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Histone Methylation Regulator PTIP Is Required for *PPAR γ* and *C/EBP α* Expression and Adipogenesis

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Summary

PPAR γ and *C/EBP α* cooperate to control preadipocyte differentiation (adipogenesis). However, the factors that regulate *PPAR γ* and *C/EBP α* expression during adipogenesis remain largely unclear. Here we show PTIP, a protein that associates with histone H3K4 methyltransferases, regulates *PPAR γ* and *C/EBP α* expression in mouse embryonic fibroblasts (MEFs) and during preadipocyte differentiation. PTIP deletion in MEFs leads to marked decreases of *PPAR γ* expression and *PPAR γ* -stimulated *C/EBP α* expression. Further, PTIP is essential for induction of *PPAR γ* and *C/EBP α* expression during preadipocyte differentiation. Deletion of PTIP impairs the enrichment of H3K4 trimethylation and RNA polymerase II on *PPAR γ* and *C/EBP α* promoters. Accordingly, *PTIP*^{-/-} MEFs and preadipocytes all show striking defects in adipogenesis. Furthermore, rescue of the adipogenesis defect in *PTIP*^{-/-} MEFs requires co-expression of *PPAR γ* and *C/EBP α* . Finally, deletion of PTIP in brown adipose tissue significantly reduces tissue weight in mice. Thus, by regulating *PPAR γ* and *C/EBP α* expression, PTIP plays a critical role in adipogenesis.

Introduction

Peroxisome Proliferator-Activated Receptor- γ (*PPAR γ*) is considered the master regulator of adipogenesis. It is a member of the nuclear receptor superfamily of ligand-activated transcription factors and is both necessary and sufficient for adipogenesis (Farmer, 2006; Rosen et al., 2002). *PPAR γ* has two isoforms, *PPAR γ 1* and *PPAR γ 2*, generated by usage of two distinct promoters and alternative splicing (Zhu et al., 1995). *PPAR γ 1* is ubiquitously expressed while *PPAR γ 2* expression is restricted to adipose tissues. However, both isoforms are strongly induced during preadipocyte differentiation *in vitro* and both are highly expressed in adipose tissues in animals. *PPAR γ 1* is induced earlier than *PPAR γ 2* and is maintained at a level higher than *PPAR γ 2* during preadipocyte differentiation (Morrison and Farmer, 1999). Data from *PPAR γ 2* isoform-specific knockout mice indicate that *PPAR γ 2* is not absolutely required for adipogenesis *in vivo* (Rosen and MacDougald, 2006), suggesting that *PPAR γ 1* may be critical for adipogenesis. Although the functional difference between endogenous *PPAR γ 1* and *PPAR γ 2* remains unclear, ectopic expression of either *PPAR γ 1* or *PPAR γ 2* is sufficient to

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stimulate immortalized, non-adipogenic MEFs to differentiate into adipocytes (Mueller et al., 2002).

C/EBP α (CCAAT/enhancer binding protein α) is another principal adipogenic transcription factor and is strongly induced in the early phase of preadipocyte differentiation. Ectopic expression of *C/EBP α* stimulates non-adipogenic MEFs to undergo adipogenesis while deletion of *C/EBP α* in mice results in almost complete absence of white adipose tissue (WAT) (Farmer, 2006). *PPAR γ* and *C/EBP α* positively regulate each other's expression and cooperate to control preadipocyte differentiation (adipogenesis) (Farmer, 2006; Rosen and MacDougald, 2006). However, the factors and the underlying mechanisms that regulate the induction of *PPAR γ* and *C/EBP α* expression during adipogenesis remain unclear.

PTIP (Pax transactivation domain-interacting protein) is a ubiquitously expressed nuclear protein that associates with active chromatin. Knockout of PTIP in mice leads to lethality by embryonic day 9.5 (Patel et al., 2007). PTIP carries six tandem BRCT domains that are predominantly found in proteins involved in DNA damage response. Indeed, ectopically expressed PTIP has been implicated in cellular response to DNA damage (Manke et al., 2003). However, the physiological function of endogenous PTIP has remained largely unclear.

Histone lysine methylation has been implicated in both gene activation and repression, depending on the specific lysine residue that gets methylated. For example, methylation on histone H3 lysine 4 (H3K4) associates with gene activation while methylation on histone H3 lysine 27 (H3K27) associates with gene repression (Li et al., 2007). We and others recently show that endogenous PTIP is a component of a histone methyltransferase (HMT) complex that contains histone H3K4 methyltransferases MLL3 and MLL4 (also known as ALR), and the JmjC domain-containing protein UTX (Cho et al., 2007; Issaeva et al., 2007; Patel et al., 2007). Further, we and others demonstrate UTX is a histone H3K27-specific demethylase (Hong et al., 2007; Swigut and Wysocka, 2007). Thus, endogenous PTIP associates with two H3K4 methyltransferases and one H3K27 demethylase. Since both methylation of H3K4 and demethylation of H3K27 presumably associate with gene activation, these data strongly suggest a role of PTIP in gene activation.

In this report, we identify PTIP as a factor that regulates *PPAR γ* and *C/EBP α* expression in MEFs as well as during preadipocyte differentiation. In MEFs, PTIP deletion leads to over 10 fold decrease of *PPAR γ* expression and over 5 fold decrease of *PPAR γ* -stimulated *C/EBP α* expression. In preadipocytes, while PTIP is dispensable for the basal level expression of *PPAR γ* and *C/EBP α* before differentiation, it is essential for the robust induction of *PPAR γ* and *C/EBP α* but not *C/EBP β* during differentiation. Further, we show PTIP is required for the enrichment of methyltransferase MLL4, H3K4 trimethylation (H3K4me3) and RNA polymerase II (Pol II) on *PPAR γ* and *C/EBP α* promoters. Accordingly, PTIP-deficient MEFs and white and brown preadipocytes all show striking defects in adipogenesis. Finally, tissue-specific deletion of PTIP significantly reduces brown adipose tissue weight and leads to cold intolerance in mice.

Results

PTIP Is Required for *PPAR γ* Expression in MEFs

To identify PTIP-regulated genes, immortalized PTIP conditional knockout *PTIP^{flox/flox}* MEFs were infected with retroviruses expressing Cre recombinase. By microarray analysis using the whole mouse genome expression arrays, we found that deletion of PTIP by Cre led to over 2.5 fold decrease of expression of ~ 60 genes. Among these, *PPAR γ* was the most significant one (Supplemental Table S1). Northern blot and quantitative reverse-transcriptase PCR (qRT-PCR) confirmed that PTIP deletion led to over ten fold decrease of *PPAR γ* expression in

PTIP^{flx/flx} cells (Figure 1A-B). Isoform-specific qRT-PCR revealed that *PPAR* γ 1 was the only isoform expressed in MEFs where *PPAR* γ 2 expression was undetectable. Accordingly, we observed over ten fold decrease of *PPAR* γ 1 expression in PTIP-deficient MEFs (Figure 1B). Expression of ectopic PTIP could fully rescue *PPAR* γ level in PTIP-deficient cells, confirming that PTIP is required for *PPAR* γ expression in MEFs (Figure 1C).

To investigate how PTIP regulates *PPAR* γ expression in MEFs, we performed chromatin immunoprecipitation (ChIP) assays. Endogenous PTIP associated with *PPAR* γ 1 promoter with a signal peaked around the transcription start site. In contrast, no significant enrichment of PTIP was found on *PPAR* γ 2 proximal promoter in MEFs (Figure 1D). Representative subunits of PTIP-associated histone H3K4 methyltransferase complex, such as PA1, RbBP5 and the enzymatic subunit MLL4 (Cho et al., 2007), were also enriched on the *PPAR* γ 1 but not the *PPAR* γ 2 proximal promoter in MEFs (Figure 1D). H3K4me3, an epigenetic mark for active genes, was highly enriched on the *PPAR* γ 1 but not the *PPAR* γ 2 proximal promoter, consistent with the lack of *PPAR* γ 2 expression (Figure 1E). PTIP deletion not only reduced enrichment of PTIP, PA1, RbBP5 and MLL4 on the *PPAR* γ 1 promoter, but also decreased H3K4me3 signal and the recruitment of Pol II to the same promoter (Figure 1D-E). Interestingly, PTIP deletion also led to significant increase of histone H3K27 trimethylation (H3K27me3), an epigenetic mark for repressed genes, on *PPAR* γ 1 promoter (Figure 1F). These data indicate that PTIP and the associated HMT complex directly regulate *PPAR* γ 1 expression in MEFs, and suggest that PTIP regulates *PPAR* γ 1 expression by modulating the recruitment of histone H3K4 methyltransferase and Pol II to the target gene promoters.

PTIP Is Required for Adipogenesis in Primary MEFs and White and Brown Preadipocytes

Since PTIP was required for *PPAR* γ expression in MEFs, we tested whether PTIP was required for adipogenesis. Primary *PTIP*^{flx/flx} MEFs were infected with adenovirus expressing Cre (Ad-Cre) to acutely delete *PTIP* gene (Figure 2A). In adipogenesis assays, approximately 20–30% of primary MEFs infected with the control virus differentiated into adipocytes as determined by Oil Red O staining. Deletion of PTIP led to a severe adipogenesis defect and significantly reduced expression of adipogenesis markers including *PPAR* γ and *C/EBP* α (Figure 2B-C).

The white preadipocyte cell line 3T3-L1 has been extensively used to study adipogenesis (Farmer, 2006). Stable knockdown of PTIP expression in 3T3-L1 led to severe defects in adipogenesis and the associated expression of adipogenesis markers, indicating that PTIP was also important for differentiation of 3T3-L1 (Figure 2D-F).

Further, we investigated whether PTIP was required for differentiation of primary preadipocytes. Primary white and brown preadipocytes isolated from *PTIP*^{flx/flx} mice were infected with Ad-Cre (Figure 2G and 2J). PTIP deletion by Cre led to a nearly complete block of adipogenesis in both white (Figure 2H) and brown preadipocytes (Figure 2K). Consistent with the morphology, PTIP deletion blocked expression of markers for white (Figure 2I) and brown adipocytes (Figure 2L). These results are consistent with the requirement of PTIP for *PPAR* γ expression in MEFs, and indicate that PTIP is required for adipogenesis in primary MEFs and preadipocytes.

Both PTIP-Deficient and C/EBP α -Deficient MEFs Show Defects in PPAR γ -Stimulated Adipogenesis

Ectopic PPAR γ is sufficient to stimulate immortalized, non-adipogenic wild type MEFs to differentiate into adipocytes even in the absence of synthetic PPAR γ ligand (Ge et al., 2002; Rosen et al., 2002). To investigate whether the severe adipogenesis defects in PTIP-deficient

cells was solely due to the defective expression of endogenous PPAR γ , we tested whether ectopic PPAR γ could stimulate adipogenesis in PTIP-deficient MEFs.

The immortalized, non-adipogenic *PTIP*^{flox/flox} MEFs were sequentially infected with retroviruses expressing PPAR γ and Cre (Figure 3A-B). Adipogenesis was induced in the presence of synthetic PPAR γ ligand rosiglitazone (Rosi) or vehicle DMSO alone. Consistent with the previous report (Ge et al., 2002), ectopic PPAR γ induced about 60–70% of cells to undergo adipogenesis in the presence of DMSO. Rosi treatment further stimulated differentiation (Figure 3C left panels). Surprisingly, ectopic PPAR γ failed to induce adipogenesis in PTIP-deficient MEFs treated with DMSO. In the presence of Rosi, ectopic PPAR γ induced about 60–70% of PTIP-deficient MEFs to differentiate into immature adipocytes that mainly contained small lipid droplets (Figure 3C right panels). However, the expression of several adipocyte markers including *C/EBP α* was impaired (Figure 3D). Thus, PTIP-deficient MEFs show a severe defect in PPAR γ -stimulated adipogenesis in the absence of Rosi, and a partial defect in the presence of Rosi.

MEFs have the capacity to differentiate into a variety of cell types besides adipocytes, as exemplified by MyoD-stimulated myogenesis (Ge et al., 2002). We found that PTIP was dispensable for MyoD-stimulated myogenesis in MEFs (Figure S1). Thus, PTIP is selectively required for PPAR γ -stimulated adipogenesis, relative to MyoD-stimulated myogenesis, and indicate the differential involvement of PTIP in different fibroblast differentiation pathways.

C/EBP α is required for adipogenesis *in vivo* (Farmer, 2006). Ectopic PPAR γ can induce adipogenesis in *C/EBP α* ^{-/-} MEFs in the presence of synthetic PPAR γ ligand. However, *C/EBP α* ^{-/-} cells show relatively immature phenotype and consistently accumulate less lipid in smaller droplets than the *C/EBP α* ^{+/+} cells (Wu et al., 1999), suggesting that *C/EBP α* ^{-/-} cells have a partial defect in PPAR γ -stimulated adipogenesis in the presence of synthetic PPAR γ ligand. Expression of ectopic PPAR γ from MSCVpuro vector induces adipogenesis of MEFs even in the absence of synthetic PPAR γ ligand (Ge et al., 2002; Rosen et al., 2002). Using MSCVpuro to express PPAR γ in *C/EBP α* ^{+/-} and *C/EBP α* ^{-/-} MEFs, we found that *C/EBP α* ^{-/-} cells showed a severe defect in PPAR γ -stimulated adipogenesis in the absence of synthetic PPAR γ ligand Rosi, and a partial defect in the presence of Rosi. Such defects could be rescued by ectopic *C/EBP α* (Figure 3E-G). These results were remarkably similar to what we observed in PTIP-deficient MEFs, and suggest that the defective PPAR γ -stimulated adipogenesis in PTIP-deficient MEFs may be due to the defective *C/EBP α* expression.

PTIP Directly Regulates *C/EBP α* Expression in MEFs

Next we investigated how PTIP regulates *C/EBP α* gene expression. The expression of PPAR γ -regulated genes including *C/EBP α* during adipogenesis results from combined action of multiple adipogenic transcription factors including PPAR γ (Farmer, 2006). In addition, there exists a possibility that the defective *C/EBP α* expression in PTIP-deficient, immature adipocytes may be secondary to the adipogenesis defect. Therefore, it was unclear from Figure 3D whether the defective PPAR γ -stimulated adipogenesis in PTIP-deficient MEFs was directly due to the failure in *C/EBP α* expression. We have shown previously that in subconfluent, undifferentiated MEFs, ectopic PPAR γ activates expression of endogenous target genes including *C/EBP α* and *aP2* in a receptor- and ligand-dependent manner (Ge et al., 2008). Using this approach, we found that PTIP-deficient MEFs show a severe defect in PPAR γ - and ligand-stimulated expression of endogenous *C/EBP α* but not *aP2* (Figure 4A-B). By ChIP assay, we found that endogenous PTIP associated with the *C/EBP α* promoter with a signal peaked around the transcription start site (Figure 4C). H3K4me3 signal was highly enriched on the *C/EBP α* proximal promoter but was reduced upon PTIP deletion (Figure 4D). These results indicate that PTIP directly regulates *C/EBP α* expression in MEFs.

Rescue of the Adipogenesis Defect in *PTIP*^{-/-} MEFs Requires Co-expression of PPAR γ and C/EBP α

Ectopic C/EBP α is unable to stimulate adipogenesis in PPAR γ -deficient MEFs (Rosen et al., 2002). Consistent with the requirement of PTIP for *PPAR* γ expression in MEFs, we found that PTIP was required for C/EBP α -stimulated adipogenesis and the associated expression of adipogenesis markers including endogenous C/EBP α and *PPAR* γ in MEFs (Figure S2). Thus, PTIP-deficient MEFs showed significant defects in both PPAR γ - and C/EBP α -stimulated adipogenesis.

Next, we tested whether co-expression of ectopic PPAR γ and C/EBP α could rescue the adipogenesis defects in PTIP-deficient MEFs. For this purpose, *PTIP*^{-/-} cell lines were established by infecting immortalized *PTIP*^{flox/flox} MEFs with Ad-Cre to permanently delete *PTIP* gene. Cells were serially diluted and single colonies were isolated. After confirming complete deletion of *PTIP* gene, *PTIP*^{-/-} MEFs were infected with retroviruses expressing PPAR γ , followed by infection with retroviruses expressing C/EBP α or PTIP (Figure 5A). Ectopic PTIP could rescue the PPAR γ - and ligand-stimulated expression of endogenous C/EBP α in subconfluent, undifferentiated *PTIP*^{-/-} MEFs (Figure 5B). As expected, *PTIP*^{-/-} MEFs were refractory to PPAR γ -induced adipogenesis. However, such defects could be rescued by ectopic expression of either C/EBP α or PTIP (Figure 5C-D). These results indicate that rescue of the adipogenesis defect in *PTIP*^{-/-} MEFs requires co-expression of PPAR γ and C/EBP α , and suggests that the adipogenesis defect in PTIP-deficient cells is mainly due to the defective expression of both *PPAR* γ and C/EBP α .

PTIP Is Required for Induction of PPAR γ and C/EBP α during Adipogenesis

We next investigated how PTIP regulates *PPAR* γ and C/EBP α expression during adipogenesis. Interestingly, in contrast to the requirement of PTIP for *PPAR* γ expression in MEFs, PTIP deletion in undifferentiated preadipocytes had little effect on the basal level of *PPAR* γ , which was ~ 5.4 fold lower in undifferentiated preadipocytes than in MEFs (Figure 6A and data not shown). Expression of PPAR γ and C/EBP α is markedly induced during 3T3-L1 white preadipocyte differentiation (Morrison and Farmer, 1999) (Figure S3A). Similarly, during brown preadipocyte differentiation, expression of PPAR γ 1 and C/EBP α was markedly induced as early as day 1, well before the appearance of immature adipocytes on day 3, and peaked at day 4. PPAR γ 2 was induced relatively late (day 3) and remained a minor isoform throughout differentiation. PTIP deletion by Cre in brown preadipocytes almost completely prevented the induction of PPAR γ 1, PPAR γ 2 and C/EBP α , but had little effect on the induction of C/EBP β , which is another adipogenic transcription factor that works upstream of both PPAR γ and C/EBP α (Farmer, 2006) (Figure 6B-C).

We performed ChIP assays to investigate how PTIP regulates *PPAR* γ and C/EBP α genes during preadipocyte differentiation. In 3T3-L1 cells before differentiation (day -3), PTIP and H3K4me3 were enriched around the transcription start sites on the *PPAR* γ 1, *PPAR* γ 2 and C/EBP α proximal promoters. In differentiating 3T3-L1 cells (day 4) that expressed the peak levels of PPAR γ 1, PPAR γ 2 and C/EBP α , we observed increased enrichment of PTIP and H3K4me3 on the three promoters (Figure S3B-C). PTIP expression did not change significantly during preadipocyte differentiation (Figure S3A), suggesting that the increased binding of PTIP to the three promoters is likely due to gene-specific recruitment of PTIP. Similarly, in differentiating brown preadipocytes (day 4) that expressed the peak levels of PPAR γ 1, PPAR γ 2 and C/EBP α , we observed enrichment of PTIP, MLL4 and H3K4me3 around the transcription start sites on the three promoters. PTIP deletion not only reduced enrichment of PTIP and MLL4 on these promoters, but also decreased H3K4me3 signal and the recruitment of RNA Pol II to the same promoters (Figure 6D-G). Taken together, these results indicate that PTIP is required for the robust induction of *PPAR* γ and C/EBP α during preadipocyte differentiation, and suggest

the involvement of the PTIP-associated histone H3K4 methyltransferase in regulation of *PPAR γ* and *C/EBP α* expression during adipogenesis.

Deletion of PTIP in Brown Adipose Tissue (BAT) Reduces Tissue Weight and Leads to Cold Intolerance in Mice

To investigate PTIP function in adipocytes *in vivo*, adipose-specific PTIP knockout mice (KO) were generated by crossing PTIP conditional knockout mice (Patel et al., 2007) with aP2-Cre mice expressing Cre under the control of adipose-specific *aP2* promoter. The aP2-Cre mice used were from line 1, which expressed Cre predominantly in BAT (Abel et al., 2001). As PTIP is required for *PPAR γ* expression and *aP2* is a downstream target of *PPAR γ* , this approach is expected to delete PTIP after formation of fat depots, allowing largely normal adipogenesis in mice (He et al., 2003).

Although KO mice had similar body weight as the control mice, they displayed over 50% decrease of BAT weight (Figure 7A-B and Figure S4). Expression of markers common to WAT and BAT (*PPAR γ* , *C/EBP α* , *aP2*), BAT-selective genes (*PRDM16*, *cidea*, *eva1*, *ntrk3*) (Seale et al., 2007), brown fat thermogenic genes (*UCP1* and *PGC-1 α*) and mitochondrial components (*cox5b* and *cox8b*) also decreased significantly in the residual BAT of KO mice (Figure 7D). In contrast, no significant change of WAT weight was found in the KO mice, consistent with the ~ 9.3 fold higher level of Cre expression in BAT than in WAT of KO mice, and with the lack of *PTIP* gene deletion in the WAT of KO mice (Figure 7A-C and Figures S4 and S5). These results are consistent with previous reports that deletion of *PPAR γ* in BAT leads to marked decrease of tissue weight in mice (He et al., 2003).

The main function of BAT is to burn fat for heat generation (thermogenesis). The decrease of BAT size in KO mice did not lead to significant change of serum parameters such as blood glucose, free fatty acid, triglyceride and insulin levels, likely because BAT only accounts for a small percentage of the fat mass in mice (data not shown). However, when exposed to environmental cold, the KO mice were unable to maintain body temperatures and were cold intolerant (Figure 7E), consistent with previous report that ablation of BAT led to cold intolerance in mice (Lowell et al., 1993). In the residual BAT of KO mice, cold-induced expression of genes that are important for fatty acid catabolism, such as *CPT1a*, *LPL* and *MCD* (Yu et al., 2002), was impaired (Figure 7F), suggesting that the cold intolerance of the KO mice is due to, at least in part, the combined effects of the reduced BAT size and the impaired expression of cold-induced genes important for fatty acid catabolism.

Discussion

Elucidating the mechanisms that regulate *PPAR γ* and *C/EBP α* expression is crucial to understanding the regulation of adipogenesis. In this paper, we report the histone methylation regulator PTIP is required for *PPAR γ* and *C/EBP α* expression and adipogenesis. We show (i) that PTIP deletion leads to severe defects in *PPAR γ* expression and *PPAR γ* -stimulated *C/EBP α* expression in MEFs (Figures 1 and 4); (ii) that similar to *C/EBP α* -deficient MEFs, PTIP-deficient MEFs show significant defects in *PPAR γ* -stimulated adipogenesis (Figure 3); (iii) that rescue of the adipogenesis defect in *PTIP*^{-/-} MEFs requires co-expression of *PPAR γ* and *C/EBP α* (Figure 5); (iv) that PTIP is required for induction of *PPAR γ* and *C/EBP α* during preadipocyte differentiation. PTIP deletion impairs the enrichment of H3K4 methyltransferase MLL4, H3K4me3, and Pol II to the *PPAR γ* and *C/EBP α* promoters (Figures 6 and S3); (v) that PTIP is required for adipogenesis of primary MEFs and white and brown preadipocytes (Figure 2); and (vi) that deletion of PTIP in BAT significantly reduces tissue weight and leads to cold intolerance in mice (Figures 7, S4 and S5). Thus, by regulating *PPAR γ* and *C/EBP α* expression, PTIP plays a critical role in adipogenesis.

Regulation of *PPAR γ* Expression by PTIP

Using the mouse whole genome expression arrays, we find that PTIP deletion in MEFs leads to over 2.5 fold decrease of expression of ~ 60 genes, which account for less than 0.5% of total genes expressed in MEFs. Consistent with the specific association of PTIP with the MLL3- and MLL4-containing HMT complex, PTIP deletion has no effect on expression of *Hoxa7*, *Hoxa9*, *Foxc2* and *p27/Kip1*, which are known targets of the homologous MLL1- and MLL2-containing HMT complexes (Figure 1B) (Milne et al., 2005). PTIP is required for *PPAR γ 1* expression in MEFs where *PPAR γ 2* expression is undetectable. These results strongly suggest a gene-specific regulatory role of PTIP in MEFs. In undifferentiated preadipocytes, PTIP is dispensable for the basal level expression of *PPAR γ* and *C/EBP α* . However, PTIP is essential for the induction of *PPAR γ* and *C/EBP α* but not *C/EBP β* during preadipocyte differentiation (Figure 6), suggesting that PTIP plays a specific role in regulating *PPAR γ* and *C/EBP α* expression during adipogenesis.

Several transcription factors, such as SREBP1c/ADD1 and E2F family members, have been shown to regulate *PPAR γ 1* expression (Farmer, 2006). Multiple subunits of the PTIP-associated HMT complex are capable of interacting directly with sequence-specific transcription factors. For example, PTIP interacts with PAX2 (Patel et al., 2007). Nco6 interacts directly with multiple transcription factors and nuclear receptors including *PPAR γ* *in vitro* or in cells (Lee et al., 2006; Mahajan and Samuels, 2005). It is possible that PTIP and the associated HMT complex may be recruited to the *PPAR γ 1* promoter through their direct interactions with promoter-bound transcription factors.

C/EBP α directly binds to and activates *PPAR γ 2* promoter and is required for the induction of *PPAR γ 2* during adipogenesis (Farmer, 2006). *In silico* analysis predicts that PTIP may use its BRCT repeats to interact with phosphorylated *C/EBPs* (Miller, 2006). It remains to be determined whether phosphorylated *C/EBP α* and *C/EBP β* recruit PTIP and the associated HMT complex to the *PPAR γ 1* and *PPAR γ 2* promoters during adipogenesis.

Regulation of *C/EBP α* Expression by PTIP

Activation of *C/EBP α* expression by *PPAR γ* is considered a critical step in the transcriptional cascade that leads to adipogenesis (Farmer, 2006; Rosen and MacDougald, 2006). However, it is unclear how *PPAR γ* activates *C/EBP α* expression. It has been suggested that *PPAR γ* may directly activate *C/EBP α* gene expression (Hamm et al., 2001). The recent genome-wide analysis of *PPAR γ* -binding sites in 3T3-L1 cells has identified several putative *PPAR γ* target regions downstream of the *C/EBP α* gene but the functional *PPAR γ* response elements remain to be identified (Nielsen et al., 2008).

Although PTIP deletion in MEFs only leads to 1.9 fold decrease of basal level of *C/EBP α* expression, the *PPAR γ* - and ligand-stimulated 146 fold increase of *C/EBP α* expression is severely impaired (Figure 4A-B). Interestingly, the recruitment of PTIP to the *C/EBP α* promoter is independent of *PPAR γ* (Figure 4C), suggesting that PTIP may not act through *PPAR γ* to regulate *C/EBP α* expression. *PPAR γ* may work with another transcription factor that works upstream of *C/EBP α* and requires PTIP to activate *C/EBP α* expression.

The induction of *C/EBP α* and *PPAR γ 1* expression appears to be synchronized in the early phase of differentiation of both white and brown preadipocytes (Figure 6B and (Morrison and Farmer, 1999)). As PTIP directly regulates expression of both *PPAR γ 1* and *C/EBP α* , our data suggest a possibility that PTIP may work with yet-to-be-identified upstream transcription factor (s) to coordinate and synchronize the induction of *C/EBP α* and *PPAR γ 1* during preadipocyte differentiation. Future work will be needed to identify the upstream transcription factor(s) that may work with PTIP to regulate *PPAR γ 1* and *C/EBP α* expression.

Regulation of Adipogenesis by PTIP

Preadipocyte differentiation (adipogenesis) is under the control of cooperative actions between PPAR γ and C/EBP α , both of which are required for adipogenesis *in vivo*. Studies using preadipocyte differentiation in cell culture and gene knockout in mice have suggested a transcriptional cascade that controls adipogenesis. In the early phase of preadipocyte differentiation, adipogenic stimuli induce low level expression of PPAR γ and C/EBP α , which then stimulate each other's expression in a positive feed back loop. The resulting high levels of PPAR γ and C/EBP α activate adipocyte gene expression and lipid accumulation and ultimately lead to phenotypic conversion from fibroblasts to adipocytes (Rosen et al., 2002) (also see Figure 6H). PTIP is required for the induction of both PPAR γ and C/EBP α during preadipocyte differentiation (Figures 6). In addition, PTIP-deficient MEFs show over 5 fold decrease of PPAR γ -stimulated C/EBP α expression (Figure 4), which would impair the positive feedback loop between PPAR γ and C/EBP α during adipogenesis. Thus, PTIP appears to regulate multiple steps in the transcriptional cascade that controls adipogenesis. Consistently, PTIP is required for adipogenesis in all *in vitro* differentiation systems that we have tested, including adipogenesis of primary MEFs, PPAR γ - and C/EBP α -stimulated adipogenesis of immortalized MEFs, differentiation of 3T3-L1 preadipocytes as well as primary white and brown preadipocytes (Figures 2, 3, 5 and S2). Taken together, our data support a model that PTIP regulates adipogenesis through direct modulation of PPAR γ and C/EBP α expression (Figure 6H).

Several transcriptional coactivators, such as Nco6 and TRAP220/Med1, have been shown to be required for PPAR γ -stimulated adipogenesis in MEFs (Ge et al., 2002; Qi et al., 2003). Nco6 and PTIP are both subunits of the MLL3/MLL4 histone H3K4 methyltransferase complex. The adipogenesis defects seen in both PTIP- and Nco6-deficient MEFs suggest that the MLL3/MLL4 complex likely plays an important role in regulation of adipogenesis. TRAP220 is a subunit of the general transcription coactivator complex Mediator. TRAP220^{-/-} MEFs show a severe defect in PPAR γ -stimulated adipogenesis even in the presence of synthetic PPAR γ ligand. However, unlike PTIP, TRAP220 is dispensable for PPAR γ -stimulated C/EBP α expression in subconfluent MEFs (Ge et al., 2008), indicating that the adipogenesis defect in TRAP220-deficient MEFs likely involves a mechanism distinct from that in the PTIP-deficient cells.

Epigenetic Regulation of PPAR γ Expression and Adipogenesis

Considerable evidence suggests that epigenetic mechanisms including histone methylation are critical for lineage specification of pluripotent stem cells. Recent mappings of histone methylation in pluripotent and lineage-committed cells have revealed an unexpected high frequency of colocalization of the "activating" H3K4me3 and the "repressive" H3K27me3 on promoters of developmental regulators. These bivalent modifications were proposed to poise key developmental genes for lineage-specific activation or repression (Bernstein et al., 2006). Interestingly, H3K4me3 and H3K27me3 also colocalize on the PPAR γ 1 promoter in MEFs (Mikkelsen et al., 2007). We previously show that endogenous PTIP associates with both histone H3K4 methyltransferases MLL3 and MLL4 and histone H3K27 demethylase UTX (Cho et al., 2007; Hong et al., 2007). The PTIP-associated HMT complex directly regulates PPAR γ 1 gene in MEFs. Deletion of PTIP in MEFs leads to significant decrease of H3K4me3 and increase of H3K27me3 on PPAR γ 1 proximal promoter (Figure 1D-F). Since both methylation of H3K4 and demethylation of H3K27 presumably associate with gene activation, it will be particularly interesting to investigate the roles of MLL3, MLL4 and UTX, and the potential synergy between the associated H3K4 methyltransferase and H3K27 demethylase activities, in regulation of PPAR γ expression during adipogenesis.

Experimental Procedures

Plasmids and Antibodies

The following retroviral plasmids have been described: MSCVpuro-PPAR γ 2, MSCVhygro-PPAR γ 2, MSCVhygro-C/EBP α , MSCVpuro-MyoD (Ge et al., 2008; Ge et al., 2002). MSCVpuro-F-PTIP expresses FLAG-tagged PTIP (Cho et al., 2007). Cre cDNA was cloned into MSCVpuro and MSCVhygro (Clontech) to generate MSCVpuro-Cre and MSCVhygro-Cre. The PTIP knockdown construct targeting mouse PTIP sequence TGACAGAGATGACCTGAAG was constructed in retroviral vector pSUPER.retro.puro (OligoEngine). All plasmids were confirmed by DNA sequencing.

The following antibodies were generated by immunizing rabbits with His-tagged recombinant proteins and were affinity purified with antigens: anti-PTIP#3 was generated against 274–472aa of mouse PTIP; anti-PA1#2 was generated against full length human PA1; anti-MLL4#3 was generated against 3020–3192aa of human MLL4. Anti-MHC antibody MF20 was described (Ge et al., 2002). Mouse IgG (sc-2025), anti-C/EBP α (sc-61), anti-MyoD (sc-760), anti-myogenin (sc-52903), anti-PPAR γ (sc-7273), and anti- α -tubulin (sc-5286) were from Santa Cruz. Rabbit IgG (I-5006) was from Sigma. Anti-RbBP5 (A300–109A) was from Bethyl. Anti-H3K4me3 (ab8580) and anti-RNA Pol II (ab5408) were from Abcam. Anti-H3K27me3 (07–449) was from Millipore.

Isolation of Primary Cells, Cell Culture, and Virus Infection

3T3-L1 preadipocyte cell line was cultured in DMEM + 10% bovine serum (Invitrogen). Primary brown preadipocytes were cultured in DMEM containing 25 mM glucose, 20 mM HEPES (pH7.5), and 20% fetal bovine serum (FBS). All other cells were routinely cultured in DMEM plus 10% FBS (Hyclone). All chemicals were from Sigma unless indicated.

Primary MEFs isolated from E13.5 *PTIP*^{fl α /fl α} embryos (Patel et al., 2007) were immortalized by following the 3T3 protocol (Ge et al., 2002). To isolate primary white preadipocytes, inguinal WAT from 4–8 week *PTIP*^{fl α /fl α} mice was excised, minced, and digested with 1 mg/ml collagenase I in the isolation buffer (123 mM NaCl, 5 mM KCl, 1.3 mM CaCl₂, 5 mM glucose, 100 mM HEPES (pH7.5), 4% BSA and 100 units/ml penicillin and streptomycin) at 37°C for 45 min. After digestion, the slurry was passed through a 100 μ m mesh cell strainer (Falcon) and cells were collected by centrifugation. Primary brown preadipocytes were isolated from interscapular BAT of 1–2 days old *PTIP*^{fl α /fl α} pups following a published protocol (Klein et al., 1999).

Retrovirus infection of MEFs was done as described (Ge et al., 2002). Adenoviruses expressing Cre recombinase and GFP (Ad5CMV-Cre-GFP) or GFP alone (Ad5CMV-GFP) were obtained from Baylor Vector Development Laboratory. Adenovirus infection of primary MEFs and white and brown preadipocytes that had limited life span in culture were done at 100 moi as described (Ge et al., 1999).

Adipogenesis and Myogenesis Assays

PPAR γ -stimulated adipogenesis and MyoD-stimulated myogenesis in immortalized MEFs were performed as described (Ge et al., 2002). Adipogenesis was induced in the presence of 0.5 μ M rosiglitazone (Rosi) (Cayman Chemical) or vehicle DMSO alone for 8 days. After adipogenesis, cells were either stained for lipid droplets with Oil Red O or total RNA was extracted for gene expression analysis by Northern blot or qRT-PCR.

Adipogenesis of 3T3-L1 was induced as described (Nielsen et al., 2008). For adipogenesis of primary MEFs, two days after cells reached confluence, cells were treated with medium

supplemented with 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX), 1 μ M dexamethasone (DEX), 10 μ g/ml insulin and 0.5 μ M Rosi. Two days later, cells were changed to medium containing insulin and Rosi. The medium was replenished at 2-day intervals for 12 days.

For adipogenesis of primary white preadipocytes, cells were plated at a density of 1×10^6 per 10cm dish four days before induction of adipogenesis. At day 0, cells were treated with medium supplemented with 0.5 mM IBMX, 1 μ M DEX, 5 μ g/ml insulin, 0.2 nM T3 and 0.5 μ M Rosi. Two days later, cells were changed to medium containing insulin, T3 and Rosi. The medium was replenished at 2-day intervals. At day 4, small lipid droplets appeared in differentiating cells. Adipogenesis of primary brown preadipocytes was carried out as described (Klein et al., 1999). At day 6 – 8 post induction, cells were either stained with Oil Red O or subjected to gene expression analysis by qRT-PCR.

Western Blot, Northern Blot, Microarray and qRT-PCR

Western blot and Northern blot were done as described (Ge et al., 2008). For microarray analysis, total RNA was sequentially purified with Trizol (Invitrogen) and RNeasy kit (Qiagen) and analyzed on Mouse Genome 430 2.0 Array (Affymetrix) at local microarray facility following standard protocols. Microarray data have been deposited in NCBI GEO data base (accession number GSE16263). For qRT-PCR, purified total RNA was reverse transcribed using random hexamers and SuperScript First-Strand Synthesis System (Invitrogen). The resulting first-strand cDNAs were quantified with Taqman or Sybr-Green assays on PRISM 7900HT System using standard curve and relative quantitation method with 18S rRNA as control (Applied Biosystems). The sequences of Sybr-Green primers are available upon request. *PPAR γ 1*- and *PPAR γ 2*-specific Taqman probes and primers were custom-synthesized: *PPAR γ 1* forward primer 5'-AAAGAAGCGGTGAACCACTGATA-3', Taqman probe 5'-FAM-ACCCTTTACTGAAATTACC-3', and reverse primer 5'-AATGGCATCTCTGTGTCAACCA-3'; *PPAR γ 2* forward primer 5'-CGCTGATGCACTGCCTATGA-3', Taqman probe 5'-FAM-CACTTCACAAGAAATTAC-3'; *PPAR γ 2* reverse primer is the same as that of *PPAR γ 1*. Data are presented as means \pm SD.

Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described (Nelson et al., 2006) with minor modifications. The cross-linked chromatin was sheared to \sim 400 bp fragments with a Bioruptor UCD-200TM (Diagenode). Each immunoprecipitation was performed with 1–2 μ g antibody in the presence of secondary antibody conjugated to Dynabeads (Invitrogen). PCR quantitation of precipitated genomic DNA relative to inputs was performed in triplicate using Taqman probes. The sequences of Taqman probes and primers are available upon request.

Mouse Breeding, Genotyping and Cold Tolerance Test

Mice carrying two floxed *PTIP* alleles (*PTIP*^{fllox/fllox}) or one floxed and one null alleles (*PTIP*^{fllox/-}) (Patel et al., 2007) were crossed with *aP2-Cre* transgenic mice line 1 (Abel et al., 2001). The progeny were intercrossed to generate *PTIP*^{fllox/-} mice carrying *aP2-Cre* transgene (*PTIP*^{fllox/-}; *Cre*), which were used as adipose-specific *PTIP* knockout mice (KO). Other littermates, such as wild type (*PTIP*^{fllox/fllox} and *PTIP*^{fllox/+}), heterozygous (*PTIP*^{fllox/-} and *PTIP*^{+/-}), and *PTIP*^{fllox/+}; *Cre* mice, were used as control (Con). No statistically significant differences in the body and tissue weights were found among the 3 groups of control mice (Figure S4). Genotyping of *PTIP* conditional knockout mice was done as described (Patel et al., 2007). Mice carrying *aP2-Cre* transgene were detected by PCR using primers 5'-CCTGTTTTGCACGTTCCACCG-3' and 5'-ATGCTTCTGTCCGTTTGCCG-3' and the PCR product was 250 bp. The efficiency of *aP2-Cre*-mediated deletion of *PTIP* gene in adipose tissues of KO mice was measured by quantitative PCR of genomic DNA using a Taqman probe

specific for the floxed exon I of *PTIP* gene: 5'-FAM-CTGTCAGGTGGCCGCACG-3', and a Taqman probe specific for *GAPDH* gene as internal control. After normalizing with *GAPDH* allele, the residual *PTIP* allele in KO mice (*PTIP*^{lox/-}; *Cre*) was compared with that in the *PTIP*^{lox/-} mice.

Mice were fed with standard rodent chow ad libitum. Twelve to sixteen week-old, age- and gender-matched mice with a mixed 129Sv and C57BL/6J background were used for this study. For cold tolerance test, mice that were housed singly and unrestrained and had free access to food and water were moved to the cold room with an ambient temperature of 4°C. Body temperature were measured every hour using mouse rectal probe (Thermalert TH-5). When the body temperature drops below 24°C, mice were moved out of the cold room. At the end of experiments, mice were euthanized and tissues were collected. Data are presented as means ± SEM. Differences were analyzed with Student's *t* test. Similar results were obtained from male and female mice but only male mice data are shown. All mouse work is approved by the Animal Care and Use Committee of NIDDK, NIH.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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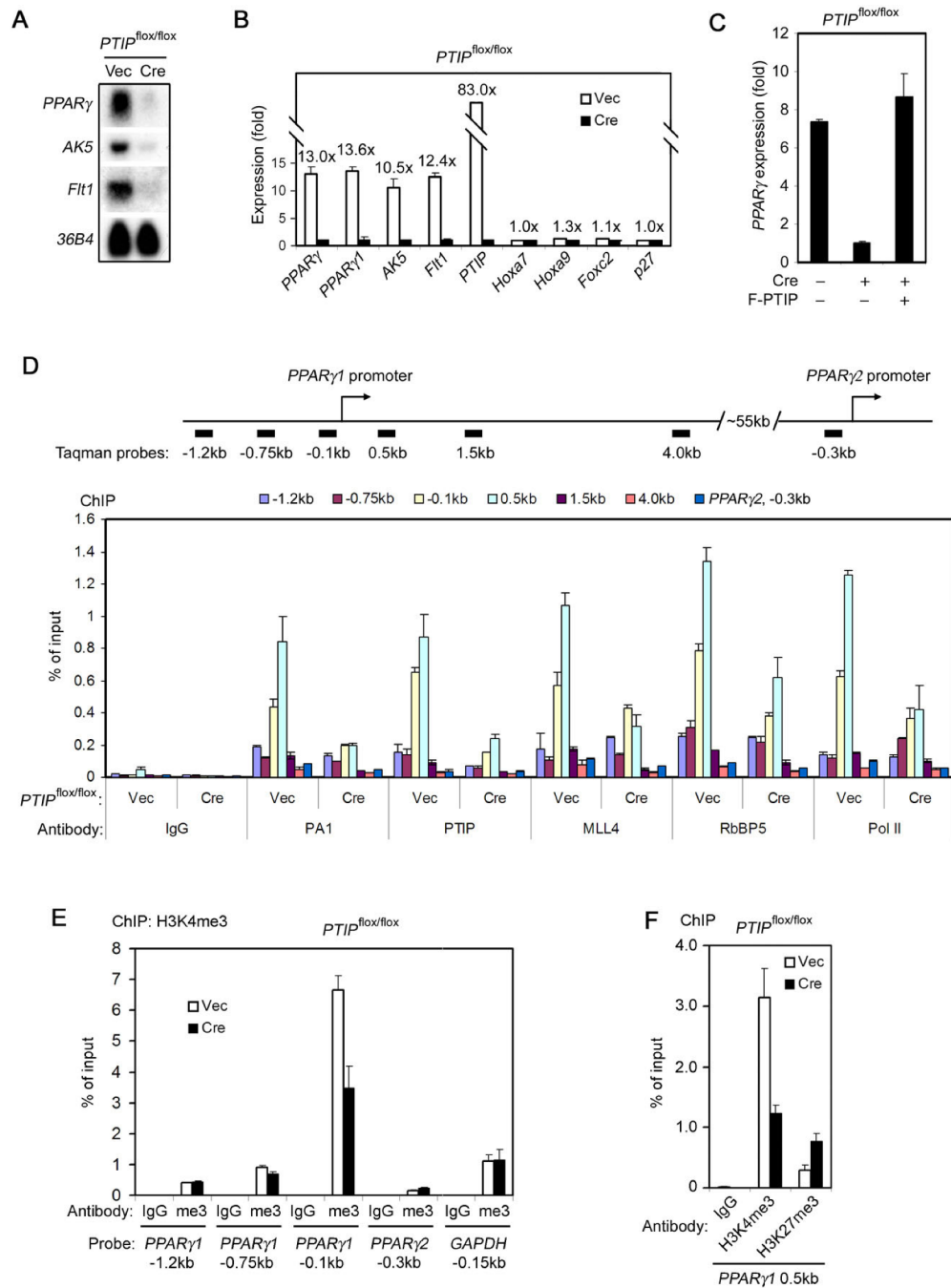


Figure 1. PTIP Is Required for PPAR γ Expression in MEFs

(A) – (B) Immortalized *PTIP^{flox/flox}* MEFs were infected with retrovirus MSCVpuro expressing Cre or vector (Vec) alone. Total RNA was extracted for confirmation of putative PTIP-regulated genes identified in microarray analysis (Supplemental Table S1). (A) Northern blot; (B) qRT-PCR. *PPAR γ 2* mRNA level is below detection limit in qRT-PCR. *AK5* and *Flt1* stand for *Adenylate Kinase 5* and *FMS-like tyrosine kinase 1*, respectively. *36B4* is the loading control.

(C) Ectopic PTIP can rescue *PPAR γ* expression in PTIP-deficient cells. *PTIP^{flox/flox}* MEFs were sequentially infected with retroviruses MSCVhygro expressing Cre and MSCVpuro expressing FLAG-tagged PTIP (F-PTIP). *PPAR γ* expression was analyzed by qRT-PCR.

(D) ChIP assay of binding of the PTIP-associated HMT complex subunits to *PPAR γ 1* and *PPAR γ 2* promoters in *PTIP^{fllox/fllox}* MEFs. Top panel, schematic of mouse *PPAR γ 1* and *PPAR γ 2* promoters. The black bars indicate locations of Taqman probes used for PCR quantitation of ChIP assays. Pol II, RNA polymerase II.

(E) ChIP assay of H3K4me3 on *PPAR γ 1* and *PPAR γ 2* promoters in *PTIP^{fllox/fllox}* MEFs. me3, H3K4me3.

(F) ChIP assay of H3K4me3 and H3K27me3 on *PPAR γ 1* promoter in *PTIP^{fllox/fllox}* MEFs.

All results are representative of two – four independent experiments.

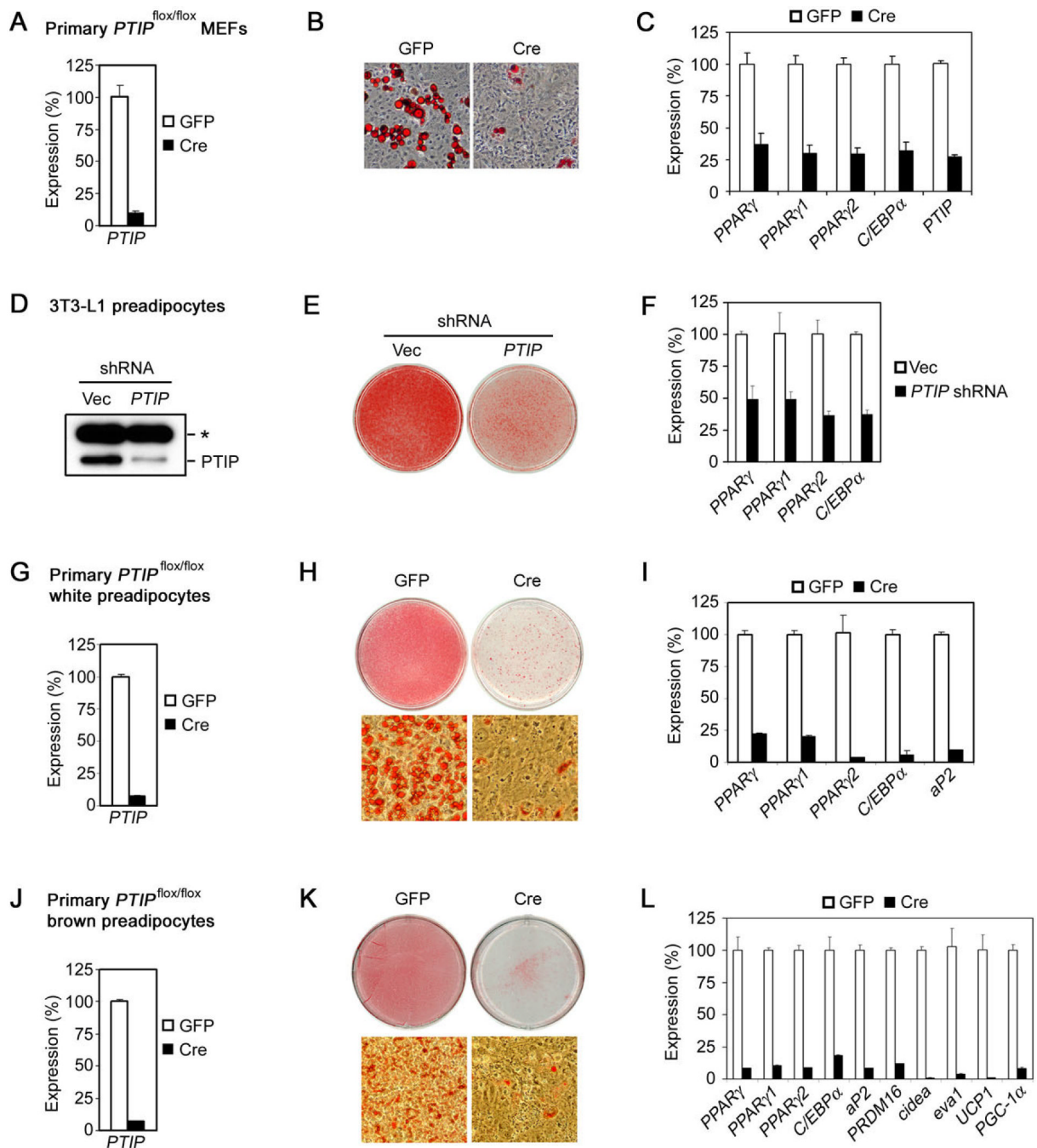


Figure 2. PTIP Is Required for Adipogenesis of Primary MEFs and Preadipocytes

(A) – (C) PTIP is required for adipogenesis of primary MEFs. Primary *PTIP*^{flx/flx} MEFs were infected with adenovirus expressing Cre (Ad-Cre) or control virus expressing GFP alone, followed by adipogenesis assay.

(A) To determine Ad-Cre-mediated gene deletion efficiency, *PTIP* expression was analyzed by qRT-PCR before induction of adipogenesis.

(B) Morphological differentiation under the phase-contrast microscope (Oil Red O staining).

(C) qRT-PCR analysis of gene expression after adipogenesis.

(D) – (F) PTIP is required for differentiation of 3T3-L1 white preadipocyte cell line. 3T3-L1 cells were infected with pSUPER.retro-based retroviral shRNA construct targeting *PTIP* or vector alone. After puromycin selection, cells were induced to undergo adipogenesis for 8 days. (D) Western blot of PTIP expression before induction of differentiation. The asterisk indicates a non-specific band.

(E) Morphological differentiation.

(F) qRT-PCR analysis of gene expression 8 days after induction of differentiation.

(G) – (L) PTIP is required for differentiation of primary white (G – I) and brown (J – L) preadipocytes. Primary white and brown preadipocytes isolated from *PTIP*^{fl^{ox}/fl^{ox}} mice were infected with Ad-Cre or control virus expressing GFP, followed by adipogenesis assay.

(G) and (J) qRT-PCR analysis of Cre-mediated *PTIP* gene deletion before differentiation.

(H) and (K) Morphological differentiation. Top panels, stained dishes. Lower panels, representative fields under the microscope.

(I) and (L) After differentiation, total RNA was isolated and subjected to qRT-PCR analysis of expression of genes common to white and brown adipocytes (*PPAR* γ , *PPAR* γ 1, *PPAR* γ 2, *C/EBP* α , *aP2*) and genes specific for brown adipocytes (*PRDM16*, *cidea*, *eva1*, *UCP1*, *PGC-1* α) (Seale et al., 2007). All results are representative of two – four independent experiments.

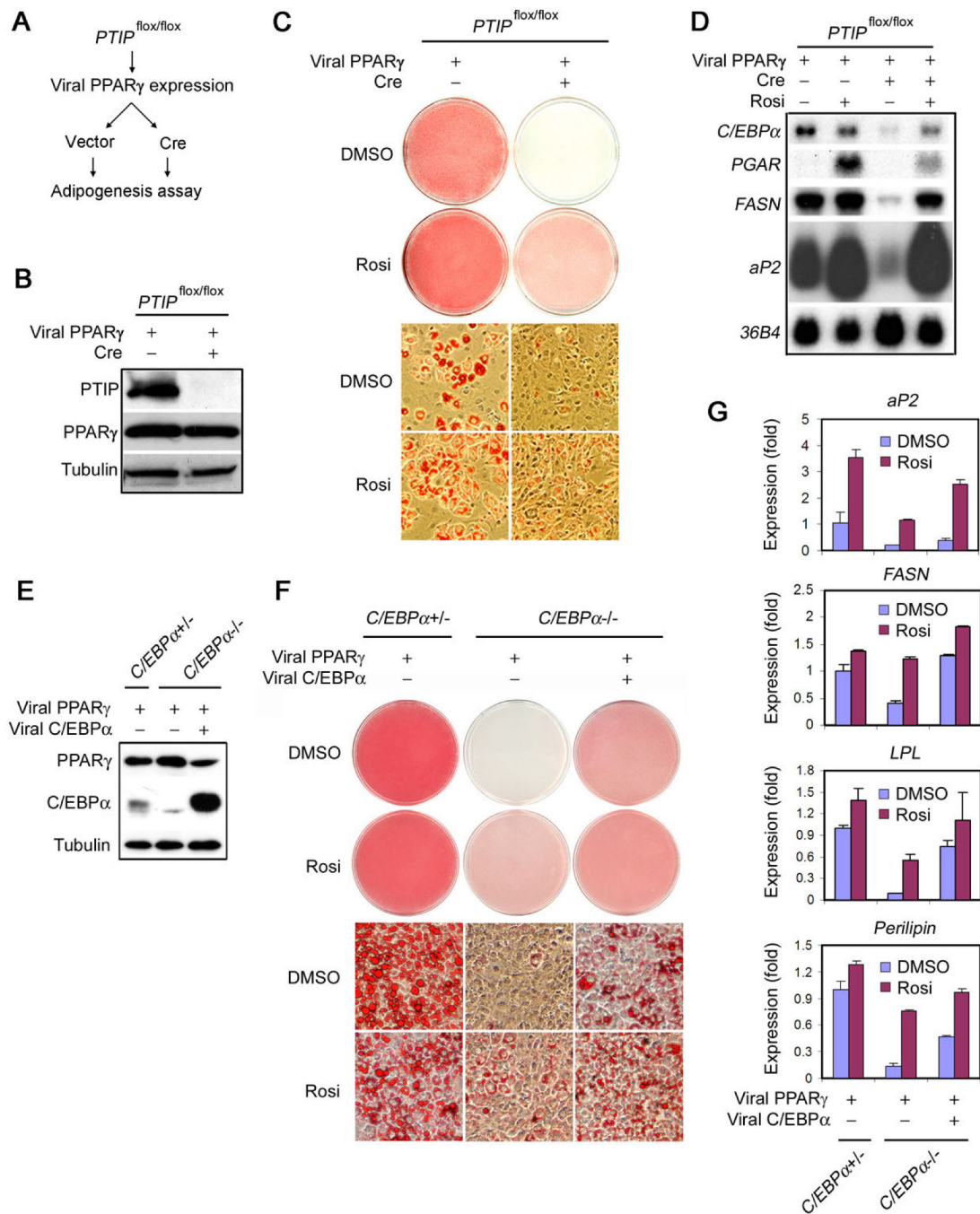


Figure 3. Both PTIP-Deficient and C/EBP α -Deficient MEFs Show Defects in PPAR γ -Stimulated Adipogenesis

(A) – (D) PTIP-deficient MEFs show significant defects in PPAR γ -stimulated adipogenesis. Immortalized *PTIP*^{flox/flox} MEFs expressing ectopic PPAR γ were infected with MSCVpuro expressing Cre or vector alone, followed by adipogenesis assay in the presence or absence of synthetic PPAR γ ligand Rosi.

(A) Schematic of the PPAR γ -stimulated adipogenesis assay.

(B) Western blot of endogenous PTIP and ectopic PPAR γ expression before adipogenesis.

(C) Morphological differentiation (Oil Red O staining). Top panels, stained dishes. Lower panels, representative fields under the microscope.

- (D) Northern blot analysis of gene expression after adipogenesis.
- (E) – (G) *C/EBPα*-deficient MEFs show significant defects in $\text{PPAR}\gamma$ -stimulated adipogenesis. Immortalized *C/EBPα*^{+/-} and *C/EBPα*^{-/-} MEFs infected with MSCVpuro- $\text{PPAR}\gamma$ 2 were then infected with MSCVhygro expressing *C/EBPα*, followed by adipogenesis assay for 8 days in the presence or absence of Rosi.
- (E) Western blot of $\text{PPAR}\gamma$ and *C/EBPα* expression before adipogenesis.
- (F) Morphological differentiation.
- (G) qRT-PCR analysis of gene expression after adipogenesis. *PGAR* is also known as *Angiopoietin-like 4 (Angptl4)*. *FASN* stands for *Fatty acid synthase*. *LPL* stands for *Lipoprotein lipase*. All results are representative of two – four independent experiments.

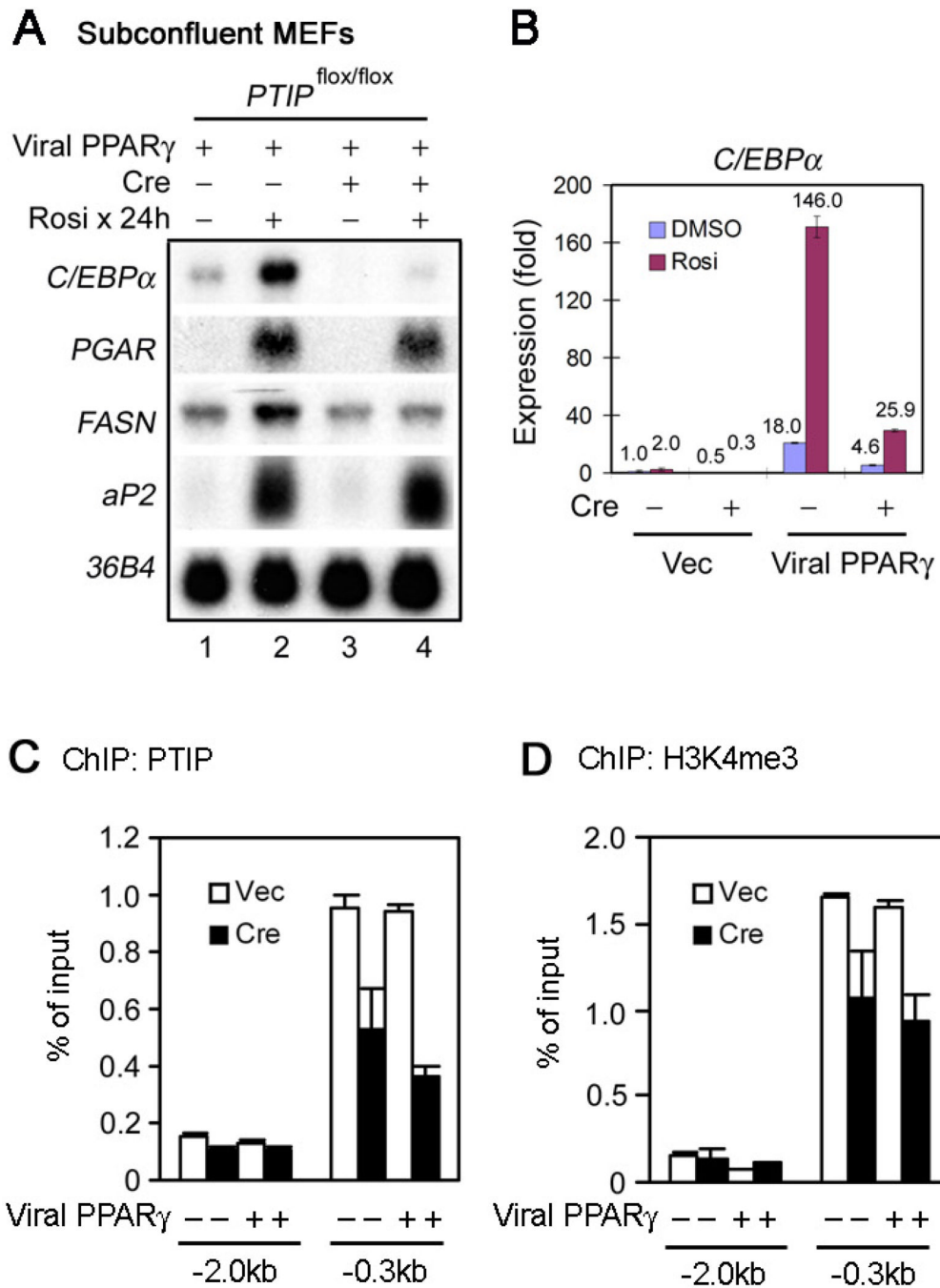


Figure 4. PTIP Directly Regulates *C/EBP α* Expression in MEFs
 (A) – (B) PTIP-deficient MEFs show over 5 fold decrease of PPAR γ - and ligand-stimulated expression of endogenous *C/EBP α* . MEFs described in Figure 3A-D were treated with 0.5 μ M Rosi or DMSO for 24 h, followed by Northern blot (A) and qRT-PCR analysis (B). When RNA samples were collected, cell density was less than 80–90%.
 (C) ChIP assay of PTIP recruitment to the *C/EBP α* promoter in *PTIP*^{flx/flx} MEFs.
 (D) ChIP assay of H3K4me3 enrichment on the *C/EBP α* promoter in *PTIP*^{flx/flx} MEFs. All results are representative of three independent experiments.

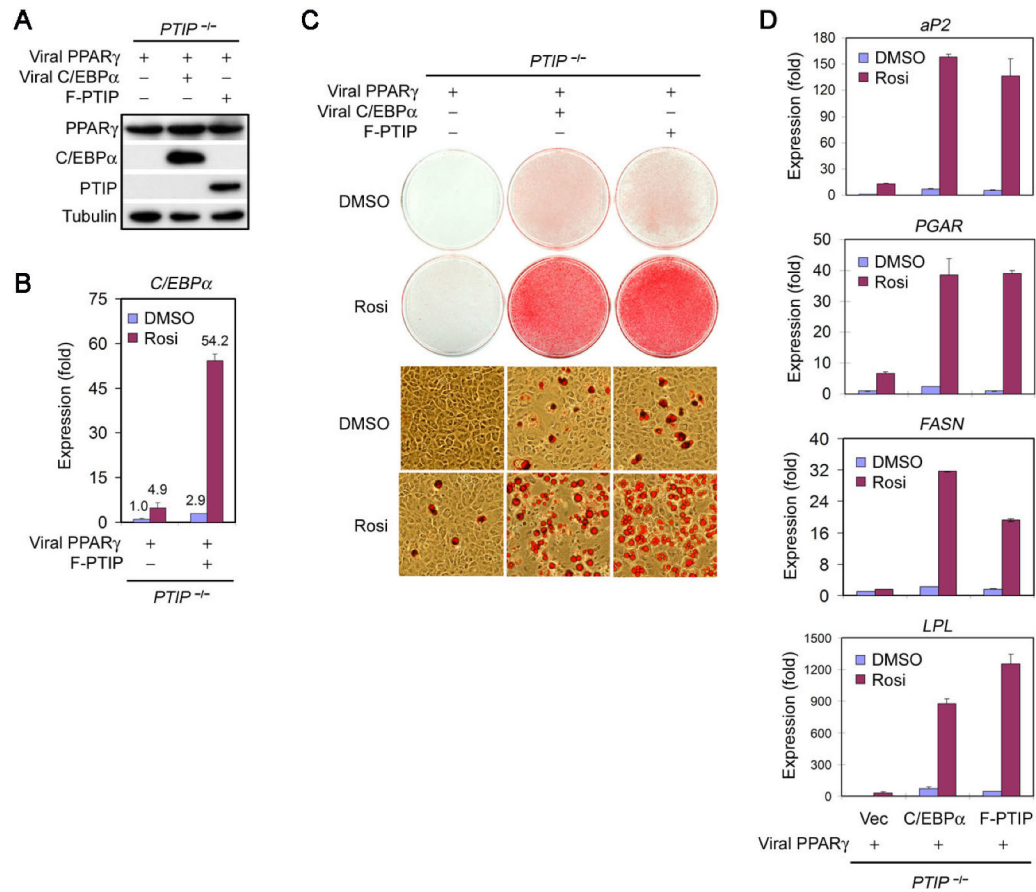


Figure 5. Rescue of the Adipogenesis Defect in *PTIP*^{-/-} MEFs Requires Co-expression of PPAR γ and C/EBP α

PTIP^{-/-} MEFs were established by infecting immortalized *PTIP*^{fllox/fllox} MEFs with Ad-Cre, followed by isolation of single colonies. *PTIP*^{-/-} MEFs were infected with MSCVpuro expressing PPAR γ , followed by infection with MSCVhygro expressing C/EBP α or F-PTIP. (A) Western blot analysis of ectopic PPAR γ , C/EBP α and PTIP expression before induction of adipogenesis. Cells were cultured in the absence of Rosi. (B) Ectopic PTIP rescues PPAR γ - and ligand-stimulated expression of endogenous *C/EBP α* in subconfluent, undifferentiated *PTIP*^{-/-} MEFs. Cells were treated with 0.5 μ M Rosi or DMSO for 24 h. *C/EBP α* expression was analyzed by qRT-PCR. (C) Cells were induced to undergo adipogenesis for 8 days and were stained with Oil Red O. (D) qRT-PCR analysis of gene expression after adipogenesis. All results are representative of two – four independent experiments.

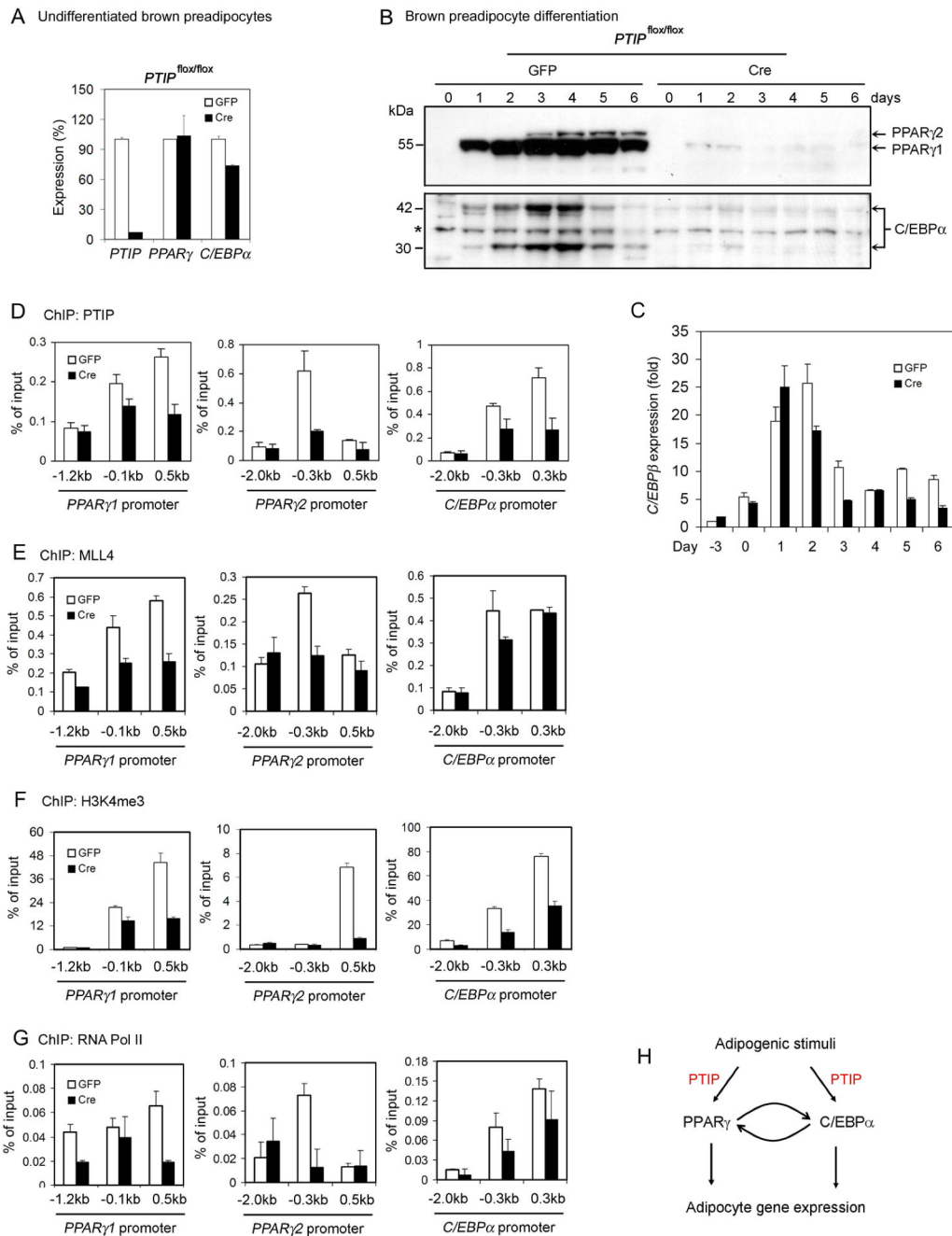


Figure 6. PTIP Is Required for Induction of *PPARγ* and *C/EBPα* during Adipogenesis
 (A)–(B) PTIP is essential for induction of *PPARγ* and *C/EBPα* expression during preadipocyte differentiation. Adipogenesis of primary *PTIP*^{fllox/fllox} brown preadipocytes was performed as in Figure 2J-L. The basal level of *PPARγ* and *C/EBPα* expression in undifferentiated preadipocytes was determined by qRT-PCR (A). *PPARγ* and *C/EBPα* protein expression during preadipocyte differentiation was analyzed by Western blot (B). Whole cell extracts were collected every 24 h following induction of differentiation with IBMX, DEX and indomethacin at day 0. The 54.5 kDa *PPARγ*1 and 57.5 kDa *PPARγ*2 bands, as well as the 30 kDa and 42 kDa *C/EBPα* isoforms are indicated by arrows. The asterisk indicates a non-specific band.

(C) qRT-PCR analysis of *C/EBPβ* expression during differentiation of *PTIP^{flox/flox}* brown preadipocytes. RNA samples were collected every 24 h starting from three days before induction of adipogenesis (d-3).

(D) – (G) ChIP assays of the recruitment of PTIP, MLL4 and Pol II, and H3K4me3 signal on *PPARγ1*, *PPARγ2* and *C/EBPα* promoters in differentiating (day 4) *PTIP^{flox/flox}* brown preadipocytes.

(H) Model depicting the role of PTIP in transcriptional regulation of preadipocyte differentiation (adipogenesis). PTIP regulates adipogenesis through direct modulation of *PPARγ* and *C/EBPα* expression. All results are representative of two – four independent experiments.

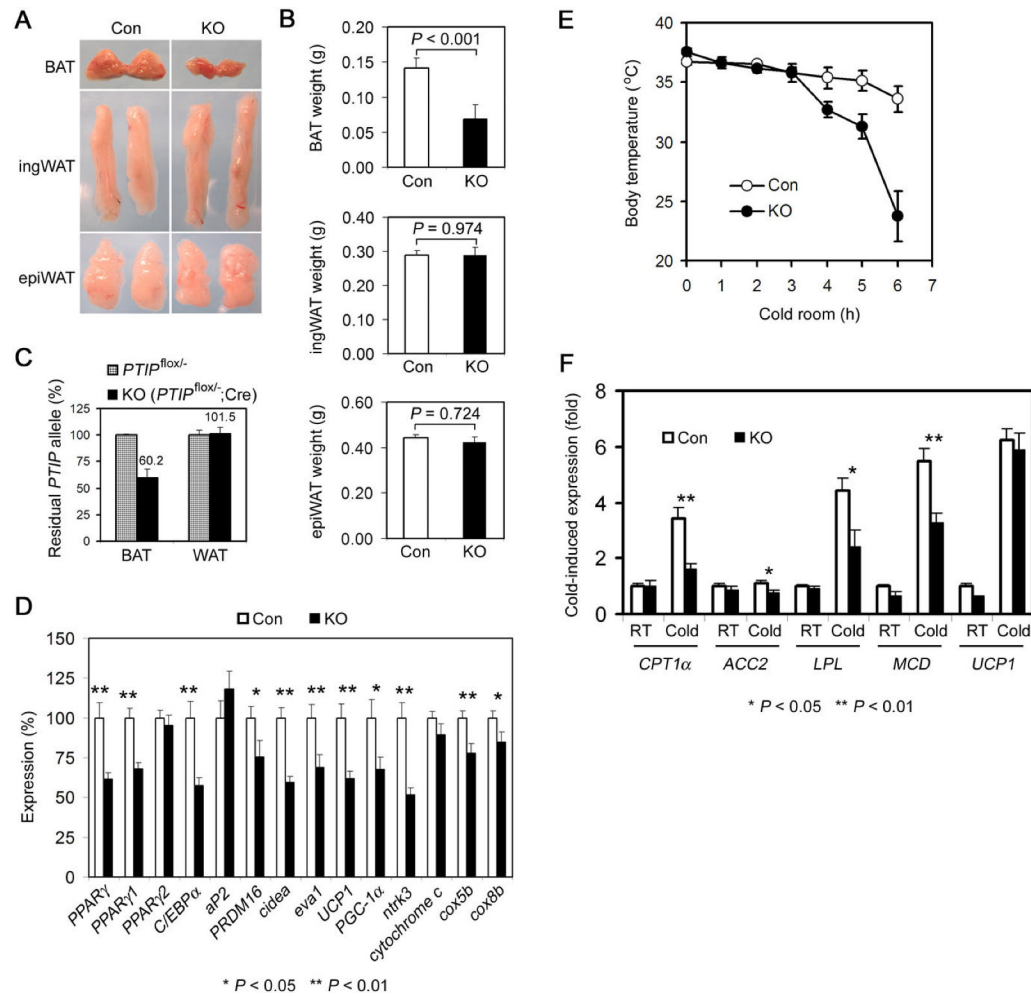


Figure 7. Deletion of PTIP in Brown Adipose Tissue Reduces Tissue Weight and Leads to Cold Intolerance in Mice

(A) – (D) PTIP deletion in brown adipose tissue (BAT) decreases both tissue weight and expression of brown adipocyte-enriched genes. Adipose-specific PTIP knockout mice (KO) carried the genotype of *PTIP*^{fllox/-}; Cre. Other littermates were used as control (Con) (see Experimental Procedures). All data were from 12–16 week-old mice.

(A) Representative pictures of interscapular BAT, inguinal WAT (ingWAT) and epididymal WAT (epiWAT) isolated from Con and KO mice.

(B) Quantitation of BAT and WAT weights in Con ($n = 22$) and KO ($n = 8$) mice. No significant differences in body weight, BMI and adipose tissue weights were found among groups of control mice (Figure S4).

(C) Lack of aP2-Cre-mediated deletion of *PTIP* gene in WAT of KO mice as determined by quantitative PCR ($n = 8$).

(D) qRT-PCR analysis of gene expression in BAT of Con ($n = 12$) and KO ($n = 8$) mice.

(E) – (F) PTIP deletion in BAT leads to cold intolerance in mice.

(E) The body temperatures of mice housed in 4 $^{\circ}$ C were measured every hour for 6h ($n = 7-9$).

(F) qRT-PCR analysis of cold-induced gene expression in BAT of mice after cold treatment for 6h ($n = 6$).

All values are the mean \pm SEM. * $P < 0.05$; ** $P < 0.01$. Similar results were obtained from male and female mice but only the male mice data are shown here.