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

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Abstract

The network of NF-κB-dependent transcription that activates both pro- and anti-inflammatory genes in mammals is still unclear. As NF-κB factors are evolutionarily conserved, we used *Drosophila* to understand this network. The NF-κB transcription factor Relish activates effector gene expression following Gram-negative bacterial immune challenge. Here, we show, using a genome-wide approach, that the conserved nuclear protein Akirin is a NF-κB cofactor required for the activation of a subset of Relish-dependent genes correlating with the presence of H3K4ac epigenetic marks. A large-scale unbiased proteomic analysis revealed that Akirin orchestrates NF-κB transcriptional selectivity through the recruitment of the Osa-containing-SWI/SNF-like Brahma complex (BAP). Immune challenge in *Drosophila* shows that Akirin is required for the transcription of a subset of effector genes, but dispensable for the transcription of genes that are negative regulators of the innate immune response. Therefore, Akirins act as molecular selectors specifying the choice between subsets of NF-κB target genes. The discovery of this mechanism, conserved in mammals, paves the way for the establishment of more specific and less toxic anti-inflammatory drugs targeting pro-inflammatory genes.

Keywords Chromatin remodeling; *Drosophila*; Innate immune response; NF-κB
Subject Categories Chromatin, Epigenetics, Genomics & Functional Genomics; Immunology; Signal Transduction
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Introduction

In mammals, the NF-κB family is composed of five related transcription factors, namely p50, p52, p65, REL, and RELB, which regulate gene expression following various stimuli. NF-κB factors are conserved among metazoans, and the *Drosophila* NF-κB transcription factors, DIF and Relish, are homologous to human REL and p52/p50, respectively (Hetru & Hoffmann, 2009). Inflammatory stimuli induce gene expression programs that are almost entirely NF-κB dependent (Ghosh & Hayden, 2012). Aberrant regulation of NF-κB signaling is strongly suspected in numerous cancers, inflammatory, and autoimmune diseases (Maeda & Omata, 2008). Moreover, activation of NF-κB signaling in response to commensal bacteria in the gut has been shown to be required for optimal intestinal homeostasis (Mukherji *et al*, 2013). Massive efforts in drug development have been aimed at targeting NF-κB signaling during inflammatory diseases. However, interfering with the NF-κB pathway can potentially lead to numerous adverse effects. Commonly used anti-inflammatory agents act through inhibition of the NF-κB pathway to exert both therapeutic and adverse side effects (Oeckinghaus *et al*, 2011; Hayden & Ghosh, 2012). NF-κB factors act mainly to trigger inflammation, but recent studies suggest that they also function during the resolution of inflammation (Lawrence *et al*, 2001; Hayden & Ghosh, 2012), emphasizing the need for the development of specific drugs switching on, or off, particular subsets of NF-κB target genes. Identifying this new generation of drug targets requires a comprehensive, large-scale dissection of NF-κB-regulated pathways to identify factors able to restrict the range of NF-κB target activities. It has been proposed that the selective activation of NF-κB target genes depends on chromatin remodeling factors (Kawahara *et al*, 2009; Smale, 2010). These selector molecules represent a 'missing link' in our understanding of both the complexity and selectivity of NF-κB signaling.

In *Drosophila*, the NF-κB transcription factors Relish and DIF (dorsal-related immunity factor) are activated upon an immune challenge downstream of the immune deficiency (IMD) and Toll pathways, respectively. Direct recognition of Gram-negative bacterial DAP-type peptidoglycan, by the peptidoglycan recognition protein-LC (PGRP-LC), occurs at the cell surface to activate the IMD pathway. Gram-positive or fungal microbial patterns, however, are recognized by circulating proteins, which trigger the activation of the Toll pathway (Ferrandon

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et al, 2007). Both pathways culminate with the nuclear translocation of an NF-κB transcription factor and activate the expression of antimicrobial peptide (AMP) coding genes in the fat body (a functional equivalent of the mammalian liver). The Toll pathway shares significant similarities with the signaling cascades downstream of the mammalian Toll-like receptors (TLRs) and the interleukin-1 receptor (IL-1R), highlighting a common ancestry for these immune mechanisms. The IMD pathway is akin to the tumor necrosis factor receptor (TNFR) pathway in vertebrates (Hoffmann & Reichhart, 2002).

A genome-wide RNA-mediated interference screen in *Drosophila melanogaster* identified Akirin as new NF-κB modulators in the IMD pathway (Goto *et al*, 2008). Akirins have a strict nuclear localization and were shown in flies to act at the level of the NF-κB factor Relish, but to be dispensable for activation of DIF target genes. Akirin was therefore identified as a new component of the IMD pathway driving the innate immune response after an immune challenge with Gram-negative bacteria (Ferrandon *et al*, 2007; Goto *et al*, 2008). Akirins are highly conserved, and the two mouse genes (*akirin-1* and *akirin-2*) have been identified and knocked out. Analysis of Akirin-2 deficient mouse embryonic fibroblasts showed that Akirin-2 acts downstream of the TLR, TNFR, and IL-1R signaling pathways. However, Akirin-2 was required for the regulation of only a specific subset of LPS and IL-1 inducible genes (Goto *et al*, 2008), although the molecular basis for this specificity remained unclear.

We provide here a comprehensive view of Akirin function in NF-κB transcriptional selectivity during the innate immune response, using *Drosophila melanogaster* as a model. We performed a two-hybrid screen aimed at identifying Akirin partners. We found that BAP60, a component of the Brahma (SWI/SNF) ATP-dependent chromatin-remodeling complex, binds to Akirin upon immune challenge. In *Drosophila*, the Brahma complex forms the BAP complex when associated with Osa, whereas an association with both Polybromo and BAP170 defines the PBAP complex. Each complex targets a mutually exclusive subset of Brahma-dependent genes (Mohrmann *et al*, 2004; Moshkin *et al*, 2007). We show that the BAP, but not PBAP, complex is required *in vivo* for efficient antimicrobial peptide synthesis and for the survival of flies following Gram-negative bacterial infection. Upon immune challenge, Akirin is able to bind Relish, forming a link between this transcription factor and the BAP complex on the promoter of a subset of NF-κB target genes. Relish-dependent genes thus fall into two groups, either relying on Akirin and the BAP complex (and encoding mostly AMPs), or expressing most of the negative regulators of the IMD pathway and AMPs independently of Akirin.

We demonstrate here that NF-κB transcriptional selectivity relies on a tripartite relationship between Relish, Akirin, and the BAP complex, following immune stimulation in *Drosophila*. These components form an active transcription complex on promoter regions decorated with H3K4ac epigenetic marks.

Results

Akirin is required for the activation of a subset of Relish-dependent genes

Drosophila Akirin had been genetically shown to be required at the level of the NF-κB factor Relish to activate two IMD pathway effectors, the antimicrobial peptide (AMP) coding genes *attacin* and *diptericin* (Goto *et al*, 2008). We conducted a genome-wide analysis using Agilent DNA microarrays in *Drosophila* S2 cells to explore the impact of Akirin on the expression of the Relish-dependent transcriptome. *Drosophila* S2 cells were treated by dsRNA against *akirin*, *relish*, or *GFP* as a control, and the IMD pathway was activated by expressing a truncated form of Peptidoglycan receptor protein-Long Chain a (PGRP-LCa) (Goto *et al*, 2008). Total RNA was extracted from FACS-sorted transfected cells to evaluate gene expression (Supplementary Fig S1A). Microarray analysis revealed that Relish is required for the transcriptional activation of 170 genes upon challenge. The expression level of these genes showed at least a twofold reduction in the absence of Relish when compared to control *Drosophila* S2 cells. Among these 170 genes, 17 were also dependent on Akirin for their expression (Fig 1A), demonstrating that Akirin is required for the activation of only a restricted subset of Relish target genes. Upon immune challenge, Akirin *per se* is required for the activation of 31 genes independently of Relish (Fig 1A).

To understand the role of Akirin in this restricted activation, we first focused on genes encoding proteins with known immune functions (Fig 1B). In agreement with previous microarray data, Relish was required for the activation of 41 of these immune-related (IR) genes, pointing to Relish as a major immune transcription factor (Irving *et al*, 2001; De Gregorio *et al*, 2002; Pal *et al*, 2008). Akirin was only required for the activation of 9, among the 41 Relish-dependent, IR genes (Fig 1B). Among the 32 Relish-dependent, but Akirin-independent, IR genes, we found 8 genes encoding AMP effectors of the innate immune response, (*Attacin-B*, *Attacin-D*, *Cecropin-A2*, *Cecropin-B*, *Cecropin-C, Diptericin-B*, *Drosomycin*, and *Metchnikowin*) with either anti-bacterial (*Attacin-B*, *Attacin-D*, *Cecropin-A2*, *Cecropin-C*, *Diptericin-B*) or anti-fungal (*Drosomycin*, *Metchnikowin*) activities (Imler & Bulet, 2005). An additional group of 5 genes were shown to encode negative regulators of the IMD (*Pirk*, *PGRP-LB*, *PGRP-LF*, *PGRP-SB1*) or the Toll pathways (*Cactus*). The 19 remaining genes were involved in immune signal transduction (*Kenny*, *Relish*); chitin, nucleic acid, or peptidoglycan binding (*Sr-Cl, Helicase89B, Gnbp3*, *PGRP-SA*, *PGRP-SD,*); iron metabolism (*Tsf1*, *Tsf3*), or were suspected AMP (*Edin*). In contrast, we found that Akirin is almost exclusively required for the activation of genes encoding peptides with anti-bacterial activities (*Attacin-A*, *Attacin-C*, *Cecropin-A1*, *Defensin*, *Diptericin-A*, and *Drosocin*) (Imler & Bulet, 2005).

We validated the genome-wide analysis by monitoring the transcription of several IR genes upon immune challenge in S2 cells using RT–PCR (Fig 1C and Supplementary Fig S1C) and confirmed that *Pirk* and *Attacin-D* expression is Relish dependent but Akirin independent. In contrast, *Attacin-C* and *Diptericin-A* rely on both Relish and Akirin for their expression (Fig 1C). Of note, we found 8 genes that, after stimulation, had a twofold higher expression level compared to control when Relish was absent, and similarly, loss of Akirin results in the overexpression of 205 genes (Supplementary Fig S1B). Among these genes, 203 are not induced in control conditions (*dsGFP*) upon PGRP-LC stimulation, indicating a genuine derepression in absence of Akirin. As previously reported, upon immune challenge, Relish is not involved in gene repression (De Gregorio *et al*, 2002). Conversely, Akirin could function as a potent gene activator or repressor. Collectively, these data suggest that Akirin is

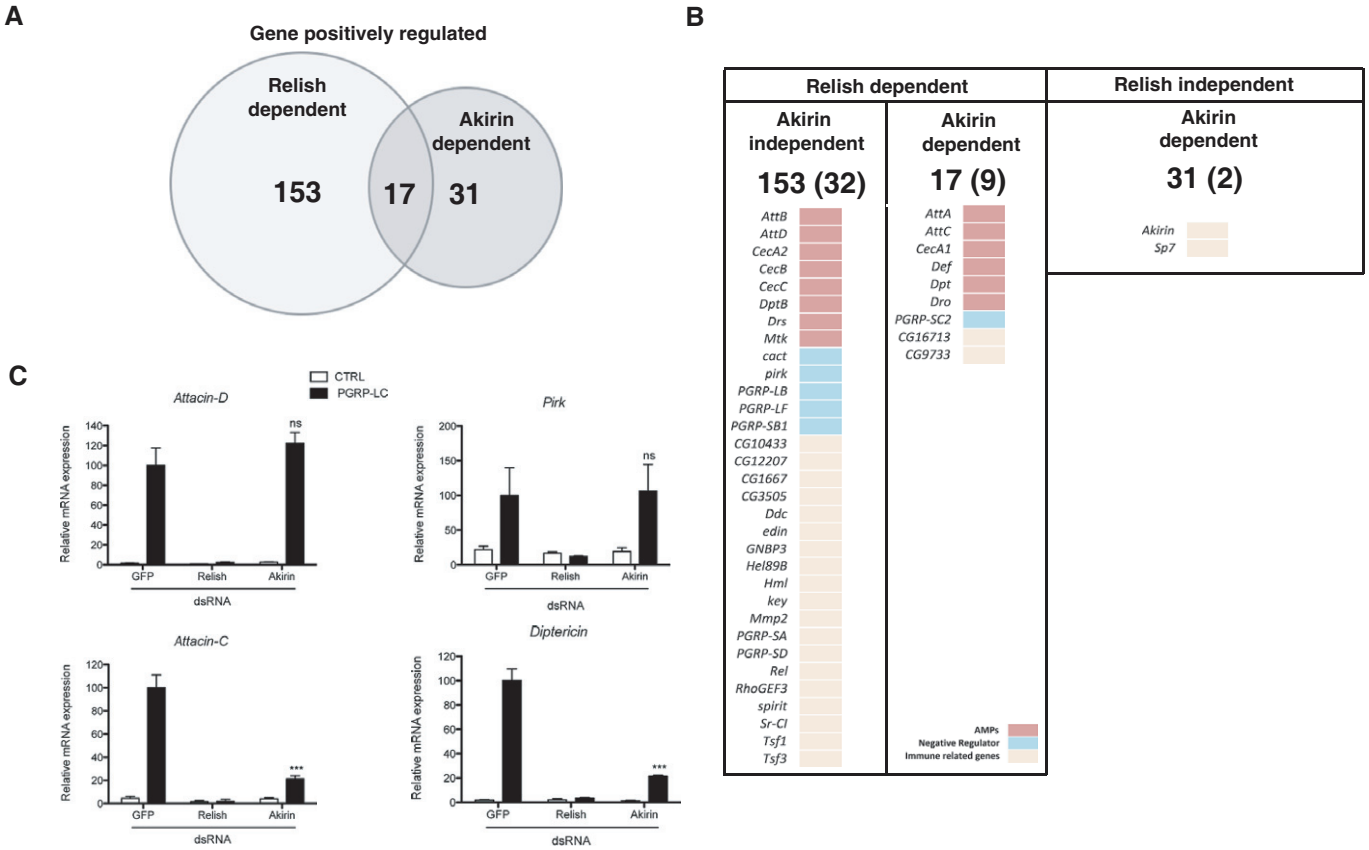


Figure 1. Akirin influences the expression of only a subset of Relish target genes.

A, B Venn diagram (A) and table representation of microarray analysis (B). Genes in PGRP-LC-stimulated S2 cells showing a twofold reduction of their expression upon knockdown of *relish* or *akirin* compared to control (dsRNA against *GFP*). Numbers in brackets correspond to genes with GO terms matching immune function. Red corresponds to anti-microbial peptides, blue to negative regulators of NF-κB pathways, and beige to other immune-related functions.

C Quantitative RT–PCR of *Pirk*, *Attacin-D*, *Attacin-C*, and *Diptericin-A* mRNA from sorted *Drosophila* S2 cells co-transfected with dsRNA against *GFP*, *relish* or *akirin*, and a PGRP-LCa overexpressing vector to stimulate the IMD pathway.

Data information: Data are represented as mean ± standard deviation of three independent experiments performed with 1–5 × 10^5 S2 cells. **P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001.

required for activation of a subset of Relish target genes mainly coding for IMD pathway effectors (AMPs) with anti-bacterial properties, whereas negative regulators of the pathway are mostly independent of Akirin.

The Brahma complex member BAP60 interacts with Akirin

To identify the molecular partners that might account for the mode of action of Akirin, we undertook a yeast two-hybrid screen. We screened a *Drosophila* embryonic cDNA library using as baits a construct corresponding to the full-length Akirin (AK) or to the highly conserved C-terminal part of the protein encompassing residues 140–201 ($AK^{\Delta 1-139}$), suspected to be important for Akirin function (Macqueen & Johnston, 2009) (Supplementary Fig S2A). These baits were not toxic for yeast and unable to drive expression of the *HIS3* reporter (Supplementary Fig S2B). Out of 200 million clones, we isolated 211 cDNAs corresponding to 38 proteins, 10 of which interacted with AK, 22 with the truncated form $AK^{\Delta 1-139}$ only, and 6 with both (Fig 2A). Unexpectedly, we observed an increased number of protein interactions with $AK^{\Delta 1-139}$ compared to AK, indicating that the N-terminal part of Akirin restricts protein binding to Akirin. We also found that Akirin was able to interact with itself.

We used *Drosophila* S2 cells to verify these 38 proteins as genuine partners of Akirin. First, we transfected S2 cells with *attacin-A-luciferase*, a reporter of the IMD pathway known to be strongly induced upon immune challenge with heat-killed *Escherichia coli* (HKE) (Tauszig *et al*, 2000). *Drosophila kenny* (*key*) is essential for IMD pathway activation (Rutschmann *et al*, 2000) and is a homolog of mammalian IKKγ. Addition of dsRNA targeting either *key* or *akirin* to the culture medium strongly reduced *attacin-A-luciferase* expression, compared to control (*GFP*) dsRNA knockdown (Fig 2B). We then evaluated the ability of dsRNAs targeting individually each of the 38 putative Akirin partners to interfere with *attacin-A-luciferase* expression in response to HKE (Supplementary Fig S2C). Using this method, we showed that 30 putative partners of Akirin were not involved in IMD pathway activation; by contrast, we found that the knockdown of *bx42*, *CG2662*, *CG15876*, *CG33229*, *CG6357*, or *kpn-α3* resulted in a significant increased *attacin-A-luciferase* response to HKE (Fig 2B). Neither the unconfirmed nor the negative regulators of the IMD pathway were analyzed further. However,

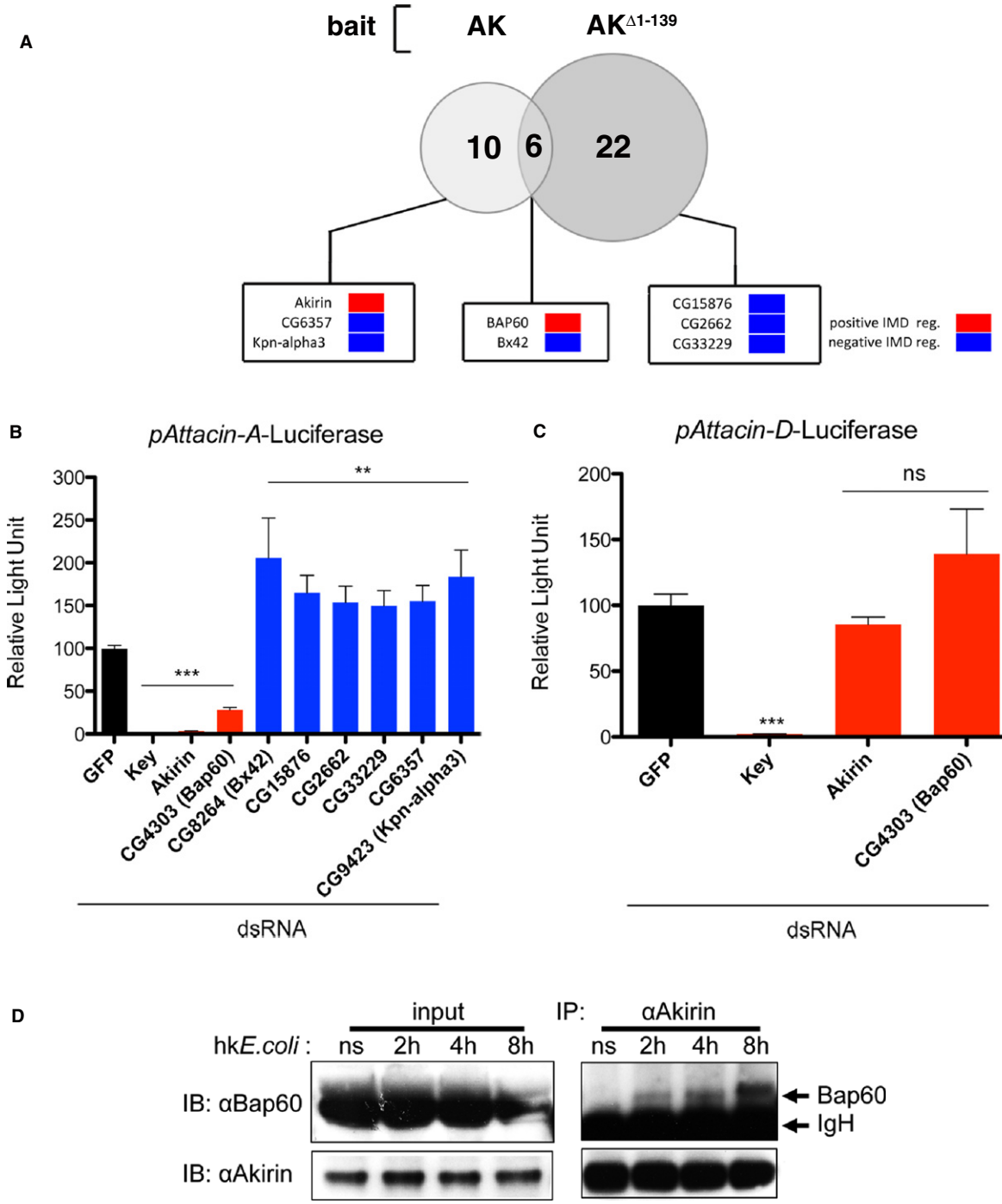


Figure 2. Akirin interacts with Bap60 upon immune challenge and activates selected Relish target gene promoters.

A Schematic representation of two-hybrid results and subsequent functional assay. Proteins interacting with full-length (AK) or N-terminally truncated ($AK^{\Delta 1-139}$) Akirin in two-hybrid assay. Proteins interacting with bait constructs encompassing full-length, or $AK^{\Delta 1-139}$ were tested for their ability to modulate the IMD pathway. Genes leading to increased IMD pathway activation when knocked down were identified as IMD negative regulators. Genes leading to decreased IMD pathway activation when knocked down were identified as positive IMD regulators.

B, C Dual luciferase assay from S2 cell co-transfected with *attacin-A-* (B) or *attacin-D-luciferase* (C) reporter plasmids and dsRNAs targeting *GFP*, *kenny* (*key*), *akirin*, and Akirin's putative partners extracts following 48 h of heat-killed *E. coli* stimulation. Data, normalized to dsRNA *GFP* controls, were from three independent experiments performed with 5×10^5 S2 cells.

D Whole-cell lysates from S2 cells stimulated with heat-killed *E. coli* at indicated time points were immunoprecipitated with anti-Bap60 or anti-Akirin antibodies. Whole-cell lysate (input, left panel) and immunoprecipitated samples (right panel) were immunoblotted and probed with antibodies against Bap60 and Akirin.

Data information: Data are represented as mean ± standard deviation from three independent experiments. *P-value < 0.05; **P-value < 0.01; ***P-value < 0.001.

attenuation of *bap60*, or *akirin*, significantly reduced *attacin-A-luciferase* expression upon HKE stimulation (Fig 2B). Thus, BAP60, a core member of the *Drosophila* Brahma SWI/SNF-like ATP-dependent chromatin-remodeling complex (Moller *et al*, 2005), is a new positive regulator of the IMD pathway.

As *attacin-D* expression required Relish, but not Akirin, we constructed an *attacin-D-luciferase* reporter, which was strongly expressed upon HKE stimulation in S2 cells (Fig 2C). Addition to the culture medium of dsRNA targeting *kenny* (*key*), but not *akirin*, strongly reduced *attacin-D-luciferase* expression when compared to control (Fig 2C), indicating that the *attacin-D-luciferase* reporter recapitulated the behavior of endogenous *attacin-D* (Fig 1C). Similarly to *akirin* knockdown, silencing of *bap60* did not affect challenge-induced expression of the *attacin-D-luciferase* reporter when compared to control (dsGFP, Fig 2C), suggesting that BAP60 and Akirin cooperate to regulate the transcription of a subset of Relish target genes, including *attacin-A* but excluding *attacin-D*.

To explore the ability of BAP60 and Akirin to physically interact, we performed immunoprecipitation (IP) experiments in S2 cells. Protein extracts from cells transfected with tagged versions of BAP60 (BAP60-Flag) and Akirin (Akirin-V5) were immunoprecipitated with an anti-Flag antibody. The corresponding blot, revealed with an anti-V5 antibody, indicated that Akirin associated with BAP60 (Supplementary Fig S2D). We then immunoprecipitated endogenous Akirin from *Drosophila* S2 cells using an anti-Akirin polyclonal antibody (Supplementary Fig S2E). We indeed detected BAP60 on blots from samples prepared 2, 4, and 8 h after challenge with HKE (Fig 2D), but we could never detect a robust endogenous interaction between Akirin and BAP60 at early time points (Supplementary Fig S3) or in the absence of an immune stimulation (Fig 2D).

Immunolocalization, both in S2 cell culture (Supplementary Fig S4) and in adult *Drosophila* (Fig 3A–J and Supplementary Fig S5), showed that Akirin is ubiquitous, thus confirming the microarray data in Flybase (Crosby *et al*, 2007). We found that endogenous Akirin is strictly localized in the nucleus (Fig 3A–J and Supplementary Figs S4 and S5) as inferred from previous overexpression experiments (Goto *et al*, 2008). Interestingly, Akirin seems excluded from heterochromatic and transcriptionally inert regions, labeled by DAPI or an anti-H3K9 di-methyl (H3K9me2) antibody (Fig 3A–J and Supplementary Fig S4, see arrowhead in Fig 3E). However, as we observed a small overlap between heterochromatin and Akirin labeling (see arrow in Fig 3E), we cannot exclude that Akirin may also be involved in gene repression. In contrast, Akirin distribution within the nucleus matched H3S10 phosphorylation (H3S10p) and partially H3K9 acetylation marks (H3K9ac) (see Fig 3J and Supplementary Figs S4 and S6), indicating a pre-eminent role in active gene transcription. The NF-κB factor Relish is a 110-kD protein localized in the cytoplasm, cleaved upon immune challenge into 49 kD (Rel-49) and 68 kD (Rel-68) peptide, the latter being relocated to the nucleus and activating gene transcription (Stoven *et al*, 2000). Rel-68 as well as BAP60 sub-nuclear distributions (Fig 3K–T and Supplementary Fig S4) partially overlapped H3K9ac labeling and was excluded from heterochromatic regions (Fig 3U–D′ and Supplementary Fig S4). Collectively, these results suggested a dynamic contribution of Akirin and BAP60 to the Brahma complex during immune challenge.

Akirin, Relish, and the Brahma complex are recruited to the vicinity of IMD target genes

Although Akirin has been shown to function downstream, or at the level of, the NF-κB transcription factor Relish, yeast two-hybrid assays, failed to identify their interaction. Using S2 cells transiently transfected with V5-tagged Akirin and a Flag-tagged constitutively active form of Relish (RelishΔS29-S45; Stoven *et al*, 2003), we immunoprecipitated Akirin with RelishΔS29-S45 (Fig 4) and reciprocally. In addition, we established a stable S2 cell line that expressed V5-tagged Akirin under the control of the copper-inducible metallothionein promoter and immunoprecipitated V5-tagged Akirin from the lysate of these cells. We detected a faint band corresponding to endogenous Rel-68 in the blot of the Akirin precipitate, but the association between Rel-68 and Akirin was significantly enhanced upon HKE stimulation (Fig 4C). Additionally, we could immunoprecipitate *in vitro* His-tagged Akirin prepared in bacteria with Flag-tagged Relish ΔS29–S45 purified from S2 cells suggesting their direct interaction (Supplementary Fig S7). Taken together, these data indicate that the interaction between Akirin and the NF-κB factor Relish depends on immune challenge.

A recent large-scale screen to isolate new interacting partners of IMD pathway core members identified the SWI/SNF Brahma complex BAP55 subunit as a putative partner of dIAP2, dTAK1, and IMD suggesting an involvement of BAP55 in the direct regulation of the IMD pathway (Fukuyama *et al*, 2013). We immunoprecipitated Flag-tagged BAP55 from transfected S2 cells and observed an interaction between Flag-BAP55 and Akirin-V5 (Supplementary Fig S2D), suggesting that the recruitment of the Brahma complex onto Akirin-dependent promoters is not triggered by a direct physical interaction with Relish.

To determine if Akirin and the Brahma (SWI/SNF) remodeling complex were physically present on the promoter of Relish target genes, we immunoprecipitated sheared cross-linked chromatin prepared from *Drosophila* S2 cells stimulated by HKE at different time points, using anti-Relish, anti-phospho-serine 5 of the RNA Pol II carboxy-terminal domain (CTD), anti-Akirin, or anti-BAP60 antibodies (Fig 5A–F and Supplementary Fig S8). Chromatin IP (ChIP) of initiating RNA Pol II (anti-RNA Pol II S5p) (Corden, 1990) showed that Pol II was gradually recruited on *attacin-A* and *attacin-D* promoter sites (Supplementary Fig S8A). Additional ChIP experiments demonstrated that Relish, Akirin, and BAP60 were recruited simultaneously to the same site on Akirin-dependent proximal promoters (*p-attacin-A*, *p-drosocin*, *p-cecropin-A1*) following immune challenge (Fig 5A, C and D). In contrast, we found that Relish, but not BAP60 or Akirin, was recruited to the promoter of Akirin-independent proximal promoters (*p-attacin-D*, *p-metchnikowin*) upon HKE stimulation (Fig 5E and F). None or weak recruitment of Relish, Akirin, or Bap60 was observed on the *attacin-A* coding sequence or on the immune-unrelated hunchback promoter (Fig 5B and Supplementary Fig S8B). We found also that H3K4ac, an epigenetic mark of active gene transcription (Guillemette *et al*, 2011), was selectively enriched on Akirin-dependent, but not on Akirin-independent promoters (Fig 5A–F). Most importantly, during an immune challenge, the removal of either Akirin or Bap60 impaired the recruitment of Relish to Akirin-dependent promoter, preventing both H3K4 acetylation (Fig 5G–J) and subsequent gene transcription. Collectively, these data demonstrate that the presence of Akirin,

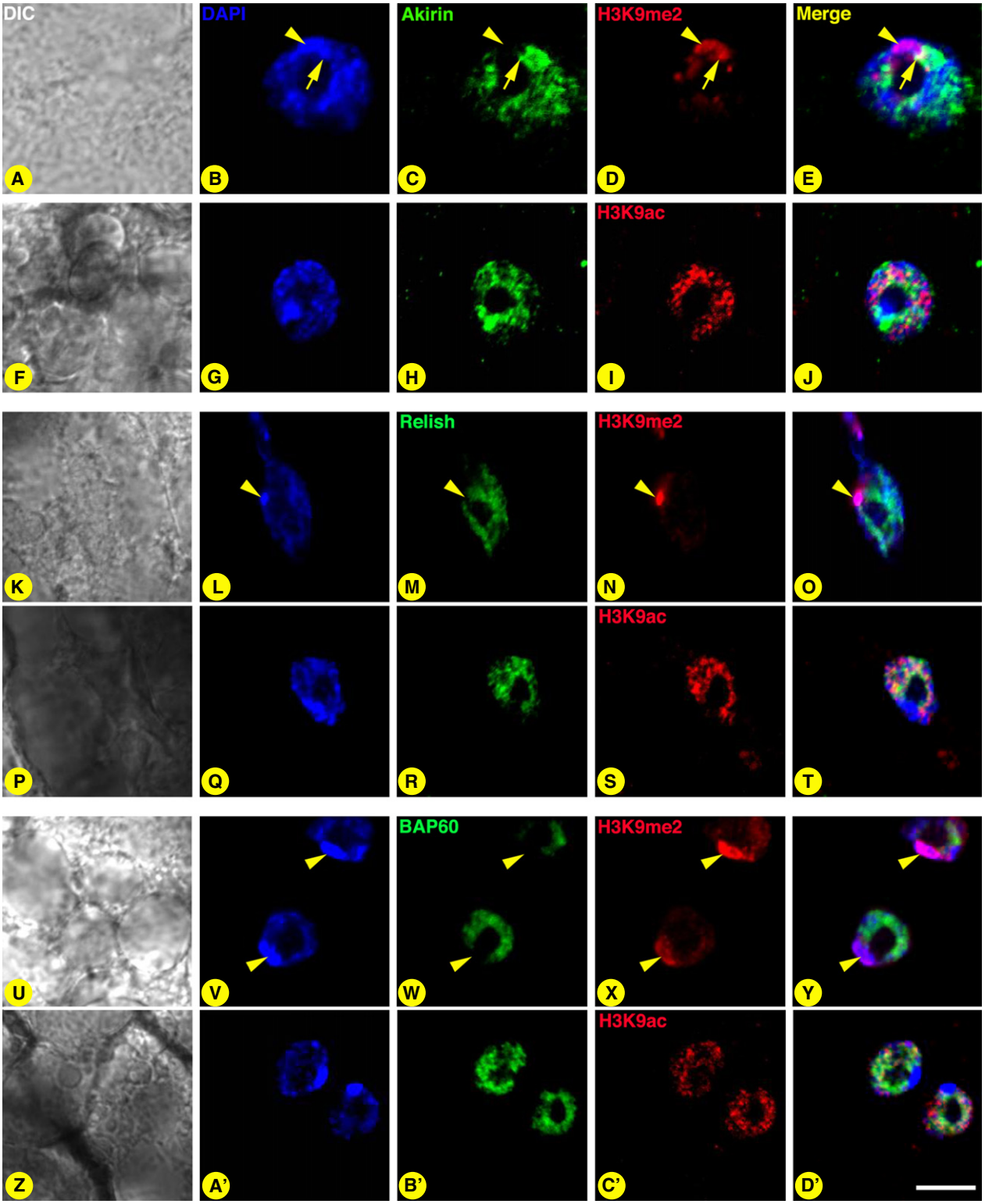


Figure 3. Akirin, Relish, and Bap60 overlap non-condensed DNA regions in fat body cells.
(A-D') Fat body cells from adult *Drosophila* were visualized by DIC (A, F, K, P, U, Z). Immunolocalization of Akirin (C and H), Relish (M and R), Bap60 (W and B'), H3K9me2 (D, N, X), the active chromatin marker H3K9ac (I, S, C'), and DAPI staining (B, G, I, Q, V, A') in whole fat body, 6 h after an immune challenge with *E. coli*. Akirin, Relish, and Bap60 sub-nuclear localizations were mostly excluded from DAPI-rich regions but partially overlapped H3K9ac regions (arrowheads) (E, J, O, T, Y, D'). In addition, Akirin systematically overlapped a small region in H3K9me2 distribution (arrows).

Data information: Images are representative of at least 3 fat body samples. Scale bars (all panels): 5 μm.

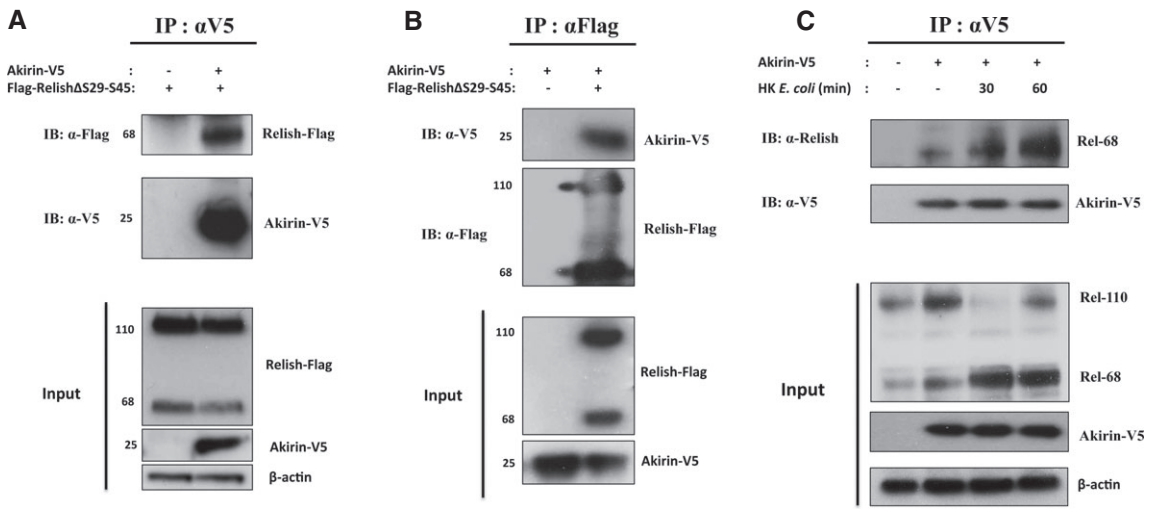


Figure 4. Heat-killed *E. coli* challenge stabilizes the interaction between Akirin and Relish.

A, B Reciprocal co-immunoprecipitation assays between ectopic Akirin and Relish in S2 cells. Wild-type S2 cells were transiently transfected with V5-tagged Akirin and Flag-tagged RelishΔS29-S45. Cell lysates were immunoprecipitated with (A) anti-FLAG coupled or (B) anti-V5 coupled agarose beads. Immunoprecipitates were analyzed by Western blotting with anti-V5 or anti-Flag antibodies.

C Heat-killed *E. coli* (HKE) promote the interaction of Akirin with Rel-68. S2 cells stably expressing V5-tagged Akirin were treated with heat-killed *E. coli* at the indicated time points. Cell lysates were immunoprecipitated using anti-V5 coupled agarose beads. Endogenous Relish was detected in Akirin immunoprecipitates using anti-Relish antibody.

Data information: Data are representative of 3 independent experiments.

BAP60, and Relish is required at the same level of the proximal promoter for an efficient transcription of Akirin-dependent genes.

Promoter regions of Akirin-dependent genes

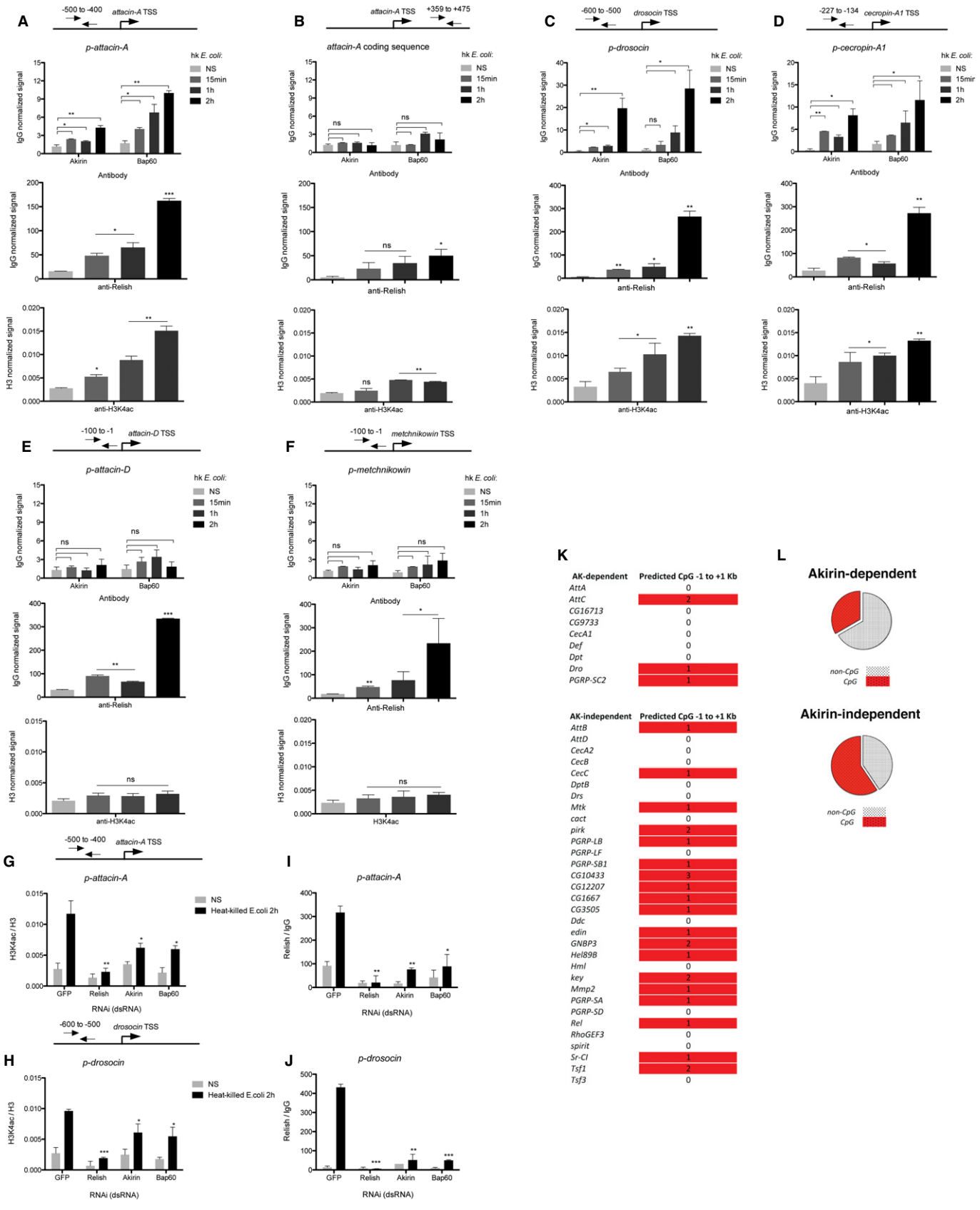
To understand the bases of Akirin specificity, we used bioinformatics to compare Akirin-dependent and Akirin-independent promoters. First, we evaluated if specific transcription factors would account for this specificity. Using MatInspector (http://www.genomatix.de/), we compared the DNA sequences of *attacin-A* and *attacin-D* promoters and identified the transcription factor binding sites specific for *attacin-A* and absent on the *attacin-D* promoter (Supplementary Fig S9A). The knockdown of these transcription factors by RNAi in S2 cells did not decrease *attacin-A-luciferase* induction upon immune challenge (Supplementary Fig S9B), ruling out a possible role for these transcription factors in Akirin-dependent transcription.

In mammalian cells, SWI/SNF-mediated nucleosome remodeling has been described to be required for the activation of immune gene promoters without CpG islands. In opposition, promoters located within CpG islands are frequently activated in a SWI/SNF-independent manner (Hargreaves *et al*, 2009; Ramirez-Carrozzi *et al*, 2009). The group of Prof. Osamu Takeuchi found that mammalian Akirin-2 interacts with the SWI/SNF complex upon immune challenge and activates preferentially target genes with low CpG content. Conversely, mammalian Akirin-2-independent genes were enriched in CpG islands (Tartey *et al*, 2014). Along the same line, we used a bioinformatics prediction tool (Cpgplot; EMBOSS); we listed the CpG-rich regions predicted to be present in the vicinity (−1 kb to +1 kb) of the *Drosophila* IMD-dependent transcription start sites (Fig 5K). We observed that most Akirin-independent promoters were CpG-enriched (Fig 5L) and that Akirin-dependent promoters displayed a low CpG content, suggesting that CpG-rich regions in *Drosophila* would somehow mimic mammalian CpG islands.

The BAP complex fine-tunes the IMD-dependent innate immune response in *Drosophila*

As in human and yeast, distinct SWI/SNF-type ATP-dependent chromatin remodelers target two non-overlapping sets of genes in *Drosophila*, namely the BAP and the PBAP complexes (Wang, 2003; Mohrmann *et al*, 2004). In the fly, we find that RNAi-mediated silencing of the Osa-associated BAP complex genes, *bap55*, *bap60*, *brm*, *moira*, *osa*, *and snr-1*, reduced *attacin-A-luciferase* expression after HKE treatment to levels similar to those observed following *kenny* or *akirin* knockdown (Fig 6A). In contrast, with respect to the Polybromo-associated PBAP complex, we find that *polybromo* knockdown led to a significant increase of reporter expression. Importantly, we show that neither the BAP nor the PBAP complex was required for *attacin-D-luciferase* expression upon HKE treatment in S2 cells (Fig 6B). Collectively, these results established that the BAP complex, but not PBAP, was required for activation of the Akirin-dependent subset of Relish target genes.

Drosophila S2 cells, transfected with a constitutively active Toll receptor ($Toll^{\Delta LRR}$), showed strong activation of a *drosomycin-luciferase* reporter, fully blocked by the knockdown of *myd88*, a critical Toll receptor adaptor (Fig 6C) (Tauszig *et al*, 2000) (Tauszig-Delamasure *et al*, 2002). These basal levels of *drosomycin-luciferase* reporter expression were strongly enhanced upon Brahma complex component knockdown (*bap55*, *bap60*, *brahma*, *moira*, *osa*, *snr-1*, or *polybromo*), demonstrating that the SWI/SNF complex negatively regulated expression of Toll pathway target genes in *Drosophila* S2 cells as previously observed (Kuttenkeuler *et al*, 2010).



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AK-dependent	Predicted CpG -1 to +1 Kb
AttA	0
AttC	2
CG16713	0
CG9733	0
CecA1	0
Def	0
Dpt	0
Dro	1
PGRP-SC2	1

AK-independent	Predicted CpG -1 to +1 Kb
AttB	1
AttD	0
CecA2	0
CecB	0
CecC	1
DptB	0
Drs	0
Mtk	1
cact	0
pirk	2
PGRP-LB	1
PGRP-LF	0
PGRP-SB1	1
CG10433	3
CG12207	1
CG1667	1
CG3505	1
Ddc	0
edin	1
GNBP3	2
Hel89B	1
Hml	0
key	2
Mmp2	1
PGRP-SA	1
PGRP-SD	0
Rel	1
RhoGEF3	0
spirit	0
Sr-CI	1
Tsf1	2
Tsf3	0

L

Figure 5. Akirin, Bap60, and Relish bind on Akirin-dependent immune gene promoters.

A–F Chromatin IP with anti-Akirin, anti-Bap60, anti-Relish, and anti-H3K4ac antibodies on sheared chromatin from S2 cells following heat-killed *E. coli* stimulation at indicated time points. The graphs show recruitment of Akirin, Bap60, and Relish, relative to the values obtained with rabbit control IgG, or of H3K4ac relative to the values obtained with anti-H3 antibody on Akirin-dependent (A, *p-attacin-A*; C, *p-drosocin*; D, *p-cecropin-A1*), Akirin-independent (E, *p-attacin-D*; F, *p-metchnikowin*) genes proximal promoter, or on *attacin-A* coding sequence (B) as an internal control.

G–J Chromatin IP with anti-Relish and anti-H3K4ac antibodies on sheared chromatin from S2 cells knocked down for *GFP*, *relish*, *akirin*, or *bap60* following heat-killed *E. coli* stimulation at indicated time points. The graphs show recruitment of Relish (I, J) relative to the values obtained with rabbit control IgG, or of H3K4ac (G, H) relative to the values obtained with anti-H3 antibody on two Akirin-dependent (G, I, *p-attacin-A*; H, J, *p-drosocin*) proximal promoters.

K Bioinformatical CpG-rich region analysis of Akirin-dependent and Akirin-independent promoters. Predicted CpG-rich regions were counted on the genomic regions −1 kb to +1 kb relative to the transcription start site for Akirin-dependent and Akirin-independent genes with Cpgplot (EMBOSS). Red squares annotate genes containing at least one CpG-rich region within its promoter.

L Pie chart representation of CpG-rich region analysis of Akirin-dependent and Akirin-independent promoters. Red areas annotate genes containing at least one CpG-rich region within its promoter.

Data information: Data are represented as mean ± standard deviation of three independent experiments performed on 1.5×10^6 (A–F) or 5×10^5 cells (G–J) per IP. Hk *E. coli*: heat-killed *E. coli*. TSS: transcriptional start site. Statistical significance was established by comparing values from stimulated (15 min, 1 h, 2 h of hk *E. coli*) with unstimulated conditions (NS) (A-F) or comparing Relish, Akirin, and Bap60 knockdown with GFP dsRNA control in stimulated (2 h hk *E. coli*) conditions (G-J). **P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001.

We next investigated if Akirin and the Brahma complex were similarly required for transcriptional selectivity *in vivo*. As *Drosophila* embryonic development is impaired in absence of Akirin or functional Brahma complex, we used the *C564-Gal4* (Hrdlicka *et al*, 2002) or *Hml-Gal4* (Goto *et al*, 2001) transgenes to express RNAi constructs targeting *akirin*, *brahma*, *moira*, *relish*, and *polybromo*, respectively, in the adult fat body (Supplementary Fig S10A) or in larval hemocytes (Supplementary Fig S11A and B). Of note, even restricted to the fat body, the knockdown of *osa* was lethal to the flies. Following *E. coli* immune challenge, expression of *Attacin-A*, *Attacin-C*, and *Diptericin-A* was significantly reduced in the absence of Akirin or a functional Brahma complex, when compared to *Attacin-D*, *Cecropin-A2*, *Cecropin-B*, and *Pirk* or control (*RNAi-GFP*, Fig 6D–I, Supplementary Fig S11C and D). However, all these IMD pathway effector genes were dependent on Relish, but independent of Polybromo (Fig 6D–I).

Flies depleted of Akirin (*C564 > RNAi-akirin*), Relish (*C564 > RNAi-relish*), or members of the Brahma complex (*C564 > RNAi-brahma* or *C564 > RNAi-moira*) had a significant decrease in survival following *Enterobacter cloacae (E. cloacae)* (Bou Aoun *et al*, 2011) or *Erwinia carotovora* Ecc15 (*E. carotovora* Ecc15) (Vidal *et al*, 2001) infections when compared to control flies (*C564 > RNAi-GFP*) or flies lacking a functional PBAP complex (*C564 > RNAi-polybromo*) (Fig 6J, K, M and N). Flies carrying a single functional copy of *relish* and *brahma* or *relish* and *moira* also showed a significant decrease in survival after *E. carotovora* Ecc15 infection (Fig 6L and O). In addition, flies lacking Akirin, Relish, or components of the Brahma complex were not susceptible to the entomopathogenic fungus *Beauveria bassiana* (*B. bassiana*), a classical agonist of the Toll pathway (Supplementary Fig S10C–E) (Lemaitre *et al*, 1997). Taken together, our results demonstrate that Akirin and the BAP complex dynamically interact to selectively activate a subset of Relish target genes during the immune response, allowing *Drosophila* to survive a Gram-negative bacterial challenge.

Discussion

The IMD pathway in *Drosophila* regulates the systemic immune response against Gram-negative bacteria, and the molecular cascade from the PGRP-LC receptor down to the activation of the NF-κB factor Relish has been extensively studied. The Akirin molecule is required for IMD target gene activation by the Relish transcription factor (Goto *et al*, 2008), and this finding suggests that IMD effector gene transcription might depend on additional factors that remained to be identified. In order to further elucidate NF-κB-dependent gene activation, we re-explore the IMD pathway using Akirin as a starting point. We undertook an unbiased two-hybrid screen that identified BAP60 as an Akirin transcriptional partner during the innate immune response, confirming the data of the protein-interaction map of the fly proteome (Giot *et al*, 2003). Additionally, we show that BAP55, an Actin-related component of the SWI/SNF Brahma complex (Papoulas *et al*, 1998; Armstrong *et al*, 2002), engages Akirin upon immune challenge, as does the NF-κB factor Relish itself.

BAP60 is a core component of the SWI/SNF-like BAP complex, conferring site-specific anchoring properties at specific promoter sites, via direct binding to transcription factors such as SisterlessA or Scute (Moller *et al*, 2005). Although BAP60, Relish and Akirin are part of the same complex (Figs 2 and 4, and Supplementary Figs S2D and S3), we could detect a direct interaction between Akirin and BAP60, probably between Akirin and Relish, but not between BAP60 and Relish. We speculate that Akirin might act as a bridge between Relish and BAP60 in order to recruit the SWI/SNF complex to the vicinity of Relish target genes. Alternatively, we cannot exclude that Akirin and the SWI/SNF complex are recruited on the promoter of Relish target genes independently of Relish itself. Consistent with this SWI/SNF-dependent chromatin remodeling process, it was recently suggested that DNA-methyltransferase associated protein 1 (DMAP1), also known to interact with BAP55 (Guruharsha *et al*, 2012), would associate with Akirin (Goto *et al*, 2014). The possibility that methyl groups on H3K4 are replaced by acetyl groups to allow full transcription would fit with our finding that H3K4ac is a hallmark of active Akirin-dependent promoters. It has been shown that Akirin links BAP60 to the transcription factor Twist during *Drosophila* myogenesis (Nowak *et al*, 2012). Thus, Akirin might act as a molecular bridge between BAP60 and several other transcription factors. Notably, this interaction between BAP60 and Akirin is conserved during evolution as mouse Akirin-2 binds all three BAF60s, the mammalian homologs of *Drosophila* BAP60 (Prof. Osamu Takeuchi personal communication).

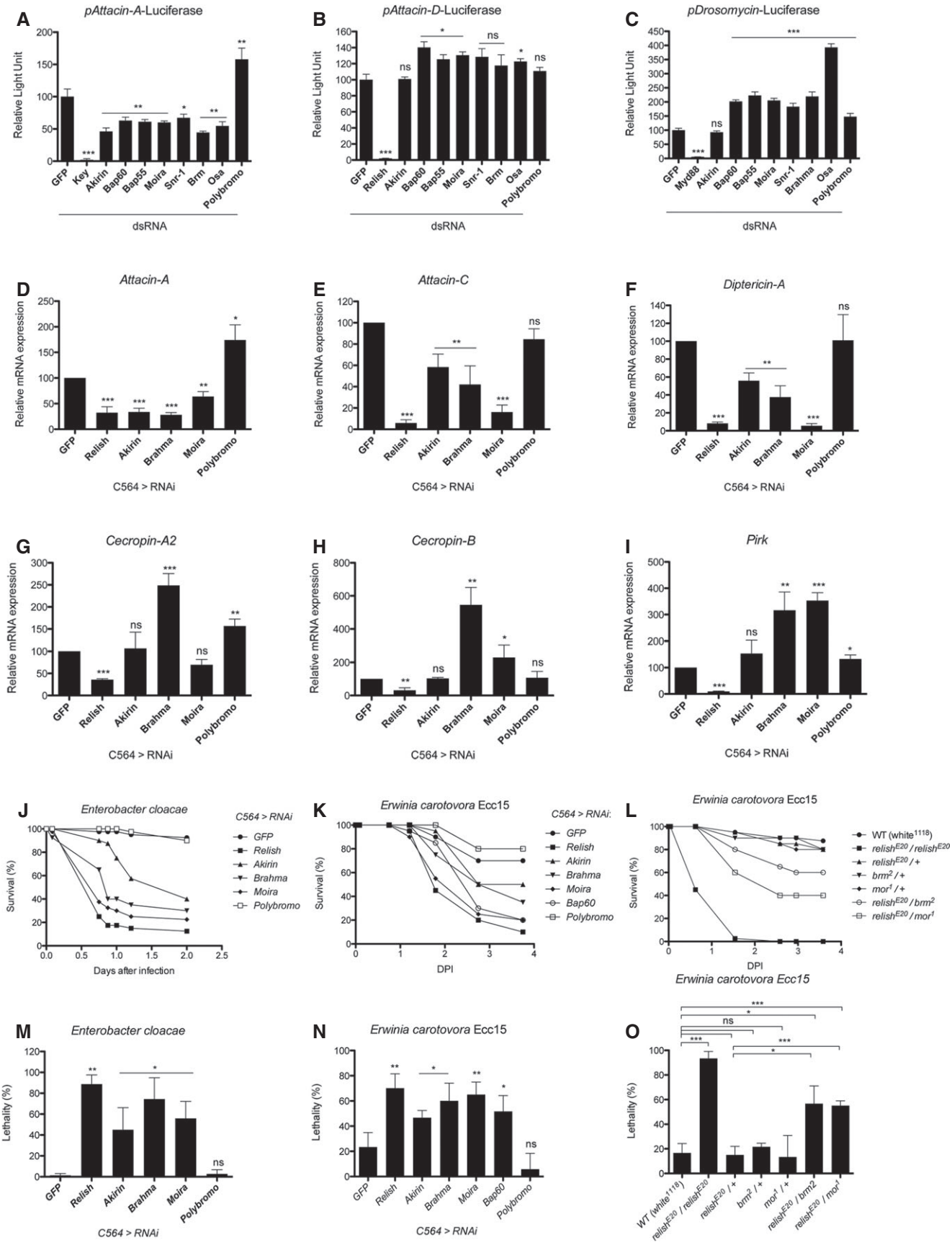

Figure 6. The Brahma BAP complex is required for Akirin-dependent immune response against Gram-negative bacteria.

A, B Dual luciferase assay from S2 cell extracts co-transfected with *attacin-A*- (A) or *attacin-D-luciferase* (B) reporter plasmids and dsRNAs against *GFP*, *kenny* (*key*), *akirin*, or Brahma complex members following 48 h of heat-killed *E. coli* stimulation.

C Dual luciferase assay from S2 cell extracts co-transfected with *drosomycin-luciferase*, Toll$^{\Delta LRR}$ *pActin5C* expressing vector, and dsRNAs against *GFP*, *kenny* (*key*), *akirin*, or Brahma complex members. Results were normalized to the dsRNA GFP controls.

D–I Quantitative RT–PCR of *Attacin-A*, *Attacin-C*, *Diptericin-A*, *Cecropin-A2*, *Cecropin-B*, and *Pirk* mRNA on *C564-gal4/UAS-RNAi* flies following an *E. coli* 6-h challenge.

J, K Survival assays following *E. cloacae* (J) or *E. carotovora* Ecc15 (K) septic infection of *C564-gal4/UAS-RNAi* flies.

L Survival assay from *E. carotovora* Ecc15 septic infection of *relish*^{E20}, *brahma*^{2}, and *moira*^{1} heterozygous or trans-heterozygous mutant flies.

M, N Lethality calculations following *E. cloacae* (M) or *E. carotovora* Ecc15 (N) septic infection of *C564-gal4/UAS-RNAi* flies.

O Lethality calculation from *E. carotovora* Ecc15 septic infection of *relish*^{E20}, *brahma*^{2}, and *moira*^{1} heterozygous or trans-heterozygous mutant flies.

Data information: Data are represented as mean ± standard deviation of three independent experiments performed with three batches of 15–20 flies. **P*-value < 0.05; ***P*-value < 0.01; ****P*-value < 0.001.

Unlike the Polybromo/BAP170 containing SWI/SNF complex (PBAP), the BAP complex is required during the immune response against Gram-negative bacterial infections, to coordinate the transcription of IMD pathway effector genes. In contrast, during embryonic myogenesis, Akirin interacts genetically with both the BAP and PBAP complexes (Nowak *et al*, 2012). In addition, both the PBAB and BAP complexes are involved in the negative regulation of the Toll pathway (Fig 6C), suggesting that the specificity of Akirin toward BAP, or PBAP, is transcription-factor dependent.

In murine macrophages depleted of functional SWI/SNF complexes, LPS stimulation results in the activation of only a subset of TLR4 target genes (Ramirez-Carrozzi *et al*, 2006). This SWI/SNF-based selectivity was recently suggested to be dependent on the differential CpG island context of NF-κB target gene promoters. Absence of CpG island results in stable nucleosome assembly at promoter sites, requiring both chromatin remodeling and transcription factors to activate gene transcription. In contrast, CpG islands appear to be responsible for unstable nucleosome assembly at promoter sites, thus explaining their SWI/SNF independence (Ramirez-Carrozzi *et al*, 2009). The genome of *D. melanogaster* is unmethylated and lacks classical CpG islands (Deaton & Bird, 2011). Even though *Drosophila* does not display CpG islands or methylation (Nanty *et al*, 2011), we undertook a bioinformatic analysis (EMBOSS, CpG plot) and identified an enrichment of the CpG content in the sequences spanning the NF-κB target genes that are independent of Akirin and the SWI/SNF complex. In contrast, the promoters of Akirin and SWI/SNF-dependent genes are depleted of CpG-rich regions. However, these data cannot be generalized as we have only analyzed immune genes. Work from our collaborators (Prof. Osamu Takeuchi personal communication) suggests similarly that mouse Akirin-2-dependent gene promoters show a low frequency of CpG island association compared to Akirin-2-independent promoters. It is tempting to speculate that, like CpG islands in vertebrates, CpG-rich sequences in *Drosophila* would establish regions of nucleosomal instability precluding any need of Akirin and the SWI/SNF complex for the control of gene transcription. However, additional factors such as H3K4ac marks must account for the observed Akirin selectivity.

In *Drosophila*, exposure to microbial cell wall proteoglycans or danger signals leads to the activation of the IMD or Toll pathways resulting in the nuclear translocation of their respective NF-κB factors and activation of the transcription of target genes (Ferrandon *et al*, 2007). These effector genes encode not only AMPs, but also molecules that feed back to regulate these pathways and dampen their response. Similarly to mammals, activation and resolution of the *Drosophila* innate immune response have to be tightly controlled in order to prevent adverse side effects (Ryu *et al*, 2008; Paredes *et al*, 2011; Bonnay *et al*, 2013). Here, we have identified Akirin as an NF-κB co-factor required for the selective transcription of a subset of direct immune effectors, that is AMPs, but dispensable for the expression of genes encoding negative regulators of the IMD pathway (except *PGRP-SC2*).

Removing Akirin or Brahma lead to an impaired expression of several antimicrobial peptide-coding genes, resulting in a weakened innate immune defense of *Drosophila* against Gram-negative bacteria. This observation suggests that the full cocktail of IMD-induced anti-microbial peptides is required to efficiently contend Gram-negative bacterial infections. The evolutionary reason why two distinct groups of AMPs coding genes, sharing similar bactericidal features, are under the transcriptional control of either Relish alone or in combination with Akirin is still an open question. As mammalian Akirin-2 similarly displays pro-inflammatory properties (Prof. Osamu Takeuchi personal communication and (Goto *et al*, 2008), Akirins represent putative therapeutic targets for small chemicals able to block the inflammatory response without interfering with the expression of genes involved in the resolution of inflammation.

Materials and Methods

Fly strains

Stocks were raised on standard cornmeal–yeast–agar medium at 25°C with 60% humidity. *w*^{1118} mutant flies were used as control. *relish*^{E20} (Hedengren *et al*, 1999) and *Myd88*^{c03881} (Tauszig-Delamasure *et al*, 2002) flies were used as mutant deficient for the IMD and Toll pathway, respectively. Flies carrying an *UAS-RNAi* transgene targeting *relish* (108469), *akirin* (109671), *brahma* (37720), *moira* (6969), *bap60* (12675) *osa* (7810), and *polybromo* (108418) were obtained from the Vienna Drosophila RNAi Center (http://stockcenter.vdrc.at/control/main). Flies carrying a *UAS-RNAi* transgene against GFP (397-05) were obtained from the Drosophila Genetic Resource Center (Kyoto, Japan; http://www.dgrc.kit.ac.jp/index.html). *moira*^{1} (3615) and *brahma*^{2} (3622) mutants and flies carrying Gal4 driver *C564* (6982) used to express *UAS* constructs in the fat body (Hrdlicka *et al*, 2002) were obtained from Bloomington Drosophila Stock Center (Bloomington, USA; http://flystocks.bio.indiana.edu/). Gal4-driven *RNAi* expression was enhanced by incubating 3-day-old flies for six further days at 29°C.

Microbial strains and infections

We used *Escherichia coli* strain DH5αGFP, *Enterobacter cloacae*, *Erwinia carotovora* Ecc15, and *Micrococcus luteus* (CIPA270) bacteria for septic injuries (Reichhart *et al*, 2011). Natural *B. bassiana* infections were performed as previously described (Lemaitre *et al*, 1997). The *E. coli* strain DH5αGFP was generated in our laboratory. Bacteria were grown in Luria broth (LB) (*E. coli*, *E. cloacae*, *E. carotovora* Ecc15) or brain–heart infusion broth (BHB) (*M. luteus*) at 29°C (*E. cloacae*, *E. carotovora* Ecc15) or 37°C (*E. coli*, *M. luteus*). Survival experiments were performed on two batches of 15–20 nine-day-old females infected by *E. cloacae* or *E. carotovora* Ecc15 septic injury or *B. bassiana* natural infection at 25°C three independent times. Control survival experiments (Supplementary Fig S10B) were made by sterile injury (Reichhart *et al*, 2011). qRT–PCR experiments were performed on three batches of 10–20 nine-day-old males infected with *E. coli* for 6 h, or *M. luteus* for 24 h, by septic injury at 25°C, three times independently. Immunostaining experiments were performed on 3-day-old control (w^{1118}) females infected with *E. coli* for 6 h.

Cell sorting and microarray analysis

To perform microarray, 2×10^6 S2 cells (10^6/ml) were transfected in 6-well plates by calcium phosphate precipitation with 1 μg of *p-actin5C-tomato*, 1 μg of *p-actin5C-PGRP-LCa* (or empty *p-actin5C* vector), and 5 μg of dsRNAs against *GFP*, *relish*, or *akirin*. After 12–16 h, the cells were washed with PBS and incubated in fresh complete Schneider's medium for 48 h. Cells were rinsed with PBS and re-suspended in serum-free Schneider's *Drosophila* Medium (Biowest) before sorting. 10^5 to 5×10^5 transfected Tomato-positive S2 cells were sorted in serum-free medium with the help of to the flow cytometry facility at Institut de Génétique et de Biologie Moléculaire et Cellulaire (Illkirch, France; http://www.igbmc.fr/technologies/6/team/64/). RNA was extracted and treated with DNAse, using RNA Spin kit (Macherey Nagel). RNA quality was checked by Eukaryote Total RNA Pico assay (Agilent) and validated with a RIN > 6,5. 200 ng of RNA were used to perform microarray (Agilent DNA microarrays Drosophila) at the GeneCore Genomics facility of EMBL (http://genecore3.genecore.embl.de/genecore3/). Total RNA was quantified on Invitrogen Qubit 2.0 Fluorometer (Q32866) and quality-checked on the Agilent Bioanalyzer 2100 (G2940CA). Samples were normalized to 100 ng in 1.5 μl working volume for the labeling reaction and were one-color Cy3-labeled using Agilent LowInput QuickAmp Labeling Kit (5190-2331). The resulting Cy3-labeled cRNAs were then hybridized onto the 4x44k Drosophila V2 microarray using Agilent GeneExpression Hyb Kit (5188–5242) for 20 h at 65°C. The microarray was scanned using Agilent Microarray Scanner (G2565CA), and data extracted with Feature Extraction Software v10.7.2. *relish* and *akirin* were reported, respectively, as Relish and Akirin-dependent genes in our assay, validating their knockdown. The GEO accession number for the microarray data is GSE54915.

Two-hybrid

Two-hybrid screens and assays were carried out using a LexA-based system (Vojtek *et al*, 1993) and yeast strains L40ΔGAL4 (Fromont-Racine *et al*, 1997) (kind gift of Drs. P. Legrain and M. Fromont-Racine) and Y187 (Clontech). A 0–24 h *Drosophila* embryo cDNA library was a generous gift of Dr. S. Elledge. Standard yeast handling techniques were used.

Immunoprecipitation and Western blot

Cells were treated for the indicated times with heat-killed *E. coli* (40:1) at 25°C. The cells were harvested, washed in PBS, and lysed in 500 μl of buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 1 mM DTT, 1% NP-40, phosphatase inhibitor cocktail (Roche), and complete protease inhibitor cocktail (Roche). Immunoprecipitations were performed overnight at 4°C with rabbit polyclonal anti-Akirin antibody coupled with Dynabeads Protein G (Invitrogen), anti-V5 agarose (Sigma), or anti-Flag agarose (Sigma). Proteins from total cell lysates and immunoprecipitates were resolved by SDS-PAGE and detected by Western blotting using anti-V5 HRP (Invitrogen), anti-Akirin, anti-Flag HRP (Sigma), anti-Bap60 (gift from Susumu Hirose), anti-Relish (gift from Tony Ip), and anti-β-actin antibodies (BD Transduction Laboratories).

Chromatin immunoprecipitation

ChIP was carried out as previously described (Batsche *et al*, 2006). S2 cells were cross-linked in phosphate-buffered saline (PBS) containing 1% formaldehyde (Sigma) for 10 min at room temperature. The crosslinking reaction was quenched with PBS containing 125 mM glycine. The chromatin was fragmented by sonication to produce average DNA lengths of 0.5 kb. 2 μg of rabbit polyclonal anti-Akirin, anti-BAP60, anti-Relish, anti-H3 (Abcam, ab1791), anti-H3K4ac (Abcam, ab113672), anti-RNA Pol II CTD repeat YSPTSPS (phospho S5) (Abcam, ab5131), and rabbit control IgG (Abcam, ab46540) were used for IP. After ChIP, the eluted DNA was detected by quantitative PCR using the primers listed in Supplementary Table S1. Levels of Akirin, BAP60, and Relish are expressed relatively to the signal obtained for ChIP using rabbit control IgG. The level of H3K4ac is expressed relatively to the signal obtained for ChIP using anti-H3 antibody. Values are averaged from three independent experiments.

Bioinformatical analysis

Predicted CpG-rich regions were counted on the genomic regions −1 kb to +1 kb relative to the transcription start site for Akirin-dependent and Akirin-independent genes with Cpgplot (EMBOSS).

Predicted transcription factors' binding sites were analyzed with MatInspector (www.genomatix.de) from the proximal 1 kb sequence of *attacin-A* or *attacin-D* 5′-promoter.

More methods are available in the Supplementary Methods section.

Supplementary information for this article is available online: http://emboj.embopress.org

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Author contributions

FB, X-HN, J-MR, and NM designed research. FB, X-HN, EC-B, EB, and NM performed research. FB, XHN, EC-B, EB, LT, JC, J-MR, and NM analyzed data. OT shared data; J-MR and NM wrote the paper.

Conflict of interest

The authors declare that they have no conflict of interest.

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