An LXR-NCOA5 gene regulatory complex directs inflammatory crosstalk-dependent repression of macrophage cholesterol efflux

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1st Editorial Decision 06 June 2014

Thank you for submitting your manuscript (EMBOJ-2014-89147) to our editorial office. I have now had a chance to read it carefully and to discuss it with my colleagues, and I am sorry to say that we cannot offer publication in The EMBO Journal.

Your analysis identifies a new component involved in the regulation of the reverse cholesterol transport pathway in macrophages. The findings show that NcoA5 is a LXR corepressor that upon TLR stimulation interacts with LXR to reduce the expression of the lipid transporter Abca1. While we find the analysis interesting, we also find that we gain too limited support for that NcoA5 modulates cholesterol homeostasis and the reverse cholesterol transport pathway as most of the readout is based upon Abca1 expression.

I have also discussed this matter further with a good expert in the field, but I am afraid that the advisor is in agreement with this view and finds that we would need more functional data to support a role of NcoA5 in cholesterol homeostasis for consideration here.

Given this assessment, I am afraid that I can't offer to consider publication here at this stage. Should further work enable you to address this point then I would be happy to take a look at the paper again.
We previously submitted a manuscript to EMBO entitled "An LXR-NCOA5 gene regulatory complex directs inflammation-dependent repression of reverse cholesterol transport." The manuscript was not sent out for review because of a lack of sufficient functional data to support our claim that NCOA5 modulates cholesterol homeostasis and the reverse cholesterol transport pathway. In our correspondence with you, you indicated that if we provide additional functional data to support NCOA5's role in cholesterol homeostasis, then you would send the paper out for review. We have now obtained this data. We performed cholesterol efflux assays in primary macrophages +/- NCOA5 and show that NCOA5 attenuates cholesterol efflux in response TLR3 signals. These results complement our other data that demonstrate NCOA5's role in regulation of expression of the lipid transporter ABCA1.

Thank you for submitting your manuscript to The EMBO Journal. Your study has now been seen by three referees and their comments are provided below.

As you can see, the referees find the characterization of the role of NCOA5 in macrophage cholesterol efflux interesting. However, they also raise many issues that would have to be sorted out for publication here. Basically more support for a role of NCOA5 as LXR co-repressor is needed. Should you be able to address the concerns raised in full then we would consider a revised manuscript. I should add that it is EMBO Journal policy to allow only a single major round of revision and that it is therefore important to resolve the raised concerns at this stage.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

REFeree REPORTS

Referee #1:

Gillespie et al use a novel approach, promoter enrichment-quantitative mass spectrometry (PE-QMS), to gain insight into what factors regulate LXR activity in the context of ABCA1 expression. They identify NCOA5 as a transcriptional repressor recruited by LXR in response to ligand and propose NCOA5 to mediate TLR3 antagonism of LXR function. The authors generate an interesting hypothesis using PE-QMS but provide poor validation of NCOA5 relevance. The quality of much of the data, in particular the data comprising Figs 3-6, falls short of standards for publication in this journal.

Major concerns:

1. What happens to the protein levels of LXRa and LXRb with T1317+PIC treatment?
2. The authors offer no explanation for the ability of NCOA5 to reduce basal LXR activity in an LXR-independent manner (Fig 3C). In the same figure, NCOA5 overexpression reduces ligand-dependent LXR activity on ABCA1 promoter-reporter construct ~70% yet for the LXREmut construct activity is still reduced ~50%. The data stand in contrast with the author's claim that the loss of LXRE negates the ligand-dependent repressive effects of NCAO5.
3. Show loading control for Fig 4B. With what antibody is LXR-ProtC detected? NCOA5 levels are significantly lower in the control lane. The experiment should be repeated with equal loading. Similarly, judging from the Western blot, the level of LXR is less in the absence of ligand, while for the IP the image is overexposed. Thus, increased NCOA5 pulldown for the ligand treated sample is simply explained by unequal staring material and is not the result of ligand enhanced interaction.
Stronger evidence should be provided for NCOA5 as a novel interacting partner of LXR. Direct interaction should be demonstrated using GST pulldowns, accompanied by mapping of respective interaction domains.

4. It is unacceptable to present control and sh knockdown samples on separate images (Fig 5B).

5. Fig 6E does not implicate NCOA5 in any way, and offers no insight into the proposed mechanism. It only shows that T compound activates transcription and it is antagonized by polyIC treatment. What happens if NCOA5 is knocked down?

6. LXR-/- macrophages should be used in experiments 6D and 6E as additional controls.

7. What happens to endogenous ABCA1 expression upon NCOA5 overexpression?

8. The authors use creative presentation techniques in Figures 5A, 6A, 6B, 6C. There is a concern that these figures do not present the underlying data accurately. The authors should clearly present on a single scale the data for vehicle, stimulation, control and target sh.

Minor comments:

1. The authors incorrectly refer to LXR protein as NR1H3 and NR1H2. This notation can only be used in a gene context.

2. Provide Western blots for Fig 1A and Fig 5D in supplementary data.

3. Fig 2B-left shows two tracks both labelled T1317/Veh. It is not clear to the reader what is the difference between the two.

4. Relabel Fig 2B. Ligand-stimulated subset contains 59proteins that show no change, which then by definition are no longer a ligand-stimulated subset. Figure should be split into “ligand-dependence” and “LXRE-dependence”.

5. Fig 2C does not provide a clear indication as to what protein set is represented by each column.

Referee #2:

This manuscript describes the use of PE-QMS to characterize the proteins at the ABCA1 promoter after stimulation of macrophages with with an LXR agonist, identifying NCOA5 as an LXR co-repressor facilitating downregulation of ABCA1 expression in the setting of TLR3 stimulation.

Specific comments:

1. The experiments are largely in the setting of LXR activation with the synthetic TO compound LXR ligand. Some of the key experiments should be performed with LXR activation in the setting of cholesterol loading, which also upregulates ABCA1 expression.

2. Why were BMMs used for some experiments, but RAW cells used for the PE-QMS and functional studies?

3. It is unclear why among the 79 proteins the authors chose to focus on NCOA5 for detailed functional studies.

4. Interaction of NCOA5 with LXR is shown after transfection of a tagged exogenous LXR construct. Can interaction of endogenous NCOA5 with endogenous LXR be demonstrated?

5. Does ChIP-seq for NCOA5 reveal binding to promoters of other LXR-responsive genes?

6. While recruitment of NCOA5 correlated with reduced activation of RNAPII, the authors have not formally shown that NCOA5 is responsible for the reduced Ser2 phosphorylation. For example, does knockdown of NCOA5 in the setting of PolyIC rescue the activation of RNAPII?

Referee #3:

In the manuscript "An LXR-NCOA5 regulatory complex directs inflammation-dependent repression of macrophage cholesterol efflux" (EMBOJ-2014-89819) Gillespie et al. use a proteomic approach to identify 79 proteins bound to the promoter of the Abca1 gene. LXR is a known transcriptional regulator of Abca1 and the authors go on to determine which of the 79 proteins are influenced by LXR agonist treatment and which require an intact LXR binding site. The work by Gillespie and colleagues provides an important advance both technically and scientifically that could have a significant impact on the field. There are, however, a number of questions and concerns (described in detail below) that need to be addressed. Notably, the manuscript is difficult to read. Perhaps because of an attempt to economize on space and words many of the experiments are poorly...
described in the text, figure legends and methods making the data difficult to understand.

Specific Comments
The authors rightly focus on the "LXR-dependent" subset of proteins because validation of this subset is the most straightforward. Nevertheless, a large number of proteins that bind independent of LXR but that could play important roles in the regulation of Abca1 expression are not addressed. It would be useful to know if any of these proteins show selectivity for Abca1 promoter sequence relative to a random DNA sequence; for instance the Abca1 upstream region use for ChIP in Figure 4A. Also, 2 of the proteins in the LXR dependent group, Rif1 and Ino80, have been associated with DNA repair and telomeres. Perhaps the proteins have an affinity for the ends of the promoter fragment used for the pulldowns. Again, the control described above could address this question.

Based on the recovery of LXR and RXR determined by mass spectrometry is it possible to estimate the percentage of biotinylated promoters that are actually occupied by LXR-RXR heterodimers in the pull-down experiments? Perhaps the complexity of the proteins bound to promoter reflects a subset that is transcriptionally active/LXR-RXR bound and a subset that is transcriptionally inactive without receptors. Including recombinant LXR-RXR such that the biotinylated promoter sequences were saturated with receptors may decrease the complexity and increase the specificity of the promoter-interacting proteins. A similar approach was used by the O'Malley group (Foulds et al. 2013) to identify proteins on estrogen-dependent promoters.

The region of the Abca1 used in the pull-down experiments contains the TATA box. Nevertheless only 1 TATA associated factor (Taf9) was identified. Similarly, only 1 subunit of the mediator complex (Med15) was recovered. Does the failure to purify additional Tafs and mediator components suggest that these complexes are not stable under the conditions used? If so, does this result suggest that even with 79 proteins on a 321 basepair strength of DNA that we still have an incomplete catalog of proteins at the Abca1 promoter? The authors should discuss this result as a potential limitation of the study.

There are a significant number of known or potential RNA binding proteins in the list of 79 proteins as well as in the LXR-dependent subgroup (e.g. Nhp2, Sart1). Is binding of these proteins to the Abca1 promoter changed if the nuclear extract is pretreated with RNase?

In Figure 4A, treatment with T0901317 for 18 hours results in increased binding of Ncoa5 to the ABCA1 promoter. On the other hand, in Figure 6D little or no Ncoa5 binding is measured after 3 hours of T0901317 unless PolyIC is included. Does this data suggest a time-dependent recruitment of NcoA5 to the ABCA1 promoter by LXR agonist treatment that is accelerated by PolyIC? The authors should perform a time course to examine the binding of Ncoa5 to ABCA1 promoter after agonist treatment. If possible it would also be informative to examine the binding of Snd1, Sart1 and Hmbox1 in the same time course to determine if the kinetics for each factor is the same.

In Figure 6C-E, PolyIC treatment alone should be included to determine if the binding and repressive activity of Ncoa5 requires LXR agonist treatment. Additionally the authors should determine if inflammatory signals acting through other TLRs influence Ncoa5 recruitment. Finally the authors should discuss potential mechanisms for how PolyIC treatment promotes the binding of Ncoa5 to the Abca1 promoter.

In Figure 1B, the timing of PolyIC treatment relative to T0901317 addition is not clear.

In Figure 2B on the left side, the red/green heat map has 2 columns both with the same heading. What does each column represent?

The authors state and provide data indicating that the region of the Abca1 promoter under investigation is nucleosome free. Nevertheless a histone chaperone (Hira) and subunits of 2 histone remodeling complexes (Smarc2 and Ino80) bind to this region and binding of each is stimulated by LXR agonist treatment. The authors should discuss the potential role of chromatin remodeling in LXR activation.
Gillespie et al. EMBOJ-2014-89819
Response to Reviewers

We thank all the Reviewers for their insightful comments. We feel the data and the conclusions of our manuscript are much stronger after addressing their concerns. We have detailed our point-by-point responses below.

Referee #1:

Gillespie et al use a novel approach, promoter enrichment-quantitative mass spectrometry (PE-QMS), to gain insight into what factors regulate LXR activity in the context of ABCA1 expression. They identify NCOA5 as a transcriptional repressor recruited by LXR in response to ligand and propose NCOA5 to mediate TLR3 antagonism of LXR function. The authors generate an interesting hypothesis using PE-QMS but provide poor validation of NCOA5 relevance. The quality of much of the data, in particular the data comprising Figs 3-6, falls short of standards for publication in this journal.

Major concerns:

1. What happens to the protein levels of LXRa and LXRb with T1317+PIC treatment?

The reviewer raises an interesting concern. We are presently unaware of any antibody that can accurately distinguish between endogenous LXRa and LXRb in mouse macrophages. Previously, we attempted to generate specific antibodies but these performed inconsistently. We attempted to address this concern using quantitative selected reaction monitoring (SRM) targeted mass spectrometry, whereby we program the mass spectrometer to monitor peptides of a specific mass and retention time, and use heavy isotope labeled peptide standards to confirm their identity. Given the high homology between LXRa and LXRb (~80%), there are only a small number of peptides that can potentially distinguish these proteins. Unfortunately, only a single LXRb peptide produced any signal in our mass spectrometer.

2. The authors offer no explanation for the ability of NCOA5 to reduce basal LXR activity in an LXR-independent manner (Fig 3C). In the same figure, NCOA5 overexpression reduces ligand-dependent LXR activity on ABCA1promoter-reporter construct ~70% yet for the LXREmut construct activity is still reduced ~50%. The data stand in contrast with the author's claim that the loss of LXRE negates the ligand-dependent repressive effects of NCOA5.

We thank the reviewer for bringing this to our attention. We observed binding of NCOA5 to the wild type promoter by PE-QMS in both the presence and absence of ligand. While there was a ~2-fold increase in binding in the presence of ligand, NCOA5 still retained the capacity to bind in the absence of ligand in Raw cells. We hypothesize that the fact NCOA5 is capable of binding the wild type promoter in the absence of ligand, albeit to a lesser degree, could explain why exogenous NCOA5 can repress basal LXR activity in the reporter assays in Raw cells.

We have added the following statement to the Discussion on page 16 to reflect this: “In our PE-QMS experiments, NCOA5 binding was 2-fold lower but not undetectable in the absence of LXR ligand stimulation. This may explain the reduction in Abca1-luciferase expression by NCOA5 even in the absence of ligand (Figure 2C). Notably, our primary BMM ChIP and expression analyses indicate a ligand-dependent NCOA5 occupancy and function at the Abca1 locus when LXR is present.”

We also present several new pieces of data showing that NCOA5 can bind and function in the absence of LXR (Figures 3D, 6C-6E). The most relevant to the reviewers concern would be Figure 3D, which shows ligand-independent binding of NCOA5 to the Abca1 promoter by ChIP in LXR−/− macrophages. This can explain why we observed a reduction in the activity of the LXRE-mutant reporter in a ligand-independent manner upon overexpression of NCOA5. As such, we have
removed any claim that the loss of the LXRE negates the ligand-dependent repressive effects of NCOA5.

The text on page 9 of the Results has been updated as follows:

“However, NCOA5 retained repressive activity after mutation of the LXRE (Figure 2C), suggesting it can still function in the absence of LXR binding.”

3. Show loading control for Fig 4B. With what antibody is LXR-ProtC detected? NCOA5 levels are significantly lower in the control lane. The experiment should be repeated with equal loading. Similarly, judging from the Western blot, the level of LXR is less in the absence of ligand, while for the IP the image is overexposed. Thus, increased NCOA5 pulldown for the ligand treated sample is simply explained by unequal staring material and is not the result of ligand enhanced interaction. Stronger evidence should be provided for NCOA5 as a novel interacting partner of LXR. Direct interaction should be demonstrated using GST pulldowns, accompanied by mapping of respective interaction domains.

We have now included Tubulin blots in Figure 3B (previously Figure 4B). We used the LXR sc1000 rabbit polyclonal antibody from Santa Cruz to detect LXR. We find it reacts well with overexpressed LXR. We have added this information to the Materials and Methods.

We agree with the reviewer that the difference in NCOA5 interaction can be explained by unequal levels of LXR bait protein. We feel the levels of bait protein are the most relevant in determining whether there is a ligand-induced change in NCOA5 binding. We have revised the text to reflect that there is a LXR-NCOA5 interaction, but removed any reference to this interaction being altered by ligand.

The following statement has been added to the Results on page 10 to reflect this:

“To confirm that NCOA5 functions as an LXR cofactor, we examined their interaction in macrophage nuclear extracts. Indeed, endogenous NCOA5 immunoprecipitated with a Protein C-tagged version of LXRa expressed in RAW 264.7 macrophages (Figure 3B). Similar to the in vitro pulldowns, this in vivo interaction also occurred both in the presence and absence of LXR ligand.”

As suggested by the reviewer, we have also included in vitro pulldown assays which show that NCOA5 directly binds LXR. We used purified GST-LXRa to pulldown in vitro translated NCOA5. We also show that an NCOA5-deletion mutant lacking the C-terminus can still bind to GST-LXR, whereas an N-terminal deletion mutant cannot bind GST-LXR. This is especially interesting given the nuclear receptor interacting domain of NCOA5 described by Sauve et al. 2001 MCB 21:343 as the Bifunctional Interaction Determinant is contained within the C-terminus. This suggests the LXR-NCOA5 interaction is through a non-canonical domain in the N-terminus of NCOA5. There are previously documented reports of direct interactions between nuclear receptors and cofactors occurring independently of the LXXLL interaction motif, including LXR-GPS2 (Jakobsson 2009 Mol Cell 34:510) and PPARG-CCPG (Li 2007 Mol Endocrinol. 21:2320).

This data can now be found in Figure 3A, and the Results section has been updated on pages 9-10 as follows:

“To determine whether NCOA5 directly interacts with LXR, we performed in vitro pulldown assays. Full length in vitro translated NCOA5 protein specifically bound to GST-LXRa in a ligand-independent manner (Figure 3A). Notably, deletion of the NH2-terminus (D1-280aa) prevented this interaction, while the NH2-terminal fragment strongly bound GST-LXRa (Figure 3A). Given the nuclear receptor interaction motif on NCOA5 is found within its COOH-terminus (Sauve et al, 2001), these results indicate NCOA5 directly interacts with LXR through a non-canonical domain in the NH2-terminus.”

4. It is unacceptable to present control and sh knockdown samples on separate images (Fig 5B).

We completely agree with the reviewer and thank them for bringing this oversight to our attention. The images were originally from the same blot but were cropped to fit into the corner of the old Figure 5B. This has now been corrected and the data can be found in Expanded View Figure E7D.

5. Fig 6E does not implicate NCOA5 in any way, and offers no insight into the proposed mechanism.
It only shows that T compound activates transcription and it is antagonized by polyIC treatment. What happens if NCOA5 is knocked down?

We have now included data showing that the reduction in pSer2 RNAPII levels at Abca1 promoter upon LXR and TLR3 ligand treatment is prevented upon knockdown of NCOA5. We performed ChIP assays for pSer2/activated RNAPII in control shRNA and Ncoa5 shRNA knockdown macrophages stimulated with T1317, T1317+PolyIC, or vehicle control. In control shRNA knockdown macrophages, T1317 increased occupancy of RNAPII on the Abca1 promoter, while treatment with T1317+PolyIC returned RNAPII occupancy to the basal levels observed following vehicle control treatment. However, in Ncoa5 shRNA knockdown macrophages treated with T1317+PolyIC, RNAPII maintains occupancy close to levels observed with T1317 stimulation and importantly, significantly above the basal levels observed following vehicle control treatment. These results suggest that NCOA5 represses Abca1 expression in response to TLR3-LXR signal crosstalk by interfering with RNAPII function. This new data can now be found in Figure 6F.

Page 14 of the Results section has been updated to reflect this as follows:

“To determine if TLR3-LXR signal crosstalk prevents RNAPII function through NCOA5, we performed ChIP assays following silencing of Ncoa5 expression. With a non-silencing control, we observed increased RNAPII pSer2 occupancy at the Abca1 TSS in response to LXR ligand (Figure 6F). The addition of both LXR and TLR3 ligands returned RNAPII pSer2 occupancy to basal levels (P = 0.33 vs. Veh; Figure 6F). Following Ncoa5 gene silencing, RNAPII pSer2 binding remained elevated, failing to return to basal levels, in response to both LXR and TLR3 ligands (P = 0.004 vs. Veh; Figure 6F). Taken together, these data establish NCOA5 as a critical downstream mediator of the crosstalk between the pro-inflammatory TLR3 and anti-inflammatory LXR pathways, and in response to these signals, NCOA5 represses macrophage cholesterol efflux through inhibition of RNAPII function at the Abca1 gene locus.”

6. LXR-/− macrophages should be used in experiments 6D and 6E as additional controls.

We thank the reviewer for this suggestion as it has allowed us to gain further insight into NCOA5 function. We have added several new pieces of data that support an additional role for NCOA5 in the absence of LXR. We show that NCOA5 binds to the Abca1 promoter in an LXR ligand-dependent manner in LXR-/− macrophages (Figure 3D). We also show that T1317+PolyIC treatment still reduces Abca1 expression compared with T1317 treatment in LXR-/− macrophages (Figure 6E). Lastly, we show that RNAPII occupancy is still reduced by T1317+PolyIC treatment compared to T1317 treatment alone in LXR-/− macrophages (Figures 6C-6D). For this last ChIP experiment, we used antibodies directed against the pSer2 form as well as an antibody that recognizes both the unmodified and pSer5 form (involved in early events of transcription). These data suggest that NCOA5 can still bind the promoter and repress Abca1 expression in the absence of LXR by blocking RNAPII function. We hypothesize that NCOA5 and LXR are part of a multi-protein complex and that NCOA5 binds LXR directly as well as another unidentified protein. This may explain the preserved NCOA5 binding and function in the absence of LXR. Importantly, our data show that NCOA5 directly binds LXR, and in the presence of LXR, NCOA5 recruitment and repression of the Abca1 locus is LXR ligand-dependent. We have updated the Results section in several places to reflect these changes.

Results pages 10-11 (for Figure 3D):

“To assess the requirement of LXR for NCOA5 binding at the Abca1 promoter, we performed ChIP assays in LXR−/− BMMs. Notably, we detected ligand-independent occupancy of the Abca1 promoter by NCOA5 in the absence of LXR (Figure 3D), suggesting an additional mechanism exists for NCOA5 recruitment under these conditions. Taken together, these results identify NCOA5 as an LXR corepressor, which localizes to the Abca1 promoter in vivo following LXR ligand treatment to repress Abca1 expression. In the absence of LXR, NCOA5 can still occupy the Abca1 promoter and repress its expression through an undetermined mechanism.”

Results pages 13-14 (for Figures 6C-6E):

“To ascertain the role of LXR in this RNAPII defect, we performed the above experiments in LXR−/− BMMs. We did not detect an LXR ligand-dependent increase in occupancy of either unmodified/pSer5 or pSer2 RNAPII at the Abca1 TSS (Figures 6C-6D). However, we still observed a reduction in RNAPII occupancy following treatment with T0901317 and PolyIC (Figures 6C-6D).
In support of this, we also detected decreased *Abca1* mRNA expression in response to both treatments in LXR–/– BMMs (Figures 6E, E8E), further suggesting that NCOA5 can function even in the absence of LXR.

We have also addressed these results in the Discussion, on page 18 as follows:

“We also discovered that NCOA5 binds the *Abca1* promoter and retains a repressive function in the absence of LXR. An intriguing hypothesis is these proteins are part of a larger transcriptional complex and that NCOA5 might associate with LXR and another constituent. This additional interaction would be sufficient to recruit NCOA5 in the absence of LXR, such as in LXR–/– BMMs. Future experiments will focus on identifying this factor, using our list of candidate regulatory proteins identified by PE-QMS.”

7. What happens to endogenous ABCA1 expression upon NCOA5 overexpression?

We overexpressed NCOA5 in primary macrophages using retroviral infections. We observed that NCOA5 inhibited induction of *Abca1* mRNA expression by T1317 compared with infection of control retrovirus. We did not observe any effect on *Abca1* mRNA expression following vehicle control treatment. This agrees with our ChIP assays showing NCOA5 binding to the *Abca1* promoter in wild type primary macrophages only in response to LXR ligand. This data is presented in Figure 2D.

The Results on page 9 have been updated as follows:

“In addition, over-expression of NCOA5 in primary BMMs confirmed its ability to repress *Abca1* expression (Figures 2D, E7A).”

8. The authors use creative presentation techniques in Figures 5A, 6A, 6B, 6C. There is a concern that these figures do not present the underlying data accurately. The authors should clearly present on a single scale the data for vehicle, stimulation, control and target sh.

We thank the authors for pointing out these data were not clearly presented. We have now presented these data as requested, with separate bars for all different stimulations, including vehicle controls, for the shRNA data. These newly presented figures can now be found in Figure E7C (old Figure 5A), Figure E8A (old Figure 6A), Figure 5A (old Figure 6B), and Figure 5G (old Figure 6C). The conclusions remain unchanged.

Minor comments:

1. The authors incorrectly refer to LXR protein as NR1H3 and NR1H2. This notation can only be used in a gene context.

We thank the authors for bringing this to our attention. We have made the necessary changes for LXR protein but not mRNA as requested.

2. Provide Western blots for Fig 1A and Fig 5D in supplementary data.

We have now included these data as requested. The blots for Figure E1A (old Figure 1A) can be found in Figure E1B. The blots for Figure 4B (old Figure 5D) can be found in Figure E7E. Note that ligand-induced ABCA1 values were normalized to Vehicle controls run on the same blot.

3. Fig2B-left shows two tracks both labelled T1317/Veh. It is not clear to the reader what is the difference between the two.

We performed orthogonal quantitative mass spec strategies for this experiment. The left lane was data from the label free quantitation and the right lane was data from isotopically labeled samples. This has been updated in Figure 1C (old Figure 2B).

4. Relabel Fig2B. Ligand-stimulated subset contains 59 proteins that show no change, which then by definition are no longer a ligand-stimulated subset. Figure should be split into “ligand-dependence” and “LXRE-dependence”.
We have changed the label in Figure 1C (old Figure 2B) to Ligand-Dependent as requested.

5. **Fig 2C does not provide a clear indication as to what protein set is represented by each column.**

Figure 2C has been removed from the manuscript. The original data is still presented in Extended View Table E2.

**Referee #2:**

*This manuscript describes the use of PE-QMS to characterize the proteins at the ABCA1 promoter after stimulation of macrophages with with an LXR agonist, identifying NCOA5 as an LXR corepressor facilitating downregulation of ABCA1 expression in the setting of TLR3 stimulation.*

**Specific comments:**

1. **The experiments are largely in the setting of LXR activation with the synthetic TO compound LXR ligand. Some of the key experiments should be performed with LXR activation in the setting of cholesterol loading, which also upregulates ABCA1 expression.**

We thank the reviewer for this suggestion. However, we are concerned about the different pathways stimulated by lipids used for cholesterol loading in macrophages, especially in the context of interpreting signal crosstalk data. While oxLDL upregulates ABCA1 through LXR and PPAR (Nagy 1998 Cell 93:229, Tontonoz 1998 Cell 93:241, Chawla 2001 Mol Cell 7:161), it also upregulates TLR4 expression and signaling as part of a CD36-TLR4-TLR6 complex (Xu 2001 Circulation 104:3103, Stewart 2010 Nature Immunol 11:155). By using oxLDL in our signal crosstalk experiments, we could be stimulating additional TLR receptors in addition to TLR3, which could profoundly impact signaling crosstalk and gene expression.

Other modified lipids can also affect inflammatory signaling. Minimally modified LDL (mmLDL) can induce the expression and signaling downstream of TLR2 and TLR4 (Choi 2009 Circ Res 104:1355, Chavez-Sanchez 2010 Hum Immunol 71:737). In addition, CD36 is a primary scavenger receptor for modified LDLs (Endemann 1993 JBC 268:11811, Kunjathoor 2002 JBC 277:49982). CD36 promotes activation of the NLRP3 inflammasome by facilitating the conversion of LDL into cholesterol crystals (Sheedy 2013 Nature Immunol 14:812). By using these methods, we feel we could be promoting the unnecessary and potentially unknown activation of additional inflammatory pathways.

2. **Why were BMMs used for some experiments, but RAW cells used for the PE-QMS and functional studies?**

We used Raw cells for the PE-QMS experiment due to the large number of cells required. Current mass spectrometers are considerably faster and exhibit greater sensitivity, which may allow us to use BMMs for future PE-QMS experiments. We addressed any concern about using Raw cells for this experiment by filtering our PE-QMS results to only include proteins whose genes were expressed in unstimulated BMMs. We also chose to validate our NCOA5 results in the more physiologically relevant primary BMMs, to ensure our conclusions were accurate. We attempted to perform all the functional validation experiments we could in BMMs. The exceptions to this were the luciferase reporter assays and the co-IP experiments. For the luciferase assays, we needed to transfected multiple plasmids into the same cell. This would be a severe limitation if BMMs were used. For the Co-IPs, we needed to exogenously express a tagged version of LXR since we lack an antibody for its immunoprecipitation or detection. This overexpression was not feasible in BMMs as we can only culture them for a short period of time (7-9 days). This approach of using Raw cells to substitute for experiments not readily feasible in primary macrophages has been employed previously (see Heinz 2010 Mol Cell 38:576 or Lam 2013 Nature 498:511 for examples).

3. **It is unclear why among the 79 proteins the authors chose to focus on NCOA5 for detailed functional studies.**
We thank the reviewer for letting us know this point was not well stated. We chose to focus on NCOA5 for the following reasons: (1) it had previously been described as a nuclear receptor cofactor, (2) it was linked to the TLRs in a protein interaction network, (3) its locus contains polymorphisms in humans associated with inflammatory and metabolic disease, and (4) when overexpressed, it repressed Abca1 transcription at a time corresponding to the natural decline in Abca1 expression in BMMs following T1317 stimulation.

We have updated the text of the Results section on page 9 to clarify this point:

“Interestingly, NCOA5 has previously been described as a cofactor for a small subset of nuclear receptors, including estrogen receptor 1 (ESR1), however its role in transcription seems to be context dependent (Gao et al, 2013; Jiang et al, 2004; Sarachana & Hu, 2013; Sauve et al, 2001). In addition, polymorphisms in Ncoa5 are associated with both chronic inflammatory disease and metabolic disease (Bento et al, 2008; Lewis et al, 2010; Zervou et al, 2011). To our knowledge, NCOA5 has never previously been implicated in LXR regulated gene expression, cholesterol efflux, or atherosclerosis. This, together with its repression of Abca1 transcription and its potential involvement in TLR signaling, make NCOA5 an ideal candidate for further investigation.”

4. Interaction of NCOA5 with LXR is shown after transfection of a tagged exogenous LXR construct. Can interaction of endogenous NCOA5 with endogenous LXR be demonstrated?

The major limitation of performing a co-IP on endogenous proteins is the lack of a quality antibody which specifically and efficaciously recognizes either the folded or denatured form of LXR in mouse macrophages. We have been unable to detect an endogenous interaction between these proteins for this reason. As the reviewer mentions, we do show an interaction between endogenous NCOA5 and exogenous LXR to support our claims. Moreover, we have now added in vitro pulldown data demonstrating a direct interaction between LXR and NCOA5. Specifically, we have narrowed down the LXR interacting domain to the N-terminus of NCOA5, which represents a non-canonical interacting domain. We described these results in response to Reviewer #1 Comment #3. This data can be found in Figures 3A-3B in the manuscript.

5. Does ChIP-seq for NCOA5 reveal binding to promoters of other LXR-responsive genes?

We thank the reviewer for this suggestion. However, we feel this is beyond the scope of the current manuscript. This manuscript focuses on the transcription mechanisms controlling Abca1 expression, for which we demonstrate an important role for NCOA5. We do agree with the reviewer that this is an important point and will yield critical insights in NCOA5 regulation. We do plan to perform these experiments as part of a future manuscript.

6. While recruitment of NCOA5 correlated with reduced activation of RNAPII, the authors have not formally shown that NCOA5 is responsible for the reduced Ser2 phosphorylation. For example, does knockdown of NCOA5 in the setting of PolyIC rescue the activation of RNAPII?

We have now included data showing that NCOA5 mediates the loss of RNAPII from the Abca1 promoter in response to LXR and TLR3 ligands. Therefore, knockdown of NCOA5 in the setting of T1317+PolyIC does rescue the activation of RNAPII. These data can now be found in Figure 6F. This was also a concern of Reviewer 1. Please see our response to Reviewer 1 Point 5 for further explanation.

Referee #3:

In the manuscript "An LXR-NCOA5 regulatory complex directs inflammation-dependent repression of macrophage cholesterol efflux" (EMBOJ-2014-89819) Gillespie et al. use a proteomic approach to identify 79 proteins bound to the promoter of the Abca1 gene. LXR is a known transcriptional regulator of Abca1 and the authors go on to determine which of the 79 proteins are influenced by LXR agonist treatment and which require an intact LXR binding site. The work by Gillespie and colleagues provides an important advance both technically and scientifically that could have a significant impact on the field. There are, however, a number of questions and concerns (described in detail below) that need to be addressed. Notably, the manuscript is difficult to read. Perhaps
because of an attempt to economize on space and words many of the experiments are poorly described in the text, figure legends and methods making the data difficult to understand.

We have provided more details in the Figure Legends and Materials and Methods. We have also included an Extended View Materials and Methods with a full description of the PE-QMS technique.

Specific Comments
1. The authors rightly focus on the "LXR-dependent" subset of proteins because validation of this subset is the most straightforward. Nevertheless, a large number of proteins that bind independent of LXR but that could play important roles in the regulation of Abca1 expression are not addressed. It would be useful to know if any of these proteins show selectivity for Abca1 promoter sequence relative to a random DNA sequence; for instance the Abca1 upstream region use for ChIP in Figure 4A. Also, 2 of the proteins in the LXR dependent group, Rif1 and Ino80, have been associated with DNA repair and telomeres. Perhaps the proteins have an affinity for the ends of the promoter fragment used for the pulldowns. Again, the control described above could address this question.

We thank the reviewer for this suggestion, which will be very informative for future experiments to expand our knowledge of Abca1 gene regulatory complexes. As the reviewer acknowledges, we chose to restrict our analysis to those proteins which bind in an LXR-dependent manner. We focused the majority of our validation efforts on NCOA5 itself, but did provide reporter assays and network analysis for SND1, SART1, and HMBOX1. We agree with the reviewer that proteins which bind independently of LXR are likely playing important roles in Abca1 expression.

Both RIF1 and INO80 were detected differentially bound in response to LXR ligand. However, their binding did not change in response to mutation of the LXRE. In addition, INO80 is also involved in chromatin remodeling for transcription (Conaway 2009 Trends Biochem Sci 34:71). We discuss the potential role of chromatin related proteins in response to your later comment (#9). It has also been reporter that INO80 can be recruited by the transcription factor YY1 to a promoter to function as a coactivator (Cai 2007 Nature Struct Mol Biol 14:872). Regardless, if these proteins were binding only to our constructs because of their affinity for ends, we would have not observed changes in their binding in response to ligand. Transcriptomic analysis indicated the genes encoding these two proteins do not appreciably change their mRNA expression in response to ligand. These data are presented in Figure E6.

2. Based on the recovery of LXR and RXR determined by mass spectrometry is it possible to estimate the percentage of biotinylated promoters that are actually occupied by LXR-RXR heterodimers in the pull-down experiments? Perhaps the complexity of the proteins bound to promoter reflects a subset that is transcriptionally active/LXR-RXR bound and a subset that is transcriptionally inactive without receptors. Including recombinant LXR-RXR such that the biotinylated promoter sequences were saturated with receptors may decrease the complexity and increase the specificity of the promoter-interacting proteins. A similar approach was used by the O'Malley group (Foulds et al. 2013) to identify proteins on estrogen-dependent promoters.

This is an interesting suggestion. It is not possible to estimate the percentage of biotinylated promoters occupied by LXR-RXR in the mass spectrometry experiment presented here. We could estimate the percentage of promoters occupied in future experiments by performing quantitative MS or quantitative western blotting. It is likely that we do not have 100% occupancy of promoters by LXR-RXR.

We thank the authors for the valuable suggestion about adding recombinant LXR-RXR to our PE-QMS experiments. When we designed the experiments, we wanted to maintain endogenous protein levels and preserve the stoichiometry of protein complexes interacting with the Abca1 promoter. We also had two LXR family members and three RXR family members to work with as potential protein combinations to add. We do think this suggestion will be an avenue to explore for future PE-QMS experiments.

3. The region of the Abca1 used in the pull-down experiments contains the TATA box. Nevertheless only 1 TATA associated factor (Taf9) was identified. Similarly, only 1 subunit of the mediator complex (Med15) was recovered. Does the failure to purify additional Tafs and mediator
components suggest that these complexes are not stable under the conditions used? If so, does this result suggest that even with 79 proteins on a 321 basepair strength of DNA that we still have an incomplete catalog of proteins at the Abca1 promoter? The authors should discuss this result as a potential limitation of the study.

As the reviewer notes, we identified TAF9 and MED15 interactions with the TATA box-containing Abca1 promoter. Previous reports have indicated that TFIIID functions primarily at TATA-less promoters and it is depleted at TATA-containing promoters (Vermeulen 2007 Cell 131:58, Rhee 2012 Nature 483:295). Moreover, TFIIID functions through interactions with H3K4me3 nucleosomes (Vermeulen 2007 Cell 131:58, Lauberth 2013 Cell 152:1021, Foulds 2013 Mol Cell 51:185). Therefore, we were surprised to detect even a single TAF protein. TAFs have been reported to interact with transcription factors, including MYOD and EKLF (Deato 2008 Mol Cell 32:96, Sengupta 2009 PNAS 106:4213), which could explain the detection of TAF9.

As for detecting only a single Mediator protein, we cannot discount the possibility this reflects complex stability on the promoter under the conditions used or the possibility that some proteins were missed because of duty cycle limitations of the MS analysis. It should be noted that Foulds et al (2013 Mol Cell 51:185) detected four of the 26 Mediator subunits in their experiments. In addition, yeast MED15 can regulate OAF1p, a orthologue of PPARα. This suggests it might regulate metabolic nuclear receptors in mammalians, such as LXR.

We have added this paragraph to the Discussion on page 19 to address the reviewers concern:

"Another limitation of this study is we cannot be completely certain the full compendium of proteins bound to the Abca1 promoter were identified. For instance, MED15 is part of the large multi-subunit Mediator complex. The lack of detection of other subunits in this complex may reflect their instability on this promoter under our experimental conditions, or duty cycle limitations of the data-dependent mass spectrometry strategy employed. Another recent study using a similar enrichment strategy identified four Mediator subunits bound to their promoter sequences (Foulds et al, 2013). Recent advances in mass spectrometry based protein identification technologies with improved sensitivity and reproducibility, such as targeted (Mirzaei et al, 2013) and data-independent peptide identification approaches (Egertson et al, 2013; Gillet et al, 2012; Lambert et al, 2013) hold great promise for overcoming these limitations, and are currently being explored."

4. There are a significant number of known or potential RNA binding proteins in the list of 79 proteins as well as in the LXR-dependent subgroup (e.g. Nhp2, Sart1). Is binding of these proteins to the Abca1 promoter changed if the nuclear extract is pretreated with RNase?

There is increasing evidence that RNA interactions are important in controlling transcription factor activity. For instance, lincRNAs are critical for regulating nuclear receptor mediated transcriptional control, including the regulation of the Androgen Receptor, Estrogen Receptor, and PPARγ (Yang 2013 Nature 500:598, Li 2013 Nature 498:516, Sun 2013 PNAS 110:3387). Furthermore, enhancer RNAs are required for transcription of a subset of macrophage genes (Lam 2013 Nature 498:511). We are therefore very interested in the binding of NHP2 and SART1 and look forward to exploring how they regulate transcription in future manuscripts. Given the importance of RNA and its binding proteins in regulating transcription factor binding and the transcriptional response, and the unknown importance of their effect on protein complex interactions, we did not want to potentially remove these interactions by pre-treating the extracts with RNase. We do think these experiments will be informative for future characterization of Abca1 promoter interacting proteins. We thank the reviewer for this suggestion.

5. In Figure 4A, treatment with T0901317 for 18 hours results in increased binding of Ncoa5 to the ABCA1 promoter. On the other hand, in Figure 6D little or no Ncoa5 binding is measured after 3 hours of T0901317 unless PolyIC is included. Does this data suggest a time-dependent recruitment of Ncoa5 to the ABCA1 promoter by LXR agonist treatment that is accelerated by PolyIC? The authors should perform a time course to examine the binding of Ncoa5 to ABCA1 promoter after agonist treatment. If possible it would also be informative to examine the binding of Snd1, Sart1 and Hmbox1 in the same time course to determine if the kinetics for each factor is the same.

We agree with the reviewer that this suggests a time-dependent recruitment of NCOA5. To address this, we performed a time course for NCOA5 binding by ChIP in response to LXR ligand. We
observed a small but significant ligand-stimulated binding of NCOA5 to the Abca1 promoter at 8h, followed by binding at 18 h. We did not detect binding at 4h, which agrees with the 3h results the reviewer is referring to. We have included these data as Figure 3C. For this manuscript, we focused the validation experiments on NCOA5. We plan to examine the binding of these other factors as part of a future manuscript.

We have updated the text of the Results section on page 10 to reflect these changes:

“To determine whether NCOA5 localizes to the Abca1 promoter in vivo, we performed chromatin IP (ChIP) assays in primary BMMs in the presence or absence of LXR ligand stimulation. Using primers spanning the proximal LXRE just upstream of the Abca1 transcriptional start site (TSS), which is contained within the same region analyzed by PE-QMS, we detected ligand-dependent occupancy of NCOA5 on the Abca1 promoter beginning at 8 h (Figure 3C).”

6. In Figure 6C-E, PolyIC treatment alone should be included to determine if the binding and repressive activity of Ncoa5 requires LXR agonist treatment. Additionally the authors should determine if inflammatory signals acting through other TLRs influence Ncoa5 recruitment. Finally the authors should discuss potential mechanisms for how PolyIC treatment promotes the binding of Ncoa5 to the Abca1 promoter.

We thank the reviewer for the suggestion. We have now included these experiments as the reviewer requested. Interestingly, while PolyIC treatment on its own represses basal Abca1 expression, it does so independently of NCOA5. This suggests that LXR ligand stimulation is required for NCOA5 function. We hypothesize this could be the result of a post-translational modification or induction of an intermediate protein that only occurs as a result of the crosstalk between LXR ligand and TLR3 ligand signals. We have included this data in Figure 5C.

Previous studies have suggested a role for TLR3 and TLR4 in repressing Abca1. To address the reviewers concern, we treated BMMs with the TLR4 agonist LPS following control shRNA or Ncoa5 shRNA knockdown. We observed a decrease in Abca1 expression in response to LPS, whether given in combination with T1317 or on its own. We also determined that NCOA5 was not involved in mediating any aspect of LPS-induced repression of Abca1. This data is now found in Figures 5B and 5D. This result is interesting as it suggests a level of specificity for the signals regulating NCOA5 function. We have updated the Results and Discussion section as indicated.

Results on page 12:

“The TICAM1/TRIF-dependent inflammatory receptors TLR3 and TLR4 have a profound antagonistic effect on ligand-induced LXR target gene expression (Castrillo et al, 2003). To investigate whether NCOA5 is involved in signal crosstalk between these pro-inflammatory TLRs and the anti-inflammatory LXR pathway, we silenced Ncoa5 expression in primary BMMs and stimulated with LXR ligand, the TLR3 agonist polyinosinic-polycytidylic acid (PolyIC), and the TLR4 agonist bacterial lipopolysaccharide (LPS). In non-silencing shRNA controls, we detected an LXR ligand-dependent increase in Abca1 expression at 4 h (Figures 5A-5B). The addition of PolyIC or LPS, either in the presence (Figures 5A-5B) or absence (Figures 5C-5D) of LXR ligand significantly reduced Abca1 expression, together with some elevation of Ncoa5 mRNA (Figures E8A-E8D). Strikingly, knockdown of Ncoa5 (Figures E8A-E8D) abolished the ability of PolyIC but not LPS to attenuate LXR ligand-dependent Abca1 expression (Figures 5A-5B). Moreover, loss of NCOA5 failed to prevent either PolyIC- or LPS-mediated reduction of basal Abca1 expression in the absence of LXR ligand in LXR+/− BMMs (Figures 5C-5D).”

Discussion on pages 17-18:

“Notably, the repression of Abca1 following PolyIC treatment alone is independent of NCOA5. Moreover, LPS mediated repression of Abca1 is completely independent of NCOA5. One possible explanation for this is a regulatory event, such as a post-translational modification of NCOA5, which specifically occurs when both the TLR3 and LXR pathways, but not the TLR4 and LXR pathways, are simultaneously activated. TLR4 induction results in widespread phosphoproteome changes (Weintz et al, 2010), so it is reasonable to hypothesize that phosphoproteome changes would occur downstream of TLR3. Alternatively, there could be distinct but complementary events mediated by the TLR3 and LXR pathways that target NCOA5 function. For instance, activation of TLR3 signaling could result in modification of NCOA5, while ligand binding to LXR could permit binding of modified NCOA5 to LXR. Castrillo et al. (2003) observed the reduction in Abca1
expression by these signals was independent of IFNαβ, which may further restrict potential candidates. Future quantitative mass spectrometry studies will be critical in delineating these mechanisms.”

7. In Figure 1B, the timing of PolyIC treatment relative to T0901317 addition is not clear.

We thank the reviewer for bringing this to our attention. Both ligands were administered at the same time in all experiments. We ended up removing Figure 1B from the manuscript due to redundancy.

We have added this statement to the Materials and Methods on page 21 to indicate this and avoid any future confusion:

“All ligands were administered simultaneously for combined treatments.”

8. In Figure 2B on the left side, the red/green heat map has 2 columns both with the same heading. What does each column represent?

This has now been corrected. Please see our response to Reviewer #1 Minor Concern #3.

9. The authors state and provide data indicating that the region of the Abca1 promoter under investigation is nucleosome free. Nevertheless a histone chaperone (Hira) and subunits of 2 histone remodeling complexes (Smarcc2 and Ino80) bind to this region and binding of each is stimulated by LXR agonist treatment. The authors should discuss the potential role of chromatin remodeling in LXR activation.

The reviewer raises an interesting point. We did use a region of DNA free from nucleosomes for PE-QMS. However, as shown in Figure E2B, nucleosomes were detected on either side of this region. HIRA, SMARCC2, and INO80 may be important in regulating these regions in vivo. Given the location of nucleosomes, we would predict remodeling is necessary for LXR ligand-induced transcription.

We have added the following text to the Discussion on page 18 to address this point:

“Several LXR cofactors reported to modulate its activity also regulate chromatin structure, including p300, SMARCA4, NCOA6, and the NCOR complex (Castrillo et al, 2003; Huuskonen et al, 2004; Huuskonen et al, 2005; Jakobsson et al, 2009; Lee et al, 2008; Wagner et al, 2003). We detected LXR ligand-stimulated binding of the chromatin regulators SMARCC2, INO80, and HIRA to the Abca1 promoter by PE-QMS (Figure 1C). Despite the fact we targeted a region of the Abca1 locus devoid of nucleosomes for PE-QMS, nucleosomes are present adjacent to this region (Figure E2), suggesting the binding of SMARCC2, INO80, and HIRA may be important for remodeling these regions or perhaps for long-distance interactions. However, we cannot discount the possibility of a non-chromatin-related role for these proteins, such as that described for the NCOR complex component HDAC3 (Sun et al, 2013).”

Thank you for submitting your revised manuscript. Your study has now been re-reviewed by referees #1 and 3.

As you can see below the referees appreciate that the introduced changes and both are supportive of publication here. Referee #3 has a few remaining concerns that would be good to sort out before publication here. Most of them should be straightforward to address. I am happy to discuss the points further.

REFEREE REPORTS

Referee #1:

The authors have provided an overall reasonable response. It is somewhat disappointing that LXRA and LXRb protein levels are not addressed. The argument provided that they cannot be
"distinguished" by antibodies does not really make sense. The proteins are not the same size and they do not comigrate on gels. Nevertheless, most concerns have been addressed and the revised paper is acceptable.

Referee #3:

In the revised manuscript "An LXR-NCOA5 regulatory complex directs inflammation-dependent repression of macrophage cholesterol efflux" (EMBOJ-2014-89819) Gillespie et al. use a proteomic approach to identify proteins that bind to the Abca1 promoter in a LXR-dependent fashion. As I stated in my initial review, the work by Gillespie and colleagues provides an important advance both technically and scientifically that could have a significant impact on the field. In particular the strength of the manuscript is the description and validation of approaches to identify proteins that bind to genomic control regions such as promoters and enhancers in a transcription factor-dependent, sequence-specific or tissue-specific fashion. I would have like to see the authors devote more of the manuscript validating the approach and exploring the general utility of the method. Overall the authors did a reasonable job in addressing the concerns and comments in my initial review. The studies describing the potential role of NCOA5 in LXR activity are, however, quite confusing and I have tried to summarize the main conclusions for my own benefit.

1. NCOA5 is recruited to the ABCA1 promoter in an LXR agonist-dependent manner at relatively late times after initiating agonist treatment and appears to play a role in attenuating ABCA1 expression.
2. In LXR null cells NCOA5 appears to interact constitutively with the ABCA1 promoter (if one is comfortable with the ChIP data in Figure 3D) and based on transfection experiments NCOA5 can repress expression of ABCA1 even in the absence of LXR binding (Figure 2C). Nevertheless, in vehicle treated cells ABCA1 mRNA levels are higher in LXR null cells compared to LXR positive cells (Figure 1A) even though NCOA5 is bound (Figure 3D)?
3. PolyIC appears to accelerate the LXR agonist-dependent recruitment of NCOA5 to the ABCA1 promoter and NCOA5 contributes to the polyIC-dependent repression of LXR agonist-dependent up-regulation of ABCA1 expression. On the other hand NCOA5 is not required for polyIC to repress ABCA1 expression in vehicle treated cells.

Including a testable model that provides a hypothesis to explain why the recruitment of NCOA5 is delayed and how it is accelerated by polyIC would go a long way to helping me try to make sense of the data. Also, data demonstrating that recruitment of other cofactors can be detected at earlier times points would be a nice control.

Additional Comments
In Figure 3B it looks like over expression of the LXR-ProtC fusion protein increases the level of NCOA5 compared to control RAW cells. Is this simply a loading artifact?

In Figure 3C it looks like there is ligand-dependent recruitment of NCOA5 (i.e. relative to vehicle treated cells) at 18 hours but not at 8 hours as mentioned in the text. Also, if 1-way ANOVA was used to compare all the groups in Figure 3C to the IgG control I am not convinced that one would conclude that was any specific association of NCOA5 with the ABCA1 promoter.

In Figure 5G why is there no LXR-agonist stimulated cholesterol efflux in the shControl cells? Once again wouldn’t it be more appropriate to use 1-way ANOVA to compare the 3 treatment groups to each other and 2-way ANOVA to examine the effect of ligand + shRNA? I am not confident that there is any effect of ligand or shRNA on cholesterol efflux in this experiment.

The Pol.2 ChIP data appears to confirm the mRNA measurements and does not really provide much insight regarding mechanism.

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Response to Reviewers
Referee #3:

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1. NCOA5 is recruited to the ABCA1 promoter in an LXR agonist-dependent manner at relatively late times after initiating agonist treatment and appears to play a role in attenuating ABCA1 expression.
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Including a testable model that provides a hypothesis to explain why the recruitment of NCOA5 is delayed and how it is accelerated by polyIC would go a long way to helping me try to make sense of the data.

The Reviewer is correct in point #1 and point #3 of their summary above. For point #2, NCOA5 occupies the Abca1 promoter and can repress Abca1 expression in the absence of LXR, as the Reviewer states. In Figure 1A, we did not see higher Abca1 expression in LXR-/- vs LXR+/+ macrophages at times when NCOA5 was bound (i.e. 18 h). We did observe higher Abca1 expression in LXR-/- at 0 h, which can be explained by the absence of an LXR-NCOR complex as reported in Wagner et al. 2003 MCB 23:5780.

We have now included a model summarizing our finding in Figure E9. We propose that regulatory modifications on NCOA5, such as phosphorylation, are responsible for the differential recruitment of NCOA5 in response to sterol signals or LXR-TLR3 crosstalk. However, we feel it is important to note that when NCOA5 is present on the Abca1 promoter, gene expression is inhibited. We have discussed this model in the Discussion on page 17 as follows:

"Notably, the repression of Abca1 following PolyIC treatment alone is independent of NCOA5. Moreover, LPS mediated repression of Abca1 is completely independent of NCOA5. One possible explanation for this is a regulatory event, such as a post-translational modification of NCOA5, which specifically occurs when both the TLR3 and LXR pathways, but not the TLR4 and LXR pathways, are simultaneously activated (Figure E9). TLR4 induction results in widespread phosphoproteome changes (Weintz et al, 2010), so it is reasonable to hypothesize that phosphoproteome changes would occur downstream of TLR3."

Also, data demonstrating that recruitment of other cofactors can be detected at earlier times points would be a nice control.

We thank the Reviewer for this suggestion. As we have mentioned in the Introduction (page 4) and Discussion (page 18), there are several cofactors reported to interact with and control the activity of LXR at the Abca1 promoter. We plan to study the temporal recruitment of these cofactors, and
NCOA5, to the Abca1 promoter in the future. We do not feel these experiments will significantly change our conclusions about NCOA5 and its function as an LXR corepressor.

Additional Comments

In Figure 3B it looks like over expression of the LXR-ProtC fusion protein increases the level of NCOA5 compared to control RAW cells. Is this simply a loading artifact?

The Reviewer is correct in noting this observation. We do detect increased NCOA5 following overexpression of LXRalpha. This is not a loading artifact. However, we do not observe consistent changes in NCOA5 expression in primary macrophages in response to LXR ligand. Therefore, we think this may be an effect specific to the overexpression of LXRalpha or the cell line used. As a result, we never pursued this observation.

In Figure 3C it looks like there is ligand-dependent recruitment of NCOA5 (i.e. relative to vehicle treated cells) at 18 hours but not at 8 hours as mentioned in the text. Also, if 1-way ANOVA was used to compare all the groups in Figure 3C to the IgG control I am not convinced that one would conclude that was any specific association of NCOA5 with the ABCA1 promoter.

We thank the Reviewer for bringing this oversight to our attention. We have now removed this statement concerning NCOA5 binding at 8h from the text, and revised Figure 3C. The text in the Results section on page 10 now reads:

“Using primers spanning the proximal LXRE just upstream of the Abca1 transcriptional start site (TSS), which is contained within the same region analyzed by PE-QMS, we detected ligand-dependent occupancy of NCOA5 on the Abca1 promoter at 18 h (Figure 3C).”

We used the Student’s t-test to determine if the difference between two conditions was significant. ANOVA on the full dataset does not provide this specific information. We therefore feel this was the appropriate statistical test to perform.

In Figure 5G why is there no LXR-agonist stimulated cholesterol efflux in the shControl cells? Once again wouldn’t it be more appropriate to use 1-way ANOVA to compare the 3 treatment groups to each other and 2-way ANOVA to examine the effect of ligand + shRNA? I am not confident that there is any effect of ligand or shRNA on cholesterol efflux in this experiment.

For this experiment, we observed a 1.3-fold increase in efflux in response to LXR ligand. In our hands, we do not normally observe very large increases in efflux in response to LXR ligand. At the time point used, the difference between ACBA1 protein expression in response to LXR ligand or vehicle control is not very large. Moreover, we often observe variability in this assay, which accounts for the large error bars. We have discussed our protocol with an expert in the field, and despite their suggestions, we did not observe any enhancement of efflux from these cells. For the statistical analysis, we were asking whether there was a significant difference between only two of the treatments, T1317 and T1317+PolyIC. Similar to our response to the above comment, we think that the t-test is the appropriate test for this specific type of comparison.

The Pol.2 ChIP data appears to confirm the mRNA measurements and does not really provide much insight regarding mechanism.

The ChIP data provides evidence that the repression of gene expression in response to LXR and TLR3 ligands correlates with reduced RNA pol II recruitment to the promoter and reduced RNA pol II activation. It is possible that RNA pol II recruitment was unaffected which would suggest that the defect correlates with a downstream function of Pol II. Furthermore, we added novel data showing NCOA5 knockdown reverses this effect on Pol II activation/function. We therefore think the Pol II result is an important mechanistic finding that provides significant insight into how NCOA5 functions as a repressor.