**Original Paper**

# CCR4-NOT2 Promotes the Differentiation and Lipogenesis of 3T3-L1 Adipocytes via Upregulation of PPARγ, CEBPα and Inhibition of P-GSK3α/β and β-Catenin

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**Key Words**
CNOT2 • Adipogenesis • 3T3-L1 • PPARγ • CEBPα • Obesity

**Abstract**
*Background/Aims:* Though CCR4-NOT2 (CNOT2), one of CCR4-NOT complex subunits, was known to be involved in metastasis and apoptosis through transcription and mRNA degradation, its other biological function is poorly understood so far. The aim of this study is to elucidate the molecular role of CNOT2 in the differentiation process of 3T3-L1 preadipocytes. *Methods and Results:* CNOT2 was overexpressed during the differentiation process of 3T3-L1 preadipocytes. Consistently, mRNA levels of CNOT2, adiponectin, adiponectin 2, PPARγ and CEBPα were enhanced in 3T3-L1 adipocytes. Conversely, CNOT2 depletion by siRNA transfection also reversed the activation of PPARγ and CEBPα and inhibition of GSK3α/β and β-catenin at the protein level in 3T3-L1 preadipocytes. Immunofluorescence assay revealed that CNOT2 was colocalized with PPARγ, but not with CEBPα in 3T3-L1 adipocyte. Consistently, IP western blots revealed that CNOT2 interacted with PPARγ in 3T3-L1 adipocyte. *Conclusion:* Our findings demonstrate that CNOT2 promotes the differentiation of 3T3-L1 preadipocytes via upregulation of PPARγ, and CEBPα and inhibition of GSK3α/β and β-catenin signaling as a potent molecular target for obesity.

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## Introduction

Obesity from the accumulation of white adipose tissue is known to be closely associated with insulin resistance, hyperlipidemia, or type 2 diabetes, heart disease, osteoarthritis and tumor progression [1, 2]. General managements for obesity are bariatric surgery, dieting,

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physical exercise and medications such as or listat (Xenical), lorcaserin (Belviq) and a combination of phentermine and topiramate [3, 4]. Nevertheless, a new effective therapy is still required for obesity because of side effects of classical treatments [5]. Thus, recently customized therapies are attractive, targeting adipogenic molecules such as PPARγ [6], C/EBPα, -β, and –δ, Krox20 [7], Krüppel-like factors [8], adiponectin [9], sterol-regulatory element-binding protein (SREBP)-1c [10], Stat5 [11], GSK3β [12], methyl-CpG binding domain protein 4 (MBD4) [13], microRNA 378 [14], microRNA 221 [15] and ADIPOQ [16], BMP-smad signaling pathway [17]. There are accumulating evidences that Wnt signaling inhibits adipogenesis. Thus, overexpression of GSK3β phosphorylation inhibited adipogenesis in 3T3-L1 preadipocytes [18] and pharmacological inhibitors of GSK3β blocked adipocyte differentiation [19].

CCR4-NOT complex (CNOT), a large (>2 MDa) multi-complex consisting of eleven subunits is a master regulator of mRNA stability, mRNA export, transcription and translation [20, 21]. Among subunits of CCR4-NOT, human CNOT2 regulates the deadenylase activity and structural integrity of the CCR4–NOT complex and controls embryonic development in *C. elegans* and *D. melanogaster* [22]. Not-Box in the C-terminus of CNOT2 mediates repression of promoter activity [23] and also the recruitment of HDACs to the promoter of CNOT2 is responsible for the repression of transcription [24]. Furthermore, deficiency of CNOT2 regulated apoptotic cell death [25] and metastasis [26]. Nevertheless, the other biological activity of CNOT2 regardless of tumor progression still remains unclear until now. Thus, in the present study, we demonstrate for the first time the critical role of CNOT2 during the adipogenic differentiation of 3T3L-1 preadipocytes in association with PPARγ, CEBPα, GSK3α/β and β-catenin signaling.

## Materials and Methods

### Cell culture

Preadipocyte 3T3-L1 cells were obtained from the Korean Cell Line Bank (KCLB, Korea) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS at 37 °C in an atmosphere of 95% air and 5% $CO_2$. The cells were incubated at 37°C in an atmosphere of 5% $CO_2$ in a humidified incubator.

### Adipogenic differentiation induction

The preadipocyte 3T3-L1 cells were incubated onto 6 well plates to confluent status. To induce differentiation, postconfluent 3T3-L1 adipocytes (designated day 0) were incubated in DMEM with 1 $\mu$M dexamethasone, and 1 μg/ml of insulin, and 0.5 mM isobutylmethylxanthine (IBMX) (Sigma-Aldrich, St. Louis, MO, USA) for 2 days and were replaced by fresh normal medium containing 1 μg/ml of insulin.

### Oil Red O staining and quantification

Preadipocyte 3T3-L1 cells were fixed with 2% paraformaldehyde at room temperature for 1 h. After fixation, the cells were washed with 60% isopropanol for 5 min and then subject to be completely dry at room temperature. The cells were stained for 10 min at room temperature by immersion with Oil Red O solution (Sigma-Aldrich, St. Louis, MO, USA) for 30 min followed by washing with distilled water several times. The cells were observed for the stained intracellular lipid droplets, and photographed using bright field inverted Nikon microscope and Nikon software. Quantification of the stained area was carried out by eluting the stain off the plate after incubation with 100% isopropanol for 1 h, followed by measuring the absorbance of the elutes at 500 nm using an EnSpire multimode plate reader (Perkin Elmer, Waltham, Massachusetts, USA).

### RT-qPCR analyses

Total RNA was isolated using the QIAzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. First-strand cDNA synthesis from one microgram of total RNA was performed by Super-

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script reverse transcriptase and amplified by Platinum *Taq* polymerase with Superscript One Step RT-PCR kit (Invitrogen, Carlsbad, CA, USA). For RT-qPCR analysis, RT-qPCR was performed with the LightCycler TM instrument (Roche Applied Sciences, Indianapolis, IN) with following primers, mouse cnot2 (forward)5'- tgc tca gca agc ctt ctaaa-3', (reverse) 5'-gcc aga tcc tca agg gatta-3', mouse C/EBPα (forward) 5'-gtg ctg gag ttg acc agtga-3', (reverse) 5'-cca agg agc tct cag gcag-3', mouse C/EBPβ (forward) 5'- gca aga gcc gcg acaag-3', (reverse) 5'-gct cgg gca gct gctt-3', mouse adiponectin (forward) 5'-aag aag gac aag gcc gtt ctc-3', (reverse) 5'-gag tcc att gtg gtc ccc-3', mouse adiponectin 2 (forward) 5'-gca tga gcc aaa gga agagg-3', (reverse) 5'-ccg act gac tat tgt agtg-3', mouse PPARγ (forward) 5'- aag gcg agg gcg atc ttgac-3', (reverse) 5'-ctt tgc tct gct cct gcagg-3', mouse GAPDH (forward) 5'- gct tgt cat caa cgg gaag-3', (reverse) 5'-gat gtt agt ggg gtc tcg-3'.

### siRNA transfection assay

Small interfering RNA (siRNA) CNOT2 (Thermo scientific, USA) or control vector (40 nM, Bioneer, Korea) was transfected using INTERFERin® reagent (Polyplus, IIIkirch, France) in 3T3-L1 adipocytes according to the manufacturer's instructions.

### Western blotting

Whole cell lysates from 3T3-L1 adipocytes were extracted using lysis buffer (20 mM Tris, pH 7.4, 250 mM NaCl, 2 mM EDTA, pH 8.0, 0.1% Triton X-100, 0.01 mg/mL aprotinin, 0.003 mg/mL leupeptin, 0.4 mM phenylmethylsulfonyl fluoride (PMSF), and 4 mM NaVO4). The lysates were resolved in 10% SDS-PAGE gels and the proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies against CNOT2, PPARγ, C/EBPα, GSK3α/β and β-catenin (Cell Signaling Tech., Danvers, MA, USA), and β-actin (Sigma, St. Louis, MO, USA) overnight. After washing, the membrane was incubated with horseradish peroxidase- (HRP-) conjugated secondary antibodies and protein expression was examined by enhanced chemiluminescence (ECL) (GE Health Care Biosciences, Piscataway, NJ, USA).

### Immunofluorescence assay

3T3L-1 adipocyte cells were fixed with 3% paraformaldehyde and permeabilized in 0.1% Triton X-100 and incubated with monoclonal anti-CNOT2 (Santa Cruz, CA, USA), C/EBPα, PPARγ antibodies (Cell Signaling Tech., Danvers, MA, USA) overnight and followed by incubation for 30 min with Alex Flour 488 Goat Anti-mouse IgG, Alex Fluor 568 goat rabbit-IgG antibody (Invitrogen, Carlsbad, CA, USA)(1 : 500 dilution). After washing, the fixed cells were stained with 5 mg/ml 4,6-diamidino-2-phenylindole (DAPI; Sigma-Aldrich, St. Louis, MO,USA). The images for colocalization between CNOT2 and PPARγ or CEBPα were taken by using a Delta Vision imaging system (Applied Precision. Issaquah, WA, USA).

### Immunoprecipitation and western blot (IP western blotting)

Adipocyte cells were solubilized in lysis buffer (50 mM Tris–HCl pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, 1 mM NaF, and 1 mM $Na_3VO_4$) for 30 min on ice. After centrifugation, the supernatants were incubated with protein A agarose (Santa Cruz, CA, USA) and mixed with anti-rabbit IgG or α-CNOT2 (Santa Cruz) overnight at 4°C. Protein A agarose was washed three times with lysis buffer, and western blotting was carried out.

### Statistical analysis

For statistical analysis of the data, Sigmaplot version 12 software (Systat Software Inc., San jose, CA, U.S.A) was used. All data were expressed as means ± standard deviation (SD). All experiments were performed at least three times.
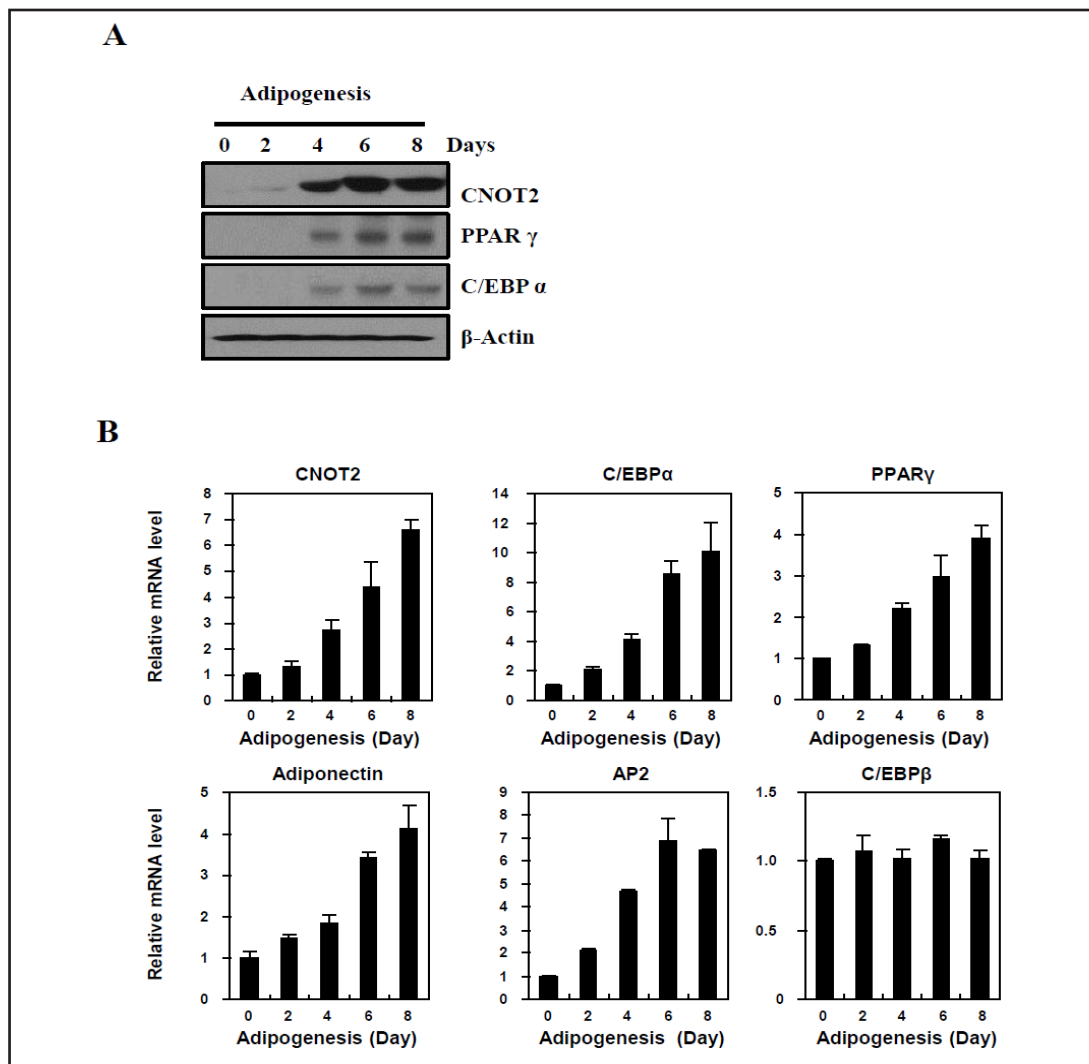
## Results

### CNOT2 and other adipogenic genes were overexpressed at the protein and mRNA levels in a time dependent fashion during the differentiation process of 3T3-L1 preadipocytes

There is accumulating evidence that several molecules such as adiponectin, PPARγ, CEBP family are involved in adipogenesis. We found that CNOT2 was overexpressed in 3T3-L1 adipocyte cells along with PPARγ and CEBPα by Western blotting (Fig. 1A). Consistently,

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RT-qPCR revealed that mRNA levels of CNOT2, adiponectin, adiponectin 2 (AP2), PPARγ and CEBPα except CEBPβ were enhanced during the differentiation process of 3T3-L1 preadipocytes (Fig. 1B).

### Silencing of CNOT2 reduced the number of lipid droplets during the differentiation process of 3T3-L1 preadipocytes

To confirm whether or not CNOT2 depletion affected adipogenesis, Oil Red O staining was performed in CNOT2 siRNA construct transfected 3T3-L1 adipocytes. As shown in Fig. 2, CNOT2 depletion by siRNA transfection reduced lipid accumulation in 3T3-L1 adipocytes, while the number of lipid droplets with dark brown color was increased in a time dependent manner from Day 4 to Day 8 after culture.



**Fig. 1.** CNOT2 and other adipogenic genes were overexpressed at the protein and mRNA levels in a time dependent fashion during the differentiation process of 3T3-L1 preadipocytes. The 3T3-L1 preadipocytes were incubated to confluent status. Confluent 3T3-L1 preadipocytes (designated as Day 0) were induced into differentiation as shown in Material and methods. (A) CNOT2, PPARγ and C/EBPα were overexpressed at protein level during the adipogenic differentiation process of 3T3-L1 preadipocytes in a time course (Day 0, 2, 4, 6 and 8) by Western blotting. (B) The mRNA expression of CNOT2, adiponectin, AP2, PPARγ, CEBPα, and CEBPβ was upregulated during differentiation process of 3T3-L1 preadipocytes by qRT-PCR analysis. GAPDH was used as the internal control. Data are presented as means ± SD for triplicate experiment.

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**Fig. 2.** Silencing of CNOT2 reduced the number of lipid droplets during the differentiation process of 3T3-L1 preadipocytes. After transfection of CNOT2 or control siRNA plasmids in 3T3-L1 adipocytes, the cells were cultured in differentiation media containing adipogenic cocktail for 8 days. The effect of CNOT2 knockdown on lipid accumulation was evaluated during the differentiation process of 3T3-L1 preadipocytes. Graphs represent lipid accumulation in 3T3-L1 cells by Oil Red staining. ** $p < 0.01$ *** $p < 0.001$ *vs.* control. Data are presented as means ± SD for triplicate experiment.
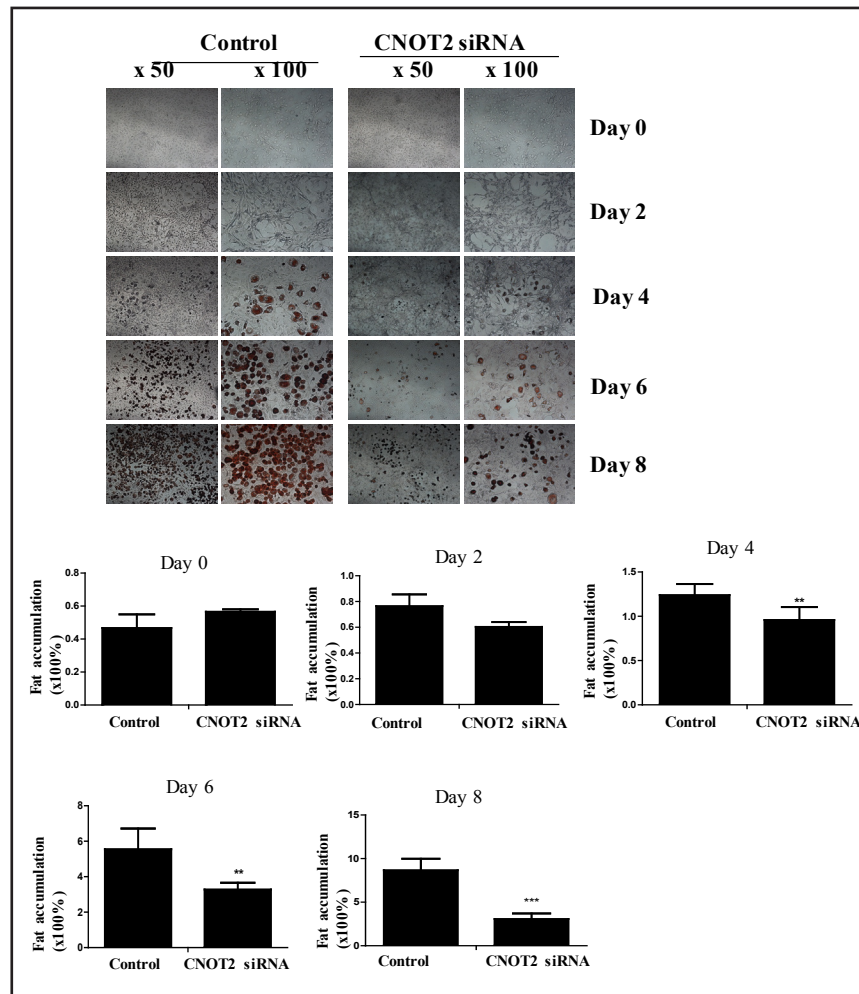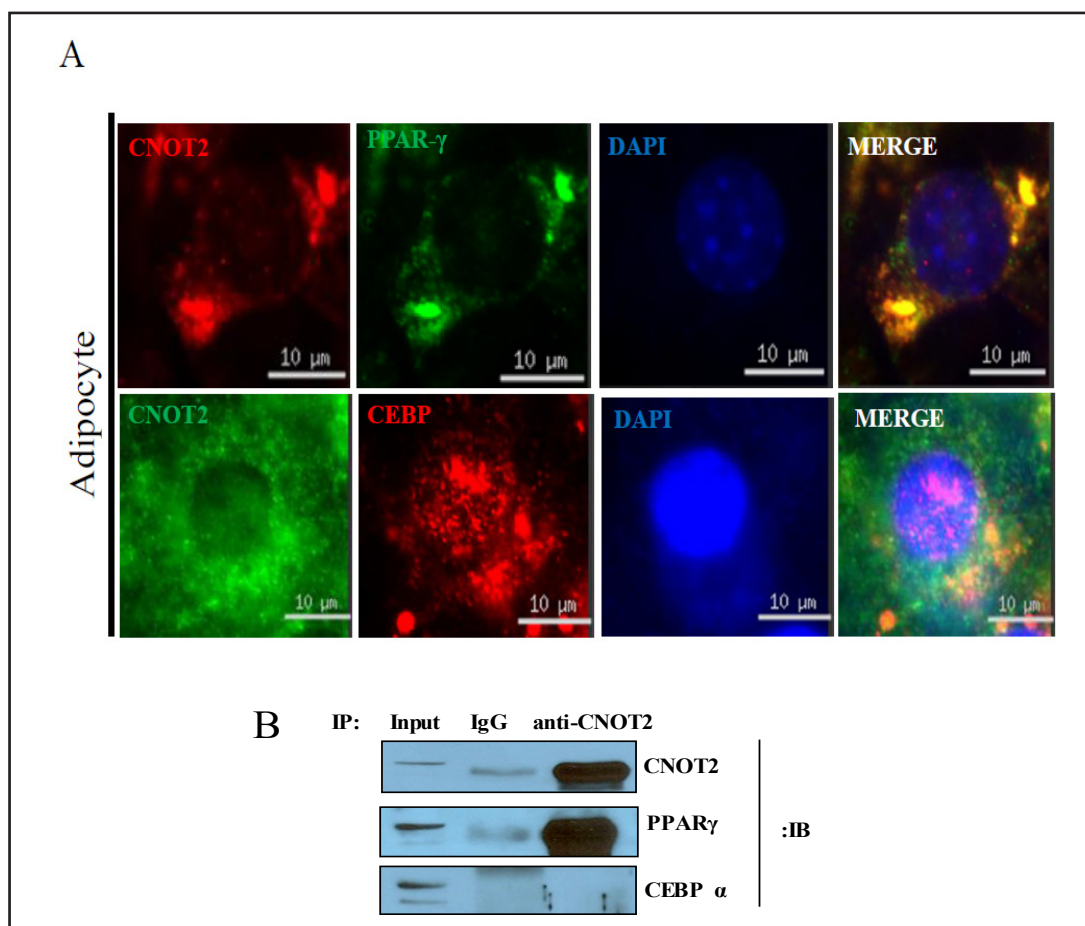


**Fig. 3.** Silencing of CNOT2 reversed upregulation of PPARγ and CEBPα and downregulation of GSK3α/β and β-catenin during the differentiation process of 3T3-L1 preadipocytes. After transfection of CNOT2 or control siRNA plasmids in 3T3-L1 adipocytes, the cells were cultured in differentiation media containing adipogenic cocktail for 4 or 6 days. Cell lysates were collected and subjected to Western blotting to check the expression of PPARγ, C/EBPα, GSK3α/β and β-catenin.
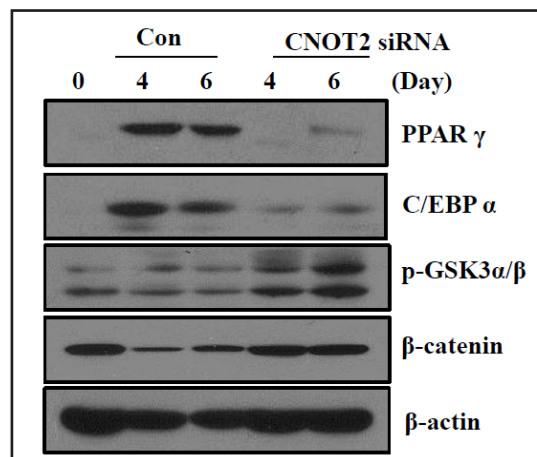


*Silencing of CNOT2 reversed upregulated expression of PPARγ and CEBPα and down regulated expression of GSK3α/β and β-catenin during the differentiation process of 3T3-L1 preadipocytes*

To confirm whether CNOT2 knockdown affected adipogenesis, siRNA transfection assay with CNOT2 siRNA construct was performed in 3T3-L1 adipocytes. As shown in Fig. 3,

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**Fig. 4.** CNOT2 was colocalized and interacted with PPARγ in 3T3 L1 adipoctyes. (A) CNOT2 was colocalized with PPARγ, but not with CEBPα in 3T3-L1 adipocyte. Adipocyte cells were fixed with 3% paraformaldehyde and permeabilized in 0.1% Triton X-100 and incubated with monoclonal anti-CNOT2 (Santa Cruz), C/EBPα, PPARγ antibodies (Cell signaling) overnight and followed by incubation for 30 min with Alex Flour 488 Goat Anti-mouse IgG, Alex Fluor 568 goat rabbit-IgG antibody. The fixed cells were stained DAPI and the images for colocalization were taken by using a Delta Vision imaging system. (B) CNOT2 was interacted with PPARγ in 3T3-L1 adipocytes. Adipoctye cells were immunoprecipitated with α-CNOT2 antibody and immunoblotted with α-PPARγ or CEBPα.

silencing of CNOT2 attenuated the activated expression of PPARγ and CEBPα at the protein level in a time dependent manner during the differentiation process of 3T3-L1 preadipocytes but increased the p-GSK3α/β and β-catenin.

*CNOT2 was colocalized and interacted with PPARγ, but not with CEBPα in 3T3-L1 adipocytes*

Immunofluorescence assay revealed that CNOT2 was colocalized with PPARγ but not with CEBPα in 3T3 L1 adipocytes (Fig. 4A). Also, IP-western blot showed that CNOT2 interacted with PPARγ, but not with CEBPα in 3T3-L1 adipocytes (Fig. 4B).

### Discussion

Though CCR4-NOT is involved in gene regulation, chromatin modification, transcription elongation and RNA export [22, 23, 27], the function of CCR4-NOT is poorly understood

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so far. In the current study, the role of CNOT2 during the adipogenic differentiation of 3T3-L1 preadipocytes was examined. CNOT2 is one of CCR4-NOT complex subunits with CNOT1 and CNOT3. Depletion of CNOT1, the largest one of the CCR4–NOT complex, induced caspase dependent apoptosis through ER stress in HeLa cells [25]. In contrast, CNOT2 was involved in transcriptional regulation through an interaction with histone deacetylase 3 (HDAC3) [24]. Here we found that CNOT2 was overexpressed in a time course during the differentiation process of 3T3-L1 preadipocytes by Western blotting, implying that CNOT2 is critically involved in adipogenic differentiation process.

There are accumulating evidences that the peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein alpha (CEBPα) are two key transcriptional factors for initiating adipocyte differentiation [28] and also adiponectin, adiponectin 2 (AP2), and CEBPβ are involved in glucose and lipid metabolism [29, 30]. Consistently, we confirmed that mRNA levels of CNOT2, adiponectin, adiponectin 2, PPARγ and CEBPα, except only CEBPβ were upregluated during the differentiation process of 3T3-L1 preadipocytes by RT-qPCR, indicating the changes of several mRNA levels during adipogenesis. Also, we can speculate that the factor for not affecting CEBPβ in our study can be different times after the induction of differentiation in 3T3-L1 adipocytes, since CEBPβ is induced within 2–4 h [31].

To validate the association of CNOT2 with adipogenic molecules, the siRNA transfection assay was performed in 3T3-L1 adipocytes. Western blotting revealed that CNOT2 depletion reversed activation of PPARγ and CEBPα as well as inactivation of GSK3α/β and β-catenin of Wnt/β-catenin signaling pathway during the differentiation process of 3T3-L1 preadipocytes, demonstrating the important role of CNOT2 in adipogenic metabolism. In the present study, immunofluorescence assay showed that CNOT2 was colocalized with PPARγ. Furthermore, IP-western blot showed that CNOT2 interacted with PPARγ, but not with CEBPα, indicating that CNOT2 closely binds to PPARγ. It is also well documented that the Wnt signaling pathway negatively regulates adipogenesis through the inhibition of C/EBPα and PPARγ [12, 19]. Given that disruption of GSK3β as a key component of Wnt/β-catenin signaling pathway by inhibitors repressed preadipocyte differentiation [19], the more detailed mechanism of CNOT2 with CEBPα or PPARγ via Wnt/ β-catenin should be further elucidated in the near future.

Collectively, CNOT2 was overexpressed at protein and mRNA expression levels and CNOT2, adiponectin, AP2, PPARγ and CEBPα were enhanced during the differentiation process of 3T3-L1 preadipocytes. However, CNOT2 depletion blocked the activation of PPARγ and CEBPα, as well as inactivation of GSK3α/β and β-catenin. Furthermore, CNOT2 was colocalized and interacted with PPARγ, but not with CEBPα, in 3T3-L1 adipocytes. Overall, our findings suggest that CNOT2 promotes the differentiation of 3T3-L1 preadipocytes via upregulation of PPARγ, and CEBPα and inhibition of GSK3α/β and β-catenin signaling as a potent molecular target for obesity.

### Disclosure Statement

The authors declare no competing financial interests.

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