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Akirin2 is critical for inducing inflammatory genes by bridging IκB-ζ and the SWI/SNF complex

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Editor: Alexander Kohlmaier

1st Editorial Decision 24 April 2014

Thank you for submitting your manuscript " Akirin2 is critical for inducing inflammatory genes by bridging IκB-ζ and the SWI/SNF complex" to The EMBO Journal editorial office.

We have now received the comments from all referees copied below for your information. As you will see, all the referees consider your findings interesting and significant. We shall therefore be happy to consider this manuscript further, and I would at this stage like to invite you to revise your manuscript according to the referees' suggestions.

The referees' comments appear constructive and self-explanatory, and I will not repeat them in detail here. I will briefly mention just some major points commonly raised by more than one referee: referee 1 and 3 concur in the request for more direct experiments testing whether p50 and p56 are part of the Akirin2-SWI/SNF-IkB-zeta complex, and whether p50/56's role is to recruit this complex.
Secondly, all four referees asked for potential further experiments and explanations that could help understand why Akirin2 controls only a subset of NF-kB target genes. To us, the question whether a CpG island-containing Akirin2-dependent target would require SWI/SNF activity for activation pertains to this request. Similarly, and most clearly articulated by referee 2 (specific point 1), more definitive experiments might be thought of to discern whether the reduced accessibility, e.g. of the Il-6 locus in akirin 2 mutant macrophages, is cause or consequence of impaired RNAPII activity at this locus. Please do not hesitate to contact me to potentially discuss feasibility of this specific request.

I am certain that modifications requested by the referees would result in an improved study, as already indicated by some of the referees' encouraging remarks. I would, therefore, be pleased if you would invest the necessary time and efforts to address the reviewers' concerns.

We generally allow three months as standard revision time. Should you foresee a problem in meeting this three-month deadline, please let us know in advance. When preparing your letter of response, please be also reminded that our policy to allow only a single round of major revision will necessitate comprehensive answering, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version. Also bear in mind that this letter will form part of the Peer Review Process File available online to our readers in the case of publication. For more details on our Transparent Editorial Process, please visit our website: http://emboj.embopress.org/about#Transparent_Process

Please also note that during our standard three months revision time, any competing manuscripts published here or elsewhere will have no negative impact on our final assessment of your revised study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed.

Should you have any additional questions regarding this revision, please do contact me.

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

REFEREE COMMENTS

Referee #1:

NF-kB transcription factor plays a central role in innate immunity from flies to mammals. In this thorough analysis, Tartey et al successively showed that (1) Using microphages of conditional Akirin2 KO mice, Akirin2 is required for the induction of some subset of immune genes such as IL6, IL12b, and Ifnb following LPS and Poly I:C challenge, (2) Akirin2 is required for host response to Listeria, (3) Akirin2 is involved in chromatin remodeling and histone modification at the IL6 promoter, (4) Akirin2 interacts with BAF60 family members, which is required for IL6 expression, (5) Akirin2 interacts with IkB-zeta, forming a IkB-zeta/Akirin2/Baf60 complex, (6) IkB-zeta/Akirin2 is required for the recruitment of Brg1 to the IL6 promoter. They concluded that Akirin2-SWI/SNF-IkB-zeta complex regulates the expression of a subset of inflammatory genes with non-CpG island promoters during NF-kB activation in innate immune cells.

This is very exciting study proposing detailed mechanism of chromatin remodeling during NF-kB activation. Notably, the power of genetic animal model system together with biochemistry and omics technology allow them to clearly address some important issues in the field of innate immunity. The proposed model is important and may also open new avenue to treat important disorders associated with inflammation. Based on the scientific and technical qualities of the present manuscript, I recommend this paper for the publication following appropriate revision. I have just two comments that should be addressed before publication.
1)It is important to show experimentally (e.g. by performing Co-IP) that Akirin2-SWI/SNF-IkB-zeta complex contains NF-kB p50.
2)What is the biological significance of Akirin-controlled NF-kB target gene activation? Why are only some subsets of NF-kB target genes controlled in an Akirin-dependent manner? What is the advantage for host? What are the differences in terms of biological functions between Akirin-dependent immune genes and Akirin-independent immune genes? This issue should be discussed in the Discussion section.

Referee #2:

In this study Tartey et al report that akirin2, a protein previously reported to control the expression of inflammatory genes in flies and mice, interacts with a subunit of the Swi/Snf chromatin remodeling complex (BAF60) and with the IkB-zeta coactivator, thus enabling the recruitment of

chromatin remodelers to a subset of inflammatory gene promoters and stimulating transcriptional activation. The authors first investigated the effects of a myeloid-specific deletion of akirin2 in cells and mice. Akirin2 deletion impaired the expression of selected cytokine genes and reduced the resistance of infected mice to an intracellular pathogen (L. monocytogenes). As expected (because of the selectivity of the effects of akirin2 deletion) signaling pathways activated by LPS were not impaired. Conversely, LPS-inducible recruitment of RNA polymerase II to the Il6 gene promoter was abrogated. A transcriptomic analysis of the effects of akirin2 deletion identified a panel of genes whose activation by LPS was reduced in akirin2-deficient macrophages. As a group, akirin2-dependent genes tended to be less frequently associated with a CpG island, although this correlation was far from perfect. Loss of akirin2 strongly reduced LPS-induced restriction enzyme accessibility of the Il6 gene promoter as well as the gain of histone marks characteristic of gene activation (H3K4me3 and H3K9Ac). In search of a mechanism for akirin2 activity the authors explored its possible association with Swi/Snf complex subunits, which was suggested by previous global Drosophila interaction data as well as by more recent results reporting akirin interaction with Brm (PMID 22396663). The authors identified a complex formed by Baf60a/b/c and akirin2 that was also to some extent LPS-inducible. Depletion of Baf60b and c in HeLa cells reduced the activation of IL6 but not of IL8 and this effect was accompanied by reduced RNA polymerase II recruitment and H3K9Ac induction. Knockdown of the same genes in the J774 macrophage cell line similarly impaired IL1b-mediated activation of IL6. Finally the authors mapped the interaction surfaces involved in akirin2 binding to IkB-zeta and found that recruitment of IkB-zeta and akirin2 to the IL6 gene promoter was interdependent. Moreover, both IkB-zeta and akirin2 were required for Brg1 recruitment to the Il6 and Il12b gene promoters.

Overall, this study provides a more complete description of the role of akirin2 in the induction of inflammatory genes and a partial mechanistic explanation of its effects. Nevertheless, there are some aspects of this study that require substantial improvements or refinement before most of the conclusions can be considered definitive.

1. The requirement for chromatin remodeling at genes without a CpG island is less obvious than claimed by the authors. This assumption is based on a 2009 publication (PMID 19596239) whose conclusions have been refined by the same authors in recent genome-wide study (PMID 22817891). In this study it was shown that in fact CpG island-containing genes include also many genes activated with very slow kinetics and requiring new protein synthesis (and maybe remodeling?) for activation. While it is clear that CpG islands are nucleosome-depleted, this does not necessarily imply that remodeling is not required. The only direct evidence of an impairment of chromatin remodeling in this study comes from the restriction enzyme accessibility assay shown in Fig. 4C. However, the increased RE accessibility at the Il6 gene promoter may well occur after gene activation (or at least gene activation and RNA Polymerase II recruitment likely contribute to this increased accessibility). Therefore the reduced accessibility in akirin2 mutant macrophages may simply be a consequence (and not a cause) of the impaired Il6 gene induction. This important aspect should therefore be further investigated and corroborated by additional definitive data.

2. The data on the impact of Baf60 subunit depletion on the activation of Il6 should also be improved and strengthened. First of all, the effects of the siRNAs used on the actual protein levels of the targeted proteins are not shown anywhere in the manuscript. In the specific case of the J774 cells (which are definitely more relevant than HeLa cells for the response being studied) the efficiency of the depletion, as evaluated by semi-quantitative RT-PCR (suppl. Fig. 8), appears to be rather modest. Second, there is no basic control for off-target effects of the siRNAs used. Third, the Il6 gene has been shown to have a different configuration in different cell types, which explains for instance its much faster inducibility in fibroblasts than in macrophages. In addition to that, it is clear that the epigenome and the gene regulatory networks in HeLa cells are completely different from those of macrophages. Therefore, activation of any given gene in HeLa cells may not reflect at all the sequence of events leading to the activation of the same gene in macrophages. This also implies that the mechanistic data obtained in HeLa cells may have little relevance to macrophages. This is also pertinent to the data shown in Fig. 7a, in which the authors used MEFs, in which Il6 gene induction is much faster than in macrophages and myeloid cells in general.

3. The association of IkB-zeta with akirin2 should be corroborated using antibodies against the endogenous proteins.

4. The anti-Flag ChIPs shown in fig 7F are not convincing at all, first of all because it is not clear if the level of expression of akirin2 is comparable to the endogenous one, and second because the enrichment over the control is in some cases very modest.

Referee #3:

Akirin2 was first identified as a potential NF-kB regulator in a Drosophila RNAi screen for new components of the Imd pathway. Moreover, a 2-hybrid screen had also previously identified Akirin2 as interacting with BAP60, a component of the Drosophila SWI/SNF chromatin remodeling complex. In this report the authors have demonstrated the functional importance of Akirin2 as a gene specific regulator of NF-kB function and also defined the mechanistic basis underlying this. A key feature of this report is a mouse model with a conditional deletion of Akirin2 that has allowed the authors to demonstrate both the physiological significance of this pathway in the cellular response to TLR activation and viral infection. Using this model they have also been able to define the subset of genes, including IL-6 and IL-12, regulated by Akirin2. They show that these form a subset of NF-kB targets that lack CpG islands and so require SWI/SNF chromatin remodeling activity. Using a variety of co-IPs and in vitro pull down experiments the authors further determined the domains of Akirin2 that mediates its interaction with the SWI/SNF complex as well as its interaction with IkBzeta, an atypical IkB that functions as a nuclear coactivator for the p50 NF-kB subunit. ChIP analysis demonstrated that Akirin2 and IkBzeta are both required to recruit the SWI/SNF complex to these promoters.

Overall this is a very interesting and convincing report. The experiments are clearly explained and well controlled. However, there are some areas where additional some additional experimentation would help to clarify and strengthen some of the conclusions.

Specific Comments

(1) The obvious assumption, based on the stimuli used and the genes analyzed, is that NF-kB complexes recruit the IkBzeta/Akirin2/SWI/SNF complex to the promoter. However this has not been demonstrated. Moreover, it is not clear if it is a p50 homodimer complex that mediates these effects (as might be expected given the involvement of IkBzeta) or whether a p50/p65 heterodimer is required. The authors could resolve this using the cell lines they have analyzed, though a combination of siRNA depletion of p50 and p65 followed by ChIP analysis of IkBzeta/Akirin2/SWI/SNF.

(2) ReChIP analysis would help to confirm that the components of the putative complex identified by the authors can be found on the promoter simultaneously.

(3) What happens with recruitment of this complex at an Akirin2 dependent promoter that does have a CpG island in its promoter region? Is this dependent or independent of SWI/SNF activity?

(4) In Fig. 3B, deletion of Akirin2 appears to result in a significant increase in the level of NF-kB complex seen after LPS treatment. Can the authors comment on whether this is a consistent effect and if so, why it might be occurring. For example, Fig. 3A appears to show reduced levels of IkBalpha in Akirin deleted cells, although as the overall protein loading of these samples also seems to be lower it is hard to be sure.

Minor points

(5) Fig. 6D seems to be wrongly cited in the text on page 12, which refers to an alignment of Akirin proteins. The actual Fig. 6D is not cited and the relevant figure showing the alignment referred to does not seem to have been included.

(6) In Supp Fig S8C, the type of cells used should be stated in the legend.

Referee #4:

In this report, Tartey et. al. perform extended analyses on their Akirin2 conditional knock out mice. They showed that Akrin2 is required for robust induction of Il6 and Il12b by various TLR, RIG-I agonists in macrophages and effective clearance of Listeria infection in vivo (Fig1 and 2).
A combined analysis of microarray and bioinformatics reveals that Akirin-dependent genes tend to have lower frequency of CpG-islands. The authors clearly show that changes in histone modification occur in the Il6 and IL-12, but not Cxcl,1promoter regions, in an Akirin dependent manner, as they predict. Also, interaction between Akirin2, IkB-zeta and BAF60 containing SWI/SNF complex and recruitment of this complex to the Il6 promoter are essential for histone modification and transcription of Il6 gene.

Although role of Akirin on promoters of immune induced genes with CpG-island still remains unclear (for 20% of the Akirin dependent genes), the main claim of this work offers the beginning of an explanation for a mechanism by which non-CpG island promoters drive transcription following immune stimulation in Akirin-dependent manner. What remains, is the question of how Akirin is specifically recruited to these promoters? Or if it is only at these promoters? The accompanying Drosophila paper argues that Akirin is recruited to only those promoters where it is required. If it is the same in mammals, where does the specificity for this recruitment arise? And why IkBzeta seems to be necessary?

Related to some of these questions is the signal dependence of the Akirin complexes characterized here. For example, on page 12, the authors report that the interaction between NF-kB family members and Akirin2 was not observed? But, what about during immune activation?. In the same sense, the transfection approaches used to not proved any information about the signal-dependence of the interaction between NF-κB, IkB-zeta, Akirin and BAF60. Overall, the reliance on transfected proteins in non-immune cells is a major weakness of the interactions characterized here.

1st Revision - authors' response 03 June 2014

We thank the reviewers for their interest in our study and for providing valuable comments and questions. Below, please find our point-by-point responses to these comments and questions. As suggested, we have performed additional experiments, added appropriate data, examined endogenous protein interactions and clarified all the issues raised during the review process. Again, we would like to thank all the reviewers for taking the time to carefully read our manuscript. We are confident that this constructive criticism and our responses have made this a much stronger paper.

Referee #1:

NF-kB transcription factor plays a central role in innate immunity from flies to mammals. In this thorough analysis, Tartey et al successively showed that (1) Using microphages of conditional Akirin2 KO mice, Akirin2 is required for the induction of some subset of immune genes such as IL6, IL12b, and Ifnb following LPS and Poly I:C challenge, (2) Akirin2 is required for host response to Listeria, (3) Akirin2 is involved in chromatin remodeling and histone modification at the IL6 promoter, (4) Akirin2 interacts with BAF60 family members, which is required for IL6 expression, (5) Akirin2 interacts with IkB-zata, forming a IkB-zeat/Akirin2/Baf60 complex, (6) IkB-zeta/Akirin2 is required for the recruitment of Brg1 to the IL6 promoter. They concluded that Akirin2-SWI/SNF-IkB-zeta complex regulates the expression of a subset of inflammatory genes with non-CpG island promoters during NF-kB activation in innate immune cells.
This is very exciting study proposing detailed mechanism of chromatin remodeling during NF-kB activation. Notably, the power of genetic animal model system together with biochemistry and omics technology allow them to clearly address some important issues in the field of innate immunity. The proposed model is important and may also open new avenue to treat important disorders associated with inflammation. Based on the scientific and technical qualities of the present manuscript, I recommend this paper for the publication following appropriate revision. I have just two comments that should be addressed before publication.
Answer:
We thank the reviewer for these constructive comments and for recognizing that our findings have the potential to provide a breakthrough in our understanding of how 'signaling transcription factors'

link to chromatin remodeling in a gene-specific manner and for recognizing the fact that our proposed model is important for understanding inflammation-related disorders.

1) It is important to show experimentally (e.g. by performing Co-IP) that Akirin2-SWI/SNF-IkB-zeta complex contains NF-kB p50.

Answer:

We performed the suggested Co-IP experiments and found that overexpressed Akirin2 in HeLa cells co-precipitated endogenous NF-κB p50 upon IL-1b stimulation, but not in unstimulated cells. Consistent with the data already shown, overexpressed Akirin2 could also precipitate endogenous IκB-ζ with and without stimulation, further confirming that in the basal state, Akirin2 and IκB-ζ exist in a complex irrespective of stimulation. Whereas Akirin2 is a nuclear protein, NF-κB p50 translocates from the cytoplasm to the nucleus upon IL-1b/TLR stimulation. In contrast, NF-κB p65 was not co-precipitated with Akirin2. Therefore, the results indicate that the complex of IκB-ζ-Akirin2-SWI/SNF further interacts with NF-κB p50 in the nucleus after IL-1b stimulation. We show these data in new **Figure 6C**.

2) What is the biological significance of Akirin-controlled NF-kB target gene activation? Why are only some subsets of NF-kB target genes controlled in an Akirin-dependent manner? What is the advantage for host? What are the differences in terms of biological functions between Akirin-dependent immune genes and Akirin-independent immune genes? This issue should be discussed in the Discussion section.

Answer:

We have shown the biological significance of Akirin2-mediated gene expression in macrophages by *in vivo Listeria* infection experiment (**Figure 2**). Even when Akirin2 is lacking only in macrophages and neutrophils, the mice showed a defect in clearing infected *Listeria* and impaired production of cytokines (**Figure 2F**), indicating that Akirin2 expressed in macrophages and neutrophils is important for host defence against bacterial infection. We mentioned the role of Akirin2 in host defence against bacterial infection in the discussion section.

As the reviewer points out, Akirin2 controls only a set of genes among all TLR-inducible genes such as *Il6*, *Il12b* and *Ifnb*. In contrast, *Tnf* and *Cxcl1* are not regulated by Akirin2. It has been shown that *Tnf* gene expression is rapidly inducible compared with *Il6* or *Il12b*. *Cxcl1* was also reported to be rapidly induced in response to TNF stimulation (*Nat Immunol. 2009 10:281-8*). This suggests that Akirin2 is critical for the control of relatively slowly induced genes in the course of macrophage activation. We added these supporting arguments to the discussion section.

Referee #2:

Overall, this study provides a more complete description of the role of akirin2 in the induction of inflammatory genes and a partial mechanistic explanation of its effects. Nevertheless, there are some aspects of this study that require substantial improvements or refinement before most of the conclusions can be considered definitive.

1. The requirement for chromatin remodeling at genes without a CpG island is less obvious than claimed by the authors. This assumption is based on a 2009 publication (PMID 19596239) whose conclusions have been refined by the same authors in recent genome-wide study (PMID 22817891). In this study it was shown that in fact CpG island-containing genes include also many genes activated with very slow kinetics and requiring new protein synthesis (and maybe remodeling?) for activation. While it is clear that CpG islands are nucleosome-depleted, this does not necessarily imply that remodeling is not required. The only direct evidence of an impairment of chromatin remodeling in this study comes from the restriction enzyme accessibility assay shown in Fig. 4C. However, the increased RE accessibility at the Il6 gene promoter may well occur after gene activation (or at least gene activation and RNA Polymerase II recruitment likely contribute to this increased accessibility). Therefore the reduced accessibility in akirin2 mutant macrophages may simply be a consequence (and not a cause) of the impaired Il6 gene induction. This important aspect should therefore be further investigated and corroborated by additional definitive data.

Answer:

We agree that a change in restriction enzyme accessibility in the absence of Akirin2, by itself, does not prove that Akirin2 is critical for inducing chromatin remodelling. However, in addition to a clear change in the restriction enzyme accessibility in response to LPS in the absence of Akirin2 in macrophages, we found that the recruitment of Brg1 to the *Il6* promoter region was impaired in

Akrin2-deficient macrophages (**Figure 7D**), indicating that Akirin2 is required for the recruitment of the SWI/SNF complex to the *Il6* promoter. In contrast, NF-κB DNA binding activity was not impaired in the absence of Akirin2. Given that Brg1 is critical for chromatin remodeling on promoters, including *Il6*, the data shown in this manuscript clearly demonstrate that Akirin2 controls chromatin remodeling by recruiting SWI/SNF complex to the *Il6* promoter.

As the reviewer points out, a recent paper (*Cell. 2012 Jul 20;150(2):279-90*) from Dr. Smale's group showed that both CpG island and low CpG promoters were abundant among genes induced at late times based on genome-wide analysis, in contrast to their previous studies. They further showed that low CpG promoters were highly enriched among the most potently induced genes, and CpG islands were more prevalent among weakly induced genes. However, the results shown in the paper indicate that genes that require a large dynamic range of expression often contain low CpG promoters, suggesting that the low CpG promoter may help limit basal transcription and may facilitate tight regulation by conferring a requirement for an inducible nucleosome remodeling event. Overall, the low CpG content found in Akirin2-regulated genes is not in conflict with the requirement of inducible chromatin remodeling for their expression.

However, we found several genes, such as *Cmpk2*, *Akap12* and *Parp14*, which harbour a CpG island but are apparently regulated by Akirin2 (**Figure 4A**). To address if these genes also require chromatin remodelling for the induction to LPS, we examined the expression of these genes in Brg1 knockdown J774 macrophage cells. As shown in the following **Figure A**, the expression of *Cmpk2*, *Akap12* and *Parp14* in response to LPS was impaired in Brg1 knockdown cells. These results further support our claim that Akirin2 functions to induce its target genes by controlling chromatin remodelling. These data have been added to **Supplementary Figure S9**.

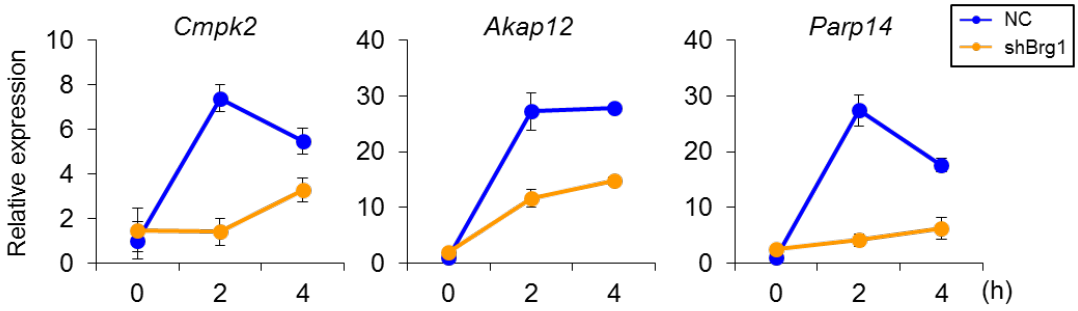


Figure A SWI/SNF Dependency of CpG island harbouring Akirin2 target genes. Brg1-shRNA knockdown J774 cells were stimulated with LPS for indicated time periods. Total RNA was prepared and quantitative PCR analysis was performed for the expression of *Cmpk2*, *Akap12* and *Parp14* mRNAs. All the samples were normalized to *Actinb* mRNA. Error bars indicate mean ± std. deviation. Results are representative of two independent experiments.

Since we mention in the introduction section that primary and secondary response genes were classified based on CpG island promoters, citing the previous paper from Dr. Smale's group (*Cell. 2009 Jul 10;138(1):114-28*), we modified this description and indicated that both CpG island and low CpG island promoters are found in late-inducible genes.

2. The data on the impact of Baf60 subunit depletion on the activation of Il6 should also be improved and strengthened. First of all, the effects of the siRNAs used on the actual protein levels of the targeted proteins are not shown anywhere in the manuscript. In the specific case of the J774 cells (which are definitely more relevant than HeLa cells for the response being studied) the efficiency of the depletion, as evaluated by semi-quantitative RT-PCR (suppl. Fig. 8), appears to be rather modest. Second, there is no basic control for off-target effects of the siRNAs used. Third, the Il6 gene has been shown to have a different configuration in different cell types, which explains for instance its much faster inducibility in fibroblasts than in macrophages. In addition to that, it is clear that the epigenome and the gene regulatory networks in HeLa cells are completely different from those of macrophages. Therefore, activation of any given gene in HeLa cells may not reflect at all the sequence of events leading to the activation of the same gene in macrophages. This also implies that the mechanistic data obtained in HeLa cells may have little relevance to macrophages. This is also pertinent to the data shown in Fig. 7a, in which the authors used MEFs, in which Il6 gene induction is much faster than in macrophages and myeloid cells in general.

Answer:

We agree that this is an important point and now we show the efficiency of shRNA knockdown by performing immunoblotting using endogenous antibodies with endogenous actin as an internal control. Appropriate changes have been made to the text and new data has been added to **Supplementary Figure 8C**. As pointed out by the reviewer, regarding the basic control for off-target effects of the shRNAs used, we would like to refer to **Supplementary Figure 8B**, where we have already shown that one particular shRNA knockdown does not have any effects on the expression of other candidate genes.

3. The association of IkB-zeta with akirin2 should be corroborated using antibodies against the endogenous proteins.

Answer:

Following the reviewer's comment, we performed endogenous interaction experiments between Akirin2 and IκB-ζ and successfully showed their interaction (new **Figure 6D**). Appropriate changes have been made to the main text.

4. The anti-Flag ChIPs shown in fig 7F are not convincing at all, first of all because it is not clear if the level of expression of akirin2 is comparable to the endogenous one, and second because the enrichment over the control is in some cases very modest.

Answer:

To address the question if Akirin2 is recruited to the *Il6* promoter in response to LPS stimulation, we retrovirally expressed Akirin2 in mouse BM macrophages. It is generally accepted that the levels of expression of transfected genes by retroviruses are lower than those induced by lipofection. Furthermore, we tested the expression of *Akirin2* mRNA in control and Akirin2-expressed cells by RT-PCR. As shown in new **Supplementary Figure S11**, the expression of *Akirin2* increased 1.1-1.2 fold in Akirin2-expressed cells compared with control. These results indicate that the levels of retrovirally expressed Flag-tagged Akirin2 were not very high.

Although the reviewer argues that the enrichment over the control is modest, we think the difference is significant and consistent with previous reports. First, the difference between stimulated versus unstimulated and Flag-Akirin2 expressed and non-expressed samples is statistically significant ($p < 0.05$). Furthermore, the observed levels are similar to those reported by Ramirez-Carrozzi et al., who demonstrated the recruitment of Brg1 and Mi-2b to *Il6* and *Il12b* promoters by ChIP assays (*Genes Dev. 2006 Feb 1;20(3):282-96*), and found that the fold increase in the enrichment of Brg1 and Mi-2b on these promoters was 2-5 fold, depending on the experiments. The ratio of enrichment between their studies and ours is similar, thus we believe that the fold enrichment found in our study is reasonable and consistent with the mechanism presented in this study.

Referee #3:

Overall this is a very interesting and convincing report. The experiments are clearly explained and well controlled. However, there are some areas where additional some additional experimentation would help to clarify and strengthen some of the conclusions.

Answer:

We thank the reviewer for showing interest in this study and for providing constructive comments to improve the conclusions.

Specific Comments

(1) The obvious assumption, based on the stimuli used and the genes analyzed, is that NF-kB complexes recruit the IkBzeta/Akirin2/SWI/SNF complex to the promoter. However this has not been demonstrated. Moreover, it is not clear if it is a p50 homodimer complex that mediates these effects (as might be expected given the involvement of IkBzeta) or whether a p50/p65 heterodimer is required. The authors could resolve this using the cell lines they have analyzed, though a combination of siRNA depletion of p50 and p65 followed by ChIP analysis of IkBzeta/Akirin2/SWI/SNF.

Answer:

We show that Akirin2 is recruited to the *Il6* promoter, and we and other researchers showed that IκB-ζ and Brg1 are recruited to the *Il6* promoter in response to TLR/IL-1R stimulation. We also show that Akirin2 interact with IκB-ζ and Baf60 proteins, components of the Swi/Snf complex, indicating that Akirin2, IκB-ζ and Swi/Snf form a complex that is recruited to the *Il6* promoter

following TLR/IL-1R stimulation. As suggested, we next determined if the complex of Akirin2 could interact with NF-κB p50. As shown in new **Figure 6C**, Akirin2 clearly co-precipitated NF-κB p50, and only upon IL-1b stimulation. This result unambiguously shows that NF-κB p50 and Akirin2 are in the same complex, probably by interacting with IκB-ζ. In contrast, previous studies reported that IκB-ζ does not interact with NF-κB p65 (*J Biol Chem. 2001 Jul 20;276(29):27657-62*).

Furthermore, we investigated if NF-κB p50 is required for the recruitment of Akirin2 to the *Il6* promoter. We retrovirally expressed Flag-Akirin2 in control and NF-κB p50-deficient BM macrophages, and performed a ChIP assay using anti-Flag Ab followed by LPS stimulation. As shown in new **Figure 7F**, the recruitment of Akirin2 to the *Il6* promoter was severely impaired in NF-κB p50-deficient BM macrophages. These results indicate that NF-κB p50 is essential for the recruitment the IκB-ζ/Akirin2 complex.

IκB-ζ has been shown to interact with NF-κB p50, but not p65. Consistent with this notion, we also found that Akirin2 coprecipitated NF-κB p50, but not p65. As the reviewer points out, NF-κB family members have been shown to function by forming homo- or heterodimers. In addition to p65/p50 heterodimers, dimeric complexes of p65/p65, p65/c-Rel, p65/p52, c-Rel/c-Rel, p52/c-Rel, p50/c-Rel, p50/p50, RelB/p50, and RelB/p52 have been described. However, the specific physiological role of individual NF-κB dimers is not fully understood. Even the relationship between NF-kB p65/p50 and p50/p50 is not fully understood. The deficiency in NF-kB p65 (RelA) in mice leads to a defect in the embryonic development because of TNF-mediated apoptosis, while NF-kBp50-deficient mice develop normally. NF-kB p65-deficient macrophages show impaired expression of various cytokine genes including *Il6*, *Tnf*, *Il12b* and *Il1b* in response to LPS (*PNAS. 2000 Nov 7;97(23):12705-10*). Consistent with this result, NF-kB p65 was reported to be recruited to the promoters of various cytokine genes such as *Tnf*, *Il6* and *Il1b* in a macrophage cell line (*J Immunol. 2010 Sep 15;185(6):3685-93*). In contrast, the expression of *Il6* and *Il12b*, but not *Tnf* or *Il1b*, mRNA was impaired in NF-kB p50-deficient macrophages. Taken together, these previous results suggest that TNF expression requires NF-kB p65, but not p50, whereas *Il6* expression requires both NF-kB p65 and p50.

In this manuscript, we wish to focus on the functional role and mechanism of Akirin2 in the regulation of inflammatory gene expression. As pointed out by the reviewer, the requirement of NF-kB p50 in the IκB-ζ-Akirin2-SWI/SNF-mediated *Il6* gene expression is important and interesting. However, we believe that investigation of the oligomeric state of NF-kB family members is beyond the scope of the current manuscript and should be addressed in a future study.

(2) ReChIP analysis would help to confirm that the components of the putative complex identified by the authors can be found on the promoter simultaneously.

Answer:

The reviewer suggests that we perform a "re-ChIP" experiment to investigate the relationship between Akirin2 and IκB-ζ on the *Il6* promoter. Although the results of such an experiment would be very interesting, it is technically difficult, since ChIP grade Akirin2 and IκB-ζ antibodies are not available. The expression of differentially tagged Akirin2 and IκB-ζ in macrophages followed by re-ChIP is also technically very challenging and may not be feasible.

However, we believe that the new data, showing that NF-kB p50 co-precipitates with Akirin2 upon Il-1b stimulation, provides important related information. Given that Akirin2 interacts with IκB-ζ, and IκB-ζ interacts with p50, these three proteins form a complex after IL-1b stimulation. Furthermore, we show that both Akirin2 and IκB-ζ are recruited to the *Il6* promoter after TLR stimulation in macrophages. In addition to IκB-ζ, we found that Akirin2 required NF-kB p50 to be recruited to the *Il6* promoter (new **Figure 7F**). Furthermore, we show that IκB-ζ is required for the recruitment of Akirin2, and vice versa (**Figure 7E and 7G**). These results clearly demonstrate the mutual relationship between NF-kB p50, Akirin2 and IκB-ζ for regulating chromatin remodeling at the promoter regions of their target mRNAs, and strongly suggest that Akirin2 and IκB-ζ are recruited to the *Il6* promoter simultaneously.

(3) What happens with recruitment of this complex at an Akirin2 dependent promoter that does have a CpG island in its promoter region? Is this dependent or independent of SWI/SNF activity?

Answer:

As the reviewer points out, there are Akirin2-dependent genes harboring CpG islands (**Figure 4A**). These include *Cmpk2*, *Akap12* and *Parp14*. To address the reviewer's concern, we examined the expression of these genes in response to LPS in Brg1 knockdown J774 macrophage cell line (**Supplementary Figure S8**). As shown in **Figure A** in our response to Reviewer 2's comment (and

Supplementary Figure S9), the expression of *Cmpk2*, *Akap12* and *Parp14* was impaired in Brg1 knockdown cells.

In a previous report (*Cell. 2009 Jul 10;138(1):114-28*), the authors picked up a CpG island containing gene *Peli1*, whose expression depends on Brg1/Brm. The *Peli1* expression was also reduced in the absence of Akirin2 based on the microarray data presented in **Table S1**. We generated a graph for the expression of *Peli1* in Control and Akirin2-deficient macrophages to LPS stimulation as shown below in **Figure B**.

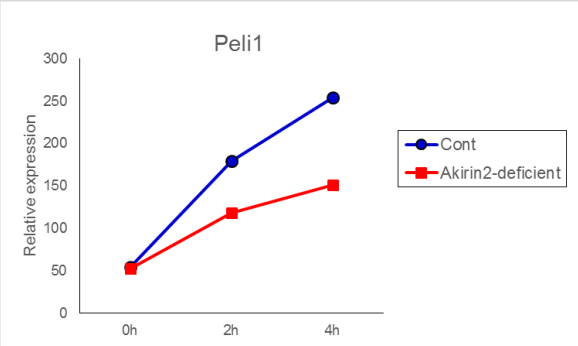


Figure B. The expression of CpG island-containing SWI/SNF-dependent gene, *Peli1*, in macrophages lacking Akirin2.

These data demonstrate that the Akirin2-dependent CpG-containing genes also required chromatin remodeling for their expression, and further strengthen the relationship between Akirin2 and SWI/SNF-mediated chromatin remodeling.

(4) In Fig. 3B, deletion of Akirin2 appears to result in a significant increase in the level of NF-kB complex seen after LPS treatment. Can the authors comment on whether this is a consistent effect and if so, why it might be occurring. For example, Fig. 3A appears to show reduced levels of IkBalpha in Akirin deleted cells, although as the overall protein loading of these samples also seems to be lower it is hard to be sure.

Answer:

As the reviewer points out, the induction of NF-κB-DNA binding activity appears to increase in *Akirin2*-deficient cells in macrophages. Although we do not have a clear explanation, this is a very consistent effect and we have also observed the same phenomenon in MEFs (*Nat Immunol. 2008 Jan;9(1):97-104*). Furthermore, the increase in DNA-NF-κB binding activity does not explain the impaired expression of a set of NF-κB target genes observed in *Akirin2*-deficient macrophages. It is intriguing to speculate that this phenomenon can be explained by feedback due to impaired expression of NF-κB- and IRF3/7-target genes. Although the underlying causes are interesting, we believe that investigation of the mechanism is beyond the scope of the current study.

Minor points

(5) Fig. 6D seems to be wrongly cited in the text on page 12, which refers to an alignment of Akirin proteins. The actual Fig. 6D is not cited and the relevant figure showing the alignment referred to does not seem to have been included.

Answer:

We apologize for the inconvenience and have made necessary corrections.

(6) In Supp Fig S8C, the type of cells used should be stated in the legend.

Answer:

We agree and have made the necessary additions.

Referee #4:

Although role of Akirin on promoters of immune induced genes with CpG-island still remains unclear (for 20% of the Akirin dependent genes), the main claim of this work offers the beginning of an explanation for a mechanism by which non-CpG island promoters drive transcription following immune stimulation in Akirin-dependent manner. What remains, is the question of how Akirin is specifically recruited to these promoters? Or if it is only at these promoters? The accompanying Drosophila paper argues that Akirin is recruited to only those promoters where it is required. If it is

the same in mammals, where does the specificity for this recruitment arise? And why IkBzeta seems to be necessary?

Answer:

First, we would like to thank the reviewer for posing these important questions. We believe that the recruitment of Akirin2 to the promoters is mediated by its interacting proteins. As we show in **Figure 7E**, Akirin2 is recruited to *Il6* and *Il12b* promoters depending on the presence of IκB-ζ, an Akirin2 binding protein. In this revised manuscript, we also show that NF-kBp50, an IκB-ζ interacting protein, is also required for the recruitment of Akirin2 to the *Il6* promoter.

Although innate immune signaling pathways are relatively conserved, there are multiple differences between the mammalian and *Drosophila* innate immune systems. In this regard, it is not surprising that Akirin2 controls gene expression by distinct mechanisms. IκB-ζ is not found in the *Drosophila* genome, although the ankyrin repeat domain is found in several proteins such as *Drosophila* NF-κB. Given that Akirin2 interacts with IκB-ζ through the ankyrin-repeat domain, it is possible that the ankyrin-repeat domain is an evolutionarily conserved target for Akirin2 interaction. Because the focus of the current study is on mammalian immune cell signaling, we would like to defer discussion of the evolution of Akirin2 until such hypothesis can be tested.

Related to some of these questions is the signal dependence of the Akirin complexes characterized here. For example, on page 12, the authors report that the interaction between NF-kB family members and Akirin2 was not observed? But, what about during immune activation?.

Answer:

Following the reviewer's suggestion, we investigated the interaction between Akirin2 and NF-kB p50, since this NF-kB family member is known to interact with IκB-ζ. As shown in new **Figure 6C**, NF-kB p50 co-precipitated with Akirin2 in response to IL-1β stimulation. This result is consistent with the notion that IκB-ζ interacts with NF-kB p50 following TLR/IL-1R stimulation.

In the same sense, the transfection approaches used to not proved any information about the signal-dependence of the interaction between NF-κB, IkB-zeta, Akirin and BAF60. Overall, the reliance on transfected proteins in non-immune cells is a major weakness of the interactions characterized here.

Answer:

According to the reviewer's comment, we examined the interaction between endogenous Akirin2 and IκB-ζ. As shown in new **Figure 6D**, endogenous Akirin2 was precipitated with endogenous IκB-ζ even in the resting cells. We have shown that endogenous Akirin2 interact with endogenous Baf60a (**Figure 5B and 5C**). Therefore, these results clearly demonstrate that endogenous IκB-ζ, Akirin2 and Baf60a form a complex even in resting cells. Since the interaction between NF-kB p50 and IκB-ζ/Akirin2 is induced after TLR/IL-1R stimulation, it is likely that the IκB-ζ-Akirin2-SWI/SNF complex in recruited to the *Il6* promoter by interacting with NF-kB 50, which translocate to the nucleus following stimulation, and binds with the *Il6* promoter.

2nd Editorial Decision 26 June 2014

Thank you for submitting your revised manuscript to The EMBO Journal.
I have now received all four referees' comments that you will find pasted below. I appreciate very much the progress you made in your revision, and I am very pleased to see that the referees' remarks are very positve. I congratulate you on your work!

Only a small additional touch-up will be needed at this point: That is, before we formally accept your manuscript for publication, we would like to invite you to address referee #2's points 1 and 2 in the form of a minor final revision step. In addition, only a small textual change to the discussion was requested by referee #4. While an additional bioinformatic analysis of the NF-kappa-B elements in the Akirin2-dependent and -independent target gene (referee #4) would possibly be interesting, we are not requesting this analysis to be performed, and we leave it open to you whether you decide to add additional information on any p50-specific signature in this respect.

Together, I am certain that addressing the indicated reviewers' points by resolving these last few open points will be straight forward.

REFEREE REPORTS

Referee #1:

I feel that all the issues are clarified. I recommend this manuscript for publication.

Referee #2:

In this revised version of the manuscript the authors only partially addressed my concerns, and some relevant issues that I already raised in my original review still require their attention and additional experimental work.

1. The interaction between endogenous IkBZ and akirin shown in Fig 6D is not convincing. Specifically, while in Fig. 6C the authors show that IL1b stimulation increased the akirin-IkBZ interaction (possibly due to an increase in IkBZ expression) the opposite is seen when looking at the endogenous proteins. Do the authors have an explanation for this unexpected behaviour? Moreover, this experiment is lacking appropriate controls: while it is obvious that IkBZ cannot coprecipitate akirin in cells that are akirin-deficient, the authors should carry out the anti-IkBZ co-immunoprecipitation in cells lacking IkBZ to demonstrate that the co-immunoprecipitation is indeed dependent on IkBZ (rather than a non-specific pull-down).

2. The Baf60 knockdown experiments are still lacking appropriate (standard) controls for off-target effects of the siRNAs used (lack of effects on other family member is not such a control).

3. Baf60 depletion experiments carried out in HeLa cells likely have limited relevance to macrophages.

Referee #3:

The authors have addressed my original concerns.

Referee #4:

In this revised manuscript, Tartey et al. provide thoughtful responses, key new data, and a revised manuscript that addresses many of the issues raised by the first round of 4 critiques. Overall, the manuscript is much stronger, with a much clearer picture of how the Akirin2 - SWI/SNF complex pre-exists in cells but is recruited by p50 transcription factor complexes following stimulation. This adds a significant piece of clarity. Likewise, the revised version downplays a bit the association of Akirin2 with CpG-less genes, as this correlation is not particular tight and does not provide a mechanistic insight into how specificity is generated. This is appropriate given the state of the field, as well as the data presented. Instead, they point out, in responses to 2 of the reviewers, that Akrin2 complexes are specifically recruited to p50 complexes via IkBzeta. Overall, this signal dependent interaction between Akirin2 and p50, but not p65, is very intriguing, and suggests that different dimers of NFkB can provide specificities for Akirin2-dependent genes through differential interaction with NFkB/Ikbzeta. These findings on Akirin2 seem to address critiques by 2 different reviewers - on the source of specificity and the relation of Akirin2 to different NF-κB dimers. The manuscript would be improved with, at least, a discussion of p50-specific connection as the possible source of specificity for Akirin2 dependency, in the Discussion for example. Moreover, it may be worthwhile to perform a comparative analysis of the kappa-B elements in the Akirin2-dependent and -independent target genes. Can a p50-specific signal be identified?

2nd Revision - authors' response 05 July 2014

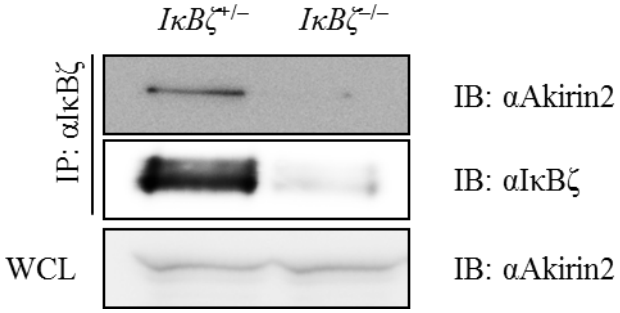
We thank the reviewers for providing valuable comments and questions. Below, please find our point-by-point responses to these comments and questions. As suggested, we have performed an additional experiment and clarified all the issues raised during the review process. We are confident that this constructive criticism and our responses have made this a much stronger paper.

Referee #2:

1. *The interaction between endogenous IkBZ and akirin shown in Fig 6D is not convincing. Specifically, while in Fig. 6C the authors show that IL1b stimulation increased the akirin-IkBZ interaction (possibly due to an increase in IkBZ expression) the opposite is seen when looking at the endogenous proteins. Do the authors have an explanation for this unexpected behaviour? Moreover, this experiment is lacking appropriate controls: while it is obvious that IkBZ cannot coprecipitate akirin in cells that are akirin-deficient, the authors should carry out the anti-IkBZ co-immunoprecipitation in cells lacking IkBZ to demonstrate that the co-immunoprecipitation is indeed dependent on IkBZ (rather than a non-specific pull-down).*

As the reviewer states, we show that the interaction between endogenous Akirin2 and IκB-ζ is observed even in resting cells in **Figure 6D**. However, we show that the interaction between Akirin2 and NF-kB p50 subunit is highly induced in response to IL-1b stimulation in Figure 6C. And these data indicate that Akirin2 and IκB-ζ form a complex in the nucleus even in resting cells, whereas the NF-kB p50 subunit is present in the cytoplasm. Upon IL-1b stimulation, the NF-kB p50 subunit translocate into the nucleus where it can interact with the preformed complex of Akirin2 and IκB-ζ. On the other hand, the interaction between Flag-Akirin2 and IκB-ζ was observed even in unstimulated cells (**Figure 6C**), consistent with the interaction between endogenous Akirin2 and IκB-ζ (**Figure 6D**). When Flag-Akirin2 was abundantly expressed, the interaction between Flag-Akirin2 and IκB-ζ looks to be modestly increased probably due to the increase in the expression of IκB-ζ. As shown in **Supplementary Figure S1**, the expression of endogenous Akirin2 was modestly decreased in response to LPS. This phenomenon may cause the modest difference in the interaction between Akirin2 and IκB-ζ, depending on the levels of Akirin2 expression. However, we believe that exploring the mechanisms of this subtle difference is beyond the scope of this manuscript.

In addition, the reviewer asked about the specificity of anti-IκB-ζ Ab and therefore suggested us to perform anti-IκB-ζ co-immunoprecipitation in cells lacking IκB-ζ. According to the reviewer's suggestion, we performed an immunoprecipitation assay with anti-IκB-ζ Ab using IκB-ζ–deficient macrophages. As shown in the Figure below, we confirmed that the anti-IκB-ζ Ab failed to immunoprecipitate IκB-ζ or co-precipitate Akirin2 in the absence of IκB-ζ protein.



2. The Baf60 knockdown experiments are still lacking appropriate (standard) controls for off-target effects of the siRNAs used (lack of effects on other family member is not such a control).
Answer: The reviewer asked us to show appropriate controls for the Baf60 knockdown experiments for the off target effects of the siRNAs used. We purchased siRNAs used in this study from Life technologies (Invitrogen or Ambion). In the revised version of this manuscript, we indicated the IDs of these siRNA in **Supplementary Table S2** to facilitate the reproduction of the siRNA knockdown experiments. When we ran a BLAST search using the available sequences, no genomic sequences

except the target sequence showed more than an 80% match, suggesting that the siRNA sequences are specific to the target genes.

To eliminate the possibility of the off-target effects completely, whole genome transcriptome analysis of all knockdown samples might be required. However, whole genome analysis is not commonly performed in knockdown experiments, and we believe this is unnecessary.

We found that BAF60 genes and BRG1 knockdown did not show a defect in the expression of *IL8* gene expression in response to IL-1β, although the *IL6* gene expression was severely impaired. These results suggest that the knockdown of BAF60 genes and BRG1 did not affect the signalling pathways leading to the activation of NF-kB or general transcription. Therefore, we believe that the effect of BAF60 and BRG1 knockdown experiments was gene specific and not due to off-target effects.

3. *Baf60 depletion experiments carried out in HeLa cells likely have limited relevance to macrophages.*
We knocked down Baf60 proteins both in HeLa cells and J774 macrophage cells. Both in HeLa cells and J774 cells, Baf60 proteins contribute to the expression of *Il6*, but not *Tnf* or *IL8*, gene expression in response to TLR/IL-1R stimulation. Therefore, we believe that the data both in HeLa cells and J774 macrophages are absolutely meaningful and compensate in each other.

Referee #4:

In this revised manuscript, Tartey et al. provide thoughtful responses, key new data, and a revised manuscript that addresses many of the issues raised by the first round of 4 critiques. Overall, the manuscript is much stronger, with a much clearer picture of how the Akirin2 - SWI/SNF complex pre-exists in cells but is recruited by p50 transcription factor complexes following stimulation. This adds a significant piece of clarity. Likewise, the revised version downplays a bit the association of Akirin2 with CpG-less genes, as this correlation is not particular tight and does not provide a mechanistic insight into how specificity is generated. This is appropriate given the state of the field, as well as the data presented. Instead, they point out, in responses to 2 of the reviewers, that Akrin2 complexes are specifically recruited to p50 complexes via IkBzeta. Overall, this signal dependent interaction between Akirin2 and p50, but not p65, is very intriguing, and suggests that different dimers of NFkB can provide specificities for Akirin2-dependent genes through differential interaction with NFkB/Ikbzeta. These findings on Akirin2 seem to address critiques by 2 different reviewers - on the source of specificity and the relation of Akirin2 to different NF-κB dimers. The manuscript would be improved with, at least, a discussion of p50-specific connection as the possible source of specificity for Akirin2 dependency, in the Discussion for example. Moreover, it may be worthwhile to perform a comparative analysis of the kappa-B elements in the Akirin2-dependent and -independent target genes. Can a p50-specific signal be identified?

Answer: We thank the reviewer for the constructive comments.
First, according to the reviewer's comment, we further discussed that NF-kB p50 recruitment is possibly determining the specificity for Akirin2-dependent gene expression in the discussion section.

The reviewer also suggests analysing NF-kB p50 elements comprehensively to compare with Akirin2–dependent and –independent genes. Although this analysis could be intriguing, we believe that this is beyond the scope of this study. Since this study aims to characterize the role of Akirin2 in macrophages and its mechanisms controlling inflammatory gene expression, we would like to leave further comprehensive identification of NF-kB p50 target genes for future study.

3rd Editorial Decision 09 July 2014

Thank you for submitting your revised manuscript ("Akirin2 is critical for inducing inflammatory genes by bridging IκB-ζ and the SWI/SNF complex" to The EMBO Journal. I appreciate the introduced changes and I am pleased to inform you that we will accept your manuscript for publication.

Before transferring your paper to our publisher I would please ask you to attend to the following points:

Concerning your response to ref #2's point 1, I would please ask you to incoporate the Western blot comprising the new data (IP α−IκBζ > IB α−Akirin2 in the IκBζ -/- background) into Fig. 6D. We would also appreciate if you could briefly add a short sentence at this or another point of the results section pointing out the overall changes to Akirin2 (decreased) and IκBζ (increased) expression levels during e.g. LPS stimulation (referring to the existing data), which will be of general interest and also allow the reader to easier interpret the quantitative aspect in Figures 6C/6D. We agree with you of course that it is beyond the scope of the current manuscript to experimentally dissect the specific relevance of quantitative changes in the abundance of the IκBζ :Akirin2 complex when comparing resting and stimulated cells.

Please send the amended manuscript text file (Word .doc) and the relevant modified figure (Fig. 6), as well as synopsis figure, summary statement and bullet points to me via email.

