Figure 1. In Vitro Reconstitution of the RIG-I Pathway and Regulation of RIG-I by RNA and Ubiquitination

(A) Purification of RIG-I protein from Sendai virus-infected (+SeV) or untreated (−SeV) HEK293T cells stably expressing RIG-I containing a C-terminal Flag epitope.

(B) Homogenates of culture cells were centrifuged at 1,000 x g for 30 min, followed by centrifugation at 5,000 x g for 30 min. The supernatants were then used for ubiquitination assays.

(C) The ubiquitination assays were performed with P5 (-SeV) and S5 (+SeV) proteins, and the ubiquitination of RIG-I was analyzed by native gel electrophoresis.

(D) Ubiquitination assay with His6-RIG-I and ubiquitin (Ub) labeled with 35S. The ubiquitination of RIG-I was detected by Coomassie Blue staining.

(E) The expression of IRF3 was analyzed by native gel electrophoresis after ubiquitination assay.

(F) The expression of IRF3 was analyzed by native gel electrophoresis after ubiquitination assay.

(G) The expression of IRF3 was analyzed by native gel electrophoresis after ubiquitination assay.

(H) The expression of IRF3 was analyzed by native gel electrophoresis after ubiquitination assay.

To dissect the signaling mechanisms in the RIG-I pathway, we establish a cell-free system that mimics viral infection in intact cells. This in vitro reconstitution system contains recombinant RIG-I protein, mitochondria, cytosol, RNA, and ubiquitination enzymes including TRIM25. We demonstrate that 5’-pppRNA and dsRNA, which resemble viral RNA, can activate the entire RIG-I signaling cascade in vitro, leading to activation of IRF3 and NF-κB. Interestingly, we find that upon incubation with both RNA and unanchored K63-polyubiquitin (polyUb) chains, RIG-I is fully activated to cause IRF3 dimerization. We show that the N terminus of RIG-I, termed RIG-I(N), which contains tandem CARDs, binds specifically to K63-polyUb chains, and that this binding is both necessary and sufficient to activate RIG-I(N). Furthermore, we devise a protocol to isolate endogenous ubiquitin chains associated with RIG-I in a human cell line and demonstrate that these are unanchored K63-polyUb chains with potent ability to activate RIG-I. Our results support a model in which RIG-I binds to two ligands, viral RNA and endogenous ubiquitin chains, in a sequential manner through the C-terminal RD and N-terminal CARDs, respectively. The binding of both ligands is required for full activation of RIG-I.

**RESULTS**

**In Vitro Reconstitution of a RIG-I Signaling Cascade from RNA to IRF3 Activation**

To reconstitute the RIG-I pathway in vitro, we first examined whether RIG-I isolated from virus-infected cells could cause IRF3 activation in the presence of mitochondria, which contain MAVS, and cytosolic extracts, which contain TBK1 and many other components. A hallmark of IRF3 activation is its dimerization, which depends on its phosphorylation by TBK1 and can be measured by native gel electrophoresis (Yoneyama et al., 2002). To set up the assay, HEK293T cells stably expressing full-length RIG-I with a C-terminal Flag tag were infected with Sendai virus (SeV) or uninfected, then RIG-I was affinity purified (Figure 1A). Crude mitochondria (P5) and cytosolic extracts (S5) were prepared from uninfected HEK293T cells by differential centrifugation (Figure 1B), and 35S-labeled IRF3 protein was synthesized by in vitro translation. As shown in Figure 1C, dimerization of IRF3 was observed when RIG-I from virus-infected cells was incubated with mitochondria (P5) and cytosolic extracts (S5) in the presence of ATP (lane 3). In contrast, RIG-I from mock-treated cells did not promote IRF3 dimerization (lane 2). The activation of IRF3 required both cytosol and mitochondria (lanes 5 and 6). Mitochondria isolated from cells depleted of MAVS by RNAi could not support IRF3 dimerization (Figure S1A available online), confirming that MAVS is essential for activating the downstream pathway in this in vitro assay. When RIG-I was isolated from cells depleted of TRIM25 by RNAi, its ability to promote IRF3 dimerization in the in vitro assay was greatly reduced (Figure S1B), supporting an important role of TRIM25 in RIG-I activation. As we have shown recently, mitochondria isolated from virus-infected cells activated IRF3 in the absence of RIG-I (Figure 1C, lane 1) (Zeng et al., 2009).

Transfection of cells with synthetic RNAs, such as the dsRNA analog poly(I:C) and 5’-pppRNA, potently induces IRF3 dimerization. To test if RNA could activate the entire RIG-I pathway in vitro, we expressed and purified full-length RIG-I from insect cells (Sf9; Figure 1D, lower left) and incubated it with ATP and a 79 nucleotide (79 nt) 5’-pppRNA (Figure S1C). This RNA strongly induced IFN-β when transfected into HEK293T-IFN-β-luciferase reporter cells (Figure S1D). However, incubation of this RNA with the recombinant RIG-I protein did not cause IRF3 dimerization in the reconstituted system (Figure 1D, lane 1 in lower right panel). As ubiquitination has been shown to be important in the RIG-I pathway, we incubated RIG-I with E1, Ubc5c (E2), TRIM25 (E3), and ubiquitin together with ATP and RNA. Remarkably, this condition caused RIG-I to activate the entire pathway, resulting in IRF3 dimerization (Figure 1D, lane 3 in lower right panel). A K270A mutation in the ATPase domain of RIG-I, which abrogates the ability of RIG-I to induce interferons in vivo (Yoneyama et al., 2004), also abolished its ability to induce IRF3 dimerization in vitro. Poly(I:C) stimulated IRF3 dimerization in this reconstituted system in a manner dependent on ubiquitin, E1, Ubc5c, TRIM25, and RIG-I (Figure 1E). Activation of IRF-1 was also observed with another 5’-pppRNA containing 135 nucleotides (135 nt) (Figure 1F). Diphosphorylation of the 5’-pppRNAs with shrimp alkaline phosphatase (SAP) destroyed their ability to activate IRF3 in vitro (Figure 1F) and IFN-β induction in HEK293T cells (Figure S1D). In contrast, treatment of poly(I:C) with SAP did not inhibit its activity, consistent with the previous report that this

(B) Procedures for isolation of crude mitochondria (P5) and cytosol (S5) by differential centrifugation.
(C) Virus-activated RIG-I induced IRF3 dimerization in vitro. The reconstitution reaction contained mitochondria (P5), RIG-I isolated from virus-infected or untreated cells, cytosolic extracts (S5) from uninfected cells, 35S-IRF3, and ATP. Dimerization of IRF3 was analyzed by native gel electrophoresis.
(D) In vitro activation of RIG-I by 5’-pppRNA and ubiquitination. His6-tagged RIG-I (wild-type or WT) or its ATPase mutant (K270A) was purified from Sf9 cells (lower left panel), then incubated with 5’-pppRNA (79 nucleotides), ATP, and ubiquitination enzymes as outlined in the diagram. After incubation, aliquots of the reaction mixtures were further incubated with mitochondria (P5) and cytosol (S5) from uninfected cells together with 35S-IRF3 and ATP, and then IRF3 dimerization was analyzed by native gel electrophoresis.
(E) In vitro activation of RIG-I by poly(I:C) and ubiquitination. Similar to (D), except that poly(I:C) was used and the dependency on ubiquitin and ubiquitination enzymes was tested.
(F) The role of 5’-triphosphate for RNA to activate RIG-I in vitro. Similar to (D), except that the RNA was pretreated with or without shrimp alkaline phosphatase (SAP).
(G) 5’-pppRNA and viral RNA are potent activators of the RIG-I pathway. Total RNA was extracted from HEK293T cells from viral-infected or untreated HEK293T cells, then incubated with RIG-I as in (D), followed by IRF3 dimerization assay (lanes 1–3). To measure the potency of 5’-pppRNA in RIG-I activation, increasing amounts of the RNA (135 nt) (0.07 to 70 nM, at 3-fold increments) were incubated with RIG-I in the presence (lanes 12–18) or absence of cellular RNA from uninfected cells (lanes 5–11), then IRF3 dimerization assay was performed.

See also Figure S1.
Figure 2. K63 Polyubiquitination Is Essential for RIG-I Activation

(A) Ubc5 and Ubc13/Uev1a activate RIG-I in vitro. RIG-I was incubated with E1, different E2s as indicated, TRIM25, ubiquitin, RNA, and ATP, followed by IRF3 dimerization assay as described in Figure 1D. The E2 proteins (2 μg) were analyzed by Coomassie blue staining (lower panel).

(B) Ubc5 and Ubc13 are required for viral activation of MAVS in the mitochondria. U2OS cells stably integrated with tetracycline-inducible shRNA against Ubc5b/c and Ubc13 were treated with or without tetracycline (Tet). After viral infection for the indicated time, mitochondrial fraction (P5) was prepared and the MAVS activity was measured by IRF3 dimerization assay.
dsRNA analog activates RIG-I and MDA5 in a manner independent of 5’-triphosphate (Kato et al., 2008). As expected, the DNA poly(dI:dC) did not activate the RIG-I pathway in vitro or in cells (Figure 1F and Figure S1D). In vitro reconstitution of the RIG-I pathway also led to site-specific phosphorylation of IRF3 and IκBα (Extended Results and Figures S1E–S1G).

To determine if RIG-I could be activated by naturally occurring viral RNA, we incubated purified RIG-I protein with total RNA from HEK293T cells infected with Sendai virus. Indeed, RNA from virus-infected but not mock-treated cells stimulated RIG-I to activate IRF3 (Figure 1G, lanes 1–3). To estimate the sensitivity of 5’-pppRNA detection by RIG-I, we incubated RIG-I with different amounts of 5’-pppRNA in the presence or absence of HEK293T cellular RNA (Figure 1G, lanes 4–18). The half-maximal effective concentration (EC50) of the 5’-pppRNA was estimated at 1.2 nM and 0.4 nM in the absence and presence of the cellular RNA, respectively (Figure 1H). It is not clear how the cellular RNA enhances the potency of 5’-pppRNA, but one possibility is that these RNAs reduce the nonspecific loss of very small amounts of 5’-pppRNA in the reactions. In any case, the fact that the presence of a large excess of cellular RNA does not interfere with the specific recognition of 5’-pppRNA by RIG-I underscores the remarkable specificity of viral RNA detection by RIG-I. Assuming that the cytoplasm of a human cell has a volume of ~500 μm3, we estimated that less than 20 molecules of viral RNA in a cell (equivalent to ~0.07 nM) are sufficient to trigger detectable IRF3 dimerization. Thus, our in vitro reconstitution recapitulates the entire RIG-I pathway with exquisite sensitivity and specificity for 5’-pppRNA (see Discussion).

**Ubc5 and Ubc13 Are Required for the Activation of RIG-I and MAVS**

Ubc5 is a family of E2s comprising highly homologous isoforms (Ubc5a, b, and c; putative Ubc5d in human) that catalyze the synthesis of polyUb chains linked through various lysines of ubiquitin, including K63 (Xu et al., 2009). In contrast to Ubc5, the E2 complex consisting of Ubc13 and Uev1A is highly specific in synthesizing K63-polyUb chains (Deng et al., 2000; Hofmann and Pickart, 1999). To determine the E2 involved in RIG-I activation, we examined a panel of E2s for their ability to stimulate RIG-I in the presence of TRIM25 and RNA (Figure 2A). The Ubc5 isoforms (Ubc5a, b, and c) and Ubc13/Uev1A were capable of stimulating RIG-I to promote IRF3 dimerization. In contrast, Ubc3, Ubc7, and E2-25K had no activity (Figure 2A), despite the ability of these E2s to form thioesters with ubiquitin (data not shown; see also Zeng et al., 2009). To test if Ubc5 and/or Ubc13 are involved in the activation of MAVS by RIG-I, we established human osteosarcoma U2OS cell lines stably integrated with tetracycline-inducible shRNA vectors targeting both Ubc13 and two isoforms of Ubc5 (Ubc5b and c) (Xu et al., 2009). As shown in Figure 2B, the mitochondria from the cells depleted of Ubc13, Ubc5b, and Ubc5c lost the ability to activate IRF3. These results suggest that Ubc5a and Ubc5d, which are not targeted by the Ubc5 shRNAs, play a minor role in IRF3 activation in U2OS cells. RNAi of Ubc13 or Ubc5 alone partially inhibited the IRF3-stimulatory activity of the mitochondria from virus-infected cells (Figure S2A; Figure S3 in Zeng et al., 2009). These results suggest that both Ubc5 and Ubc13 are involved in the activation of MAVS in the mitochondria by RIG-I in response to viral infection.

**K63 Polyubiquitination Is Essential for the Activation of RIG-I and MAVS**

To determine if K63 of ubiquitin is required for RIG-I activation in the in vitro system, we incubated various ubiquitin lysine mutants with RIG-I in the presence of RNA, ATP, E1, TRIM25, and Ubc5c or Ubc13/Uev1A. The reaction mixture was then incubated with the mitochondrial fraction (P5) to activate MAVS. The activated mitochondria were isolated and then tested for their ability to stimulate IRF3 dimerization in the presence of cytosolic extracts (Figure 2C). The ubiquitin proteins containing a lysine at position 63 (wild-type, K48R and K63-only) were capable of activating RIG-I, whereas those containing a substitution at K63 (K63R, K48-only, KO, and methylated ubiquitin) had no activity (Figure 2C; see Figure S2C for an illustration of ubiquitin mutants). Interestingly, although Ubc5c and TRIM25 catalyze the synthesis of polyUb chains from K63R (Figure S2D, lane 2), these chains did not stimulate RIG-I (Figure 2C, lane 2), indicating that K63-polyUb chain synthesis is specifically required for RIG-I activation in vitro.

To investigate the role of K63 polyubiquitination in the activation of MAVS in cells, we used recently developed U2OS cell lines in which endogenous ubiquitin is replaced with wild-type or K63R mutant of ubiquitin through a tetracycline-inducible strategy (Xu et al., 2009). As shown in Figure 2D, depletion of endogenous ubiquitin severely impaired the ability of the mitochondria to promote IRF3 dimerization, but this activity was rescued by the expression of the wild-type ubiquitin transgene. In contrast, when the endogenous ubiquitin was replaced with the K63R mutant, the mitochondria isolated from these cells failed to activate IRF3 in the in vitro assay, strongly suggesting that K63 polyubiquitination is essential for viral activation of MAVS in the mitochondria.

**K63-Polyubiquitin Chains Activate RIG-I through Its N-terminal CARDs**

The N terminus of RIG-I contains tandem CARDs, which, when overexpressed in mammalian cells, constitutively activate IRF3 (Yoneyama et al., 2004). To determine if this N-terminal fragment
[RIG-I(N), see Figure 3A] could activate IRF3 in vitro, we expressed the protein in *E. coli* and purified it to near homogeneity (Figure 3B). When the protein was incubated with mitochondria and cytosolic extracts, it did not promote IRF3 dimerization (Figure 3C, lane 2). However, when the incubation mixtures contained ubiquitination components, including E1, Ubc5c, TRIM25, and ubiquitin, robust IRF3 dimerization was detected even in the absence of RNA (lane 3).

To determine if ubiquitination of RIG-I(N) is required for IRF3 activation in the in vitro system, we carried out a ubiquitination reaction in the presence of different pairs of E2 and E3 and then treated the reaction mixture with the chemical N-ethylmaleimide (NEM), which alkylates the active site cysteine of E1 and E2. The reaction mixtures were then incubated with GST-RIG-I(N) before further incubation with the mitochondrial and cytosolic fractions to measure IRF3 dimerization (Figure 3D). Under these conditions, RIG-I(N) was not ubiquitinated because E1 and E2 had been inactivated by NEM. Remarkably, when TRIM25 or another RING domain E3 TRAF6 was incubated with either Ubc13/Uev1A or Ubc5c, robust IRF3 dimerization was detected (lanes 2–5).

When K48 polyUb chains were synthesized by Ubc3 and an E3 complex consisting of Skp1, Cul-1, Rbx1, and β-TrCP2 (SCF-β-TrCP2), these chains did not activate the RIG-I pathway. Similarly, linear polyUb chains generated by Ubc5c and an E3 complex consisting of HOIL-1 and HOIL-1L (termed LUBAC) did not lead to significant IRF3 dimerization. To further test if...
ubiquitin chains of other linkages could support IRF3 dimerization, we used a panel of ubiquitin mutants harboring a single lysine (Figure 3E). Although all the ubiquitin mutants were capable of forming polyUb chains in the presence of UbC5c and TRIM25, the only mutants capable of supporting IRF3 activation were those containing a lysine at position 63 (K63 only and His6-K63 only). Collectively, these results clearly demonstrate that polyUb chains containing the K63 linkage, but not other linkages, specifically activate the RIG-I pathway. We also found that unanchored polyUb chains, but not ubiquitinated TRIM25, mediate RIG-I activation (Extended Results and Figure S3).

**Short, Unanchored, K63-Linked Ubiquitin Chains Activate RIG-I**

To determine if short ubiquitin chains are capable of activating RIG-I, we incubated GST-RIG-I(N) with a ubiquitin polymer containing four units of ubiquitin linked through K63 (K63-UB4; Figure 4A). Strikingly, incubation of GST-RIG-I(N) with K63-UB4 led to robust activation of IRF3 in this assay (lane 2), whereas GST-RIG-I(N) or K63-UB4 alone had no activity (lanes 1 and 3). We then tested a panel of ubiquitin chains of different lengths and linkages for their ability to activate RIG-I(N) (Figure 4B). Interestingly, K63-UB chains containing more than two ubiquitin moieties potentently activated RIG-I(N) (Figure 4B, lanes 3–8). K63-UB2 had very weak activity (lane 2), whereas monomeric ubiquitin and K48-linked ubiquitin chains were inactive (lanes 11–14). Ub4 containing mixed linkages of K48 and K63 also had greatly reduced activity (lanes 9–10). We then carried out titration experiments to quantify the relative potency of different ubiquitin chains in the activation of RIG-I(N) (Figures 4C and 4E). Similar titration experiments were also carried out using full-length RIG-I in the presence of 5′-pppRNA (Figures 4D and 4F). Among the ubiquitin chains tested, K63-UB4 was the most potent activator, with an EC50 of ~25 nM for RIG-I(N) activation. The potency of K63-UB3 was about 27-fold less (EC50 = 680 nM), whereas K63-UB2 was nearly inactive even at the highest concentration (1 μM). Ubiquitin, K48-UB3, and linear-UB3 were inactive at all the concentrations tested. These results demonstrate that short, unanchored K63-ubiquitin chains containing at least three ubiquitin moieties are potent and specific activators of RIG-I.

**The Tandem CARDs of RIG-I Bind to K63-Ubiquitin Chains**

The potent activation of RIG-I(N) by K63-ubiquitin chains implies that the N terminus of RIG-I binds to these chains. Indeed, GST-RIG-I(N) was able to pull down K63-UB3, but not K48-UB3 or linear Ub3 (Figure 5A). Both CARDs are required for ubiquitin binding because the fragments containing residues 11–200 or 2–180 of RIG-I failed to bind K63-UB4 or activate IRF3 (Figures 5B and 5C; see also Figures S5A and S5B). We also generated a RIG-I(N) protein containing a T55I mutation, which was previously shown to render RIG-I defective in interferon induction (Sumpter et al., 2005). Interestingly, T55I mutation severely impaired the ability of RIG-I(N) to bind K63-UB4 and activate IRF3 (Figure 5C, lane 10), suggesting that the signaling defect of this mutant may be due to its failure to bind K63-ubiquitin chains.

To determine if ubiquitin chain binding induces oligomerization of RIG-I(N), we incubated RIG-I(N) with K63-UB4 and then performed gel filtration analysis using Superdex-200. K63-UB4 and RIG-I(N) have predicted molecular masses of ~200 and ~24 kDa, respectively; however, they formed an active complex with an apparent molecular mass of ~200 kDa (Figure 5D). When each protein alone was fractionated on the same column, K63-UB4 and RIG-I(N) eluted at positions corresponding to ~25 and ~50 kDa, respectively. These results suggest that the binding of K63-UB4 to RIG-I(N) promotes the formation of an oligomeric complex (e.g., a tetramer).

**Full-length RIG-I Binds to K63-Ubiquitin Chains in a Manner that Depends on RNA and ATP**

As full-length RIG-I is regulated by RNA binding and ATP hydrolysis, we tested its binding to K63-ubiquitin chains in the presence or absence of RNA and ATP. Immunoprecipitation of His6-RIG-I-Flag led to coprecipitation of K63-UB4 and long K63-polyUb chains in the presence of 5′-pppRNA and ATP (Figure 5E, lanes 2 and 6). This binding was lost when the RNA was absent or when EDTA was added to chelate Mg2+, which is required for ATP binding. Two different mutations that abrogate the ATPase activity of RIG-I, K270A and D372N, also disrupted the ability of RIG-I to bind K63 polyUb (Figure 5F).

To determine if RIG-I binds to RNA and K63 polyUb in a sequential manner, we incubated RIG-I with 5′-pppRNA and K63 polyUb in reciprocal orders (Figure 5G). If RIG-I was preincubated with 5′-pppRNA in the first step and then with K63 polyUb in the second step, it was capable of activating IRF3 dimerization in the reconstitution assay (lane 4). In contrast, if RIG-I was preincubated with K63 polyUb first, then immunoprecipitated to remove unbound polyUb, its subsequent incubation with 5′-pppRNA failed to activate it (lane 6). Thus, the binding of RIG-I to RNA precedes its binding to K63 polyUb.

**Binding of K63-Ubiquitin Chains Is Necessary for RIG-I Activation**

Previous studies have shown that RIG-I is ubiquitinated at K172 and that a mutation of this residue (K172R) impairs its ability to induce IFN-β (Gack et al., 2007). Consistent with an important role of K172 in RIG-I activation, we found that GST-RIG-I(N) containing the K172R mutation failed to activate IRF3 in the in vitro reconstitution assay (Figure 6A, lanes 4–6). A GST-RIG-I(N) protein containing mutations at six lysines (K99, 169, 172, 181, 190, 193; herein referred to as 6KR) was also inactive (lanes 10–12), but this activity was rescued by keeping the lysine at position 172 (K172-only; lanes 7–9). Interestingly, the K172R, 6KR, and T55I mutants were greatly compromised in their ability to bind K63 polyUb, whereas K172-only was fully capable of binding to these chains (Figure 6B). These results show that the IRF3-activating function of RIG-I correlates well with its ubiquitin-binding activity.

To determine if ubiquitination or ubiquitin binding of RIG-I is important for its activation in cells, we expressed GST-RIG-I(N) and various mutants in HEK293-IFN-β-luciferase reporter cells (Figure 6C), then pulled down these proteins with glutathione

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**Cell** 141, 315–330, April 16, 2010 ©2010 Elsevier Inc.
Figure 4. Short, Unanchored K63-Ubiquitin Chains Potently Activate RIG-I

(A) K63-Ub4 activates RIG-I(N). GST-RIG-I(N) (0.2 μM) was incubated with or without K63-Ub4 (0.3 μM), followed by IRF3 dimerization assay as outlined.

(B) K63, but not K48, ubiquitin chains activate RIG-I(N). Ubiquitin chains of defined lengths and linkages were incubated with GST-RIG-I(N), then IRF3 dimerization assay was carried out as in (A). The quality of the ubiquitin chains was evaluated by immunoblotting (lower panel) or silver staining (see Figure S4).

(C) K63-ubiquitin chains potently activate RIG-I(N) in a chain length- and linkage-dependent manner. Different concentrations of Ub chains (0.01–1 μM) or ubiquitin (0.1–10 μM) were tested for RIG-I(N) activation using the IRF3 dimerization assay.

Se phosphate-buffered saline (PBS) or a more stringent buffer containing RIG-I, the Sepharose beads were washed with either phosphate-buffered saline (PBS) or a more stringent buffer containing 0.1% SDS and 1% deoxycholate (RIPA; Figure 6D). Immunoblotting with a GST antibody showed that whereas the wild-type GST-RIG-I(N) was conjugated by ubiquitin chains, no apparent ubiquitination of the mutants, including K172R and K172-only, was detected (Figure 6D, lower panel). Immunoblotting of the pull-down proteins with a ubiquitin antibody showed an interesting difference depending on the wash buffers (upper panel). Whereas GST-RIG-I(N) containing K172-only was able to pull down polyUb chains from the cells when the beads were washed with PBS (lane 9), these chains were largely washed away with the RIPA buffer (lane 4), indicating that this mutant was defective in ubiquitination but retained the ability to bind polyUb chains. Because GST-RIG-I(N)-K172-only was active in inducing IFN-β (Figure 6C), these results suggest that polyUb binding by RIG-I is responsible for its function. The other RIG-I mutants, including K172R, 6KR, and T551L, were unable to bind ubiquitin chains in cells, consistent with their inability to induce IFN-β.

To further evaluate whether RIG-I ubiquitination is required for its function, we treated GST-RIG-I(N) isolated from HEK293T cells with a deubiquitination enzyme (DUB), which contains the OTU domain of the Crimean Congo hemorrhagic fever virus (CCHFV) large (L) protein (Figure 6E) (Frias-Staheli et al., 2007; Xia et al., 2009). The viral OTU (vOTU) can cleave polyUb chains from target proteins as well as unanchored polyUb chains. In contrast, another DUB, isopeptidase T (IsoT), only cleaves unanchored polyUb chains (Reyes-Turcu et al., 2006). Following treatment with vOTU or IsoT, GST-RIG-I(N) was tested for its ability to activate IRF3 in the in vitro reconstitution assay. Unlike GST-RIG-I(N) expressed in E. coli, which does not have the ubiquitin system, the same protein expressed in HEK293T cells was able to activate IRF3 without preincubation with ubiquitin chains (Figure 6E, bottom panel), probably because RIG-I(N) isolated from human cells is already bound to endogenous polyUb chains (e.g., lane 9 in Figure 6D). Interestingly, although vOTU removed most of the ubiquitin chains from GST-RIG-I(N) (Figure 6E, lanes 6 and 9 in upper panel), the deubiquitinated RIG-I protein was as active in causing IRF3 dimerization as the protein that was mock treated (lanes 1–4 and 9–12, lower panel). These results suggest that ubiquitination of the RIG-I CARDs may be dispensable for its activity.

Unanchored K63-Polyubiquitin Chains Isolated from Human Cells Are Potent Activators of RIG-I

In Figure 6E, we noted that IsoT treatment did not remove much of the polyUb chains from GST-RIG-I(N) (lane 5 in upper panel), indicating that RIG-I(N) protects the ubiquitin chains from degradation by IsoT (see Extended Results and Figure S6). The protection of polyUb by RIG-I(N) offers an opportunity to detect the otherwise labile ubiquitin chains in cells but poses another challenge of how to release these chains while preserving their functions. We devised a protocol to isolate the RIG-I-bound ubiquitin chains from HEK293T cells by employing two strategies (Figure 7A). First, we lysed the cells in the presence of NEM to inactivate most of the deubiquitination enzymes. Ubiquitin contains no cysteine, therefore evading modification by NEM. Second, as ubiquitin is known to be relatively stable at high temperatures, we heated the RIG-I(N)-polyUb complex at different temperatures to identify a condition that denatures RIG-I(N) but preserves the structure and function of ubiquitin chains. Following centrifugation that precipitates denatured protein aggregates, the supernatant is expected to contain polyUb chains and can be tested for its ability to activate IRF3 in the presence of fresh RIG-I(N). Because GST-RIG-I(N)-K172-only appears to capture more polyUb chains, we expressed this protein in HEK293T cells (Figure 7B). In control experiments, we incubated GST-RIG-I(N)-K172-only protein with K63-polyUb chains in vitro, carried out GST pull-down, and then heated the complex (lanes 1–6). Five minutes of heating at 70°C-80°C was sufficient to inactivate the RIG-I protein (lane 1, 3, and 5) and release polyUb chains into the supernatant, which were capable of activating IRF3 when supplied with fresh GST-RIG-I(N) (lanes 2, 4, and 6). When the GST-RIG-I(N) protein expressed in HEK293T cells was heated at 70°C, it was still capable of activating IRF3 in the absence of fresh RIG-I(N) (lane 7), indicating that the activated RIG-I(N) complex was resistant to NEM and 70°C treatment. However, raising the temperatures to 75°C and 80°C inactivated the GST-RIG-I(N) protein (lanes 9 and 11). Strikingly, the supernatant containing polyUb chains released from the RIG-I(N) complex at the high temperatures were capable of activating IRF3 when supplied with fresh GST-RIG-I(N) (lanes 10 and 12).

To determine whether the supernatant of the heated GST-RIG-I(N) complex contained unanchored polyUb chains and whether these chains were responsible for activating the RIG-I pathway, we performed two sets of experiments. First, we incubated the supernatant with E1 to determine if the endogenous ubiquitin chains could form thioesters with E1. Indeed, in the presence of E1 and ATP, substantial fractions of both synthetic and endogenous ubiquitin chains formed thioesters with E1. In control experiments, we incubated the supernatant with IsoT to determine if the endogenous ubiquitin chains could form thioesters with IsoT. Interestingly, although IsoT removed most of the ubiquitin chains from GST-RIG-I(N) (Figure 7E, lanes 6 and 9 in upper panel), the deubiquitinated RIG-I protein was as active in causing IRF3 dimerization as the protein that was mock treated (lanes 1–4 and 9–12, lower panel). These results suggest that ubiquitination of the RIG-I CARDs may be dispensable for its activity.
Figure 5. RIG-I CARDs Bind K63-Ubiquitin Chains, and This Binding in Full-Length RIG-I Is Regulated by RNA and ATP

(A) RIG-I(N) binds specifically to K63-ubiquitin chains. GST or GST-RIG-I(N) was incubated with Ub3 containing K63, K48, or linear linkage, then pulled down with glutathione Sepharose, followed by immunoblotting. Input represents 10% of Ub3 used in the pull-down experiments.

(B) Diagram of RIG-I N terminus containing the tandem CARDs and various deletion and point mutants. The table on the right summarizes the results in (C).

(C) Both CARDs of RIG-I are required for polyUb binding and IRF3 activation. GST-RIG-I(N) and various mutants were incubated with K63-Ub4. One microliter aliquot of each mixture was used for IRF3 dimerization assay (upper panel), and the remainder was pulled down with glutathione Sepharose followed by immunoblotting with a Ub antibody (middle panel). The GST-RIG-I(N) and the mutant proteins (2 μg each) were analyzed by Coomassie blue staining (lower panel). See also Figure S5.

(D) RIG-I(N) and K63-Ub4 form an active high-molecular-weight complex. RIG-I(N) was incubated with K63-Ub4 and then the mixture was fractionated on Superdex-200. Aliquots of the fractions were assayed for their ability to stimulate IRF3 dimerization, whereas other aliquots were subjected to immunoblotting with antibodies against RIG-I and ubiquitin. K63-Ub4 or RIG-I(N) alone was also analyzed by gel filtration on the same column (lower two panels).
40 kDa band, disappeared in the IsoT-treated sample, indicating that these are unanchored ubiquitin polymers containing approximately 5–10 ubiquitin moieties. However, most of the high-molecular-weight bands remained resistant to IsoT treatment, suggesting that they represent ubiquitin chains conjugated to some protein targets. Because the IsoT treatment completely abolished the activity of the heat supernatant to promote IRF3 dimerization (Figure 7C, lane 8 on left panel), the high-molecular-weight ubiquitin conjugates remaining after IsoT treatment apparently did not contribute to RIG-I activation.

To determine if the endogenous unanchored polyUb chains are linked through K63 of ubiquitin, we treated the heat-resistant supernatant with the K63-specific deubiquitination enzyme CYLD. This treatment markedly diminished the ability of the supernatant to activate RIG-I (Figure 7D, left panel). CYLD also destroyed the ubiquitin chains with molecular masses in the range of 40–75 kDa, which could be detected with an antibody specific for the K63-ubiquitin linkage (Figure 7D, right panel).

TRIM25 and CYLD have previously been shown to be important for the activation and inactivation of the RIG-I pathway, respectively (Friedman et al., 2008; Gack et al., 2007; Zhang et al., 2008). To determine if these enzymes are involved in the synthesis and disassembly of endogenous K63-ubiquitin chains, we used siRNA to knock down the expression of TRIM25 or CYLD in HEK293T cells, then isolated the endogenous ubiquitin chains as outlined in Figure 7A. Immunoblotting experiments showed that depletion of TRIM25 diminished, whereas depletion of CYLD enhanced, the production of endogenous polyUb chains, which in turn activated IRF3 (Figure 7E).

To evaluate the potency of endogenous polyUb chains in activating RIG-I, we carried out titration experiments using different amounts of heat-resistant supernatants isolated from HEK293T cells (Figure 7F). The amount of endogenous K63-Ub6 was estimated by comparing to synthetic K63-Ub6 using semiquantitative immunoblotting (Figure S7B). Because K63-Ub6 is the dominant species of endogenous polyUb chains, its concentration was used to calculate the EC_{50} for stimulating RIG-I. Remarkably, the EC_{50} for the endogenous ubiquitin chains was estimated to be approximately 50 picomolar (pM), indicating that these ubiquitin chains are highly potent ligands of RIG-I.

Finally, we examined whether viral infection induces the formation of polyUb chains associated with full-length RIG-I in cells (Figure S7C). HEK293T cells expressing RIG-I-Flag were infected with Sendai virus or mock treated, and then the RIG-I complex was immunoprecipitated and heated at 76°C to release polyUb chains. The heat supernatant prepared from the virus-infected cells, but not mock-treated cells, stimulated IRF3 dimerization in the presence of RIG-I(N). This activity was diminished by IsoT treatment (Figure S7D), indicating that the supernatant contained unanchored polyUb chains.

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In sum, the results presented herein demonstrate that (1) the unanchored K63-ubiquitin chains are present in human cells; (2) they are bound and protected by RIG-I; and (2) they are potent activators of the RIG-I pathway. We propose that sequential binding of RIG-I to RNA and unanchored K63-polyUb chains leads to full activation of RIG-I, which in turn activates MAVS and downstream signaling cascades (Figure 7G).

**DISCUSSION**

In this report, we demonstrate that RNA with characteristics of viral RNA (5’-pppRNA and dsRNA) can activate the RIG-I pathway in vitro in a reconstitution system with exquisite specificity and sensitivity. To our knowledge, this is the first in vitro reconstitution of an immune signaling cascade from a microbial ligand (viral RNA) to activation of a transcription factor (IRF3). Our success in reconstituting the RIG-I pathway in vitro is beneficiary of at least three previous discoveries that (1) RNAs containing 5’-triphosphate are direct functional ligands of RIG-I (Hornung et al., 2006; Pichlmair et al., 2006); (2) mitochondria containing MAVS are essential for RIG-I signaling (Seth et al., 2005); and (3) ubiquitination is important for RIG-I activation (Gack et al., 2007). The in vitro reconstitution of the RIG-I pathway sets the stage for delineating the biochemical mechanism of RIG-I activation and subsequent steps of downstream signaling. Testimonial to the power of this in vitro system are the discoveries revealed in this study that the tandem CARDs of RIG-I represent a new class of ubiquitin-binding domain with specificity for K63-polyUb chains and that unanchored K63-polyUb chains are potent intracellular signaling molecules that activate the RIG-I pathway.

**Specificity and Sensitivity of Viral RNA Detection by RIG-I**

A major determinant that distinguishes viral RNA from the host RNA in mammalian cells is the presence of 5’-triphosphate in viral RNA but not in host RNA (Fujita, 2009). 5’-pppRNA is generated in the course of RNA replication by viral RNA polymerases. However, RIG-I may have very limited access to viral 5’-pppRNA for the following reasons: (1) viral RNA replication normally occurs within a membrane compartment (e.g., vesicles) that is sequestered from the cytosolic immune sensors such as RIG-I; (2) some viruses such as influenza replicate in the nucleus rather...
Figure 6. Polyubiquitin Binding Is Required for RIG-I Activation

(A) Mutations at K172 and T55 impair RIG-I(N) activation in vitro. GST-RIG-I(N) and the indicated mutants were expressed and purified from E. coli, then incubated with K63-Ub4, followed by IRF3 dimerization assay (upper panel). Aliquots of the reaction mixtures were analyzed by immunoblotting with a GST antibody (lower panel). 6KR: GST-RIG-I(N) containing six K > R mutations, including K172R. K172-only: similar to 6KR except that K172 is not mutated.

(B) Mutations at K172 and T55 impair polyUb binding by RIG-I(N) in vitro. GST-RIG-I(N) and mutant proteins were incubated with K63-polyUb chains, then pulled down with glutathione Sepharose and analyzed by immunoblotting.

(C) Mutations at K172 and T55 impair RIG-I(N)'s ability to induce IFN-β in cells. Different amounts (30 ng and 100 ng) of mammalian expression vectors for GST-RIG-I(N) and mutants were transfected into HEK293-IFN-β-Luc cells, then luciferase activity was measured. Error bars represent the variation range of duplicate experiments.

(D) GST-RIG-I(N) and mutant proteins as described in (C) were pulled down with glutathione Sepharose beads, washed with PBS or RIPA buffer, then analyzed by immunoblotting.

(E) GST-RIG-I(N) was expressed in HEK293T cells, then purified using glutathione Sepharose. The purified protein was treated with isopeptidase T (IsoT) or viral OTU (vOTU) or mock-treated, then immunoblotted with an antibody against ubiquitin or GST (upper panel; N.S indicates a nonspecific band). Aliquots of the treated GST-RIG-I(N) were tested for their ability to promote IRF3 dimerization in the reconstitution assay (lower panel).

See also Figure S6.
than in the cytosol; (3) the majority of viral RNA is in complex with viral capsid proteins to form ribonucleoprotein particles (RNPs), and the 5’ end of viral RNA can be blocked by viral proteins; (4) like their mammalian counterparts, most viral mRNAs also contain 5’-cap or other 5’-modifications. Thus, RIG-I must be capable of detecting very tiny amounts of 5’-pppRNA in the presence of abundant cellular RNA and trigger a signaling cascade leading to IRF3 activation. Thus, our reconstitution system recapitulates the specificity and sensitivity of viral RNA recognition by the RIG-I pathway in cells.

**RIG-I CARDs Represent a New Type of Ubiquitin Sensor**

CARDs are protein interaction domains that play key roles in many cellular processes including inflammation and apoptosis (Park et al., 2007). Unexpectedly, we found that the tandem CARDs of RIG-I bind specifically to K63-linked polyUb chains. We also found that the tandem CARDs of MDA5, but not the CARDs of NOD2 or MAVS, bind to K63-polyUb chains (K.J., and Z.C., unpublished data; Figure S5C). Thus, a subset of CARDs appears to have the specialized ability to bind K63-polyUb chains. Importantly, mutations that disrupt ubiquitin binding of RIG-I also impair its ability to activate IRF3, indicating that polyUb binding plays a key role in RIG-I activation. It will be of significant interest to identify other CARDs capable of ubiquitin binding and determine whether proteins harboring these CARDs require ubiquitin binding for their function.

**Polyubiquitin Binding Is Required for RIG-I Activation**

An important role of RIG-I ubiquitination in its activation was suggested based on the observations that K172R mutation of RIG-I diminishes its ubiquitination and its ability to induce IFN-β, and that the K172-only mutant can induce IFN-β, but not the CARDs of MDA5, but not the CARDs of NOD2 or MAVS, bind to K63-polyUb chains (K.J., and Z.C., unpublished data; Figure S5C). Thus, a subset of CARDs appears to have the specialized ability to bind K63-polyUb chains. Importantly, mutations that disrupt ubiquitin binding of RIG-I also impair its ability to activate IRF3, indicating that polyUb binding plays a key role in RIG-I activation. It will be of significant interest to identify other CARDs capable of ubiquitin binding and determine whether proteins harboring these CARDs require ubiquitin binding for their functions.

**Sequential Binding to RNA and Unanchored K63-Polyubiquitin Chains Leads to Full Activation of RIG-I**

Our study reveals unanchored K63-polyUb chains as the second ligand of RIG-I and demonstrates that full activation of RIG-I requires an orderly binding of viral RNA followed by polyUb chains. Based on available evidence, we propose a multistep model of RIG-I activation (Figure 7G). After viral infection, RIG-I binds to viral 5’-pppRNA through its C-terminal RD domain and may bind to the double-stranded segments of the RNA through the central helicase domain (Fujita, 2009). The RNA binding causes dimerization of RIG-I and activates its ATPase activity (Cui et al., 2008). The energy derived from ATP hydrolysis propels the translocation of RIG-I along the viral RNA (Myong et al., 2009). The translocation of RIG-I on viral RNA may help displace viral proteins from the RNA, facilitating further RIG-I binding. The translocating RIG-I dimer likely has an “open” conformation that exposes the N-terminal CARDs, which form a cluster consisting of four CARDs. This cluster of CARDs binds to K63-polyUb chains. This binding may cause additional conformational changes and/or oligomerization of RIG-I, forming a new signaling platform to activate MAVS. The dependency of RIG-I
Figure 7. Endogenous Unanchored K63-Polyubiquitin Chains Activate the RIG-I Pathway

(A) A protocol for isolating functional endogenous polyUb chains in human cells.

(B) Endogenous polyUb chains bound to RIG-I can be released by heat treatment and remain functional. GST-RIG-I(N)-K172-only was expressed in HEK293T cells and isolated as described in (A), then heated for 5 min at the indicated temperatures. After centrifugation, the supernatant containing heat-resistant ubiquitin chains was incubated with GST-RIG-I(N)-K172-only (lanes 1–6). Endo.polyUb: endogenous polyUb.

(C) Endogenous unanchored polyUb chains activate RIG-I(N). PolyUb chains associated with GST-RIG-I(N)-K172-only were captured and released at 75°C. The heat-resistant supernatant was incubated with GST-RIG-I(N)-K172-only, which was then pulled down and heated in parallel experiments (lanes 1–6). Endo.polyUb: endogenous polyUb.
activation on binding to unanchored K63-ubiquitin chains may provide another level of regulation of antiviral immune responses.

**Unanchored K63-Polyubiquitin Chains Are Intracellular Signaling Molecules**

Our previous study has shown that unanchored polyUb chains directly activate the protein kinases TAK1 and IKK by binding to the essential regulatory subunits TAB2 and NEMO, respectively (Xia et al., 2009). We now show that unanchored polyUb chains bind and activate RIG-I, suggesting that these ubiquitin chains may have more general functions in cell signaling. However, unlike TAK1 and IKK activation, which requires very long polyUb chains, RIG-I can be activated by short K63 chains consisting of only 3 or 4 ubiquitin moieties. Indeed, K63-UB4 and endogenous Ub chains are very potent activators of RIG-I (Xia et al., 2009). We now show that unanchored polyUb chains and deviate from the paradigm that ubiquitin functions for the standard assay with RIG-I (N), each μl mixture contained 100 ng GST-RIG-I(N) and 50 to 100 ng ubiquitin chains in a buffer containing 20 mM HEPES-KOH (pH 7.0) and 10% (v/v) glycerol. After incubation at 4°C for 10 min or up to 1 hr, the mixture was directly used in the IRF3 activation assay.

**Ubiquitin-Binding Assay**

For ubiquitin binding by full-length RIG-I, His6-RIG-I-Flag (1 μg) or the ATPase mutant (K270A or D372N) was incubated with K63-UB4 or K63-polyUb (1 μg), and 5′-pppRNA (0.3 μg for 135nt RNA) in 10 μl Buffer A at 4°C for 1 hr. For reactions lacking ATP, EDTA (10 mM) was added. The mixture was then diluted 5-fold in Buffer D (20 mM Tris-HCl [pH 7.5], 100 mM NaCl, 0.5% [w/v] NP-40, and 10% [v/v] glycerol) and immunoprecipitated with anti-Flag or anti-RIG-I antibody. The bound proteins were eluted with Flag peptide or SDS sample buffer and subjected to immunoblotting analysis with antibodies specific for RIG-I or ubiquitin.

For ubiquitin binding by RIG-I(N), 2 μg of wild-type or mutant GST-RIG-I(N) was incubated with 1 μg ubiquitin chains in 10 μl buffer containing 20 mM HEPES-KOH (pH 7.0) and 10% (v/v) glycerol at 4°C for 1 hr. The mixture was then diluted 5-fold in Buffer D before the GST proteins were pulled down with glutathione Sepharose. The bound proteins were analyzed by immunoblotting.

To analyze RIG-I(N):K63-UB4 complex, GST-RIG-I(N)-Flag was cleaved with Tewe protease and then RIG-I(N) was purified on Q-Sepharose and Superdex-200 columns. Five micrograms each of purified RIG-I(N) and K63-UB4 were incubated at 4°C for 60 min, and then the mixture was separated on Superdex-200 PC (3.2/30) using the SMART purification system. The elution buffer contained 20 mM Tris-HCl (pH 7.5), 0.1 mM EGTA, 0.5 mM DTT, and 0.02% CHAPS. Aliquots of the fractions were analyzed in the in vitro IRF3 dimerization assay and also by immunoblotting using antibodies against RIG-I or ubiquitin.

**Isolation and Functional Analysis of Endogenous Polyubiquitin Chains**

HEK293T cells were transfected in 10 cm dishes with 5 μg of pEF-GST-RIG-I(N)-K172-only. The cells were lysed 48 hr later in Buffer E (20 mM Tris-HCl at pH 7.5, 150 mM NaCl, 10% [v/v] glycerol, 1% [w/v] Triton X-100, 200 mM β-glycerophosphate, 1 mM Na3VO4, 0.1 mM NaF, 0.1 mM phenylmethylene sulfonyl fluoride, and EDTA-free protease inhibitor cocktail) supplemented with 10 mM NEM. After centrifugation at 20,000 g for 15 min, 5 mM DTT was added to the supernatant to quench NEM, then the GST proteins were isolated with glutathione Sepharose. The proteins on the beads were incubated in 30 μl of 20 mM HEPES-KOH (pH 7.0, 10% [v/v] glycerol, and 0.02% [w/v] CHAPS) at 70°C, 75°C, or 80°C for 5 min. Following a brief centrifugation, the supernatant containing the dissociated polyUb chains was incubated with 100 ng of bacterially expressed GST-RIG-I(N) at 4°C for 1 hr, followed by IRF3 dimerization assay. For deubiquitination enzyme treatment, the heated supernatant was incubated with 10 ng/μl IsoT or CYLD at 30°C for 1 hr. The concentration of endogenous polyUb chains was estimated by semiquantitative immunoblotting using synthetic K63-UB6 (Boston Biochem) as the standard (Figure S7B).

unanchored K63-polyUb chains were incubated with GST-RIG-I(N) and IsoT in sequential orders as indicated. In the right panel, the heat supernatant containing endogenous polyUb from HEK293T cells was incubated with or without IsoT, then analyzed by immunoblotting with a ubiquitin antibody. The arrow denotes an ~40 kDa band that is likely K63-UB6 (see Figure S7B).
SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Results, Extended Experimental Procedures, and seven figures and can be found with this article online at doi:10.1016/j.cell.2010.03.029.

ACKNOWLEDGMENTS

We thank Francesco Melandri and Dr. Kai Lapan (Boston Biochem) for generously providing some of the ubiquitin chains and Dr. Jae Jung (University of Southern California) for the mammalian expression plasmids of GST-RIG-I(N), K172R, K172-only, and 6KR mutants. We also thank Brian Skaug for reading the manuscript. This work was supported by grants from NIH (ROI-AI09919 and ROI-GM63692) and the Welch Foundation (I-1389).

Z.J.C. is an Investigator of Howard Hughes Medical Institute.

Received: December 20, 2009
Revised: February 22, 2010
Accepted: March 23, 2010
Published: April 15, 2010

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NLRC5 Negatively Regulates the NF-κB and Type I Interferon Signaling Pathways

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DOI 10.1016/j.cell.2010.03.040

SUMMARY

Stringent control of the NF-κB and type I interferon signaling pathways is critical to effective host immune responses, yet the molecular mechanisms that negatively regulate these pathways are poorly understood. Here, we show that NLRC5, a member of the highly conserved NOD-like protein family, can inhibit the IKK complex and RIG-I/MDA5 function. NLRC5 inhibited NF-κB-dependent responses by interacting with IKKa and IKKβ and blocking their phosphorylation. It also interacted with RIG-I and MDA5, but not with MAVS, to inhibit RLR-mediated type I interferon responses. Consistent with these observations, NLRC5-specific siRNA knockdown not only enhanced the activation of NF-κB and its responsive genes, TNF-α and IL-6, but also promoted type I interferon signaling and antiviral immunity. Our findings identify NLRC5 as a negative regulator that blocks two central components of the NF-κB and type I interferon signaling pathways and suggest an important role for NLRC5 in homeostatic control of innate immunity.

INTRODUCTION

The innate immune response, elicited through the detection of pathogen-associated molecular patterns (PAMPs), provides the first line of defense against invading microorganisms. PAMP recognition depends on several classes of pattern recognition receptors (PRRs), including Toll-like receptors (TLRs), NOD-like receptors (NLRs), and RIG-I-like receptors (RLRs) (Akira et al., 2006; Honda and Taniguchi, 2006; Inohara et al., 2005; Medzhitov, 2007; Meylan et al., 2006; Ting et al., 2006). Activation of most TLRs leads to the recruitment of a common adaptor, MyD88, and in turn to a series of downstream signaling events that culminate in NF-κB activation (Akira et al., 2006; Chen, 2005; Hayden and Ghosh, 2008). By contrast, activation of RLRs (RIG-I and MDA5) by double- and single-stranded RNAs or certain viruses (Hornung et al., 2006; Kato et al., 2006; Pichlmair et al., 2006) results in recruitment of the MAVS protein (mitochondrial antiviral signaling; also called VISA, IPS-1 and Cardif), which further activates the downstream signaling molecules TBK1/IKKi and IRF3 for type I interferon responses, as well as IKK molecules for NF-κB activation (Meylan et al., 2006). Besides their roles in innate immunity and inflammation, TLR-mediated signaling pathways have been shown to play an important role in the control of regulatory T cell function (Liu et al., 2006; Peng et al., 2005, 2007; Sutmuller et al., 2006).

Because uncontrolled immune responses can be harmful, even fatal, to the host (Liew et al., 2005), NF-κB activation and type I interferon signaling must be tightly regulated to maintain immune balance in the organism. Despite the importance of the IKK complex as a central transducer of signaling from various stimuli, leading to the activation of the NF-κB pathway, and of RLRs as critical receptors in type I interferon signaling (Chen, 2005; Honda and Taniguchi, 2006), the molecular mechanisms responsible for IKK activation and RLR-mediated signaling remain poorly understood.

NLRs represent a large family of intracellular PRRs that are characterized by a conserved nucleotide-binding and oligomerization domain (NOD) and a leucine-rich repeat (LRR) region, and are involved in the activation of diverse signaling pathways (Akira et al., 2006; Inohara et al., 2005; Meylan et al., 2006). Several NLRs, such as NOD1, NOD2 and NALP3, have been extensively studied and shown to activate signaling pathways once they encounter relevant PAMPs (Akira et al., 2006; Chen, 2005; Inohara et al., 2005; Meylan et al., 2006). NALP3 inflammasome, for example, functions as a crucial component in the adjuvant effect of aluminum and asbestos (Dostert et al., 2008; Eisenbarth et al., 2008). More recently, NLRX1 was demonstrated to function as a mitochondrial protein that interacts with the mitochondrial adaptor MAVS to inhibit the RIG-I-mediated signaling pathway and triggers the generation of reactive oxygen species as well (Moore et al., 2008; Tattoli et al., 2008). These studies suggest that understanding the function and mechanisms of these innate immune receptors or regulators may aid in developing more effective strategies for the immunological treatment...
of inflammation-associated diseases (Karin et al., 2006; Wang et al., 2008).

Given that the NLR protein family is involved in many biological processes and functions as proinflammatory receptors as well as negative regulators, we hypothesized that some NLR members may play a critical regulatory role in the control of NF-kB and type I interferon signaling. Here we report the identification of NLRC5 as a potent negative regulator of NF-kB and IRF3 activation. It strongly inhibits NF-kB-dependent responses by interacting with IKKa and IKKb and blocking their phosphorylation. It also interacts with RIG-I and MDA5, but not with MAVS, to potently inhibit RLR-mediated type I interferon responses.

As a key negative regulator of NF-kB and type I interferon signaling, NLRC5 may serve as a useful target for manipulating immune responses against infectious or inflammation-associated diseases, including cancer.

RESULTS

Molecular Cloning and Characterization of NLRC5

As a member of the NLR protein family, NLRC5 contains a CARD-like domain, a central NOD domain and a large LRR region (Figure 1A), but its biological function remains unknown (Chen et al., 2009; Dowds et al., 2003). To determine the function of NLRC5, we cloned full-length human and murine NLRC5 complementary DNAs (cDNAs) by rapid amplification of cDNA ends (RACE) (Figure 1A). There was a 64% amino acid sequence identity between the human and murine proteins.

Expression of both HA-tagged NLRC5 and mNLRC5 was demonstrated by western blotting with an anti-HA antibody (Figure 1B). Both human and murine NLRC5 mRNAs were strongly expressed in spleen, thymus, and lung (Figure 1C), suggesting that this molecule is biologically conserved in these tissues.

To further demonstrate the protein expression of NLRC5 in different cell types, we generated polyclonal antibodies against endogenous NLRC5 and mNLRC5 (Figures S1A–S1E available online), which readily detected these proteins in human HEK293T (293T) cells, human THP1 cells or mouse RAW264.7 cells (Figure S1F). To determine whether NLRC5 expression could be regulated in response to TLR stimulation or virus infection, we treated RAW264.7 cells with LPS, intracellular poly(I:C), or infection with vesicular stomatitis virus-enhanced green fluorescent protein (VSV-eGFP), which are known to activate the NF-kB or type I IFN pathway (Yoneyama and Fujita, 2009). Real-time PCR and western blot analyses revealed strong upregulation of mNLRC5 at both the mRNA and protein levels, which peaked at 6 hr after LPS treatment (Figures 1D and 1E and Figure S1G). In contrast, we observed only a weak increase of mNLRC5 mRNA and little or no change in mNLRC5 protein after intracellular poly(I:C) treatment or VSV infection (Figures 1D and
consistent with a previous study showing that VSV infection is a weak NF-κB inducer (Ishikawa and Barber, 2008). To further explore the mechanisms by which NLRC5 is regulated, we performed similar experiments with wild-type and MyD88 knock- out (KO) macrophages and found that upregulation of mNLRC5 was completely abolished when MyD88 KO macrophages were stimulated with LPS (Figure 1F), indicating that the expression of mNLRC5 itself is controlled by the NF-κB signaling pathway.

To determine the cellular localization of NLRC5, we transfected 293T cells with NLRC5-GFP fusion DNA and found that NLRC5-GFP was present in the cytoplasm, but not in the nucleus or mitochondria (Figures S1H–S1I), suggesting that NLRC5 is a cytosolic protein. No change in the cellular localization of NLRC5 was observed after LPS treatment (Figures S1I–S1K).

The NLRC5 plasmid, and then treated them with interleukin (IL)-1β, TNF-α, or LPS. Results in Figure 2A show that NLRC5 potently inhibited NF-κB activation induced by IL-1β, TNF-α, or LPS. We next sought to identify potential signaling molecules that activated the NF-κB-luc reporter. Expression of MyD88, TRAF6, IKKα, IKKβ, or p65, along with NF-κB-luc, strongly induced NF-κB-luc activity, but such activity was inhibited when NLRC5 was cotransfected at increasing concentrations (Figure 2B). In contrast, NLRC5 did not inhibit p65-mediated NF-κB-luc activity, suggesting that it may interact with IKK signaling molecules upstream of p65 to block NF-κB activation (Figure 2B). The control plasmids NOD1 and NOD2 enhanced rather than inhibited NF-κB activity (Figure 2C), consistent with previous studies (Inohara et al., 1999; Ogura et al., 2001).

Since LPS can also activate the type I IFN pathway through an adaptor TRIF molecule, we asked whether NLRC5 can inhibit LPS-induced IFN-β-luc activity (which requires cooperation between IRF3 and NF-κB activation) or interferon-stimulated response element (ISRE)-luc activity (which requires IRF3 activation only) (Zhong et al., 2008). Functional assays showed that NLRC5 inhibited LPS- or TRIF-induced NF-κB-luc and IFN-β-luc activities, but had no effects on ISRE-luc activity (Figures S2A and S2B). Further experiments demonstrated that NLRC5 strongly inhibited R848-induced NF-κB-luc activity and weakly inhibited IFN-β-luc activity, but did not inhibit ISRE-luc activity (Figure S2C). Thus, NLRC5 strongly inhibits NF-κB activation induced by different TLR ligands and weakly blocks IFN-β activation mainly due to its requirement for NF-κB activity, but has no apparent effect on ISRE activity. 

Figure 2. NLRC5 Inhibits NF-κB Activation Induced by IL-1β, LPS, TNF-α, and Their Downstream Signaling Molecules

(A) 293T cells were transfected with an NF-κB-luc reporter plasmid and TLR4 plasmid (for LPS treatment), together with an empty vector or NLRC5 construct, and analyzed for NF-κB-dependent luciferase activity (fold induction) after treatment with IL-1β, LPS, or TNF-α. 

(B) 293T cells were transfected with MyD88, TRAF6, IKKα, IKKβ, or p65, along with NF-κB-luc.

(C) 293T cells were transfected with NOD1 or NOD2, along with NF-κB-luc.

(D) Detection of endogenous NF-κB DNA binding activity in a gel-mobility shift assay. Oct-1/DNA-binding complexes served as a loading control for nuclear extracts.

(E) Human THP-1 cells and murine embryonic fibroblasts (MEF) were transfected with the NF-κB-luc reporter plasmid, together with (or without) NLRC5 or mNLRC5 plasmid, and then analyzed for NF-κB-dependent luciferase activity after LPS treatment (**p < 0.001). See also Figure S2.
To determine whether the observed NLRC5-mediated inhibition of NF-κB-luc activity is associated with endogenous NF-κB activity, we performed experiments with a gel-mobility shift assay. IKKβ expression allowed endogenous NF-κB to bind to the biotin-HRP-labeled DNA with NF-κB binding sites, but this activity was completely inhibited when NLRC5 was cotransfected. However, NLRC5 failed to inhibit p65-mediated NF-κB activation (Figure 2D), consistent with results obtained with the NF-κB-luc reporter assay. To substantiate these findings, we found that like its human homolog, mNLRC5 strongly inhibited NF-κB activation by MyD88, TRAF6, IKKα and IKKβ, but not by p65 (Figure S2D). Moreover, NLRC5- or mNLRC5-mediated NF-κB inhibition in 293T cells could be extended to THP-1 cells and murine embryonic fibroblasts (MEFs) (Figure 2E). Taken together, these results suggest that NLRC5 functions as a negative regulator of NF-κB activation induced by TNF-α, IL-1β, LPS or by their downstream signaling molecules, and that its biological function is conserved between humans and mice as well as among multiple cell types.

**NLRC5 Interacts with IKKβ/IKKβ in Unstimulated and Stimulated Cells**

Results presented in Figures 2B and 2D suggest that NLRC5 may directly interact with IKKα, IKKβ or NEMO to inhibit NF-κB activation. To test this prediction, we transfected 293T cells with HA-tagged NLRC5 together with Flag-tagged IKKα, Flag-tagged IKKβ or Flag-tagged NEMO expression plasmids. Coimmunoprecipitation and western blot analyses revealed that NLRC5 interacted with IKKα and IKKβ subunits, but not with NEMO, although the corresponding proteins were readily detected in whole-cell lysates (Figure 3A). Notably, NLRC5 did not interact with either TAK1 or MEKK3 upstream of IKK (Figure S3A). To further test whether NLRC5 can interact with endogenous IKKα/β, we immunoprecipitated 293T cell lysates with an isotype IgG or anti-NLRC5 antibody, followed by western (Figure S3A). To further test whether NLRC5 can interact with endogenous IKKα/β, we immunoprecipitated 293T cell lysates with an isotype IgG or anti-NLRC5 antibody, followed by western blot analysis with the anti-IKKα/β, anti-NEMO or anti-NLRC5 antibody. As shown in Figure 3B, the IKKα/β, but not NEMO, could be detected in the anti-NLRC5 or anti-mNLRC5-immunoprecipitants. Such an interaction between NLRC5 and IKKα/β was also observed when anti-IKKβ/α and anti-NEMO antibodies were used for immunoprecipitation (Figure S3B). These results suggest that NLRC5 can interact with IKKα/β, but not with NEMO, under physiological conditions.

Since IKKα/β generally forms a complex with NEMO, a key question is whether there are two distinct complexes in unstimulated cells: IKKα/β/NEMO and IKKα/β/NLRC5. To address this issue, we used different antibodies (anti-IKKα, anti-Flag and anti-NEMO) to immunoprecipitate the IKK complexes containing either NLRC5 or NEMO, and then determined the components of their complexes. We found NEMO and NLRC5 in the anti-IKKα immunoprecipitants, NLRC5 and IKKα/β (but not NEMO) in the anti-Flag-NLRC5 immunoprecipitants, and IKKα/β and NEMO (but not NLRC5) in the anti-NEMO immunoprecipitants (Figure S3C), suggesting that the IKKα/β/NEMO and IKKα/β/NLRC5 complexes coexist in unstimulated cells. To definitively demonstrate the presence of two distinct complexes, we fractionated cell extracts of RAW264.7 on a size-exclusion column (HiPrep 16/60 Sephacryl S-300 HR). Immunoblotting of fractions collected from chromatography showed that IKKα/β mainly coeluted with NEMO in fractions 22–31 (molecular mass about 700 kDa) (Figure 3C), consistent with a previous report (Rothwarf et al., 1998). In contrast, NLRC5 mainly eluted in fractions 15–18 (molecular mass about 1100 kDa), which also contained IKKα/β but no NEMO (Figure 3C), suggesting that NLRC5 forms a larger complex with IKKα/β than previously reported for the IKKα/β/NEMO complex, thus confirming the coexistence of IKKα/β/NLRC5 and IKKα/β/NEMO complexes in unstimulated cells.

To determine the dynamics of NLRC5 interaction with IKKα/β after stimulation, we treated RAW264.7 cells with LPS, collected them at different time points and performed immunoprecipitation and western blot analyses. The interaction between NLRC5 and IKKα/β was reduced at 30 min, but was restored at 60 min after LPS stimulation (Figure S4A). This oscillating pattern of interaction correlated inversely with IKK phosphorylation (p-IKK) during the first 60 min posttreatment and then diminished or even disappeared after 2 hr of treatment (Figures S4A–S4C), suggesting that the negative regulatory activity of NLRC5 is signal-dependent, but is not affected exclusively by protein concentration.

**NLRC5 Competes with NEMO for IKKα/β and Inhibits Their Phosphorylation and Kinase Activity**

We next tested whether NLRC5 and NEMO compete each other for binding to IKKα/β. 293T cells were transfected with a fixed concentration of Flag-NLRC5 and HA-IKKα/β DNAs, together with increasing concentrations of HA-NEMO DNA. Coimmunoprecipitation and immunoblot revealed that the binding between NLRC5 and IKKβ was markedly decreased with increasing concentrations of NEMO (Figure 3D). Although the phosphorylation and kinase activity of IKKα/β could be detected in the IKKα/β/NEMO complex after LPS stimulation, they decreased with increasing amounts of transfected NLRC5. There were no detectable phosphorylation and kinase activity of IKKα/β in the IKKα/β/NLRC5 complex (Figure 3E). Importantly, increasing amounts of NLRC5 had more pronounced effects on the phosphorylation and kinase activity of IKKα/β in the total and NEMO-immunoprecipitated IKKα/β fraction than on its changes at the protein level (Figure 3E). These results suggest that NLRC5 not only competes with NEMO for IKKα/β binding, but also inhibits IKKα/β phosphorylation and its ability to phosphorylate IkB or free IKKα/β. To test this possibility, we performed experiments with constitutively active IKKα (SS176/180→EE) or IKKβ (SS177/181→EE) mutants and found that NLRC5 interacted with constitutively active IKKα (EE) and IKKβ (EE) (Figure S4D). Kinase assays revealed that expression of NLRC5 inhibited the ability of IKKα (EE) and IKKβ (EE) to phosphorylate IkB (i.e., 32p-GST-IkBα), as well as their ability to autophosphorylate IKK (i.e., 32p-IKK) (Figure S4E). These observations were further supported by NF-κB-luc assays showing that NLRC5 can inhibit NF-κB activation by the constitutively active IKKα (EE) and IKKβ (EE) (Figure S4F). These results suggest that NLRC5 can inhibit the ability of active IKKα/β/IKKα to phosphorylate IkBα or free IKKα/β.

To determine how NLRC5 inhibits the phosphorylation of IKK, we sought to identify the domain of IKKβ responsible
Figure 3. NLRC5 Interacts with IKKα and IKKβ to Inhibit Their Phosphorylation

(A) 293T cells transfected with Flag-IKKα, Flag-IKKβ, Flag-NEMO and HA-NLRC5. HA-tagged NLRC5 protein was immunoprecipitated with anti-HA beads, and blotted with anti-Flag.

(B) 293T and RAW264.7 cell extracts were immunoprecipitated with IgG, anti-NLRC5 or anti-mNLRC5 antibody, respectively, and then analyzed together with whole-cell extracts by western blot with specific antibodies. The elution positions of calibration proteins with known molecular masses (kDa) were used to determine the size of complexes.

(C) Cell extracts of RAW264.7 cells were fractionated on a size-exclusion column (HiPrep 16/60 Sephacryl S-300 HR). The collected factions with an equal volume were used for western blot analysis with specific antibodies. The elution positions of calibration proteins with known molecular masses (kDa) were used to determine the size of complexes.

(D) 293T cells were transfected with the indicated doses of Flag-NLRC5, HA-IKKβ and HA-NEMO. Whole-cell extracts were immunoprecipitated with anti-Flag beads and blotted with anti-HA.

(E) 293T/TLR4 cells were transfected with empty vector or different doses of HA-NLRC5 (0, 50 or 200 ng) DNA and then treated with LPS. Cell extracts were collected at 30 min poststimulation and prepared for immunoprecipitation with anti-HA and anti-NEMO, followed by immunoblot (IB) with indicated antibodies or kinase assay (KA).

(F) The domain structure of IKKβ. Numbers in parentheses indicate amino acid position in construct. LZ, leucine zipper; HLH, helix-loop-helix.

(G) 293T cells were transfected with HA-NLRC5 and Flag-IKKβ or various Flag-IKKβ mutants. Whole-cell extracts were immunoprecipitated with anti-Flag beads, and blotted with anti-HA.

(H and I) 293T/TLR4 cells transfected with IKKα, IKKβ, JNK1, JNK2, and p38 with or without HA-NLRC5 were used to analyze the phosphorylation of IKKα/β, JNK and p38.

(J) RAW264.7 cells were treated with LPS and collected at the indicated time points. Cell extracts were prepared for immunoprecipitation with anti-mNLRC5 or anti-NEMO, followed by immunoblot (IB) to determine the IKK phosphorylation with anti-p-IKK or anti-IKKα/β antibody and kinase activity of IKK.

See also Figure S3 and Figure S4.
for interacting with NLRC5. Because NEMO is known to bind to the C terminus of IKKβ (May et al., 2000), we generated deletion mutants encompassing the amino-terminal kinase domain (KD), leucine zipper domain (LZ) and a C-terminal helix-loop-helix (HLH) domain of IKKβ, and tested them for their ability to interact with NLRC5 in an immunoprecipitation assay (Figure 3F). Like the full-length IKKβ, the IKKβ-KD construct strongly interacted with NLRC5, while neither the IKKβ-LZ nor the IKKβ-HLH construct showed appreciable binding activity with NLRC5 (Figure 3G), indicating that NLRC5 specifically binds to the IKKβ-KD domain. However, because of the large size of the NLRC5 protein, we considered that its binding to IKKβ might physically block any binding of NEMO to IKK (Figures 3D and 3E).

We next tested the specificity of NLRC5-mediated inhibition of IKK phosphorylation. As shown in Figures 3H and 3I, NLRC5 markedly inhibited the IKKα/β phosphorylation, but not p38 or JNK phosphorylation, consistent with the observation that NLRC5 did not interact with TAK1 or MEKK3 (Figure S3A). Finally, we further tested the status of the phosphorylation and kinase activity of IKKα/β in IKKα/β/NLRC5 and IKKα/β/NEMO complexes in RAW264.7 cells under physiological conditions. IKKα/β in the IKKα/β/NLRC5 (NLRC5-IP) complex was not phosphorylated and showed no kinase activity during LPS stimulation. By contrast, IKKα/β in the IKKα/β/NEMO (NEMO-IP) complex was phosphorylated and exhibited a strong kinase activity after LPS stimulation (Figure 3J), suggesting that NLRC5 inhibits its phosphorylation and kinase activity in the IKKα/IKKβ/NLRC5 complex under physiological conditions.

LRR Region Is Responsible for NLRC5-Mediated Inhibition of IKK Activation
To identify the functional domains of NLRC5, we generated four deletion constructs: NLRC5-D1, containing the CARD and NOD domains (aa 1–517); NLRC5-D2, containing the LRR region and LRR-R2 (aa 900–1329); and NLRC5-D3, containing the linker region and LRR-R2 (aa 900–1329); and NLRC5-D4, containing the CARD domain and LRR-R3 (aa 1–215 plus 1471–1866) (Figure 4A). While all four NLRC5 deletion constructs could interact with the full-length IKKβ protein as well as IKKβ-KD (Figures 4B and 4C), NLRC5-D3, like the full-length NLRC5, strongly inhibited NF-κB-luc activity (Figure 4D). Other NLRC5 deletions showed either partial or no inhibitory effect on NF-κB-luc activity. These results suggest that NLRC5-D3 is required for the observed inhibition of NF-κB activity by NLRC5.

To further investigate the molecular mechanism by which NLRC5-D3 inhibits NF-κB activity, we tested whether these deletions can inhibit the phosphorylation of IKKα/β. We found that NLRC5-D3 strongly inhibited IKKα/β phosphorylation, while NLRC5-D1, -D2 and -D4 produced little or weak inhibition (Figure 4E). Notably, the inhibitory activity of these NLRC5 deletion mutants on the phosphorylation of IKKα/β correlated with their ability to inhibit NF-κB activation (Figure 4D), suggesting that NLRC5-D3 inhibits NF-κB activation by blocking the phosphorylation of IKK complexes. Unlike the full-length NLRC5, however, NLRC5-D3 failed to compete with NEMO for binding to IKKα/IKKβ (Figure S4G), suggesting that the size of full-length NLRC5 is critical for its ability to physically block NEMO from interacting with IKKα/β.

Knockdown of NLRC5 Enhances NF-κB Activation as Well as the Inflammatory Response
Since NLRC5 specifically interacts with IKKα/β and inhibits NF-κB activation, we reasoned that knockdown of NLRC5 would release IKKα/β for increased NF-κB activation under physiological conditions. To test this prediction, we first demonstrated the
knockdown efficiency and specificity of NLRC5 or mNLRC5 at both the mRNA and protein levels in various cell types with at least two corresponding siRNAs, but not scrambled siRNAs (Figure 5A and Figures S5A–S5C). We next tested the effect of mNLRC5 knockdown on the phosphorylation of IKK, IκB, and other kinases, including JNK, ERK, and p38. As shown in Figure 5B and Figure S5D, the phosphorylation of IKK (p-IKK) and IκB (p-κB) in the mNLRC5 knockdown cells was at least 3-fold higher than that in the scrambled siRNA-transfected (control) cells at 30 min after LPS treatment, although the total amounts of IKK and IκB proteins were comparable between mNLRC5 knockdown and control cells. More importantly, we did not observe any appreciable differences in p-JNK, p-ERK and p-p38 between mNLRC5 knockdown and control cells, clearly indicating the specific inhibitory effect of mNLRC5 on the phosphorylation of IKK, but not on JNK, ERK, and p38 phosphorylation. Similar results were obtained with human THP-1 cells (Figure S5E). Collectively, these results suggest that specific knockdown of NLRC5 or mNLRC5 strongly enhances the phosphorylation of the IKK complexes, but does not affect the phosphorylation of JNK, ERK, and p38 after LPS stimulation.

To further determine the molecular mechanisms by which NLRC5 knockdown affects NF-κB signaling, we performed two-step immunoprecipitations to obtain NEMO-associated IKK (IP-1) and NEMO-free IKK (free IKK and NLRC5-associated IKK, IP-2) (Figure 5C) and then compared the amounts of IKKα/β in each complex and their phosphorylation (p-IKKα/β) in mNLRC5 knockdown versus control cells. We found that the total IKKα/β proteins in the NEMO-free IKK fraction (i.e., IP-2) of mNLRC5 knockdown cells was slightly lower than that of control cells, while IKKα/β in the IKKα/β/NEMO fraction (i.e.,
IP-1) in mNLRC5 knockdown cells was slightly higher than that in control cells, suggesting that the reduction of IKKα/β/NLRC5 complex in mNLRC5 knockdown cells decreases the IKKα/β proteins in the NEMO-free IKK fraction, but increases the IKKα/β proteins in the NEMO/IKK complex (Figure 5C). Consistent with this observation, we found that the phosphorylation of IKK (p-IKK) in the NEMO-containing IP fraction (IP-1) in the mNLRC5 knockdown cells was higher than that in control cells. Importantly, even though the total IKKα/β in the NEMO-free fraction (IP-2) of mNLRC5 knockdown cells was less than that of control cells, its phosphorylation (p-IKK) level in mNLRC5 knockdown cells was higher than that in control cells (Figure 5C), suggesting that mNLRC5 knockdown reduces the inhibition of phosphorylation of free IKKα/β. This notion agrees with data, showing that NLRC5 interacts with constitutively active IKKα (EE) and IKKβ (EE) and inhibits their phosphorylation and kinase activity (Figures S4D and S4E).

We next sought to determine whether the enhanced IKK phosphorylation seen with NLRC5 knockdown promotes NF-κB activation and NF-κB-dependent gene expression. Using the NF-κB luc reporter assay, we found that NLRC5 knockdown markedly increased NF-κB-luc activity in 293T/TLR4 and RAW264.7 cells after LPS treatment (Figure 5D). To directly demonstrate that mNLRC5 knockdown enhances endogenous NF-κB activation, we examined the DNA-binding activity of endogenous NF-κB in control cells. Consistent with this observation, knockdown of mNLRC5 knockdown reduces the inhibition of phosphorylation of free IKKα/β. This notion agrees with data, showing that NLRC5 interacts with constitutively active IKKα (EE) and IKKβ (EE) and inhibits their phosphorylation and kinase activity (Figures S4D and S4E).

**NLRC5 Negatively Regulates Type I Interferon Signaling by Interacting with RIG-I and MDA5**

Recent studies show that NLRX1 inhibits the RIG-I-mediated signaling pathway by targeting MAVS (Moore et al., 2008). To determine whether NLRC5 might also be involved in the regulation of type I interferon signaling, we performed functional assays in TLR3-deficient 293T cells and found that intracellular poly(I:C)-activated IFN-β signaling, although such activation was strongly inhibited by NLRC5 (Figure 6A), suggesting that NLRC5 functions as a negative regulator of this antiviral pathway.

To determine the molecular mechanisms by which NLRC5 could inhibit the IFN-β response, we performed similar experiments with different signaling molecules and found that RIG-I- and MDA5-induced IFN-β-luc activities could be markedly inhibited by increasing concentrations of NLRC5. However, NLRC5 weakly inhibited MAVS- and TBK1-induced IFN-β-luc activity and did not inhibit Iκκα/β-induced IFN-β luciferase activity (Figure 6A and Figure 6A). Furthermore, NLRC5 markedly inhibited NF-κB-luc activity induced by intracellular poly(I:C), RIG-I, MDA5, MAVS or TBK1 (Figure 6B). To determine whether the weak inhibition of MAVS- and TBK1-induced IFN-β-luc activity by NLRC5 might be due to the inhibitory effect of NLRC5 on IKK complexes, but not due to any direct effect on MAVS or TBK1, we transfected 293T cells with ISRE-luc, together with RIG-I, MDA5, MAVS, or TBK1 in the presence or absence of NLRC5. Both RIG-I- and MDA5-induced ISRE-luc activities were potently inhibited, while MAVS- and TBK1-induced ISRE-luc activities were not (Figure 6C), suggesting that NLRC5 inhibits IFN-β activation by directly interacting with RIG-I and MDA5, but not with MAVS or TBK1. Indeed, we found that NLRC5 was strongly associated with RIG-I and MDA5, but did not interact with MAVS, IKKα, TBK1, TRIF, TRAF3, or IRF3 (Figures 6D and 6E and Figure S6B). Thus, NLRC5 specifically binds to the RIG-I and MDA5 proteins to inhibit the IFN-β response.

To further define the interaction between RIG-I and mNLRC5 under physiological conditions, we infected RAW264.7 cells with VSV-eGFP and performed immunoprecipitation with anti-mNLRC5 at different time points after VSV-eGFP infection. We detected only a weak RIG-I protein band in anti-mNLRC5 immunoprecipitates at 4 hr postinfection; however, it peaked at 6 hr and then became weak at 8 and 10 hr postinfection (Figure 6F). Notably, RIG-I protein expression was also increased at 6 hr postinfection (Figure 6F), consistent with previous observations (Honda and Taniguchi, 2006). Importantly, IRF3 phosphorylation was observed at 4 hr and further increased at 6 hr postinfection, indicating that VSV-eGFP infection activated the IRF3-dependent signaling pathway. However, Iκκα phosphorylation and IκB degradation were weak after VSV-eGFP infection (Figure 6F). These results suggest that interaction between NLRC5 and RIG-I is inducible after VSV-eGFP infection.

Because both the RIG-I protein level and IRF3 phosphorylation peaked at 6 hr postinfection, coincident with the peak interaction between mNLRC5 and RIG-I in RAW264.7 cells, we considered that this interaction might critically rely on activating signal. To test this possibility, we generated RIG-I-CARD and RIG-I-helicase constructs and tested their ability to interact with NLRC5. Interestingly, NLRC5 strongly bound to the CARD domain of RIG-I, but not to the helicase domain, while binding of NLRC5 to the CARD domain of RIG-I markedly exceeded that to the full-length RIG-I (Figure 6G), suggesting that the CARD domain of RIG-I is critical for a productive interaction between NLRC5 and RIG-I, and that such interaction may also be influenced by the relative concentrations of MAVS and NLRC5.

To confirm the inhibitory effect of NLRC5 on the IRF3 phosphorylation, we assessed the phosphorylation states of IRF3 by overexpressing NLRC5 in 293T cells and found that NLRC5 potently blocked the phosphorylation of endogenous IRF3 induced by RIG-I, but not by MAVS (Figure 6). In contrast, NLRC5 or mNLRC5 knockdown in THP-1 and RAW264.7 cells markedly increased IRF3 phosphorylation (Figure 6J). Thus, NLRC5 can inhibit IFN-β signaling by blocking the binding of MAVS to RIG-I and the IRF3 phosphorylation.

**Knockdown of NLRC5 Enhances Innate and Antiviral Immunity**

To further demonstrate the effects of NLRC5 knockdown on the expression of IFN-responsive genes, we knocked down...
Figure 6. NLRC5 Negatively Regulates IFN-β Activation by Inhibiting RIG-I and MAVS Function

(A–C) 293T cells were transfected with NF-κB-luc, INF-β-luc or ISRE-luc, NLRC5 plus poly(I:C)/LyoVEC, RIG-I, MAVS, TBK1, or IKK plasmids and analyzed for INF-β or ISRE luciferase activity. Values are means ± SEM of three independent experiments (*p < 0.05, **p < 0.01, ***p < 0.001).

(D) 293T cells were transfected with HA-NLRC5 plus RIG-I, MAVS or IKK. After immunoprecipitation with anti-HA beads, specific proteins were analyzed by western blot with anti-Flag.

(E) 293T cells were transfected with MAVS or without HA-NLRC5. After immunoprecipitation with anti-HA beads, specific proteins were analyzed by western blot with anti-MAVS.

(F) RAW264.7 cells were infected with VSV-eGFP, and cell extracts were harvested at different time points, immunoprecipitated with anti-mNLRC5 antibody and analyzed by western blot with anti-RIG-I.

(G) NLRC5 binds to the CARD domain of RIG-I. 293T cells were transfected with HA-NLRC5 plus Flag-RIG-I, Flag-RIG-I CARD domain and Helicase domain (HD).

(H) NLRC5 competitively binds to RIG-I with MAVS. HEK293T cells were transfected with Flag-MAVS-HA plus Flag-RIG-1, or Flag-NLRC5. After immunoprecipitation with anti-HA beads, specific proteins were analyzed by western blot with anti-Flag.

(I) 293T cells were transfected with Flag-RIG-I and Flag-MAVS, with or without HA-NLRC5, and used for western blot analysis with anti-phospho-IRF3 and IRF3 antibodies.

(J) RAW264.7 and THP-1 cells were transfected with NLRC5/mNLRC5-specific siRNA or scrambled siRNA, respectively, and then infected with VSV-eGFP. The cell extracts were harvested for western blot with anti-phospho-IRF3 and IRF3 antibodies.

See also Figure S6.
Figure 7. Knockdown of NLRC5 Enhances Cytokine Response and Antiviral Immunity

(A and B) RAW264.7 cells or THP-1 cells were transfected with NLRC5-specific siRNA or scrambled siRNA, followed by poly(I:C)/Lyovec treatment. ISG-54, ISG-56, IFN-β mRNA and IFN-β protein were determined by real-time RT-PCR or ELISA.

(C) NLRC5 or mNLRC5 knockdown and control cells were infected with VSV-eGFP. Cell supernatants were used to measure IFN-β protein secretion by ELISA.

(D) RAW264.7 cells were transfected with mNLRC5 siRNA or scrambled siRNA for 36 hr and then treated with LPS, poly (I:C)/LyoVec (1 μg/ml) or VSV-eGFP infection. Total RNAs from the treated cells were harvested at different time points and used for real-time PCR analysis to determine the expression of TNF-α, IL-6, IFN-α, and IFN-β. The data in (A–D) are reported as means ± SEM of three independent experiments. Asterisks indicate significant differences between groups (*p < 0.05, **p < 0.01, ***p < 0.001 determined by Student's t test analysis).
endogenous NLRC5 or mNLRC5 and then treated the cells with poly(I:C)/Lyovec or infected them with VSV-eGFP. Real-time PCR analysis revealed that poly(I:C)/Lyovec treatment or infection with VSV-eGFP strongly increased mRNA levels of IFN-β and the interferon-stimulating genes ISG54 and ISG56 in cells transfected with mNLRC5- or NLRC5-specific siRNAs (Figure 7A and Figure S7A), consistent with a previous study suggesting the upregulation of IFN-β mRNA in A549 cells with NLRC5 knockdown (Opitz et al., 2006). Furthermore, we found that poly(I:C)/Lyovec treatment or VSV-eGFP infection led to a large increase in the production of IFN-β protein in THP-1, RAW264.7, primary murine macrophages and primary human monocytes transfected with mNLRC5 or NLRC5-specific siRNA (Figures 7B and 7C and Figure S7B). We next determined their expression patterns over time after stimulation or VSV infection, and found that the NF-κB-responsive genes TNF-α and IL-6 in mNLRC5-knockdown cells were upregulated as early as 2–4 hr after LPS treatment, and IL-6 continued in that state for another 10 hr (Figure 7D). No difference was found between mNLRC5 knockdown and control cells, with the exception of IFN-β expression at 8 hr after LPS treatment (Figure 7D). Similar experiments with intracellular poly(I:C) treatment or VSV infection showed strong upregulation of IRF3-responsive IFN-α and IFN-β expression, but little or no effect on TNF-α and IL-6 expression (Figure 7D). Consistent with these observations, we found that LPS treatment resulted in more IL-6 and TNF-α than IFN-β protein, while VSV-eGFP infection led to more IFN-β than TNF-α and IL-6 proteins in mNLRC5 knockdown RAW264.7 cells (Figure S7C). It appears that stimulation with LPS leads to more pronounced effects on NF-κB-regulated genes than does either poly(I:C) treatment or VSV-eGFP infection, while the converse is seen for IRF-3-regulated genes.

To demonstrate a link between increased innate cytokine responses and antiviral immunity in NLRC5-silenced cells, we showed that knockdown of endogenous NLRC5 or mNLRC5 rendered cells remarkably resistant to viral infection and reduced the levels of VSV-eGFP-positive cells among 293T, THP-1, and RAW264.7 cells, as well as MEFs and human monocytes (Figure 7E and Figure S7D). To monitor VSV-eGFP propagation in both mNLRC5 knockdown and control cells, we performed time course experiments and showed that GFP expression from VSV-eGFP could be observed at 8 hr postinfection with rapid propagation at 10 hr in control cells. More than 90% of the cells were GFP positive at 12 hr. By contrast, GFP expression from VSV-eGFP viruses could rarely be observed at 10 hr, became visible at 12–14 hr, and remained limited to a few cells even at 18 hr in mNLRC5-silenced cells (Figure S7E). At 15 hr, the morphology of the cells was markedly changed in control cells, but not in mNLRC5 knockdown cells (Figure S7F). At 20 hr, most of the cells in control group had died with a striking reduction in the GFP signal, while the cells treated with mNLRC5 siRNA remained alive and appeared normal (Figure S7G). We conclude that NLRC5 knockdown can significantly increase innate cytokine production and antiviral immunity against VSV-eGFP infection and propagation.

**DISCUSSION**

Activation of innate immune receptors (TLRs, NLRs, and RLRs) by their corresponding ligands initiates several key signaling pathways, leading to the production of proinflammatory cytokines, such as IL-6 and TNF-α, which in turn induce profound positive feedback for adaptive immune responses (Akira et al., 2006; Honda and Taniguchi, 2006). Increasing evidence indicates that many inflammation-associated diseases may result from dysregulated innate immunity (Inohara et al., 2005; Ting et al., 2006). More recently, IL-1, IL-6, and TNF-α produced by innate immune cells in chronic inflammation conditions have been shown to promote cancer development and progression (Karina et al., 2006). Thus, an understanding of the molecular mechanisms by which innate immunity is held in check through negative regulators appears critical for developing novel and more effective treatments for inflammation-induced autoimmune diseases and cancer (Wang et al., 2008).

Both NF-κB and type I interferon signaling are controlled at multiple levels by distinct mechanisms, whose regulatory proteins may themselves be direct transcriptional targets of NF-κB and type I interferon signaling, contributing to a negative regulatory feedback loop (Komuro et al., 2008). For example, expression of the A20, CYLD, and DUBA negative regulators is controlled by NF-κB activity, while the Rig-I and MDA5 genes are transcriptionally regulated by type I interferon signaling (Komuro et al., 2008). We similarly observed the upregulation of NLRC5 or mNLRC5 at both the mRNA and protein levels after 6 hr of treatment with LPS. Such upregulation was abolished in MyD88-deficient macrophages, suggesting that expression of NLRC5 itself is under the control of MyD88-NF-κB pathways and forms a negative regulatory feedback loop.

It has been demonstrated that the deubiquitinating enzymes A20 and CYLD inhibit NF-κB signaling by targeting TRAF6 upstream of IKK (Kovalenko et al., 2003; Liew et al., 2005; Trompouki et al., 2003; Wertz et al., 2004), while the deubiquitinating protein DUBA inhibits type I interferon activity by targeting TRAF3 (Kayagaki et al., 2007). Despite the importance of IKK as a central transducer of signaling from cytokines, TLRs and RLRs, leading to NF-κB activation, relatively little is known about its negative regulation. Our findings show that NLRC5 blocks IKK phosphorylation and thus NF-κB activation by interacting with IKKα and IKKβ, but not with the regulatory subunit NEMO. To elucidate the mechanism(s) by which NLRC5 inhibits IKK function, we provide evidence that NLRC5 forms a large complex with IKKα/β/NEMO complex that seems to be dominant in unstimulated cells (Rothwarf et al., 1998). The interaction between NLRC5 and IKKα/β appears to be dynamic during the early phase (1–2 hr) of LPS stimulation and correlates inversely with

(E) 293T cells, THP-1 cells and RAW264.7 cells were transfected with NLRC5- or mNLRC5-specific siRNA or scrambled siRNA, and then infected with VSV-eGFP. Viral infections were analyzed by fluorescence microscopy (with phase contrast as a control) as well as FACS analysis.

(F) Proposed model illustrating how NLRC5 negatively regulates both NF-κB and type I IFN signaling pathways. Auto-p, autophosphorylation. See also Figure S7.
the phosphorylation of IKKβ (p-IKK). This oscillation pattern diminishes or even disappears with time after LPS stimulation, suggesting the importance of this activating signal in the regulation of the interaction between NLRC5 and IKKβ. NLRC5 knockout experiments showed increased cytokine responses in NLRC5-silenced cells compared with control cells, suggesting that the NLRC5 protein concentration is also an important factor in the regulation of the IKK activity. Overall, it appears that both the LPS-induced activating signals and the relative protein concentration of NLRC5 influence the interaction between NLRC5 and IKKβ, as well as the phosphorylation of IKKβ. Although NLRC5 is ubiquitinated at about 30–40 min after LPS stimulation (data not shown), further studies are needed to determine whether the ubiquitination of NLRC5 plays a role in the interaction between NLRC5 and IKKβ.

A recent study shows that CUEDC2 interacts with IKKα, but not NEMO, and recruits PP1c to deactivate the IKK complex by dephosphorylating IKKα (Li et al., 2008). In resting cells, CUEDC2 binds to IKKα (but not NEMO) and undergoes transient disassociation and reassociation steps after TNF-α treatment (Li et al., 2008), similar to the interaction we described between NLRC5 and IKKα after LPS stimulation. We also show that IKKα/β in the IKKα/β/NLRC5 complex is not phosphorylated and lacks kinase activity, in contrast to IKKα/β in the IKKα/IKKβ/NEMO complex (Figures 3E and 3J). These studies clearly indicate that besides NEMO, other important regulatory proteins such as NLRC5 can control the phosphorylation and kinase activity of IKK complex through sequestering active IKKα/β.

Although the kinase domain of IKKβ specifically recognized by NLRC5 differs from the NEMO-binding site at the C terminus of IKKβ (May et al., 2000), we show that NLRC5 physically blocks the binding of NEMO to IKKα/β due to its large size (Figure 3E and Figure 5G). Our results also show that NLRC5 can interact with constitutively active forms of IKKα (EE) and IKKβ (EE) and inhibit their ability to phosphorylate IkBα, as well as their ability to autophosphorylate IKKα (Figures 4D and 4E). Importantly, experiments with two-step immunoprecipitation provide further evidence that mNLRC5 knockdown has striking effects on IKKα and NF-κB signaling (Figure 5C), probably through a modulating dynamic balance between /IkKα/β/NLRC5 and /IkKα/β/NEMO complexes as well as sequestering the active IKKα/β/NLRC5 complex. Furthermore, it has been suggested that the phosphorylation of Ser740 in IKKβ and Ser68 in NEMO by active IKK may disrupt the interaction between IKK and NEMO (Hayden and Ghosh, 2008), thus allowing other proteins, including NLRC5 or phosphatases, to interact with active IKK and terminate NF-κB signaling. These studies suggest that NLRC5 plays a critical role in inhibiting the phosphorylation and kinase activity of IKKα/β in NF-κB activation after LPS stimulation.

RIG-I and MDA5 are key receptors for triggering type I interferon signaling pathways, and are controlled by positive and negative regulators, such as TRIM25 and RNF125, through ubiquitination (Arimoto et al., 2007; Gack et al., 2007). To test the hypothesis that other proteins are involved in the regulation of RLRs, we present evidence showing that NLRC5 can bind to both RIG-I and MDA5 but not to their downstream signaling molecules such as MAVS, TBK1, IKKi, TRAF3, or IRAF. These findings support a dual regulatory role for NLRC5 that encompasses both NF-κB and type I interferon signaling. Importantly, our results show that NLRC5 binds more strongly to the CARD domain of RIG-I than to the full-length RIG-I, suggesting that NLRC5 specifically recognizes the CARD domain when it becomes accessible after viral infection or stimulation by its ligands. This interpretation agrees with the proposed model for RIG-I action, in which the CARD domain of inactive RIG-I is masked by its intramolecular binding with the repressor domain in the C terminus (Cui et al., 2008; Yoneyama and Fujita, 2009). Once RIG-I is activated, the CARD domain is exposed for binding to MAVS or NLRC5.

Based on the experimental data discussed above, we propose a model to illustrate how NLRC5 could negatively control both NF-κB and type I interferon signaling (Figure 7F). First, the expression of NLRC5 itself is controlled by NF-κB activation, thus forming a negative regulatory feedback loop. Second, the key to modulating the activation of NF-κB by NLRC5 in stimulated cells lies in the dynamic balance between NLRC5/IKKα/ IKKβ and NEMO/IKKα/IKKβ/NEMO complexes, which is controlled by signaling stimulation and relative protein (NLRC5 versus NEMO) concentrations, as well as the ability of NLRC5 to inhibit IKK phosphorylation and kinase activity by sequestering active IKKα/IKKβ during signaling amplification after LPS stimulation. Thus, the striking effects of NLRC5 on NF-κB signaling and its downstream cytokine target genes cannot be explained by only a small fraction of the NLRC5/IKKα/IKKβ/NEMO complex being in an unstimulated state. Third, in contrast to its regulation of NF-κB activation, NLRC5 competes with MAVS for binding to the CARD domain of RIG-I or MDA5 only after it is exposed by ligand stimulation of these receptors, leading to dampened activation of IRF3. The interaction between NLRC5 and RIG-I is inducible after poly(I:C) treatment or VSV viral infection. Although TLR activation, cytokine stimulation and viral infection can activate both NF-κB and type I signaling pathways, but they tend to have a much more pronounced effect on either NF-κB-regulated genes (e.g., LPS stimulation) or IRF3-regulated genes (poly(I:C) or VSV infection). This selectivity appears to reflect the different mechanisms by which NLRC5 regulates IKK activity or RIG-I/MDA5 proteins. The ultimate outcome of NLRC5 inhibition is determined by whether target gene promoters require NF-κB, IRF3, or both transcription factors for gene expression.

Finally, we show that specific knockdown of NLRC5 not only enhances NF-κB and type I interferon signaling and expression of their target genes, but also increases antiviral immunity in multiple cell lines and primary cells. Because of the conserved biological function of NLRC5 in humans and mice, as well as in various cell types, it appears to play a physiologically important role in the maintenance of immune homeostasis, especially with regard to regulation of the innate immune responses. Hence, NLRC5 may provide a useful therapeutic target for enhancing immunity against microbial infections and inflammation-associated diseases.

**EXPERIMENTAL PROCEDURES**

**Molecular Cloning of Full-Length Human and Mouse NLRC5**

A full-length NLRC5 cDNA was obtained from human PBMC cDNA by two-step PCR and was then cloned into pcDNA3.1Z with HA tag sequence.
A similar strategy was used to clone mouse NLRC5. Both pcDNA-HA-NLRC5 and pcDNA-HA-mNLRC5 were sequenced to verify the correct DNA sequence and their open reading frames.

**Expression Profile and Antibody Production**
The expression profile of NLRC5 and mNLRC5 in different tissues was evaluated by reverse transcriptase (RT)-PCR analysis. NLRC5 and mNLRC5 peptides were used to generate polyclonal antibodies by standard methods.

**Luciferase Assays, Immunoblot, Immunoprecipitation, and Kinase Assay**
HEK293 cells were transfected with IFN-β, NF-κB, or ISRE luciferase plasmids and HA-NLRC5. TNF-α, IL-1β, LPS as well as exogenous MyD88, TRAF6, IKKα, IKKβ, NEMO, p65 (NF-κB), RIG-I, MDAS, MAVS, and IKKα plasmids were used as stimulators. Dual-luciferase kits (Promega) were used for subsequent analysis. For kinase assay, a fusion of glutathione S-transferase and amino acids of 1–54 of IκBα (GST-IκBα) was used as the substrate. To determine the kinase activity of immuno precipitated IKKα (EE) and IKKβ (EE) to autophosphorylate IKK, we added 32p-ATP to immunoprecipitates and incubated the mixture at 30°C for 30 min. 32p-GST-IκBα (p-GST-IκBα) and 32p-IKK (p-IKK) were detected by autoradiography.

**Protein Fractionation by Size-Exclusion Column**
Cell extracts prepared from RAW264.7 cell with lysis buffer were centrifuged at 10,000 g for 30 min, and the supernatant was loaded onto a size-exclusion column (HiPrep 16/60 Sephacryl S-300 HR) with the capacity to separate the large protein complexes. Samples were fractionated with a flow rate of 0.5 ml per min, and collected as 0.5 ml fractions after passage through the void volume. Protein fractions were separated by SDS-PAGE and detected by western blotting with antibodies against mNLRC5, IKKα/β, or NEMO.

**Electrophoretic Mobility Shift Assay**
Electrophoretic mobility shift assays were performed by using the LightShift Chemiluminescent EMSA kit from Pierce Biotechnology according to the manufacturer’s standard protocol.

**Real-Time PCR Analysis**
First-strand cDNA was generated from total RNA using oligo-dT primers and reverse transcriptase (Invitrogen). Real-time PCR was conducted with the QuantiTect SYBR Green PCR Master Mix (Qiagen) and specific primers on an ABI Prism 7000 analyzer (Applied Biosystems).

**Knockdown of NLRC5 and mNLRC5 by RNA Interference**
NLRC5-specific, mNLRC5-specific and control (2-scramble mix) siRNA oligonucleotides were obtained from Invitrogen and Integrated DNA Technologies, and transfected into 293T, THP-1, RAW264.7 and primary cells with use of Lipofactamine 2000 (Invitrogen) and various Nucleofector kits (one for each cell type) according to the manufacturer’s instruction.

**Statistical Analysis**
The results of all quantitative experiments are reported as mean ± SEM of three independent experiments. Comparisons between groups for Statistical Significance were performed with a two-tailed paired Student’s t test.

**SUPPLEMENTAL INFORMATION**
Supplemental Information includes Extended Experimental Procedures and seven figures and can be found with this article online at doi:10.1016/j.cell.2010.03.040.

**ACKNOWLEDGMENTS**
We would like to thank Drs. Michael Karin and Bing Su for IKKα, IKKβ, NEMO, and p38 constructs; Yining Hu and Feng Zhu for constitutively active IKKα/β (EE) mutants and GST-IκB (1–54) constructs; Jae U. Jung for RIG-I construct; Hongbin Shu for TRAF6; Kate Fitzgerald for TBK1 and IKKβ constructs; and John Hiscott for pISRE-luc construct. We would also like to thank Drs Shao-Cong Sun, Xin Lin, and John Gilbert for their critical reading of this manuscript. This work was supported in part by grants (P01CA094237, 05CA126752, R01CA09327, R01CA101795, R01CA16408, and R01CA121191) from National Cancer Institute, NIH, and Cancer Research Institute (to R.F.W.). J.C. and L.Z. were partially supported by NCET-06-0445 and the China Scholarship Council. Z.J.C. is an investigator of Howard Hughes Medical Institute.

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