Aryl hydrocarbon receptor (AhR) regulates adipocyte differentiation by assembling CRL4B ubiquitin ligase to target PPARγ for proteasomal degradation

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Running title: CRL4BAhR ubiquitinates PPARγ in adipocytes

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ABSTRACT
Peroxisome proliferator–activated receptor γ (PPARγ) is the central regulator of adipogenesis, and its dysregulation is linked to obesity and metabolic diseases. Identification of the factors that regulate PPARγ expression and activity is therefore crucial for combating obesity. Aryl hydrocarbon receptor (AhR) is a ligand-activated transcription factor with a known role in xenobiotic detoxification. Recent studies have suggested that AhR also plays essential roles in energy metabolism. However, the detailed mechanisms remain unclear. We previously reported that experiments with adipocyte-specific Cullin 4b (Cul4b)-knockout mice showed that CUL4B suppresses adipogenesis by targeting PPARγ. Here, using immunoprecipitation, ubiquitination, real-time PCR and Gg-pulldown assays, we report that AhR functions as the substrate receptor in CUL4B–RING E3 ubiquitin ligase (CRL4B) complex and is required for recruiting PPARγ. AhR overexpression reduced PPARγ stability and suppressed adipocyte differentiation, and AhR knockdown stimulated adipocyte differentiation in 3T3-L1 cells. Furthermore, we found that two lysine sites on residues 268 and 293 in PPARγ are targeted for CRL4B-mediated ubiquitination, indicating cross-talk between acetylation and ubiquitination. Our findings establish a critical role of AhR in regulating PPARγ stability and suggest that the AhR–PPARγ interaction may represent a potential therapeutic target for managing metabolic diseases arising from PPARγ dysfunction.

Exorbitant body fat in obese people is thought to be a major reason for insulin resistance, cardiovascular diseases and diabetes (1). Obesisty-related insulin resistance is associated with dysregulation
of lipid storage and chronic inflammation in adipose tissue (2). Insulin resistance is likely to be induced by inappropriate regulation of gene expression required for adipocyte differentiation or functions. Adipocyte differentiation from pre-adipocytes is controlled by a number of transcriptional cascades, particularly PPARγ (3). PPARγ proteins are expressed in two isoforms, PPARγ1 and PPARγ2. PPARγ1 is expressed in a number of tissues. PPARγ2 is only observed in adipocytes and crucial in maintaining normal insulin sensitivity (4).

PPARγ protein is post-translationally regulated by several modifications. Phosphorylation on Ser273 of PPARγ is modified by Cdk5 to dysregulate the expression of specific genes, such as adiponectin (5). Acetylation on Lys268 and Lys293 of PPARγ is a signal of lipid storage and cell proliferation, whereas deacetylation of these two sites results in energy expenditure and promotes insulin sensitivity (6). PPARγ degradation is linked to the regulation of its transcriptional activity. Recently, several E3s have been identified in adipocytes, including seven in absentia homolog 2 (SIAH2) (7), makorin ring finger protein 1 (MKRN1) (8), tripartite motif protein 23 (TRIM23) (9), and neural precursor cell expressed developmentally down-regulated protein 4 (NEDD4) (10). These E3s are located predominantly in the cytoplasm and not all of them target PPARγ toward proteasomal degradation.

Cullin 4B (CUL4B) acts as a scaffold protein that assembles DDB1, ROC1 and DDB1-CUL4-association-factor (DCAF) to form Cullin 4B-RING E3 ubiquitin ligases (CRL4B). CRL4B uses a variety of DCAXs to assemble different E3 ligases to specifically target substrate (11). Thus, DCAF is correlated with substrate selection and specificity. By targeting different substrates for Ub-dependent degradation or modification, CRL4B has been shown to participate in the regulation of diverse physiologically and developmentally controlled processes. Patients with CUL4B mutations manifest mental and growth retardation as well as central obesity (12,13,14,15). Using adipocyte-specific Cul4b knockout (AKO) mice, we previously showed that CUL4B functions as a negative regulator of adipogenesis (16). However, the DCAF in CRL4B complex that recruits PPARγ remains unknown.

AhR, also known as dioxin receptor, was reported to play essential roles in xenobiotic and energy metabolism (17,18,19,20). In this study, we identified AhR as a novel factor that negatively regulates PPARγ protein stability via forming the CRL4B E3 ligase complex. The physiological function of AhR in adipocyte differentiation was further demonstrated using AhR overexpressing and knockdown 3T3-L1 cell lines. Our study provides a direct link between AhR and PPARγ, and indicates that AhR-PPARγ interaction is a potential therapeutic target in PPARγ-related diseases.

Results

AhR negatively regulates adipocyte differentiation

To investigate the role of AhR in adipocyte differentiation, we first examined the expression level of AhR in mouse adipose tissues. Similar to CUL4B and PPARγ, AhR was detected in white adipocyte tissues (WAT), including inguinal (Ing-WAT, subcutaneous) and epididymal (Epi-WAT, visceral) fat pads. AhR was expressed highly in brown adipose tissue (BAT) (Fig. 1A). We also examined the mRNA and protein levels of AhR, CUL4B and PPARγ as well as its target genes at
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AhR decreases the stability of PPARγ protein via a proteasome-dependent mechanism

To determine whether AhR regulates adipogenesis via modulating the PPARγ level, we examined the expression of PPARγ when AhR expression was knocked down or overexpressed. While transcriptional levels of PPARγ in 3T3-L1 cells were not obviously changed by either AhR knockdown or overexpression (Fig. S3), knockdown of AhR expression significantly increased the endogenous protein level of PPARγ (Fig. 2A). Conversely, overexpression of AhR in 3T3-L1 cells induced the decrease of endogenous PPARγ. Importantly, the decreased PPARγ led by the overexpression of AhR was efficiently blocked by the administration of MG132, a proteasome inhibitor, suggesting that AhR may downregulate PPARγ by a proteasome-dependent degradation mechanism (Fig. 2B). To further confirm this notion, we measured the half-life of PPARγ in the AhR overexpressed HEK293T cells. As expected, overexpression of AhR significantly increased PPARγ decay (Fig. 2C and 2D). Consistently, the administration of MG132 significantly increased the accumulation of polyubiquitinated PPARγ in HEK293T cells transfected with AhR and PPARγ plasmids (Fig. 2E). Knockdown of AhR in 3T3-L1 preadipocytes also resulted in a reduction of polyubiquitinated PPARγ (Fig. 2F). Furthermore, in the presence or absence of Leptomycin B (LMB) or MG132, we observed that PPARγ was ubiquitinated and targeted for proteasomal degradation in nucleus (Fig. 2G). Immunofluorescence analysis results also showed that PPARγ was accumulated in nucleus upon treatment of MG132 (Fig. 2H). Taken together, these results suggest that AhR decreases the stability of PPARγ protein via a proteasome-dependent mechanism.

AhR functions as a substrate receptor for CRL4B-mediated PPARγ degradation

The fact that AhR decreases the stability of PPARγ protein prompted us to determine whether AhR acts as a substrate receptor for CRL4B-mediated PPARγ degradation. When overexpression of CUL4B decreased PPARγ in 3T3-L1 cells, knockdown of AhR could efficiently block the reduction of PPARγ caused by CUL4B overexpression (Fig. 3A), suggesting that AhR is required for the CRL4B-mediated degradation of PPARγ.
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To further strengthen this notion, we performed co-immunoprecipitation assays in 3T3-L1 cells to determine possible physical association among AhR, CUL4B and PPARγ. As shown in figures, when AhR was immunoprecipitated from 3T3-L1 cells, both CUL4B and PPARγ were brought down as well (Fig. 3B). Consistently, AhR was also co-immunoprecipitated with antibodies against PPARγ or CUL4B (Fig. 3C and 3D). Interactions between PPARγ and AhR were also confirmed in mouse adipose tissue using proximity ligation assay (PLA). Consistent with increased PPARγ level led by CUL4B deletion, more positive signals showing PPARγ-AhR interactions were detected in adipose tissues of adipocyte-specific knockout mice (Fig. 3E and 3F). Glutathione S-transferase (GST) pull-down experiments were performed to further examine whether AhR binds PPARγ. Our results showed that AhR directly interacts with PPARγ (Fig. 3G).

We next determined whether CUL4B-AhR complex represented an E3 ligase for PPARγ. To this end, His-FLAG-AhR was expressed in HEK293T cells, and cellular extracts were prepared by affinity purification. As expected, the components of AhR complex, including endogenous CUL4B, DDB1, and ROC1, were detected in the affinity-purified fractions, thereby forming CRL4BAhR E3 complex (Fig. 3H). Importantly, the affinity-purified AhR complex significantly increased the amount of polyubiquitinated PPARγ, as indicated by in vitro ubiquitination assay (Fig. 3I). Taken together, these results suggest that AhR functions as the substrate receptor in CRL4BAhR complex by recruiting PPARγ and facilitating its ubiquitination.

Domain-domain interactions between PPARγ and AhR

In order to map out the region where PPARγ (isoform 2) binds AhR, we designed several plasmids that expressing truncated PPARγ. Full-length AhR and PPARγ truncations were expressed in E.coli. Our results showed that PPARγ binds AhR mainly on its DNA binding domain (136-232aa). The N-terminal region of PPARγ (1-135aa) seemed to assist in binding AhR, whereas the C-terminal region of PPARγ including its ligand binding domain (233-505aa) did not contribute to binding (Fig. 4A).

Meanwhile, we investigated the region of AhR that contributes in binding PPARγ. All truncated AhR were expressed in E.coli and purified separately. Since neither AhR nor Gst antibodies recognize all of AhR truncations, we performed two sets of experiments. AhR 403-848aa was assayed in both experiments. Our results demonstrated that the C-terminal fragment of AhR (403-848aa) does not pull down PPARγ (Fig. 4B). Instead of full-length AhR, AhR fragment (188-403aa) including its ligand binding domain tends to pull down more PPARγ, suggesting that this region mainly contributes to recruiting PPARγ (Fig. 4C).

Two lysines located on the hinge domain of PPARγ are targeted for ubiquitination by CRL4BAhR

K184 and K185 on PPARγ were previously reported as ubiquitination sites mediated by MKRN1 (8). To examine whether CRL4BAhR targets the same residues, we mutated K184/185 to alanines and performed ubiquitination assay. Our results showed that K184A/K185A mutant exhibited a similar level of ubiquitination to wild-type PPARγ, suggesting that CRL4BAhR catalyzes PPARγ ubiquitination.
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on novel site(s) (Fig. 5A). PPARγ is a lysine-rich protein. To identify the amino acid residue(s) targeted by CRL4B<sup>AhR</sup>, we first mapped the ubiquitinated region using different PPARγ truncations. Truncations 1-135aa, 136-232aa, and 233-505aa were barely ubiquitinated, suggesting that the targeted lysine was not located within these regions. Truncations 136-333aa and 136-505aa that contain the major AhR-binding region (136-232aa) were both ubiquitinated with a similar level to wild-type PPARγ, indicating that the 233-333aa region of PPARγ is required for ubiquitination by CRL4B<sup>AhR</sup>. Additionally, retaining the ability to bind AhR, truncation 1-232aa was also ubiquitinated but with a lower level compared to wild-type PPARγ, possibly due to nonspecific modifications (Fig. 5B).

We next characterized the specific ubiquitination site(s) on PPARγ by mass spectrometry (MS). This can be accomplished by affinity purification of Myc-tagged PPARγ, isolating the captured proteins by SDS-PAGE, extracting the high molecular weight modified protein, and performing in-gel tryptic digestion. According to MS results, three peptide fragments gave GG-K ubiquitin-modification signals (a mass shift of +114.0429 Da) with high scores (experiments were done by Applied Protein Technology company). These peptides contain K268, K293, and K329 individually (Fig. 5C), which are also located in the 233-333aa region of PPARγ (Fig. 5D). K268 and K293 were reported as two evolutionally conserved residues in the hinge domain (6) (Fig. 5E). We then used point mutation analysis to confirm the residue(s) that are polyubiquitinated. As shown in figures, mutation on single site of these three residues did not affect substrate ubiquitination level (Fig. 5F). However, the ubiquitination levels of K268A/K293A double mutant or K268A/K293A/K329A triple mutant were significantly decreased, suggesting K268 and K293 were both targeted by CRL4B<sup>AhR</sup> (Fig. 5G). Consistently, in the presence of AhR, mutations on K268 and K293 could remarkably prolong PPARγ half-life in HEK293T cells (Fig. 5H and 5I). SirT1 was previously reported to deacetylate PPARγ on its K268 and K293 (6). In the presence of NAM, an inhibitor of SirT1 (21), acetylation of PPARγ was increased whereas ubiquitination of PPARγ was decreased (Fig. 5J), suggesting that acetylation on these two sites competes against ubiquitination. However, inhibiting ubiquitination by knockdown of AhR does not increase acetylation level of PPARγ (Fig. S4).

Discussion

In adipocytes, PPARγ protein has a short half-life (22). Several E3s were found to target PPARγ for proteasomal degradation. MKRN1 and SCF<sup>FBXO9</sup> promote PPARγ proteasomal degradation in cytoplasm (8,23). SIAH2 has been shown to be localized in nucleus and promotes PPARγ ubiquitination on dependent of TZDs (24). Some E3s were not associated with PPARγ degradation. TRIM23 regulates PPARγ ubiquitination and stabilizes it (9). NEDD4 has been reported to induce both K48 and K63-linked polyubiquitination of PPARγ (10,25). Although WDTC1 binds PPARγ in adipocytes, CRL4B<sup>WDTC1</sup> complex promotes histone H2AK119 monoubiquitination, and plays a role in transcriptional repression (26). However, our previous study demonstrate that knockdown of CUL4B and DDB1 resulted in a significant increase in the half-life of PPARγ, indicating that CRL4B complex might use a different
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DCAF, rather than WDTC1 to promote PPARγ ubiquitination and degradation. AhR, a nuclear receptor, has been shown to recruit ER-α for proteasomal degradation in a ligand-dependent manner (27). In this study, we showed here that AhR functions as the substrate receptor and recruits PPARγ onto CRL4BAhR E3 complex. Up-regulation of AhR shortens the PPARγ protein half-life via forming the CRL4BAhR E3 complex to facilitate K268- and K293-linked polyubiquitination of PPARγ, thereby blocking adipocyte differentiation. Knockdown of AhR stabilizes PPARγ and promotes adipocyte differentiation in 3T3-L1 cells.

PPARγ plays a central role during adipocyte differentiation (28,29). Increasing attention has been paid to PPARγ post-translational modifications (30,31,32). Despite the fact that PPARγ binds its E3 ligases through its N-terminal (8) or C-terminal region (10,23), our data showed that PPARγ interacts with AhR mainly on its DNA-binding domain and part of hinge region. The hinge region has been reported to regulate the subcellular distribution (33) and interacting with many nuclear receptors, such as androgen receptor (34,35), estrogen receptor (36,37), glucocorticoid receptor (38), and PPARα (39).

PPARγ is a nuclear receptor with a NLS region between amino acid 181-224 (40,41). NLS in CUL4B is located in its N terminus, between amino acid 37-40 (42). Upon binding with ligand and interaction with HSP90, AhR translocates to the nucleus (43). Other components in CRL4BAhR E3 complex were all reported to localize mainly in nucleus (44,45,46). Although ubiquitin, E1, E2, E3s and proteasomal subunits were found in nucleus, some studies suggest that nuclear export is required for the degradation of nuclear substrates (43,47,48,49). However, other studies indicated that a nuclear ubiquitin-proteasome system may also be responsible for the degradation of several transcription factors (50,51,52,53). In our case, PPARγ was ubiquitinated and targeted for proteasomal degradation in nucleus.

Acetylation, which modifies the lysine residue of target proteins including histone and non-histone proteins, is now recognized as a critical step in transcriptional regulation (54). Interestingly, many of these identified acetylated substrates are involved in ubiquitin-dependent proteolysis. Several identified acetylated lysine residues are also potential ubiquitination sites in proteins (55,56). For example, the same lysine residues at the C terminus of p53 can be modified by both acetylation and ubiquitination, implicating a role of acetylation in regulating p53 stability (57). Acetylation is also integrated with other posttranslational modifications to regulate PPARγ activity (58,59). PPARγ acetylation on Lys268 and Lys293 is a signal of lipid storage and cell proliferation. Conversely, deacetylation of PPARγ induces energy expenditure and promotes insulin sensitivity (6). We used MS analysis on trypsin-digested peptides to identify three ubiquitinated lysines at residues 268, 293 and 329. Among them, two evolutionally conserved residues Lys268 and Lys293 were main ubiquitinated sites verified by the following ubiquitination assay and CHX chasing assay. In some cases, acetylation competes with ubiquitination for the same lysine sites to prevent protein degradation (60). Deacetylation of PPARγ makes K268 and K293 available for ubiquitination by CRL4BAhR and subsequent proteasomal degradation. Therefore, it is important to keep the balance for PPARγ protein level and makes it transcriptionally activated, thus
paving the way for making PPARγ activated by post-translationally level is a potential therapeutic target.

Previously, we reported that scaffold protein CUL4B of CRL4B E3 ligase complex functions as a negative regulator of adipogenesis. We observed that depletion of CUL4B improves adipose function and protects against glucose intolerance and insulin resistance (16). A similar phenotype was observed in AhR knockout mice. Exposing AhR$^{-/-}$ mice to a high-fat diet showed lower fasting glucose levels and improved glucose tolerance (61). In addition to AhR$^{-/-}$ mice, AhR$^{+/+}$ mice that express about 30% of the AhR levels also exhibited significantly improved glucose tolerance.

Experimental procedures

Plasmids. Full-length cDNAs of AhR and PPARγ were inserted into either pcDNA3.1 (Invitrogen), pGEX4T1 (GE) or pRsfduet-1 (Novagen) vectors as indicated. Truncated mutants of AhR (1-187aa, 188-403aa, 403-848aa) and truncated mutants of PPARγ (1-135aa, 136-232aa, 1-232aa, 136-505aa, 233-505aa, 136-333aa) were amplified by PCR and cloned into vectors as indicated. Different point mutations in PPARγ or AhR were generated by site-directed mutagenesis. Other plasmids were generated and described previously (16,63).

Antibodies and chemicals. For Western blotting, the antibodies used were as follows: PPARγ (C26H12, Cell Signalling), AhR (ab2769, Abcam), CUL4B (HPA011880, Sigma), HA (rabbit D110004, mouse D199961, BBI and 600401384, Rockland), Myc (rabbit D110006, mouse D153566, BBI), β-actin (sc-69879, Santa Cruz), Acetylated-Lysine (9441, Cell Signalling). Reagents used to culture cells or perform experiments were as follows: X-tremeGene and insulin sensitivity (62).

Recent studies revealed that AhR plays critical roles in energy metabolism (17,18). Suppression of AhR activity can improve metabolic function to avoid obesity (62). Unlike the low affinity direct or indirect interaction between SIAH2 and PPARγ (24), AhR functions as a substrate receptor, directly recruits substrate PPARγ, facilitates its ubiquitination and promotes subsequent proteasomal degradation (Fig. 6). Further studies are certainly required to reveal precise mechanism on substrate selection, crosstalk between acetylation and ubiquitination, and detailed AhR-PPARγ interactions for the treatment of obesity and metabolic diseases.

HP DNA transfection reagent (Roche), X-tremeGene siRNA transfection reagent (Roche), PEI (23966, Polysciences Inc.), CHX (HY-12320, MCE), MG132 (HY-13259, MCE), leptomycin B (LMB, S1726, Beyotime), N-Ethylmaleimide (NEM, EB0450, BBI), DMSO (A503039, Sangon Biotech), DAPI (ab104139, Abcam), Glutathione reduced (A600229, BBI), and PMSF (PB0425, BBI).

Cell culture and adipocyte differentiation. HEK293T and 3T3-L1 cells were grown in DMEM (Gibco) with 10% bovine serum (Sigma-Aldrich). Adipocytes differentiated from 3T3-L1 cells were first maintained in DMEM with 10% bovine serum (Gibco) for 2-3 days. Cells were then differentiated in DMEM containing 10% fetal bovine serum, dexamethasone (1μM), IBMX (520μM), and insulin (1μg/ml) for 7 days. Differentiated cells were stained using Oil-Red-O staining (Sigma-Aldrich). The stained cells were photographed using a camera-connected microscope (Olympus).

Generation of AhR knockdown and
overexpression stable cell lines. AhR shRNA construct (LV3(H1/GFP&Puro)) and AhR overexpression plasmid (LV5(EF-1aF/GFP&Puro)) were purchased from GenePharma. Lentivirus packaging was done in GenePharma. Lentiviral supernatant of shRNA and AhR overexpression plasmids were used to infect 3T3-L1 using the manufacturer’s instructions. Infected cells were selected by puromycin (1.5μg/ml) treatment for 6 days.

**Protein stability assay and analysis.** HEK293T cells were co-transfected with Myc-PPARγ (2 μg) and AhR-Flag-His (4 μg) or the empty vector (4 μg). After 20 hours, cells were treated with 60μg/ml cycloheximide (CHX) to inhibit protein synthesis. CHX-treated cells were harvested at different time points (0, 4, 8 hours) and processed for immunoblotting with anti-PPARγ antibody. Anti-actin antibody was used as internal controls. Signals from the Western blots were analyzed by Volume Analysis of Quantity One with volumn background subtraction (Bio-Rad).

**Protein purification and binding assays.** pGEX4T1-AhR and pRsfduet-PPARγ (isoform2) were expressed in *E.coli* and purified using glutathione-sepharose 4B (GE Healthcare) or Ni²⁺-NTA (GE Healthcare) separately. The purified Gst-AhR and His-PPARγ were further purified by size-exclusion chromatography (SD200 or SD75, GE) and stored in protein buffer containing 25mM Tris 7.6, 150mM NaCl, and 1mM DTT. The binding assays were performed by mixing His-PPARγ and Gst-AhR. The mixture was then incubated with AhR antibody and protein A/G sepharose (Santa Cruz) for 2h at 4°C. Immunoprecipitates were boiled in sample loading buffer for 5 min.

**Immunoprecipitation.** HEK293T cells were lysed using lysis buffer (50mM Tris-HCl pH7.6, 150mM NaCl, 0.5% sodium deoxycholate, 0.5% NP40) with protease inhibitor cocktail (Roche) followed by sonication (8s, 12 cycles). Cell lysate supernatants were incubated with indicated antibodies and protein A/G sepharose (Santa Cruz) for 2h at 4°C. Immunoprecipitates were boiled in sample loading buffer for 5 min.

**Proximity ligation assay (PLA).** Adult male mice (WT and adipocyte-specific *Cul4b* knockout) were housed at the animal unit at Department of Genetics, Shandong University. Mouse adipose tissues were extracted and fixed for embedding in paraffin. 4μm samples of the paraffin tissue array were stained against indicated antibodies according to Duolink® in Situ PLA (Sigma) protocol. Antibodies were used in this experiment include PPARγ (mouse 95128S, Cell Signalling, 1:400), AhR (rabbit 83200S, Cell Signalling, 1:50), CUL4B (HPA011880, Sigma, 1:600). Images were collected by fluorescence microscope (Olympus BX51).

**Ubiquitination assay.** HEK293T cells or 3T3-L1 cells were first transfected with indicated plasmids or siRNAs and treated with indicated chemicals, including MG132 (20μM), NAM (100μM), or LMB (10ng/ml). Cells were then harvested with PBS containing 10μM NEM to prevent deubiquitination. Cells were lysed in 2% SDS by boiling the samples for 10 min, followed by sonication (8s, 1 cycle). Lysed sample supernatants were incubated with Myc antibody (D153566, BBI), mixed with protein A/G sepharose (Santa Cruz) for 2h at 4°C, followed by Western blotting or Coomassie Blue staining for MS.
In vitro ubiquitination assay. His-PPARγ was expressed in *E. coli* and purified with Ni²⁺-NTA (GE Healthcare), followed by size-exclusion chromatography (SD75, GE). His-PPARγ was incubated with 200ng E1 (UBE1), 500ng E2 (UbcH5c), 10μg His-Ub, 2 mM ATP (Enzo life sciences) as described previously (8). The reaction was performed in the absence and presence of AhR-Flag-His co-IP products. After the 1h incubation at 37°C, samples were quenched in 6M guanidinium-HCl (pH 8) containing 5mM NEM. His-ubiquitinated proteins were pulled down with Ni²⁺-NTA (GE Healthcare), followed by washing and elution in sample buffer (64). The mixture was then boiled in loading dye at 95°C for 10 min to disrupt protein-protein interactions.

Mass spectrometry. Mass spectrometry experiments were done by Applied Protein Technology. LC-MS/MS analysis was performed on a Q Exactive mass spectrometer (Thermo Fisher Scientific) for 60min. MS data was acquired using a data-dependent top10 method dynamically choosing the most abundant precursor ions from the survey scan (300–1800 m/z) for HCD fragmentation. Survey scans were acquired at a resolution of 70,000 at m/z 200 and resolution for HCD spectra was set to 17,500 at m/z 200, and isolation width was 2 m/z. The instrument was run with peptide recognition mode enabled. The raw data was loaded to supporting information. The mass spectrometry search parameters are as follows: The peaklist-generating software and search engine that we used is Mascot2.2. We used Uniprot database for sequence searching. We actually searched 161584 entries in the database. Trypsin was used in our experiments with specificity sites K/R. Two missed and/or non-specific cleavages were permitted. Fixed and variable modifications (including residue specificity) include Carbamidomethyl (C) and Oxidation (M); GlyGly(K), respectively. Mass tolerance for precursor ions is 20ppm and for fragment ions is 0.1 Da. Threshold score/Expectation value for accepting individual spectra is no less than 20.

Immunofluorescence analysis. HEK293T cells were co-transfected with Myc-PPARγ (2μg) and AhR-Flag-His (4μg) and treated with DMSO or MG132 for 6 hours. Cells were fixed for 30min at room temperature (P0098, Beyotime) and washed with PBS buffer three times. Cells were stained with anti-PPARγ antibody (1:100). Images were collected by fluorescence microscope (Olympus BX51).

RT-PCR analysis. RNAs were extracted using Trizol (Invitrogen) according to the manufacturer’s directions. cDNAs were synthesized from total RNA using Reverse Transcriptase (EP0441, Thermo), amplified, and analyzed using Green PCR kit and real-time PCR. The primers used in this study are described in supporting information. Each gene expression level was normalized by the GAPDH gene.

Statistical analysis. Statistical analyses on RT-PCR and PLA were performed using Prism software. Measurement data were expressed as mean ± standard deviation (SD). The paired, two-tailed Student t test was used to determine the significance between two groups. P < 0.05 was regarded as the threshold value for statistical significance. Statistical analyses on protein level and stability were performed using Quantity One (Bio-Rad).
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Data Availability: The MS proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository with the data set identifier PXD015830.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
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FOOTNOTES
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Figure legends

Figure 1. Adipogenesis is negatively regulated by AhR.

(A) The levels of indicated proteins across mouse adipose tissues. (B) Protein levels of CUL4B and its related proteins were determined in 3T3-L1 cells. Upon differentiation with induced medium, cells were harvested and lysed for Western blotting. Protein band of interest was marked as star. Signals from the Western blots were analyzed by Volume Analysis of Quantity One with volume background subtraction (Bio-Rad). (C) Adipogenesis of AhR overexpressing 3T3-L1. Cells stably expressing empty vector or AhR were constructed using a lentivirus system. At day 9 post-induction, the cells were stained for lipid droplets using Oil Red O. (D) Adipogenesis of AhR knockdown 3T3-L1. Stable cell lines were constructed using a lentivirus expressing shRNA for scrambled sequences (control) or mouse ahr. On day 9 after induction of differentiation, cells were stained with Oil Red O. (E) In AhR overexpressing 3T3-L1 cells, total RNA samples were extracted at different time points upon induction. Samples were subjected to quantitative-PCR analysis of adipogenic markers (CD36, Adipsin, and Fabp4). (F) In AhR knockdown 3T3-L1 cells, total RNA samples were extracted at different time points. Samples were subjected to quantitative-PCR analysis of adipogenic markers (CD36, Adipsin, and Fabp4). Data were presented as mean ± SD.; n=4 with *P < 0.05, **P < 0.01, ***P < 0.001 by student’s t test.
AhR affects PPARγ protein level and stability.

(A) Effect of AhR ablation on PPARγ protein level. 3T3-L1 cells were transfected with the indicated siRNAs for 8 hours. Cells were harvested, lysed, and immunoprecipitated with indicated antibodies, followed by Western blotting. (B) Effect of AhR overexpression on PPARγ protein level. 3T3-L1 cells were transfected with the indicated plasmids expressing AhR-Flag-His in the absence or presence of MG132. Cells were harvested, lysed, and immunoprecipitated with indicated antibodies, followed by Western blotting. (C) Effect of AhR on the protein stability of PPARγ. HEK293T cells were transfected with the indicated plasmids expressing Myc-PPARγ in the absence or presence of AhR-Flag-His for CHX chasing. Cells were harvested and lysed. The cell lysate was then detected with indicated antibodies. (D) Turnover of PPARγ was determined by Western blotting. Signals from immunoblots were analyzed using the Quantity One (Bio-Rad). PPARγ protein signals were normalized with the actin protein signals, and the percentage of PPARγ protein remaining was plotted against time. (E) Ubiquitination product mediated by CUL4B-AhR associated complex was accumulated in the presence of proteasome inhibitor MG132. HEK293T cells were transfected with plasmids expressing Flag-Cul4B, Myc-PPARγ, AhR-Flag-His, and HA-Ub. Cells were then treated with DMSO or MG132 for 3 hours. (F) Effect of CUL4B-AhR associated complex on PPARγ ubiquitination. 3T3-L1 cells were transfected with the indicated siRNA for 5 hours. The cells were then transfected with plasmids expressing Flag-Cul4B, Myc-PPARγ, and HA-Ub. After MG132 treatment of 4 hours, cells were harvested, lysed, and immunoprecipitated with indicated antibodies, followed by
Western blotting. (G) HEK293T cells were transfected with plasmids expressing Flag-Cul4B, AhR-Flag-His, HA-Ub and Myc-PPARγ. Cells were treated with MG132 for 3 hours and Leptomycin B (LMB) for 4 hours, followed by immunoprecipitation and Western blotting. Input (5%) was used for Western blotting. (H) HEK293T cells were transfected with plasmids expressing Myc-PPARγ and AhR-Flag-His. Cells were treated with MG132 or DMSO for 6 hours. Immunofluorescence against PPARγ was performed. Images were collected using fluorescence microscope.
AhR directly interacts with PPARγ and functions as a substrate receptor in the E3 ligase complex.

(A) CUL4B affects PPARγ stabilization dependent on AhR. 3T3-L1 cells were transfected with the indicated siRNA and plasmids. Cells were harvested, lysed, and immunoprecipitated with indicated antibodies, followed by Western blotting. (B-D) Interactions among CUL4B, AhR and PPARγ. 3T3-L1 cells were transfected with the plasmids as indicated. The transfected cells were then harvested and immunoprecipitated with the indicated antibodies individually. (E) Interactions between PPARγ and AhR were tested in adipose tissues (Epi-WAT) from WT and KO mice. PLA (Duolink, sigma) was performed and images were collected using fluorescence microscope. (F) Number of signal/nucleus were calculated and analyzed using Prism software. Data were presented as mean ± SD.; n=4 with **P < 0.01 by student’s t test. (G) Direct interactions between AhR and PPARγ. Gst-AhR and His-PPARγ were expressed in E.coli individually followed by protein purification and immunoprecipitated pulldown. The proteins were detected using indicated antibodies. Input (5%) was used for Western blotting. (H) HEK293T cells were transfected with AhR-Flag-His or Flag-His vector. Cells were then harvested and sonicated, followed by Flag pulldown. The co-immunoprecipitated products used in ubiquitination assay were detected using indicated
antibodies. (I) Ubiquitination of PPARγ targeted by the AhR complex *in vitro*. Purified human recombinant His-PPARγ (full-length) was incubated with E1, E2, Ub and ATP in the absence and presence of the AhR complex, the co-immunoprecipitated products obtained in (F). Reactions were performed at 37°C for 1 hour, followed by Flag pulldown. Ubiquitination of His-PPARγ was analyzed by Western blotting using anti-Ub antibody.
Figure 4. AhR recruits PPARγ upon domain-domain interactions.

(A) Mapping of the PPARγ domain responsible to bind AhR. Gst-AhR (full length) and His-PPARγ truncations were expressed in E.coli individually followed by protein purification. Proteins were mixed for Ni²⁺ pulldown, followed by Western blotting. Input (5%) was detected using indicated antibodies. Protein bands of interest were marked as stars. (B and C) Mapping of the AhR domain responsible to interact with PPARγ. His-PPARγ (full length) and Gst-AhR truncations were expressed in E.coli individually followed by protein purification. Proteins were mixed, followed by immunoprecipitation with indicated antibodies and Western blotting. Input (5%) was detected using indicated antibodies. Protein bands of interest were marked as stars.
Figure 5. Identification of ubiquitination site(s) in PPARγ.

(A) CRL4B\textsuperscript{AhR} targets PPARγ for ubiquitination at novel site(s). HEK293T cells were transfected with plasmids expressing Flag-Cul4B, AhR-Flag-His, HA-Ub and Myc-PPARγ variants. Cells were treated with MG132 for 3 hours and harvested for the following immunoprecipitation and Western blotting. Input (5%) was used for Western blotting. (B) Domain-domain interactions are required for PPARγ ubiquitination. HEK293T cells were transfected with plasmids expressing Flag-Cul4B, AhR-Flag-His, HA-Ub and Myc-PPARγ truncations. Cells were treated with MG132 for 6 hours and harvested for the following immunoprecipitation and Western blotting. Input (5%) was used for Western blotting. (C) List of PPARγ ubiquitination sites determined by MS. HEK293T cells were transfected with plasmids expressing Flag-Cul4B, AhR-Flag-His, HA-Ub and Myc-PPARγ. Cells were treated with MG132 for 10 hours and harvested for immunoprecipitation using anti-Myc antibody. (D) Schematic structure of PPARγ. Arrowheads indicate potential ubiquitinated sites. (E) The model of PPARγ isoform2 (created from 1PRG (65)) is demonstrated in cartoon. The AhR-binding region is colored green and the ubiquitination region is colored yellow. Potential
ubiquitination sites are shown in red spheres. (F) Single mutations on PPARγ barely affect ubiquitination. HEK293T cells were transfected with plasmids expressing Flag-Cul4B, AhR-Flag-His, HA-Ub and Myc-PPARγ variants. Cells were treated with MG132 for 3 hours and harvested for the following immunoprecipitation and Western blotting. Input (5%) was used for Western blotting. (G) Dual mutations on PPARγ defect ubiquitination. HEK293T cells were transfected with plasmids expressing Flag-Cul4B, AhR-Flag-His, HA-Ub and Myc-PPARγ variants. Cells were treated with MG132 for 3 hours and harvested for the following immunoprecipitation and Western blotting. Input (5%) was used for Western blotting. (H) Effect of dual mutations on PPARγ stability. HEK293T cells were transfected with the indicated plasmids expressing AhR-Flag-His and Myc-PPARγ variants for CHX chasing. The final concentration of CHX used in the treatment was 60μg/ml. The cell lysate was then detected with indicated antibodies. (I) Turnover of wild type and mutated PPARγ were determined by Western blotting. Signals from immunoblots were analyzed using the Quantity One (Bio-Rad). Protein signals of PPARγ variants were normalized with the actin protein signals, and the percentage of PPARγ variants remaining was plotted against time. (J) SirT1 inhibitor NAM reduced PPARγ ubiquitination level. HEK293T cells were transfected with plasmids expressing Flag-Cul4B, AhR-Flag-His, HA-Ub and Myc-PPARγ. Cells were treated with MG132 (20μM, 1.5 h) in the absence or presence of NAM (100μM, 48 h). Cells were then harvested for the following immunoprecipitation and Western blotting. Input (5%) was used for Western blotting.
Figure 6. CRL4\textsuperscript{AhR}-mediated PPAR\textsubscript{γ} ubiquitination regulates adipocyte differentiation.
Aryl hydrocarbon receptor (AhR) regulates adipocyte differentiation by assembling CRL4B ubiquitin ligase to target PPAR γ for proteasomal degradation

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