



# Cysteine dioxygenase type 1 promotes adipogenesis via interaction with peroxisome proliferator-activated receptor gamma



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## ABSTRACT

Mammalian cysteine dioxygenase type 1 (CDO1) is an essential enzyme for taurine biosynthesis and the biodegradation of toxic cysteine. As previously suggested, Cdo1 may be a marker of liposarcoma progression and adipogenic differentiation, but the role of Cdo1 in adipogenesis has yet been reported. In this study, we found that the expression of Cdo1 is dramatically elevated during adipogenic differentiation of 3T3-L1 pre-adipocytes and mouse bone marrow-derived mesenchymal stem cells (mBMSCs). Conversely, knockdown of Cdo1 inhibited expression of adipogenic specific genes and lipid droplet formation in 3T3-L1 cells and mBMSCs. Mechanistically, we found Cdo1 interacted with Pparg in response to adipogenic stimulus. Further, depletion of Cdo1 reduced the recruitment of Pparg to the promoters of C/EBP $\alpha$  and Fabp4. Collectively, our finding indicates that Cdo1 may be a co-activator of Pparg in adipogenesis, and may contribute to the development of disease associated with excessive adipose tissue.

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## 1. Introduction

Adipogenesis refers to the process whereby mesenchymal stem cells (MSCs) or preadipocytes differentiate into lipid-laden and insulin-responsive adipocytes, which may involve several stages as defined previously, including mesenchymal precursors, committed pre-adipocytes, growth-arrested pre-adipocytes, mitotic clonal expansion, terminal differentiation and mature adipocytes [1,2]. Adipocytes play an important role in energy balance by storing or mobilizing triacylglycerol according to energy level in body [3]. In addition, adipocytes can synthesize and secrete numerous cytokines and hormones that are involved in overall energy homeostasis [4]. However, accumulation of adipose tissue is associated with various diseases, such as obesity [5], osteoporosis [6], and cardiac steatosis [7].

Adipogenesis is a highly controlled process involving several positive and negative regulators, such as a cascade of transcription factors, cell-cycle proteins, and Wntless and INT-1 proteins (Wnts) [8,9]. Among these, peroxisome proliferator-activated receptor gamma (Pparg) is considered as a master transcription factor of adipogenesis, which has been shown to directly promote

expression of the adipocyte gene program. In particular, no factor has been discovered that can rescue adipogenesis in the absence of Pparg [9]. To initial transcription of target genes, Pparg with retinoid X receptor (RXR) binds the promoter as a heterodimer [2]. Co-activators may also be recruited with ligand stimulation. For example, PPAR $\gamma$  is constitutively associated with steroid receptor co-activators (SRCs) in the promoter of fatty acid binding protein 4 (Fabp4) in adipocytes [10,11]. In addition, it is recently reported that CCAAT/enhancer binding protein  $\alpha$  (C/EBP $\alpha$ ) is required for the binding of Pparg to the promoters of several key metabolic adipocyte genes [12].

Cysteine dioxygenase type 1 (Cdo1) is a non-heme structured, iron-containing metalloenzyme, which plays a vital role in taurine biosynthesis by catalyzing the oxidation of cysteine to cysteine sulfinic acid [13,14]. Previous studies have shown that CDO1 is expressed in adipose tissue, liver, brain, and kidney [15]. Specifically, Well-differentiated liposarcomas (WDLs) has a significantly higher CDO1 expression level than dedifferentiated liposarcomas (DDLs) [16]. Further, expression of CDO1 is up-regulated during adipogenic differentiation of human bone marrow-derived MSCs and adipose tissue-derived pre-adipocytes [16,17], which suggests that CDO1 may play a role in regulation of adipogenesis.

In this study, we found up-regulation of Cdo1 expression during adipogenic differentiation of 3T3-L1 pre-adipocytes and mBMSCs in vitro. Further, siRNA-mediated depletion of Cdo1 significantly

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suppressed adipogenesis of 3T3-L1 and mBMSCs. Through IP and ChIP assay, we found that Cdo1 is required for Ppar $\gamma$  binding to the target gene promoters.

## 2. Materials and methods

### 2.1. Cell culture

3T3-L1 (murine embryonic fibroblast) cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA), and were maintained in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% heat-inactivated fetal bovine serum (FBS), 2 mM L-Glutamine, plus 100 U/ml of K-Penicillin G and 100 mg/ml of Streptomycin sulfate at 37 °C with a humidified atmosphere of 5% CO<sub>2</sub> (all from Gibco). Primary MSCs from mouse bone marrow (mBMSCs) were isolated and cultured as described previously [18]. All animal procedures were conducted in accordance with the guidelines for the care and use of laboratory animals of State Key Laboratory of Oral Diseases, West China Hospital of Stomatology, Sichuan University.

### 2.2. Induction of adipogenic differentiation

Adipogenic induction (AI) medium was comprised of 90% DMEM high glucose (Gibco), 10% FBS, 1  $\mu$ M dexamethasone, 10  $\mu$ g/mL insulin, 0.5 mM 3-isobutyl-1-methylxanthine, and 0.2 mM indomethacin (all from Sigma). After 7–10 days differentiation, Oil Red O staining was performed to detect the lipid droplet formation according to the manufacturer's instructions (Diagnostic Biosystems). Then, the plates were photographed, and the stained cultures were destained by isopropanol. The absorbance of the solution was read at 450 nm using a microplate reader (Bio-Rad).

### 2.3. Small interfering RNA (siRNA) and transfection

All the siRNAs were purchased from Invitrogen (Carlsbad, CA, USA), including a scrambled siRNA (Scr) as a control, and two siRNAs: si1 (5'-AUGCCAAAUUCGAUCAUAUU-3'), and si2 (5'-CUGCAAAGGUGUGU CCUAUU-3'), targeting mRNA of Cdo1. Transfection was performed using Lipofectamine RNAiMAX reagent (Invitrogen) according to manufacturer's instructions. The cells were transfected with 30–60 pmol siRNA and incubated for 48 h, and the knockdown efficiency was determined by RT-PCR and western blot.

### 2.4. RNA isolation and reverse transcription-PCR (RT-PCR)

Total RNA was extracted using the Trizol reagent (Invitrogen), and complementary DNA was then synthesized from 1  $\mu$ g of total RNA, using PrimeScript RT Reagent Kit (Takara). Quantitative real-time PCR was performed using SYBR Premix Ex Taq (Takara). The real-time PCR conditions were 40 cycles at 94 °C for 10 s and 60 °C for 60 s. The primer sequences used for this analysis were: 5'-AATGATTCCATTGGCTTACACCG-3' (forward) and 5'-GGCATGTATCGAAGGGTGGAC-3' (reverse) for Cdo1; 5'-AACTGGATGACAGTGACATTCCC-3' (forward) and 5'-CCCCTCTGCAACTTCTCAAT-3' (reverse) for Ppar $\gamma$ ; 5'-GTCACTGGTCAACTCCAGCA-3' (forward) and 5'-TGGACAAGAACACGACAG-3' (reverse) for C/EBP $\alpha$ ; 5'-AAGGTGAAGAGCATATAACCT-3' (forward) and 5'-TCACGCCTTTCATAACACA TTCC-3' (reverse) for Fabp4; 5'-ACAACCTTGGCATTGTGGAA-3' (forward) and 5'-GATGCAGGGATGATGTTCTG-3' (reverse) for GAPDH.

### 2.5. Western blot

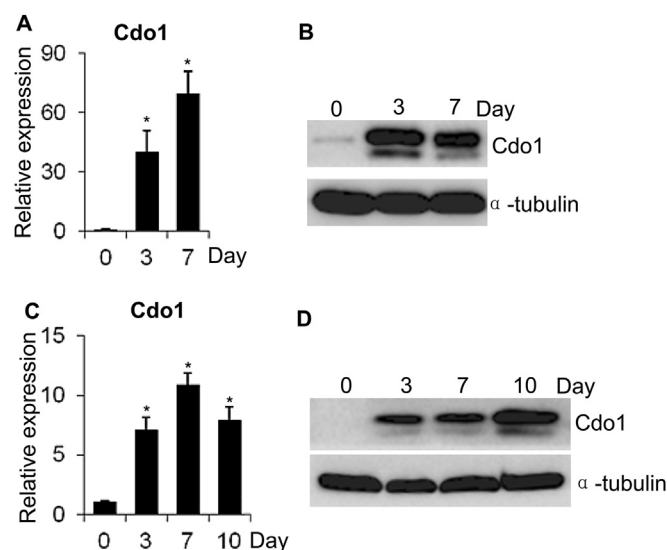
Cells were harvested and lysed on ice for 30 min in CelLytic MT solution (Sigma), supplemented with protease inhibitor cocktail (Pierce Biotechnology), and centrifuged at 18,000 g for 15 min at 4 °C. Aliquots of the lysates were electrophoresed on a 12.5% sodium dodecyl sulfate-polyacrylamide gel. The resolved proteins were then transferred onto nitrocellulose membranes (Bio-Rad), which were subsequently incubated with primary antibodies followed by a horseradish peroxidase-conjugated secondary antibody (Boster, Wuhan, China). Protein bands were detected using an enhanced chemiluminescence western blotting detection kit (Thermo). Antibodies for western blot were purchased from the following commercial sources: mouse polyclonal anti-Ppar $\gamma$  (Santa Cruz), rabbit monoclonal anti-Cdo1 (Abcam).

### 2.6. Immunoprecipitation (IP) assay

Protein was collected after 3 day of adipogenic differentiation. Aliquots of the protein sample were incubated with Ppar $\gamma$  antibodies or Cdo1 antibody overnight at 4 °C with gentle orbital rotation. Then 30  $\mu$ l of protein G-Sepharose beads (Pierce Biotechnology) were added and the incubation was extended for another 2 h. The antibody/antigen complexes were eluted from the beads and subjected to 12.5% SDS-PAGE followed by western blot procedures.

### 2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assay was performed using a Simple ChIP Assay Kits (Cell Signaling Technology) according to the manufacturer's protocol. All precipitated DNA samples were quantified with Real-time PCR. Data are expressed as the percentage of input DNA. Same antibodies for western blot are used for ChIP assay. The primer sets were designed around the Ppar $\gamma$  binding elements in the mouse C/EBP $\alpha$  and Fabp4 gene: 5'-CTGAGCTACACCTCGGCTC-3' (forward) and 5'-TCCCCACCGGAGGGCATGAG-3' (reverse) for C/EBP $\alpha$ ; 5'-AAATGCACATT



**Fig. 1.** Cdo1 is up-regulated during adipogenesis. (A) The mRNA expression levels of Cdo1 during adipogenesis of 3T3-L1 pre-adipocytes at 0, 3, 7 days. (B) The protein expression levels of Cdo1 during adipogenesis of 3T3-L1 pre-adipocytes at 0, 3, 7 days. (C) The mRNA expression levels of Cdo1 during adipogenesis of mBMSCs at 0, 3, 7, 10 days. (D) The protein expression levels of Cdo1 during adipogenesis of mBMSCs at 0, 3, 7, 10 days. \* $p < 0.05$ .

CACCCAGA-3' (forward) and 5'-GGGCTGTGACACTTCCACTA-3' (reverse) for Fabp4. As negative control, 7 kb downstream for C/EBP $\alpha$ : 5'-CGGAGCTGAAGTGTGTGTT-3' (forward), and 5'-CATGCATAGTCCCAAACCTG-3' (reverse); 4 kb downstream for Fabp4: 5'-AAGCTTTGTTTCTGCCCTGT-3' (forward) and 5'-ACGCATGAATGCTATGGA GA-3' (reverse).

### 2.8. Statistical analysis

Data shown are representative of at least three experiments and are represented as mean  $\pm$  SD. For single comparisons, an unpaired student's t-test was used. For multiple comparisons, the one-way analysis of variance (ANOVA) was used to assess the statistical significances of differences among pair of data sets. A *p* value <0.05 was considered to be statistically significant.

## 3. Results

### 3.1. Cdo1 is up-regulated during adipogenesis

We first evaluated Cdo1 expression levels during adipogenesis of 3T3-L1 pre-adipocyte cell line. As determined by RT-PCR and western blot, the mRNA and protein expression of Cdo1 was up-regulated in response to adipogenic induction (Fig. 1A, B). Consistently, Cdo1 was expressed at a low level, but highly induced by adipogenic stimulation in primary mBMSCs, (Fig. 1C, D). These results indicate that Cdo1 may play a role in adipogenesis.

### 3.2. Depletion of Cdo1 inhibits adipogenesis in 3T3-L1 pre-adipocytes

To investigate the role of Cdo1 in adipogenesis, siRNAs were used to knockdown Cdo1 in 3T3-L1 cells. The knockdown efficiency

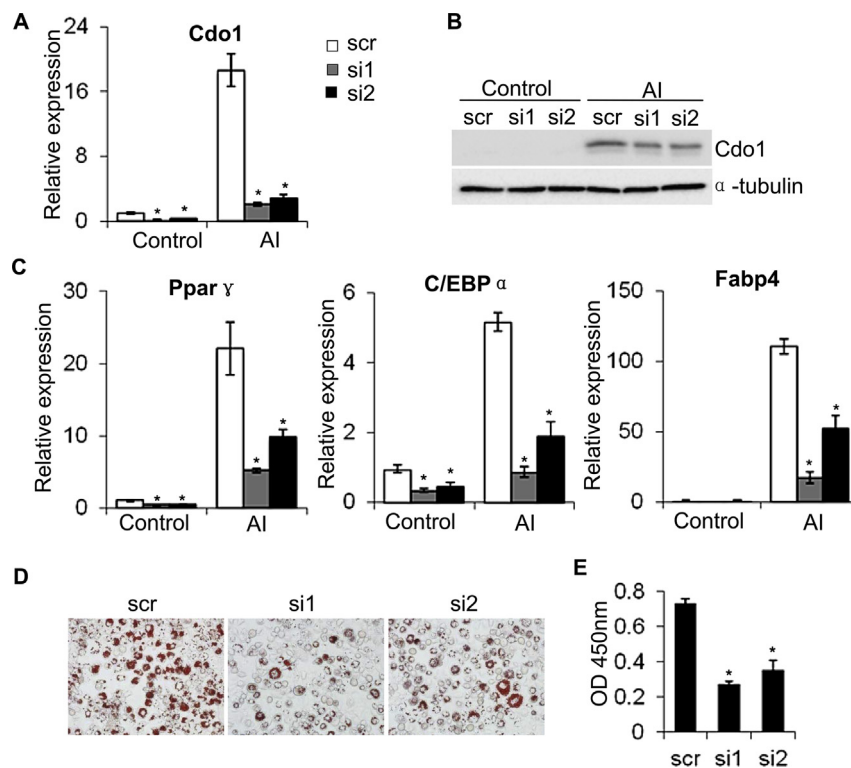
in the presence or absence of adipogenic induction (AI) was confirmed by RT-PCR and western blot (Fig. 2A, B). Interestingly, siRNA-mediated depletion of Cdo1 significantly inhibited expression of adipose-related genes, such as Ppar- $\gamma$ , Cebpa, Fabp4 (Fig. 2C). The lipid droplet formation was also reduced by knockdown of Cdo1, as determined by Oil red O staining after 7 days of adipogenesis in 3T3-L1 cells (Fig. 2D, E).

### 3.3. Depletion of Cdo1 inhibits adipogenic differentiation in mouse primary BMSCs

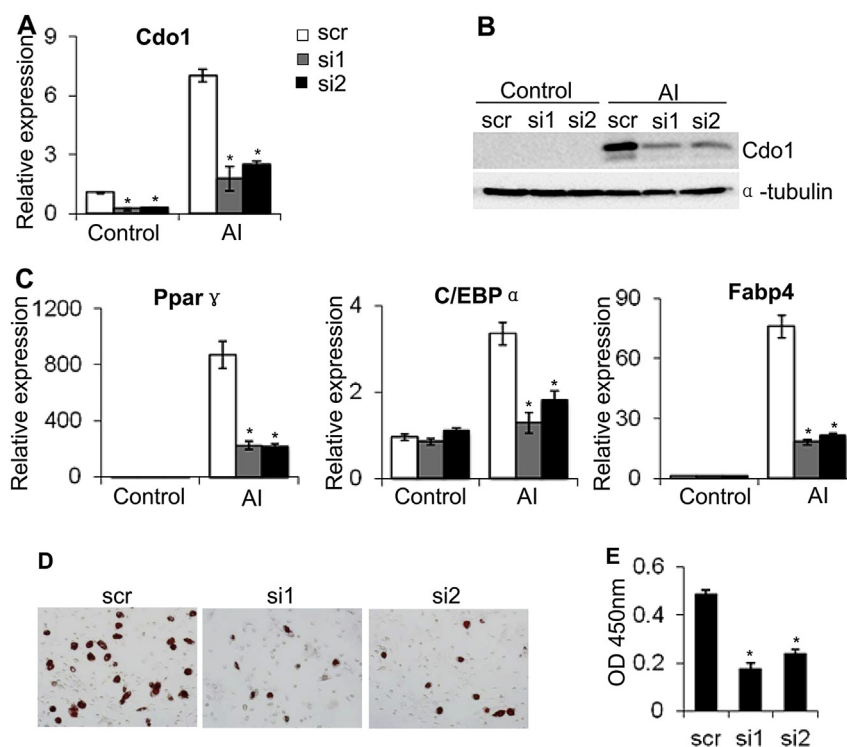
To determine if Cdo1 is involved in the early stage of adipogenic differentiation of MSCs, similar approaches were performed on mouse primary BMSCs. As shown in Fig. 3A and B, the knockdown efficiency was evaluated 48 h post-transfection in the presence or absence of adipogenic induction. Consistently, knockdown of Cdo1 suppressed adipose-related gene expression and lipid droplet formation in mBMSCs stimulated by adipogenic inducing media (Fig. 3C–E).

### 3.4. Cdo1 interacts with Ppar $\gamma$ during adipogenesis

Ppar $\gamma$  is regarded as a master regulator of adipogenic differentiation in mammals. To assess whether Cdo1 interacts with Ppar $\gamma$ , IP assay was performed after 3 days of adipogenic induction in 3T3-L1 cells. We found that Cdo1 co-immunoprecipitated with Ppar $\gamma$  as shown in Fig. 4A. And reverse co-immunoprecipitation confirmed that Ppar $\gamma$  co-immunoprecipitated with Cdo1 (Fig. 4B). Further, knockdown of Cdo1 reduced Ppar $\gamma$  binding to the promoters of C/EBP $\alpha$  induced by adipogenic stimulus (Fig. 4C). As a control, Ppar $\gamma$  was not present 7 kb downstream of the transcription start site (Fig. 4C). Consistently, the binding of Ppar $\gamma$  to the promoter of Fabp4 was also inhibited by depletion of Cdo1 (Fig. 4D).



**Fig. 2.** siRNA-mediated depletion of Cdo1 inhibits adipogenesis in 3T3-L1 pre-adipocytes. (A) The knockdown of Cdo1 was confirmed by RT-PCR in the presence or absence of adipogenic induction. (B) The knockdown of Cdo1 was confirmed by western blot in the presence or absence of adipogenic induction. (C) Knockdown of Cdo1 inhibited expression levels of Ppar $\gamma$ , C/EBP $\alpha$ , and Fabp4 as determined by RT-PCR in 3T3-L1 cells. (D) Knockdown of Cdo1 inhibited lipid droplet formation as determined by Oil Red O staining. (E) Quantification of Oil red O staining in D. \**p* < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)



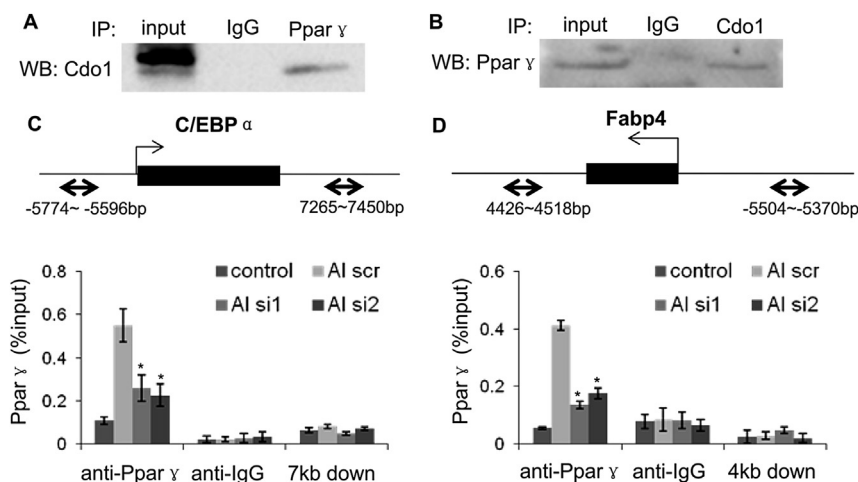
**Fig. 3.** siRNA-mediated depletion of Cdo1 inhibits adipogenesis in mBMSCs. (A) The knockdown of Cdo1 in mBMSCs was confirmed by RT-PCR in the presence or absence of adipogenic induction. (B) The knockdown of Cdo1 in mBMSCs was confirmed by western blot in the presence or absence of adipogenic induction. (C) Knockdown of Cdo1 inhibited expression levels of Pparγ, C/EBPα, and Fabp4 as determined by RT-PCR in mBMSCs. (D) Knockdown of Cdo1 inhibited lipid droplet formation as determined by Oil Red O staining. (E) Quantification of Oil red O staining in D. \**p* < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

#### 4. Discussion

In this study, we investigated the expression pattern of Cdo1 during adipogenesis of 3T3-L1 cells and mBMSCs *in vitro*. To explore the mechanism by which Cdo1 regulates adipogenic differentiation of mBMSCs and 3T3-L1 cells, we further performed immunoprecipitation assay and ChIP assay. And we found that Cdo1 interacts with Pparγ in presence of adipogenic induction.

Knockdown of Cdo1 significantly inhibited the recruitment of Pparγ to the promoters of C/EBPα and Fabp4.

Our findings strongly suggest that Cdo1 is required for adipogenesis. Adipogenesis involves a two-step differentiation process: undifferentiated mesenchymal progenitors differentiate into pre-adipocytes, a secondary differentiation from pre-adipocyte to mature adipocytes [1]. In our study, mBMSCs and 3T3-L1 pre-adipocytes were used to investigate the role of Cdo1 during



**Fig. 4.** Cdo1 interacts with Pparγ during adipogenesis. (A) Western blot analysis using Cdo1 antibodies after immunoprecipitation from 3T3-L1 cell lysates using Pparγ or IgG antibodies. (B) Western blot analysis using Pparγ antibodies after immunoprecipitation from 3T3-L1 cell lysates using Cdo1 or IgG antibodies. (C) Representative results of qPCR analyses to quantify the relative amounts of DNA obtained by ChIP for the regulatory sites of Pparγ in the promoter region of C/EBPα. (D) Representative results of qPCR analyses to quantify the relative amounts of DNA obtained by ChIP for the regulatory sites of Pparγ in the promoter region of Fabp4. ChIP assay was performed 2 day after transfection followed by 1 day of adipogenic induction. Control group indicates sample from 3T3-L1 cells with no treatment. \**p* < 0.05.



adipogenesis. Although the basal level of Cdo1 expression is extremely low in both mBMSCs and 3T3-L1 cells, it highly increased in response to adipogenic stimulus. Then two siRNAs were used to knockdown expression of Cdo1, which target the encoding region and 3' UTR region of Cdo1 mRNA, respectively. We found that depletion of Cdo1 significantly suppressed the expression adipogenesis related genes, such as Ppar $\gamma$ , C/EBP $\alpha$ , and Fabb4. As determined by Oil Red O staining, knockdown of Cdo1 also led to a decrease in lipid droplet formation in both mBMSCs and 3T3-L1 cells.

We also found that the role of Cdo1 in regulation of adipogenesis is mediated by Ppar $\gamma$ . mBMSCs can differentiate into either osteoblasts or adipocytes, and there is an inverse relationship between osteogenesis and adipogenesis [19,20]. Ppar $\gamma$  is key transcription factor in MSC lineage specification, which inhibits osteoblastogenesis and stimulates adipogenesis [21,22]. In our study, we demonstrated the interaction between Cdo1 and Ppar $\gamma$ , as determined by immunoprecipitation assay. And the result of ChIP assay showed that knockdown of Cdo1 inhibited Ppar $\gamma$  binding to the promoters of C/EBP $\alpha$  and Fabb4, indicating Cdo1 may be required for the transcriptional activity of Ppar $\gamma$ . On the other hand, previous studies have shown that Cdo1 is a target gene of Ppar $\gamma$ , which is facilitated by C/EBP $\alpha$  [12]. Taken together, Cdo1 and Ppar $\gamma$  may be integrated as a positive feedback loop, in which Ppar $\gamma$  initiates transcription of Cdo1, and Cdo1 in turn facilitates Ppar $\gamma$  binding to the promoters of target genes. In addition, expression of CDO1 was tightly controlled by promoter methylation [23,24], epigenetic mechanism should also be taken into account in the future studies.

In summary, we found that Cdo1 promotes adipogenesis via interaction with Ppar $\gamma$ . Our findings suggest that Cdo1 might be a co-activator of Ppar $\gamma$ , and is required for the recruitment of Ppar $\gamma$  to the promoters of target genes. Thus, Cdo1 may represent a novel therapeutic target of lipotoxic diseases caused by excessive adipose tissue or adipose tissue dysfunction.

### Conflict of interest

The authors declare no conflicts of interest.

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### Transparency document

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