Histone H3K9 Methyltransferase G9a Represses PPAR Expression and Adipogenesis

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1st Editorial Decision 18 July 2012

Thank you for submitting your research manuscript (EMBOJ-2012-82375) to our editorial office. It has now been seen by three referees and their comments are provided below.

All reviewers appreciate your study and are in general supportive of publication in The EMBO Journal. Nevertheless, they do raise a number of key concerns, which should be addressed appropriately. Specifically, I would like to emphasize one important issue related to comment 1. of referee #1, in which s/he stresses that the increased adiposity that you observe in aP2-Cre x G9afl/fl mice is not related to altered adipogenesis and suggests to remove this data. I appreciate her/his point, and am curious to hear your opinion. Principally, I would prefer to retain in-vivo data in the manuscript, especially as they are valued by the other referees. However, given the striking concern of reviewer #1, it might be necessary to analyze additional features of the aP2-Cre x G9afl/fl mice that do address adipocyte differentiation phenotypes directly. I would be happy to discuss this possibility either on the phone or by e-mail according to your preference.

Overall, I would like to invite you to submit a suitably revised manuscript to The EMBO Journal that addresses all raised concerns in full. I should add that it is our policy to allow only a single major round of revision and that it is therefore important to address the raised concerns at this stage.

When preparing your letter of response to the referees’ comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html
Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1

The studies in this manuscript center on the function of H3K9me2 and G9a in the repression of PPARγ and adipogenesis. The key findings are:
1. The repressive chromatin mark H3K9me2 was highly enriched at the PPARγ locus in L1 preadipocytes, but not other known adipogenic transcription factors. 2. Global levels of H3K9me2 and of the H3K9 methyltransferase G9a decrease during adipogenesis of 3T3-L1 and immortalized brown preadipocytes. 3. G9a deficient brown preadipocytes showed reduced total H3K9me2 and increased H3K9ac. Inhibiting the activity or decreasing expression of G9a resulted in enhanced adipogenic potential while adding back G9a to knock-out cells reversed the phenotype. 4. G9a deletion in brown preadipocytes enhanced PPARγ expression prior to differentiation and enhanced PPARγ induction after adipogenic stimuli with increased C/EBPβ recruitment to the PPARγ locus. 5. Wnt10a expression was selectively reduced in G9a deficient cells and this inhibition did not require the enzymatic activity of G9a. 6. Adipose-specific deletion of G9a using aP2-Cre results in increased adipose mass, adipocyte size, and hepatic lipid accumulation in mice. This is an interesting paper that presents information about the dual nature of the G9a methyltransferase as it relates to adipogenesis. The experiments appear to be well-done, and the paper is nicely written and logical.

My concerns are:

1. The in vivo data is cute, but utterly irrelevant to the rest of the paper. The adipose-G9a KO mice are fat—what does this have to do with adipogenesis? Are the authors claiming that the increased adiposity of the mice is due to increased adipose differentiation? This cannot be true, as obesity is caused by increased food intake and/or reduced energy expenditure. There might be an interesting story in there about the role of G9a and thermogenesis or something similar, but it has nothing to with adipogenesis. These experiments should be removed from the manuscript.

2. The authors did a genome-wide analysis of H3K9me2, but they really don't present much data beyond the Pparg and Wnt10a loci. The authors need to do a much more comprehensive job of presenting the results of the H3K9me2 data. What genes and gene sets are associated with this mark? What is relationship between H3K9me2 and transcript levels globally? What other marks and TFs are associated with H3K9me2 (perhaps comparing to other published 3T3-L1 data sets)?

3. This paper extends previous findings that G9a can act in nonenzymatic ways, possibly by acting as a transcriptional co-factor. Some genes (like Pparg), require methyltransferase activity, while others (like Wnt10a) do not. This should be expanded upon. One way to do this would be to perform transcriptional profiling in G9a null cells, with and add-back of WT G9a or mutant G9a lacking enzymatic activity. 4. What is the role of GLP in all of this? Does add back of GLP to the G9a MEFs rescue the phenotype?

5. Why is global H3K9me3 unaffected by the loss of G9a? Isn't H3K9me1/2 a required substrate for H3K9me3?

6. Do G9a null cells spontaneously differentiate? Why do they need cocktail? 7. The equivalent of Fig 5H should be done for PPARγ, to show that the methyltransferase activity of G9a is required to repress PPARγ.
Referee #2

In this manuscript, Wang et al. have studied the role of the methyltransferase G9a in the regulation of adipogenesis. Through comparative analysis of H3K9me2, H3K27me3 and H3K4me3 genomic profiles in 3T3-L1 preadipocytes, they identified high levels of H3K9me2 (but not H3K27me3) throughout the PPARg locus, which is a positive regulator of adipogenesis. In contrast they do not detect H3K9me2 on negative regulators of adipogenesis such as Wnt10a. Furthermore, they found that during induction of adipogenesis there is a decrease in G9a levels, which correlates with increased transcription of the PPARg gene as well as a decrease in H3K9me2 (both bulk levels of H3K9me2 and H3K9me2 levels on the PPARg genes), suggesting that G9a might repress PPARg in preadipocytes. Next, using immortalized G9a-/- brown preadipocytes, the authors showed that the KO of G9a promotes the induction of adipogenic transcription factors (e.g. PPARg) and enhances adipogenesis. These effects can be rescued by ectopically expressing G9a, and overexpression of G9a inhibits adipogenesis. Looking at some specific genes, the authors found that in G9a KO cells, transcription of the PPARg gene is increased. Furthermore, this is accompanied by a decrease in the H3K9me2 mark on both PPARg1 and g2. Finally, the authors show by ChIP that G9a is bound to the promoters of both PPARg1 and g2. This suggests that G9a directly represses PPARg1 and 2 in preadipocytes. Furthermore, the authors show by FAIRE analysis that the KO of G9a is accompanied by an increase in chromatin accessibility at a specific region on the PPARg2 promoter, and an increase in binding of CEBPb to this region. In contrast to PPARg, RTqPCR data seems to indicate that transcription of the Wnt10a gene is decreased in G9a-/- preadipocytes, although this result is surprising/questionable (see Point1 below). This would suggest that G9a could be involved in activating the Wnt10a gene. Furthermore, rescue experiments using G9a and a mutant with a deletion in its SET domain appear to suggest that this activating function is independent of G9a methyltransferase activity. Finally, G9a is bound to Wnt10a gene as shown by ChIP suggesting direct regulation.

The BIX inhibitor of G9a was also used to confirm the role of G9a in repressing PPARg. Finally, an adipose-specific G9a KO mouse was generated, which displays enhanced adipose tissue development. This last data is a nice in vivo confirmation that G9a represses adipogenesis. Overall, this manuscript provides important new insights into the role of G9a in controlling adipogenesis. Data obtained both in cell lines and in transgenic mice complement each other. Mechanistically, the authors provide convincing data to show that G9a's role in repressing adipogenesis is mediated (at least partially) through the repression of PPARg. However, the role of G9a in the activation of Wnt10a gene is less convincing (see Point1 below). This is a major point to address as it relates directly to the main model proposed by the authors. In addition, several additional points should be addressed (detailed below).

Major Points:

1) In Fig.1F ChIP-seq data show that in preadipocytes, the Wnt10a gene is highly enriched in the repressive mark H3K27me3 with no enrichment in H3K4me3. Similarly in Fig2 A ChIPqPCR result shows a high level of H3K27me3 on this gene. According to these data, the Wnt10a gene should be completely turned off in preadipocytes. However this contradicts the RTqPCR results on Fig.5 F and H, which appear to indicate that the Wnt10a gene is active in preadipocytes. Could the authors clarify this discrepancy?

This is a very important point to address since one of the main conclusions of this paper is that G9a activates the Wnt10a gene in preadipocytes. If the Wnt10a gene is not transcribed in these cells, this would contradict the model shown on Fig.7I.

2) It has now been firmly established that in addition to H3K9, G9a is able to mediate dimethylation of H3K27, both in vitro (Tachibana JBC 2001 Jul 6;276(27):25309-17) and in vivo (Wu et al. Cell Res. 2011 Feb;21(2):365-7). Furthermore, a decrease in H3K27me2 bulk levels is visible on Fig.3E. At minimum, the authors should examine the H3K27me2 mark by ChIP on the PPARg gene locus during adipogenesis in WT and G9a-/- cells. Ideally, H3K27me2 ChIP-seq data in the preadipocyte cell line could be added to Fig.1 (to remain consistent with this figure), but this is not absolutely necessary since the paper focuses mostly on the PPARg locus.

3) A rescue experiment is performed on Fig.5H to show that G9a can activate the Wnt10a gene in a
methyltransferase-independent manner. However, no experiment is performed to test whether the methyltransferase activity of G9a is required for the repression of the PPARγ genes. This conclusion is only inferred indirectly by measuring the H3K9me2 mark. The authors should test directly if the SET domain of G9a is required for 1) the repressive function of G9a towards PPARγ and 2) for the rescue of adipogenesis (in parallel to the rescue done with wild type G9a in Figure S5). Importantly, this last experiment will also allow the authors to address the fundamental question of the regulation of the Wnt10a gene by G9a (raised in Point1). Indeed, if the model presented on Fig7I is correct, one would expect only a partial rescue of the phenotype by G9a-deltaSET with the Wnt10a gene not being rescued.

4) A detailed analysis was performed on the PPARg2 gene in G9a-/- preadipocytes, including FAIRE analysis and CEBPβ binding, suggesting that G9a prevents chromatin opening on the PPARg2 promoter (Fig.5). However the authors have not tested if chromatin opening also occurs on the PPARg1 gene in G9a-/- cells (using FAIRE analysis) even though they have studied the binding of G9a and various histone marks on both PPARg1 and g2 (Fig.5B). A FAIRE analysis should be performed on PPARg1.

Minor Points:

1) p.6 lane 4: "corrected" should be "correlated"

2) Fig.1D: the figure legend indicates that the horizontal line is the gene expression level. On the figure, it seems to be the opposite.

3) p.9 Figure S7 should be Figure S6.

Referee #3

This is an interesting, scientifically well-designed study demonstrating essential roles of histone methyltransferase (HMT) G9a in adipogenesis as a regulator of PPARγ. The critical point of the study is that, among various adipogenic regulators, PPARγ is uniquely characterized by repressive H3K9me2 mark in its entire gene locus, and H3K9me2 and G9a levels decrease during adipogenesis. The authors demonstrate the decrease of G9a levels plays a crucial role in the induction of PPARγ expression during adipogenesis by using a combination of gain-of-function and loss-of-function studies as well as a chemical inhibitor of HMT. Another interesting finding of the paper is the dual roles of G9a in the regulation of adipogenesis: One is HMT-dependent repression of PPARγ expression and the other is HMT-independent activation of Wnt genes. Finally, the regulatory action of G9a in adipogenesis in vivo is demonstrated by using aP2-Cre-specific G9a conditional knock-out mice. All experiments were carefully performed and evidences are strong. There are several points, however, that need to be addressed in order to justify the conclusions of the study.

Major points:

1) Figure 1E and S1 show clear enrichment of H3K9me2 spanning the entire PPARγ locus. There is, however, relatively large region that is devoid of the H3K9me2 signal around the PPARγ1 promoter region. In the subsequent ChIP-qPCR experiments (Fig. 1G, 2, 5), the authors examined H3K9me2 around the PPARγ1 promoter (+/- 2kb), but not in distal regions. Therefore, the authors are not showing the same genomic region in the ChIP-seq and ChIP-qPCR experiments. Reduction of H3K9me2 enrichment (Fig. 2A) is one of the critical findings of this study. Is this reduction of the enrichment located in the empty promoter region in Fig. 1E? Is the reduction of H3K9me2 enrichment also seen in the entire PPARγ locus? To examine this possibility, it is necessary to do ChIP-qPCR at multiple distal regions. It is also important to show more zoomed ChIP-seq data around the PPARγ1 promoter. How relevant is the relatively low H3K9me2 enrichment at PPARγ promoters and its reduction, when compared to very strong enrichment observed in the rest of the gene locus?
2) Related to #1, there is no description about the locations of ChIP-qPCR measurement near PPARγ1 and PPARγ2 genes (Fig. 1G, 5B, 6). Information has to be included in the figure or in the legends.

3) In aP2-Cre:G9a fl/fl mice, the G9a will be deleted relatively at late phase of adipogenesis. Since the reduction of G9a is so robust (it goes down to almost undetectable levels, Fig. 2D and 4B), one can imagine that the deletion of G9a in the late phase of adipogenesis may not be as effective as in vitro experiments with G9a deleted from the beginning (Fig. 4-6). Furthermore, as the authors examined, it is reported that G9a deletion affect senescence, which was demonstrated to be linked to adipocyte size and adipose tissue inflammation (10.1038/nm.2014), which may suggest contribution of an indirect action of G9a deletion on the adipose tissue phenotype through the modulation of senescence. To address these issues, the following experiments are recommended.

- **In vitro experiment:** Deletion of G9a at later phase. This can be performed by adding Ad-Cre at later time points of adipogenesis of immortalized MEF.

- **Ex vivo experiment:** More simply, the authors can perform adipogenesis assay using MEFs or adipose tissue SVF directly isolated from aP2-Cre:G9a fl/fl mice.

- **In vivo experiment,** aP2-Cre:G9a fl/fl mice: The authors should measure senescence-associated factors such as Ink4a, p53 etc and to perform senescence-associated b-gal staining.

**Minor points:**

4) Food intake measurement must be presented.

5) G9a protein levels completely decreased to zero whereas G9a mRNA level was more than half. Are they differentially regulated?

6) Fig. 6A. It is better to include G9a protein blot as a control.

1st Revision - authors' response 13 October 2012

**Response to Referees**

We thank referees for the favorable and highly constructive comments. We have addressed all of referees’ comments in the revised version. Here is a summary of major changes.

1. Using the new-generation illumina sequencer HiSeq 2000, we have done ChIP-Seq of H3K9me2 in 3T3-L1 adipocytes (day 7) and repeated ChIP-Seq of H3K9me2 in 3T3-L1 preadipocytes (day 0). The new ChIP-Seq data in preadipocytes (day 0) are highly consistent with the previous one but provide 4-5-fold higher sequencing reads and thus much better coverage. Using the new ChIP-Seq data, we re-plotted Figure 1A-F and Figure 2 (previously Figure S1). We also show the inverse correlation of global H3K9me2 levels with H3K36me3 and H3K27ac levels (the new Figure S1), loss of H3K9me2 on the entire PPARγ locus in adipocytes (the new Figure 3B), and GO analysis of H3K9me2 enriched genes in preadipocytes (the new Figure S1B).

2. We have done RNA-Seq in 3T3-L1 preadipocytes. The semi-quantitative microarray data in Figure 1D was replaced with the quantitative RNA-Seq data. The new plot shows stronger inverse correlation between gene expression and H3K9me2 levels.
3. We confirmed that G9a represses PPARγ expression in an enzymatic activity-dependent manner (the new Figure 6C).

4. We performed microarray analysis of gene expression in G9aΔ/Δ and G9a KO preadipocytes (the new Figure S5A). We also examined G9a enzymatic activity-dependent and -independent gene expression by qRT-PCR (the new Figure S5B).

5. In the new Figure S6, we show that aP2 expression is induced at ~day 1-2 of adipogenesis, well before PPARγ expression reaches the maximal level. In ex vivo experiments, we induced adipogenesis in primary white preadipocytes isolated from G9aΔ/Δ,aP2-Cre mice. The results indicate that aP2-Cre-mediated deletion of G9a clearly contributes to the enhanced PPARγ expression and adipogenesis (the new Figure 8).

6. We analyzed additional features of the G9a KO mice.
1) Food intake data (the new Figure S7E).
2) We observed moderate increases of cell senescence markers in the white adipose tissues of a subset of G9a KO mice, suggesting that senescence may contribute to the increased adiposity in G9a KO mice (the new Figure S7G-H).

**Referee #1**

“The studies in this manuscript center on the function of H3K9me2 and G9a in the repression of PPARγ and adipogenesis. The key findings are:
1. The repressive chromatin mark H3K9me2 was highly enriched at the PPARγ locus in L1 preadipocytes, but not other known adipogenic transcription factors. 2. Global levels of H3K9me2 and of the H3K9 methyltransferase G9a decrease during adipogenesis of 3T3-L1 and immortalized brown preadipocytes. 3. G9a deficient brown preadipocytes showed reduced total H3K9me2 and increased H3K9ac. Inhibiting the activity or decreasing expression of G9a resulted in enhanced adipogenic potential while adding back G9a to knock-out cells reversed the phenotype. 4. G9a deletion in brown preadipocytes enhanced PPARγ1 expression prior to differentiation and enhanced PPARγ2 induction after adipogenic stimuli with increased C/EBPβ recruitment to the PPARγ2 locus. 5. Wnt10a expression was selectively reduced in G9a deficient cells and this inhibition did not require the enzymatic activity of G9a. 6. Adipose-specific deletion of G9a using aP2-Cre results in increased adipose mass, adipocyte size, and hepatic lipid accumulation in mice. This is an interesting paper that presents information about the dual nature of the G9a methyltransferase as it relates to adipogenesis. The experiments appear to be well-done, and the paper is nicely written and logical.”

1. “The in vivo data is cute, but utterly irrelevant to the rest of the paper. The adipose-G9a KO mice are fat-what does this have to do with adipogenesis? Are the authors claiming that the increased adiposity of the mice is due to increased adipose differentiation? This cannot be true, as obesity is caused by increased food intake and/or reduced energy expenditure. There might be an interesting story in there about the role of G9a and thermogenesis or something similar, but it has nothing to with adipogenesis. These experiments should be removed from the manuscript.”

RE: We greatly appreciate referee #1’s comments. However, we do not agree with the point 1.

First, what we observed in the adipose-G9a KO mice is the increased adipose tissue development and tissue weight, which is fundamentally different from the high fat diet-induced obesity.
Second, several reviews from leading experts have pointed out a possible contribution of altered adipogenesis to obesity. A recent review states that "adipogenesis probably has a role in the pathology of obesity" (Cristancho & Lazar, 2011). Another one mentions that "adipocyte hyperplasia and hypertrophy can both contribute to AT (adipose tissue) expansion" (Sun et al, 2011). Another earlier review states that "increased adipogenesis is most commonly associated with obesity" (Spiegelman & Flier, 1996).

Thus, it is possible that the enhanced PPARγ expression and enhanced adipogenesis in G9a KO cells contribute to the enhanced adipose development in G9a KO mice. Please also note that we are not claiming that the phenotype of G9a KO mice is solely due to enhanced PPARγ expression and adipogenesis. Other mechanisms likely contribute to the phenotype. For example, moderately increased cell senescence is found in a subset of G9a KO mouse adipose tissues (the new Figure S7G-H), which may contribute to the increased adiposity. We have included a new paragraph in the Discussion on the possible mechanisms that contribute to the phenotype of G9a KO mice.

2. “The authors did a genome-wide analysis of H3K9me2, but they really don't present much data beyond the Pparg and Wnt10a loci. The authors need to do a much more comprehensive job of presenting the results of the H3K9me2 data. What genes and gene sets are associated with this mark?”

RE: We have done gene ontology (GO) analysis of H3K9me2-enriched genes in 3T3-L1 preadipocytes (the new Figure S1B). GO identified the immune and inflammatory response genes, which have been shown to be directly regulated by G9a and H3K9me2 (Fang et al, 2012). The raw ChIP-Seq data are deposited in GEO database under accession number GSE41455.

In Figure 2, we show that H3K9me2 levels are high on PPARγ locus but are low on gene loci encoding other adipogenic transcription factors, including C/EBPα, C/EBPβ, C/EBPδ, KLF4, Krox20 and CREB (Figure 2A-G). H3K9me2 levels are 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).

“What is relationship between H3K9me2 and transcript levels globally? What other marks and TFs are associated with H3K9me2 (perhaps comparing to other published 3T3-L1 data sets)?”

RE: As shown in the updated Figure 1D, there is an inverse correlation between H3K9me2 and transcript levels globally. We have shown in Figure 1B-C that H3K4me3 and H3K27me3 levels inversely correlate with H3K9me2 level globally. Using 3T3-L1 ChIP-Seq datasets published in (Mikkelsen et al, 2010), we now show in the new Figure S1A that H3K9me2 inversely correlates with active epigenetic marks H3K27ac and H3K36me3 globally.

In the new Figure S1C, we include ChIP-seq data of H3K4me3, H3K9me2, H3K27me3, H3K27ac, H3K36me3 and CTCF on PPARγ locus in 3T3-L1 preadipocytes (H3K27ac, H3K36me3 and CTCF data are from (Mikkelsen et al, 2010))

3. “This paper extends previous findings that G9a can act in nonenzymatic ways, possibly by acting as a transcriptional co-factor. Some genes (like Pparg), require methyltransferase activity, while others (like Wnt10a) do not. This should be expanded upon. One way to do this would be to perform transcriptional profiling in G9a null cells, with and add-back of WT G9a or mutant G9a lacking enzymatic activity.”
RE: We have done microarray analysis of gene expression in G9a KO brown preadipocytes (NCBI GEO accession# GSE41456, summarized in the new Figure S5A). Microarray data confirmed the upregulation of PPARγ and down-regulation of Wnt10a in G9a KO brown preadipocytes. Interestingly, microarray data also show increased expression of immune and inflammatory response genes, which is consistent with a recent report (Fang et al, 2012).

We picked ~15 genes each of up-regulated and down-regulated genes from the microarray data and did qRT-PCR in G9a null cells, with the add-back of WT G9a or mutant G9a lacking enzymatic activity. The results show that the majority of up-regulated genes are G9a enzymatic activity-dependent and most of the down-regulated genes are HMT-independent (representative genes are shown in Figure S5B).

4. “What is the role of GLP in all of this? Does add back of GLP to the G9a MEFs rescue the phenotype?”

RE: It has been shown that GLP and G9a need to form a hetero-dimer to methylate H3K9 and that G9a or GLP alone is incapable of catalyzing H3K9 methylation [Figure 7 in (Tachibana et al, 2008)]. Therefore, adding back GLP to G9a−/− cells will not rescue the phenotype.

5. “Why is global H3K9me3 unaffected by the loss of G9a? Isn't H3K9me1/2 a required substrate for H3K9me3?”

RE: This is due to the distinct localization of me1, me2 and me3 of H3K9 on chromatin. H3K9me1 and H3K9me2 are enriched in silent domains within euchromatin while H3K9me3 is enriched at pericentric heterochromatin (Rice et al, 2003). Previous studies have demonstrated that G9a is the histone methyltransferase (HMT) responsible for the majority of global H3K9me1/2 and that Suv39h1/h2 are HMTs crucial for the global of H3K9me3. Similar to our results in preadipocytes, deletion of G9a in ES cells has little effect on the global H3K9me3 level (Peters et al, 2003; Rice et al, 2003; Tachibana et al, 2002).


RE: G9a null cells do not differentiate spontaneously (at least in a large fraction of the population) within 2 weeks. The 2-3 fold increase of the basal PPARγ1 level in G9a null cells is insufficient to cause spontaneous adipogenesis. We have shown before that even when PPARγ is ectopically over-expressed, the adipogenic cocktail is still needed to stimulate MEFs to differentiate homogeneously within 6-10 days (Ge et al, 2002).

7. “The equivalent of Fig 5H should be done for PPARγ, to show that the methyltransferase activity of G9a is required to repress PPARγ.”

RE: We show in the new Figure 6C and S5B that the methyltransferase activity of G9a is required to repress PPARγ expression.

8. “In a follow-up correspondence with referee #1, s/he suggested that to show an effect on adipogenesis in vivo, one could analyze the mass of the BAT depot as well as the developing subcutaneous WAT in G9a KO embryos to avoid effects arising with food intake and energy expenditure. I am not certain how feasible you will deem these experiments, but I nevertheless wanted to pass on the suggestion.”
RE: Since WAT only develops after birth, we are unable to analyze the WAT in G9a KO embryos. Since BAT develops in the late stage of embryogenesis, we were able to examine the interscapular BAT in newborn pups by checking the expression of adipogenesis markers. As shown in the new Figure S7F, expression of PPARγ, C/EBPα and aP2 increases in the BAT from newborn G9a KO pups, suggesting enhanced adipogenesis. To address this comment, a better approach is the ex vivo experiment (see response to referee #3 point #3).

Referee #2

“In this manuscript, Wang et al. have studied the role of the methyltransferase G9a in the regulation of adipogenesis. Through comparative analysis of H3K9me2, H3K27me3 and H3K4me3 genomic profiles in 3T3-L1 preadipocytes, they identified high levels of H3K9me2 (but not H3K27me3) throughout the PPARγ locus, which is a positive regulator of adipogenesis. In contrast they do not detect H3K9me2 on negative regulators of adipogenesis such as Wnt10a. Furthermore, they found that during induction of adipogenesis there is a decrease in G9a levels, which correlates with increased transcription of the PPARγ gene as well as a decrease in H3K9me2 (both bulk levels of H3K9me2 and H3K9me2 levels on the PPARγ genes), suggesting that G9a might repress PPARγ in preadipocytes. Next, using immortalized G9a−/− brown preadipocytes, the authors showed that the KO of G9a promotes the induction of adipogenic transcription factors (e.g. PPARγ) and enhances adipogenesis. These effects can be rescued by ectopically expressing G9a, and overexpression of G9a inhibits adipogenesis. Looking at some specific genes, the authors found that in G9a KO cells, transcription of the PPARγ gene is increased. Furthermore, this is accompanied by a decrease in the H3K9me2 mark on both PPARγ1 and γ2. Finally, the authors show by ChIP that G9a is bound to the promoters of both PPARγ1 and γ2. This suggests that G9a directly represses PPARγ1 and γ2 in preadipocytes. Furthermore, the authors show by FAIRE analysis that the KO of G9a is accompanied by an increase in chromatin accessibility at a specific region on the PPARγ2 promoter, and an increase in binding of CEBPβ to this region. In contrast to PPARγ, RTqPCR data seems to indicate that transcription of the Wnt10a gene is decreased in G9a−/− preadipocytes, although this result is surprising/questionable (see Point1 below). This would suggest that G9a could be involved in activating the Wnt10a gene. Furthermore, rescue experiments using G9a and a mutant with a deletion in its SET domain appear to suggest that this activating function is independent of G9a methyltransferase activity. Finally, G9a is bound to Wnt10a gene as shown by ChIP suggesting direct regulation. The BIX inhibitor of G9a was also used to confirm the role of G9a in repressing PPARγ. Finally, an adipose-specific G9a KO mouse was generated, which displays enhanced adipose tissue development. This last data is a nice in vivo confirmation that G9a represses adipogenesis.

Overall, this manuscript provides important new insights into the role of G9a in controlling adipogenesis. Data obtained both in cell lines and in transgenic mice complement each other. Mechanistically, the authors provide convincing data to show that G9a’s role in repressing adipogenesis is mediated (at least partially) through the repression of PPARγ. However, the role of G9a in the activation of Wnt10a gene is less convincing (see Point1below). This is a major point to address as it relates directly to the main model proposed by the authors. In addition, several additional points should be addressed (detailed below).”

RE: We greatly appreciate referee #2’s comments.

1) “In Fig.1F ChIP-seq data show that in preadipocytes, the Wnt10a gene is highly enriched in the repressive mark H3K27me3 with no enrichment in H3K4me3. Similarly in Fig2 A ChIPqPCR result shows a high level of H3K27me3 on this gene. According to these data, the Wnt10a gene should be completely turned off in preadipocytes. However this contradicts the RTqPCR results on Fig.5 F and H, which appear to indicate that the Wnt10a gene is active in preadipocytes. Could the authors clarify this discrepancy? This is a very important point to address since one of the main conclusions of this paper is that G9a activates the Wnt10a gene in preadipocytes. If the Wnt10a gene is not transcribed in these cells, this would contradict the model shown on Fig.7I.”
RE: We appreciate the referee’s comments and would like to clarify this point.

We do not know why H3K4me3, a mark usually found on active promoters, is absent from Wnt10a proximal promoter. However, close examination of H3K4me3 ChIP-Seq profile reveals a strong H3K4me3 peak at the 3’ end of Wnt10a gene locus (Figure 1F). Whether this 3’ H3K4me3 peak regulates or reflects Wnt10a expression remains unclear. Nevertheless, our own data as well as data from the literature clearly indicate that Wnt10a is expressed in preadipocytes and is functional:

1) Although Wnt10a expression level in preadipocytes is relatively low due to the high level of H3K27me3, it is detectable. Our microarray data show that Wnt10a expression is detectable in preadipocytes but decreases after deletion of G9a (the new Figure S5A). This is confirmed by qRT-PCR using Wnt10a Taqman probe (Figures 6 and S5B).

2nd, a recent report from Ormond MacDouglad lab shows that Wnt10a is expressed in preadipocytes but decreases during adipogenesis (Figure 1A in (Cawthorn et al, 2012a)) and that knockdown of Wnt10a in preadipocytes promotes adipogenesis (Figure 4 in (Cawthorn et al, 2012a)).

3rd, several other reports from different labs also confirm that Wnt10a is indeed expressed both in brown and white preadipocytes (Cawthorn et al, 2012b; Pacenti et al, 2006; Tseng et al, 2005; Tseng et al, 2008).

2) “It has now been firmly established that in addition to H3K9, G9a is able to mediate dimethylation of H3K27, both in vitro (Tachibana JBC 2001 Jul 6;276(27):25309-17) and in vivo (Wu et al. Cell Res. 2011 Feb;21(2):365-7). Furthermore, a decrease in H3K27me2 bulk levels is visible on Fig.3E. At minimum, the authors should examine the H3K27me2 mark by ChIP on the PPARγ gene locus during adipogenesis in WT and G9a−/− cells. Ideally, H3K27me2 ChIP-seq data in the preadipocyte cell line could be added to Fig.1 (to remain consistent with this figure), but this is not absolutely necessary since the paper focuses mostly on the PPARγ locus.”

RE: We now show ChIP of H3K27me2 on PPARγ and Ey-globin promoters in G9a+/− and G9a−/− cells before and after adipogenesis (the new Figure S5D). Consistent with a previous report (Chaturvedi et al, 2009), deletion of G9a results in ~2-fold decrease of the high H3K27me2 level on Ey-globin promoter. However, H3K27me2 level is low on the PPARγ promoter and decreases very mildly after deletion of G9a. Unlike H3K9me2, which decreases dramatically on PPARγ promoter after adipogenesis (Figure 3), the low H3K27me2 level on PPARγ promoter only decreases slightly after adipogenesis. These results suggest that G9a-mediated H3K9me2, but not H3K27me2, plays a major role in repressing PPARγ expression and adipogenesis (see Discussion).

3) “A rescue experiment is performed on Fig.5H to show that G9a can activate the Wnt10a gene in a methyltransferase-independent manner. However, no experiment is performed to test whether the methyltransferase activity of G9a is required for the repression of the PPARg genes. This conclusion is only inferred indirectly by measuring the H3K9me2 mark. The authors should test directly if the SET domain of G9a is required for 1) the repressive function of G9a towards PPARγ and 2) for the rescue of adipogenesis (in parallel to the rescue done with wild type G9a in Figure S5). Importantly, this last experiment will also allow the authors to address the fundamental question of the regulation of the Wnt10a gene by G9a (raised in Point1). Indeed, if the model presented on Fig7I is correct, one would expect only a partial rescue of the phenotype by G9a-deltaSET with the Wnt10a gene not being rescued.”

RE: As shown in the new Figure 6C, HMT activity of G9a is critical for repressing PPARγ expression. Adipogenesis assay shows a partial rescue of the phenotype by G9aΔSET in G9a KO preadipocytes, suggesting that the HMT activity of G9a is critical for repressing adipogenesis (the new Figure S5C).
4) “A detailed analysis was performed on the PPARg2 gene in G9a−/− preadipocytes, including FAIRE analysis and CEBPβ binding, suggesting that G9a prevents chromatin opening on the PPARg2 promoter (Fig.5). However the authors have not tested if chromatin opening also occurs on the PPARγ1 gene in G9a−/− cells (using FAIRE analysis) even though they have studied the binding of G9a and various histone marks on both PPARg1 and g2 (Fig.5B). A FAIRE analysis should be performed on PPARg1.”

RE: We have performed FAIRE analysis around PPARγ1 locus in G9aKO brown preadipocytes. The results show that chromatin opening on PPARγ1 promoter increases moderately after G9a deletion in cells (the new Figure 6D).

“Minor Points:
1) p.6 lane 4: "corrected" should be "correlated"
2) Fig.1D: the figure legend indicates that the horizontal line is the gene expression level. On the figure, it seems to be the opposite.
3) p.9 Figure S7 should be Figure S6.”

RE: We have corrected them in the revised manuscript.

Referee #3

“This is an interesting, scientifically well-designed study demonstrating essential roles of histone methyltransferase (HMT) G9a in adipogenesis as a regulator of PPARγ. The critical point of the study is that, among various adipogenic regulators, PPARγ is uniquely characterized by repressive H3K9me2 mark in its entire gene locus, and H3K9me2 and G9a levels decrease during adipogenesis. The authors demonstrate the decrease of G9a levels plays a crucial role in the induction of PPARγ expression during adipogenesis by using a combination of gain-of-function and loss-of-function studies as well as a chemical inhibitor of HMT. Another interesting finding of the paper is the dual roles of G9a in the regulation of adipogenesis: One is HMT-dependent repression of PPARγ expression and the other is HMT-independent activation of Wnt genes. Finally, the regulatory action of G9a in adipogenesis in vivo is demonstrated by using aP2-Cre-specific G9a conditional knock-out mice. All experiments were carefully performed and evidences are strong. There are several points, however, that need to be addressed in order to justify the conclusions of the study.”

RE: We greatly appreciate referee #3’s comments.

1) “Figure 1E and S1 show clear enrichment of H3K9me2 spanning the entire PPARγ locus. There is, however, relatively large region that is devoid of the H3K9me2 signal around the PPARγ1 promoter region. In the subsequent ChIP-qPCR experiments (Fig. 1G, 2, 5), the authors examined H3K9me2 around the PPARγ1 promoter (+/- 2kb), but not in distal regions. Therefore, the authors are not showing the same genomic region in the ChIP-seq and ChIP-qPCR experiments. Reduction of H3K9me2 enrichment (Fig. 2A) is one of the critical findings of this study. Is this reduction of the enrichment located in the empty promoter region in Fig. 1E? Is the reduction of H3K9me2 enrichment also seen in the entire PPARγ locus? To examine this possibility, it is necessary to do ChIP-qPCR at multiple distal regions.

It is also important to show more zoomed ChIP-seq data around the PPARγ1 promoter.

How relevant is the relatively low H3K9me2 enrichment at PPARγ promoters and its reduction, when compared to very strong enrichment observed in the rest of the gene locus?”

RE: Because of the wide-spread distribution of H3K9me2 in the genome, a lot more sequencing reads are needed for generating H3K9me2 ChIP-Seq plots than for H3K4me3 and H3K27me3. The
H3K9me2 gap on PPARγ1 promoter in the previous manuscript was due to insufficient reads from illumina GAII sequencer. To better address this issue, we have repeated H3K9me2 ChIP-seq using the new-generation illumina sequencer HiSeq 2000. The new H3K9me2 ChIP-seq data are highly consistent with the previous one but provides 4-5 fold increase of number of reads, which has fixed the gap on PPARγ1 promoter (see the updated Figures 1E, 2A and S1D). Based on the new H3K9me2 ChIP-seq data, we have updated the plots in Figure 1A-E and Figure 2.

To confirm the decrease of H3K9me2 level on the PPARγ locus during adipogenesis, we have also done ChIP-seq of H3K9me2 in 3T3-L1 adipocytes (day 7). As shown in the new Figure 3B, there is a dramatic but rather selective decrease of H3K9me2 signals on the entire PPARγ locus after adipogenesis. We also did ChIP-qPCR at -30kb of PPARγ1 promoter and +70kb of PPARγ2 promoter. The results are consistent with the ChIP-seq data and confirm that the H3K9me2 signal is indeed lower around TSS sites comparing to the rest of gene locus (the updated Figure 3A). The zoom-in ChIP-seq data around PPARγ1 promoter is shown in the new Figure S1D.

2) “Related to #1, there is no description about the locations of ChIP-qPCR measurement near PPARγ1 and PPARγ2 genes (Fig. 1G, 5B, 6). Information has to be included in the figure or in the legends.”

RE: We have included the information in the figure legends. The detailed information of qPCR primers for ChIP is shown in Table S2 and S3.

3) “In aP2-Cre:G9a fl/fl mice, the G9a will be deleted relatively at late phase of adipogenesis. Since the reduction of G9a is so robust (it goes down to almost undetectable levels, Fig. 2D and 4B), one can imagine that the deletion of G9a in the late phase of adipogenesis may not be as effective as in vitro experiments with G9a deleted from the beginning (Fig. 4-6). Furthermore, as the authors examined, it is reported that G9a deletion affect senescence, which was demonstrated to be linked to adipocyte size and adipose tissue inflammation (10.1038/nm.2014), which may suggest contribution of an indirect action of G9a deletion on the adipose tissue phenotype through the modulation of senescence. To address these issues, the following experiments are recommended.”

“- In vitro experiment: Deletion of G9a at later phase. This can be performed by adding Ad-Cre at later time points of adipogenesis of immortalized MEF.
- Ex vivo experiment: More simply, the authors can perform adipogenesis assay using MEFs or adipose tissue SVF directly isolated from aP2-Cre:G9a fl/fl mice.”

RE: We appreciate the reviewer’s highly insightful comments. We have compared aP2 and PPARγ induction patterns during adipogenesis of 3T3-L1, primary white preadipocytes, and immortalized brown preadipocytes using qRT-PCR and/or RNA-Seq. As shown in the new Figure S6, aP2 expression is induced at ~day 1-2 of adipogenesis, well before PPARγ expression reaches the maximal level. These results suggest that aP2-Cre starts to delete G9a before PPARγ reaches maximum level and would promote PPARγ expression.

The ex vivo experiment is an excellent suggestion. We isolated primary white preadipocytes from G9a^−/−; aP2-Cre mice and littermates controls. As shown in the new Figure 8, G9a^−/−; aP2-Cre preadipocytes show enhanced adipogenesis and increased expression of adipogenesis marker genes. Further, genomic PCR reveals that aP2-Cre-mediated deletion of G9a allele in G9a^−/−; aP2-Cre preadipocytes starts from day 2 of adipogenesis (Figure 8C). Together, these results indicate that aP2-Cre-mediated deletion of G9a promotes adipogenesis and PPARγ expression.

“- In vivo experiment, aP2-Cre:G9a fl/fl mice: The authors should measure senescence-associated factors such as Ink4a, p53 etc and to perform senescence-associated b-gal staining.”
RE: we have checked expression of senescence-associated genes and performed senescence-associated b-gal staining in the white adipose tissue of G9a<sup>fl/fl</sup> aP2-Cre mice. As shown in the new Figure S7G-H, we observed moderate increases of cell senescence markers in the white adipose tissues of a subset of G9a KO mice, suggesting that senescence may contribute to the increased adiposity in G9a KO mice (see Discussion).

4) “Food intake measurement must be presented.”

RE: As shown in the new Figure S7E, G9a KO mice show trend of increased food intake (p=0.056). These results suggest that in addition to enhanced adipogenesis and cell senescence, increased food intake may also contribute to the increased adipose tissue weight in G9a KO mice (see Discussion).

5) “G9a protein levels completely decreased to zero whereas G9a mRNA level was more than half. Are they differentially regulated?”

RE: Yes, these results suggest 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 signaling triggers degradation of histone methyltransferases G9a and GLP through APC/C(Cdh1) in senescent cells and causes a global decrease in H3K9me2 (Takahashi et al, 2012). Whether a similar mechanism is employed in the down-regulation of G9a protein during adipogenesis remains to be determined.

6) “Fig. 6A. It is better to include G9a protein blot as a control.”

RE: G9a protein blot is now added to Figure 7A.

References


Cawthorn WP, Scheller EL, MacDougald OA (2012b) Adipose tissue stem cells meet preadipocyte commitment: going back to the future. J Lipid Res 53: 227-246


Thank you for submitting your revised manuscript for our consideration. I have now had a chance to look through it and to assess your responses to the comments raised by the original reviewers. I am happy to inform you that we are ready to proceed with acceptance of the paper, pending modification of a few minor points.

- It is currently not obvious how many biological replicates the data in Fig. 1 and 8B/C are based upon; please clarify this in the figure legend.

- Please add the statistical method used to derive the error bars in Fig. 6, 7 and 8. As stated in the legend, some of the results are representatives of only two independent experiments. In case any of the data including error bars are based on two biological duplicates only, I would like to ask you to remove the error bars and to blot both data points instead as it is not statistically sound to derive standard deviations from only two data points.
In light of our prior discussion regarding the phenotype of the adipose-specific G9a KO mice, please verify that the following conclusion on p10 of the results section is scientifically accurate:

"Histology analysis of adipose tissue section revealed increased size and volume of adipocytes in G9a KO mice, suggesting enhanced adipose tissue development in vivo."

Finally, we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. A ZIP archive containing these individual files can be uploaded upon resubmission (selecting "Figure Source Data" as object type) and would be published online with the article as a supplementary "Source Data" file.

To simplify the processing, you can just sent the amended text file in reply to this e-mail. After that, we should then be able to swiftly proceed with formal acceptance and production of the manuscript!

If you have any questions in this regard, please do not hesitate to contact me directly.

Yours sincerely,

Editor
The EMBO Journal

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Thank you very much for the efficient handling our manuscript. We appreciate your suggestions. Here are the response and explanations:

1. "It is currently not obvious how many biological replicates the data in Fig. 1 and 8B/C are based upon; please clarify this in the figure legend."

As indicated in Response to Reviewers, we did ChIP-Seq of H3K9me2 twice using illumina sequencers GAII and HiSeq2000 and the results are highly consistent. We show in Figure 1A-F the data obtained from the new-generation sequencer HiSeq2000 because the data provide 4-5-fold higher sequencing reads and thus much better coverage than the data from the old sequencer GAII. Figure 1G data are representative of 3 independent experiments.

In Figure 8B/C, qPCR was performed in triplicates for littermate group 1. Similar results were obtained for littermate groups 2 & 3 but are not shown. Thus, we have 3 biological replicates.

We have updated the figure legends accordingly.

2. "Please add the statistical method used to derive the error bars in Fig. 6, 7 and 8. As stated in the legend, some of the results are representatives of only two independent experiments. In case any of the data including error bars are based on two biological duplicates only, I would like to ask you to remove the error bars and to blot both data points instead as it is not statistically sound to derive standard deviations from only two data points."

Only the western blots (Figure 6I and 7A) and adipogenesis assay (Figure 7D) are representative of 2 independent experiments. All other data in Figure 6 & 7 are representative of 3-4 independent experiments. Each data point was generated from triplicates.

Standard Deviation (SD) was used to derive the error bars in Figure 6 & 7 and standard error of the mean (SEM) was used in Figure 8. We have updated the figure legends.

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We have updated the figure legends accordingly.

3. "In light of our prior discussion regarding the phenotype of the adipose-specific G9a KO mice, please verify that the following conclusion on p10 of the results section is scientifically accurate. "Histology analysis of adipose tissue section revealed increased size and volume of adipocytes in G9a KO mice, suggesting enhanced adipose tissue development in vivo."

We do observe increased adipocyte size in G9a KO mice (Figure 9F-G). Therefore, the adipocyte volume is likely increased as well. However, since we do not have ways to directly measure the cell volume, we have removed "and volume" from the sentence.

4. "Finally, we encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. I am taking this opportunity to invite you to provide a single PDF/JPG/GIF file per figure comprising the original, uncropped and unprocessed scans of all gel/blot panels used in the respective figures. These should be labeled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential."

We will provide source data for Western blots except the histone modification blots shown in Figure 4E, 5D and 7A. Because of the very low molecular weight of histone proteins (~17kDa), we only transfer proteins in the range of 10-30kDa from SDS-PAGE gels to PVDF membranes before Western blotting of histone modifications. Because of the strong signals shown in the Western blots of histone modifications, we generally do not see non-specific bands in the 10-30kDa range. Therefore, we feel it is not necessary to show the source data of histone modification blots.