transfected with HA-PPAR γ and FLAG-FBXO9 expression vectors and then treated with cycloheximide, a protein synthesis inhibitor. When FBXO9 was overexpressed, PPAR γ protein levels decreased more rapidly (Figures 3a and b), suggesting that FBXO9 accelerates PPAR γ degradation. Because FBXO9 is considered to be a ubiquitin ligase, we tested whether FBXO9 is involved in ubiquitination of PPAR γ . In the presence of MG132, a proteasome inhibitor, ubiquitin-conjugated PPAR γ was dramatically increased by FBXO9 overexpression (Figure 3c).



Figure 2 The domain of PPAR γ involved in the interaction with FBXO9. (a) Structure of the deletion mutants of PPAR γ . (b) pHA-PPAR γ AF1 (3 µg), pHA-PPAR γ DBD (4 µg) or pHA-PPAR γ LBD (3 µg) was overexpressed with pFLAG-FBXO9 (1 µg) for 24 h and treated with MG132 (10 µm) for 5 h before harvesting. The deletion mutants of PPAR γ were immunoprecipitated using anti-HA antibody-conjugated beads and immunoblotted using anti-FLAG and anti-HA antibodies. (c) GST-FBXO9 (1 µg) and PPAR γ LBD (2 µg) were mixed and incubated. Co-precipitates were immunoblotted using anti-PPAR γ or anti-FBXO9 antibodies.

These results suggest that FBXO9 is an E3 ubiquitin ligase targeting PPARy.

The F-box motif of FBXO9 is important for the regulation of PPARγ protein levels

The F-box motif in F-box family proteins is involved in the association with Skp1, which acts as bridge between F-box protein and Cullin. Therefore, F-box proteins lacking the F-box motif can bind to their substrates but are unable to induce ubiquitination of the substrates. We prepared a mutant form of FBXO9 lacking the F-box motif (Figure 4a) and tested whether the motif is important for the regulation of PPARy protein levels. As expected, the FBXO9 mutant form (FBXO9 Δ F) did not affect PPARy protein levels when it was overexpressed, while the FBXO9 wild type (FBXO9 WT) effectively decreased PPARy levels (Figure 4b). The activity of PPARy was also measured using transfection of pPPRE-TK-luc and expression vectors for the FBXO9 WT or FBXO9 Δ F. Luciferase activity was dramatically reduced when FBXO9 WT was co-transfected but was not affected by FBXO9 ΔF (Figure 4c). These results confirm that FBXO9 reduces PPARy protein level and activity through PPARy ubiquitination and that the F-box motif is required for its action.

Overexpression of FBXO9 regulates endogenous PPAR_γ levels in adipocytes

To clarify whether FBXO9 overexpression affects endogenous PPARy protein levels, FBXO9 was overexpressed using an adenoviral system in 3T3-L1 adipocytes, in which PPARy is abundantly expressed. Two days after the viral infection, PPARy protein levels were determined using immunoblotting with an anti-PPARy antibody. PPARy protein levels were reduced after overexpression of FBXO9 in a dose-dependent manner (Figures 5a and b). In contrast, PPARy protein levels were not affected by the control virus (Ad-GFP). These results suggest that endogenous PPARy levels were regulated by FBXO9 in the adipocytes. Furthermore, we investigated the effect of FBXO9 overexpression on adipogenesis. FBXO9 was overexpressed 2 days after initiation of differentiation, when endogenous PPARy expression starts to rise. Adipocyte differentiation was inhibited by overexpression of FBXO9 (Figures 6a and b). However, FBXO9 ΔF did not affect the levels of PPARy and adipogenesis. The effect of FBXO9 on adipogenesis was not influenced by the treatment of TZD. The PPARy mRNA level was not influenced by FBXO9 overexpression (Figure 6c). These findings indicate that overexpression of FBXO9 inhibits adipogenesis by reducing PPARy protein stability.

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Figure 3 FBX09 decreases the levels of PPAR γ protein by induction of ubiquitination. (a) The day before transfection, COS7 cells were plated in 12-well plates. CMV (100 ng) or FLAG-FBX09 (100 ng) was co-transfected with HA-PPAR γ (100 ng) for 24 h, and cycloheximide (5 μ M) was added for the indicated times. Whole-cell lysates were then analyzed using western blotting. Stars indicate non-specific bands. (b) The densities of the PPAR γ bands at time point 0 were set to 100%, and the remaining densities were expressed as relative values. Data represent the mean ± s.e.m. of three independent experiments. **P*<0.05 vs CMV. (c) COS7 cells were subcultured in 60 mm dishes one day before transfection. HA-PPAR γ (1 μ g), His-Ub (0.2 μ g) and FLAG-FBX09 (0.5 μ g) or CMV (0.5 μ g) were overexpressed for 12 h and treated with DMS0 or MG132 (10 μ M) 5 h before harvesting. PPAR γ was precipitated with anti-HA antibody-conjugated beads and immunoblotted with anti-Ub, anti-PPAR γ , anti-FLAG or anti- γ -tubulin antibodies.

DISCUSSION

In this study, using co-immunoprecipitation in the presence of MG132 and mass spectrometry analysis, we identified FBXO9 as a novel interacting protein of PPAR γ with E3 ligase activity. We showed that FBXO9 decreased the protein stability of PPAR γ by inducing ubiquitination and proteasomal degradation. In addition, the PPAR γ activity was also decreased by overexpression of FBXO9. The F-box motif of FBXO9 was crucial for modulating PPAR γ protein levels, and FBXO9 interacted with the AF1 and LBD of PPAR γ . Finally, we demonstrated that FBXO9 was important in regulating endogenous PPAR γ levels in 3T3-L1 adipocytes and overexpression of FBXO9 impaired adipogenesis.

Independent of activation by TZD, the level of PPAR γ protein *per se* is important in exerting its effect.⁵ Because the half-life of PPAR γ is relatively short ($t^{1/2} = 2$ h), the process of degradation is important in controlling the metabolic effects of PPAR γ .⁹ It is well known that PPAR γ is ubiquitinated and degraded by the proteasomal pathway.⁹ However, the precise mechanism and molecules involved in this process are not fully understood. We have identified a novel E3 ligase, FBXO9, which is involved in the process of PPAR γ degradation.

Overexpression of FBXO9 not only decreased the protein stability of PPAR γ but also decreased its transcriptional activity. One of the limitations of our study is that FBXO9 knockdown in 3T3-L1 adipocytes did not result in meaningful changes in PPAR γ expression (data not shown). This might be because endogenous FBXO9 expression is relatively low in the basal state of adipocytes. It is speculated that FBXO9 might play an important role when FBXO9 expression is induced by certain conditions. It would be of interest to find treatments that modulate the FBXO9 level and regulate the activity and protein level of PPAR γ , which might have clinical implications.

In our previous report, we showed that FBXO9 expression is increased at the very early stage of adipogenesis of 3T3-L1 cells, and knockdown of FBXO9 at the early stage (day 0 to day 1) significantly inhibited adipogenesis.¹⁸ Because PPAR γ is not expressed at this early stage of adipogenesis, FBXO9 might target other proteins, which could be inhibitory factors of adipogenesis. In this study, we further investigated the effect of FBXO9 overexpression on adipogenesis. We observed that overexpression of FBXO9 in 3T3-L1 cells also resulted in decreased levels of PPAR γ and impaired adipogenesis. Therefore, it is suggested that normal levels of FBXO9 are _



Figure 4 The requirement for the F-box motif on the effect of FBXO9 on PPAR_γ stability. (a) Structure of FBXO9. (b) COS7 cells were seeded in 12-well plates. The next day, cells were transfected with pHA-PPAR_γ2 (100 ng) and pFLAG-FBXO9 WT (100 ng, 300 ng) or pFLAG-FBXO9 Δ F (25 ng, 50 ng) for 24 h. The amount of transfected DNA of FBXO9 WT and Δ F was determined by similarly expressed protein levels. Whole-cell lysates were then subjected to western blot analysis. (c) COS7 cells were transfected with pFLAG-FBXO9 WT or pFLAG-FBXO9 Δ F in the presence of pPPRE-Tk-Luc (1 µg), pHA-PPAR_γ (0.3 µg) and pCMV-βgal (0.5 ug) for 18 h. Luciferase activities were normalized to β-galactosidase activity. The relative values for the luciferase activity were calculated from three independent experiments (mean ± s.e.m.). **P*<0.05 vs the transfection of HA-PPAR_γ alone.

required for normal adipocyte differentiation. As we only investigated the role of FBXO9 on adipogenesis, it would be of interest to investigate the functional role of endogenous FBXO9 in differentiated adipocytes.

Several different E3 ligases, Siah2, NEDD4-1 and MKRN1, have been reported to be involved in PPAR γ ubiquitination. In the report by Kilroy *et al.*,¹¹ TZDs increased expression of Siah2, and it was involved in TZD-dependent PPAR γ ubiquitination and degradation in adipocytes. However, it was suggested that the nuclear hormone receptor corepressor NCoR might be the direct target of Siah2. Han *et al.*¹² identified NEDD4-1 as an E3 ligase that leads to PPAR γ ubiquitination and degradation. NEDD4-1 was shown to delay cellular senescence by decreasing PPAR γ expression. A study by Kim *et al.*¹³ showed that adipogenesis was suppressed in stable



Figure 5 The effect of FBXO9 overexpression on endogenous PPAR γ protein levels in adipocytes. (a) Control adenovirus (Ad-GFP) or adenovirus-expressing FLAG-FBXO9 (Ad-Flag-FBXO9) was infected into 3T3-L1 adipocytes using the indicated multiplicity of infection (MOI). Whole-cell lysates were prepared 48 h after infection. Endogenous PPAR γ , FLAG-FBXO9, GFP or γ -tubulin was detected by specific antibodies. Stars indicate non-specific bands. (b) The intensity of PPAR γ bands in (a) was normalized to γ -tubulin. The mean value was calculated from six independent experiments (mean ± s.e.m.). **P*<0.05 vs control.

cells overexpressing MKRN1, and conversely, adipogenesis was increased when MKRN1 was knocked down. These results suggest that there are several ubiquitin ligases, including FBXO9, that are involved in the ubiquitination of PPARγ.

There are several distinct features of FBXO9 compared with the other suggested E3 ligases of PPARy. It has been reported that Siah2 gene expression is increased by TZDs, and Siah2 is involved in TZD-dependent PPARy ubiquitination and degradation. In contrast, FBXO9 expression was not regulated by TZDs, and FBXO9 was not involved in TZD-dependent PPARy degradation. It has been reported that MKRN1 overexpression suppressed adipogenesis. Regarding FBXO9, we showed that both inhibition and overexpression resulted in impaired adipogenesis, and normal levels of FBXO9 are required for normal adipocyte differentiation. These results suggest that each E3 ligase regulates PPARy stability in response to different cellular signaling or conditions in adipocytes. Further studies are required to elucidate the relative contribution of different E3 ligases to PPARy ubiquitination and their distinct roles in different settings.

F-box proteins usually recognize specific phosphorylation sites of the substrate proteins and facilitate ubiquitination of the target proteins.¹⁴ PPAR γ has several sites that are phosphorylated by various kinases.^{19–21} We tested whether two well-known phosphorylation sites of PPAR γ , S112 and S273, are required for FBXO9-mediated ubiquitination. FBXO9 was able to efficiently reduce the stability of two mutant forms



Figure 6 Adipogenesis is inhibited by overexpression of FBXO9. Adipogenesis was induced using differentiation medium containing IBMX, dexamethasone and insulin either with or without rosiglitazone. Cells were transfected with Ad-GFP (100 MOI), Ad-FBXO9 (100 MOI) or Ad-FBXO9 Δ F (100 MOI) 2 days after induction of differentiation. (a) Cell lysates were harvested at the indicated day of adipogenesis and subjected to western blot analysis. (b) Cells were stained with oil red O at day 8 of adipogenesis. (c) Total RNA was harvested at the indicated day of adipogenesis, and PPAR_Y mRNA levels were determined by real-time PCR. The PPAR_Y mRNA level of the Ad-GFP-infected cells harvested on day 3 without rosiglitazone treatment was set as a reference value of 1, and others are presented as relative values (mean ± s.e.m., n=4). G, Ad-GFP; F, Ad-FBXO9; Δ , Ad-FBXO9 Δ F.

of PPAR γ , PPAR γ S112A and PPAR γ S273A (data not shown), suggesting that these two phosphorylation sites are not important for the recognition by FBXO9. Regarding MKRN1, it has been shown that two lysine sites at 184 and 185 of PPAR γ were critical for ubiquitination. Unfortunately, these two sites were not tested for their role in PPAR γ ubiquitination in our study. Further investigation is required to understand the specific target sites of PPAR γ by FBXO9.

In conclusion, we have identified FBXO9, an E3 ligase, as a novel PPAR γ ubiquitination enzyme that regulates protein stability and transcriptional activity of PPAR γ . We hope this information on post-translational modification of PPAR γ will extend our understanding of the regulation of PPAR γ and possibly lead to the development of novel therapeutic targets to ameliorate obesity and diabetes.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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