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## Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation

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### Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision

29 July 2010

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Thank you for submitting your manuscript for consideration by The EMBO Journal. It has now been evaluated by three referees and I enclose their reports below, as you will see while they find the study to be of potential interest further experimental work is required to make it suitable for The EMBO Journal.

The referees provide mixed recommendations, with two referees finding the manuscript potentially interesting. Both these referees ask for further experiments to strengthen the conclusions, this includes ChIP of the HATs to determine their timecourse of recruitment to the promoter, and quantitative mass spec analysis of the effect of HAT deletions on K18ac and K27ac and in vitro HAT assays. One issue that remains is the novelty of the role of these HATs in nuclear receptor mediated transcription, it is extremely notable that the current manuscript lacks citations to many primary articles placing the known role of HATs in receptor mediated transcription. The EMBO Journal has announced an initiative to increase the citation of primary data (see Editorial, The EMBO Journal 2009: new initiative (EMBO J (2009) Jan 7;28(1):1-3). This must be also addressed in a revised manuscript, this is also very important given the negative comments of referee #2 and the current inability of referee #3 to judge the overall novelty of the role of the HATs in nuclear receptor mediated transcription. Therefore a judgment based on the novelty will be provided after a revised version on the manuscript, where I will ask an independent editorial advisor to comment specifically on this issue, instead of referee #2 who provides a clear opinion. If you are able to address these issues given the potential interest in the study, we would be willing to consider a revised manuscript. However, I would like to point out that if the referees are not convinced by the revised version of the manuscript, including the novelty, it may be rejected at this later stage.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments 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 initiative, 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 (Remarks to the Author):

The study by Jin and colleagues investigates the involvement of histone acetyltransferases in chromatin modification and transcriptional activation induced by nuclear receptors (NRs) and by PPAR-delta, in particular. First, the authors establish an experimental framework for the *Angptl4* induction in the mouse embryo fibroblast (MEF) system. In particular, they find that H3 and H4 acetylation occurs concomitantly with transcriptional activation and pol II recruitment, while H3K4, H3K36 and H3K79 methylations occur later. Acetylation of H3K18 and K27 appears an early event in target gene activation as these marks are insensitive to DRB, which is in contrast to H3K9, H3K14 or H4 acetylation. Next, the authors use either MEFs deleted for *Gcn5* and *Pcaf* or for *Cbp/p300*. By immunoblotting and mass spectrometry they find a global loss of H3K9ac, but no effects on transcriptional induction of NR target genes nor on histone methylation in MEFs lacking *Gcn5* and *Pcaf*. Analysis of *Cbp/p300* DKO MEFs on the other hand shows that these HATs are required for H3K18/K27 acetylation and NR target gene induction. Loss of *Cbp/p300* results in a proliferation block, but this is not the cause for gene activation defects in general as shown by the *III-beta* induction experiment in supplemental figure S6.

In general, this is a very nice and carefully controlled study providing strong evidence that *Cbp/p300* are the relevant HATs for transcriptional activation by PPAR-delta, LXR-alpha and RAR-alpha. Clearly, *Gcn5/Pcaf* are not required for activation by these NRs, This reviewer believes that in the current organization of the manuscript too much emphasis is placed on the *Gcn5/Pcaf* part, which represents 'negative' data. To counter this parts of Figure 4 and 5 should be combined into one figure, ie 4A, 4C, 4D with 5A-5D and the remaining panels should be moved to the supplementary material. The experiments excluding a general effect of *Cbp/p300* on cell proliferation in Figure S6 should be combined in with panels 5A, 5C and 5D. The other panels of 5 could go into the Supplemental.

The authors build a strong case for involvement of *Cbp* and *p300* in NR target gene activation. Nevertheless they do not show that these HATs are directly recruited to these promoters in a time frame similar to K18/K27ac. The study would be strengthened considerably when such data is provided. At present, it remains formally possible that *Cbp* and *p300* are only indirectly involved.

The authors nicely quantify the extent of loss of global H3K9ac in *Gcn5/Pcaf* MEFs. A similar mass spec analysis for H3K18/K27ac is lacking, while these are more relevant for NR target gene induction. This is quite important as significant (residual?) signals can be observed in the K18ac and K27ac ChIPs of the *Cbp/p300* DKO's. Can the authors provide quantitative data for the remaining K18ac and K27ac levels?

Minor points:

- According to the Brno nomenclature (NSMB (2005) 12, 110-112) the correct abbreviation for an acetyl group is 'ac' and not 'Ac'.
- The single-letter abbreviation for lysine is confusing in several places.
- The discussion section ends rather abruptly and a 'closing sentence' should be provided.

Referee #2 (Remarks to the Author):

The manuscript by Jin et al. reports the functional role of the histone acetyl transferases GCN5/PCAF and CBP/p300 controlling gene expression activated through PPAR $\delta$  ligand activation. In particular, these authors focus on the Angptl4 gene, a strong target of PPAR $\delta$ . Using ChIP assays and cell lines genetically deleted for different combinations of these HATs, the authors conclude that specific histone modifications catalyzed by p300/CBP are required to mediate ligand-dependent PPAR $\delta$  activation in several target genes including the Angptl4. Overall, the experiments are good and logical but there is no sufficient novelty for this manuscript to be at the level of EMBO.

It is already well established in the nuclear receptor field that CBP/p300 are key coactivators that mediate ligand-induced gene expression, so this data adds very little to what is already known.

The involvement of GCN5/PCAF is interesting but so far there is no functional data -since it is negative-linked the specific histone modifications catalyzed by GCN5/PCAF on the Angptl4 promoter and PPAR $\delta$  mediated gene expression. What is the role of H3K9 acetylation? Could it be that in other contexts or different time/dose scales this modification is also relevant?

The lack of functional/metabolic data related to the manipulation of GCN5/pCAAF and CBP/p300 also compromise the final conclusions of the manuscript. At this stage, it seems more tailored for a more specialized type of journal.

Referee #3 (Remarks to the Author):

This is an interesting paper investigating the roles of several mammalian HATs in nuclear receptor-mediated transcriptional activation. The authors monitor the histone modification profiles of several methylation and acetylation sites on lysine residues at the promoter region of the nuclear receptor PPAR $\delta$  target gene Angptl4. They find that H3K9 acetylation displays an intermediate timing with respect to the "early phase" observed with K14, 18, and 27 acetylation, and the "late phase" observed with K4, K36, and K79 methylation. They further show that K9 acetylation is regulated by GCN5/PCAF, and is dispensable for receptor-mediated transcriptional activation. In contrast, acetylation of K18 and K27 is mediated by CBP/p300, and is required for ligand-induced transcription of Angptl4, as well as several other receptor target genes.

I find the majority of the data to be of high quality and technically sound, with few exceptions. However, it is unclear to this reviewer the advancement that this manuscript presents over the current literature describing nuclear receptor-mediated transcriptional activation. The authors would benefit from highlighting the novelty of their work and emphasizing its context by better citing the current literature regarding HAT function in nuclear receptor-mediated transcription. For example, is this the first time that a GCN5-independent function has been described in receptor-mediated transcriptional activation? The first time that a p300/CBP requirement has been demonstrated? Does this study change the paradigm of how HATs function in conjunction with nuclear receptors to regulate transcription? Given the broad readership of EMBO Journal, I would also suggest the authors put more thought into their introductory description of the role of histone lysine acetylation in gene expression, as this is the central theme of their manuscript. Particularly, the authors state: "Histone acetylation neutralizes the basic charge on the K residues and therefore can unfold chromatin" (Page 3). However, there is no mention at all of the fact that only one lysine acetylation

site (H4K16) has been shown to have a measurable effect on chromatin folding (See: Science 2006, vol 311 844-847), and this residue is not one of the lysines studied in this manuscript. Furthermore, there is evidence to suggest that some of the residues in question likely do not simply function through charge mediation (See: EMBO J. 1998, vol 17 3155-3167). A few sentences on effector proteins for histone acetylation here would greatly enhance this discussion.

My specific concerns regarding experimental data are:

1) An important yet unanswered question from this manuscript is whether the HATs described display differential recruitment to the *Angptl4* promoter. Based on their data, the authors suggest that "the histone modifying enzymes responsible for depositing H3K9Ac...are neither recruited by nuclear receptors before transcription initiation nor important for transcription initiation" and that "the recruitment of these enzymes is transcription-dependent" (Page 17-18). This is a very notable conclusion that would provide insight into our understanding of GCN5/PCAF localization and function in mammals. However, the authors have not performed a single experiment to investigate the recruitment of these HATs to the *Angptl4* promoter in support of these claims. The authors should perform ChIP experiments for the four HATs in question in the absence and presence of ligand and DRB to substantiate this conclusion.

2) Another notable conclusion of this manuscript is that GCN5 and PCAF in MEFs display altered substrate specificity compared to that of the homologous Gcn5 protein in yeast and *Drosophila*. However, the authors have not performed any *in vitro* HAT assays using purified proteins to supplement their *in vivo* data. It is of course highly possible that GCN5 and or PCAF are capable of acetylating K14, perhaps even preferentially, but that this acetylation is compensated for *in vivo*. (To this point, the authors have stated incorrectly that "yeast SAGA complex preferentially acetylates H3K9 and to a lesser extent H3K14". See: J. Biol. Chem. 1999, vol 274(9) 5895-5900.) The authors cite *in vitro* studies from alternative systems, but comparable experiments in MEFs are necessary given the authors' argument that the substrate specificity of these HATs in MEFs differ from those of the systems that were cited. HAT assays along with the ChIPs described above would go a long way to highlight the proposed distinct roles of all four HAT proteins, and substantially strengthen the conclusions of this manuscript.

3) In addition to these concerns, I have 2 technical issues:

- In Figure 5E, top panel, why is there so much variation in the CBP protein levels of the vector-treated samples (compare lanes 1, 3, and 5)? There appears to be, if anything, an increase in CBP level in the CBP<sup>flox/flox</sup> strain upon Cre treatment (compare lanes 1 and 2).

- In Figure S1B, the authors did not show *Angptl4* expression upon CHX treatment in the absence of ligand. Given the ~ 10-fold increase in expression after CHX treatment (left panel, lanes 2 and 3), it is reasonable to question whether CHX treatment alone, even in the absence of ligand, causes increased expression, perhaps through decreased translation of transcriptional repressors. This is an important control as the authors use this experiment to show that *Angptl4* is a "direct target gene of PPAR $\delta$ ".

1st Revision - authors' response

12 October 2010

We have done all experiments suggested by the referees. Here is a summary of the major changes in the revised version:

1. ChIP of HATs on *Angptl4* promoter (the new **Figure 4E-F** and **Figure 6H-I**).

The time courses of ligand-induced recruitment of GCN5/PCAF and CBP/p300 to the *Angptl4* promoter correlate well with the enrichment of H3K9ac and H3K18/27ac, respectively. Further, DRB blocks the recruitment of GCN5/PCAF but not that of CBP/p300 to the *Angptl4*

promoter, which is consistent with our data that DRB blocks H3K9ac but not H3K18/27ac on the same promoter.

2. Mass spectrometry of H3K18ac and H3K27ac in CBP/p300 double knockout cells (the new **Table II**).

The results are highly consistent with the Western blot data and demonstrate that CBP/p300 are responsible for over 90% of H3K18ac and H3K27ac in MEFs. The data in **Table II** also provide useful information on the relative abundance of several histone acetylations in mammalian cells.

3. In vitro HAT assay (the new **Figure 3F** and **Figure S4**).

Both the recombinant GCN5 and the purified GCN5-associated HAT complexes (GCN5.com) are capable of acetylating multiple lysine residues on histone H3, including H3K14. These results suggest that mammalian GCN5 is capable of acetylating H3K14 but this acetylation may be compensated by other HATs in GCN5/PCAF double knockout cells.

4. Clarification on the novelties of our findings.

In the Introduction, we now cite multiple papers and introduce what has been shown in the literature on regulation of nuclear receptor (NR) target gene activation by GCN5/PCAF, CBP/p300, and associated HAT activities. In the Discussion, we compare our findings on the substrate and site specificities of GCN5/PCAF and CBP/p300 with the data shown in the literature. We also add two new sections to discuss GCN5/PCAF and H3K9ac in NR target gene activation, and CBP/p300-mediated H3K18/27ac in NR target gene activation.

5. As pointed out by referee #3, the central theme of our manuscript is the role of histone acetylation in gene expression. Therefore, we have changed the manuscript title to “**Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation**”.

6. Since the current manuscript focuses on NR-dependent transcription, we have removed the data on regulation of other inducible genes by GCN5/PCAF shown in the old Figure S3 (see the attached **Unpublished Figure 1 for referees**). Removal of the data makes the manuscript more focused and logic. These results will be reported in a new manuscript that investigates the genome-wide role of GCN5/PCAF and H3K9ac in regulating constitutive and inducible gene expression.

7. It has been reported that CBP/p300 show structural similarity with the yeast H3K56 acetyltransferase Rtt109 and that CBP/p300 are responsible for H3K56ac in human cells (Das et al, 2009). By Western blot analysis using two different sources of H3K56ac antibodies described in (Das et al, 2009), we found that CBP/p300 are dispensable for H3K56ac in MEFs (**Figure S8C**). This result emphasizes the site specificities of CBP/p300 and should be interesting to those who work on H3K56ac.

**Referee #1.**

We thank referee #1's highly insightful and constructive comments.

1. “.....in the current organization of the manuscript too much emphasis is placed on the *Gcn5/Pcaf* part, which represents 'negative' data. To counter this, parts of Figure 3 and 4 should be combined into one figure, ie 3A, 3C, 3D with 4A-4D and the remaining panels should be moved to the supplementary material.”

We have moved the myocytes data in the old Figure 4 to **Figure S5**. With the new mass spectrometry data on CBP/p300-mediated H3K18/27ac (the new **Table II**), we feel the revised manuscript no longer puts too much emphasis on the GCN5/PCAF part. Therefore, it's no longer necessary to combine Figures 3 and 4 into one figure.

2. “The experiments excluding a general effect of Cbp/p300 on cell proliferation in Figure S6 should be combined in with panels 5A, 5C and 5D. The other panels of 5 could go into the Supplemental.”

We have moved the experiments excluding a general effect of CBP/p300 on cell proliferation to **Figure 5F-G**.

3. “The authors build a strong case for involvement of Cbp and p300 in NR target gene activation. Nevertheless they do not show that these HATs are directly recruited to these promoters in a time frame similar to K18/K27ac. The study would be strengthened considerably when such data is provided. At present, it remains formally possible that Cbp and p300 are only indirectly involved.”

As shown in the new **Figure 4E** and **Figure 6H**, GCN5/PCAF and CBP/p300 are directly recruited to the *Angptl4* promoter in time frames similar to H3K9ac and H3K18/27ac, respectively. Further, DRB blocks the recruitment of GCN5/PCAF but not that of CBP/p300 to the *Angptl4* promoter (the new **Figure 4F** and **Figure 6I**), which is consistent with our data that DRB blocks H3K9ac but not H3K18/27ac on the same promoter.

These results suggest that GCN5/PCAF and CBP/p300 are directly responsible for H3K9ac and H3K18/27ac, respectively, on NR target gene promoters.

4. “The authors nicely quantify the extent of loss of global H3K9ac in *Gcn5/Pcaf* MEFs. A similar mass spec analysis for H3K18/K27ac is lacking, while these are more relevant for NR target gene induction. This is quite important as significant (residual?) signals can be observed in the K18ac and K27ac ChIPs of the Cbp/p300 DKO's. Can the authors provide quantitative data for the remaining K18ac and K27ac levels?”

Please see the new **Table II** for mass spectrometry analysis of H3K18/27ac in CBP/p300 double KO cells. The results are highly consistent with Western blot data and indicate that CBP/p300 are responsible for over 90% of H3K18ac and H3K27ac in MEFs.

5. “-According to the Brno nomenclature (NSMB (2005) 12, 110-112) the correct abbreviation for an acetyl group is 'ac' and not 'Ac'.

-The single-letter abbreviation for lysine is confusing in several places.

-The discussion section ends rather abruptly and a 'closing sentence' should be provided.”

We have changed all Ac to ac as the abbreviation for acetyl group. We also have clarified the single letter abbreviation for lysine. A closing paragraph describing our model (the new **Figure 6J**) is now provided in the Discussion.

## Referee #2.

We thank referee #2's efforts on reviewing our manuscript but we disagree with the comments.

1. *"It is already well established in the nuclear receptor field that CBP/p300 are key coactivators that mediate ligand-induced gene expression, so this data adds very little to what is already known."*

As we mention in the Introduction, CBP/p300 are important for ligand-induced nuclear receptor (NR) target gene activation. However, likely due to the early embryonic lethality of both *CBP<sup>-/-</sup>* and *p300<sup>-/-</sup>* mice as well as the potential functional redundancy between CBP and p300 in cells, the roles of CBP/p300 in expression of endogenous NR target genes were incompletely understood. More importantly, because the substrate and site specificities of CBP/p300 *in vivo* were not determined, the molecular mechanisms by which CBP/p300-mediated histone acetylations regulate NR-dependent transcription have remained largely unclear.

As pointed out by referee #3, the central theme of our manuscript is the role of histone acetylation in gene expression. We use ligand-induced NR target gene activation as a model system to address this central theme. Our data in **Figure 5**, **Figure 6** and **Table II** provide strong evidence to suggest that CBP/p300-mediated selective acetylation on H3K18 and H3K27 play critical roles in ligand-induced gene expression. Our data thus provide novel insights into the molecular mechanism by which the HATs CBP/p300 regulate nuclear receptor target gene activation.

2. *"The involvement of GCN5/PCAF is interesting but so far there is no functional data -since it is negative-linked the specific histone modifications catalyzed by GCN5/PCAF on the *Angpt4* promoter and *PPAR $\delta$*  mediated gene expression. What is the role of H3K9 acetylation? Could it be that in other contexts or different time/dose scales this modification is also relevant?"*

Histone acetylations generally correlate with gene activation. However, it has been unclear whether the increased histone acetylations are a cause or a consequence of the increased transcription in mammalian cells. We identify the substrate and site specificities of GCN5/PCAF in cells. Further, we show GCN5/PCAF and GCN5/PCAF-mediated H3K9ac correlate well with, but are dispensable for, ligand-induced NR target gene activation. Such results are surprising, because H3K9ac is a hallmark for gene activation and GCN5/PCAF have been shown to function as nuclear receptor coactivators. Our data thus provide novel insights into the role of the HATs GCN5/PCAF in NR target gene activation. In the Discussion, we discuss the possible roles of H3K9ac in transcription.

3. *"The lack of functional/metabolic data related to the manipulation of GCN5/pCAF and CBP/p300 also compromise the final conclusions of the manuscript."*

This manuscript focuses on the molecular mechanisms by which these two distinct families of HATs regulation NR-dependent gene activation. The "functional/metabolic" data are not essential for this manuscript.

**Referee #3.**

We thank referee #3's highly insightful and constructive comments.

*1. "I find the majority of the data to be of high quality and technically sound, with few exceptions. However, it is unclear to this reviewer the advancement that this manuscript presents over the current literature describing nuclear receptor-mediated transcriptional activation. The authors would benefit from highlighting the novelty of their work and emphasizing its context by better citing the current literature regarding HAT function in nuclear receptor-mediated transcription."*

We apologize for not doing a good job in citing the literature regarding HAT function in nuclear receptor (NR)-mediated transcription and in highlighting the novelties of our findings in the previous version. We now cite many primary articles and introduce what has been shown in the literature on the roles of GCN5/PCAF, CBP/p300, and associated HAT activities in regulation of NR-mediated transcription in the Introduction. In the Discussion, we highlight the novelties of our findings in the 1<sup>st</sup> paragraph and compare our findings with what's known in the literature.

*2. "For example, is this the first time that a GCN5-independent function has been described in receptor-mediated transcriptional activation? The first time that a p300/CBP requirement has been demonstrated? Does this study change the paradigm of how HATs function in conjunction with nuclear receptors to regulate transcription?"*

We show for the first time that GCN5/PCAF and GCN5/PCAF-mediated H3K9Ac correlate well with, but are dispensable for, ligand-induced NR target gene activation. Such results are surprising, given that GCN5/PCAF are recruited to NR target gene promoters during gene activation and that GCN5/PCAF function as coactivators for several NRs on reporter genes (Blanco et al, 1998; Korzus et al, 1998; Kraus & Wong, 2002; Metivier et al, 2003; Zhao et al, 2008). Our study examines the roles of GCN5/PCAF in expression of endogenous NR target genes. In contrast, most of the previous studies that implicate GCN5/PCAF as NR coactivators rely on over-expressing GCN5/PCAF in reporter gene assays without addressing the contributions from the GCN5/PCAF HAT activities. Our data suggest that GCN5/PCAF function as NR coactivators through the associated SAGA and/or ATAC complexes independent of their HAT activities. Such a possibility is supported by results from studies in yeast and *Drosophila* that the GCN5-associated SAGA complex can function as a coactivator independent of its enzymatic activity (Weake et al, 2009).

As mentioned in the Introduction, CBP and p300 are known to be important for ligand-induced NR target gene activation. However, likely due to the early embryonic lethality of both *CBP<sup>-/-</sup>* and *p300<sup>-/-</sup>* mice as well as the potential functional redundancy between CBP and p300 in cells, the roles of CBP/p300 in expression of endogenous NR target genes were incompletely understood. More importantly, because the substrate and site specificities of CBP/p300 *in vivo* were not determined, the molecular mechanisms by which CBP/p300-mediated histone acetylations regulate NR-dependent transcription have remained largely unclear. In this manuscript, we show CBP/p300 and their HAT are critical for NR target gene expression. More importantly, we provide strong evidence

to suggest that CBP/p300-mediated selective acetylation on H3K18 and H3K27 play critical roles in NR target gene expression.

Taken together, our study would change the paradigm of how the two distinct families of HATs, GCN5/PCAF and CBP/p300, regulate NR-mediated transcription.

3. “ Given the broad readership of EMBO Journal, I would also suggest the authors put more thought into their introductory description of the role of histone lysine acetylation in gene expression, as this is the central theme of their manuscript. Particularly, the authors state: "Histone acetylation neutralizes the basic charge on the K residues and therefore can unfold chromatin" (Page 3). However, there is no mention at all of the fact that only one lysine acetylation site (H4K16) has been shown to have a measurable effect on chromatin folding (See: Science 2006, vol 311 844-847), and this residue is not one of the lysines studied in this manuscript. Furthermore, there is evidence to suggest that some of the residues in question likely do not simply function through charge mediation (See: EMBO J. 1998, vol 17 3155-3167). A few sentences on effector proteins for histone acetylation here would greatly enhance this discussion.”

Exactly as this referee points out, the role of histone acetylation in gene expression is the central theme of our manuscript. We have improved the introduction on the role of histone acetylation in gene expression. Further, we have changed the manuscript title to “Distinct roles of GCN5/PCAF-mediated H3K9ac and CBP/p300-mediated H3K18/27ac in nuclear receptor transactivation”.

Since H4K16ac is not studied in this manuscript, we have removed the statement that "Histone acetylation neutralizes the basic charge on the K residues and therefore can unfold chromatin".

A few sentences on effector proteins for histone acetylation are included in the final paragraph of the Discussion (also see the model in the new **Figure 6J**).

4. “..... However, the authors have not performed a single experiment to investigate the recruitment of these HATs to the *Angptl4* promoter in support of these claims. The authors should perform ChIP experiments for the four HATs in question in the absence and presence of ligand and DRB to substantiate this conclusion.”

We have done ChIP of the four HATs on *Angptl4* promoter in the absence and presence of ligand and DRB (see the new **Figure 4E-F** and **Figure 6H-I**). The time courses of ligand-induced recruitment of GCN5/PCAF and CBP/p300 to the *Angptl4* promoter correlate well with the enrichment of H3K9ac and H3K18/27ac, respectively (**Figures 4E and 6H**). Further, DRB blocks the recruitment of GCN5/PCAF but not that of CBP/p300 to the *Angptl4* promoter (**Figures 4F and 6I**), which is consistent with our data that DRB blocks H3K9ac but not H3K18/28ac on the same promoter.

5. “Another notable conclusion of this manuscript is that GCN5 and PCAF in MEFs display altered substrate specificity compared to that of the homologous Gcn5 protein in yeast and *Drosophila*. However, the authors have not performed any in vitro HAT assays using purified proteins to supplement their in vivo data. It is of course highly possible that GCN5 and or PCAF are capable of acetylating K14, perhaps even preferentially, but that this acetylation is compensated for in vivo.

*(To this point, the authors have stated incorrectly that "yeast SAGA complex preferentially acetylates H3K9 and to a lesser extent H3K14". See: J. Biol. Chem. 1999, vol 274(9) 5895-5900.) The authors cite in vitro studies from alternative systems, but comparable experiments in MEFs are necessary given the authors' argument that the substrate specificity of these HATs in MEFs differ from those of the systems that were cited. HAT assays along with the ChIPs described above would go a long way to highlight the proposed distinct roles of all four HAT proteins, and substantially strengthen the conclusions of this manuscript."*

We have done the in vitro HAT assay (see the new **Figure 3F and Figure S4**). The results show that both the recombinant GCN5 and the GCN5-associated HAT complexes purified from MEFs are capable of acetylating multiple K residues on histone H3, including H3K14. These results suggest that mammalian GCN5 is capable of acetylating H3K14 but this acetylation may be compensated by other HATs in GCN5/PCAF double knockout cells. In addition, we now show that deletion of GCN5/PCAF in brown preadipocytes, a cell type different from MEFs, depletes H3K9ac but has no effect on the global level of H3K14ac (the new **Figure S3A**).

The statement that "yeast SAGA complex preferentially acetylates H3K9 and to a lesser extent H3K14" was from Jerry Workman's recent review (Lee & Workman, 2007), although Jerry Workman's 1999 JBC paper found that in vitro the yeast SAGA complex prefers H3K14 than H3K9 on nucleosome substrate and that yeast SAGA acetylates all 4 lysines on histone H3 (Grant et al, 1999). We have deleted this statement from the manuscript.

6. *"In Figure 5E, top panel, why is there so much variation in the CBP protein levels of the vector-treated samples (compare lanes 1, 3, and 5)? There appears to be, if anything, an increase in CBP level in the CBP<sup>flox/flox</sup> strain upon Cre treatment (compare lanes 1 and 2)."*

Likely due to the high molecular weight of CBP and the low quality of the earlier batch of CBP antibody, there is a smear at the expected CBP position in the CBP-<sup>flox/flox</sup>-Cre lane of the old Figure 5E. We have repeated the Western blot twice using a new batch of CBP antibody and confirmed the loss of CBP protein level in the CBP-<sup>flox/flox</sup>-Cre cells (see the updated **Figure 5E**).

7. *"In Figure S1B, the authors did not show Angptl4 expression upon CHX treatment in the absence of ligand. Given the ~ 10-fold increase in expression after CHX treatment (left panel, lanes 2 and 3), it is reasonable to question whether CHX treatment alone, even in the absence of ligand, causes increased expression, perhaps through decreased translation of transcriptional repressors. This is an important control as the authors use this experiment to show that Angptl4 is a "direct target gene of PPAR $\delta$ "*

As mentioned in the Introduction, *Angptl4* is a known direct target gene of PPAR $\delta$ , with PPAR response element (PPRE) being located in intron 3 (Mandard et al, 2004). Treating MEFs with GW selectively activates PPAR $\delta$  target genes in MEFs, with *Angptl4* being the most significantly induced one (Hummasti & Tontonoz, 2006; Oliver et al, 2001).

We have included CHX treatment alone in **Figure S1B**. Indeed, CHX alone in the absence of ligand causes increase expression of *Angptl4*. This is expected, because inducible gene mRNAs are generally highly unstable (for another example, please see (Hargreaves et al, 2009)). Importantly,

protein synthesis inhibitor CHX fails to inhibit GW-induced *Angptl4* expression, which confirms that *Angptl4* is a direct PPAR $\delta$  target in our MEFs.

#### References:

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2nd Editorial Decision

05 November 2010

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I have now received the report from the one referee who has re-evaluated your revised manuscript. As you can see from the comments below, this referee is now supportive and therefore I am happy to accept your manuscript for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

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