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A dysregulated Acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity

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1st Editorial Decision 07 March 2014

Thank you for submitting your study "A dysregulated Acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity" for our consideration. We have now received the reports of three expert reviewers, which you will find copied below. As you will see, all three referees acknowledge the potential interest of your findings. I am afraid to note, however, that in their reviews and during the subsequent cross-refereeing process, all three experts concur that the core model (that enhanced acetylation of FXR in obesity would cause a diminished anti-inflammatory response through a reduction of FXR sumoylation) was not yet conclusively demonstrated to warrant publication at the EMBO Journal at this point.

As reviewer #2 most clearly states (major points 1-4), the presented evidence does not rule out that only a minor fraction of FXR is sumoylated - if FXR is sumoylated at all. Specifically, this would be difficult to reconcile with the rather complete abolishment of interaction with RXR and also the rather striking SUMO-dependent association with NFkB. Together, it remains to be decisively demonstrated that these interactions are indeed SUMO-dependent.

Beyond this, especially reviewer #1 raised several independent conceptual concerns regarding experiments that test the physiological relevance of the proposed model. Finally, also other substantial questions remained open, which I will not repeat in detail here (SUMO1 vs. SUMO2, involvement of co-repressors, general significance of the findings for other members of the

nuclear receptor family). I hope you appreciate that the nature and number of concerns leave me little choice but to conclude that we will not be able to offer publication of this study at this point.

We appreciate though the comprehensive approach that goes all the way from molecular work to physiology in vivo. For this reason, I can offer that we would be willing to look at a resubmission, should you be able to address the concerns raised in full and to provide decisive evidence in support of the model. I should point out that the manuscript would be editorially evaluated afresh and that for a resubmission we consider novelty at the time of submission and, if needed, might involve new referee(s).

Thank you for the opportunity to consider you work for The EMBO Journal. I am sorry that I can't be more positive on this occasion, but I hope that you nevertheless find the comments helpful.

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REFEREE COMMENTS

Referee #1:

This study by Kim et al. was performed to evaluate the impact of DIO on FXR acetylation. The authors demonstrate that DIO mice have elevated FXR K217 acetylation levels. Using acetylation-defective and -mimicking mutants they further show that FXR acetylation leads to enhanced hepatic inflammation, TG accumulation, and impaired insulin signaling. Their mechanistic model suggests that FXR acetylation at K217 hinders SUMOylation at K277, thereby diminishing the anti-inflammatory action of FXR on NF-kB target genes. Although overall the study is well conceived and performed, some physiological and mechanistic questions remain.

Major comments:

1. Fig 6G: The authors conclude that in lean mice, FXR agonists trigger SUMO2 modification of FXR, which in turn enhances the interaction with NF-kB and CoR, but blocks the interaction with RXR. How do the authors reconcile this mechanism with the established knowledge that FXR agonists not only repress NF-kB inflammatory genes but also induce the classical FXR target genes through mechanisms that critically depend on FXR-RXR interaction?

2. Based on hepatic TG analysis (Fig 2F+I), ND mice infected with Ad-K217Q show increased hepatic lipid content. It is not very clear whether the excessive accumulation of hepatic lipids induces the inflammatory genes or if the lipid accumulation is a consequence of inflammatory activation. To clarify this point, time course experiments should be performed to assess the onset of the lipid and inflammation transcriptional programs.

3. Based on biochemical knowledge, fasting induces lipolysis and significantly increases the concentration of acetyl-CoA (substrate for acetylation) in hepatocytes. Have the authors analyzed whether differences in acetylation can be observed between fed and fasted DIO mice?

4. Have the authors compared the level of SUMO1 and SUMO2 modification of FXR in ND and HFD mice. Is SUMO1 modification also reduced upon HFD feeding or instead unchanged by HFD? What determines the differential binding of SUMO1 and SUMO2 on FXR?

5. The authors propose a model in which acetylation of K217 prevents SUMOylation at K277. This is supported by the finding that FXR-acetylation is increased during HFD, while SUMOylation is reduced (Fig 4D). It is striking however that the acetylation of FXR is only moderate under HFD (when taking the loading into account), .while SUMOylation of FXR (by SUMO2) is completely lost under HFD (Fig 4D). This raises the question as to whether the opposite is not occurring, i.e. SUMOylation hinders the acetylation under ND, but not during HFD when SUMOylation of FXR becomes abolised? This alternative scenario should be evaluated as well.

6. The authors observed a clear difference in the glucose tolerance test, while no difference on the basal fasting glucose levels was shown between WT and Ad-K217R mice (Fig 2J). However, the expression of gluconeogenic genes (PEPCK and G6Pase, Fig S2) was significantly decreased. Would the authors not expect different fasting glucose levels? In addition, plasma insulin levels at the different time points (0, 15, 30 and 60 min) should be provided.

7. P-Akt levels are reduced upon ectopic expression of FXR K217Q. What is the consequence of Ad-K217Q on glucose tolerance and insulin sensitivity?

Minor comments:

1. The analyzed gene sets in Fig 2C-E, Fig 5A and Fig 6A are inconsistent. It would be better to monitor the same genes throughout the study.

2. More information should be provided about the nature and generation of the adenoviral constructs.

Referee #2:

In this study Kemper and co-workers investigate the interplay of acetylation and sumoylation on the nuclear receptor FXR. In a previous paper by Kemper et al. it was shown that FXR undergoes acetylation, with K217 being the major site of acetylation. It was further demonstrated that this modification inhibits heterodimerization with RXRα, DNA binding, and transactivation activity. Moreover, acetylation of FXR was shown to be induced in leptin-deficient ob/ob mice or diet-induced obese mice.

In the current paper the authors propose interplay of FXR acetylation with conjugation of the ubiquitin-related SUMO modifier. They show that in analogy to other members of the nuclear receptor family FXR engages in an anti-inflammatory transrepression pathway when conjugated to SUMO2. They propose that this is mediated through enhanced binding of the FXR-SUMO2 conjugate to NFkB. They further suggest that K217 acetylation prevents sumoylation of FXR by inhibiting its interaction with the SUMO E3 ligase PIAS4. Based on these findings the authors propose a model, in which enhanced acetylation of FXR in obesity would contribute to a diminished anti-inflammatory response through a reduction of sumoylation.
Although this is an intriguing concept the data shown here are not fully conclusive and at this stage are too preliminary support the authors claims. As detailed below I am particularly concerned about the experimental part on sumoylation of FXR, which to my opinion contains a number of conceptual an technical weaknesses.

Major points:

1. In their in vitro sumoylation assays (for example Figure 3A) the authors do not really show sumoylated forms of FXR, since they only do anti-SUMO2 immunoblots after the in vitro assay. Because it is known that free SUMO2 chains can be assembled in vitro, the authors might therefore simply look on the formation on these chains rather than sumoylated FXR. Actually it would be crucial to see in anti-FXR immunoblots what fraction of FXR gets actually modified. Given that the amount of unmodified FXR (see Figure 4B, lower panel) is not significantly reduced one can assume that only a very minor fraction of FXR is converted to FXR-SUMO2.
2. The issue in point 1 becomes very crucial when it comes to the experiments shown in Figure 5E, I. Here the authors claim that sumoylation of FXR prevents binding to RXR but fosters binding of NFκB/p65. The setup for these experiments is outlined in Figure 5D. In principal, FXR was in vitro sumoylated and used for a Co-IP with RXR or NFκB/p65, respectively (both generated by in vitro transcription/translation). Considering again that only a very minor fraction of FXR is sumoylated (as judged by the equal amounts of unmodified FXR in lane 3 and 4, Figure 5E bottom panel), it is difficult to explain why the interaction of RXR is completely gone. The same issue arises when it comes to the SUMO-dependent binding to NFκB (Figure 5I). The striking differences in the cellular

Co-IP of NFκB/p65 and FXR WT vs. SUMO-deficient FXR K277R (Figure 5G) are also very surprising given that only minute amounts of FXR are modified. To confirm that the interaction is indeed SUMO-dependent (in particular SUMO2-dependent) in a cellular setting the authors should deplete cells from SUMO2/3 by RNAi. Depletion of SUMO2/3 should recapitulate the data with the FXR K277R variant.

3. Another important issue concerns the molecular basis of SUMO-dependent NFκB binding. Is there a typical SUMO interaction motif in NFκB. If yes is this specific for SUMO2 or can SUMO1 also bind. This is an important issue because the authors shown that FXR is also a substrate for SUMO1.

4. Related to the above point: So far the SUMO-mediated transrepression pathway of nuclear hormone receptors has been linked to SUMO-dependent interactions with SIM-containing co-repressor complexes (like coronin or GPS2). Therefore it is conceptually rather intriguing that here the authors show that loss of changes in FXR sumoylation directly impinge on promoter occupancy of NFκB/p65 on inflammatory genes. Is SUMO-FXR signalling independent of the above-mentioned SUMO-anchored co-repressors? This is a critical point since the authors suggest that the proposed acetyl/SUMO switch is conserved in other nuclear hormone receptors.

5. The authors propose that acetylated FXR cannot bind PIAS4 thus preventing sumoylation. The assumption that PIAS4 is the relevant SUMO ligase for FXR is only based on overexpression experiments (for example Figure 3D). Data on the role of endogenous PIAS4 are missing. Moreover, in Figure 3D expression controls for PIAS family members are missing.

6. Data obtained with the acetyl-mimicking FXR variant K217Q should be discussed more carefully. Although glutamine has been frequently used as an acetyl-mimic, it may not fully recapitulate the behaviour of the naturally acetylated protein.

Referee #3:

EMBOJ-2014-88013, corr. author Prof. Jongsook Kemper
"A dysregulated Acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity"

The study by Kemper and coworkers address the important question of how nutrients and metabolic alterations (here high-fat diet HFD linked to obesity) modulate gene expression and physiology. Using the nutrient-sensing nuclear receptor FXR as a model, they convincingly demonstrate that cross-regulated post-translational modifications (here acetylation versus SUMOylation) are subject to nutritional control. Physiological consequences are described with focus on the liver, a main site of FXR action, using a wide array of in vivo and vitro methods, including adenovirus-mediated expression of FXR WT/mutant in mouse liver and primary hepatocytes.

The main findings are the following: Obesity triggers FXR acetylation at K217, which causes increased liver inflammation and associated metabolic imbalances such as increased cytokines, triglycerides, and glucose intolerance. As underlying mechanism the authors show that obesity-associated acetylation inhibits modification of FXR K277 by SUMO2. A variety of approaches supports the model that SUMOylated FXR is required to inhibit inflammatory gene expression in liver hepatocytes, and that reduced FXR SUMOylation under HFD/obesity could be a main underlying factor that contributes to elevated liver inflammation.

The study is of general interest as the proposed mechanisms have direct implications for the understanding of mechanisms that control inflammation and that can be altered during the progression of obesity. The study is of particular interest for those concerned about the anti-inflammatory control mechanisms. To my knowledge, this is the first study that demonstrates the occurrence of SUMO-dependent anti-inflammatory transrepression by a nuclear receptor in vivo (here in mouse liver).

Conceptually and experimentally, the study is of sound quality and most of the data are well presented and controlled, with few exceptions outlined below. There is therefore no major concern but a number of issues that require the inclusion of additional data and/or additional experiments.

1. It is an attractive idea that nutrients, linked to the supply of different endogenous FXR ligands, may differentially modulate the acetylation - SUMOylation switch of FXR.
This raises the question of which endogenous FXR agonists would trigger SUMOylation in vivo and how would the change in the levels of FXR ligands (such as different bile acid species and its accumulation in the liver) during obesity progression modulate the FXR modifications? Have the authors investigated whether endogenous FXR agonists, and antagonists, induce SUMOylation to the same extent as GW4064? If not, the issue should at least be discussed in the context of current knowledge.

2. Fig. 2: Regarding liver steatosis in K217R mice: What is the underlying mechanism, less lipogenesis, increase of SREBP? Referring to Fig. 2J, it is suggested that FXR acetylation provokes impaired insulin signaling in the liver. To assess the hepatic-specific influence on insulin sensitivity, hyperinsulinemic/euglycemic clamp studies (HGP) are probalby more suitable than GTT.

3. Fig. 2A/B: What is the rationale behind the comparison ND mice K217Q vs. WT and in HFD K277R vs. WT? Doesn´t the comparison need to include all combinations. i.e. in ND mice K217Q vs. K277R vs. WT and in HFD K217Q vs. K277R vs. WT?

4. As for the working model, do the authors suggest that de-acetylation at K217 is a pre-requisite to induce ligand-dependent (GW4064) SUMO2 modification at K277? The use of structurally diverse FXR ligands may override the functional connection between K217 acetylation and K277 SUMOylation. In the experiments shown in Fig 3, the inclusion of the K217Q versus K mutants would be helpful to support the model.

5. Regarding the interdependence of the two modifications, obesity (HFD) was in previous work from the authors demonstrated to increase levels of Acetyl-CoA and the acetylase p300, while decreasing levels of the deacetylase SIRT1. At this point we cannot exclude whether critical components of the SUMOylation system are also changed (expression, modification) in obesity (HFD). Have the authors looked in their expression profiling whether any of the SUMO components change under HFD treatment, or upon treatment with FXR agonist?

6. Along these lines, Figure 4C suggests that the inverse correlation between acetylation and SUMOylation under obesity (HFD) is not limited to FXR but possibly seen with many more transcription factors. Since the arrangement of the respective acetylation and SUMOylation sites in these factors (e.g. LXRs, ER, PXR, SREBP) is likely to be very different, a generalization of the FXR model (Fig. 5F) is difficult to envisage. Thus, in the absence of further clarifying data, the authors should remove Fig. 4C from the current manuscript, as it does not add to the story and the understanding of how FXR SUMOylation is regulated in obesity.

7. Regarding the speculative FXR model (Fig. 5F) based on the PPAR/RXR structure, I wonder whether during the time of revision any better model became apparent? FXR seems closer to LXRs than to PPARs, so a comparison to LXR structures (possibly to the LXR/RXR heterodimer, expected to become public any day) may be more suitable.
I also wonder how FXR antagonists, clinically relevant, would affect the link between K217 Ac and K277 SUMO in the model?

8. Regarding the inability of SUMOylated FXR to dimerize with RXR: The model is appealing and consistent with similar suggestions made for LXRs, however the role of RXR in anti-inflammatory action by partner NRs remains controversial. So in the ChIP-SEQ data shown in Fig. 6 and S7, are these inflammatory FXR sites are actually occupied by RXR or is RXR absent? Do these sites have FXREs, in addition to the NKkB sites? Negative regulation may occur via direct negative FXREs (requiring RXR), or via trans-repression type tethering (perhaps not requiring RXR). Note that in these experiments mice were treated with GW4064 but not simultaneously with LPS or cytokines to activate inflammatory factors. So classic tethering and classic trans-repressions seems rather

unlikely to occur at these sites under these conditions. Also, were these data new generated for this study, or have they been generated in the previous study published in Lee et al. Hepatology 2012? If so, this should be stated.

9. Regarding the target range of inflammatory genes that are under FXR-SUMO control: Are the authors suggesting that all inflammatory genes in liver are affected, based on the NFkB requirement? Or is there evidence for gene-selectivity in liver? This would be expected since FXR is selectively SUMOylated by SUMO2, thus the transrepression might be more restricted (assuming the current model that SUMO1 versus SUMO2 selective SUMO binding proteins, like coronin 2A, are required for the NCOR/SMRT corepressor-dependent pathway. What are the thoughts of the authors regarding SUMO2 preference of the FXR K277 site? It does not seem to depend on HDAC4, as suggested for LXR by Glass and coworkers.

10. FigS4: Correct the figure legend: Levels of hepatic proteins.... are DECREASED (not increased). In the FigureS4, lower panel (SUMO2/3 IB) - the only difference we see is the SUMO species around 140 kDa; the species are quite distinct and may reflect a protein (or subset of proteins) that are specifically modified under control conditions but not HFD. Do the authors suggest that this is FXR? If not, this Figure does not add to this study and may be removed.

11. The FXR K217 Ac site is conserved in species, especially between rodents and humans (as shown in Kemper et al. 2009), how about the K277 SUMO2 site and the other previously identified additional SUMO sites in FXR? An additional figure panel may be included and discussed if appropriate.

Resubmission 15 July 2014

Response to Reviewers

Referee #1

This study by Kim et al. was performed to evaluate the impact of DIO on FXR acetylation. The authors demonstrate that DIO mice have elevated FXR K217 acetylation levels. Using acetylation-defective and -mimicking mutants they further show that FXR acetylation leads to enhanced hepatic inflammation, TG accumulation, and impaired insulin signaling. Their mechanistic model suggests that FXR acetylation at K217 hinders SUMOylation at K277, thereby diminishing the anti-inflammatory action of FXR on NF-kB target genes. Although overall the study is well conceived and performed, some physiological and mechanistic questions remain.

Major comments:

1. Fig 6G: The authors conclude that in lean mice, FXR agonists trigger SUMO2 modification of FXR, which in turn enhances the interaction with NF-kB and CoR, but blocks the interaction with RXR. How do the authors reconcile this mechanism with the established knowledge that FXR agonists not only repress NF-kB inflammatory genes but also induce the classical FXR target genes through mechanisms that critically depend on FXR-RXR interaction?

Response: An important aspect of the model is that only a fraction of FXR is SUMOylated so that the SUMO2-FXR targets and inhibits inflammatory genes and the unmodified FXR binds to RXR and activates classical FXR target genes containing an FXRE. To more clearly support the selective trans-repression of NF-kB target inflammatory genes by SUMOylation of FXR without affecting classical FXR/RXR target genes, we did several additional studies.

First, we repeated experiments to examine the different effects of SUMO-FXR on interaction of FXR with NF-kB and RXRa, and obtained results consistent with the initial studies. We determined that the fraction of FXR that was SUMOylated in vitro was about 60% (Fig. 6D) and this in vitro SUMO2-FXR was used in studies of the interaction of FXR with RXRa and NF-kB. When FXR was about 60% SUMOylated in vitro, interaction with RXRa was substantially decreased (Fig. 6D) and interaction with NF-kB was markedly increased (Fig. 6H). It is well known that most SUMO targets are modified at very low steady state levels. Due to its dynamic nature and

very low fraction of the targets that are SUMOylated, the fraction of SUMO2-FXR at steady state is likely to be far less than 60% in cells.

Second, to further examine interactions of FXR with RXR and NF-kB in a DNA context, we performed gel mobility shift assays utilizing the in vitro SUMOylated FXR. When about 60% of FXR was SUMOylated, binding of FXR/RXRa to the FXRE in *Shp* probe was substantially reduced, while inhibition of binding of NF-kB to the NF-kB site in *Tnfs4* by FXR was substantially enhanced (Fig. 7D-E).

Third, we examined by ChIP assay whether SUMO2-FXR is recruited to FXR/RXRa target genes like *Shp* and *Bsep*. Indeed, in these new ChIP studies, SUMO2, but not SUMO1 proteins were detected at the inflammatory gene, *Tnsf4*, (Fig. 7H) and other inflammatory genes (Supplementary Fig. S16), but not at the FXR/RXRa target genes, *Shp* (Fig. 7H) or *Bsep* (Supplementary Fig. S16).

These new data strongly support our conclusion that SUMOylation of agonist-activated FXR increased its interaction with NF-kB but not with RXRa, so that SUMOylation of FXR selectively inhibits inflammatory genes without inhibiting FXR/RXRa target genes. Importantly, only a fraction of FXR is SUMOylated so that ligand activation of FXR/RXRa target genes is not substantially affected and ligand activated FXR both inhibits inflammatory genes and activates FXR/RXRa target genes.

2. Based on hepatic TG analysis (Fig 2F+I), ND mice infected with Ad-K217Q show increased hepatic lipid content. It is not very clear whether the excessive accumulation of hepatic lipids induces the inflammatory genes or if the lipid accumulation is a consequence of inflammatory activation. To clarify this point, time course experiments should be performed to assess the onset of the lipid and inflammation transcriptional programs.

Response: Whether abnormal fat accumulation in liver causes inflammation and insulin resistance, or conversely, whether inflammation causes fatty liver is a long-standing question. With regard to the effects of K217Q, we examined expression of genes related to fatty accumulation (*Srebp1c, Fas, Dgat1*) and inflammation (*IL1b, Cxcl8, Inos*) as a function of time after expression of K217Q in hepatocytes from FXR KO mice (Supplementary Fig. S4). In general, increased expression of inflammatory genes occurred before increases in genes associated with accumulation of fat. Of course, this type of correlation does not establish causality and to do so major additional studies would be required, which are outside the central themes in this manuscript, so we have not pursued this question further.

3. Based on biochemical knowledge, fasting induces lipolysis and significantly increases the concentration of acetyl-CoA (substrate for acetylation) in hepatocytes. Have the authors analyzed whether differences in acetylation can be observed between fed and fasted DIO mice?

Response: We have previously published studies showing the acetylation of FXR (Cell Metabolism 10:392, 2009) and SREBP (J Biol Chem. 285:33959, 2010) are elevated in obese mice. We have modified the Introduction to emphasize this point. In addition, the increased acetylation of FXR after feeding a high fat diet is shown in Fig. 1F by IHC analysis using the Ac-K217-specific antibody.

4. Have the authors compared the level of SUMO1 and SUMO2 modification of FXR in ND and HFD mice. Is SUMO1 modification also reduced upon HFD feeding or instead unchanged by HFD? What determines the differential binding of SUMO1 and SUMO2 on FXR?

Response: It is not clear what determines the differential binding of FXR with SUMO1 and 2, but signaling-induced PTMs of FXR likely change protein interactions with SUMO proteins. In response to LPS and GW4064 treatment, in cell SUMO assays, SUMO2-FXR levels were increased, whereas SUMO1-FXR was not detected (Supplementary Fig. S5). Further, in the ChIP studies (Fig. 7H and Supplementary Fig. S16), occupancy of SUMO2 proteins was detected at the inflammatory genes, *Tnfsf4, IL6ra, and C1qtnf2*, but not at the FXR/RXR target genes, *Shp ad Bsep*, while SUMO1 was not detected at any of these genes (Fig. 7H, Supplementary Fig. S16). SUMO1-FXR was reported to be involved in regulation of liver bile acid levels. We, therefore, focused on SUMO2 modification of FXR in anti-inflammatory functions in this study.

5. The authors propose a model in which acetylation of K217 prevents SUMOylation at K277. This is supported by the finding that FXR-acetylation is increased during HFD, while SUMOylation is

reduced (Fig 4D). It is striking however that the acetylation of FXR is only moderate under HFD (when taking the loading into account), .while SUMOylation of FXR (by SUMO2) is completely lost under HFD (Fig 4D). This raises the question as to whether the opposite is not occurring, i.e. SUMOylation hinders the acetylation under ND, but not during HFD when SUMOylation of FXR becomes abolised? This alternative scenario should be evaluated as well.

Response: It is possible that SUMOylation of FXR inhibits acetylation in lean mice, and while we have not extensively examined this possibility, we did an additional experiment examining the effect of SUMOylation on acetylation of FXR in vitro. The results are consistent with inhibition of acetylation by SUMOylation. This observation, however, likely has little physiological relevance. It is well known that the SUMO modified fraction of a particular protein is low and transient so that SUMO-protein detected at steady state is less than 5% of total protein and it is even technically very challenging to detect endogenous SUMO-proteins. Thus, even if SUMOylation of FXR inhibits acetylation in a small fraction of FXR, it would not substantially affect levels of Ac-FXR. Further, we have shown that the acetylation level of FXR is mainly dependent on the activities and levels of the acetylase, p300, and deacetylase, SIRT1 (Cell Metabolism, 2009). In HFD obese mice, SIRT1 levels are abnormally low resulting in increased acetylation of FXR and other protein factors. While the fraction of FXR that is SUMOylated in lean mice may not be acetylated, the overall low acetylation level of FXR in these mice is governed by the ratio of p300 to SIRT1.

6. The authors observed a clear difference in the glucose tolerance test, while no difference on the basal fasting glucose levels was shown between WT and Ad-K217R mice (Fig 2J). However, the expression of gluconeogenic genes (PEPCK and G6Pase, Fig S2) was significantly decreased. Would the authors not expect different fasting glucose levels? In addition, plasma insulin levels at the different time points (0, 15, 30 and 60 min) should be provided.

Response: We would expect decreased glucose levels after an overnight fast. At the initial time point of the experiment, however, glucose levels in the ITT/GTT tests were not true fasting levels since the fasting was only for 5-6 h for these experiments and glucose levels may have been maintained in this short fasting by glycogen breakdown. Fasting for the gene expression studies was overnight or about 18 h which would be consistent with the later requirement of gluconeogenesis, after gycogenolysis, to maintain glucose levels.

7. P-Akt levels are reduced upon ectopic expression of FXR K217Q. What is the consequence of Ad-K217Q on glucose tolerance and insulin sensitivity?

Response: To address the reviewer's question, we examined in mice the effects of the Ac-mimic K217Q FXR mutant on glucose intolerance and the expression of metabolic genes in liver. Mice were injected via the tail vein with Ad-FXR WT or Ad-FXRK217Q (Ac-mimic), and 1 week later, were fasted for 5-6 h and GTT and ITT tests were performed. The results indicate that expression of K217Q resulted in significant increases in glucose tolerance (presented in Fig. 2J). In the ITT test, expression of K217Q in lean mice fed a ND showed slightly higher serum glucose levels at 15 min after insulin injection and conversely, expression of K217R in HFD induced obese mice resulted in slightly lower serum glucose levels compared to those from FXR-WT, but, although there was a trend the values were not statistically significant (Supplementary Fig. S3).

Minor comments:

1. The analyzed gene sets in Fig 2C-E, Fig 5A and Fig 6A are inconsistent. It would be better to monitor the same genes throughout the study.

Response: Inflammatory genes presented in Fig, 2C-E and Fig. 5 were identified from microarray data but these genes are not necessarily direct target genes of FXR. In contrast, NF-kB target inflammatory genes presented in Fig. 6 (original manuscript), such as *Tnfsf4, Il6ra, C1qtnf2*, were identified as direct FXR target genes from global ChIP-seq analysis. In mechanistic studies, we preferred to use these direct FXR target genes to examine the role of acetylation and SUMO of FXR on occupancy of FXR at inflammatory genes compared to occupancy at the FXR/RXR target genes like *Shp and Bsep*.

2. More information should be provided about the nature and generation of the adenoviral constructs.

Response: As suggested, more information on the construction and generation of adenoviruses expressing FXR-WT or K217R, K217Q, K277R mutants is provided in the Materials and Methods Section.

Referee #2:

In this study Kemper and co-workers investigate the interplay of acetylation and sumoylation on the nuclear receptor FXR. In a previous paper by Kemper et al. it was shown that FXR undergoes acetylation, with K217 being the major site of acetylation. It was further demonstrated that this modification inhibits heterodimerization with RXRα, DNA binding, and transactivation activity. Moreover, acetylation of FXR was shown to be induced in leptin-deficient ob/ob mice or diet-induced obese mice.

In the current paper the authors propose interplay of FXR acetylation with conjugation of the ubiquitin-related SUMO modifier. They show that in analogy to other members of the nuclear receptor family FXR engages in an anti-inflammatory transrepression pathway when conjugated to SUMO2. They propose that this is mediated through enhanced binding of the FXR-SUMO2 conjugate to NF-κB. They further suggest that K217 acetylation prevents sumoylation of FXR by inhibiting its interaction with the SUMO E3 ligase PIAS4. Based on these findings the authors propose a model, in which enhanced acetylation of FXR in obesity would contribute to a diminished anti-inflammatory response through a reduction of sumoylation. Although this is an intriguing concept the data shown here are not fully conclusive and at this stage are too preliminary support the authors claims. As detailed below I am particularly concerned about the experimental part on sumoylation of FXR, which to my opinion contains a number of conceptual and technical weaknesses.

Major points:

1. In their in vitro sumoylation assays (for example Figure 3A) the authors do not really show sumoylated forms of FXR, since they only do anti-SUMO2 immunoblots after the in vitro assay. Because it is known that free SUMO2 chains can be assembled in vitro, the authors might therefore simply look on the formation on these chains rather than sumoylated FXR. Actually it would be crucial to see in anti-FXR immunoblots what fraction of FXR gets actually modified. Given that the amount of unmodified FXR (see Figure 4B, lower panel) is not significantly reduced one can assume that only a very minor fraction of FXR is converted to FXR-SUMO2.

Response: I do appreciate the reviewer for raising this crucial and insightful issue. In the initial manuscript, the input FXR lane showed only the initial input for FXR before SUMO reaction. In the revised manuscript, we repeated these experiments twice and show the amount of unmodified FXR before and after the SUMOylation. In these experiments, the unmodified FXR was reduced by about 60% after SUMOylation, indicating that about 60% of FXR was SUMOylated after the in vitro SUMO reaction (Fig. 3B).

2. The issue in point 1 becomes very crucial when it comes to the experiments shown in Figure 5E, I. Here the authors claim that sumoylation of FXR prevents binding to RXR but fosters binding of NF-κB/p65. The setup for these experiments is outlined in Figure 5D. In principal, FXR was in vitro sumoylated and used for a Co-IP with RXR or NF-κB/p65, respectively (both generated by in vitro transcription/translation). Considering again that only a very minor fraction of FXR is sumoylated (as judged by the equal amounts of unmodified FXR in lane 3 and 4, Figure 5E bottom panel), it is difficult to explain why the interaction of RXR is completely gone. The same issue arises when it comes to the SUMO-dependent binding to NFκB (Figure 5I). The striking differences in the cellular Co-IP of NFκB/p65 and FXR WT vs. SUMO-deficient FXR K277R (Figure 5G) are also very surprising given that only minute amounts of FXR are modified. To confirm that the interaction is indeed SUMO-dependent (in particular SUMO2-dependent) in a cellular setting the authors should deplete cells from SUMO2/3 by RNAi. Depletion of SUMO2/3 should recapitulate the data with the

FXR K277R variant.

Response: Using the SUMO-FXR samples described in the response to comment 1 above, the interaction of FXR with RXRa was substantially decreased (Fig. 6D) whereas the interaction with NF-kB was markedly increased (Fig. 6H).

To further support our model that SUMO-FXR differentially interacts with RXRa and NF-kB, we examined the interactions in a DNA context in vitro using gel mobility shift assays. We selected two radiolabeled oligonucleotide probes, one from the *Shp* gene that contains the FXR/RXRa DNA binding IR1 motif, and the other from the *Tnfsf4* gene that contains the NF-kB binding site. We consistently observed that SUMOylation of FXR substantially reduced heterodimerization with RXRa and binding to the *Shp* FXRE binding site and that addition of SUMO-FXR reduced the binding of NF-kB to the *Tnfsf4* probe (Fig. 7C-E). In both cases, partial effects were consistent with the 60% extent of SUMOylation of FXR. However, it is noteworthy that due to its dynamic nature and the very low fraction of targets that are SUMOylated, the fraction of SUMO2-FXR is likely to be far much less than 60% in cells. The results from these in vitro gel shift assays are consistent with our CoIP using SUMO-FXR (Fig. 6D, H), ChIP studies (Fig. 7H), and also with predictions from structural modeling studies using both PPARg/RXR/DNA (Fig. 6E) and LXR/RXR/DNA templates (Supplementary Fig. S11).

With regard to the concern about the SUMO-deficient K277R studies (now Fig. 6F,G), in these experiments M2 was used to detect or IP FXR so only interactions with the expressed FXR variant was observed. The mutated FXR did not CoIP with p65 or SMRT, but FXR WT which could be SUMOylated even if only partially did detectably CoIP with p65 and SMRT.

Although we did not directly assess the effects of downregulation of SUMO2/3, downregulation of SUMO ligase PIASy reduced SUMO2 modification of FXR and altered gene expression as expected (presented in Fig. 3G). Also, in CoIP studies, downregulation of PIASy by siRNA decreased the interaction of FXR with p65 (Supplementary Fig. S10). Further evidence that SUMO2, rather than SUMO1, is involved in FXR SUMOylation included ChIP assays showing that SUMO2, and not SUMO1, was detected at three inflammatory genes, *Tnfsf4, IL6ra, and C1qtnf2*, after LPS or LPS/GW4064 treatment, but not at FXR/RXRa target genes, *Shp and Bsep*, (Fig. 7H, Supplementary Fig. S16). These studies, collectively, indicate that SUMO2 modification of FXR is important for its anti-inflammatory responses.

3. Another important issue concerns the molecular basis of SUMO-dependent NFκB binding. Is there a typical SUMO interaction motif in NFκB. If yes is this specific for SUMO2 or can SUMO1 also bind. This is an important issue because the authors shown that FXR is also a substrate for SUMO1.

Response: There is a SIM motif in NF-kB (Liu et al., Plos One, 2012). To further address the reviewer's question, we performed in vitro GST pull down studies using GST fusion proteins containing different regions of the NF-kB subunit, p65. NF-kB directly interacted with SUMO2 but not with SUMO1 proteins in in vitro GST pull down assays (presented in Supplementary Fig. S12). These data are consistent with the other data described above (Fig. 7H, Supplementary Fig. S16) that indicate that SUMO2 and not SUMO1 is involved in regulating the anti-inflammatory function of FXR.

Also, as noted in the comments to reviewer 1, in in-cell SUMO assays, SUMO2-FXR levels were increased, whereas SUMO1-FXR was not detected, in response to LPS and GW4064 treatment (Supplementary Fig. S5). Further, in ChIP studies (Fig. 7H and Supplementary Fig. S16), occupancy of SUMO2 proteins was detected at the inflammatory genes, *Tnfsf4, IL6ra, and C1qtnf2*, but not at the FXR/RXR target genes, *Shp ad Bsep*, while SUMO1 was not detected at any of these genes (Fig. 7H, Supplementary Fig. S16). SUMO1-FXR has been reported to be involved in regulation of liver bile acid levels, but not inflammation. We, therefore, focused on the role of SUMO2 modification of FXR in its anti-inflammatory functions in this study.

4. Related to the above point: So far the SUMO-mediated transrepression pathway of nuclear hormone receptors has been linked to SUMO-dependent interactions with SIM-containing co-repressor complexes (like coronin or GPS2). Therefore it is conceptually rather intriguing that here the authors show that loss of changes in FXR sumoylation directly impinge on promoter occupancy of NFκB/p65 on inflammatory genes. Is SUMO-FXR signalling independent of the above-mentioned SUMO-anchored co-repressors? This is a critical point since the authors suggest that the proposed

acetyl/SUMO switch is conserved in other nuclear hormone receptors.

Response: As noted by the reviewer, recent studies from the Treuter and Glass groups have shown that GPS2 and Coronin contain SIM motifs and function as key trans-repression mediators by linking SUMO-nuclear receptors and the NcoR/SMRT complex. In response to the reviewer's comment, we examined the role of GPS2 in SUMO2-FXR mediated dissociation of NF-kB. In three independent experiments in primary hepatocytes in which ChIP assays were combined with siRNA for GPS2, we found that FXR binding appears to be selectively affected by downregulation of GPS2 in a gene-specific manner, whereas NF-kB binding is not directly affected, at least at the 3 inflammatory genes we tested (Supplementary Fig. S18).

5. The authors propose that acetylated FXR cannot bind PIAS4 thus preventing sumoylation. The assumption that PIAS4 is the relevant SUMO ligase for FXR is only based on overexpression experiments (for example Figure 3D). Data on the role of endogenous PIAS4 are missing. Moreover, in Figure 3D expression controls for PIAS family members are missing.

Response: We agree with the reviewer that the role of PIASy in SUMOylation of FXR was not convincingly shown in the previous manuscript. We therefore performed additional studies using siRNA downregulation of PIASy and now show that downregulation of PIASy reduced SUMO2 modification of FXR in cells (Fig. 3G), decreased FXR interaction with p65 (Supplementary Fig. S10), and increased expression of inflammatory genes in hepatocytes (Supplementary Fig. S10). Also, the expression levels of the flag-tagged PIAS family proteins are now shown in Fig. 3E.

6. Data obtained with the acetyl-mimicking FXR variant K217Q should be discussed more carefully. Although glutamine has been frequently used as an acetyl-mimic, it may not fully recapitulate the behaviour of the naturally acetylated protein.

Response: We agree with the reviewer that caution is needed to interpret data from experiments using the Ac-mimic K217Q mutant. We have included this issue in the revised manuscript (page 7).

Referee #3:

The study by Kemper and coworkers address the important question of how nutrients and metabolic alterations (here high-fat diet HFD linked to obesity) modulate gene expression and physiology. Using the nutrient-sensing nuclear receptor FXR as a model, they convincingly demonstrate that cross-regulated post-translational modifications (here acetylation versus SUMOylation) are subject to nutritional control. Physiological consequences are described with focus on the liver, a main site of FXR action, using a wide array of in vivo and vitro methods, including adenovirus-mediated expression of FXR WT/mutant in mouse liver and primary hepatocytes.

The main findings are the following: Obesity triggers FXR acetylation at K217, which causes increased liver inflammation and associated metabolic imbalances such as increased cytokines, triglycerides, and glucose intolerance. As underlying mechanism the authors show that obesity-associated acetylation inhibits modification of FXR K277 by SUMO2. A variety of approaches supports the model that SUMOylated FXR is required to inhibit inflammatory gene expression in liver hepatocytes, and that reduced FXR SUMOylation under HFD/obesity could be a main underlying factor that contributes to elevated liver inflammation.

The study is of general interest as the proposed mechanisms have direct implications for the understanding of mechanisms that control inflammation and that can be altered during the progression of obesity. The study is of particular interest for those concerned about the anti-inflammatory control mechanisms. To my knowledge, this is the first study that demonstrates the occurrence of SUMO-dependent anti-inflammatory transrepression by a nuclear receptor in vivo (here in mouse liver).

Conceptually and experimentally, the study is of sound quality and most of the data are well presented and controlled, with few exceptions outlined below. There is therefore no major concern but a number of issues that require the inclusion of additional data and/or additional experiments.

1. It is an attractive idea that nutrients, linked to the supply of different endogenous FXR ligands, may differentially modulate the acetylation - SUMOylation switch of FXR.
This raises the question of which endogenous FXR agonists would trigger SUMOylation in vivo and how would the change in the levels of FXR ligands (such as different bile acid species and its accumulation in the liver) during obesity progression modulate the FXR modifications? Have the authors investigated whether endogenous FXR agonists, and antagonists, induce SUMOylation to the same extent as GW4064? If not, the issue should at least be discussed in the context of current knowledge.

Response: We thank the reviewer of raising the important issue of the effects of natural ligands of FXR on its SUMOylation. In mouse liver, we observed that SUMO-FXR levels were markedly increased in response to both a short CA feeding (0.5 % CA chow for 3h) and GW4064 treatment (Fig. 3H). We examined the effects of known FXR ligands, a primary BA, CDCA, secondary bile acids, such as UDCA or LCA, or a known antagonist, guggulsterone in primary mouse hepatocytes. Detection of endogenous SUMO-FXR is quite technically difficult, but the results were consistent in three independent experiments with an increase in SUMO-FXR levels after treatment with CDCA, GW4064, or UDCA, a modest increase with LCA, and conversely a substantial decrease after treatment with the antagonist gugglesterone. These results with quantitation are now presented in Supplemental Fig. S6.

2. Fig. 2: Regarding liver steatosis in K217R mice: What is the underlying mechanism, less lipogenesis, increase of SREBP? Referring to Fig. 2J, it is suggested that FXR acetylation provokes impaired insulin signaling in the liver. To assess the hepatic-specific influence on insulin sensitivity, hyperinsulinemic/euglycemic clamp studies (HGP) are probalby more suitable than GTT.

Response: We agree that the clamp experiment is preferable to GTT to examine the liver-specific role, but we do not have the capability to do these experiments. The changes in GTT in response to K217R FXR in the liver do indicate a contribution of the liver to the changes in GTT. With regard to the mechanism of liver steatosis, expression of K217R compared to WT FXR resulted in downregulation of cholesterol, sterol, and lipid biosynthetic genes (Fig. 2A) which may explain the reduced steatosis. We have not identified the gene or genes most directly responsible for the improvement in steatosis.

3. Fig. 2A/B: What is the rationale behind the comparison ND mice K217Q vs. WT and in HFD K277R vs. WT? Doesn´t the comparison need to include all combinations. i.e. in ND mice K217Q vs. K277R vs. WT and in HFD K217Q vs. K277R vs. WT?

Response: The rationale is that in lean ND mice acetylation of FXR is low so that expression of K217Q as an acetylation mimic mutant would alter the FXR status toward that of obese HFD mice and have a substantial effect while expression of an acetylation-deficient mutant in a low acetylation background would have little effect. Similarly, expression of the acetylation-deficient mutant in HFD obese mice with high FXR acetylation would examine the effects of expression of unacetylated FXR in the obese mice while the acetylation mimic mutant would have little effect in a high acetylation background. While studying all combinations would be most desirable, these are costly and difficult experiments so we examined the two situations in which the maximum effect would be obtained.

4. As for the working model, do the authors suggest that de-acetylation at K217 is a pre-requisite to induce ligand-dependent (GW4064) SUMO2 modification at K277? The use of structurally diverse FXR ligands may override the functional connection between K217 acetylation and K277 SUMOylation. In the experiments shown in Fig 3, the inclusion of the K217Q versus K mutants would be helpful to support the model.

Response: In principle, it is possible that some FXR ligands may induce SUMOylation of even acetylated FXR. In normal lean mice, this is a mute point since acetylation of FXR is low and transient so that deacetylation is not necessary for SUMOylation to occur and diverse FXR ligands induce SUMOylation (see response to Comment 1). It is a relevant question for obese mice if some FXR ligands are able to induce SUMOylation of FXR in the hyperacetylated state. At least for endogenous FXR ligands, this seems unlikely since SUMOylation of FXR is low in obese mice. For this reason, we have not directly tested the effects of various FXR ligands on SUMOylation in obese

mice. With regard to the suggestion related to Fig. 3, the experiments in this figure are directed at identifying the SUMOylation site in FXR using the K277R mutant. I am sorry, but I do not understand the rationale for the suggestion of using K217Q in these experiments to support the model.

5. Regarding the interdependence of the two modifications, obesity (HFD) was in previous work from the authors demonstrated to increase levels of Acetyl-CoA and the acetylase p300, while decreasing levels of the deacetylase SIRT1. At this point we cannot exclude whether critical components of the SUMOylation system are also changed (expression, modification) in obesity (HFD). Have the authors looked in their expression profiling whether any of the SUMO components change under HFD treatment, or upon treatment with FXR agonist?

Response: In response to the reviewer's suggestion, we examined expression of SUMO1, SUMO2, and PIASy in mouse liver during the development of diet-induced obesity. Expression of SUMO2, at least mRNA levels, was not markedly changed whereas that of PIASy was significantly reduced after 16 weeks of HFD feeding, and conversely, mRNA levels of SUMO1 gradually increased during diet-induced obesity. This data are now included in Fig. 4B.

6. Along these lines, Figure 4C suggests that the inverse correlation between acetylation and SUMOylation under obesity (HFD) is not limited to FXR but possibly seen with many more transcription factors. Since the arrangement of the respective acetylation and SUMOylation sites in these factors (e.g. LXRs, ER, PXR, SREBP) is likely to be very different, a generalization of the FXR model (Fig. 5F) is difficult to envisage. Thus, in the absence of further clarifying data, the authors should remove Fig. 4C from the current manuscript, as it does not add to the story and the understanding of how FXR SUMOylation is regulated in obesity.

Response: We agree with the reviewer that substantial additional data would be required for each of the factors with increased acetylation in obesity to link the acetylation with SUMOylation. We have moved Fig. 4C to supplemental data (Supplementary Fig. S8). We believe it is useful information to include since it shows that hyperacetylation in obesity is not unique to FXR and at least raises the possibility that the increased acetylation may be related to reduced SUMOylation in these other transcription factors.

7. Regarding the speculative FXR model (Fig. 5F) based on the PPAR/RXR structure, I wonder whether during the time of revision any better model became apparent? FXR seems closer to LXRs than to PPARs, so a comparison to LXR structures (possibly to the LXR/RXR heterodimer, expected to become public any day) may be more suitable. I also wonder how FXR antagonists, clinically relevant, would affect the link between K217 Ac and K277 SUMO in the model?

Response: I thank the reviewer for this helpful comment. The structure of the LXR/RXR heterodimer on DNA was very recently published (Lou et al., Nature Structural and Molecular Biology, 2014). The sequence of FXR is more similar to LXR than to that of PPARg, but the hinge region of FXR is longer than that of LXR or PPARg, so prediction of the position of K217 in FXR is still uncertain and speculative. We remodeled FXR using the LXR/RXR/DNA structure (Supplementary Fig. S11) and while the relationship of SUMO2 and RXR is somewhat different than obtained with the PPARg structure, SUMO2 modification would still be predicted to sterically hinder the interaction of FXR with RXR. The question of FXR antagonists is interesting and in principle their binding could alter the structural relationship between the acetylation site and the SUMO site. Antagonist binding to the ligand binding pocket should change the conformation of helix 2 (part of the ligand binding pocket) and K277 is located at the C-terminus of helix 2, so it could possibly affect the SUMO binding. However, we feel confident that Ac-FXR at K217 inhibits its SUMOylation at K277 from data from our experimental studies (Fig. 5D-H) but haven't examined the possible effects of antagonists on this interaction.
Our results from in vitro gel mobility shift assay using SUMO2-FXR, FXR, and RXR proteins (Fig. 7C, D) are consistent with the structural modeling analyses.

8. Regarding the inability of SUMOylated FXR to dimerize with RXR: The model is appealing and consistent with similar suggestions made for LXRs, however the role of RXR in anti-inflammatory action by partner NRs remains controversial. So in the ChIP-SEQ data shown in Fig. 6 and S7, are these inflammatory FXR sites are actually occupied by RXR or is RXR absent? Do these sites have

FXREs, in addition to the NKkB sites? Negative regulation may occur via direct negative FXREs (requiring RXR), or via trans-repression type tethering (perhaps not requiring RXR). Note that in these experiments mice were treated with GW4064 but not simultaneously with LPS or cytokines to activate inflammatory factors. So classic tethering and classic trans-repressions seems rather unlikely to occur at these sites under these conditions. Also, were these data new generated for this study, or have they been generated in the previous study published in Lee et al. Hepatology 2012? If so, this should be stated.

Response: This issue was also raised by other reviewer and please read the response to the comment #1 and #2 of the reviewer 2.

In summary, there is no FXRE site at the FXR binding peaks regions at inflammatory genes, *Tnfsf4*, *IL6ra*, and *C1qtnf2*. RXRa was barely detectable in ChIP assays suggesting that RXRa is not binding to these regions (Fig. 7F), and in vitro gel shift assays show direct interaction of FXR with NF-kB in the absence of RXRa which also suggests trans-repression (not requiring RXRa) (Fig. 7D-E). The ChIP-seq analysis was published by Lee et al, but the transrepression tethering data for FXR with NF-kB was generated in this study. We have tried to clearly indicate that the ChIP-seq data in the manuscript was from Lee et al.

9. Regarding the target range of inflammatory genes that are under FXR-SUMO control: Are the authors suggesting that all inflammatory genes in liver are affected, based on the NFkB requirement? Or is there evidence for gene-selectivity in liver? This would be expected since FXR is selectively SUMOylated by SUMO2, thus the transrepression might be more restricted (assuming the current model that SUMO1 versus SUMO2 selective SUMO binding proteins, like coronin 2A, are required for the NCOR/SMRT corepressor-dependent pathway. What are the thoughts of the authors regarding SUMO2 preference of the FXR K277 site? It does not seem to depend on HDAC4, as suggested for LXR by Glass and coworkers.

Response: It is not clear what determines the preference of SUMO2 over SUMO1 for the FXR K277 site, but signaling-induced PTMs of FXR likely change protein interaction with SUMO proteins, as suggested in our preliminary studies (Supplementary Fig. S5). The preference of SUMO2, rather than SUMO1, for the FXR K277 site and focusing on SUMO2 instead of SUMO1 in this study was discussed in response to comment 4 for reviewer 1 and comment 2 for reviewer 2.

For the three inflammatory genes that we studied, each contained a NF-kB binding site and was inhibited by SUMO2-FXR. We have not obtained evidence for a gene-selective effect of SUMO2-FXR on inhibition with the three inflammatory genes tested, however, it is certainly possible.

10. FigS4: Correct the figure legend: Levels of hepatic proteins.... are DECREASED (not increased). In the FigureS4, lower panel (SUMO2/3 IB) - the only difference we see is the SUMO species around 140 kDa; the species are quite distinct and may reflect a protein (or subset of proteins) that are specifically modified under control conditions but not HFD. Do the authors suggest that this is FXR? If not, this Figure does not add to this study and may be removed.

Response: We agree with the reviewer on the comment that this figure does not add too much on our conclusion. We therefore deleted this supplemental figure in the revised manuscript.

11. The FXR K217 Ac site is conserved in species, especially between rodents and humans (as shown in Kemper et al. 2009), how about the K277 SUMO2 site and the other previously identified additional SUMO sites in FXR? An additional figure panel may be included and discussed if appropriate.

Response: The FXR K277 site is also highly conserved in mammals and we have included a comparison of the sequences (Fig. 3D).

We thank all three reviewers for their constructive comments on our work. By performing extensive studies, we have addressed nearly all of their major comments and suggestions. We believe that new data in our revised manuscript provide additional strong support for our

conclusions and substantially strengthen the paper. We hope that our manuscript will now be acceptable for publication.

2nd Editorial Decision 27 August 2014

Thank you for re-submitting your manuscript " A dysregulated Acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity" to The EMBO Journal editorial office. I appreciate very much the changes you introduced, and I am glad to see that these improvements were also appreciated by the referees. We have now received the comments from the three original referees copied below for your information. All the referees consider your findings interesting and significant and concur that a majority of their concerns has been addressed. Two reviewers now fully endorse the publication of your manuscript.

Also referee # 1 acknowledges that the presented data are much more strengthened now, but still retains one last technical concern regarding sumoylation of FXR in the in vitro modification system: We feel that referee #1 makes a very constructive suggestion here how to address this concern by specifying which quality control to provide. We would, therefore, like to invite you to revise your manuscript regarding this specific point in a last small revision step, before we can formally proceed to accept publishing the manuscript.

Given your expertise I do believe that attending to this point will be straight-forward both technically as well as time-wise, and that this touch-up would certainly contribute to make the in vitro data very compelling.

Please do contact me regarding this decision.

Congratulations on your work!

REFEREE COMMENTS

Referee #1:

My major criticism on the initial version of the manuscript was related to data on SUMO-modification of FXR. My particular concern were the experiments aimed to demonstrate the impact of FXR sumoylation on binding to RXR and NFkappaB. Some aspects have now been clarified and strengthened in the revised manuscript. For example, by using an anti-FXR antibody the authors confirmed that in their in vitro modification system a fraction of FXR gets indeed modified by SUMO2 (Figure 1D). However, I do have difficulties to agree with their conclusion that 60% of FXR is converted to a SUMO-modified form. Even though the amount of unmodified FXR is reduced to 40%, the upper, SUMO-modified bands that are visible on the gel do unlikely represent 60% of FXR. Could part of FXR have been degraded? I do insist on that point, because this material from the in vitro reaction was used for the gel shift assays shown in Figure 7. To understand the significance of these results it would be very important to know whether a purification step was included after the in vitro sumoylation reaction. Otherwise not only the unmodified FXR, but also the enzymatic machinery (E1, E2) as well as unconjugated SUMO2 are still present in the reaction mix. Figure S15, which is entitled "Analysis of proteins used for gel shift assays" is not informative in this respect, because again it only shows a anti-SUMO2 immunoblot of the material after the reaction. How does a coomassie stain look like? The presence of proteins from the in vitro reaction could easily explain why addition of SUMO-FXR decreased the binding of NFκB to the Tnfsf4 probe. Along this line, it is not clear how to reconcile this new data from the band shift experiments with the model in Fig. 8. Here, SUMO-FXR is recruited to trans-repressed inflammatory genes through NFκB. This should explained.

In summary, the revisions have strengthened the work. However, there remain still some technical issues, which have to be clarified. I do acknowledge that biochemical experiments with SUMO-modified proteins are challenging due to the low abundance of the modified species. Nevertheless,

the authors have to make sure that they work with defined reagents. Eventually separation of SUMO-FXR from unmodified FXR should be considered. If this is not doable, purity of the in vitro sumoylated material used for Co-IP and band-shift assays should be carefully checked. To my opinion this is still a key aspect of the work.

Referee #2:

The authors have addressed most of my concerns / questions in a satisfactory way.

Referee #3:

The new manuscript has been substantially improved and all issues raised have been appropriately addressed. The reviewer is particularly impressed by the large amount of new data which, along with the thorough revision of data presentation, clarify the initial issues concerning FXR SUMOylation and the mechanisms of transrepression.

Minor: Supplemental Figure S21 should be corrected to S19.

1st Revision - authors' response 15 September 2014

Response to Reviewers

Referee #1:

My major criticism on the initial version of the manuscript was related to data on SUMO-modification of FXR. My particular concern were the experiments aimed to demonstrate the impact of FXR sumoylation on binding to RXR and NFkappaB. Some aspects have now been clarified and strengthened in the revised manuscript. For example, by using an anti-FXR antibody the authors confirmed that in their in vitro modification system a fraction of FXR gets indeed modified by SUMO2 (Figure 1D). However, I do have difficulties to agree with their conclusion that 60% of FXR is converted to a SUMO-modified form. Even though the amount of unmodified FXR is reduced to 40%, the upper, ***SUMO-modified bands that are visible on the gel do unlikely represent 60% of FXR.*** *Could part of FXR have been degraded? I do insist on that point, because this material from the in vitro reaction was used for the gel shift assays shown in Figure 7. To understand the significance of these results it would be very important to know whether a* ***purification step was included after the in vitro sumoylation reaction. Otherwise*** *not only the unmodified FXR, but also the enzymatic machinery (E1, E2) as well as unconjugated SUMO2 are still present in the reaction mix. Figure S15, which is entitled "Analysis of proteins used for gel shift assays" is not informative in this respect, because again it only shows a anti-SUMO2 immunoblot of the material after the reaction. How does a coomassie stain look like? The presence of proteins from the in vitro reaction could easily explain why addition of SUMO-FXR decreased the binding of NFκB to the Tnfsf4 probe. Along this line, it is not clear* ***how to reconcile this new data from the band shift experiments with the model in Fig. 8. Here, SUMO-FXR is recruited to trans-repressed inflammatory genes through NFκB.*** *This should explained.*

In summary, the revisions have strengthened the work. However, there remain still some technical issues, which have to be clarified. I do acknowledge that biochemical experiments with SUMO-modified proteins are challenging due to the low abundance of the modified species. Nevertheless, the authors have to make sure that they work with defined reagents. ***Eventually separation of SUMO-FXR from unmodified FXR should be considered.*** *If this is not doable, purity of the in vitro sumoylated material used for Co-IP and band-shift assays should be carefully checked. To my opinion this is still a key aspect of the work.*

Response: The reviewer raises three questions: 1) Can a better estimate of the percent of SUMOylated FXR be done? 2) Could other proteins from the SUMO reaction affect the NF-kB binding in gel shifts rather than SUMOylation of FXR? And 3) how can the decrease in NF-kB

binding to DNA by SUMOylated FXR in the gel shift (Fig. 7E) be reconciled with the model in which recruitment of SUMO-FXR to chromatin is facilitated by NF-kB?

1. ***Can a better estimate of the percent of FXR that is SUMOylated be done?***
   We agree that the estimate of SUMOylated FXR by simple subtraction is not the best method, but technically the estimate by protein staining is extremely difficult. We have repeated the in vitro SUMO assays twice (overexpression of flag-FXR in cells and purification, in vitro SUMOylation assay, and IB experiments) and analyzed decreasing amounts on the gel so that the amount of FXR and SUMO-FXR could be estimated from the same gel. In both analyses, about 40% of the FXR was SUMOylated which is lower limit estimate since the higher MW SUMOylated protein may be less efficiently transferred to the membrane. These results have been included in Supplemental Fig. S15. We agree that the ideal procedure would separate SUMO-modified FXR from FXR as the reviewer suggested, but that would be more technically difficult than analysis by protein staining. Finally, we feel the fraction of FXR that SUMOylated is relatively unimportant since the effect of SUMOylation on the interaction of FXR with NFkB is a positive dominant effect that increases the interaction (by CoIP, Fig. 6H), while it decreased interaction with RXRa, so even a small percentage of modification can explain the NF-kB gel shift results.

2. ***Could other proteins from the SUMOylation reaction affect the NF-kB binding in gel shifts rather than SUMOylation of FXR?***
   In the gel shift reactions, the unSUMOylated (-ATP) and SUMOylated +(ATP) FXR samples for the gel shift assays were processed in parallel so that any extraneous proteins should be the same in both reactions. Flag-FXR was overexpressed in Cos-1 cells, purified by M2 agarose, and isolated flag-FXR attached in M2 agarose beads was incubated with SUMO components. The only difference in the (-) or (+) SUMO reaction is the addition of ATP in the (+) SUMO reaction. After the SUMO reaction was done, the samples were washed multiple times before the CoIP or gel shift assays so most of the extraneous proteins including unincorporated SUMO proteins were removed. We have modified our description of the methods for in vitro SUMOylation to clarify this point (Method, page 21).

3. ***How can the decrease in NF-kB binding to DNA by SUMOylated FXR in the gel shift (Fig. 7E) be reconciled with the model in which recruitment of SUMO-FXR to chromatin is facilitated by NF-kB?***
   In the model, FXR is recruited to the NF-kB binding sites by interaction with NF-kB which results in dissociation of NF-kB from the binding site, which is consistent with the decrease in binding of NF-KB upon addition of SUMOylated FXR in gel shifts (Fig. 7E) and in the ChIP results in Fig. 7F,G. What may seem inconsistent is that FXR is recruited by NF-kB, and while NF-kB binding decreases, FXR binding remains elevated. In the model, interaction with NF-kB contributes to selective recruitment of FXR to NF-kB inflammatory target genes, but retention of FXR, once recruited, is likely enhanced by interactions with other proteins, such as, the corepressor complex. Further, before activation or SUMOylation of FXR, there is very little binding of FXR at NF-kB target promoters, so activation or SUMOylation will increase binding from a very low starting point. We have added discussion of this issue (Discussion, page 17).

Referee #2:

The authors have addressed most of my concerns / questions in a satisfactory way.

Response: We thank reviewer 2 for his/her positive comments on our work.

Referee #3:

The new manuscript has been substantially improved and all issues raised have been appropriately addressed. The reviewer is particularly impressed by the large amount of new data which, along with the thorough revision of data presentation, clarify the initial issues concerning FXR SUMOylation and the mechanisms of transrepression.

Minor: Supplemental Figure S21 should be corrected to S19.

Response: We thank reviewer 3 for his/her positive comments on our work. The supplemental figure number has been corrected.

3rd Editorial Decision 08 October 2014

Thank you for submitting your revised manuscript ("A dysregulated Acetyl/SUMO switch of FXR promotes hepatic inflammation in obesity" to The EMBO Journal. I appreciate the introduced changes and I am pleased to inform you that also referee #1 fully endorses now publication of your manuscript (see his report pasted below). Therefore, we will accept your manuscript for publication. Your article will be processed for publication in The EMBO Journal by EMBO Press and Wiley.

Congratulations to your work!

Referee #1:

The authors have now clarified the technical issues by adding information in the Material and Methods section (Page 21). Moreover, they discuss the proposed model in more detail (Page 17). To my opinion the manuscript is now suitable for publication.

