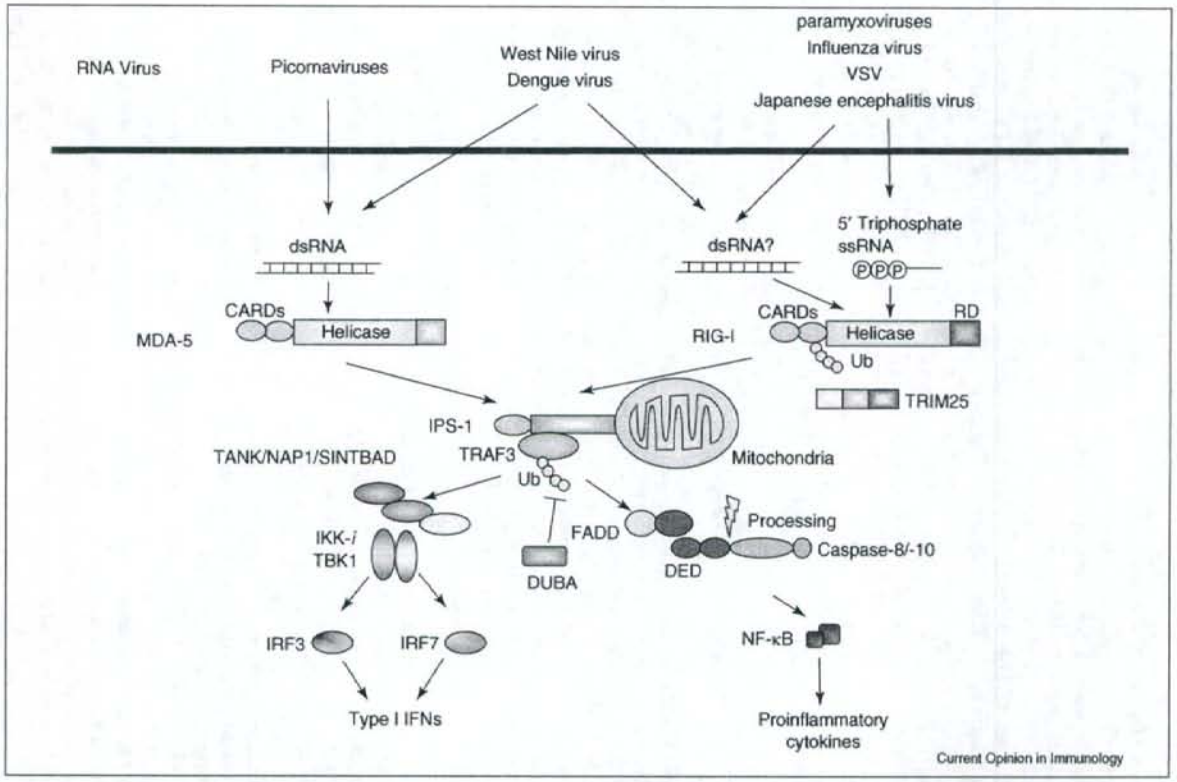
Figure 1



RLH-mediated recognition of RNA viruses. RIG-I and MDA5 recognize 5'-triphosphate RNA and dsRNA from RNA viruses, and interact with IPS-1. TRAF3 is required for activation of IPS-1 signaling pathways, and K63-type polyubiquitination, which is controlled by the presence of DUBA. Subsequently, TRAF3 recruits TANK/NAP1/SINTBAD and TBK1/IKK-*i*, which phosphorylate IRF-3 and IRF-7. Next, nuclear translocation of IRFs takes place to induce the expression of type I IFN genes. NF-κB is also activated by IPS-1 via a FADD and caspase-8/caspase-10-dependent pathway.

[32,33]. However, IKK-*i* is also activated by IFN-β to directly phosphorylate STAT1, thereby controlling a set of IFN-inducible genes such as the dsRNA-activated adenosine deaminase gene (*Adar1*) [34].

TBK1 and IKK-i interact with TRAF family member-associated NF-κB activator (TANK), NAK-associated protein 1 (NAP1) and similar to NAP1 TBK1 adaptor (SINTBAD). These molecules show similarities with each other, and knockdown of any of them was reported to impair RLH signaling [35–37].

Phosphorylation of IRF-3 and IRF-7 by these kinases induces the formation of homodimers and/or heterodimers [38], which translocate into the nucleus and bind to IFN-stimulated response elements (ISREs), thereby resulting in the expression of type I IFN genes and a set of IFN-inducible genes. In addition, FAS-associated death domain-containing protein (FADD) interacts with caspase-8, caspase-10 and IPS-1, and the FADD-dependent pathway is responsible for the activation of NF-κB downstream of IPS-1 [39].

Type I IFN-producing cells in response to viral infection

Although RLHs play essential roles in the production of type I IFNs and cytokines in various cell types, such as fibroblasts and conventional dendritic cells (cDCs), plasmacytoid DCs (pDCs) produce cytokines in the absence of RLH signaling [13]. pDCs produce huge amounts of type I IFNs in response to virus infections, and TLR signaling is essential for type I IFN production by pDCs. TLR7 and TLR9 are highly expressed on pDCs, and stimulation with viral RNA and DNA induces the recruitment of a complex of MyD88, IRAK-1, IRAK-4, IKK-α and IRF-7 to the receptor, thereby leading to the phosphorylation and nuclear translocation of IRF-7 to activate the expression of IFN-inducible genes [40,41]. Although the importance of pDCs as a source of type I IFNs *in vivo* has been emphasized, direct identification of IFN-producing cells *in vivo* has not been achieved. Recently, a reporter mouse strain monitoring IFN-α6 ($Ifna6^{GFP/+}$) has been established. Although pDCs are highly potent in expressing GFP upon systemic NDV infection, lung infection of $Ifna6^{GFP/+}$ mice with NDV results in increases in GFP^+ alveolar macrophages and cDCs, but not pDCs [42•]. These observations indicate that cells other than pDCs can act as sources of type I IFNs depending on the route of infection. Interestingly, pDCs start to produce IFN-α when alveolar macrophages are depleted, suggesting that pDCs function when the first line of defense is broken.

Roles of RLHs and TLRs in the activation of adaptive immune responses to viruses

Immediate innate responses are important for mounting acquired immune responses to viral infections. However, it was not clear how the innate PRRs are involved in the activation of acquired immunity. Recently, two different virus infection models have been tested to examine the roles of RLHs and TLRs in the activation of acquired immune responses. The first model virus is lymphocytoid choriomeningitis virus (LCMV), an ambisense ssRNA virus belonging to the *Arenaviridae* family, which induces cytotoxic T lymphocyte (CTL) responses in a type I IFN-dependent manner [43]. Using $MyD88^{-/-}$ and $IPS\text{-}1^{-/-}$ mice, the serum levels of type I IFNs and pro-inflammatory cytokines were found to mainly depend on the presence of MyD88, but not IPS-1. The generation of virus-specific CTLs was critically dependent on MyD88, but not IPS-1. Analyses of $Ifna6^{GFP/+}$ reporter mice revealed that pDCs are the major sources of IFN-α in LCMV infection. These results suggest that recognition of LCMV by plasmacytoid DCs via TLRs is responsible for the production of type I IFNs *in vivo*. Furthermore, TLRs, but not RLHs, appear to be important for mounting CTL responses to LCMV infection.

Influenza virus has also been used to study the activation of adaptive immune responses [44]. Induction of type I IFNs in response to intranasal influenza A virus infection is abrogated in the absence of both MyD88 and IPS-1, although mice lacking either of these molecules are capable of producing type I IFNs. Induction of B cells or CD4 T cells specific for viral proteins is dependent on the presence of MyD88, but not IPS-1, whereas induction of nuclear protein Ag-specific CD8 T cells is not impaired in the absence of either MyD88 or IPS-1. These results suggest that adaptive immune responses to influenza A virus infection are governed by a TLR pathway.

The virus infection models tested to date support roles for TLRs, rather than RLHs, in instructing the adaptive immune system. However, further studies are required, since these two PRR systems provide different contributions depending on the viruses involved and also may depend on the route of infection.

Conclusions

In this review, we have described the roles of RLHs, their signaling pathways and the relationships among RLHs and TLRs in responses against RNA viruses. Although recent studies have clarified the functions and signaling pathways of RLHs, the molecular structures of the RNAs recognized by MDA5 are not understood. In addition, it remains unclear whether RIG-I can recognize dsRNA in addition to 5′-triphosphate ssRNA. Although a previous report showed that small dsRNAs without 3′ overhangs induced IFN-inducible genes via RIG-I in certain cell types [45], chemically synthesized small dsRNAs (20-27 nucleotides) were only found to activate mouse fibroblasts and cDCs (OT and SA: unpublished observations). Thus, further investigations are required to clarify the structures of RIG-I and MDA5 ligands.

Furthermore, the mechanisms of DNA virus recognition are not well understood. Recently, the presence of a cytoplasmic DNA sensor was predicted [46,47], and a protein named DAI was proposed as a candidate for this sensor [48]. However, loss-of-function studies are still required to prove the importance of this protein. Although we focused on the mechanisms of innate responses and T cell activation toward RNA viruses in this review, many other cell types such as NK cells and NK T cells are involved in antiviral responses *in vivo*. Further studies are required to fully elucidate the complex regulation of antiviral responses *in vivo*.

Acknowledgements

We thank M. Hashimoto for excellent secretarial assistance. This work was supported in part by grants from the Special Coordination Funds of the Japanese Ministry of Education, Culture, Sports, Science and Technology, from the 21st Century Center of Excellence Program of Japan and from the NIH (P01 AI070167).

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Akirins are highly conserved nuclear proteins required for NF-κB-dependent gene expression in drosophila and mice

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During a genome-wide screen with RNA-mediated interference, we isolated *CG8580* as a gene involved in the innate immune response of *Drosophila melanogaster*. *CG8580*, which we called *Akirin*, encoded a protein that acted in parallel with the NF-κB transcription factor downstream of the Imd pathway and was required for defense against Gram-negative bacteria. *Akirin* is highly conserved, and the human genome contains two homologs, one of which was able to rescue the loss-of-function phenotype in drosophila cells. Akirins were strictly localized to the nucleus. Knockout of both *Akirin* homologs in mice showed that one had an essential function downstream of the Toll-like receptor, tumor necrosis factor and interleukin (IL)-1β signaling pathways leading to the production of IL-6. Thus, Akirin is a conserved nuclear factor required for innate immune responses.

The innate immune system shields all metazoans against invading microorganisms. This well conserved defense mechanism relies on host-pathogen interactions between nonclonally distributed pattern recognition receptors in the host and pathogen-associated molecular patterns in microbes[1–4]. In contrast, the acquired immune system, based on selection of lymphocytes and their antigen-specific receptors, is specific to vertebrates. Drosophila has become an attractive model organism for the study of the innate immune system due to its well established genetics, the absence of an acquired immune system and the striking conservation between its immune system and many mammalian innate immune defenses.

One of the hallmarks of the drosophila defense is the systemic response, which involves the synthesis of small cationic antimicrobial peptides by the fat body, a functional equivalent of the mammalian liver. Two distinct signaling pathways, namely the immune deficiency (Imd) and the Toll pathways, control the transcription of the antimicrobial peptide genes[2,4,5]. Fungal or Gram-positive bacterial infections activate the Toll pathway[6]. The cytokine-like peptide Spaetzle is cleaved in response to microbial challenge in the open circulatory system of the fly and binds to the transmembrane receptor Toll[7]. The subsequent intracellular cascade leads to the dissociation of the NF-κB family member Dorsal-related immunity factor (Dif)[8,9] from its inhibitor, the IκB-like protein Cactus, through the recruitment of the myeloid differentiation factor 88 homolog (MyD88)[10], the adaptor molecule Tube, and the IL-1R-associated kinase (IRAK)-like serine-threonine kinase Pelle[2]. Dif nuclear translocation then activates many genes, including the gene encoding the antifungal peptide Drosomycin (*Drs*)[4,6,9].

In contrast, Gram-negative bacterial infection activates the Imd pathway, resulting in the expression of genes encoding antimicrobial peptides such as Attacin, Cecropin and Diptericin[3–5]. Expression of these effector genes requires the signal-dependent cleavage and subsequent nuclear translocation of Relish, another member of the NF-κB family of transcription factors[11–13]. Several genetic screens have identified many players in the Imd pathway and shown striking similarities with components of the mammalian tumor necrosis factor (TNF) pathway[14]. Gram-negative bacterial peptidoglycan (PGN) binds to peptidoglycan recognition protein LC (PGRP-LC) and PGRP-LE, which are the most upstream components of the Imd pathway[15–21]. Imd itself encodes a protein with a death domain (DD) similar to that of the mammalian receptor-interacting protein (RIP) that is important in both NF-κB activation and apoptosis[22,23]. Yeast two-hybrid experiments and genetic analysis have demonstrated that Imd forms a complex with the death domain–containing adaptor Fadd and the caspase Dredd[24,25]. This upstream protein complex then activates, through a TAK1-binding protein called dTAB2 (ref. 26) and inhibitor of apoptosis protein 2 (IAP2)[27], the drosophila TGF-β–activated kinase-1 (dTAK1), a member of the MAPKKK family of kinases[28]. Both IκB kinase (IKK)-β (IKKβ) and IKKγ are also required downstream of Imd and dTAK1 for Relish activation[29,30].

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Received 12 June; accepted 23 October; published online 9 December 2007; corrected after print 11 January 2008; doi:10.1038/ni1543

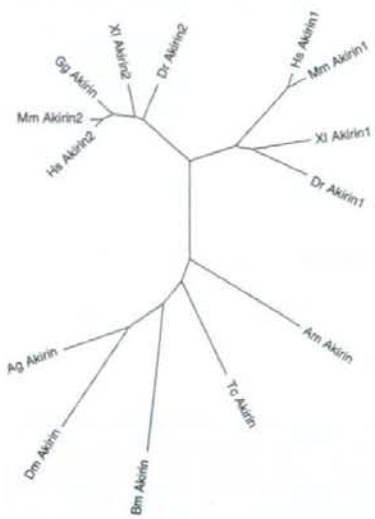

Figure 1 Unrooted evolutionary tree of Akirin homologs: Akirins are highly conserved. Dm, *Drosophila melanogaster*; Ag, *Anopheles gambiae*; Bm, *Bombyx mori*; Am, *Apis mellifera*; Tc, *Tribolium castaneum*; Gg, *Gallus gallus*; Hs, *Homo sapiens*; Mm, *Mus musculus*; Xl, *Xenopus laevis*; Dr, *Danio rerio*. In vertebrates, similarity splits *Akirins* into two groups that we have numbered 1 and 2, with *Akirin2s* more closely related to invertebrate *Akirins*.

In mammals, Gram-negative bacteria are sensed by Toll-like receptors (TLRs) that activate, similarly to the drosophila Imd pathway, an IKK complex and NF-κB. In response to TLR or IL-1R stimulation, MyD88 and IRAKs are recruited to the receptor, and then interact with TNF receptor–associated factor 6 (TRAF6), which acts as an ubiquitin protein ligase (E3). Subsequently, TRAF6, together with a ubiquitination E2 enzyme complex consisting of UBC13 and UEV1A, catalyzes the formation of a K63-linked polyubiquitin chain on TRAF6 and on IKK-γ–NF-κB essential modulator (NEMO)[31]. A complex comprising TAK1 and the TAK1-binding proteins, TAB1, TAB2 and TAB3, is also recruited to TRAF6 (ref. 32). After stimulation by TLR ligands, IκBα is phosphorylated on two serine residues by an IKK complex activated by TAK1. Phosphorylated IκBα is then ubiquitinated and degraded by the proteasome. Liberated NF-κB translocates into the nucleus, where it activates the transcription of its target genes.

Despite more than ten years of research since the initial discovery of the Imd mutation, the pathway bearing its name is still not fully understood. We undertook a functional genome-wide RNA-mediated interference (RNAi) screen in drosophila cell culture to isolate new components in the Imd pathway. We report here the isolation of *CG8580* (that we renamed *Akirin*) encoding a nuclear protein with no recognizable domain and required for NF-κB-dependent transcription. RNAi-mediated knock down of *Akirin* led to impaired Imd pathway signaling and enhanced sensitivity of flies to Gram-negative bacterial infection. Moreover, epistatic analysis allowed us to place the *Akirin* function at the level of the transcription factor itself. As *Akirin* shows striking evolutionary conservation, we generated mice deficient for *Akirin* homologs and demonstrated that one of these mouse *Akirin* homologs was required for NF-κB dependent IL-6 production after TLR agonist, IL-1β or TNF stimulation of embryonic fibroblasts. A drosophila loss of function phenotype could also be restored by expression of the human homolog of *Akirin*. We therefore propose that *Akirin* is an ancient conserved nuclear factor regulating NF-κB dependent transcription.

RESULTS

Identification of drosophila and mouse Akirin homologs

To identify new components of the Imd pathway, we performed a high-throughput RNAi screen with cultured drosophila S2 hemocyte-like cells[27,33]. Of 21,306 RNAi probes, several induced a moderate to marked effect on the expression of the Imd pathway-dependent *Attacin* gene activated by an *Escherichia coli* infection. We selected *CG8580* for further study, as the corresponding RNAi reduced the induction of Attacin expression by 90%. *CG8580* encoded a putative 201–amino acid protein with no recognizable domains. Two homologs of the *CG8580* sequence were present in zebrafish (*Danio rerio*), African clawed frog (*Xenopus laevis*), human (*Homo sapiens*) and mouse (*Mus musculus*) databases. Only one copy was present in insects (*Apis mellifera, Tenebrio molitor, Anopheles gambiae, D. melanogaster*) and in birds (*Gallus gallus*); none was found in plants, yeast or bacteria. The similarities allowed the sequences to be split into discrete groups, one in insects and two in vertebrates (**Fig. 1**). The conservation was highest for the putative C- and N-terminal domains. All sequences showed a clear nuclear localization signal (NLS) located between residues 24 and 29 near the N terminus (**Supplementary Fig. 1** online). We renamed the gene *Akirin* (*Akirin1* and *Akirin2* in the case of vertebrates) from the Japanese 'akiraka ni suru', which means 'making things clear'.

Akirins are ubiquitously expressed nuclear proteins

Microarray data in Flybase[34] indicate that *D. melanogaster Akirin* expression is ubiquitous. Similarly, an analysis based on a blot with human RNA points to almost ubiquitous expression of human *Akirins* (**Supplementary Fig. 2** online). To monitor the cellular localization of drosophila Akirin, we fused the *D. melanogaster Akirin* coding sequence to a V5 tag and transfected S2 cells. Immunoblot analysis with antibody to V5 (anti-V5) showed that drosophila *Akirin* was expressed as a single ~27-kDa protein that was not modified after *E. coli* stimulation (**Supplementary Fig. 3** online). Antibody staining of the S2 cells established that drosophila Akirin had a strict nuclear localization, which was dependent on the presence of the N-terminal NLS (**Fig. 2a**) and did not change after *E. coli* treatment (data not shown). Similarly, we fused the *H. sapiens Akirin1* and *Akirin2* sequences to a Flag tag and transfected HeLa cells. Antibody staining of the human cells clearly showed the nuclear localization of human Akirin1 and human Akirin2, which was again dependent on the NLS (**Fig. 2b**).

Akirin function in drosophila

To analyze the effects of drosophila *Akirin* on the Imd pathway, we used an RNAi-mediated knock down strategy in S2 cells. A truncated form of PGRP-LCa (containing only the transmembrane and intracellular segment) can induce a robust expression of an Attacin-luciferase (Att-Luc) reporter (refs. 10,15,16,27 and A.G., unpublished data). Compared with GFP RNAi controls, the induction of the Att-Luc reporter was strongly suppressed by double-stranded RNA (dsRNA) against *Akirin* (**Fig. 3a,b**), in keeping with reduced *Akirin* mRNA abundance (**Supplementary Fig. 4** online). The degree of reduction was similar to that obtained with dsRNA against *Imd* (**Fig. 3b**). In further experiments we confirmed the specificity of the suppression with two different, nonoverlapping dsRNAs directed against *Akirin*, which both produced a considerable reduction in

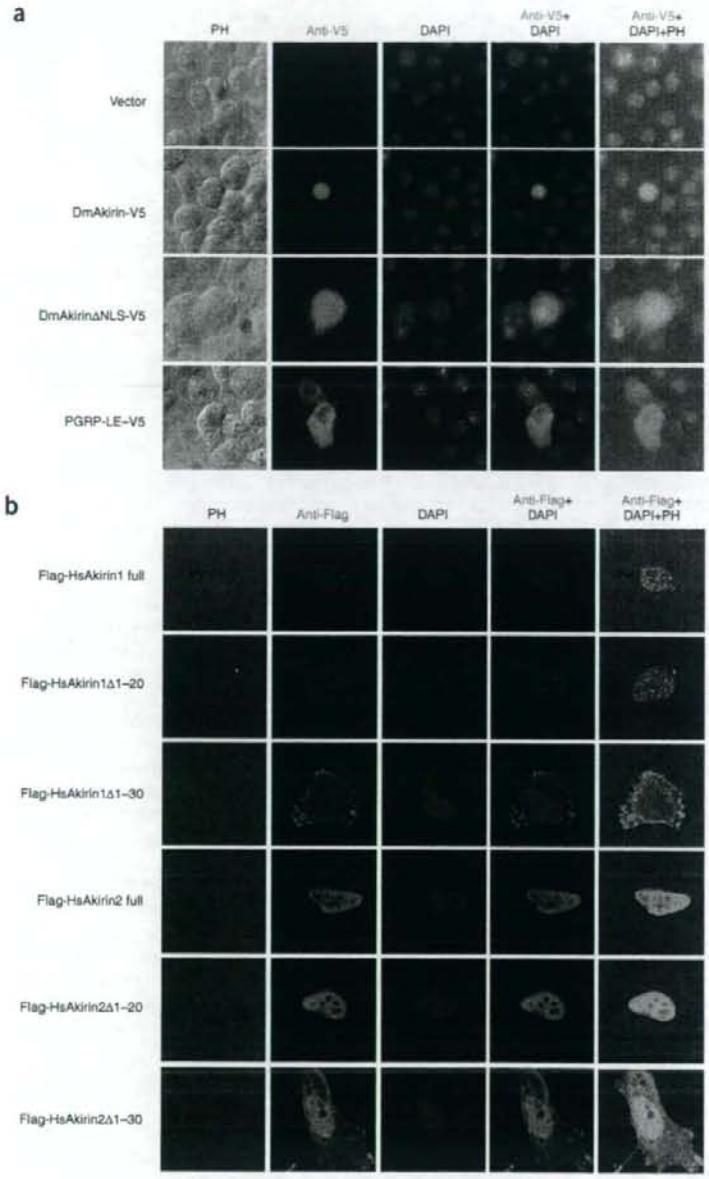

Figure 2 Nuclear localization of Akirins. (**a**) S2 cells transfected with constructs encoding V5-tagged drosophila Akirin, NLS-deleted drosophila Akirin or PGRP-LE. Cells transfected with an empty vector were used as control. Nuclei were visualized with DAPI (blue). Akirin, NLS-deleted Akirin and PGRP-LE were visualized by V5 antibody (green). The merged fields including phase contrast (PH) showed nuclear localization of drosophila Akirin (anti–V5+DAPI+PH), in contrast to the cytoplasmic localization of PGRP-LE. This nuclear localization was abrogated when the NLS was deleted from Akirin. Results are representative of three independent experiments. (**b**) HeLa cells transfected with Flag-tagged full-length or N-terminally deleted (amino acids 1–20 or 1–30) *H. sapiens Akirin1* or *H. sapiens Akirin2*. Nuclei were visualized with DAPI (blue) and human Akirins were visualized with an anti-Flag antibody (green). The merged fields (anti–Flag+DAPI+PH) showed NLS-dependent (amino acids 20–30; **Supplementary Fig. 1**) nuclear localization for both human Akirin1 and human Akirin2.

Att-Luc, similar to that of the original dsRNA, demonstrating that the suppression is gene specific (**Fig. 3a,c**).

The Imd pathway responds to Gram-negative bacteria, but the Toll pathway is predominantly activated by Gram-positive bacteria or fungi and culminates in the expression of many genes, including the antifungal peptide Drs[5]. To address whether *Akirin* is also involved in the Toll pathway, we transfected an expression construct encoding *D. melanogaster* TollΔLRR, a constitutively active form of Toll lacking its extracellular leucine-rich repeat (LRR) domain, into S2 cells together with a Drs-luciferase reporter. As expected, transfection of this constitutively active TollΔLRR resulted in a marked luciferase expression[10], which was reduced by dsRNA targeting *Pelle*, a gene encoding a serine-threonine kinase required in the Toll pathway (**Fig. 3d** and ref. 10). However, dsRNA against either *Akirin* or *Imd* did not affect Drs-Luciferase expression, demonstrating that drosophila *Akirin* is not involved in the Toll pathway and eliminating the possibility that dsRNA against drosophila *Akirin* might affect luciferase expression itself.

We next undertook epistatic experiments to analyze the position of drosophila *Akirin* within the Imd pathway. For this, we transfected S2 cells with expression constructs encoding several genes of the Imd pathway—*PGRP-LE*, *Imd*, *Fadd*, *Dredd* and *Relish*—and monitored Att-Luc expression. Transfection of *PGRP-LE*, *Imd* and *Relish* constructs led to abundant Att-Luc expression (**Fig. 4a–c**). *Fadd* transfection led to a dominant-negative effect on *E. coli*–induced Att-Luc expression, whereas *Dredd* expression resulted in lower cell viability (data not shown). Notably, in *PGRP-LE*-transfected S2 cells, the enhanced Att-Luc expression was significantly decreased by transfection of dsRNA against either *Imd* or *Akirin* ($\sim$60% ($P = 0.001$)) and $\sim$80% ($P = 0.007$), respectively; **Fig. 4a**). Expression of *Imd* also resulted in a robust Att-Luc expression that could be suppressed by both dsRNAs against *Akirin*, indicating that *Akirin* acts downstream of *Imd* (**Fig. 4b**).

As expression of Fadd and Dredd in S2 cells did not cause any Att-Luc expression, we decided to transfect the cells with a construct encoding the NF-κB family member Relish, which acts downstream in the Imd pathway. As shown earlier, transfection of a construct encoding full-length Relish only moderately activated the Imd

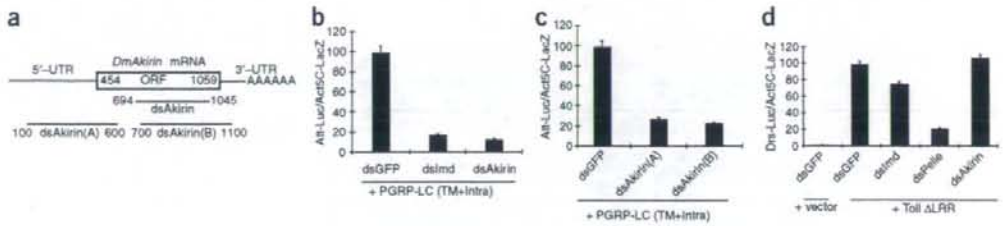

Figure 3 Effect of RNAi knock-down of drosophila *Akirin* on the activation of the Imd and Toll pathways in drosophila S2 cells. (**a**) Structure of *D. melanogaster Akirin* mRNA and of the dsRNAs against drosophila *Akirin*. An original dsRNA (dsAkirin) covering nucleotides 694–1045 was used for the screen. We synthesized two more dsRNAs, (dsAkirins(A) and (B)) covering nucleotides 100–600 and 700–1100, respectively. ORF, open reading frame. (**b**) S2 cells transfected with PGRP-LC (TM+Intra) constitutively express the Attacin-Luciferase (Att-Luc) reporter gene as an indicator of activation of the Imd pathway; this expression is lower in cells treated with dsAkirin than in control cells treated with dsGFP and is similar to that in cells treated with dsImd. (**c**) Both dsAkirin(A) and (B) suppressed the Att–Luc induction in the same way as the original dsAkirin. (**d**) S2 cells transfected with TollDLRR constitutively express the Drosomycin-Luciferase (Drs-Luc) reporter gene as an indicator of activation of the Toll pathway. In contrast to the expression in cells treated with dsPelle, this expression is unchanged in cells treated with dsImd and dsAkirin relative to that of control cells treated with dsGFP. Each bar represents the mean of three independent experiments. Error bars are s.d.

pathway, but a Relish construct deleted for the nucleotides encoding a serine-rich region (ΔS29–S45) led to a strong Att-Luc expression[11]. We confirmed this result (**Fig. 4c**) and further noted that the strong RelishΔS29–S45-dependent reporter gene induction was significantly suppressed by both dsRNAs against *Akirin* ($P = 0.0003$). This result indicated that *Akirin* acts downstream of or at the level of *Relish* (**Fig. 4c**), which is in agreement with the nuclear localization of Akirin.

Drosophila *Akirin* expression in S2 cells by itself did not activate the Imd pathway, as monitored by expression of Att-Luc, nor result in lower cell viability. Further, it did not show any dominant-negative effect against *E. coli* treatment (data not shown). To ascertain that the expressed *Akirin* construct was functional, we set up a rescue experiment. dsRNA against the *Akirin* 5′ untranslated region (UTR) was synthesized and shown to suppress activation of the Imd pathway in PGRP-LC transfected cells that actively expressed the reporter gene. However, when the coding sequence of *Akirin* devoid of its 5′ UTR—that is, the target of the dsRNA sequence—was coexpressed in the same cells, Att-Luc expression was rescued such that it was equivalent to wild-type expression. We could also rescue this phenotype with the human ortholog of *D. melanogaster Akirin*, *H. sapiens Akirin2*, clearly indicating that Akirin is functionally and evolutionary conserved (**Fig. 4d**).

To analyze the *in vivo* function of drosophila *Akirin*, we first generated null mutants by imprecise excision of EY08097, a P element located in the first intron of *CG8580*. Out of 430 lines, we isolated seven representing a deletion removing the *Akirin* gene. However, all deletion lines were homozygous embryonic lethal, indicating that *Akirin* is critically required during drosophila embryonic development (see Discussion). We next tried to knock down *Akirin* through a transgenic RNA interference (RNAi) approach[35]. We generated UAS-Akirin RNAi transgenic flies and crossed them with different GAL4 drivers (**Fig. 5**). *Akirin* knock down with heat-shock–GAL4 and yolk-GAL4 resulted in reduction of Imd pathway–dependent *Diptericin* gene expression after infection with a mix of Gram-positive and Gram-negative bacteria (**Fig. 5a,b**). Consistent with cell culture data (**Fig. 2d**), *Drs* expression was unchanged in these experiments (**Fig. 5a,c**), indicating that Toll pathway activation does not require *Akirin* function. Finally, RNAi-mediated knock-down of *Akirin* in whole flies led to enhanced sensitivity to Gram-negative bacterial infection (**Fig. 5d**).

Akirin loss-of-function mouse embryonic fibroblasts

To investigate whether the function of *Akirins* is conserved in the immune response between drosophila and mammals, we generated mice deficient in either the mouse *Akirin1* or the mouse *Akirin2* gene. A gene-targeting vector was constructed by placing two *lox*P sites flanking the first coding exon of the *Akirin1* gene and inserting a *lox*P site–flanked ('floxed') *neo*^{r} gene into intron 1 of the *Akirin1* gene

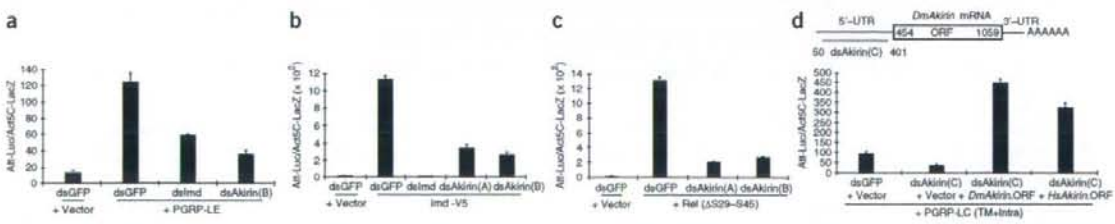

Figure 4 Epistatic analysis of *D. melanogaster* Akirin position within the Imd pathway. Constitutive activation of the Imd pathway induced by the transfection of S2 cells with PGRP-LE-V5 (**a**), Imd-V5 (**b**), Rel (ΔS29-S45; **c**) or PGRP-LC (TM+Intra; **d**) is highly compromised when cells are also treated with dsAkirin, as demonstrated by expression of the Att-Luc reporter gene ($P < 0.05$). (**d**) The compromised expression is restored by the coexpression in the same cells of the coding sequence of *D. melanogaster Akirin or of H. sapiens Akirin2*. Cells treated with vector alone serve as a control. Each bar represents the mean of three independent experiments (error bars, s.d.).

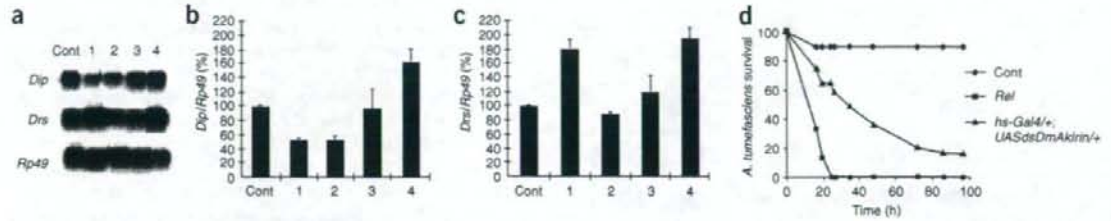

Figure 5 *In vivo* function of *D. melanogaster* Akirin. (**a**) The Imd and Toll pathway activations were monitored by RNA blot analysis of the *Diptericin* (*Dip*) and *Drosomycin* (*Drs*) messengers expression 6 h and 24 h, respectively, after infection with a mixture of Gram-positive and Gram-negative bacteria. The *Rp49* messenger was used as loading control. (**b**,**c**) Quantification of *Dip* (**b**) and *Drs* (**c**) normalized with *Rp49*. 1: *hs-GAL4/+;UAS-dsDmAkirin/+*, 2: *yolk-GAL4/+; UAS-dsDmAkirin/+* (females), 3: *CyO/+; UAS-dsDmAkirin/+*, 4: *yolk-GAL4/+; UAS-dsDmAkirin/+* (males). Homozygous *white*^{1118} flies were used as a control (cont). Each bar represents the mean of three independent experiments. Error bars are s.d. (**d**) Survival of adult flies infected with a Gram-negative bacterium (*Agrobacterium tumefasciens*). The Imd pathway mutant flies, *Relish*^{E20} (*Rel*), are highly sensitive to this bacterial infection. Compared with control (*white*^{1118}) flies, flies in which drosophila *Akirin* was knocked down showed an increased sensitivity to infection. Results are representative of three independent experiments.

(**Supplementary Fig. 5** online). We transiently transfected the targeted embryonic stem cells with a plasmid encoding the Cre protein to excise the *neo*^{r} gene. We then crossed *Akirin1*$^{flox/+}$ mice with a transgenic mouse line expressing Cre in germ cells (CAG-Cre mice). The deletion of the *Akirin1* gene was confirmed by Southern blot analysis (**Supplementary Fig. 5**). *Akirin1*$^{-/-}$ mice were born in a mendelian ratio, grew healthily and did not show gross developmental abnormalities. *Akirin1* mRNA was not expressed in mouse embryonic fibroblasts (MEFs) obtained from *Akirin1*$^{-/-}$ mice (**Supplementary Fig. 5**).

To generate an *Akirin2* flox allele, we constructed a targeting vector inserting two *lox*P sites flanking the first coding exon of the mouse *Akirin2* gene, with a *lox*P site–flanked *neo*^{r} gene (**Supplementary Fig. 6** online). The targeted embryonic stem cells were transiently transfected with a plasmid encoding Cre to eliminate *neo*^{r}. *Akirin2*$^{+/-}$ mice were obtained by mating *Akirin2*$^{flox/+}$ mice with CAG-Cre mice. In contrast to *Akirin1*$^{-/-}$, *Akirin2*$^{-/-}$ was embryonic lethal, and we did not find *Akirin2*$^{-/-}$ embryos even on embryonic day 9.5, indicating that the *Akirin2* gene is essential for normal embryonic development in mice (**Supplementary Table 1** online). Thus, we generated MEFs from *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ embryos and excised the *lox*P-flanked genomic fragment by retroviral expression of the Cre protein together with the puromycin resistance gene (*Puro*). We examined puromycin-resistant cells for the expression of *Akirin2* by RT-PCR. The expression of *Akirin2* was suppressed in Cre-transduced *Akirin2*$^{flox/-}$ (*Akirin2*$^{-/-}$) MEFs (**Supplementary Fig. 6**). This enabled us to analyze MEFs specifically lacking *Akirin1* or *Akirin2*.

Mouse *Akirin2* in IL-1β- and TLR-mediated responses

As drosophila Akirin was critical for the Imd pathway, which activates NF-κB through the IKK complex similarly to the mammalian TNF signaling pathway, we hypothesized that mouse Akirins could likewise be involved in TLR-, IL-1β- and TNF-mediated responses. We first examined the production of cytokines in *Akirin1*$^{-/-}$ MEFs in response to TLR ligands, IL-1β and TNF. The production of IL-6 was similar in wild-type and *Akirin1*$^{-/-}$ MEFs in response to all stimuli tested (**Fig. 6a**). However, when *Akirin2*$^{-/-}$ MEFs were stimulated with TLR ligands (MALP-2 and lipopolysaccharide (LPS)), IL-1β and TNF, production of IL-6 was much less than in control *Akirin2*$^{+/-}$ MEFs (**Fig. 6b**). Thus, Akirin2, but not Akirin1, was responsible for the production of IL-6 in response to TLR or IL-1R activation.

Next we examined whether Akirin2 regulated IL-6 production at the level of gene expression. LPS-induced expression of genes encoding IL-6, IP-10, RANTES and BCL3 two hours after challenge was severely impaired in *Akirin2*$^{-/-}$ MEFs relative to that in control cells, indicating that Akirin2 is critical for the expression of several LPS-inducible genes (**Fig. 7a**). However, the induction of genes encoding IκBα, IκBζ and the CXCL1 chemokine KC was similar in *Akirin2*$^{-/-}$ and control MEFs. The gene induction in response to IL-1β stimulation was similarly impaired in *Akirin2*$^{-/-}$ MEFs (**Fig. 7b**). Thus, mouse Akirin2 regulates the expression of a set of LPS- and IL-1β-inducible genes.

As drosophila Akirin acts together with or downstream of Relish, we next examined the IL-1β- and LPS-dependent activation of NF-κB

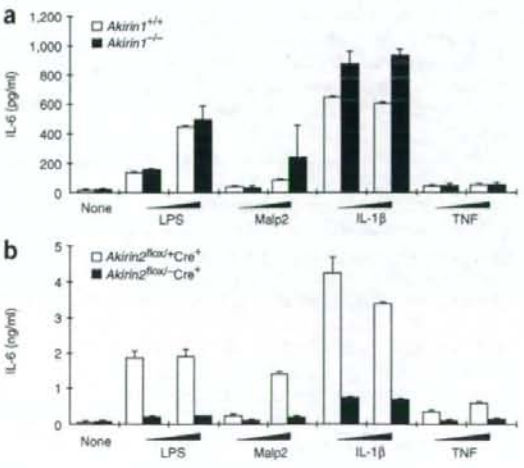

Figure 6 TLR-, IL-1β- and TNF-induced IL-6 production in *Akirin1*$^{-/-}$ and *Akirin2*$^{-/-}$ mouse embryonic fibroblasts (MEFs). (**a**,**b**) IL-6 concentrations in *Akirin1*$^{+/+}$ and *Akirin1*$^{-/-}$ MEFs (**a**) and Cre-transduced *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ MEFs (**b**) stimulated with increasing concentrations of LPS (1, 10μg/ml), MALP-2 (1, 10 nM), IL-1β (1, 10 ng/ml) and TNF (1, 10 ng/ml) for 24 h. Unlike IL-6-induced production in *Akirin*$^{-/-}$ MEFs, that in *Akirin2*$^{-/-}$ MEFs is reduced compared with corresponding wild-type control cells. Each bar represents the mean of three independent experiments. Error bars are s.d.

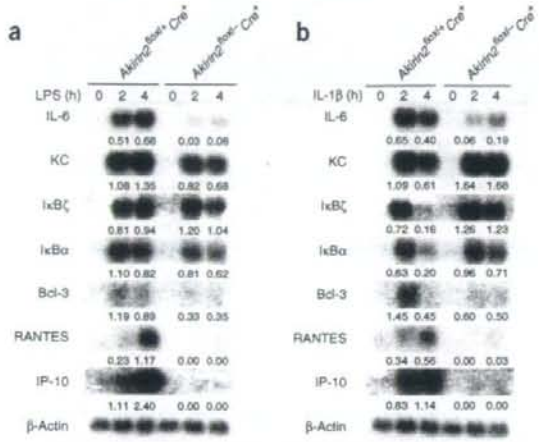

Figure 7 LPS- and IL-1β-induced gene expression in *Akirin2*$^{-/-}$ MEFs. (**a**,**b**) Total RNA blot analysis of the expression of IL-6, KC, IκBζ, IκBα, BCL3, RANTES and IP-10 in Cre-transduced *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ MEFs stimulated with LPS (10 μg/ml) (**a**) or IL-1β (10 ng/ml) (**b**) for 2 and 4 h. The β-actin messenger is used as loading control. Signals were quantified, and values indicate relative density compared with the corresponding loading control. The expression of several LPS- and IL-1β-inducible genes is reduced in *Akirin2* deficient MEFs compared with wild-type control cells. Results are representative of three independent experiments.

in *Akirin2*$^{-/-}$ MEFs. In response to these stimuli, neither degradation of IκBα (**Fig. 8a**,**b**) nor induction of NF-κB DNA binding (**Fig. 8c**,**d**) was impaired in *Akirin2*$^{-/-}$ MEFs. These data indicated that mouse Akirin2 acts together with or downstream of NF-κB in the control of TLR- and IL-1β-inducible gene expression.

DISCUSSION

Akirins represent previously unknown, extremely conserved, nuclear factors that are involved in the metazoan innate immune system. Akirins function during immune and inflammatory responses in drosophila as well as in mice, most likely at the level of the transcription factor NF-κB. We demonstrate here that *D. melanogaster Akirin* encodes a nuclear protein that is required downstream in the Imd pathway at the level of the transcription factor Relish in flies. The function of the mammalian homolog of Akirin is conserved, as mouse Akirin2 was required downstream of TLR, TNF and IL-1β signaling, again at the level of NF-κB, for the production of IL-6.

Akirins are highly conserved among different animal species and show two conserved domains, respectively at the N and C termini, separated by a stretch of less conserved residues. The presence of a nuclear localization signal explains the N-terminal conservation and the nuclear staining that we have noted. Akirins are most probably nuclear resident proteins, as we did not see any change in drosophila Akirin subcellular localization after overexpression or *E. coli* infection.

Drosophila, like other insects, has only one *Akirin* gene, but the vertebrate genomes that we analyzed, except for that of birds, contain two copies of the *Akirin* gene (mouse *Mus musculus Akirin1* and *Akirin2* show 34% and 39% amino acid identity, respectively, with the unique *D. melanogaster Akirin*). All *Akirin1* genes were similar and segregated from the group containing the *Akirin2* genes, indicating an early duplication event followed by divergence in the evolution of vertebrates. Birds would then have secondarily lost the *Akirin1* gene. The diverging function between Akirin1 and 2 was attested to by the contrasting phenotypes of mouse *Akirin* knockouts. Mouse *Akirin2* was essential for embryogenesis and the cytokine response to TLR and IL-1R stimulation, whereas *Akirin1* knockout mice showed no obvious phenotype. Mouse *Akirin2* would be functionally closer to the single gene in drosophila, as the homozygously null *D. melanogaster Akirin* mutants show a similar, mid- to early embryonic death. The function of mouse Akirin1, which is clearly an Akirin on the basis of its sequence conservation, is unknown. It is possible that mouse Akirin1 and Akirin2 work redundantly in the regulation of target gene expression in MEFs. Generation of cells lacking both *Akirin1* and *Akirin2* will help to elucidate the function of Akirin1 *in vivo*.

Both drosophila Akirin and mammalian Akirin2 regulate the expression of a set of genes together with or downstream of NF-κB. These results imply that both drosophila and mammalian Akirins associate with similar protein(s) for controlling gene expression in the nucleus. Transcription by RNA polymerase II involves the cooperative assembly of an initiation complex, which is restrained by the incorporation of promoter DNA into nucleosomes and other chromatin structures. Transcription is then modulated by chromatin remodeling cofactors targeting the nucleosomes or by general cofactors that associate with the basal transcription machinery. It is unlikely that Akirins regulate transcription by binding directly to DNA, as Akirin sequences show no obvious DNA- or RNA-binding motifs. According to Occam's razor principle, the prediction would be that Akirins act as cofactors to regulate or fine-tune NF-κB transcriptional activity by interacting with components of the chromatin or the transcriptional engine. We tested the hypothesis of a direct interaction of drosophila Akirin with DNA or Relish, but we could not precipitate DNA in chromatin immunoprecipitation assays with tagged Akirin or Akirin with a tagged Relish (data not shown), which means that the postulated associations are either weak or most probably require intermediary components. The notion that Akirins could function to modulate transcriptional factors in several other immune-related processes is strengthened by the report that drosophila *Akirin* was found as interacting genetically with *pannier*, one of the GATA factors involved in heart and blood cell development[36]. Along the same line, after another genome-wide RNAi screen, drosophila *Akirin* appeared in a list of putative modulators of the Wingless pathway[37], which was recently shown to be involved in the inflammatory response[38]. Taken

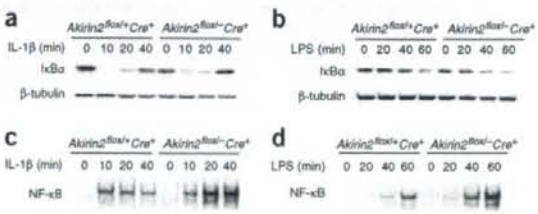

Figure 8 LPS- and IL-1β-induced activation of NF-κB in *Akirin2*$^{-/-}$ MEFs. (**a**,**b**) IκBα expression in the whole cell lysates, analyzed by immunoblotting. (**c**,**d**) NF-κB-DNA binding activity in the nuclear extracts, determined by electrophoretic mobility-shift assay. Cre-transduced *Akirin2*$^{flox/+}$ and *Akirin2*$^{flox/-}$ MEFs were stimulated with IL-1β (10 ng/ml) (**a**,**c**) or LPS (10 μg/ml) (**b**,**d**) for the indicated periods. IκBα degradation and NF-κB-DNA binding activity were similar in wild-type and *Akirin2*-deficient cells after IL-1β and LPS stimulations. Results are representative of three independent experiments.

together, these results indicate that Akirins are previously unknown, important nuclear cofactors regulating the transcriptional activities of main transactivators. However, further studies are needed to clarify how Akirins control gene expression in the Imd and the TLR–IL-1R pathways.

METHODS

DNA constructs. The expressed sequence tag clone of *PGRP-LCa* (LP06704) was obtained from MRC geneservice. We subcloned all constructs into the *Bam*HI–*Kpn*I sites of the expression vector pPAC (ref. 39). A PCR fragment of *PGRP-LCa* was amplified with forward 5′-CCCCGGATCCGATAATTCCC GCCATGCCTTTTAGCAATGAAACG-3′ and reverse 5′-GGGGGGTACCTCA GTTCAACGTCTTTCCGAAGAG-3′ primers. The *PGRP-LE-V5* fragment was obtained from *UAS-PGRP-LE* transgenic flies[20] with forward 5′-CCCCGG ATCCGATAATTCCCGCCATGTCCGAATCGGGAATC-3′ and reverse 5′-GGGG GGTACCTCAGGTGGAATCCAGGCCCAGCAGGGGGTTGGGGATGGCTTG CCTTGTTCCTCCTCCTCGATATTG -3′ primers. The V5-tagged *D. melanogaster Akirin* vector was constructed with forward 5′-CCCCGGATCCGATAAT TCCCGCCATGGCCTGTGCAACCCTGAAAC-3′ and reverse 5′-GGGGGGTA CCTCAGGTGGAATCCAGGCCCAGCAGGGGGTTGGGGATGGGCTTGCCC GACAGGTAGCTAGGCGCTG-3′ primers. The NLS was deleted from V5-tagged drosophila *Akirin* with the 5′-CTAGACTGGGAGTCGATCAACCGTTG CAATCCCTTTGGCCAG-3′ primer. The Imd-V5 construct was obtained by exchanging the tag in an Imd-hemagglutinin construct[25]. *H. sapiens Akirin2* was amplified with forward 5′-CCCCGGATCCGATAATTCCCGCCATGGCGTGC GGAGCCACTCTG-3′ and reverse 5′-GGGGGGTACCTCATGAAACATAGCTA GCAGGC-3′ primers. *Relish* constructs were from ref. 11 and *TollALRR* construct from ref. 10. *UAS-dsDmAkirin* fly stocks were established as in ref. 35 with 5′-GGGGCCGGATCCATGGCCTGTGCAACCC-3′ and 5′-GGGG CCGCTAGCTTACGACAGGTAGC-3′ primers. N-terminal deletions from *H. sapiens Akirin1* and *H. sapiens Akirin2* were constructed by PCR with the following primers: 5′-AGCTTGGCTCCCCGAAGCGGCGGCGCTGC-3′ (Δ20), 5′-AAGCTTCTGCCCGGCCCCACTCCGGGCCTC-3′ (Δ30), 5′-AAGCTTTCC CCGAAGCGCAGGCGATGTGCG3′ (Δ20) and 5′-AAGCTTTCGGCGCCCACC TCGGCCGCTGCC-3′ (Δ30), respectively.

Sequence analysis. We retrieved sequences by homology search with BLAST with the *D. melanogaster CG8580* from the US National Center for Biotechnology Information (NCBI) database, except for *Bombyx mori*, for which we used SilkBase (http://morus.ab.a.u-tokyo.ac.jp/). The sequences were as follows: *D. melanogaster* Akirin, NP_648113; *Anopheles gambiae* Akirin, XP_308938, modified; Akirin for *Bombyx mori* Akirin, wdS20131; *Apis mellifera* Akirin, XP_395252; *Tribolium castaneum* Akirin, XP_971340; *Gallus gallus* Akirin, XP_419845; *H. sapiens* Akirin1, NP_078871; *Mus musculus* Akirin1, NP_075912; *Xenopus laevis* Akirin1, AAH72831; *Danio rerio* Akirin1, NP_001007187; *H. sapiens* Akirin2, NP_060534; *Mus musculus* Akirin2, NP_001007590; *Xenopus laevis* Akirin2, AAH72831; and *Danio rerio* Akirin2, NP_998707. Sequences were aligned with MULTALIN[40] (**Supplementary Fig. 1**). Subsequent assembly into a majority consensus minimum evolution bootstrap tree was made with the MEGA3 software[41].

Cell culture and transfection assays. Akirin was identified in a large-scale RNAi screen as previously described[27,33]. In brief, 384-well screening plates were prespotted with approximately 75 nM dsRNA in 5 μl of 1 mM Tris at pH 7. Hemocyte-like Kc167 cells were batch-transfected with an IMD-specific *mtk*-luciferase reporter[27], a truncated form of PGRP-LC and a constitutive expressed *Renilla* luciferase and transferred to dsRNA-containing screening plates. Then 15,000 cells in 20 μl were dispensed per well and incubated for 1 h before the addition of serum-containing medium. After 5 d, medium was removed, cells were lysed and both firefly and *Renilla* luciferase activities were determined.

Akirin was also identified in IMD-pathway experiments in S2 cells (as described in ref. 27). S2 cells (Invitrogen and DGRC) were grown at 23 °C in Schneider's medium (Biowest) supplemented with 10% FCS. Cells (1.2×10^6/ml) were transfected in 24-wells plates by calcium phosphate precipitation with 10 μg of *Attacin (Att)*-luciferase or *Drosomycin (Drs)*-luciferase reporter vector, 10 μg of an *Actin5C-lacZ* transfection control vector and dsRNAs (1.0 μg/well). After 12–16 h, the cells were washed with PBS and incubated in fresh medium. Cells were stimulated by heat-killed *E. coli* (~20–30 bacteria per cell) the next day. After 12–16 h of *E. coli* stimulation, cells were lysed and luciferase activity was measured in a luminometer (BCL Book, Promega) immediately after addition of the substrate (luciferin, Promega). β-Galactosidase activity was measured with *O*-nitrophenyl-β-D-galactoside as a substrate, and the values were used to normalize variability in transfection efficiency. For epistatic analysis various amounts (0.001, 0.002, 0.01, 0.02, 0.2 or 0.5 μg per well) of expression vectors were used. For rescue experiments, 0.75 μg of Akirin, 0.025 μg of *PGRP-LC* and 0.25 μg of dsRNAs were transfected. All experiments were done more than twice independently with duplicate wells.

dsRNA preparation. Templates for dsRNA preparation were PCR-derived fragments between two T7 promoter sequences. Fragments for each gene were as follows: *GFP* (nucleotides 35–736, GenBank accession L29345), *Key* (nucleotides 222–744, NCBI accession NM_079132), *Imd* (nucleotides 331–1015, NCBI accession NM_133166) *Akirin* (nucleotides 50–401, 100–600, 694–1045, 700–1100; GenBank accession number AY095189) and *PGRP-LCa*: LP06704 (nucleotides 318–1028, NCBI accession AY119048). Single-stranded RNAs were synthesized with the MEGAscript T7 transcription kit (Ambion). Annealed dsRNAs were ethanol precipitated and dissolved in injection buffer (0.1 mM sodium phosphate, pH 6.8; 5 mM KCl).

Cell staining. S2 cells were fixed 3 d after transfection with 2% paraformaldehyde in PBS for 15 min. Cells were then permeabilized with 0.1% Triton X-100, 1% BSA, PBS for 1 h, incubated overnight with monoclonal antibody to V5 (Invitrogen; 500-fold dilution in PBT: PBS containing 0.1% Tween 20), washed and incubated with fluorescein isothiocyanate–conjugated anti–mouse IgG (500-fold dilution in PBS, Jackson ImmunoResearch). Cells were stained with DAPI in PBS to visualize nuclei and examined with a Zeiss Axioskop 2 microscope.

Microbial infection, survival experiments and RNA blot analysis. We used the following bacterial strains: *E. coli* (1106), *Micrococcus luteus* (CIP A270) and *Agrobacterium tumefasciens*. Survival experiments were carried out as previously described[42]. For RNA blot analysis, flies were challenged with a thin tungsten needle previously dipped into a concentrated culture of mixed Gram-positive (*M. luteus*) and Gram-negative (*E. coli*) bacteria. After 6 h (for *Dip*) or 24 h (for *Drs*), flies were collected. MEFs (1×10^6) were stimulated with 10 ng/ml of IL-1β or 10 mg/ml of LPS for 2 or 4 h. Total RNA was extracted with TRIzol (Invitrogen). RNA (20 μg for flies; 10 μg for MEFs) was electrophoresed, transferred to nylon membrane (Hybond N+; Amersham Pharmacia Biotech) and hybridized with specific cDNA probes for *Dip*, *Drs*, *Il6*, *Nfkbia*, *Nfkbiz*, *Bcl3*, *Ccl5*, *Cxcl1* and *Cxcl10*. The same membrane was stripped and rehybridized with an *Rp49* (flies) or an *Actb* cDNA probe as internal control. Signals were quantified with BAS 2000 Image Analyzer (Fuji) for fly RNA data and with NIH Image software (US National Institutes of Health) for mouse RNA data.

Fly strains and crosses. Flies were grown on standard medium at 25 °C. Drosophila Gal4 driver stocks are described in ref. 43. We used $Relish^{E20}$ and $white^{1118}$ as Imd pathway mutant and wild-type control, respectively. Transgenic w^{1118}; +/+; *UAS-dsDmAkirin/TM3* males were crossed with either w^{1118}; *heat-shock (hs)-GAL4/CyO*; +/+, or w^{1118}; +/+; *yolk-GAL4* females and the progeny kept at 29 °C.

Establishment of *Akirin2*$^{-/-}$ MEFs. We obtained MEFs from embryonic day 13.5 $Akirn2^{flox/+}$ or $Akirin2^{flox/-}$ embryos. To excise the floxed genomic fragment containing exon 1, we infected the MEFs with retrovirus expressing Cre protein together with puromycin-resistance gene product. At 24 h after infection, we added 3 mg/ml of puromycin (Invivogen) and grew the cells under this selection for 72 h. Then the MEFs were used for analysis. All animal experiments were done with the approval of the Animal Research Committee of the Research Institute for Microbial Diseases (Osaka University, Osaka, Japan).

Measurement of IL-6 production. MEFs (2×10^4) were stimulated with 0.1 and 1 μg/ml of recombinant mouse IL-1β (R&D Systems), 10 μg/ml of LPS

(Sigma), 1 and 10 nM of MALP-2 or 1 and 10 ng/ml of recombinant mouse TNF (R&D Systems) for 24 h. We collected culture supernatants and measured IL-6 concentrations with the ELISA kit (R&D Systems).

Immunoblot analysis. MEFs (2×10^6) preincubated in FBS-free medium for 1 h were stimulated with 10 ng/ml of IL-1β in FBS-free medium or 10 mg/ml of LPS in medium containing 0.3% FBS for various periods. MEFs were then lysed in a lysis buffer containing 1.0% Nonidet-P40, 150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA and a protease inhibitor cocktail (Roche). Lysates were separated by SDS-PAGE and transferred onto polyvinylidene difluoride membranes (BioRad). Membranes were probed with antibodies and visualized with an enhanced chemiluminescence system (Perkin-Elmer). Polyclonal antibody to IκBα (anti-IκBα and HRP-conjugated monoclonal anti-β-tubulin (clone D-10) were purchased from Santa Cruz. Monoclonal anti–phosho-p65 (Ser536) (clone 7F1) was purchased from Cell Signaling.

Electrophoretic mobility-shift assay. MEFs (2×10^6) preincubated in FBS-free medium for 1 h were stimulated with 10 ng/ml of IL-1β in FBS-free medium or 10 mg/ml of LPS in medium containing 0.3% FBS for various periods. Nuclear extracts were purified from cells, incubated with a probe specific for NF-κB DNA-binding sites, separated by electrophoresis and visualized by autoradiography.

Additional methods. Information on multiple-tissue RNA blot analysis and the generation of *Akirin*$^{-/-}$ and *Akirin*$^{flox/flox}$ mice is available in the **Supplementary Methods** online.

Statistical analysis. Mean values and s.d. were calculated with Excel software (Microsoft).

Note: Supplementary information is available on the Nature Immunology website.

ACKNOWLEDGMENTS

We thank S. Stöven for Relish constructs; J.L. Imler for reporter constructs; S. Kurata for support to A.G. and discussions; M. Shiokawa, Y. Fujiwara, L. Troxler, A. Meunier and R. Walther for technical help; M. Hashimoto for secretarial assistance; and our colleagues for discussions and suggestions. Supported by the Japan Society for the Promotion of Science (A.G.), the Centre National de la Recherche Scientifique, the Ministère de l'Education Nationale de la Recherche et de la Technologie, Special Coordination Funds, the Japanese Ministry of Education, Culture, Sports, Science and Technology, the US National Institutes of Health (AI070167 and AI44220) and the Emmy-Noether Program of the Deutsche Forschungsgemeinschaft.

AUTHOR CONTRIBUTIONS

A.G., V.G., L.E.C. and D.K. did the drosophila experiments. K.M. and O.T. did the mouse experiments. S.A., M.B., O.T. and J.-M.R. conceived and directed the experiments. A.G., O.T., J.A.H. and J.-M.R. wrote the paper. All authors contributed to manuscript criticism.

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Corrigendum: Akirins are highly conserved nuclear proteins required for NF-κB-dependent gene expression in drosophila and mice

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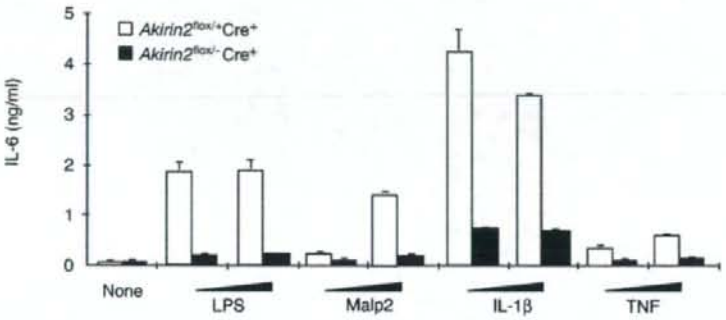
Nat. Immunol. 9, 97–104 (2008); published online 9 December 2007; corrected after print 11 January 2008

In the version of this article initially published, the bars for the LPS samples in Figure 6b are incorrect. The correct data are presented here. The error has been corrected in the HTML and PDF versions of the article.



LETTERS

TANK-binding kinase-1 delineates innate and adaptive immune responses to DNA vaccines

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Successful vaccines contain not only protective antigen(s) but also an adjuvant component that triggers innate immune activation and is necessary for their optimal immunogenicity[1,2]. In the case of DNA vaccines[3], this consists of plasmid DNA; however, the adjuvant element(s) as well as its intra- and inter-cellular innate immune signalling pathway(s) leading to the encoded antigen-specific T- and B-cell responses remain unclear. Here we demonstrate *in vivo* that TANK-binding kinase 1 (TBK1), a non-canonical IκB kinase, mediates the adjuvant effect of DNA vaccines and is essential for its immunogenicity in mice. Plasmid-DNA-activated, TBK1-dependent signalling and the resultant type-I interferon receptor-mediated signalling was required for induction of antigen-specific B and T cells, which occurred even in the absence of innate immune signalling through a well known CpG DNA sensor—Toll-like receptor 9 (TLR9) or Z-DNA binding protein 1 (ZBP1, also known as DAI, which was recently reported as a potential B-form DNA sensor[4]). Moreover, bone-marrow-transfer experiments revealed that TBK1-mediated signalling in haematopoietic cells was critical for the induction of antigen-specific B and $CD4^+$ T cells, whereas in non-haematopoietic cells TBK1 was required for $CD8^+$ T-cell induction. These data suggest that TBK1 is a key signalling molecule for DNA-vaccine-induced immunogenicity, by differentially controlling DNA-activated innate immune signalling through haematopoietic and non-haematopoietic cells.

To develop optimal vaccines for clinical applications, it is important to understand the mechanisms of their actions on immune systems in terms of efficacy as well as safety. In particular, the innate immune recognition of the adjuvant element of vaccine formulations had been shown to be critical for its immunogenicity[2]. Many adjuvants, such as monophosphoryl lipid A and CpG DNA, seem to be ligands for TLRs[5,6]. In contrast, some conventional adjuvants, including aluminium hydroxide (alum) and incomplete Freund's adjuvant, as well as unconventional adjuvant-containing vehicles, such as apoptotic cells, are free of TLR ligand[7,8], suggesting that multiple innate immune recognition and signalling pathways are required for an adjuvant to function.

In the case of DNA vaccines, which have been shown to elicit humoral[9] and cellular[10] immune responses, unmethylated CpG motifs expressed within a plasmid backbone have been considered to be 'built-in' adjuvants, owing to their ability to activate the innate immune system by means of TLR9 (ref. 11). TLR9-deficient mice, however, mounted humoral and cellular immune responses to the encoded antigen comparable to those of wild-type mice[12,13]. Although another report showed a partial reduction of immune responses to a DNA vaccine in TLR9-deficient mice[14], the molecular and/or cellular mechanisms underlying the adjuvant effect and element(s) of DNA vaccines have not been fully clarified[15]. To address this issue, we used an optimized immunization protocol for DNA vaccination by electroporation as described previously[16,17]. After DNA vaccination, mice lacking TLR9 or its essential adaptor, MyD88, mounted both humoral and cellular immune responses to DNA vaccines comparable to those of wild-type mice (Supplementary Figs 1a–d and 2). Moreover, plasmid DNA electroporation activated dendritic cells to produce type-I interferons (IFNs) and inflammatory cytokines in a TLR9-independent manner (Supplementary Fig. 1e and data not shown). Although the immunogenicity of DNA vaccines may vary due to many factors such as the quality of plasmid DNA, injected sites, injection methods or modification of CpG motifs within plasmid DNA[11], our results support previous findings[12,13] indicating that TLR9-mediated recognition of plasmid DNA and subsequent signalling are not essential for optimal DNA vaccination.

Recent accumulating evidence, in contrast, suggests that the double-stranded structure of DNA, independently of CpG motifs, possesses immunomodulatory effects when introduced into the cytosol or its homeostatic clearance is hampered[18]. Intracellular administration of double-stranded B-form DNA (B-DNA) triggers TLR-independent, TBK1- and interferon regulatory factor 3 (IRF3)-dependent innate activation of both immune and non-immune cells to produce type-I IFNs and their inducible genes[19–21]. On the basis of these results, we hypothesized that the immunogenicity of DNA vaccines may be controlled by these TLR9-independent immunostimulatory activities of B-DNA as 'built-in' adjuvant(s), thereby prompting us to investigate whether TBK1-mediated innate immune activation contributes to DNA vaccine immunogenicity.

Because induction of type-I IFNs is a hallmark of TLR9-independent innate immune activation by B-DNA[19–21], which has been shown to have an important role in the following adaptive immune responses[22,23], we initially examined the effect of type-I IFNs on the immunogenicity of DNA vaccines. IFN-αβR-deficient mice that lack type-I IFN-mediated signalling (*Ifnar2*$^{-/-}$) and wild-type mice were immunized with plasmid DNA encoding LacZ or the influenza NP protein. After immunization, wild-type but not *Ifnar2*$^{-/-}$ mice elicited strong T- and B-cell responses to LacZ, including serum LacZ-specific immunoglobulin (Ig)Gs (Fig. 1a, b), spleen $CD8^+$ T-cell frequency and IFN-γ secretion (Fig. 1c, d). Similarly, NP-specific IFN-γ production by $CD4^+$ or

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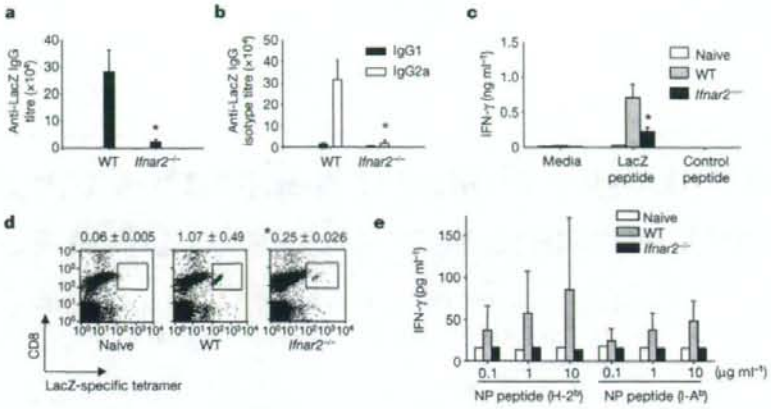

Figure 1 | Optimal DNA vaccine immunogenicity requires type-I interferons. Mice lacking type-I IFN signalling (*Ifnar2*$^{-/-}$) or wild-type (WT) mice were immunized twice by i.m. electroporation with a DNA vaccine encoding LacZ (**a**–**d**) or influenza A virus NP (**e**) protein at intervals of 4 weeks. The serum titres of anti-LacZ IgG (**a**), of IgG1 or IgG2a (**b**), and of spleen LacZ-antigen-specific IFN-γ production (**c**), as well as the frequency of CD8$^+$ T cells (**d**; average % of the gated population ± s.d.), were measured two weeks after the second immunization. Similarly, NP-specific IFN-γ production in the immunized spleen in response to NP peptides (I-A^b or H-2^b) for CD4$^+$ or CD8$^+$ T cells, respectively, was also measured (**e**). Data are the averages ± s.d. of 3–5 mice per group; *$P < 0.01$ against wild-type mice.

CD8$^+$ T-cell-specific peptide was dependent on IFN-αβR (Fig. 1e). These results indicated that signalling by type-I IFNs is indispensable among the critical factors for DNA-vaccine-induced immunogenicity, although minimal immune responses were still observed in *Ifnar2*$^{-/-}$ mice.

We confirmed *in vitro* that plasmid DNA electroporation activated bone-marrow-derived dendritic cells to produce type-I IFNs in a TBK1-dependent manner (Supplementary Fig. 3a, b and data not shown), consistent with our previous findings with cationic liposomes[20]. To examine the role of TBK1 in DNA vaccination *in vivo*, we used TBK1-deficient mice on a *Tnf*$^{-/-}$ background[24]. Although *Tbk1*$^{-/-}$ mice die *in utero*, this lethal effect can be resuscitated in the absence of tumour necrosis factor (TNF)[24]. An advantage of using these mice is that deficiency of TBK1 or of TNF does not influence TLR9-dependent innate immune activation and vice versa[20]. Wild-type and *Tnf*$^{-/-}$ mice elicited comparable antigen-specific immune responses to the encoded antigen after DNA vaccination (Figs 2a and 3a, and data not shown). In contrast, however, *Tbk1*$^{-/-}$ mice on a *Tnf*$^{-/-}$ background failed to increase the frequency and cytotoxicity of antigen-specific CD8$^+$ T cells and spleen IFN-γ production

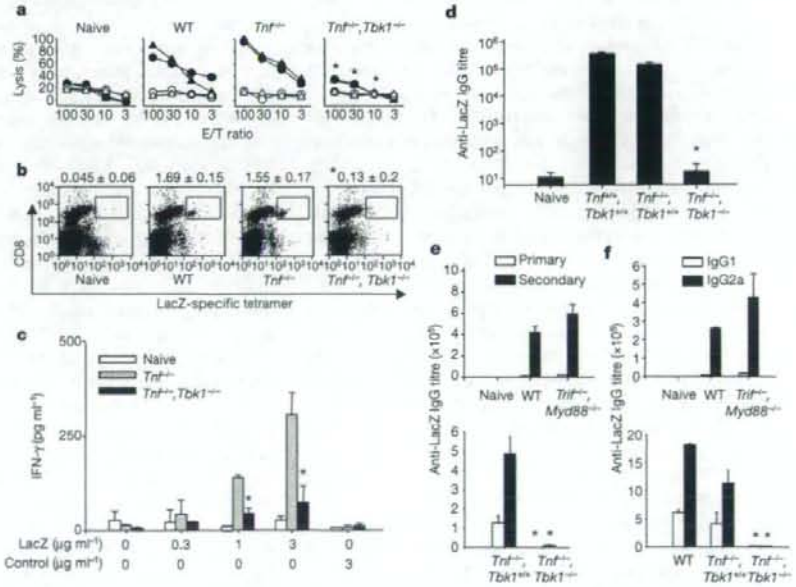

Figure 2 | *Tbk1*$^{-/-}$ mice failed to elicit antigen-specific T- and B-cell responses after DNA vaccination. Mice (five per group) deficient for TNF, TNF and TBK1 or MyD88 and TRIF were immunized with a DNA vaccine encoding LacZ protein on days 0 and 28 by intramuscular electroporation, as described in the Methods. Fourteen days after the second immunization, the antigen-specific CTL activity (**a**, representative individual data with (filled symbol) or without (open symbol) LacZ peptide), the frequency of CTL (**b**, positive for LacZ-specific tetramer, in which the number is the average percentage of the gated population ± s.d.) and the LacZ-protein-specific IFN-γ production (**c**) by spleen cells were analysed. LacZ-specific IgG titre (**d**–**f**), including their isotypes, were analysed by ELISA. Data are the averages ± s.d. of five mice per group; *$P < 0.01$ against control (*Tbk1*$^{+/+}$) mice. E/T ratio, effector to target ratio.

(Fig. 2a–c). Moreover, *Tbk1*$^{-/-}$, but not control (*Tnf*$^{-/-}$) or *Myd88*$^{-/-}$,*Trif*$^{-/-}$ (deficient for any known TLR signalling), mice immunized with a different DNA vaccine encoding influenza A virus NP protein also failed to induce IFN-γ-producing spleen cells in response to NP peptides specific to both CD4$^+$ and CD8$^+$ T cells (Supplementary Fig. 4a). To examine whether *Tbk1*$^{-/-}$ T cells are able to induce TLR9-adjuvanted immune responses or not, we immunized control (*Tnf*$^{-/-}$) and *Tbk1*$^{-/-}$ mice with a vaccine consisting of a NP peptide and CpG ODN, the adjuvant effect of which is totally dependent on TLR9 (ref. 25). Both control (*Tnf*$^{-/-}$) and *Tbk1*$^{-/-}$ immunized mice showed antigen-specific IFN-γ production and cytotoxicity of spleen cells (Supplementary Fig. 4b, c), suggesting that the TLR9-mediated adjuvant effects of CpG ODN are intact in the absence of TBK1 and TNF. The normal functions of *Tbk1*$^{-/-}$ T cells and dendritic cells were further confirmed by several assays, because *Tbk1*$^{-/-}$ T cells respond normally to anti-CD3 and anti-CD28 (Supplementary Fig. 5a and b), and *Tbk1*$^{-/-}$ dendritic cells had intact antigen-presenting functions (Supplementary Fig. 5c). These data clearly demonstrate that TBK1-dependent signalling, but not TLR signalling, is essential for DNA-vaccine-induced T-cell responses to the encoded antigen.

We next examined the role of TBK1 in the humoral responses elicited by DNA vaccination. When wild-type and control (*Tnf*$^{-/-}$) mice were immunized with DNA vaccine, their IgG titres against the encoded LacZ protein were significantly augmented in serum; however, titres in *Tbk1*$^{-/-}$ mice were reduced to the level observed in naive mice, nearly 4 log lower than those in control (*Tnf*$^{-/-}$) or wild-type mice (Fig. 2d). This was the case for either primary or secondary immune responses including isotypes (Fig. 2e, f), whereas those in *Myd88*$^{-/-}$ or *Trif*(also called Ticam 1)$^{-/-}$ mice were comparable to those in wild-type mice (Fig. 2e, f). This was not due to malfunction of *Tbk1*$^{-/-}$ B cells, because the levels of total serum IgG, including IgG1 and IgG2a, were at comparable levels to those of wild-type mice (Supplementary Fig. 5d). Taken together, these results strongly suggest that TBK1 is required for the induction of both humoral and cellular immune responses by DNA vaccination *in vivo*.

To elucidate further the intercellular mechanism(s) by which the TBK1-mediated signalling contributes to DNA-vaccine immunogenicity, we next examined the role of TBK1 signalling in haematopoietic and non-haematopoietic cells by transferring the bone marrows of *Tbk1*$^{+/+}$ or *Tbk1*$^{-/-}$ mice into *Tbk1*$^{-/-}$ or *Tbk1*$^{+/+}$ mice on a *Tnf*$^{-/-}$ background, respectively. When *Tbk1*$^{+/+}$ or *Tbk1*$^{-/-}$ chimaeric mice with *Tbk1*$^{-/-}$ bone marrow were immunized with DNA vaccines, the antigen-specific IgG and IFN-γ production were significantly impaired compared with those in *Tbk1*$^{+/+}$ or *Tbk1*$^{-/-}$ chimaeric mice with *Tbk1*$^{+/+}$ bone marrow (Fig. 3a–d). It has been shown that direct transfection of dendritic cells with DNA vaccines can prime both humoral and cellular immune responses to the encoded antigen[26]. To examine whether TBK1-mediated signalling of dendritic cells directly transfected with DNA vaccine is involved in DNA-vaccine-induced immune responses, splenic dendritic cells from wild-type, control (*Tnf*$^{-/-}$) or *Tbk1*$^{-/-}$ mice were transfected with DNA vaccine by electroporation *in vitro*, and were then transferred to naive, control (*Tnf*$^{-/-}$) mice. Serum IgG and IgG2a titers were significantly increased in mice that received wild-type or control (*Tnf*$^{-/-}$) dendritic cells, but not in those that received *Tbk1*$^{-/-}$ (*Tnf*$^{-/-}$) dendritic cells (Fig. 3e), suggesting that TBK1 signalling in dendritic cells is sufficient to prime antigen-specific antibody responses. Taken together, TBK1 signalling in bone-marrow-derived cells, probably dendritic cells, and to a lesser extent that in non-haematopoietic cells, is critical for the optimal humoral response as well as for helper T (Th)1 cytokine production after DNA vaccination.

We also examined the roles of TBK1 in haematopoietic and non-haematopoietic cells, in the induction of antigen-specific CD4$^+$ and

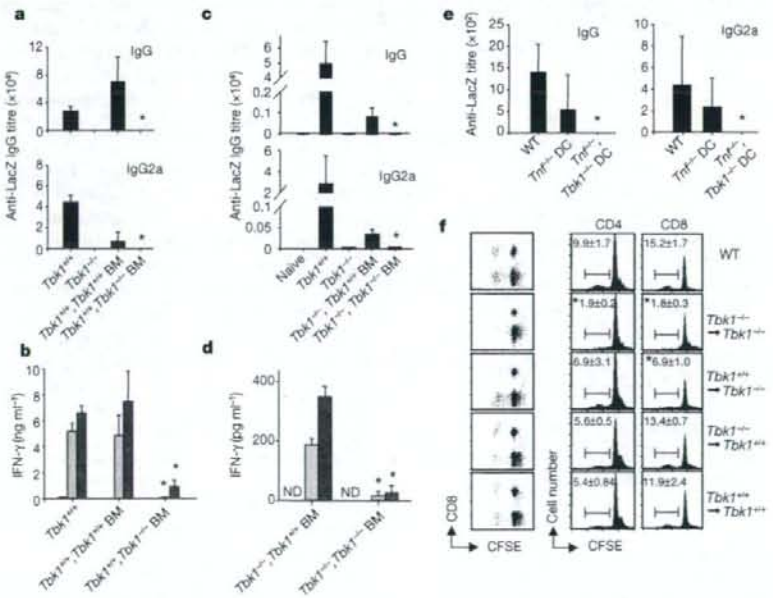

Figure 3 | Contribution of haematopoietic and non-haematopoietic cells to DNA-vaccine-induced immunogenicity. Bone marrow (BM) chimaeric mice with TNF-deficient or TNF and TBK1-double-deficient bone marrow were immunized with a DNA vaccine encoding LacZ, as described in the Methods. Fourteen days after the second immunization, sera from the chimaeric mice (TNF-deficient (**a**, **b**) or TNF and TBK1-double-deficient mice (**c**, **d**)) were analysed for LacZ-specific IgG titre (**a**, **c**), and spleen cells were analysed for their antigen-specific IFN-γ production (**b**, **d**) as well as for their antigen-specific CD4$^+$ or CD8$^+$ T-cell proliferation (**f**) in response to LacZ antigen. Splenic dendritic cells from wild-type, control (*Tnf*$^{-/-}$) or *Tbk1*$^{-/-}$ mice were transfected with DNA vaccine by electroporation *in vitro*, and were then transferred to naive, control (*Tnf*$^{-/-}$) mice (**e**). Serum LacZ-specific IgG titre was analysed 3 weeks after dendritic-cell transfer (**e**). Data are the averages ± s.d. of three mice per group (except **f**, which is representative of two experiments); $^*P < 0.01$ against wild-type mice. DC, dendritic cell. N.D., not detected.

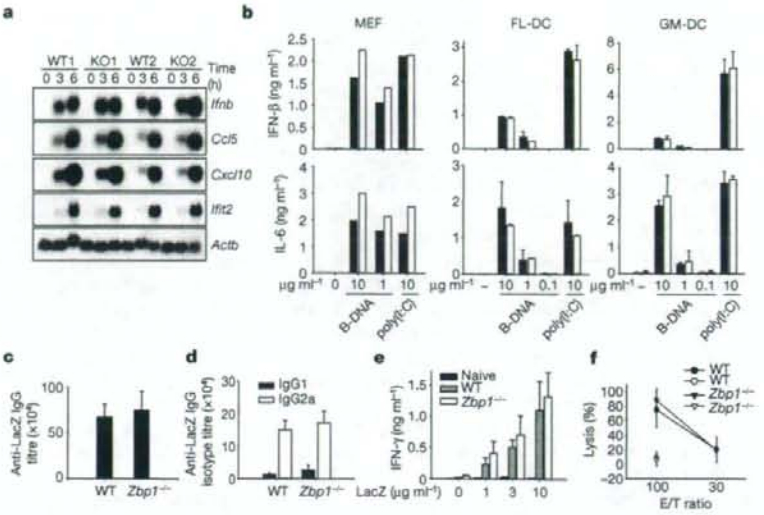

Figure 4 | Effects of ZBP1 deficiency on the innate immune activation by B-DNA and the adaptive immune responses to DNA vaccine. MEFs or bone marrow dendritic cells generated by GM-CSF (GM-DC) or Flt3 (Fms-like tyrosine kinase 3) ligand (FL-DC) were stimulated with poly(dA-dT)•poly (dT-dA) by transfection. Three and six hours later, the MEF messenger RNA expression of *Ifnb*, *Ccl5*, *Cxcl10*, *Ifit2* and *Actb* was determined by northern blot analysis (**a**), and IFN-β and IL-6 concentrations at 24 h stimulation were measured by ELISA (**b**, black bars, wild type; white bars, $Zbp1^{-/-}$). Mice lacking ZBP1 were immunized with a DNA vaccine, and two weeks after the second immunization, antigen-specific serum IgG (**c**), IgG1 and IgG2a (**d**) and IFN-γ concentration (**e**) as well as percentage lysis of the immunized mice spleen with (filled symbol) or without (open symbol) LacZ antigen (**f**) were measured. Data are the averages ± s.d. of three mice per group (except **f**, which is representative of two experiments); $^{*}P < 0.01$ against wild-type mice.

$CD8^+$ T cells after DNA vaccination. Wild-type, control ($Tnf^{-/-}$) and $Tbk1^{-/-}$ mice received $Tbk1^{+/+}$ or $Tbk1^{-/-}$ bone marrow cells and were immunized with a DNA vaccine. Proliferation of antigen-specific $CD4^+$ and CD8 T^+ cells was analysed by a CFSE (5-(and 6-)carboxyfluorescein diacetate succinimidyl ester)-based division assay using flow cytometry. In wild-type mice immunized with DNA vaccine, a significant number of antigen-specific $CD4^+$ and $CD8^+$ T cells proliferated after five days in response to LacZ antigen (Fig. 3f). Although $Tbk1^{+/+}$ chimaeric mice with $Tbk1^{-/-}$ bone marrow displayed a comparable number of proliferating antigen-specific $CD4^+$ and $CD8^+$ T cells to $Tbk1^{+/+}$ chimaeric mice with $Tbk1^{+/+}$ bone marrow, $Tbk1^{-/-}$ chimaeric mice with $Tbk1^{+/+}$ or $Tbk1^{-/-}$ bone marrow had significantly fewer proliferating antigen-specific $CD8^+$ T cells (Fig. 3f). Interestingly, $Tbk1^{-/-}$ chimaeric mice with $Tbk1^{-/-}$ bone marrow, but not those with $Tbk1^{+/+}$ bone marrow, had significantly fewer proliferating antigen-specific $CD4^+$ T cells (Fig. 3f), suggesting that non-haematopoietic cells (or radiation-resistant cells), but not bone-marrow-derived, most probably haematopoietic cells, are required for optimal antigen-specific $CD8^+$ T-cell proliferation, whereas both are required for proliferation of $CD4^+$ T cells after DNA vaccination.

Our results revealed that the TLR9 ligand activity of plasmid DNA seems minimal for its adjuvant effects, and that the double-stranded B-form of plasmid DNA might be the critical adjuvant element for DNA vaccine, especially when introduced into the cytoplasm and/or nucleus by transfection such as electroporation. We carefully excluded possibilities that RNA generated during DNA vaccination acts as an adjuvant by activating TBK1-dependent signalling, because mice deficient for TRIF, MyD88 and IPS-1 are essential for TLR3-, TLR7/8- and RIG-I/MDA5-mediated RNA recognition, respectively, were intact in inducing DNA vaccine immunogenicity (Supplementary Figs 2 and 6). We also evaluated a possibility of ZBP1 (renamed as DAI in ref. 4 which was recently demonstrated *in vitro* as a candidate for a B-DNA receptor[4], by generating its knockout mice (Supplementary Fig. 7). The results *in vitro* and *in vivo*, however, showed that ZBP1 was not essential for either innate or adaptive responses to B-DNA or DNA vaccination, respectively (Fig. 4).

Mouse embryonic fibroblasts (MEFs), two type of bone-marrow dendritic cells and macrophages responded to B-DNA and plasmid DNA as well as DNA virus infection normally to produce type-I IFNs, IL-6 and the other IFN-inducible chemokines, evaluated by northern blot, PCR with reverse transcription (RT–PCR) and ELISA (Fig. 4a, b and data not shown). In addition, ZBP1 was dispensable for inducing DNA vaccine immunogenicity including both T and B cells specific to the encoded antigen (Fig. 4c–f). Thus, ZBP1 is dispensable for both innate and adaptive immune responses to B-DNA and DNA vaccine, respectively, although its redundant role(s) is not formally excluded.

The importance of TBK1-mediated innate immune signalling for adjuvant effect, possibly through type-I IFNs, has been implicated because TRIF-dependent signalling was the major contributor to the adjuvant activity of monophosphoryl lipid A[5], and co-administration of the *Irf3*, *Irf7* or *Trif* gene as a genetic adjuvant for a DNA vaccine augmented the immunogenicity[17,27]. It will be of interest to investigate whether activation of TBK1-dependent pathway is involved in the immunogenicity of the other vaccines and to develop novel vaccine adjuvants that activate the TBK1-dependent signalling pathway. Although further studies are needed to clarify the factors including a potential DNA sensor(s) that mediates DNA-activated, TBK1-mediated innate immune activations towards adaptive immune responses or, ultimately, memory, our results may provide insights into the molecular and cellular mechanisms by which DNA vaccines trigger innate and adaptive immune responses to the encoded antigen.

METHODS SUMMARY

Mice, cells and reagents. Mutant mice lacking TNF, TBK1, IKK-i (encoded by $Ikbke^{-/-}$), TLR9, MyD88, TRIF, IFN-αβR (encoded by $Ifnr2^{-/-}$) or IPS-1, either on a 129/Ola × C57/BL6 or on a C57/BL6 background, have been described previously[20,28]. Mice lacking ZBP1 (also known as DLM-1 or DAI) were generated as described in Supplementary Fig. 7a and in the Methods. Spleen cells, MEFs and dendritic cells (GM-DCs or FL-DCs) were prepared as described previously[20]. Cells were stimulated in the presence of the indicated stimuli, and supernatants or total RNAs were collected for cytokine ELISA or for northern blot or RT–PCR, respectively, performed as described previously[20,24].

DNA vaccination. Immunization of mice (3–5 mice per group) with a DNA vaccine encoding LacZ or influenza A virus NP proteins was performed by intramuscular (i.m.) electroporation (100 μg per mouse), as described previously[17]. Mice were immunized twice, on days 0 and 28, followed by immunological assays two weeks after the second immunization unless otherwise indicated. In some experiments, splenic dendritic cells were electroporated with DNA vaccine *in vitro* and transferred intravenously into naive mice as described previously[29]. In some experiments, bone marrow was transferred approximately 1–2 months before DNA immunization as described previously[30]. All animal experiments were approved by the institutional animal care and welfare committee, and the mice were treated in accordance with the animal care guidelines of the Research Institute for Microbial Diseases, Osaka University, Japan.

Measurements of LacZ- or NP-specific immune responses. The serum anti-LacZ antibody titre was measured by ELISA as described previously[17]. A cytotoxic T lymphocyte (CTL) assay was performed as described previously[17]. Antigen (LacZ or NP)-specific IFN-γ production was analysed as described previously[17,28]. The number of Lac-Z-specific $CD8^+$ T cells was also measured using phycoerythrin (PE)-conjugated H-$2D^b$/LacZ(96–103) tetramer reagent (MBL)[28]. To analyse the proliferation of $CD4^+$ and $CD8^+$ T cells, spleen cells were stained with CFSE (Molecular Probes) and were cultured in the presence of LacZ protein (10 μg ml^{-1}) for 5 days. Spleen cells were stained with anti-CD4, anti-T cell receptor β (anti-TCRβ) and anti-CD8 antibody and analysed with a FACS Calibur instrument (BD) using CellQuest software (BD).

Statistical analysis. Differences between groups were analysed for statistical significance by the Student's *t*-test or ANOVA, using SigmaStat 3.0 software.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 25 October; accepted 29 November 2007.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements The authors thank T. Horii, K. Suzuki and S. Sasaki for suggestions, and Y. Fujita for technical support. This study was supported by Grant-in-Aid for Scientific Research (B) (to K.J.I.) from the Ministry of Education, Culture, Sports, Science and Technology in Japan.

Author Contributions K.J.I., C.C. and S.A. designed the research and analysed data. K.J.I., S.K. and C.C. performed most experiments. T.K. generated ZBP-1-deficient mice and performed the related experiments. K.M. and O.T. performed the bone-marrow-transfer experiments. S.U., T.K. and H.K. provided mutant mice. F.T. provided critical materials and advice. K.J.I., C.C. and S.A. prepared the manuscript.

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METHODS

Mice, cells and reagents. In brief, bone marrow cells were cultured in DMEM medium supplemented with 10% FCS, 100 mM 2-mercaptoethanol (2-ME) and 100 ng ml^{-1} human FLT3 ligand (PeproTech) or 10 ng ml^{-1} mouse GM-CSF (PeproTech) for 7–9 days to use as FL-DCs or GM-DCs, respectively. For liposomal transfection, DNA was mixed with Lipofectamine 2000 (Invitrogen) at a 1:1 (v/w) ratio in OptiMEM for 15 min before use in the stimulation experiments. In some experiments, splenic dendritic cells (1×10^5) were electroporated with DNA vaccine (150 μF, 300 V, BioRad) *in vitro*. Synthetic polydeoxynucleotides (B-DNA; poly(dA-dT)•(dT-dA) and poly(I:C); poly(rI)•poly(rC)) and CpG ODNA were purchased from Amersham Biosciences and Gene Design. All DNA used was tested and was free of endotoxin (<0.001 U mg^{-1} DNA).

RT–PCR. RT–PCR was performed as described previously[20]. In brief, total RNA was extracted using TRIzol reagent (Invitrogen), and then 1 μg total RNA was reverse transcribed with SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's protocol. Complementary DNA was generated by standard PCR of 28 cycles using primers as described previously.

Northern blot. MEFs were transfected with 1 μg ml^{-1} poly(dA-dT)•poly(dT-dA) for three and six hours, and total RNA was extracted using TRIzol reagent (Invitrogen). RNA was electrophorated, transferred to nylon membranes and then hybridized with the indicated cDNA probes. To detect the expression of *Zpb1* mRNA, a 372-bp fragment (503–875) was used as a probe. The same membrane was re-hybridized with a β-actin probe.

ELISA. Cell culture supernatants were collected and analysed for IFN-α, IFN-β, IFN-γ and IL-6 concentration by ELISA according to the manufacturer's protocol. ELISA kits for mouse IFN-α and IFN-β were purchased from PBL Biomedical Laboratories; those for IFN-γ and IL-6 were obtained from R&D Systems.

Measurement of serum IgG. The serum anti-LacZ antibody titre was measured by ELISA as described previously[17,28]. In brief, 96-well plates were coated with a LacZ protein at 1 μg ml^{-1} in a carbonate buffer (pH 9.6), and incubated for 18 h at 4 °C. Plates were then washed with PBS containing 0.05% Tween 20. Serial dilutions of serum in PBS/Tween containing 5% skimmed milk were applied and incubated for 2 h at room temperature. After washing, antibodies were detected using goat anti-mouse total IgG, IgG1 or IgG2a conjugated to horseradish peroxidase (Southern Biotech). After an additional washing step, the plates were stained using 3,3′,5,5′-tetramethylbenzidine (TMB, Sigma) as a substrate. The reaction was stopped with 1 M H_2SO_4 and the absorbance was measured. For serum total IgG, IgG1 and IgG2a were measured by coating the plates with anti-immunoglobulin, followed by same methods as described previously. The cut-off value was defined as the mean value of absorption of negative control serum plus two standard deviations.

Measurements of T-cell responses. Spleens were extracted 14 days after the second immunization and 5×10^5 spleen cells were seeded on 96-well plates and then stimulated with synthetic peptides (Operon) specific for LacZ (H-2D^b DAPIYTNV) or influenza A virus NP protein (I-A^b ARSALILRGSVAHKSCLPACVYGP or H-2^b ASNENMETM) at 0.1, 1 and 10 μg ml^{-1}. Seventy-two hours later, the cell culture supernatants were collected and analysed for the IFN-γ concentration by ELISA. A CTL assay was performed as described previously[17]. In brief, single-cell suspensions of spleen cells were prepared from mice 14 days after the second infection, and were seeded onto 24-well plates and cultured in RPMI complete medium in the presence of a peptide specific to NP (H-2^b; 1 μg ml^{-1}) for five days; these were then used as effector cells. Ten-thousand target EL4 cells pulsed with the same peptide were incubated with increasing numbers of effector cells for 4 h at 37 °C in 5% CO_2, and then the lactate dehydrogenase (LDH) levels in cell culture supernatants were measured according to the manufacturer's protocol (Promega). Tetramer assays for measuring NP-specific CD8 T-cell precursors were performed as described previously[17]. In brief, spleen cells were collected and incubated with H-2D^b tetramer specific to LacZ (DAPIYTNV), PE-conjugated TCRβ, allophycocyanin (APC)-conjugated CD8 and anti-CD16/32 (Fc block) for 30 min at room temperature, and then washed with PBS. Pellets were then fixed with 0.5% paraformaldehyde–PBS and analysed with FACS Calibur (Becton Dickinson) using CellQuest software (Becton Dickinson). To analyse the proliferation of CD4$^+$ and CD8$^+$ T cells, a single suspension of immunized mice spleen cells was isolated, stained with CFSE (Molecular Probes) and cultured in the presence of LacZ protein (10 μg ml^{-1}) for 5 days. Spleen cells were then collected, washed and incubated with PE-conjugated CD4, PE–Cy5-conjugated anti-TCRβ, APC-conjugated anti-CD8 antibody and anti-CD16/32 (Fc block), and were analysed as described above.

Generation of ZBP1-deficient mice. The *Zbp1* gene was isolated from genomic DNA extracted from embryonic stem cells (GSI-I) by PCR. The targeting vector was constructed by replacing a 1.6-kb fragment encoding the *Zbp1* open reading frame with a neomycin-resistance gene cassette (neo) and a herpes simplex virus thymidine kinase (HSV-TK). After the targeting vector was transfected into embryonic stem cells, G418 and gancyclovir doubly resistant colonies were selected and screened by PCR and were further confirmed by Southern blotting. Homologous recombinants were micro-injected into blastocysts from C57BL/6 female mice, and heterozygous F1 progenies were intercrossed to obtain *Zbp1*$^{-/-}$ mice.

Statistical analysis. Differences between groups were analysed for statistical significance by the Student's *t*-test or ANOVA, using SigmaStat 3.0 software.

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Nonself RNA-Sensing Mechanism of RIG-I Helicase and Activation of Antiviral Immune Responses

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DOI 10.1016/j.molcel.2007.11.028

SUMMARY

A DExD/H protein, RIG-I, is critical in innate antiviral responses by sensing viral RNA. Here we show that RIG-I recognizes two distinct viral RNA patterns: double-stranded (ds) and 5′ppp single-stranded (ss) RNA. The binding of RIG-I with dsRNA or 5′ppp ssRNA in the presence of ATP produces a common structure, as suggested by protease digestion. Further analyses demonstrated that the C-terminal domain of RIG-I (CTD) recognizes these RNA patterns and CTD coincides with the autorepression domain. Structural analysis of CTD by NMR spectroscopy in conjunction with mutagenesis revealed that the basic surface of CTD with a characteristic cleft interacts with RIG-I ligands. Our results suggest that the bipartite structure of CTD regulates RIG-I on encountering viral RNA patterns.

INTRODUCTION

Viral infections provoke various host responses, including early innate and subsequent adaptive immune responses. Innate responses are genetically programmed to detect a wide range of viral infections and activate a set of genes encoding humoral factors known as cytokines and chemokines. The most important cytokines in viral infection are type I interferons (IFNs), which are secreted and confer antiviral activity to the host (Joklik, 1991; Samuel, 2001). Moreover, IFN and other cytokines critically contribute to the successful activation of acquired immunity. Genomes of higher eukaryotes encode receptors for detecting pathogen molecules called pathogen-associated molecular patterns (PAMPs). Toll-like receptors (TLRs) detect PAMPs of extracellular origin either at the cell surface or in the endosome (Akira et al., 2006). TLR3, TLR7/8, and TLR9 are receptors for viral polynucleotides and trigger the production of cytokines. Unlike bacteria and fungi, viruses strictly require host cells, in which they replicate; therefore, cytoplasmic receptors to detect viral infection and subsequent replication have been hypothesized to exist since the discovery of the IFN system. Recent screening of an expression cDNA library identified an RNA helicase, RIG-I, as a cytoplasmic receptor for viral replication (Yoneyama and Fujita, 2007; Yoneyama et al., 2004).

RIG-I consists of an N-terminal *c*aspase *r*ecruitment *d*omain (CARD), a domain with the signature of DExD/H box helicase (helicase domain), and a C-terminal repression domain (RD) (Saito et al., 2007; Yoneyama et al., 2004). Functional analyses revealed that the helicase domain and RD are required for detecting viral RNA and the CARD triggers the activation of a downstream signaling cascade, including the activation of transcription factors, NF-κB, interferon regulatory factor (IRF)-3, and IRF-7. RD interacts with the helicase domain (helicase linker region) and CARD. A model is proposed that, in the absence of dsRNA, RIG-I adopts a "closed" conformation but upon binding to dsRNA changes to an "open" structure, exposing the CARD. Human and mouse genomes encode another CARD-containing helicase, termed MDA5, and a structurally related helicase without a CARD, LGP2 (Yoneyama et al., 2005). Studies in knockout mice have revealed that both RIG-I and MDA5 function as cytoplasmic sensors and are physiologically critical for antiviral defense (Gitlin et al., 2006; Kato et al., 2005, 2006). Moreover, RIG-I and MDA5 function differently in the recognition of RNA viruses (Gitlin et al., 2006; Kato et al., 2006).

IFN-inducing compounds have been extensively screened in the past, and it was shown that dsRNA, particularly the synthetic cohomopolymer poly I:C, exhibits comparative activity to viral infections (Joklik, 1991; Samuel, 2001). It has been widely accepted that dsRNA is a viral PAMP because it is normally absent in mammalian cells, due to the absence of RNA-dependent RNA polymerase. Poly I:C not only activates TLR3, but when directly introduced into the cytoplasm, preferentially activates MDA5 (Alexopoulou et al., 2001; Kato et al., 2006). Conversely, dsRNA with a nonbiased sequence, prepared by in vitro transcription and annealing, preferentially activates RIG-I (Kato et al., 2006). Recently, it was discovered that ssRNA with 5′ triphosphate (5′ppp ssRNA) is a ligand for RIG-I (Hornung et al.,