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# Epigenetic Regulation of Adipogenesis by Histone Methylation

**Kai Ge***
Adipocyte Biology and Gene Regulation Section, Laboratory of Endocrinology and Receptor Biology, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA

## Abstract

Histone methylation is implicated in both gene activation and repression, depending on the specific lysine residue that gets methylated. Recent years have witnessed an explosive expansion of the list of remarkably site-specific histone methyltransferases and demethylases, which greatly facilitates the study on the biological functions of histone methylation in gene expression and cell differentiation in mammalian cells. Adipogenesis represents an excellent model system to understand transcriptional and epigenetic regulation of gene expression and cell differentiation. While transcriptional regulation of adipogenesis has been extensively studied, the roles of epigenetic mechanisms in particular histone methylation in regulation of adipogenesis have just begun to be understood. This review will summarize the recent progress on epigenetic regulation of adipogenesis by histone methylation, with a focus on histone H3K4 and H3K27. The available evidence suggests that site-specific histone methylations play critical roles in adipogenesis and control the expression of both positive and negative master regulators of adipogenesis.

## Introduction

Type 2 diabetes, which accounts for 90–95% of all diabetes, is one of the leading causes of morbidity and mortality worldwide. Obesity is the single most important risk factor for type 2 diabetes. Understanding the molecular mechanisms underlying adipogenesis (generation of fat tissue) may lead to novel approaches to the treatment of obesity and lipodystrophy, the two diseases that are tightly associated with type 2 diabetes. Transcriptional regulation of adipogenesis has been extensively reviewed [1, 2]. This review will focus on the role of histone lysine methylation in regulation of adipogenesis. I start with an introduction on the dynamic regulation of histone methylations by site-specific histone methyltransferases and demethylases. After a brief overview of the major positive and negative regulators of adipogenesis, I discuss the roles of histone methylations in particular histone H3K4 and H3K27 methylations, and the associated histone methyltransferases and demethylases, in controlling the expression of the master positive and negative regulators of adipogenesis.

## 1. Dynamic regulation of histone methylation by site-specific methyltransferases and demethylases

Epigenetic mechanisms, including histone modifications (such as acetylation, methylation and phosphorylation) (Figure 1), chromatin remodeling, histone variant incorporation, non-coding RNAs and DNA methylation, play critical roles in regulating both global and tissue-

*Corresponding author: Tel.: +1 301 451 1998; Fax: +1 301 480 1021. kaig@niddk.nih.gov (K. Ge).

and developmental stage-specific gene expression [3]. Histone acetylation occurs on lysine (K) residues and is dynamically regulated by histone acetyltransferases (HATs) and deacetylases [4]. Recent evidences suggest that although HATs are often capable of acetylating multiple K residues *in vitro*, they possess remarkable site-specificities in cells. For example, the paralogous HATs CBP and p300 are redundant and are specifically required for H3K18ac and H3K27ac in cells while another pair of paralogous HATs GCN5 and PCAF are also redundant but are specifically required for H3K9ac in cells [5]. Histone acetylation generally correlates with gene activation, with some histone acetylations, such as H3K18ac and H3K27ac, are likely the cause of gene activation, while other histone acetylations, such as H3K9ac, are likely the consequence of gene activation [5].

Unlike histone acetylations, histone lysine (K) methylations can be correlated with either gene activation or gene repression, depending on the specific K residue that becomes methylated [3, 6]. Methylations on histone H3K4, H3K36 and H3K79 are generally associated with gene activation, whereas methylations on histone H3K9 and H3K27 are generally associated with gene repression (Figure 1).

Histone lysine methylation is dynamically regulated by site-specific methyltransferases and demethylases [7, 8]. In mammals, the Drosophila Trithorax-related Set1-like histone methyltransferase (HMT) complexes specifically methylate H3K4 [9, 10]. The Polycomb repressive complex 2 (PRC2) is the predominant H3K27 methyltransferase in mammalian cells [11]. Multiple site-specific histone demethylases have also been identified. These enzymes are capable of removing methylations on H3K4, H3K9, H3K27 and H3K36 in a site-specific manner. Figure 2 lists the HMTs and histone demethylases that have been identified so far [7]. Almost all of these enzymes were identified within the last decade. While their biochemical properties have been extensively studied *in vitro*, their substrate- and site-specificities in cells are incompletely understood. More importantly, the biological functions of these histone modifying enzymes in regulating gene expression, cell differentiation and animal development have remained largely unexplored. Adipogenesis provides an excellent model system to study the biological functions of these enzymes.

## 2. Positive and negative regulators of adipogenesis

Adipocytes are believed to derive from multipotent mesenchymal stem cells (MSCs). Differentiation of MSCs to adipocytes involves two stages: determination and terminal differentiation. Determination refers to the commitment of MSCs to the adipocyte lineage, which results in the conversion of MSCs into preadipocytes. In the terminal differentiation stage, the fibroblast-like preadipocytes differentiate and convert into fat-laden adipocytes [2].

Much of our knowledge on adipogenesis comes from studies on differentiation of preadipocytes or mouse embryonic fibroblasts (MEFs) in cell culture. The immortalized white preadipocyte cell line 3T3-L1 is widely used. Primary white and brown preadipocytes can also be easily isolated from the stromal vascular fractions of the inguinal white adipose tissue (WAT) of young adult mice and the interscapular brown adipose tissue (BAT) of new born mouse pups, respectively. The primary brown preadipocytes can further be immortalized using SV40T antigen [12]. Differentiation of these primary and immortalized preadipocytes towards mature adipocytes is efficient in cell culture and appears to faithfully recapitulate adipogenesis in mice [2].

In a standard adipogenesis assay, differentiation is induced by treating confluent preadipocytes with an adipogenic cocktail of isobutylmethylxanthine (IBMX), dexamethasone (DEX) and insulin [1]. IBMX increases intracellular cAMP level to activate protein kinase A, which phosphorylates and activates cAMP response element-binding

protein (CREB). DEX binds and activates glucocorticoid receptor (GR). Phosphorylated CREB (pCREB, the active form) and DEX-bound GR serve as initiating adipogenic transcription factors and induce the expression of early adipogenic transcription factors C/EBPβ, KLF4, Krox20 and C/EBPδ within hours of initiation of adipogenesis [1, 13]. The elevated levels of these early adipogenic transcription factors induce expression of two principal adipogenic transcription factors, PPARγ (Peroxisome Proliferator-Activated Receptor-γ) and C/EBPα. PPARγ belongs to the nuclear receptor super family of ligand-activated transcription factors. It is considered the master regulator of adipogenesis and is both necessary and sufficient for adipogenesis [1, 14]. PPARγ cooperates with another principal adipogenic transcription factor C/EBPα to directly and synergistically activate expression of hundreds of adipocyte genes responsible for establishing the mature adipocyte phenotype [15, 16]. Thus, adipogenesis is positively regulated by a cascade of sequentially expressed adipogenic transcription factors (Figure 3).

Multiple negative regulators of adipogenesis have also been identified [2]. The Wnt/β-catenin signaling is one of the best studied and appears to play a major role in negative regulation of adipogenesis. The Wnt family of secreted proteins regulates cell proliferation, differentiation, and fate determination during embryonic development and adult tissue homeostasis [17]. The Wnt family has nineteen members in humans and mice. Among them, Wnt1, Wnt6, Wnt10a and Wnt10b have been shown to inhibit adipogenesis [18, 19]. Activation of Wnt/β-catenin signaling by over-expression of Wnt1 or Wnt10b prevents the induction of PPARγ and C/EBPα but not C/EBPβ, which works upstream of PPARγ and C/EBPα [20]. In addition, β-catenin interacts with and inhibits the activity of PPARγ, the master regulator of adipogenesis [21]. Conversely, inhibition of Wnt/β-catenin signaling promotes adipogenesis [20].

Thus, adipogenic transcription factors in particular PPARγ and C/EBPα promote adipogenesis and Wnt/β-catenin signaling inhibits adipogenesis. Recent studies suggest that site-specific histone methylations control the expression of these positive and negative master regulators of adipogenesis (see below).

## 3. Regulation of adipogenesis by H3K4 methylation

Mono-, di- and tri-methylations on histone H3K4 (H3K4me1, H3K4me2 and H3K4me3, respectively) are generally correlated with gene activation. Genome-wide analyses show that H3K4me1 and H3K4me2 are associated with open chromatin and are often enriched on cis-regulatory regions [22]. H3K4me1, along with H3K27ac, is often enriched on enhancers [23]. H3K4me3 is enriched around transcription start sites and correlates well with gene expression level [24]. PPARγ has mainly two isoforms, PPARγ1 and PPARγ2, which are transcribed from two different promoters [25]. While the two isoforms are expressed at comparable levels in white adipocytes, PPARγ1 is the predominant one in brown adipocytes [26, 27]. PPARγ1 is expressed at low level in preadipocytes and its expression increases markedly during adipogenesis. PPARγ2 is absent in preadipocytes but is dramatically induced during adipogenesis. H3K4me3 levels on *PPARγ1* and *PPARγ2* promoters correlate remarkably well with both the dynamic changes and the relative levels of *PPARγ1* and *PPARγ2* expression [22, 27].

The enzymes responsible for H3K4 methylation have been identified. In yeast, a single Set1/COMPASS complex, through its enzymatic subunit Set1, is responsible for all mono-, di- and tri-methylations on histone H3K4. Drosophila has three Set1-like H3K4 methyltransferase complexes, which use dSet1, Trithorax (Trx), or Trithorax-related (Trr) as the enzymatic subunit. Mice and humans have six Set1-like histone H3K4 methyltransferase complexes. Based on the protein sequence homologies among the enzymatic subunits and

the subunit compositions, the six complexes can be categorized into three subgroups: SET1A and SET1B, MLL1 and MLL2, and MLL3 and MLL4 (MLL4 is also known as ALR and KMT4D and is sometimes named as MLL2 in the literature), which correspond to the Drosophila dSet1, Trx and Trr complexes, respectively. The two members of each subgroup share identical subunit composition except for the enzymatic subunits [9, 10, 28].

PTIP and a novel protein PA1 are both unique components of the MLL3/MLL4-containing histone H3K4 methyltransferase complexes [10, 29, 30]. PTIP is required for *PPARγ* and *C/EBPα* expression in MEFs. Further, PTIP is required for the robust induction of *PPARγ* and *C/EBPα* during adipogenesis of preadipocytes. Deletion of PTIP reduces H3K4me3 levels on *PPARγ* and *C/EBPα* promoters, which correlate well with the reduced gene expression levels. Accordingly, PTIP-deficient MEFs and white and brown preadipocytes all show severe defects in adipogenesis. Rescue of the adipogenesis defect in PTIP-null MEFs requires co-expression of PPARγ and C/EBPα. Finally, deletion of PTIP in mouse adipose tissue significantly reduces tissue weight. Thus, by controlling the induction of PPARγ and C/EBPα, the two principal adipogenic transcriptional factors, histone methylation regulator PTIP plays a critical role in adipogenesis [27].

Several other unique components of the MLL3/MLL4 complexes are also required for adipogenesis. Deletion of the Ncoa6 subunit leads to defect in PPARγ-stimulated adipogenesis in MEFs [31]. Ncoa6 interacts directly with PPARγ and is likely mediating the interaction between PPARγ and MLL3/MLL4 complexes [32]. Consistently, deletion of MLL3 leads to a significantly decreased amount of white adipose tissue in mice [33]. Together, these results suggest a critical role of the MLL3/MLL4-containing histone H3K4 methyltransferase complexes in adipogenesis.

Several questions remain to be answered on the precise mechanism by which PTIP and associated MLL3/MLL4 complexes regulate adipogenesis. First, besides their association with MLL3/MLL4 complexes, PTIP and PA1 also form a small and separate complex that exists outside of the MLL3/MLL4 complexes [34]. It remains to be determined whether regulation of *PPARγ* and *C/EBPα* expression by PTIP is mediated by the associated MLL3/MLL4 complexes. Second, the role of the novel protein PA1 in adipogenesis remains to be shown. Third, the molecular mechanism by which H3K4 methyltransferases MLL3/MLL4 regulate adipogenesis remains to be defined. Is the increase of H3K4me3 on *PPARγ1* and *PPARγ2* promoters during adipogenesis a cause or a consequence of gene activation? Fourth, the MLL3/MLL4 complexes not only contain H3K4 methyltransferases MLL3/MLL4 but also histone H3K27 demethylase UTX [10, 29, 35, 36]. Since *PPARγ* promoter is enriched with H3K4me3 but lacks H3K27me3 during adipogenesis [22], it will be interesting to investigate whether H3K4 methyltransferases MLL3/MLL4 synergize with H3K27 demethylase UTX to facilitate *PPARγ* expression and adipogenesis. Finally, the roles of SET1A/B- and MLL1/2-containing H3K4 methyltransferase complexes in adipogenesis remain to be understood.

## 4. Regulation of adipogenesis by H3K27 methylation

Tri-methylation on H3K27 (H3K27me3) is a repressive epigenetic mark important for Polycomb-mediated gene silencing [11]. The mammalian Polycomb repressive complex 2 (PRC2) uses its enzymatic subunit Ezh2 to specifically methylate H3K27. Ezh2 is responsible for the majority of H3K27me2 and H3K27me3 in cells [37, 38]. Genome-wide analyses have shown that Ezh2 and H3K27me3 are enriched on a large number of developmental regulators in embryonic stem cells and other cell types [39, 40]. In preadipocytes, Ezh2 and H3K27me3 levels are low on *PPARγ* gene locus but are high on multiple *Wnt* gene loci [22, 38]. Deletion of Ezh2 in preadipocytes dramatically decreases

H3K27me3 on PRC2 target genes including *Wnt1, Wnt6, Wnt10a* and *Wnt10b*, which are negative regulators of adipogenesis. The resulting de-repression and increased expression of *Wnt1, Wnt6, Wnt10a* and *Wnt10b* leads to activation of Wnt/β-catenin signaling, which inhibits adipogenesis by preventing the induction of principal adipogenic transcription factors PPARγ and C/EBPα. The adipogenesis defect in Ezh2 null preadipocytes can be rescued by over-expression of PPARγ and C/EBPα [38]. Deletion of Ezh2 also increases expression of other PRC2 target genes including known adipogenesis inhibitors *Pref-1* and *GATA3*. However, the adipogenesis defect in Ezh2 null cells can be partially rescued by over-expression of inhibitors of Wnt/β-catenin signaling, indicating that the de-repression of *Wnt* genes is responsible at least in part for the differentiation defect in Ezh2 null preadipocytes. Importantly, the HMT activity of Ezh2 is essential for repression of *Wnt* genes and for adipogenesis [38]. Together, these results indicate that Ezh2 is required for adipogenesis and that the H3K27 methyltransferase PRC2, through the enzymatic activity of Ezh2, directly represses *Wnt* genes to facilitate adipogenesis. These results also establish a direct, functional link between Polycomb and Wnt proteins, which are two important classes of developmental regulators.

It should be noted that the Polycomb targeted *Wnt1* and *Wnt10b* genes are expressed at significant levels in preadipocytes [18]. Ezh2 protein level in cells, as well as the H3K27me3 level on *Wnt* genes, remains constant during adipogenesis. However, *Wnt1* and *Wnt10b* expression decreases rapidly during differentiation of both wild-type and Ezh2 null preadipocytes [38]. These results suggest that PRC2 constitutively represses *Wnt* genes during adipogenesis and that transcription repressors other than PRC2 actively decrease *Wnt1* and *Wnt10b* levels during adipogenesis.

Interestingly, deletion of Ezh2 in cells leads to a marked increase of H3K27ac along with the marked decrease of H3K27me3, not only in whole cell extracts but also on Ezh2-regulated *Wnt* promoters, in Ezh2 null preadipocytes [38]. Since HATs CBP and p300 are responsible for H3K27ac in cells [5], these results suggest that Ezh2-mediated H3K27 methylation represses *Wnt* expression by blocking CBP/p300-mediated H3K27ac.

Several questions remain to be answered on the role of H3K27 methylation in adipogenesis. First, Ezh2 is responsible for both H3K27me2 and H3K27me3 but not H3K27me1 in cells. It is currently unclear whether H3K27me2 or H3K27me3 or both are involved in repressing *Wnt* genes to facilitate adipogenesis. Second, how PRC2 complex is recruited to the *Wnt* genes is unclear. Ezh2 has been reported to bind long non-coding RNAs (ncRNAs), which may recruit PRC2 to target gene promoters [41]. The potential involvement and the identities of ncRNAs or transcription factors that recruit PRC2 to the *Wnt* genes remain to be investigated.

## 5. Summary

Methylations on histone H3K4 are generally associated with gene activation whereas methylations on H3K27 are generally associated with gene repression. PTIP, a protein that associates with histone H3K4 methyltransferases MLL3/MLL4 and histone H3K27 demethylase UTX, is required for *PPARγ* and *C/EBPα* expression and adipogenesis [27]. The histone H3K27 methyltransferase PRC2 uses its enzymatic subunit Ezh2 to repress *Wnt* genes and facilitate adipogenesis [38]. Together, these results provide an initial view of epigenetic regulation of adipogenesis by histone H3K4 and H3K27 methylations, and suggest that site-specific histone methylations control expression of both positive and negative master regulators of adipogenesis (Figure 4).

## 6. Future directions

PPARγ and Wnts are master positive and negative regulators of adipogenesis, respectively. The H3K4 methylation regulator PTIP promotes *PPARγ* expression while the H3K27 methyltransferase Ezh2 represses *Wnt* expression during adipogenesis (Figure 4). However, the epigenetic factors that repress *PPARγ* but promote *Wnt* expression in preadipocytes have not been identified. Analyzing the enrichment and/or the dynamic changes of histone methylation patterns on *PPARγ* and *Wnt* promoters in preadipocytes and in the early phase of adipogenesis may provide clues to solving this issue.

It has been reported that knockdown of histone demethylase LSD1 increases histone H3K9 dimethylation (H3K9me2) on *C/EBPα* promoter and leads to decreased adipogenesis. Conversely, knockdown of histone H3K9 methyltransferase SetDB1 (also known as ESET) decreases H3K9me2 on *C/EBPα* promoter and leads to increased adipogenesis. These results suggest opposing roles of LSD1 and SetDB1 in regulating *C/EBPα* expression and adipogenesis [42]. However, LSD1 mainly demethylates H3K4me1/2 and SetDB1 mainly performs trimethylation on histone H3K9 [7, 43]. The H3K9me2 level in cells is predominantly controlled by the euchromatin-associated H3K9 methyltransferase G9a [44]. Thus, it will be important to determine whether G9a-mediated H3K9me2 plays any role in regulation of *PPARγ* expression and adipogenesis.

Within hours of initiation of adipogenesis, cAMP-induced pCREB and Dex-bound GR rapidly induce expression of early adipogenic transcription factors C/EBPβ, KLF4, Krox20 and C/EBPδ, which are essential for induction of *PPARγ* and *C/EBPα* expression and for adipogenesis (Figure 3). How histone methylations regulate the rapid induction of these early adipogenic transcription factors is completely unknown.

Another important question is how H3K4 or H3K27 methylation targets *PPARγ* or *Wnt* genes, respectively. In other words, how H3K4 methyltransferase complexes are recruited to *PPARγ* genes and how H3K27 methyltransferase complex PRC2 is recruited to *Wnt* genes are unclear. These histone modifying complexes may be recruited by sequence-specific transcription factors that directly bind to *PPARγ* or *Wnt* gene loci. Alternatively, sequence-specific non-coding RNAs may directly recruit these HMT complexes to *PPARγ* or *Wnt* genes. Since subunits of these histone modifying complexes contain multiple histone modification-binding domains, it is possible that these HMT complexes are recruited to *PPARγ* or *Wnt* genes by recognizing pre-existing histone modifications on target genes, i.e. through cross-talk between histone modifications.

PPARγ is a nuclear receptor and thus a ligand-activated transcription factor. Correlating with ligand-induced nuclear receptor target gene activation, ligand induces sequential enrichment of H3K18/27ac, RNA Pol II, and several histone methylations including H3K4me3, H3K36me3 and H3K79me2 on nuclear receptor target genes [5]. While the exact roles of these gene activation-associated histone methylations remain to be determined, gene repression-associated histone methylations have been implicated in regulating nuclear receptor target gene expression [45]. One good example is the role of histone methyltransferase SetDB1, which is activated by non-canonical Wnt signaling to methylate histone H3K9 to repress target gene activation by PPARγ [46]. The contributions of other site-specific histone methylations to the transcriptional activation or repression of PPARγ target genes important for adipogenesis remain to be defined.

The majority of histone methyltransferases and demethylases were identified in the 21$^{st}$ century and their biological functions are poorly understood. Adipogenesis provides an excellent model system to study the roles of histone methyltransferases and demethylases, and the dynamics of site-specific histone methylations, in regulation of gene expression and

cell differentiation. In addition to histone H3K4 and H3K27 methylations described above, it will be important to understand the roles of methylations on histone H3K9, H3K36 and H3K79, and the related histone methyltransferases and demethylases, in regulation of adipogenesis. Straightforward knockout (KO) of these enzymes usually leads to embryonic lethality. Fortunately, conditional KO mouse strains (floxed mice) for many of these histone modifying enzymes and associated factors are becoming available. Preadipocytes carrying conditional KO of histone modifying enzymes can be easily isolated from these mice, thus providing an excellent model system to study epigenetic regulation of cell differentiation *in vitro* [27, 38]. The results should be verified *in vivo* by crossing floxed mice with tissue-specific Cre mice to specifically delete the gene-of-interest in adipose tissue [47].

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## Highlights

- Histone methylations regulate gene expression and cell differentiation
- Histone methylations are regulated by methyltransferases and demethylase
- This review focuses on regulation of adipogenesis by histone methylation
- H3K4 and H3K27 methylations control expression of master regulators of adipogenesis


**Figure 1. Histone modifications**

Acetylation (ac) generally correlates with gene activation. Histone lysine (K) methylations (me) that correlate with gene activation are shown in green while those correlated with gene repression are shown in red.

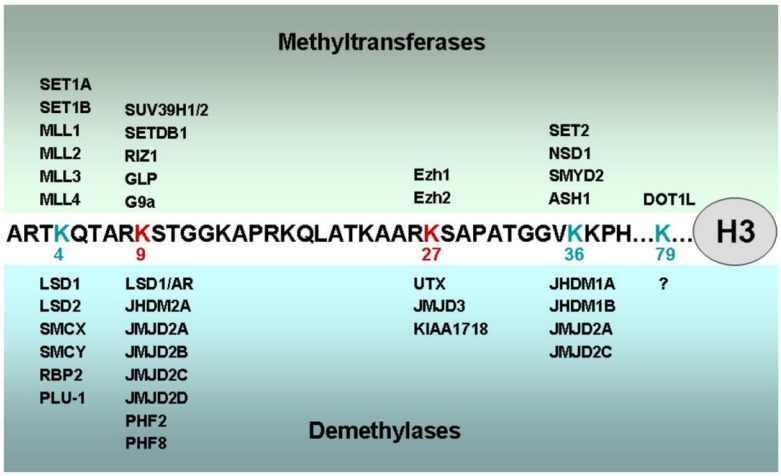


**Figure 2. Histone lysine methyltransferases and demethylases**

Histone lysine methylation is dynamically regulated by site-specific methyltransferases and demethylases.

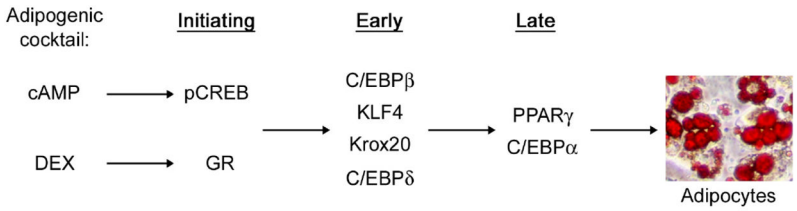


**Figure 3. Adipogenesis is positively regulated by a cascade of sequentially expressed adipogenic transcription factors**

Adipocytes have been stained with Oil Red O and show red color. pCREB, phosphorylated CREB.

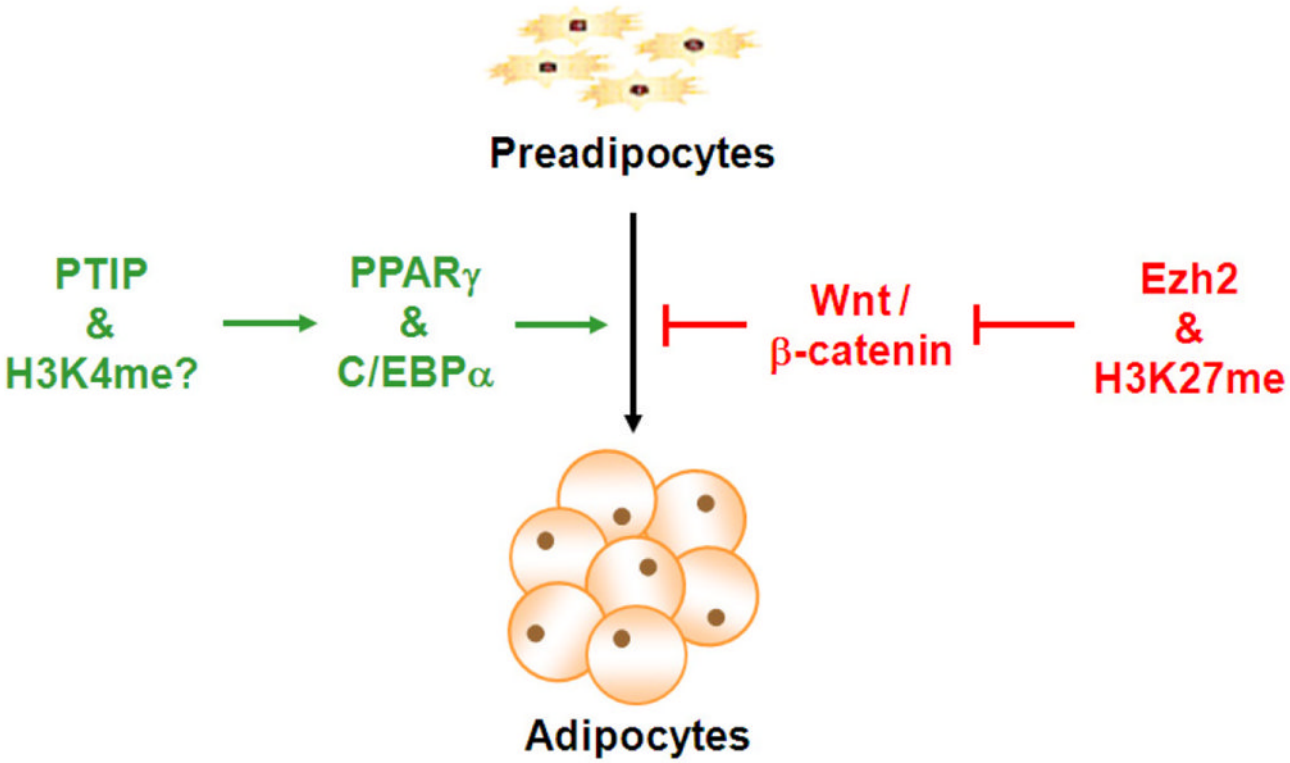


**Figure 4. Epigenetic regulation of adipogenesis by histone H3K4 and H3K27 methylations**

PTIP, a protein that associates with histone H3K4 methyltransferases MLL3/MLL4, is required for *PPARγ* and *C/EBPα* expression and adipogenesis. Ezh2 uses its histone H3K27 methyltransferase activity to constitutively repress *Wnt* genes and facilitate adipogenesis.