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Akirin specifies NF-κB selectivity of *Drosophila* innate immune response via chromatin remodeling

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

Editor: Alexander Kohlmaier

1st Editorial Decision 24 April 2014

Thank you for submitting your manuscript "Akirin specifies NF-κB selectivity of Drosophila innate immune response via chromatin remodeling" 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 would just like to mention that referees 1, 2 and 3 commonly requested a number of experiments addressing the selectivity of Akirin for a subset of Relish target genes and the biological significance of this selectivity. But also the other concerns raised are well taken.

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 invested 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

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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:

It is well-known that NF-kB activation leads to the expression of both pro- and anti-inflammatory genes. In this manuscript, Bonnay et al. investigated how NF-kB differentially regulates between pro- and anti-inflammatory genes. Using Drosophila model system, they showed that Relish (a Drosophila homologue of mammalian NF-kB), Akirin and the BAP form a complex following immune challenge. This tripartite complex forms an active transcription machinery on promoter regions of some subset of NF-kB target genes (especially, some class of antimicrobial peptide genes such as attacin-A, attacin-C, ceropin-A1, defensin, diptericin, and drosocin) carrying CpG-low sequence. However, this complex does not affect the transcription of many other antimicrobial peptide genes as well as genes involved in the negative regulation of IMD pathway (Drosopihla NF-kappaB pathway). Flies depleted of Akirin (C564/RNAi-akirin) or members of the Brahma complex (C564/RNAi-brahma or C564/RNAi-moira) are susceptible to E. cloacae infection when compared to control flies. They concluded that Akirin and the BAP complex dynamically interact to selectively activate a subset of Relish target genes during the immune response for host survival against Gram-negative bacterial infection.

General comment:
Overall, this study identifies an evolutionarily conserved molecular regulator of NF-kB and demonstrates its in vivo roles. The paper is clearly written and most of the experiments were designed in a logical way to provide experimental support for their proposed model. I believe that this work is novel and of interest to the broad audience. However, I have few concerns that should be clarified before publication.

Major points.
1. They claimed that Akirin complex controls immune effector genes without affecting anti-inflammatory genes. I understand that this concept is attractive but it is only partly true because Akirin controls only small subset of antimicrobial peptides (attacin-A, attacin-C, ceropin-A1, defensin, diptericin, and drosocin). As they showed in the manuscript, many other types of antimicrobial peptides are not affected by Akirin. Therefore, the text should be modified.
2. Why does Akirin control some antimicrobial peptides (e.g. Attacin-A) but not others (e.g. Attacin-D)? What is the biological significance? Does Akirin control target genes differently in a tissue-specific manner? For example, is there any difference between hemocytes and fat body?
3.How do they explain that knockdown flies for Akirin or members of the Brahma complex are susceptible to E. cloacae infection? What happen when they use other bacterial species for infection experiment? One possibility is that Akirin complex is required for host resistance against some bacterial species (i.e. host requires Akirin-controlled antimicrobial peptides to kill these bacterial species). I think that this point is important and should be addressed by performing infection experiment with different types of bacteria.

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4. Can the lethality of Akirin or Brahma Knockdown flies be rescued by UAS-Akirin or UAS-Brahma?

Minor point.

Page 6, 42 Relish-dependent genes instead of 41 Relish-dependent genes.

Referee #2:

In this study Bonnay et al. investigated the mechanisms implicated in akirin-dependent activation of Relish target genes in the context of the IMD pathway in Drosophila. The authors first used a microarray analysis to globally determine the impact of akirin deficiency on the activation of immune response genes. Of the 170 Relish-dependent genes, 19 (mainly antibacterial genes) were also akirin dependent, suggesting a highly specific and restricted role of akirin in this response. To identify direct mediators of akirin activity the authors used a yeast two-hybrid screen and after functional validation they retrieved only a single partner (BAP60) whose depletion impaired the activation of an attacinA-luc reporter. BAP60 and akirin interacted only upon immune stimulation, indicating that this is not a preformed and stable complex. Similarly, BAP60 interacted with the activated form of Relish (Rel-68) after immune challenge. Akirin and BAP60 were recruited to the attacinA gene promoter with kinetics that were apparently faster than those of RNA polymerase II, which may indirectly suggest a role of BAP60-dependent remodeling events in preparing this gene for activation. A sequence analysis of gene promoters showed that Akirin-dependent genes were more frequently characterized by the absence of a CpG island compared to those unaffected by akirin depletion. Finally, silencing of components of the BAP complex genes reduced both attacinA-luc expression and survival of flies challenged with E. Cloacae.

Overall, this manuscript strengthens previous data on the possible role of akirin in the induction of immune response genes, defines in a more precise manner the spectrum of transcriptional activities regulated by akirin and provides some initial mechanistic clues on this functional activity. The notion that BAP60 is a bona fide akirin binding and functional partner is rather well supported by the data. Nevertheless, there are some important limitations of this study that require additional efforts to be clarified.

The most important issue is the lack of a clear and solid explanation of the selectivity of akirin for a subset of Relish target genes. The first question to be addressed is whether recruitment of akirin is restricted to some Relish targets or not. Data shown in Fig. 5 B are not conclusive because they are very noisy, but they seem to suggest that akirin was not recruited to an akirin-independent Relish target (Attacin D). If akirin binds Relish after immune challenge but it is recruited only to a subset of Relish target genes, there must be a specific explanation of such a selective recruitment. This issue cannot be solved based on anecdotal data on individual genes but requires either a genome-wide analysis of akirin recruitment or at least the investigation of a consistent number of genes with distinct dependency on akirin. Second, the correlation between CpG islands and akirin/BAP60 dependence is interesting but far from being conclusive. Indeed, this correlation is very imperfect because 3/10 akirin-dependent genes contain and 12/32 akirin-independent ones do not contain, a CpG island. Irrespective of the possible (and in this context irrelevant) statistical significance of this difference, from a mechanistic point of view the presence or absence of a CpG island (which would imply a different nucleosomal stability and therefore a different requirement for remodeling to increase accessibility of the underlying regulatory sequences) clearly does not account for the selective requirement of akirin at a subset of Relish-dependent genes. Even with a stronger correlation, the mechanistic explanation of the selective requirement would have been unclear. An additional important issue related to this point is how the authors defined CpG islands in D. melanogaster, since obvious clusters of CpG dinucleotides do not exist in this genome (PMID 21177961).

Additional issues:
1. Fig. 2, coimmunoprecipitation of BAP60 and akirin: it would greatly help to include additional controls in this analysis and specifically to know what is the fraction of BAP60 that is associated with akirin and what is the fraction of akirin that is associated with BAP60.

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2. Fig. 3. While I do agree that akirin is excluded form dense DAPI-stained regions, the overlap with acetylation is not so obvious, being many akirin-stained nuclear regions completely devoid of acetylated chromatin.
3. Fig. 5. Recruitment of akirin to attacinA is very modest (3 fold increase over IgG). The authors should optimize this ChIP and/or use primers for additional flanking regions to better demonstrate the specificity of this binding.

Referee #3:

In this report the authors have investigated the role of Akirin as regulator of NF-kB transcriptional activity in Drosophila. Akirin had previously been identified as regulators of NF-kB activity in a genome wide RNAi screen in Drosophila, although the mechanisms involved were not clear. Here the authors find that Akirin regulates only a subset of NF-kB target genes that generally lack CpG islands in their promoters. Moreover, by performing a yeast 2 hybrid screen they identify BAP60, a component of the Drosophila SWI/SNF complex as an Akirin interacting protein. They confirm the importance of these proteins for Relish driven expression of the subset of Akirin dependent genes and use ChIP assays to demonstrate recruitment to the promoter. Finally they demonstrate the importance of this pathway to the IMD-dependent innate immune response in Drosophila.

This is an interesting report that identifies an important transcriptional regulator mechanism controlling NF-kB activity in Drosophila that should also be relevant to mammalian cells. Generally the experiments are clear and well performed although there are a number of areas where mechanistic insight is lacking.

Specific comments

(1) In the ChIP analysis, what happens with recruitment of Relish/Akirin/BAP60 at Akirin dependent promoters that do have a CpG island (e.g. Attacin C). Similarly, do immune induced, Akirin independent genes that lack CpG islands still recruit these proteins (it is possible that there is functional redundancy for Akirin at these targets).

(2) In the ChIP analysis, what is the effect of recruiting the components of this complex if the other members are depleted by RNAi?

(3) In the absence of co-IPs of the endogenous proteins, it should be demonstrated that these proteins simultaneously occupy their target promoters by performing Re-ChIP analysis.

(4) The authors should perform an in vitro interaction assay to determine if Relish binds Akirin directly?

Minor points

(5) BAP60 had been potentially identified as an interactor of Akirin in a Drosophila whole genome 2 hybrid screen (Giot et al, Science 2003). This manuscript should be cited.

(6) On page 10, the text refers to Figs 4A-D

Referee #4:

Bonnay et. al. investigated Akirin-dependency of immune induced genes using microarrays in S2 cells. Interestingly, of a subset of immune inducible genes were Akirin dependent. Like the companion paper, the Akirin dependent genes were enriched for promoters that lacked CpG island. The authors also note that the induction of 4 negative regulators (out of 5) are Akirin-independent, while the AMP genes were split almost evenly, undermining their claim "that Akirin is required for the transcription of effector genes, but dispensable for the transcription of genes that are negative

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regulators." Signal-dependent interaction between Relish, Akirin and Bap60, a component of Drosophila SWI/SNF complex, is a major finding of this work and this is significant addition to the understanding of Drosophila innate immunity. The sub-nuclear colocalization of these factors in areas devoid of HK9me2 is particularly novel and impressive. But a few important points should be addressed appropriately to merit publication of this work.

* Major points
- Signal-dependent interaction of Akirin and Bap60 is a major finding of this work, but protein-protein interaction between Akirin and Bap60 was already reported in Giot et.al. (2003) A protein interaction map of Drosophila melanogaster. Science 302: 1727-1736. This work needs to be cited.

- In Fig2, the slow kinetics of the coimunoprecipitation between Bap60 and Akirin is surprising, especially given the rapidity of the signal observed in ChIP assays. Earlier time points and some comment on this apparent discrepancy would be informative. in Furthermore, Fig2D and Fig 4C simultaneously using same lysates to show the dynamics of interactions between Relish, Akirin and Bap60, altogether. In figure 2, it is also disappointing that an inducible interaction between Relish and endogenously expressed Akirin is not demonstrated. The ChIP studies suggest an Akirin antibody is available.

- Regarding Fig3, presence or absence of colocalization between Akirin, Relish, and Bap60 with histone modification markers suggest chromatin remodeling mediated by Bap60 containing SWI/SNF complex but are not strong enough to full support the authors' claim. Chromatin-IP assays, with histone marks, should be provided.

- Also, the authors seem to be mis-citing work from their own group. In the Fukuyama paper, Bap55 seems to be a putative interactor of Iap2, Tak1 and Imd, but not Relish, at least according to the diagram therein. Apart from it, an independent co-immunoprecipitation experiments need to be added to confirm/validate to show interaction between Relish, Akirin, Bap60, and more of the Brahma components in a signal-dependent manner.

- In Fig6D-I, Bap60-RNAi should be included in parallel, as with S2 cells experiments, as this factor is a major focal point of this manuscript.

* Minor points
-
- In FigS1, Akirin does not show an increase after stimulation with heat-killed E.coli induction, but it is reported as an inducible Relish-independent gene in Figure 1. Which one is true?

- Resolution of Supplementary figures were so poor, but Drs seems to be in the immune-repressed genes in FigS1B, while in other reports Drs is robustly induced by IMD pathway stimulation of S2 cells.

- On page 9, the authors wrote "showed that Akirin is ubiquitously expressed". Is there data or citation to support this claim?

1st Revision - authors' response 22 July 2014

We appreciate the constructive reviews from the reviewers and the opportunity of improving the quality of our manuscript. We are now providing an enhanced version of the main text, two remodeled main figures and six new supplementary figures.

You will find below a point-by-point answer to questions and comments. We believe that the reviewers will share our enthusiasm about the new version of the manuscript. In our opinion, this study, together with the accompanying paper of Pr. Takeuchi demonstrate that Akirins are involved in an evolutionary conserved regulation of a subset of NF-kB target genes, which is mandatory for the innate immune response.

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Referee #1:
It is well-known that NF-kB activation leads to the expression of both pro- and anti-inflammatory genes. In this manuscript, Bonnay et al. investigated how NF-kB differentially regulates between pro- and anti-inflammatory genes. Using Drosophila model system, they showed that Relish (a Drosophila homologue of mammalian NF-kB), Akirin and the BAP form a complex following immune challenge. This tripartite complex forms an active transcription machinery on promoter regions of some subset of NF-kB target genes (especially, some class of antimicrobial peptide genes such as attacin-A, attacin-C, ceropin-A1, defensin, diptericin, and drosocin) carrying CpG-low sequence. However, this complex does not affect the transcription of many other antimicrobial peptide genes as well as genes involved in the negative regulation of IMD pathway (Drosopihla NF-kappaB pathway). Flies depleted of Akirin (C564/RNAi-akirin) or members of the Brahma complex (C564/RNAi-brahma or C564/RNAi-moira) are susceptible to E. cloacae infection when compared to control flies. They concluded that Akirin and the BAP complex dynamically interact to selectively activate a subset of Relish target genes during the immune response for host survival against Gram-negative bacterial infection.

General comment:
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Major points.
1. They claimed that Akirin complex controls immune effector genes without affecting antiinflammatory genes. I understand that this concept is attractive but it is only partly true because Akirin controls only small subset of antimicrobial peptides (attacin-A, attacin-C, ceropin-A1, defensin, diptericin, and drosocin). As they showed in the manuscript, many other types of antimicrobial peptides are not affected by Akirin. Therefore, the text should be modified.

Answer
Thank you for this comment. The text has been modified accordingly; we now clearly state that Akirin is required for the transcriptional control of a subset of immune effectors.

2. Why does Akirin control some antimicrobial peptides (e.g. Attacin-A) but not others (e.g. Attacin-D)? What is the biological significance? Does Akirin control target genes differently in a tissue-specific manner? For example, is there any difference between hemocytes and fat body?

Answer
We had already shown the biological significance of the molecular dichotomy in antimicrobial peptide (AMP) gene expression orchestrated by Akirin because in its absence, flies succumb to the Gram-negative bacterium *E. cloacae* (new Figure 6J, M). We have now added survival assays following *Erwinia carotovora* Ecc15 infection (new Figure 6K, N), which demonstrates that *Akirin* is required together with NF-kB for the full immune response that is mandatory for an efficient survival of Drosophila against at least two different Gram-negative bacteria.
Following another suggestion from reviewer 1, we have investigated the function of Akirin in the hemocyte antimicrobial response. By pricking third instar larvae with *E. coli* and bleeding them into PBS (new Supplementary Figure S11A), we could recover a pure population of Hml > GFP positive cells (new Supplementary Figure S11B). Inactivating Akirin in these cells using the hemocyte-specific Hml-gal4 driver led to a significant decrease of Akirin-dependent AMP mRNA level (attacin-A, diptericin-A and attacin-C) (new Supplementary Figure S11C), but did not alter significantly the expression of Akirin-independent AMPs (attacin-D, pirk and cecropin-A2) (new Supplementary Figure S11D). We have therefore now shown that Akirin and Brm are required in a similar manner for selective Relish-target gene expression in three different biological contexts -embryonic S2 cells, adult fat-body and larval hemocytes.
Nevertheless, we agree with reviewer 1: the evolutionary reason why two groups of antimicrobial peptide coding genes are under the transcriptional control of either the NF-kB factor alone or Relish in combination with Akirin is still an open question. We have now more clearly addressed this issue in the discussion of our manuscript.

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3.How do they explain that knockdown flies for Akirin or members of the Brahma complex are susceptible to E. cloacae infection?

Answer
We have shown in our study that removing Akirin or Brahma leads to an impaired expression of several antimicrobial peptide-coding genes. This most probably results in a weakened innate immune defense of the flies against Gram-negative bacteria. This observation suggests for the first time that the full cocktail of IMD-induced anti-microbial peptides is required to efficiently oppose a Gram-negative bacterial threat. We have now included this point in the discussion of our manuscript.

What happen when they use other bacterial species for infection experiment? One possibility is that Akirin complex is required for host resistance against some bacterial species (i.e. host requires Akirin-controlled antimicrobial peptides to kill these bacterial species). I think that this point is important and should be addressed by performing infection experiment with different types of bacteria.

Answer
We thank reviewer 1 for this valuable suggestion. This comment fits with the question of the biological significance of the dual transcriptional control operated by Akirin and Relish during the innate immune response.
To address this issue, we have specifically knocked down Akirin, BAP-complex (brahma, moira, bap60) or PBAP-specific (polybromo) genes in the fat-body and infected the flies with another pathogenic bacterium (*Erwinia carotovora* Ecc15). While GFP and Polybromo-RNAi flies displayed 10 to 20% lethality following this infection, Relish, Akirin and BAP-complex RNAi flies died within 4 days with a lethality-rate of about 50 to 70% (new Figure 6K, N). We therefore assume that Akirin and Brahma complex-dependent AMP transcription is required for an efficient survival of Drosophila against at least two different Gram-negative bacteria.
4. Can the lethality of Akirin or Brahma Knockdown flies be rescued by UAS-Akirin or UASBrahma?

Answer
The inverted-repeat-based RNAi-constructs (VDRC) that we used for Akirin, Brahma and Moira, target their respective coding-sequences (and not the 5'UTR), preventing a rescuing construct to be expressed. Therefore, to further illustrate the requirement of the Brahma complex for the survival following Gram-negative bacterial infection, we generated trans-heterozygous $relish^{E20}$ / $brahma^{2}$ and $relish^{E20}$ / $moira^{1}$ flies. Unlike $relish^{E20}$ / +, $brahma^{2}$ / + or $moira^{1}$ / + single heterozygous, $relish^{E20}$ / $brahma^{2}$ and $relish^{E20}$ / $moira^{1}$ transheterozygous flies showed an increased susceptibility to *Erwinia carotovora* Ecc15 (new Figure 6L, O). We believe that the demonstration of the genetic interaction between the Brahma complex and Relish to prevent lethality after infection strengthens our conclusions. Of note, previous work from our group has already shown that in S2 cells, the immune phenotype observed when removing Akirin (using an RNAi sequence targeting specifically its 5'UTR) can be rescued by over-expressing *Drosophila* Akirin or human Akirin-2 (Goto *et al.*, 2008, PMID: 18066067).

Minor point. Page 6, 42 Relish-dependent genes instead of 41 Relish-dependent genes.

Answer
The text has been modified accordingly.

Referee #2:
Overall, this manuscript strengthens previous data on the possible role of akirin in the induction of immune response genes, defines in a more precise manner the spectrum of transcriptional activities regulated by akirin and provides some initial mechanistic clues on this functional activity. The notion that BAP60 is a bona fide akirin binding and functional partner is rather well supported by the data. Nevertheless, there are some important limitations of this study that require additional efforts to be clarified.
The most important issue is the lack of a clear and solid explanation of the selectivity of akirin for a subset of Relish target genes. The first question to be addressed is whether recruitment of

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akirin is restricted to some Relish targets or not. Data shown in Fig. 5 B are not conclusive because they are very noisy, but they seem to suggest that akirin was not recruited to an akirin-independent Relish target (Attacin D). If akirin binds Relish after immune challenge but it is recruited only to a subset of Relish target genes, there must be a specific explanation of such a selective recruitment. This issue cannot be solved based on anecdotal data on individual genes but requires either a genome-wide analysis of akirin recruitment or at least the investigation of a consistent number of genes with distinct dependency on akirin.

Answer
We thank reviewer 2 for his constructive comments. We share his analysis and have performed a set of experiments to explore the molecular basis of Akirin selectivity. A genome-wide analysis of the distribution of Akirin-binding on the genome after an immune challenge would have provided an interesting cartography. However since Akirin is not a direct DNA binding protein, we doubted that we could perform, together with the relevant controls, these chromatin-IP experiments in a reasonable time schedule. We therefore decided to focus on three interesting additional genes, which we analyzed by chromatin-IP.

We performed chromatin-IP experiments on three Akirin-dependent target promoters (*attacin-A*, *drosocin*, *cecropin-A1*), two Akirin-independent target promoters (*attacin-D*, *metchnikowin*) and one immune-unrelated promoter (*hunchback*). We observed an inducible recruitment of Akirin and Bap60 with a peak at 2 hours post-stimulation on *p-attacin-A* (4-fold / 10-fold), *p-drosocin* (20-fold / 28-fold) and *p-cecropin-A1* (8-fold / 11-fold) (new Figure 5A, C and D, upper panels), but not on *attacin-A* coding-sequence, *p-attacin-D*, *p-metchnikowin* or *p-hunchback* (new Figure 5B, E, F, new Supplementary Figure S8B upper panels). Collectively these results indicate that Akirin and Bap60 are recruited to Akirin-regulated gene promoters and not to Akirin-independent gene promoters.

Second, the correlation between CpG islands and akirin/BAP60 dependence is interesting but far from being conclusive. Indeed, this correlation is very imperfect because 3/10 akirin-dependent genes contain and 12/32 akirin-independent ones do not contain, a CpG island. Irrespective of the possible (and in this context irrelevant) statistical significance of this difference, from a mechanistic point of view the presence or absence of a CpG island (which would imply a different nucleosomal stability and therefore a different requirement for remodeling to increase accessibility of the underlying regulatory sequences) clearly does not account for the selective requirement of akirin at a subset of Relish-dependent genes. Even with a stronger correlation, the mechanistic explanation of the selective requirement would have been unclear. An additional important issue related to this point is how the authors defined CpG islands in D. melanogaster, since obvious clusters of CpG dinucleotides do not exist in this genome (PMID 21177961).

Answer
The regions we define as CpG–rich were found using cpgplot (EMBOSS). This tool scores a region as CpG-rich when its GC content is higher than 60% and its average of G plus C content is more than 50% on a minimal length of 200nt, which is a classical tool used to identify CpG islands in other species (Blankenberg *et al.*, 2007, PMID: 17568012).
We are aware that the definition of CpG-rich regions is controversial for *Drosophila* as its genome lacks classical methylated CpG islands, which we had mentioned in our discussion (Deaton and Bird, 2011, PMID: 21576262).

Accordingly, we did not use the term “CpG islands” when referring to *Drosophila* CpG-rich regions. We have now removed the last confusing mention of “Predicted CpG islands” from the materials and methods section.

We agree that the CpG content of the promoter is not likely to be the only factor responsible for the observed Akirin and SWI/SNF selectivity and have accordingly modified the corresponding headline in the results section and the discussion. The fact that the correlation between CpG content and Akirin requirement is observed in both *Drosophila* and mice (see the accompanying manuscript from Pr. Takeuchi) strengthens our hypothesis of an evolutionary conserved promoter-sequence design combined with Akirin recruitment. This is the major point of our discussion, and would require further studies to be fully understood.
Of note, the group of Dr. Smale previously showed that in immune challenged mice CpG

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islandcontaining
promoters correlate with SWI/SNF independent genes (Ramirez-Carrozzi et al., 2009, PMID: 19596239).

Additional issues:
1. Fig. 2, coimmunoprecipitation of BAP60 and akirin: it would greatly help to include additional controls in this analysis and specifically to know what is the fraction of BAP60 that is associated with akirin and what is the fraction of akirin that is associated with BAP60.

Answer
To address this issue, we repeated co-immunoprecipitation of Akirin and Bap60 (new Supplementary Figure S3). We then measured the fraction of Bap60 bound to Akirin compared to the total amount of immunoprecipitated Bap60 and plotted it in new Supplementary Figure S3B. However, we were unable to observe this endogenous interaction by a reciprocal experiment (new Supplementary Figure S3A).

2. Fig. 3. While I do agree that akirin is excluded form dense DAPI-stained regions, the overlap with acetylation is not so obvious, being many akirin-stained nuclear regions completely devoid of acetylated chromatin.

Answer
To further investigate the relationship between Akirin and active transcription, we performed a co-labeling of Akirin with another epigenetic marker of transcriptional activity and we did observe a very good overlap between phosphorylated H3S10 and Akirin staining in fat body cells (new Supplementary Figure S6). This colocalization had also been observed in larval polytene chromosomes (Nowak *et al.*, 2012, PMID: 22396663). Of note, it is known that H3S10 phosphorylation is mediated by IkB kinase-alpha on NF-kB-target promoters in response to an immune stimulation in mammals (Anest *et al.*, 2003, PMID: 12789343).
Nevertheless, we do not rule out a role for Akirin in transcriptional repression as we observed a small overlap between heterochromatin and akirin labeling (see text and Figure 3E). This observation is also consistent with our microarray data (Supplementary Fig S1B).

3. Fig. 5. Recruitment of akirin to attacinA is very modest (3 fold increase over IgG). The authors should optimize this ChIP and/or use primers for additional flanking regions to better demonstrate the specificity of this binding.

Answer
To increase the quality of our ChIP data, we performed additional experiments showing 8-fold and 20-fold recruitment of Akirin on two other Akirin-dependent genes (p-*cecropin-A1* and *pdrosocin*
respectively in Figure 5A, C, D, upper panels). Additionally, we did not observe any recruitment of Akirin or Bap60 on the *attacin-A* coding sequence (new Figure 5B, upper panel). We hope that these additional results will answer the question of reviewer 2.

Referee #3:
This is an interesting report that identifies an important transcriptional regulator mechanism controlling NF-kB activity in Drosophila that should also be relevant to mammalian cells. Generally the experiments are clear and well performed although there are a number of areas where mechanistic insight is lacking.
Specific comments
(1) In the ChIP analysis, what happens with recruitment of Relish/Akirin/BAP60 at Akirin dependent promoters that do have a CpG island (e.g. Attacin C). Similarly, do immune induced, Akirin independent genes that lack CpG islands still recruit these proteins (it is possible that there is functional redundancy for Akirin at these targets).

Answer
We thank reviewer 3 for this suggestion. We performed ChIP on CpG-rich Akirin-dependent promoter (*p-drosocin*) and observed a significant recruitment of Akirin and Bap60 (20-fold and 28-fold respectively) at 2h post-stimulation (new Figure 5C, upper panel). Additionally, we did not observe the recruitment of Akirin and Bap60 on Akirin-independent promoters irrespective of

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the CpG content (*p-metchnikowin* and *p-attacin-D* respectively), (new Figure 5E, F, upper panels). These results suggest that factors additional to the CpG content are involved in the selective recruitment of Akirin and Bap60 to promoters. We have consequently monitored the presence of several transcriptionally active histone marks and found that H3K4ac was specifically observed on Akirin-binding promoters (new Figure 5A, C, D, lower panels) but not on Akirin-non-binding promoters (new Figure 5E, F, lower panels) despite Relish being always recruited (new Figure 5A, C, D, E, F, middle panels). Consistent with these results, knockingdown Akirin or Bap60 significantly decreased H3K4ac marks on *attacin-A* and *drosocin* promoters following immune-stimulation (new Figure 5G, H). We now propose H3K4ac as a potential hallmark of Akirin-dependent promoter activation.

(2) In the ChIP analysis, what is the effect of recruiting the components of this complex if the other members are depleted by RNAi?

Answer
We thank reviewer 3 for this suggestion. We performed a knock-down of Akirin and Bap60 in S2 cells and observed a significant decrease of Relish binding on Akirin-dependent promoters (new Figure 5I, J), indicating that Akirin and the Brahma complex are required to recruit and/or stabilize Relish on promoters. Unfortunately, we could not perform anti-Akirin and anti-Bap60 ChIP in these conditions because our antibodies were not sensitive enough to detect the low amount of chromatin material from RNAi-KD-cells.

(3) In the absence of co-IPs of the endogenous proteins, it should be demonstrated that these proteins simultaneously occupy their target promoters by performing Re-ChIP analysis.

Answer
The suggestion of Reviewer 3 was to perform Re-ChIP experiments to study the relationship between Relish, BAP60 and Akirin. While these results would have been bringing valuable information, we were unable to perform properly this experiment, most probably due to the lack of sensitivity of our antibodies for Re-ChIP. However, the observation of an interaction between Akirin and Bap60 upon immune challenge together with the absence of Relish recruitment without Akirin and Bap60 strongly suggest that these proteins gather simultaneously at Akirin proximal promoters sites (Figure 2, new Figure 5I, J).

(4) The authors should perform an in vitro interaction assay to determine if Relish binds Akirin directly?

Answer
Following the reviewer's comment, we performed *in vitro* interaction experiments between Akirin and Relish. Although GST-tagged Relish and his-tagged Akirin were successfully expressed in bacteria and purified by affinity chromatography, our *in vitro* binding assays was unable to detect the interaction of these purified proteins. Since the bacterial expressed proteins might lack the proper post-translation modification required for the protein-protein interaction, Flag-tagged Relish ΔS29-S45 was expressed in S2 cells and purified by immunoprecipitation with anti-Flag agarose beads. As shown in new Supplementary Figure S7 this purified Flagtagged Relish ΔS29-S45 interacts with His-tagged Akirin in an *in vitro* binding assay, suggesting that Relish binds Akirin directly.

Minor points
(5) BAP60 had been potentially identified as an interactor of Akirin in a Drosophila whole genome 2 hybrid screen (Giot et al, Science 2003). This manuscript should be cited.

Answer
Thank you for this comment. The discussion has been modified accordingly.

(6) On page 10, the text refers to Figs 4A-D

Answer
Thank you for your careful reading. The text has been modified accordingly.

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Referee #4:
Bonnay et. al. investigated Akirin-dependency of immune induced genes using microarrays in S2 cells. Interestingly, of a subset of immune inducible genes were Akirin dependent. Like the companion paper, the Akirin dependent genes were enriched for promoters that lacked CpG island. The authors also note that the induction of 4 negative regulators (out of 5) are Akirin-independent, while the AMP genes were split almost evenly, undermining their claim "that Akirin is required for the transcription of effector genes, but dispensable for the transcription of genes that are negative regulators." Signal-dependent interaction between Relish, Akirin and Bap60, a component of Drosophila SWI/SNF complex, is a major finding of this work and this is significant addition to the understanding of Drosophila innate immunity. The sub-nuclear colocalization of these factors in areas devoid of HK9me2 is particularly novel and impressive. But a few important points should be addressed appropriately to merit publication of this work.

** Major points*
- Signal-dependent interaction of Akirin and Bap60 is a major finding of this work, but protein-protein interaction between Akirin and Bap60 was already reported in Giot et.al. (2003) A protein interaction map of Drosophila melanogaster. Science 302: 1727-1736. This work needs to be cited.

Answer
Thank you for this comment. The discussion has been modified accordingly.

- In Fig2, the slow kinetics of the coimunoprecipitation between Bap60 and Akirin is surprising, especially given the rapidity of the signal observed in ChIP assays. Earlier time points and some comment on this apparent discrepancy would be informative.

Answer
In agreement with this comment, we performed endogenous co-immunoprecipitation at an early time point (15min after heat-killed *E. coli* stimulation, new Supplementary Figure S3) but were not able to observe a significant interaction between Akirin and BAP60, perhaps due to the sensitivity of the method. We observed a weak interaction at 2 hours post-stimulation, increasing with time at 4h and 8h (as documented in Figure 2D). Our ChIP assays reveal a much stronger recruitment of Akirin and Bap60 2 hours post-stimulation when compared to 15min and even 1h, in particular on the promoter of *drosocin* and *cecropin-A1* (new Figure 5C, D, upper panels). We believe therefore that Akirin and Bap60 are gradually recruited to their target sites as does Relish.

Furthermore, Fig2D and Fig 4C simultaneously using same lysates to show the dynamics of interactions between Relish, Akirin and Bap60, altogether. In figure 2, it is also disappointing that an inducible interaction between Relish and endogenously expressed Akirin is not demonstrated. The ChIP studies suggest an Akirin antibody is available.

Answer
By performing endogenous co-IP, we were not able to find an interaction between Akirin and Relish or between Bap60 and Relish, even following immune stimulation. Our study suggests that these interactions are occurring in one fifth of the immune-induced promoters since Akirin is required for a small portion of Relish-targets, thus other Relish targets would require Relish without Akirin and the Brahma complex. Therefore, one explanation for these negative results would be a lack of sensitivity of our method of endogenous co-IP. We have however now shown a direct interaction between Akirin and Relish in an *in vitro* binding assay (new Supplementary Figure S7).

- Regarding Fig3, presence or absence of colocalization between Akirin, Relish, and Bap60 with histone modification markers suggest chromatin remodeling mediated by Bap60 containing SWI/SNF complex but are not strong enough to full support the authors' claim. Chromatin-IP assays, with histone marks, should be provided.

Answer
Following the suggestion of reviewer 4, we have now monitored the presence of several transcriptionally active histone marks and found that H3K4ac was specifically observed on Akirin-binding promoters (new Figure 5A, C, D, lower panels) but not on Akirin-non-binding

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promoters (new Figure 5E, F, lower panels) despite Relish being always recruited (new Figure 5A, C, D, E, F, middle panels). Consistent with these results, knocking-down Akirin or Bap60 significantly decreased H3K4ac marks on *attacin-A* and *drosocin* promoters following immune-stimulation (new Figure 5G, H). We now propose H3K4ac as a potential hallmark of Akirin-dependent promoter activation (See comments to reviewer 3). The new results have been included in the main text.
Of note, a very recent publication shows that *Drosophila* DNA-methyltransferase associated protein 1 (DMAP1) is able to associate with Akirin to positively regulate Relish-dependent AMPs in S2 cells, possibly by acting on epigenetic regulation (Goto *et al.*, 2014, PMID: 24947515).

- Also, the authors seem to be mis-citing work from their own group. In the Fukuyama paper, Bap55 seems to be a putative interactor of Iap2, Tak1 and Imd, but not Relish, at least according to the diagram therein.

Answer
Thanks a lot to reviewer 4 for this comment. We have changed this part of the manuscript to be more explicit. We agree that claiming a putative interaction between BAP55 and Relish without this background information was misleading.

Apart from it, an independent co-immunoprecipitation experiments need to be added to confirm/validate to show interaction between Relish, Akirin, Bap60, and more of the Brahma components in a signal-dependent manner.

Answer
Following reviewer's suggestion, we performed an additional co-immunoprecipitation between endogenous Akirin and Bap60 that validated this immune signal dependent interaction (new Supplementary Figure S3). The interaction between Akirin and Relish could only be visualized *in vitro* (new Supplementary Figure S7, see answer to reviewer 3).

- In Fig6D-I, Bap60-RNAi should be included in parallel, as with S2 cells experiments, as this factor is a major focal point of this manuscript.

Answer
We thank Reviewer 4 for this improvement. We performed fat-body specific RNAi knock-down of Bap60 and observed the expected immune impaired survival following Gram-negative bacterial infection (new Figure 6K, N).

** Minor points*
-
- In FigS1, Akirin does not show an increase after stimulation with heat-killed E.coli induction, but it is reported as an inducible Relish-independent gene in Figure 1. Which one is true?
- Resolution of Supplementary figures were so poor, but Drs seems to be in the immune-repressed genes in FigS1B, while in other reports Drs is robustly induced by IMD pathway stimulation of S2 cells.

Answer
Thanks to reviewer 4 comments, we realized that the way we presented our microarray data was confusing. We have now improved the way these data are documented: in the figure headline we are now presenting genes positively (in new Figure 1A-B) or negatively (in new Supplementary Figure S1B) regulated in the absence of Relish or Akirin during an immune-challenge, rather than immune-induced (former Figure 1B) or immune-repressed (former Supplementary Figure S1B) genes.

Reviewer 4 is correct: *drosomycin* (*Drs*) is slightly induced by PGRP-LC overexpression as shown in our microarray data (Figure 1B). However, silencing Akirin results in an increase of *Drs* gene induction (new Supplementary Figure S1B), which led us to categorize *Drs* as negatively regulated by Akirin.

Concerning the Akirin gene expression data, we confirm that Akirin is not induced during an

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immune challenge. In Figure 1B, the repression observed for *akirin* (and for *relish*) is due to their RNAi-mediated knockdown and is used as internal control, which is now mentioned in the materials and methods section. We thank reviewer 4 for helping us to improve the readability of our manuscript.

- On page 9, the authors wrote "showed that Akirin is ubiquitously expressed". Is there data or citation to support this claim?

Answer
As mentioned in our manuscript, Crosby et al., 2007 described that Akirin is expressed in a large variety of tissues (flyatlas.org, new Supplementary Figure S5A); in addition, we have checked Akirin distribution by immuno-staining and found a nuclear localization in all investigated adult tissues (new Supplementary Figure S5B-G).

2nd Editorial Decision 06 August 2014

Thank you for submitting your revised manuscript to The EMBO Journal. Your submission has been handled by Alexander Kohlmaier, but as Alexander is away on vacation this week, I have stepped in as 2nd editor to help speed things up.

We have now heard back from the four referees and as you can see below the referees appreciate the introduced change and support publication here. There are just some minor points to sort out before final acceptance here. I have provided the link below for you to upload the amended version. Please also make sure that microarray accession numbers are provided in the text.

Given that the related paper has been accepted and transferred to our publisher, I am keen on getting the revised version back as soon as possible.

I think that should be it. Let me know if you have any questions.

Referee #1:

Most of this reviewer's concerns have been addressed. I recommend this manuscript for publication.

Referee #2:

In this revised version of the manuscript the authors addressed my previous comments. Although the Akirin ChIP data are restricted to a very small number of targets, which in my opinion does not allow drawing any definitive mechanistic conclusion, the manuscript is now improved and almost ready for publication.

The only issue left to amend relates to the CpG island. The authors state that " The genome of D. melanogaster is unmethylated and therefore lacks classical, methylated, CpG islands". This is completely wrong: CpG islands are unmethylated by default. In Drosophila, the lack of DNA methylation implies that there is no loss of cytosines (in a CpG context in the genome) and therefore there are no regions where CpG dinucleotides are relatively enriched compared to the rest of the genome (namely, CpG islands). Since this is an important issue with mechanistic implications, it is necessary to show the distribution of CpG dinucleotides over stretches of representative regions (promoters) and flanks, rather than providing (Fig. 5K-L) a digital description (presence or absence) of something (the CpG island) that in fact does not exist in this organism. In other words, it is not formally correct that there are groups of genes that are + or - for a feature if the properties of this feature are not defined.

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Referee #3:

The authors have comprehensively addressed the issues I raised in my previous review. I have no further concerns.

Referee #4:

- Overall, the authors nicely improved their manuscript and most of the points raised were properly addressed. Importantly, they found that H3K4 acetylation is selectively enriched on Akirin-dependent promoters and dependent on Akirin/Bap60/Relsh. This provide a significant mechanistic insight into the underlying mechanisms involved in Akirin-dependent immune gene expression.

One major concern remains. Based on over expression, co-ip experiments the authors wish to conclude that Relish interacts with and recruits Akirin and the Bap60 complex, driving H3K4ac at target promoters. Yet they fail to see either a direct interaction, with fully recombinant or purified or non-Drosophila derived proteins, or with endogenous proteins, even upon signaling.
While the RNAi and ChIP studies clearly show that Relish recruitment to Akirin-dependent genes was indeed Akirin dependent, they do not provide the reverse experiment (for technical reasons). Thus, it remains possible that Akirin is recruited to these promoters independent of Relish, perhaps by a pioneer transcription factor, while Relish recruitment is demonstrated to require Akirin.
While it may be difficult to experimentally address this concern, with currently available tools, the Discussion might include the possibility that link between Relish and Akirin recruitment to target promoters is still a bit fuzzy, as an alternative to their preferred model.

Minor points

- Double check the header ; In Fig1A, "negatively regulated", whereas in Supp Fig1 "positively regulated" used. Should be corrected the other way around.

- In fact, grouping of genes in the Fig1B and S1B is still confusing.
In Fig1A, 170 genes were induced by over expression of truncated PGRP-LC. Most of them are "Relish-dependent", whereas there are some "Akirin-dependent" genes, too.

In FigS1B, I understand that 216 genes are negatively regulated by Akirin, but it's still unclear to me if those genes are induced/repressed or neutral upon PGRP-LC over expression. Need more clarification.

- In Fig1A, "Relish-dependent" is easier to understand than "in absence of Relish". Same with Akirin-dependent genes.

- In Fig1A, if reduction in Akirin level comes from the RNAi effect, it should be removed from the table.

- Indicate the statistics used to acquire p-values in the method section. One way Anova, for example. Also, indicate the compared pairs in Fig 5G-J for clarification.

2nd Revision - authors' response 07 August 2014

Referee #1:
Most of this reviewer's concerns have been addressed. I recommend this manuscript for publication.

Referee #2:
In this revised version of the manuscript the authors addressed my previous comments. Although the Akirin ChIP data are restricted to a very small number of targets, which in

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my opinion does not allow drawing any definitive mechanistic conclusion, the manuscript is now improved and almost ready for publication.
The only issue left to amend relates to the CpG island. The authors state that " The genome of D. melanogaster is unmethylated and therefore lacks classical, methylated, CpG islands". This is completely wrong: CpG islands are unmethylated by default. In Drosophila, the lack of DNA methylation implies that there is no loss of cytosines (in a CpG context in the genome) and therefore there are no regions where CpG dinucleotides are relatively enriched compared to the rest of the genome (namely, CpG islands). Since this is an important issue with mechanistic implications, it is necessary to show the distribution of CpG dinucleotides over stretches of representative regions (promoters) and flanks, rather than providing (Fig. 5K-L) a digital description (presence or absence) of something (the CpG island) that in fact does not exist in this organism. In other words, it is not formally correct that there are groups of genes that are + or - for a feature if the properties of this feature are not defined.

Answer:
We have taken into account the comment of referee #2 and performed significant changes in the text. We removed any mention of the influence of the CpG content towards the selectivity of Akirin, in the abstract and in the introduction. However we still mention this analysis in the result and discussion sections with appropriate care. Instead of CpG content, we mention the H3K4ac marks, because as commented by reviewer 4 this "*provide a significant mechanistic insight into the underlying mechanisms involved in Akirin-dependent immune gene expression"*.

Referee #3:
The authors have comprehensively addressed the issues I raised in my previous review. I have no further concerns.

Referee #4:
- Overall, the authors nicely improved their manuscript and most of the points raised were properly addressed. Importantly, they found that H3K4 acetylation is selectively enriched on Akirin-dependent promoters and dependent on Akirin/Bap60/Relsh. This provide a significant mechanistic insight into the underlying mechanisms involved in Akirin-dependent immune gene expression.
One major concern remains. Based on over expression, co-ip experiments the authors wish to conclude that Relish interacts with and recruits Akirin and the Bap60 complex, driving H3K4ac at target promoters. Yet they fail to see either a direct interaction, with fully recombinant or purified or non-Drosophila derived proteins, or with endogenous proteins, even upon signaling.
While the RNAi and ChIP studies clearly show that Relish recruitment to Akirindependent genes was indeed Akirin dependent, they do not provide the reverse experiment (for technical reasons). Thus, it remains possible that Akirin is recruited to these promoters independent of Relish, perhaps by a pioneer transcription factor, while Relish recruitment is demonstrated to require Akirin.
While it may be difficult to experimentally address this concern, with currently available tools, the Discussion might include the possibility that link between Relish and Akirin recruitment to target promoters is still a bit fuzzy, as an alternative to their preferred model.

Answer:
As previously mentioned in the manuscript, our results suggest that a direct interaction between Akirin and Relish occurs. We thought that using the word "suggest" was enough to highlight the fuzziness of this result.
However, we agree with referee #4 and added in the discussion section an alternative scenario where Akirin and Bap60 could be recruited to relish-dependent promoters independently of Relish.

Minor points
- Double check the header ; In Fig1A, "negatively regulated", whereas in Supp Fig1 "positively regulated" used. Should be corrected the other way around.

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Answer:
Appropriate changes have been made.

- In fact, grouping of genes in the Fig1B and S1B is still confusing.
In Fig1A, 170 genes were induced by over expression of truncated PGRP-LC. Most of them are "Relish-dependent", whereas there are some "Akirin-dependent" genes, too.

Answer:
We understand the concern of the reviewer, but it was the most clear way of presenting the results..

In FigS1B, I understand that 216 genes are negatively regulated by Akirin, but it's still unclear to me if those genes are induced/repressed or neutral upon PGRP-LC over expression. Need more clarification.

Answer:
We reanalyzed our data and indeed found that 205 genes *are negatively regulated by Akirin instead of 216.* We apologize for this mistake. We showed that 203 of the 205 genes are “neutral” upon PGRP-LC induction in control conditions. This is now mentioned in the text.
Additionally we reanalyzed our microarray data in the light of this comment and found also a slight error in the presentation of the positively regulated genes, that we have now corrected. This does not impact the text of the manuscript.

- In Fig1A, "Relish-dependent" is easier to understand than "in absence of Relish". Same with Akirin-dependent genes.

Answer: done

- In Fig1A, if reduction in Akirin level comes from the RNAi effect, it should be removed from the table.

Answer: We do not agree with this point. As stated in the materials and methods section, this represents a complementary validation of our microarray results.

- Indicate the statistics used to acquire p-values in the method section. One way Anova, for example. Also, indicate the compared pairs in Fig 5G-J for clarification.

Answer:
P-values were calculated with a two-tailed student t test. This has been added to the Supplementary Material and Method.
We have now indicated which values were compared for statistical significance in the legend of Figure 5.

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