Histone H3K9 methyltransferase G9a represses *PPAR* γ expression and adipogenesis

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PPAR γ promotes adipogenesis while Wnt proteins inhibit adipogenesis. However, the mechanisms that control expression of these positive and negative master regulators of adipogenesis remain incompletely understood. By genome-wide histone methylation profiling in preadipocytes, we find that among gene loci encoding adipogenesis regulators, histone methyltransferase (HMT) G9a-mediated repressive epigenetic mark H3K9me2 is selectively enriched on the entire *PPAR* γ locus. H3K9me2 and G9a levels decrease during adipogenesis, which correlates inversely with induction of *PPAR* γ . Removal of H3K9me2 by G9a deletion enhances chromatin opening and binding of the early adipogenic transcription factor C/EBP β to *PPAR* γ promoter, which promotes *PPAR* γ expression. Interestingly, G9a represses *PPAR* γ expression in an HMT activity-dependent manner but facilitates *Wnt10a* expression independent of its enzymatic activity. Consistently, deletion of G9a or inhibiting G9a HMT activity promotes adipogenesis. Finally, deletion of G9a in mouse adipose tissues increases adipogenic gene expression and tissue weight. Thus, by inhibiting *PPAR* γ expression and facilitating *Wnt10a* expression, G9a represses adipogenesis.

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Rosen and MacDougald, 2006). *PPAR* γ has two isoforms, *PPAR* γ 1 and *PPAR* γ 2, which are produced by differential promoter usage and alternative splicing. *PPAR* γ 1 is expressed at low level in preadipocytes where *PPAR* γ 2 is undetectable. Both isoforms are strongly induced during adipogenesis (Rosen and MacDougald, 2006). *PPAR* γ expression is positively regulated by a cascade of sequentially expressed adipogenic transcription factors. Expression of early adipogenic transcription factors C/EBP β , C/EBP δ , Krox20, and KLF4 is induced within hours of initiation of adipogenesis (Farmer, 2006; Birsoy *et al*, 2008; Ge, 2012). C/EBP β directly binds to –0.3 kb of *PPAR* γ 2 promoter and activates *PPAR* γ 2 expression (Salma *et al*, 2006). C/EBP β and C/EBP δ also activate expression of another principal adipogenic transcription factor C/EBP α . *PPAR* γ cooperates with C/EBP α in a positive feedback loop to promote terminal differentiation of adipocytes (Farmer, 2006; Rosen and MacDougald, 2006).

The Wnt family of secreted proteins is important developmental regulators. The canonical Wnt signalling, also known as Wnt/ β -catenin signalling, is a major negative regulator of adipogenesis (Prestwich and Macdougald, 2007). Among the 19 Wnt family members in mice and humans, Wnt1, Wnt6, Wnt10a, and Wnt10b have been shown to inhibit adipogenesis. Expression of endogenous Wnt6, Wnt10a, and Wnt10b decreases during adipogenesis. Activation of Wnt/ β -catenin signalling by overexpression of Wnt1, Wnt6, Wnt10a, and Wnt10b prevents the induction of *PPAR* γ and C/EBP α and inhibits adipogenesis through a β -catenin-dependent mechanism (Cawthorn *et al*, 2012). Conversely, inhibiting the Wnt/ β -catenin signalling promotes adipogenesis (Ross *et al*, 2000). In mice, *Wnt1* and *Wnt10b* genes are clustered on chromosome 15 while *Wnt6* and *Wnt10a* genes are clustered on chromosome 1 in a head-to-tail manner.

Epigenetic mechanisms in particular histone modifications play important roles in regulating gene expression and cell differentiation. Histone acetylation on lysine (K) residues generally correlates well with gene activation. However, histone methylation on K residues can be correlated with either gene activation or repression, depending on which K residue gets methylated. Genome-wide analyses have revealed that trimethylation on histone 3 lysine 4 (H3K4me3) is enriched on active gene promoters, while trimethylation on histone 3 lysine 27 (H3K27me3) is enriched on repressed genes (Boyer *et al*, 2006; Barski *et al*, 2007; Mikkelsen *et al*, 2007). Histone H3 lysine 9 dimethylation (H3K9me2) is enriched in silent regions on euchromatin and correlates with gene silencing (Rice *et al*, 2003; Barski *et al*, 2007). Histone methyltransferase (HMT) G9a mainly functions on euchromatin and is responsible for the majority of H3K9me2 in cells (Tachibana *et al*, 2002; Rice *et al*, 2003). Studies using G9a knockout (KO) or knockdown cells and mice suggest that G9a and G9a-mediated H3K9me2 mainly associate with transcriptional silencing (Shinkai and Tachibana, 2011). Interestingly, recent reports also show that G9a is involved

Introduction

Peroxisome proliferator-activated receptor γ (*PPAR* γ) is a member of the nuclear receptor superfamily of ligand-activated transcription factors. *PPAR* γ is considered as the master positive regulator of preadipocyte differentiation towards adipocyte (adipogenesis) and is both necessary and sufficient for adipogenesis (Rosen and Spiegelman, 2001; Farmer, 2006;

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in activation of specific genes in an HMT activity-independent manner (Lee *et al*, 2006; Chaturvedi *et al*, 2009). Thus, G9a plays a dual role in regulating gene expression.

Previous reports suggest that H3K4me3 positively regulates *PPAR γ* expression while H3K27me3 negatively regulates *Wnt* expression during adipogenesis (Cho *et al*, 2009; Mikkelsen *et al*, 2010; Wang *et al*, 2010; Ge, 2012). However, the mechanisms that negatively regulate *PPAR γ* expression while positively regulate *Wnt* expression during adipogenesis remain incompletely understood. Further, the roles of G9a and G9a-mediated H3K9me2 in adipogenesis have not been shown. In this paper, we report genome-wide profiling of H3K9me2 in preadipocytes. At the genome-wide level, H3K9me2 correlates inversely with both H3K4me3 and H3K27me3. Among gene loci encoding adipogenesis regulators, H3K9me2 is selectively enriched on the entire *PPAR γ* locus, where H3K27me3 is absent. We show that G9a represses *PPAR γ* expression by adding H3K9me2 to the entire *PPAR γ* gene locus and that G9a facilitates *Wnt10a* expression independent of its enzymatic activity. Finally, deletion of *G9a* promotes adipogenesis in cell culture and increases adipose tissue weight in mice.

Results

H3K9me2 is enriched on the entire *PPAR γ* locus in preadipocytes

To investigate the roles of site-specific histone methylations in adipogenesis, we performed chromatin immunoprecipitation followed by deep sequencing (ChIP-Seq) of H3K4me3, H3K9me2, and H3K27me3 in undifferentiated 3T3-L1 white preadipocytes. The H3K4me3 and H3K27me3 ChIP-Seq results were highly consistent with the reported genome-wide profiling data using the same cell line (Mikkelsen *et al*, 2010). Unlike H3K4me3 and H3K27me3, both of which peaked around the transcription start sites (TSSs), H3K9me2 level was generally low around the TSSs and in the gene body regions but was enriched in upstream and downstream untranscribed regions (Figure 1A). At the genome-wide level, we observed mutually exclusive occupancies between the repressive mark H3K9me2 and the active marks including H3K4me3 (Figure 1B), H3K27ac, and H3K36me3 (Supplementary Figure S1A). Interestingly, H3K9me2 also correlated inversely with H3K27me3 globally (Figure 1C), suggesting that the two repressive epigenetic marks target distinct sets of genes in preadipocytes. Consistent with the association of H3K9me2 with gene repression, gene expression levels correlated inversely with H3K9me2 intensities at genome-wide level (Figure 1D). Gene ontology (GO) analysis of H3K9me2-enriched genes identified the immune and inflammatory response genes (Supplementary Figure S1B), which have been shown to be directly regulated by G9a and H3K9me2 (Fang *et al*, 2012).

We next examined H3K9me2 levels on gene loci encoding known positive and negative regulators of adipogenesis (Farmer, 2006; Rosen and MacDougald, 2006). High levels of H3K9me2 were found on the entire 129 kb gene locus of the master adipogenic transcription factor *PPAR γ* (Figure 1E). In contrast, H3K9me2 levels were low on gene loci encoding other adipogenic transcription factors, including C/EBP α , C/EBP β , C/EBP δ , KLF4, Krox20 and CREB (Figure 2A–G). Interestingly, H3K9me2 levels were also low on gene loci encoding negative regulators of adipogenesis, including

Wnt6–Wnt10a, Wnt1–Wnt10b, Pref-1 (also known as DLK1), GATA2, GATA3, KLF2 and Chop10 (Figures 1F and 2H–N). The selective enrichment of H3K9me2 on *PPAR γ* locus in preadipocytes was confirmed by manual ChIP (Figure 1G). *PPAR γ* is expressed at low level in preadipocytes. Accordingly, H3K27ac and H3K36me3 levels were low on the *PPAR γ* locus in preadipocytes (Supplementary Figure S1C and D). ChIP-Seq data confirmed our previous observation that H3K27me3 was enriched on the *Wnt* loci (Figures 1F, 2H and I; Wang *et al*, 2010). Consistent with a previous report (Mikkelsen *et al*, 2010), H3K27me3 was excluded from the *PPAR γ* locus where H3K9me2 was enriched (Figure 1E), suggesting that H3K9me2, rather than H3K27me3, is involved in repressing *PPAR γ* expression in preadipocytes.

H3K9me2 and G9a levels decrease during adipogenesis

We next examined histone methylation changes on the *PPAR γ* locus before and after adipogenesis by ChIP assay. Consistent with previous reports (Cho *et al*, 2009; Mikkelsen *et al*, 2010; Wang *et al*, 2010), H3K4me3 levels on *PPAR γ* 1 and γ 2 promoters increased after differentiation of 3T3-L1 white preadipocytes (Figure 3A), which correlated well with both the induction and the relative expression levels of *PPAR γ* 1 and γ 2 (Figure 3D). H3K27me3 remained very low on *PPAR γ* locus but remained high on *Wnt10a* locus throughout adipogenesis (Figure 3A). Interestingly, H3K9me2 levels on the entire *PPAR γ* locus decreased markedly, which was confirmed by ChIP-Seq of H3K9me2 before and after adipogenesis of 3T3-L1 cells (Figure 3A and B). The decrease in H3K9me2 levels on *PPAR γ* locus correlated inversely with the marked induction of *PPAR γ* during adipogenesis. G9a expression levels also decreased during adipogenesis, mainly at the protein level (Figure 3C and D). Accordingly, global H3K9me2 level decreased during 3T3-L1 adipogenesis (Figure 3D). Similar results were obtained during adipogenesis of brown preadipocytes (Supplementary Figure S2). Consistent with the results from 3T3-L1 adipogenesis in cell culture, G9a protein level was much lower in adipocytes than in preadipocytes of white adipose tissue (WAT) (Figure 3E). The global as well as *PPAR γ* locus-specific decreases in H3K9me2 suggest that G9a and H3K9me2 negatively regulate *PPAR γ* expression and adipogenesis.

Characterization of SV40T-immortalized *G9a*^{−/−} preadipocytes

G9a is required for cell growth as knockdown of G9a in cells leads to senescence (Takahashi *et al*, 2012). Deletion of *G9a* in primary mouse embryonic fibroblasts (MEFs) and primary white preadipocytes also led to severe growth defect and senescence due to derepression of tumor suppressor genes such as *p16^{Ink4a}* (Supplementary Figure S3). To investigate the role of G9a in adipogenesis and also to distinguish the roles of G9a in cell differentiation versus proliferation, we immortalized primary *G9a^{flox/flox}* brown preadipocytes with SV40 large T antigen (SV40T) following an established protocol (Wang *et al*, 2010). SV40T functionally inactivates *p16^{Ink4a}* but does not interfere with differentiation of brown preadipocytes (Wang *et al*, 2010). The immortalized cells were infected with retrovirus expressing Cre to generate *G9a*^{−/−} brown preadipocytes (Figure 4A and B). Unlike the case of primary preadipocytes, deletion of *G9a* had no significant effects on the morphology or the growth rate of the

Figure 2 Histone methylation profiles on adipogenesis regulator genes in preadipocytes. ChIP-Seq profiles of H3K4me3, H3K9me2 and H3K27me3 on gene loci encoding positive (A–G) and negative (H–N) regulators of adipogenesis in 3T3-L1 preadipocytes (day 0) are visualized using the UCSC genome browser. Panels (A) and (H) are shown as controls.

immortalized cells (Figure 4C and D). Consistent with previous reports on *G9a*^{−/−} embryonic stem (ES) cells (Peters *et al*, 2003), *G9a*^{−/−} brown preadipocytes showed marked reduction of H3K9me1 and H3K9me2 but retained robust H3K9me3. Among the histone methylation and acetylation

marks that we examined, H3K9 acetylation (H3K9ac) increased markedly in *G9a*^{−/−} preadipocytes (Figure 4E). The decrease of H3K9me2 and the increase of H3K9ac in *G9a*^{−/−} cells could be reversed by ectopic expression of G9a (Supplementary Figure S4A).

Figure 3 H3K9me2 and G9a levels decrease during adipogenesis. (A) ChIP analyses of H3K4me3, H3K9me2 and H3K27me3 on *PPARγ1* and *PPARγ2* promoters before (day 0) and after (day 6) 3T3-L1 adipogenesis. The schematic of *PPARγ1* and *PPARγ2* promoters and the locations of Taqman probes for PCR quantitation of ChIP are shown at the top. *Wnt10a* promoter serves as a control. (B) ChIP-Seq profiles of H3K9me2 on *PPARγ* locus (highlighted) before (day 0) and after (day 7) 3T3-L1 adipogenesis. (C) qRT-PCR analysis of *PPARγ* and *G9a* expression before and after 3T3-L1 adipogenesis. (D) Western blot analysis of G9a, PPARγ and H3K9me2 levels during 3T3-L1 adipogenesis. The 54.5-kDa PPARγ1 and 57.5-kDa PPARγ2 bands as well as the 30-kDa and 42-kDa C/EBPα isoforms are indicated. The p85α subunit of phosphoinositol-3-phosphate kinase is used as a loading control. (E) Western blot analysis of protein levels in preadipocytes and adipocytes isolated from mouse inguinal white adipose tissue. Figure source data can be found in Supplementary data.

G9a represses adipogenesis

Among the three SV40T-immortalized *G9a^{flox/flox}* brown preadipocyte cell lines that we established, #1 cell line showed poor differentiation potential, with ~40% of cells in the population differentiating into relatively immature adipocytes under the standard induction condition. However, deletion of *G9a* in the #1 cell line accelerated and enhanced adipogenesis, with over 90% of cells in the population differentiating into lipid-laden adipocytes (Figure 5A). The markedly enhanced adipogenesis could be reversed by ectopic expression of G9a (Supplementary Figure S4B). Consistent with the morphological differentiation, deletion of *G9a* in the #1 cell line promoted induction of master adipogenic transcription factors PPARγ and C/EBPα and increased expression of other adipocyte markers such as aP2, PGC1α, Prdm16, UCP1 and adiponectin (Figure 5B and C; Supplementary Figure S4C). The other two immortalized *G9a^{flox/flox}* brown preadipocyte cell lines (#2 and #3) showed full adipogenesis potential under the standard induction condition (Supplementary

Figure S4D). However, enhanced adipogenesis was also observed in #2 and #3 *G9a^{-/-}* brown preadipocyte cell lines when differentiation was induced under suboptimal condition (Supplementary Figure S4E). Consistently, in 3T3-L1 white preadipocytes, knockdown of *G9a* decreased global H3K9me2 level and promoted PPARγ expression and adipogenesis (Figure 5D–G), while overexpression of G9a inhibited adipogenesis (Figure 5H and I). These results indicate that G9a represses adipogenesis in both white and brown preadipocytes.

G9a directly represses PPARγ expression but facilitates Wnt10a expression

The enrichment of G9a-mediated H3K9me2 on *PPARγ* locus and the accelerated PPARγ induction during adipogenesis of *G9a^{-/-}* cells suggest that G9a may directly repress PPARγ expression. Indeed, microarray analysis followed by qRT-PCR confirmation showed that deletion of *G9a* in preadipocytes increased basal level PPARγ expression but had little

Figure 4 Characterization of immortalized *G9a*^{flox/flox} brown preadipocytes. SV40T-immortalized *G9a*^{flox/flox} brown preadipocyte cell line #1 was infected with retroviruses MSCVhygro expressing Cre (MSCVhygro-Cre) or vector (Vec) alone. After selection with 150 µg/ml hygromycin for 2 weeks, cells were maintained at subconfluence. (A, B) Confirmation of *G9a* deletion by qRT-PCR (A) and western blot (B). (C) Cell morphology under the microscope. (D) To analyse the short-term cell growth rates, 1×10^5 cells were plated at day 0 and the cumulative cell numbers were determined every day for 5 days. (E) Western blot analysis of histone methylation and acetylation in nuclear extracts. me1, me2 and me3 refer to mono-, di- and trimethylation, respectively. ac, acetylation. Figure source data can be found in Supplementary data.

effects on basal level expression of other positive regulators of adipogenesis including C/EBP α , C/EBP β , C/EBP δ , Krox20, KLF4, SREBP1, STAT5A, CREB and GR (Supplementary Figures S5A, B and 6A). *G9a* was enriched on both *PPAR* γ 1 and γ 2 promoters in preadipocytes. Deletion of *G9a* decreased H3K9me2 level but increased levels of RNA polymerase II (Pol II) and H3K9ac on *PPAR* γ 1 and γ 2 promoters (Figure 6B). The increased *PPAR* γ level in *G9a*^{-/-} preadipocytes could be reversed by ectopic expression of wild-type *G9a* but not the enzyme-dead mutant Δ SET (Figure 6C; Supplementary Figure S5B). Using FAIRE (Formaldehyde Assisted Isolation of Regulatory Elements) analysis to monitor chromatin opening (Simon *et al*, 2012), we observed moderately increased chromatin opening on *PPAR* γ 1 promoter in *G9a*^{-/-} preadipocytes (Figure 6D).

The increased *PPAR* γ expression in *G9a*^{-/-} preadipocytes mainly came from *PPAR* γ 1, as *PPAR* γ 2 level was very low before differentiation. However, deletion of *G9a* and thus the removal of H3K9me2 significantly enhanced *PPAR* γ 2 induction in the early phase of adipogenesis (Figure 6E). C/EBP β directly binds to -0.3 kb of *PPAR* γ 2 promoter and stimulates *PPAR* γ 2 expression in the early phase of adipogenesis (Salma *et al*, 2006; Nielsen *et al*, 2008). Deletion of *G9a* had no effect on C/EBP β induction but promoted C/EBP β binding to -0.3 kb of *PPAR* γ 2 promoter in the early phase of adipogenesis (Figure 6E and F). C/EBP β binding has been shown to correlate with, and be required for, the efficient opening of chromatin region surrounding the C/EBP β -binding site on *PPAR* γ 2 promoter (Siersbaek *et al*, 2011). By FAIRE analysis, we observed increased opening of chromatin region surrounding the -0.3 kb C/EBP β -binding site on *PPAR* γ 2 promoter in *G9a*^{-/-} cells 2 h after induction of differentiation, which remained open in the early phase of adipogenesis (Figure 6G). These results suggest that elimination of H3K9me2 by *G9a* deletion promotes chromatin opening and binding of C/EBP β , which directly activates *PPAR* γ 2 expression.

Multiple negative regulators of adipogenesis have also been described, including Wnt1, Wnt6, Wnt10a, Wnt10b, Pref-1, GATA2, GATA3, KLF2 and Chop10 (Rosen and MacDougald, 2006). Among them, *Wnt10a* expression decreased markedly and expression of *Wnt1* and *Wnt6* decreased about two-fold in *G9a*^{-/-} preadipocytes (Figure 6H; Supplementary Figure S5A and B). Consistent with the decreased expression of *Wnt* genes, the cytosolic β -catenin level also decreased in *G9a*^{-/-} preadipocytes (Figure 6I), indicating attenuated *Wnt* signalling with *G9a* deletion. Interestingly, the decreased expression of *Wnt10a* in *G9a*^{-/-} preadipocytes could be rescued by ectopic expression of both wild-type *G9a* and the enzyme-dead mutant Δ SET (Figure 6J; Supplementary Figure S5B), indicating that *G9a* facilitates *Wnt10a* expression in preadipocytes independent of its enzymatic activity. Consistently, ChIP assays revealed recruitment of *G9a* and *G9a*-dependent recruitment of Pol II to *Wnt10a* promoter where H3K9me2 level was absent (Figures 6K and 1F). Compared to the wild-type *G9a*, the enzyme-dead mutant Δ SET showed reduced ability to repress adipogenesis in *G9a* KO preadipocytes, suggesting that the HMT activity of *G9a* is critical for repressing adipogenesis (Supplementary Figure S5C). Together, these results indicate that *G9a* directly represses *PPAR* γ expression through H3K9me2 while facilitates *Wnt10a* expression independent of its methyltransferase activity.

Inhibiting *G9a* methyltransferase activity promotes *PPAR* γ expression and adipogenesis

To confirm the role of *G9a* methyltransferase activity in repressing *PPAR* γ expression and adipogenesis, we treated preadipocytes with *G9a* inhibitor BIX01294 (BIX) (Kubicek *et al*, 2007). To minimize the potential toxicity to cells, confluent preadipocytes were treated with 8 µM BIX for 48 h before the induction of adipogenesis. BIX treatment decreased H3K9me2 levels but increased H3K9ac levels not

Figure 5 G9a represses adipogenesis. (A–C) G9a represses adipogenesis of brown preadipocytes. *G9a^{flx/flx}* brown preadipocytes described in Figure 4 were infected with MSCVhygro-Cre. Adipogenesis was induced at standard condition at day 0. Whole cell extracts and RNA samples were prepared at indicated time points for western blot and qRT-PCR, respectively. (A) Morphological differentiation at day 6. Cells were stained with Oil Red O. Top panels, stained dishes; lower panels, representative fields under the microscope. (B) Western blot analysis of expression of adipogenesis makers and G9a during adipogenesis. (C) qRT-PCR analysis of expression of adipogenic makers and G9a during adipogenesis. (D–G) Knockdown of *G9a* promotes adipogenesis of 3T3-L1 white preadipocytes. 3T3-L1 cells were infected with lentiviral shRNA targeting *G9a* or control (Con), followed by puromycin selection for 7 days. (D) Western blot analysis of undifferentiated, subconfluent cells using antibodies indicated on the right. (E) qRT-PCR analysis of *G9a* and *PPAR γ* expression in undifferentiated, subconfluent 3T3-L1 cells. (F, G) Adipogenesis was induced at suboptimal condition using 1/3 of adipogenic cocktail. After differentiation, cells were stained with Oil Red O (F) or collected for qRT-PCR analysis of adipocyte marker expression (G). (H, I) Overexpression of G9a inhibits 3T3-L1 adipogenesis. Cells were infected with MSCVhygro expressing FLAG-tagged G9a (F-G9a). Adipogenesis was induced at standard condition. (H) Oil Red O staining after differentiation. (I) qRT-PCR analysis of adipocyte marker expression before and after adipogenesis. Figure source data can be found in Supplementary data.

only globally but also on *PPAR γ 1* and γ 2 promoters in preadipocytes (Figure 7A and B). BIX treatment increased *PPAR γ* expression in preadipocytes but did not affect *Wnt10a*

expression (Figure 7C), indicating that G9a represses *PPAR γ* expression through H3K9me2 while facilitates *Wnt10a* expression independent of its enzymatic activity. Further,

Figure 6 G9a directly represses *PPARγ* expression but facilitates *Wnt10a* expression. *G9a^{flx/flx}* brown preadipocytes described in Figure 4 were infected with MSCVhygro-Cre. **(A, B)** G9a directly represses *PPARγ* expression in preadipocytes. Cells maintained at subconfluence were collected for **(A)** qRT-PCR analysis of expression of adipogenic transcription factors and **(B)** ChIP assays of G9a, H3K9me2, H3K9ac and RNA polymerase II (Pol II) levels on *PPARγ1/2* promoters (+0.5 kb). **(C)** G9a enzymatic activity is required for repressing *PPARγ* expression in preadipocytes. *G9a^{flx/flx}* brown preadipocytes were infected with MSCVhygro-F-G9a expressing FLAG-tagged G9a (F-G9a) or with MSCVhygro-F-G9aΔSET expressing G9a lacking the SET domain. After hygromycin selection, cells were infected with WZLneo expressing Cre (WZLneo-Cre), followed by selection with G418. Gene expression in subconfluent preadipocytes was analysed by qRT-PCR. **(D)** FAIRE analysis of chromatin opening on *PPARγ1* promoter. **(E–G)** Deletion of *G9a* promotes chromatin opening of, and C/EBPβ binding to, *PPARγ2* promoter in the early phase of adipogenesis. Adipogenesis of Vec- and Cre-infected *G9a^{flx/flx}* brown preadipocytes was induced under standard condition. Samples were collected at indicated time points for qRT-PCR of *PPARγ2* and *C/EBPβ* expression **(E)**, ChIP of C/EBPβ and H3K9me2 around -0.3 kb C/EBPβ-binding site on *PPARγ2* promoter **(F)**, and FAIRE analysis of chromatin opening on *PPARγ2* promoter **(G)**. **(H)** *Wnt10a* expression in preadipocytes requires G9a. Cells maintained at subconfluence were collected for qRT-PCR. **(I)** Western blot analysis of β-catenin levels in the cytosolic and membrane fractions. β-Actin serves as the loading control. **(J)** G9a facilitates *Wnt10a* expression in preadipocytes independent of its enzymatic activity. The experiment was done as in **(C)**. **(K)** ChIP of G9a and Pol II levels on *Wnt10a* promoter in subconfluent preadipocytes. qPCR data are presented as means ± s.d. and are representative of 3–4 independent experiments. Figure source data can be found in Supplementary data.

treating preadipocytes with BIX for 48 h before the induction of differentiation promoted adipogenesis and expression of adipocyte markers *PPARγ*, *C/EBPα* and *aP2*, although not as much as *G9a* deletion (Figure 7D and E). These results indicate that H3K9 methyltransferase activity of G9a is required for repressing *PPARγ* and adipogenesis.

Deletion of *G9a* increases adipose tissue weight in mice

G9a is essential for early embryogenesis (Tachibana *et al*, 2002). To investigate *G9a* function in adipose tissue *in vivo*, adipose-specific *G9a* KO mice were generated by crossing *G9a^{flx/flx}* with *aP2-Cre* mice expressing Cre under the control of adipocyte-specific *aP2* promoter (He *et al*, 2003;

Figure 7 Inhibiting G9a methyltransferase activity promotes *PPAR γ* expression and adipogenesis. *G9a^{flox/flox}* brown preadipocytes described in Figure 4 were infected with MSCVhygro-Cre or Vec alone, followed by BIX treatment and adipogenesis assay. In all, 8 μ M of BIX was used except as indicated in (A). (A–C) Inhibiting G9a methyltransferase activity promotes *PPAR γ* expression in preadipocytes. BIX was added when cells reached confluence. Forty-eight hours later, cells were collected for western blot of H3K9me2 and H3K9ac (A), ChIP of H3K9me2 and H3K9ac levels on *PPAR γ 1/2* promoters at +0.5 kb (B), and qRT-PCR of *PPAR γ* and *Wnt10a* expression (C). (D, E) Inhibiting G9a methyltransferase activity promotes adipogenesis. BIX was added when cells reached confluence. Forty-eight hours later, BIX was removed and adipogenesis was induced under standard condition. Six days later, cells were stained with Oil Red O (D) or collected for qRT-PCR of adipocyte marker expression (E). qPCR data are presented as means $\pm$ s.d. and are representative of three independent experiments. Figure source data can be found in Supplementary data.

Li *et al*, 2011). *aP2* is a direct *PPAR γ* target gene and is robustly induced when preadipocytes (such as 3T3-L1) have differentiated into immature adipocytes (Cho *et al*, 2009).

Analyses of the time courses of *aP2* expression during adipogenesis of primary white preadipocytes, 3T3-L1, and immortalized brown preadipocytes revealed that *aP2* was induced at days 1–2, well before *PPAR γ* expression reached the maximal level (Supplementary Figure S6). Thus, *aP2* promoter-driven Cre was expected to start deleting *G9a* in immature adipocytes before the completion of adipogenesis. To confirm this, we performed *ex vivo* experiments. As shown in Figure 8A and B, primary white preadipocytes directly isolated from *G9a* KO (*G9a^{flox/flox};aP2-Cre*) mice showed enhanced adipogenesis and increased expression of adipogenesis marker genes than the littermate controls (*G9a^{flox/flox}*). Further, genomic PCR revealed that *aP2*-Cre-mediated deletion of *G9a* allele in *G9a^{flox/flox};aP2-Cre* preadipocytes started from day 2 of adipogenesis (Figure 8C). These results indicate that *aP2*-Cre-mediated deletion of *G9a* promotes adipogenesis and *PPAR γ* expression.

In the adipose-specific *G9a* KO mice, deletion of *G9a* allele was found in epididymal and inguinal WATs and interscapular brown adipose tissue (BAT) but not in other tissues

examined (Supplementary Figure S7A). Under normal chow, adipose-specific *G9a* KO mice weighed more than the littermate controls starting from 12 weeks age (Figure 9A and B; Supplementary Figure S7B). Body composition test showed increases in fat mass but not lean mass in *G9a* KO mice compared to the controls (Figure 9C). Consistently, *G9a* KO mice showed increases in size and weight of both WAT and BAT but not other tissues (Figure 9D and E; Supplementary Figure S7C). Histology analyses of adipose tissue sections revealed increased size of adipocytes in *G9a* KO mice, suggesting enhanced adipose tissue development *in vivo* (Figure 9F and G). Serum chemistry analyses showed that *G9a* KO mice had statistically higher levels of triglyceride, total cholesterol, insulin and leptin in blood (Supplementary Table S1). Consistently, fatty liver was found in a high percentage of male *G9a* KO mice although no *G9a* deletion was detected in liver (Supplementary Figure S7D). *G9a* KO mice showed the trend of increased food intake compared with the littermate controls (Supplementary Figure S7E). Expression of adipogenic markers *PPAR γ* , *C/EBP α* and *aP2* increased significantly in *G9a* KO mice while *Wnt10a* expression decreased (Figure 9H). Moderately increased expression of adipogenic marker genes was also observed in the BATs isolated from *G9a* KO newborn pups, suggesting en-

Figure 8 *aP2*-Cre-mediated deletion of *G9a* promotes adipogenesis *ex vivo*. Primary white preadipocytes were isolated from inguinal white adipose tissues of littermate control (Con, with the genotype *G9a*^{flox/flox}) and *G9a* knockout mice (KO, with the genotype *G9a*^{flox/flox}; *aP2*-Cre). (A) Adipogenesis assay. (B) qRT-PCR of gene expression during adipogenesis. (C) *aP2*-Cre-mediated deletion of *G9a* allele was determined by quantitative PCR analysis of tissue genomic DNA. qPCR was performed in triplicates for littermate group 1. Similar results were obtained for littermate groups 2 and 3 (data not shown). qPCR data are presented as means $\pm$ s.e.m.

hanced adipogenesis (Supplementary Figure S7F). These results are consistent with the increased adipogenesis in *G9a* KO preadipocytes *in vitro* and *ex vivo* and indicate that deletion of *G9a* in mouse adipose tissue promotes adipogenic gene expression and increases fat accumulation and tissue weight.

Discussion

PPAR γ is the master adipogenic transcription factor while Wnt proteins are major negative regulators of adipogenesis. Using ChIP-Seq, we show that among gene loci encoding major adipogenesis regulators, *G9a*-mediated repressive epigenetic mark H3K9me2 is selectively enriched on the entire *PPAR* γ gene locus. *G9a* and H3K9me2 decrease both globally as well as on the *PPAR* γ locus during adipogenesis. Deletion of *G9a* or inhibiting its enzymatic activity removes H3K9me2 and promotes both basal level expression and induction of *PPAR* γ . On the other hand, *G9a* facilitates *Wnt10a* expression in preadipocytes independent of its enzymatic activity. Deletion of *G9a* in mouse adipose tissues increases *PPAR* γ expression but decreases *Wnt10a* expression. Consistently, deletion of *G9a* or inhibiting its enzymatic activity promotes adipogenesis. These results suggest a model that by inhibiting *PPAR* γ expression through H3K9me2 while promoting *Wnt10a* expression independent of its methyltransferase activity, *G9a* represses adipogenesis (Figure 9I).

Profile of H3K9me2 in preadipocytes

G9a-mediated H3K9me2 covers the entire 129 kb *PPAR* γ gene locus in preadipocytes, which is consistent with the observations in ES cells that *G9a*-mediated H3K9me2 covers large chromatin regions (Wen *et al*, 2009; Lienert *et al*, 2011). Different from the active epigenetic mark H3K4me3 which

is enriched around TSSs and associates with active genes during adipogenesis (Mikkelsen *et al*, 2010; Ge, 2012), H3K9me2 is generally excluded from active genes and is mutually exclusive with H3K4me3. Consistent with the established role of H3K9me2 in gene repression and with the results from ES cells (Lienert *et al*, 2011), H3K9me2 correlates inversely with gene expression levels in preadipocytes. Genes carrying high levels of H3K9me2 are expressed at very low levels, an observation also seen in ES cells (Wen *et al*, 2009). On the other hand, genes carrying low levels of H3K9me2 show varying levels of expression, suggesting that loss of the repressive H3K9me2 alone is insufficient for gene activation and that transcription activators and/or active histone marks are needed for gene activation.

We observe an inverse correlation between H3K9me2 and H3K27me3 at genome-wide scale in preadipocytes, similar to the observation in ES cells (Lienert *et al*, 2011). H3K9me2 level is high on *PPAR* γ locus but very low on *Wnt6-Wnt10a* locus. In contrast, H3K27me3 level is absent on *PPAR* γ locus but high on *Wnt6-Wnt10a* locus (Figure 1E and F). Consistently, removal of H3K9me2 by inhibiting *G9a* methyltransferase activity in preadipocytes de-represses *PPAR* γ expression but has no effect on *Wnt10a* expression (Figure 7C); removal of H3K27me3 by deletion of H3K27 methyltransferase *Ezh2* de-represses *Wnt* genes but has no effect on *PPAR* γ expression in preadipocytes (Wang *et al*, 2010). These results suggest that although both H3K9me2 and H3K27me3 associate with gene repression, they silence distinct sets of target genes in preadipocytes. Consistently, despite the loss of H3K9me2 during adipogenesis, H3K27me3 level remains low on the *PPAR* γ locus (Figure 3A).

Interestingly, removal of H3K9me2 by *G9a* deletion leads to marked upregulation of H3K9ac both globally and on *PPAR* γ

Figure 9 Deletion of *G9a* increases adipose tissue weight in mice. Adipose-specific *G9a* knockout (KO) mice carried the genotype of $G9a^{flox/flox};aP2-Cre$. Other age-matched littermates were used as control (Con) (see Materials and methods). All data were from 16- to 22-week-old male mice. Female mice data are shown in Supplementary Figure S7B and C. (A) Representative pictures of Con and KO mice. (B) Growth curves of Con ($n = 10$) and KO ($n = 7$) mice fed with normal chow. (C) Increased fat mass in KO mice. Body composition was measured in non-anaesthetized mice using the Bruker Minispec NMR analyzer. (D–G) Increased adipose tissue weights and adipocyte sizes in KO mice. (D) Representative pictures of epididymal WAT (epi-WAT), inguinal WAT (ing-WAT) and interscapular BAT isolated from Con and KO mice. (E) The average tissue weights in Con ($n = 8$) and KO ($n = 6$) mice are presented as % of body weight. (F, G) H&E staining of paraffin sections of ing-WAT from Con and KO mice are shown in (F). The cell sizes in the sections were quantified with ImageJ (G). (H) qRT-PCR analysis of gene expression in ing-WAT of Con ($n = 8$) and KO ($n = 8$) mice. (I) Model on how *G9a* represses adipogenesis. *G9a* inhibits *PPAR* γ expression by adding H3K9me2 on the entire *PPAR* γ locus while facilitates *Wnt10a* expression independent of HMT activity. All values are presented as the mean $\pm$ s.e.m. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$. Similar results were obtained from male and female mice, but only the male mice data are shown here.

promoter (Figures 4E and 6B). We have shown previously that H3K9ac correlates well with, but is dispensable for, nuclear receptor target gene expression, suggesting that H3K9ac is a consequence, rather than a cause, of gene activation (Jin *et al*, 2011). Whether the increased H3K9ac in $G9a^{-/-}$ cells promotes *PPAR* γ expression or is simply a marker for the active *PPAR* γ promoter remains to be investigated.

Selective enrichment of H3K9me2 on *PPAR* γ locus in preadipocytes

Among gene loci encoding the major positive and negative regulators of adipogenesis, *G9a*-mediated H3K9me2 is selectively enriched on the *PPAR* γ locus in preadipocytes, suggesting a rather specific role of *G9a*-mediated H3K9me2 in repressing *PPAR* γ expression. *PPAR* γ is a direct target of *G9a* and deletion of *G9a* in preadipocytes increases expression of *PPAR* γ but not other positive regulators of

adipogenesis (Figure 6A and B). Consistently, inhibiting *G9a* activity decreased H3K9me2 level on *PPAR* γ locus and increased *PPAR* γ expression in preadipocytes. Future studies will be needed to identify the mechanism that targets *G9a* to *PPAR* γ locus in preadipocytes. Such a mechanism likely plays an important role in repressing *PPAR* γ expression and adipogenesis.

G9a-mediated H3K9me2 represses not only the basal-level expression of *PPAR* γ but also the induction of *PPAR* γ . Removal of H3K9me2 by *G9a* deletion increases H3K9ac and chromatin opening, which facilitate binding of upstream adipogenic transcription factor C/EBP β to the proximal *PPAR* γ 2 promoter to promote *PPAR* γ 2 expression. These results correlated well with the recent observations that active acetylation mark, chromatin opening and C/EBP β recruitment are critical for *PPAR* γ 2 induction in the early phase of adipogenesis (Steger *et al*, 2010; Siersbaek *et al*, 2011). A similar mechanism may work on the *PPAR* γ 1

promoter because of the increased chromatin opening on both *PPAR* γ 1 and *PPAR* γ 2 promoters and the markedly enhanced induction of both *PPAR* γ 1 and *PPAR* γ 2 during adipogenesis of *G9a*^{-/-} preadipocytes, although it is presently unclear which upstream adipogenic transcription factor directly activates *PPAR* γ 1 promoter during adipogenesis.

Maintaining *Wnt10a* expression by *G9a* in preadipocytes

Although *Wnt10a* expression is repressed by H3K27me3 in preadipocytes (Wang *et al*, 2010), it is detectable and functional. It has been shown that overexpression of *Wnt10a* inhibits adipogenesis while knockdown of *Wnt10a* in preadipocytes promotes adipogenesis (Cawthorn *et al*, 2012). Our microarray and qRT-PCR data show that *Wnt10a* expression is detectable in preadipocytes but decreases after deletion of *G9a* (Supplementary Figure S5A and B).

Several studies have shown that in addition to gene repression through its H3K9 methyltransferase activity, *G9a* also promotes gene activation independent of its enzymatic activity (Lee *et al*, 2006; Chaturvedi *et al*, 2009). In preadipocytes, *G9a* maintains *Wnt10a* expression independent of its HMT activity. Consistently, deletion of *G9a* in preadipocytes decreases *Wnt10a* expression while inhibiting *G9a* enzymatic activity has no effect. *Wnt10a* expression decreases during adipogenesis (Cawthorn *et al*, 2012), which correlates well with the decrease in *G9a* protein level during adipogenesis. *G9a* has been shown to cooperate with transcription co-activators GRIP1, CARM1 and p300 (Lee *et al*, 2006). Whether *G9a* cooperates with these co-activators to promote *Wnt10a* expression in preadipocytes remains to be investigated. Nonetheless, the decreased expression of *Wnt10a* and other *Wnts* in *G9a*^{-/-} preadipocytes, which reduces cytosolic β -catenin protein level and attenuates *Wnt* signalling (Figure 6H and I), would synergize with the increased expression of *PPAR* γ to promote adipogenesis.

Repression of adipogenesis by *G9a* and H3K9me2

During adipogenesis, *G9a* protein level decreases markedly whereas *G9a* mRNA level decreases about 40% (Figure 3C and D), suggesting that *G9a* protein and *G9a* mRNA are differentially regulated and that *G9a* is mainly regulated at the protein level during adipogenesis. A recent paper shows that DNA damage signalling triggers degradation of HMTs *G9a*/GLP through APC/C in senescent cells and causes a global decrease in H3K9me2 (Takahashi *et al*, 2012). Whether a similar mechanism is employed in the downregulation of *G9a* protein during adipogenesis remains to be determined. While H3K9me2 is largely invariant between ES cells and derived neurons (Lienert *et al*, 2011), H3K9me2 decreases markedly during adipogenesis, not only on *PPAR* γ locus but also globally. This is due to, at least in part, the global decrease in H3K9 di-methyltransferase *G9a* protein.

Our data do not exclude the involvement of demethylases in removal of H3K9me2 on *PPAR* γ locus and induction of *PPAR* γ especially in the early phase of adipogenesis. Consistent with this possibility, a recent paper reported that knockdown of H3K4/K9 demethylase LSD1 in 3T3-L1 preadipocytes leads to increased H3K9me2 on *C/EBP* α promoter,

decreased *C/EBP* α expression in preadipocytes and impaired adipogenesis (Musri *et al*, 2010), although it was unclear whether LSD1 regulates H3K9me2 on *PPAR* γ promoter. Our study also does not rule out the possibility that *G9a* and/or H3K9 methylation may regulate expression of *PPAR* γ target genes important for adipogenesis. Consistent with this possibility, SetDB1, the enzyme that performs H3K9me3, has been shown to methylate H3K9 to repress *PPAR* γ target gene activation (Takada *et al*, 2007).

In addition to H3K9me2, *G9a* has been shown to mediate H3K27me2 (Tachibana *et al*, 2001). We found that consistent with a previous report (Chaturvedi *et al*, 2009), deletion of *G9a* results in ~2-fold decrease in the high H3K27me2 level on *Ey-globin* promoter. However, H3K27me2 level is low on the *PPAR* γ promoter and decreases very mildly after deletion of *G9a* (Supplementary Figure S5D). Unlike H3K9me2, which decreases dramatically on *PPAR* γ promoter after adipogenesis (Figure 3B), the low H3K27me2 level on *PPAR* γ promoter only decreases slightly after adipogenesis (Supplementary Figure S5D). These results suggest that *G9a*-mediated H3K9me2, but not H3K27me2, plays a major role in repressing *PPAR* γ expression and adipogenesis.

Increase of adipose tissue weight in *G9a* KO mice

By comparing *PPAR* γ and *aP2* expression patterns during adipogenesis of white and brown preadipocytes, we show that *aP2* expression is markedly induced well before *PPAR* γ expression reaches the maximal level (Supplementary Figure S6). Consistently, data from *ex vivo* experiments show that *aP2*-Cre starts to delete *G9a* allele before *PPAR* γ expression reaches the maximal level during adipogenesis (Figure 8B and C). The increased expression of *PPAR* γ and the decreased expression of *Wnt10a* were also found in the adipose tissue of *G9a* KO mice (Figure 9H). These results indicate that *aP2*-Cre-mediated deletion of *G9a* promotes *PPAR* γ expression and adipogenesis. Increased adipogenesis has been suggested to contribute to adipose tissue expansion and obesity (Spiegelman and Flier, 1996; Cristancho and Lazar, 2011; Sun *et al*, 2011). The enhanced adipogenesis in *G9a* KO cells likely contributes to the enhanced adipose development and increased adipose tissue weight in *G9a* KO mice. Importantly, the increased adipose tissue development and tissue weight observed in *G9a* KO mice is fundamentally different from the high fat diet-induced obesity.

Other mechanisms likely contribute to the phenotype in *G9a* KO mice. Cell senescence has been linked to adipocyte size and adipose tissue inflammation (Minamino *et al*, 2009). Consistent with our observation that *G9a* deletion leads to senescence in primary cells (Supplementary Figure S3), we observed moderate increases in cell senescence markers in the WATs of a subset of *G9a* KO mice (Supplementary Figure S7G and H). In addition, *G9a* KO mice show trend of increased food intake (Supplementary Figure S7E, $P = 0.056$). These results suggest that in addition to enhanced adipogenesis, cell senescence and increased food intake may also contribute to the increased adipose tissue weight in *G9a* KO mice. Thus, *G9a* deletion leads to alterations in multiple factors/pathways *in vivo*, which collectively contribute to the phenotype observed in *G9a* KO mice.

We previously show that the histone H3K4 methylation regulator PTIP is required for *PPAR* γ and *C/EBP* α induction

and adipogenesis (Cho *et al*, 2009), and that histone H3K27 methyltransferase Ezh2 uses its enzymatic activity to directly and constitutively repress *Wnt* genes to facilitate adipogenesis (Wang *et al*, 2010). Our current study indicates that H3K9 methyltransferase *G9a* represses adipogenesis by repressing *PPAR γ* expression and facilitating *Wnt10a* expression. Together, these data suggest that site-specific HMTs control the expression of master positive and negative regulators of adipogenesis.

Materials and methods

Plasmids and antibodies

Retroviral plasmids MSCVhygro-Cre and WZLneo-Cre have been described (Wang *et al*, 2010). The SV40T-expressing retroviral plasmid pBabepuro-large T was from Addgene (#14088). Full-length (1210aa) or truncated (1–1048aa) human G9a (also known as EHMT2) cDNAs with N-terminal FLAG tag were amplified by PCR from MGC clone BC020970 to add the missing N-terminal 14aa and were cloned into MSCVhygro to generate MSCVhygro-F-G9a and MSCVhygro-F-G9a Δ SET, respectively. The lentiviral shRNA plasmid pLKO.1 targeting *G9a* (clone ID TRCN0000054545) and shRNA control plasmid were purchased from Sigma. All plasmids were confirmed by DNA sequencing.

Mouse IgG (sc-2025), anti-C/EBP α (sc-61), anti-C/EBP β (sc-150x), anti-PPAR γ (sc-7273) and anti-p85 α subunit of PI3-kinase (sc-1637) antibodies were from Santa Cruz. Rabbit IgG (I-5006) and anti-FLAG (F3165) were from Sigma. Anti-RNA Pol II (Ab5408) and H3K9me1 (ab8896) were from Abcam. Anti-G9a for ChIP was kindly provided by Yoshihiro Nakatani (Harvard Medical School). Anti-G9a for western blot (09-071) and anti-GAPDH (mAb374) were from Millipore. Anti-H3K4me3 (07-473), anti-H3K9me2 (17-648) and anti-H3K27me3 (07-449) for ChIP and ChIP-Seq were from Millipore. Anti-H3K27me2 (#9728) was from Cell Signaling. Other histone methylation and acetylation antibodies have been described (Jin *et al*, 2011). All chemicals were from Sigma unless indicated.

Isolation of primary preadipocytes, immortalization, virus infection and adipogenesis assay

Primary brown preadipocytes were cultured in DMEM plus 20% fetal bovine serum (FBS). Immortalized brown preadipocytes and all other cells were routinely cultured in DMEM plus 10% FBS. Primary white preadipocytes were isolated as described (Cho *et al*, 2009). Primary brown preadipocytes were isolated from interscapular BATs of newborn *G9a^{flox/flox}* pups and immortalized using retroviral plasmid pBabepuro-large T as described (Wang *et al*, 2010). Retroviral infection of preadipocytes was done as described (Wang *et al*, 2010). Infection of primary MEFs and preadipocytes with adenoviruses expressing Cre and GFP (Ad5CMV-Cre-GFP) or GFP alone (Ad5CMV-GFP) was performed at 50 m.o.i. as described (Cho *et al*, 2009).

Adipogenesis of primary white preadipocytes and immortalized white preadipocyte cell line 3T3-L1 was carried out as described (Wang *et al*, 2010). For adipogenesis of brown preadipocytes, cells were plated at a density of 5×10^5 per 10 cm dish in differentiation medium (DMEM plus 10% FBS, 0.1 μ M insulin and 1 nM T3) 4 days before induction of adipogenesis. At day 0, cells were treated with differentiation medium supplemented with inducing reagents 0.5 mM IBMX, 2 μ g/ml dexamethasone, and 0.125 mM indomethacin (standard induction condition) or with 1/5 of inducing reagents (suboptimal condition). Two days later, cells were changed to the differentiation medium alone. The medium was replenished at 2-day intervals. At days 6–8 post induction, fully differentiated cells were either stained with Oil Red O or subjected to qRT-PCR of gene expression or western blot analysis.

ChIP-Seq and RNA-Seq

Two days after 3T3-L1 preadipocytes reached confluence (day 0), cells were treated with 1% formaldehyde for 10 min at room temperature. ChIP was performed by following a protocol from R Myers' laboratory (<http://www.hudsonalpha.org/myers-lab/protocols>). The sequencing libraries were constructed with ChIP-Seq DNA Sample Prep kit (Illumina). RNA-Seq libraries were prepared

as described (Wei *et al*, 2011). Both ChIP-Seq and RNA-Seq libraries were sequenced on Illumina HiSeq 2000 system. The ChIP-enriched regions were identified using SICER (Zang *et al*, 2009), an algorithm designed to capture domains of diffuse signals. The ChIP-Seq and RNA-Seq raw data sets were deposited in GEO database (accession no. GSE41455). GO analysis of H3K9me2-enriched genes was done using the FunNet program (Transcriptional Networks Analysis) (www.funnet.info). The details of the bioinformatic data analysis are described in Supplementary data.

Western blot, qRT-PCR, microarray and ChIP

Western blot and qRT-PCR were done as described (Cho *et al*, 2009). The cytosolic fraction of β -catenin was prepared as described (Wang *et al*, 2010). Taqman probes for qRT-PCR were from Applied Biosystems: *PPAR γ* (Mm00440945_m1), *C/EBP α* (Mm00514283_s1), *Wnt1* (Mm01300555_g1), *Wnt6* (Mm00437351_m1), *Wnt10a* (Mm00437325_m1) and *Wnt10b* (Mm00442104_m1). Data are presented as means $\pm$ s.d. Microarray analysis in SV40T-immortalized *G9a^{flox/flox}* brown preadipocytes was performed in duplicate on Mouse Genome 430 2.0 Array (Affymetrix) as described (Cho *et al*, 2009). Microarray data are deposited in NCBI GEO database (accession no. GSE41456). ChIP was performed as described (Wang *et al*, 2010). PCR quantitation of precipitated genomic DNA relative to inputs was performed in triplicates using Taqman or SYBR Green kits (Applied Biosystems). The sequences of Taqman and SYBR Green primers are listed in Supplementary Tables S2 and S3.

Generation of adipose tissue-specific *G9a* KO mice

Mice carrying two floxed *G9a* alleles (*G9a^{flox/flox}*) (Sampath *et al*, 2007) were crossed with an *ap2-Cre* transgenic mice line (He *et al*, 2003). The progeny was intercrossed to generate *G9a^{flox/flox}* mice carrying *ap2-Cre* transgene (*G9a^{flox/flox}; ap2-Cre*), which were used as adipose-specific *G9a* KO mice. Other littermates, *G9a^{flox/flox}* and *G9a^{flox/+}*, were used as control (Con). Mice were analysed after weaning. No statistically significant differences in the body and tissue weights were found among the control mice. Genotyping of *G9a^{flox/flox}* mice was described (Sampath *et al*, 2007). Mice carrying *ap2-Cre* transgene were detected by PCR using primers 5'-CTGT TTTTGACGTTTACCG-3' and 5'-ATGCTTCTGTCCGTTTGCCG-3' and the PCR product was 250 bp. The efficiency of *ap2-Cre*-mediated deletion of *G9a* gene in adipose tissues of KO mice was measured by western blot or quantitative PCR of genomic DNA using primers specific for the floxed exon 24 of *G9a* gene, and a Taqman probe specific for the *GAPDH* gene as internal control. After normalizing with *GAPDH* allele, the residual *G9a* allele in KO mice (*G9a^{flox/flox}; ap2-Cre*) was compared with that in the control mice. All mouse work was approved by the Animal Care and Use Committee of NIDDK, NIH.

Body composition, histology and blood chemistry

Body composition was measured in non-anaesthetized mice using the Bruker Minispec NMR analyzer (Bruker Optics). For RNA isolation, the adipose tissues dissected from mice were put into Trizol followed by homogenization and preparation. Dissected adipose tissues were directly fixed in 10% formalin and embedded in paraffin. The sliced 5 μ m sections were stained with hematoxylin and eosin and examined under Nikon Eclipse 80i microscope. β -Galactosidase staining of adipose tissue was performed using the Senescence Cells Histochemical Staining Kit (Sigma CS0030) except the fixation time was increased to 40 min and samples were stained for 3–4 h. Blood serum chemistry tests were done by NIDDK Mouse Metabolism Core following standard procedures.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Author contributions: KG conceived research; LW and KG designed research; LW, JEL and AB performed research; LW and KG analysed the data; SX, SG and WP analysed the ChIP-seq, RNA-Seq

and microarray data; LW and KG wrote the paper and all authors reviewed the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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