During infection with an RNA virus, the DExD/H-box RNA helicases RIG-I (retinoic acid–inducible gene I) and MDA5 (melanoma differentiation–associated gene 5) activate the interferon regulatory factor 3 (IRF3), nuclear factor κB (NF-κB), c-Jun amino-terminal kinase (JNK), and p38 mitogen-activated protein kinase (MAPK) signaling pathways through an unknown mechanism involving the adaptor protein MAVS (mitochondrial antiviral signaling). We used a Drosophila misexpression screen to identify DExD/H-box polypeptide 15 (DHX15) as an activator of the p38 MAPK pathway. Human DHX15 contributed to the activation of the NF-κB, JNK, and p38 MAPK pathways, but not the IRF3 pathway, in response to the synthetic double-stranded RNA analog poly(I:C) (polynosinic-polycytidylic acid), and DHX15 was required for optimal cytokine production in response to poly(I:C) and infection with RNA virus. DHX15 physically interacts with MAVS and mediated the MAVS-dependent activation of the NF-κB and MAPK pathways. Furthermore, DHX15 was required for poly(I:C)- and RNA virus–dependent, MAVS-mediated apoptosis. Thus, our findings indicate that, in RIG-I–like receptor signaling, DHX15 specifically stimulates the NF-κB and MAPK pathways downstream of MAVS and contributes to MAVS-mediated cytokine production and apoptosis.

INTRODUCTION

The innate immune response is the first line of host defense against microbial infection. Upon recognition of pathogen-associated molecular patterns, germ-line encoded pattern recognition receptors induce the transcription of genes encoding immunomodulatory cytokines to eliminate microbes (1). In the case of infection with RNA viruses, Toll-like receptors (TLRs) and retinoic acid–inducible gene I (RIG-I)–like receptors (RLRs) sense viral nucleic acids and lead to the production of type I interferons (IFNs) and proinflammatory cytokines. Whereas TLR3, TLR7, and TLR8 detect viral double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) in the endosomes of immune cells such as plasmacytoid dendritic cells, RLRs function as sensors of cytoplasmic viral RNA in most cell types (2–4).

DExD/H-box RNA helicases play important roles in antiviral signaling by RLRs. RIG-I (also known as DDX58) and MDA5 (melanoma differentiation–associated gene 5; also known as IFIH1/Helicard), two RLRs for cytosolic viral RNA, belong to the DExD/H-box helicase family (5, 6). RIG-I is responsible for the detection of short dsRNA and ssRNA, whereas MDA5 senses long dsRNA (7). RIG-I and MDA5 both contain a caspase activation and recruitment domain (CARD) at their N termini. Upon binding to viral RNA, RIG-I and MDA5 activate the downstream adaptor protein mitochondrial antiviral signaling (MAVS; also known as IPS-1, VISA, and Cardif) through a CARD-CARD interaction (8–11). Activated MAVS then recruits tumor necrosis factor (TNF) receptor–associated factor (TRAF) family proteins, leading to the production of type I IFNs and proinflammatory cytokines through activation of the IFN regulatory factor 3 (IRF3), nuclear factor κB (NF-κB), and mitogen-activated protein kinase (MAPK) pathways (12–14).

In addition to RIG-I and MDA5, several other DExD/H-box RNA helicases are involved in antiviral innate immune signaling. For example, DDX3 binds to MAVS to enhance the production of IFN-β (15). Other reports indicate that DDX3 is phosphorylated by TRAF-associated NF-κB activator (TANK)–binding kinase 1 (TBK-1) and that it is required for the production of IFN-β (16, 17). DDX60 physically interacts with RIG-I and MDA5 and promotes RLR signaling (18). Moreover, other reports showed that several family members, including DDX1, DHX9, DHX21, DHX33, and DHX36, directly recognize dsRNA and activate antiviral signaling (19–21). These findings indicate divergent roles of DExD/H-box RNA helicases in antiviral immune responses.

Programmed cell death, apoptosis, is the other response of the defense system against viral infection. Apoptosis induced by foreign RNA is mediated by RIG-I or MDA5 independently of the production of type I IFN (22). Several proteins, including MAVS, are required for RNA virus–induced apoptosis, although the mechanism through which RLR and MAVS induce apoptosis is not fully understood (23). Apoptosis signal–regulating kinase 1 (ASK1) is a member of the family of MAPK kinase kinases (MAPKKs; also known as MAP3Ks). ASK1 is activated in response to various stresses, including reactive oxygen species (ROS) and inflammatory cytokines, thereby leading to apoptosis and other stress responses through the activation of c-Jun N-terminal kinase (JNK) and p38 MAPK signaling. ASK1 is required for the TLR4-mediated activation of p38 and the production of cytokines in response to the TLR4 ligand lipopolysaccharide, which suggests the involvement of ASK1 in innate immune responses (24). Our previous gene misexpression screen with Drosophila melanogaster identified DEAH (Asp-Glu-Ala-His)–box polypeptide 15 (DHX15; also known as DDX15) as an activator of ASK1-MAPK signaling (25). Here, we report that DHX15 mediates RNA virus–induced cytokine production and apoptosis by facilitating the MAVS-mediated activation of NF-κB and MAPK signaling.
RESULTS

DHX15 is an activator of the MAPK cascade

We previously reported that a mutant Drosophila ASK1 protein that lacks its N terminus (DASK1ΔN) constitutively activates the Drosophila p38 (Dp38) MAPK pathway, and that ectopic expression of DASK1ΔN at the dorsal midline of the fly thorax through a pnr-GAL4–UAS system, in which the transcription factor GAL4 promotes the expression of a transgene downstream of the UAS sequence, induces the accumulation of melanin pigment in a Dp38-dependent manner (26). Although we have not revealed the physiological importance of this melanization, we used this phenotype as a visible readout of Dp38 MAPK activity in flies. Through the Gene Search (GS) misexpression system, we searched for genes whose expression led to melanin accumulation at the fly thorax and that would be expected to activate Dp38 signaling (fig. S1) (25). As a result of this screen, we found nine lines, designated key upswing in melanin accumulation 1 (kuma1) to kuma9, that exhibited induced melanization. We previously reported that KLHDC10, a mammalian ortholog of the gene product induced in the kuma1 line, stimulates ASK1-p38 MAPK signaling in response to ROS (25), suggesting that this screen worked effectively in identifying activators of the MAPK pathway. Here, we focused on analysis of the gene responsible for melanization in the kuma2 (GS11266) line.

The GS vector, which induces expression of the endogenous downstream gene, was inserted 236 bases upstream of the gene CG11107 in the kuma2 line (Fig. 1A). The amino acid sequence of CG11107 had the greatest homology to DHX15 among human proteins (fig. S2); therefore, we named it DmelDHX15. We measured the expression of DmelDHX15 in the kuma2 line under the control of heat shock (hs)–inducible GAL4 (displayed as hs>kuma2). The abundance of DmelDHX15 mRNA was greater in hs>kuma2 flies than in hs>GFP flies (Fig. 1B). In addition, coexpression of an inverted repeat (IR) RNA targeting DmelDHX15 abolished melanization in pnr>kuma2 flies (Fig. 1, C and D), indicating that DmelDHX15 was responsible for the melanization in the kuma2 line. Next, we assessed the requirement for Dp38 in DmelDHX15-induced melanization. Coexpression of a dominant-negative (DN) mutant of Dp38a (Dp38aDN) in pnr>kuma2 flies resulted in the attenuation of melanization, suggesting that the misexpression of DmelDHX15 induced melanin accumulation in a Dp38 MAPK–dependent manner (Fig. 1E). Although we tested the requirement for DASK1 in DmelDHX15–induced melanization, knockdown of DASK1 in pnr>kuma2 flies did not alter the phenotype (Fig. 1F).

Because, in addition to DASK1, other MAP3Ks are thought to activate the Dp38 MAPK pathway (27), this result may indicate the redundancy of endogenous DASK1. A single mutation in the pnr promoter that is constitutively active (pnr>kuma2) results in the attenuation of melanization, which suggests that the pnr promoter contains a mutant enhancer that is required for melanization. The physiological importance of this melanization, we used this phenotype as a visible readout of Dp38 MAPK activity in flies. Through the Gene Search (GS) misexpression system, we searched for genes whose expression led to melanin accumulation at the fly thorax and that would be expected to activate Dp38 signaling (fig. S1) (25). As a result of this screen, we found nine lines, designated key upswing in melanin accumulation 1 (kuma1) to kuma9, that exhibited induced melanization. We previously reported that KLHDC10, a mammalian ortholog of the gene product induced in the kuma1 line, stimulates ASK1-p38 MAPK signaling in response to ROS (25), suggesting that this screen worked effectively in identifying activators of the MAPK pathway. Here, we focused on analysis of the gene responsible for melanization in the kuma2 (GS11266) line.

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MAP3Ks that mediate the DmelDHX15-induced activation of Dp38 in the fly thorax.

To examine whether DHX15 activated the MAPK pathway in cultured cells, we expressed Drosophila or human DHX15 together with either DASK1 or human ASK1 in Drosophila S2 cells or human embryonic kidney (HEK) 293 cells, respectively. Coexpression of DmelDHX15 promoted the phosphorylation of Thr747 in DASK1, which is essential for the activation of DASK1 in S2 cells (Fig. 1G). We also reproducibly observed a modest increase in the phosphorylation of ASK1 Thr838, which is essential for the activation of human ASK1, by coexpression of DHX15 in HEK 293 cells (Fig. 1H). These results suggest that DHX15 is an activator of the ASK1-MAPK pathway, not only in Drosophila but also in mammalian cells.

**DHX15 is involved in the innate immune response to poly(I:C)**

DHX15 is a putative DEAH-box RNA helicase that contains the Asp-Glu-Ala-Asp motif in its RNA helicase domain. Prp43p, a yeast ortholog of DHX15, is involved in pre-mRNA splicing and ribosome biogenesis in an adenosine triphosphatase (ATPase) activity–dependent manner (28). Human DHX15 physically interacts with the RNA chaperone La (SS-B), the RNA binding motif protein RBM5, and other helicases and splicing factors (29–31), which suggests that DHX15 functions in RNA-related biological processes. However, the function of DHX15 in stress-responsive signaling has not been elucidated. Because several DExD/H-box RNA helicases, including RIG-I and MDA5, play important roles in the antiviral innate immune response and because MAPK signaling is required for the RIG-I-dependent and MDA5-mediated production of antiviral cytokines, we examined the possible involvement of DHX15 in the innate immune response to infection with RNA viruses.

To assess the requirement for DHX15 in RNA virus–induced cytokine production, we performed small interfering RNA (siRNA)–mediated knockdown of DHX15 in HeLa cells (Fig. 2A). Transfection of HeLa cells with poly(I:C) (polynosinic-polycytidylic acid), a synthetic dsRNA mimetic, induced an increase in the abundance of Ifnb mRNA, which was substantially inhibited by knockdown of DHX15 with either of two independent siRNAs (Fig. 2B). The abundance of Gapdh mRNA was not substantially affected in DHX15 knockdown cells and was used as an internal control (fig. S3A). Furthermore, increases in the abundances of mRNAs for genes encoding inflammatory cytokines, including Tnfa, Il6, and Cxcl10, in response to transfection with poly(I:C) were inhibited by siRNA-dependent targeting of Dhx15 (Fig. 2C to E). The amounts of IFN-β, TNF-α, and IL-6 (interleukin-6) secreted into the culture medium upon transfection with poly(I:C) by cells treated with DHX15-specific siRNA were reduced compared to those secreted by cells treated with control siRNA (Fig. 2F and G).
These results suggest that DHX15 is required for optimal poly(I:C)-induced production of antiviral cytokines. Because transfection of HeLa cells with poly(I:C) also induced apoptosis, we examined the possible involvement of danger signals triggered by neighboring apoptotic cells in the poly(I:C)-induced production of antiviral cytokines. Although the expression of cytokine-encoding genes upon transfection with poly(I:C) was slightly increased in cells treated with the pan-caspase inhibitor zVAD-fmk, which was probably because less cytokine-producing cells succumbed to apoptosis, there were no decreases in cytokine production by zVAD-fmk–treated cells, which excluded the possibility of apoptosis-dependent secondary signals in our experimental conditions (fig. S3C).

**DHX15 activates NF-κB and MAPK signaling in response to poly(I:C)**

The induction of Ifnb expression requires the assembly of the transcription factors IRF3, NF-κB, and AP-1 on its promoter region (32, 33). To further elucidate the role of DHX15 in the antiviral response, we examined the involvement of DHX15 in the activation of JNK, p38 MAPK, NF-κB, and IRF3 signaling. Although we could not show statistical significance, the extent of phosphorylation of JNK and p38 MAPK in response to transfection of HeLa cells with poly(I:C) was reproducibly reduced by knockdown of DHX15 to a similar extent as that in MAVS-silenced cells. The poly(I:C)-dependent degradation of inhibitor of κBα (IkBα), a protein that prevents NF-κB activation, was also reduced in extent in DHX15-silenced cells (Fig. 3A). In contrast, the poly(I:C)-induced phosphorylation of IRF3 was not affected by knockdown of DHX15, but was suppressed by knockdown of MAVS (Fig. 3, A and B). These results suggest that DHX15 may be selectively required for the activation of NF-κB and MAPKs, but not IRF3, during poly(I:C)-induced responses.

Because overexpression of DHX15 resulted in increased activation of the ASK1-MAPK signaling pathway (Fig. 1, G and H), we also examined the ability of DHX15 to stimulate the activation of NF-κB and IRF3. We found that overexpression of DHX15 resulted in activation of the NF-κB reporter (IgxB-Luc) similar to that induced by overexpression of MAVS (Fig. 3C). In contrast, overexpression of DHX15 did not activate p55C1B-Luc, a reporter containing tandem repeats of the IRF-binding motif, whereas overexpression of MAVS led to substantial activation of the reporter (Fig. 3D). These findings suggest that DHX15 promotes the transcriptional activity of NF-κB, but not IRF3. To assess whether the ATPase activity of DHX15 was required for the activation of NF-κB and MAPK, we generated alanine substitution mutants of DHX15, which are homologous to yeast Prp43p mutants that lack ATPase activities, and confirmed that they lacked ATPase activities (fig. S4, A and B). Overexpression of the ATPase-deficient mutants of DHX15 resulted in activation of the NF-κB reporter similarly to that of wild-type DHX15 (Fig. 3E). Mutant Δn/eDHX15 proteins corresponding to ATPase-deficient yeast and human DHX15 mutants also activated the DASK1-MAPK pathway (fig. S4, A and C), suggesting that DHX15 activates NF-κB and MAPK signaling in an ATPase-independent manner.

**DHX15 is required for the activation of RLR signaling downstream of MAVS**

Human DHX15 is thought to be localized in the nucleus and nucleolus in a manner similar to that of the yeast ortholog Prp43p (34, 35). However, our results showing that DHX15 was required for the cytoplasmic dsRNA–dependent degradation of IkBα and the phosphorylation of JNK and p38 MAPK suggested that DHX15 might also be localized at the extranuclear region during antiviral responses. Therefore, we performed subcellular fractionations to investigate the localization of DHX15 in detail (Fig. 4A). We observed that DHX15 was found not only in the nuclear fraction (which was identified by the presence of ATF-2) but also in the cytosolic fraction (indicated by the presence of β5B) and the mitochondrial fraction (marked by the presence of VDAC and CORE1) (Fig. 4B). The amount of DHX15 in each fraction was not substantially changed in response to transfection with poly(I:C) (Fig. 4B), suggesting that DHX15 was located in the cytosol and mitochondria under steady-state conditions.

**DHX15 mediates dsRNA- and MAVS-induced apoptosis**

In addition to cytokine secretion, apoptosis is also induced during RNA virus infection to limit the spread of the infection. Transfection of cells with poly(I:C) induces apoptosis in HeLa cells (37). Consistent with this previous report, we confirmed that transfection with poly(I:C) induced apoptosis in HeLa cells as determined by detection of caspase-3 activation and analysis of the apoptotic morphology of the dying cells (fig. S6, A and B). We also found that the amount of lactate dehydrogenase (LDH) released into the culture medium by cells in response to transfection with poly(I:C) was reduced by treatment with the pan-caspase inhibitor zVAD-fmk, which suggested that the extent of poly(I:C)-induced apoptosis of HeLa cells could be monitored by measuring the amount of released LDH (Fig. 5A). We first examined whether JNK and p38 MAPK were required for poly(I:C)-induced apoptosis. The poly(I:C)-induced increase in the DEVase activity of caspase-3 was partly, but statistically significantly, suppressed by SB202190 and SP600125, inhibitors of p38 MAPK and JNK activity.
respectively (Fig. 5B). We next assessed the requirement for DHX15 in poly(I:C)-induced apoptosis. Knockdown of DHX15 resulted in an attenuation of caspase-3 activity and LDH release in response to transfection with poly(I:C) (Fig. 5, C and D). The reduction in the amount of cellular ATP (adenosine 5′-triphosphate) because of the lack of new ATP synthesis, another indicator of cell death, was also suppressed by knockdown of DHX15 (fig. S6C).

An increase in the abundance of MAVS induces apoptosis in an IRF3- and NF-κB–independent manner (38). We found that either SB202190 or SP600125 attenuated the caspase-3 activity induced by overexpression of
MAVS, although treatment with SB202190 also reduced the amount of exogenously expressed MAVS protein (Fig. 5E). Furthermore, knockdown of DHX15 suppressed the MAVS-dependent activation of caspase-3 (Fig. 5F). These results suggest that the DHX15-MAPK axis, at least in the case of JNK, is required for poly(I:C)-induced and MAVS-mediated apoptosis.

**DHX15 is required for antiviral responses during infection with Sendai virus or encephalomyocarditis virus**

Our data from experiments with poly(I:C) suggested that DHX15 might be required for the dsRNA-induced innate immune response and apoptosis. We further assessed the requirement for DHX15 in these responses during the infection of HeLa cells with the RNA viruses encephalomyocarditis virus (EMCV) and Sendai virus (SeV). Knockdown of DHX15 in HeLa cells resulted in attenuated induction of Ifnb and Tnfa expression after infection with SeV and EMCV compared to that in cells in which DHX15 was intact (Fig. 6, A to D). Furthermore, reporter analysis revealed that knockdown of DHX15 suppressed the SeV-induced activation of the Ifnb promoter in HEK 293 cells (Fig. S7). These results suggest that DHX15 is required for RNA virus–dependent antiviral signaling. We also examined whether DHX15 was required for RNA virus–induced apoptosis. Knockdown of DHX15 in HeLa cells suppressed the activation of caspase-3 during EMCV infection, suggesting that DHX15 is required for EMCV-induced apoptosis (Fig. 6E).
DISCUSSION

Several reports demonstrated that DExD/H-box RNA helicases play important roles in the innate immune response to infection with RNA viruses. Here, we found that DHX15 was required for optimal cytokine production and apoptosis during RNA virus infection (fig. S8). Because DHX15 was required for the poly(I:C)-induced degradation of IκB and phosphorylation of JNK and p38 MAPK, and because both the NF-κB and MAPK pathways are required for cytokine production in response to infection with an RNA virus (39, 40), we believe that the marked reduction in cytokine production by cells in which DHX15 was knocked down is attributable to suppression of the activities of NF-κB and MAPK.

Coexpression and reporter analysis revealed that DHX15 physically interacted with MAVS through its RNA helicase domain and that it activated the NF-κB and MAPK pathways downstream of MAVS. These findings provide new insights into the mechanism of NF-κB and MAPK activation in RLR signaling; however, several questions, including the precise mechanism of DHX15-mediated activation of NF-κB and MAPK, remain to be answered. Downstream of MAVS, TRAF6 stimulates the activities of the NF-κB and MAPK pathways, but not that of IRF3 (14), which depends on TRAF3 for its activation (12). TRAF6 stimulates NF-κB and MAPK signaling through the activation of MAP3Ks, such as TAK1, MEKK1, and ASK1, in response to various cellular stresses. TAK1 and MEKK1 are involved in RLR signaling and activate the NF-κB and MAPK pathways (14, 39). Our finding that DHX15 specifically activated the NF-κB and MAPK pathways raises the possibility that DHX15 interacts with TRAF6, but not TRAF3, during RNA virus infection, thereby activating the MAP3Ks. Supporting this hypothesis, human DHX15 has consensus binding sequences for TRAF6 (P-X-E-X-X-acidic/aromatic), but not TRAF3 (P-X-Q-X-T/S). Our data showed the interdependency between DHX15 and TRAF6 for activation of the NF-κB reporter. It is possible that DHX15 may function as an adapter protein that promotes the activation of MAVS-TRAF6-MAP3K signaling, although a potential interaction between TRAF6 and DHX15 requires further investigation.

Prp43, a yeast ortholog of DHX15, is involved in pre-mRNA splicing in an ATPase activity–dependent manner. Although RIG-I and MDA5 require their ATPase activities.

Fig. 5. DHX15 is required for poly(I:C)-induced and MAVS-mediated apoptosis. (A) HeLa cells were transfected with poly(I:C) (5 μg/ml) and then were treated for 24 hours with dimethyl sulfoxide (DMSO) or 10 μM zVAD-fmk. The amounts of LDH released into the cell culture medium were then measured. (B) HeLa cells were treated with DMSO, 10 μM SB202190, or 10 μM SP600125 followed by transfection with poly(I:C) (10 μg/ml). Four hours later, the caspase-3 DEVDase activities of the samples were measured. (C) HeLa cells transfected with the indicated siRNAs were then transfected with poly(I:C) (10 μg/ml). Four hours later, the caspase-3 DEVDase activities of the samples were measured. (D) HeLa cells transfected with the indicated siRNAs were then transfected with poly(I:C) (5 μg/ml). Twenty-four hours later, the amounts of LDH released by the cells into the culture medium were measured. (E) HeLa cells were transfected with plasmid encoding FLAG-tagged MAVS and then were treated with 5 μM SB202190 or 5 μM SP600125. Twenty-four hours later, the caspase-3 DEVDase activities of the samples were measured. The abundance of exogenous MAVS protein in each sample was determined by Western blotting (IB) analysis. (F) HeLa cells transfected with the indicated siRNAs were then transfected with increasing concentrations of plasmid encoding FLAG-MAVS. Twenty-four hours later, caspase-3 DEVDase activities were measured. The abundance of exogenous MAVS protein in each sample was determined by Western blotting (IB) analysis. Graphs in (A) to (F) show means ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.
Ifnb cytosol and mitochondrial fractions support the idea that DHX15 has an indicating that DHX15 localized not only to the nucleus but also to the independent of its role in pre-mRNA splicing. In addition, our findings no intron, these results suggest that the role of DHX15 in RLR signaling is activate NF-kB to recognize RNA viruses, DHX15 did not require its ATPase activity to

Fig. 6. DHX15 is required for antiviral responses during SeV and EMCV infection. (A and B) HeLa cells transfected with the indicated siRNAs were infected with (A) SeV or (B) EMCV. Eight hours later, the abundance of Ifnb mRNA in each sample was determined by quantitative RT-PCR analysis. (C and D) HeLa cells transfected with the indicated siRNAs were infected with (C) SeV or (D) EMCV. Eight hours later, quantitative real-time PCR analysis of the abundance of Tnfa mRNA was performed. (E) HeLa cells transfected with the indicated siRNAs were infected with EMCV for 8 hours before the DEVDase activity of caspase-3 in each sample was measured. Data in (A) to (E) are means ± SEM of three independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001.

to recognize RNA viruses, DHX15 did not require its ATPase activity to activate NF-kB and MAPK. Together with the fact that Ifnb mRNA has no intron, these results suggest that the role of DHX15 in RLR signaling is independent of its role in pre-mRNA splicing. In addition, our findings indicating that DHX15 localized not only to the nucleus but also to the cytosol and mitochondrial fractions support the idea that DHX15 has an additional function to its role in nuclear pre-mRNA splicing. Mitoma et al. (19) showed that the nucleolar RNA helicase DHX33 senses cytosolic dsRNA to activate the NLPR3 inflammasome independently of its function in the biogenesis of 47S ribosomal RNA. These findings demonstrate the multifunctionality of DEXD/H-box helicase family members depending on their localization and interacting molecules.

Apoptosis is one type of host defense against viral infection. RIG-I and MDA5 are required for RNA virus-induced apoptosis (22). In addition, (25). Briefly, cells and flies were lysed in IP lysis buffer [50 mM tris-HCl (pH 8.0), 150 mM NaCl, 1% deoxycholate, 1% Triton X-100, 10 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), leupeptin (5 mg/ml)] or radioimmunoprecipitation assay (RIPA) buffer [150 mM NaCl, 50 mM tris-HCl (pH 8.0), 1% NP-40, 0.5% deoxycholate, 0.1% sodium dodecyl] with or without PhosSTOP (Roche), respectively. The lysates were separated by SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and were transferred onto polyvinylidene difluoride (PVDF) membranes (PALL). The membranes were incubated with the antibodies indicated in the legends, and binding was detected with the ECL system (GE Healthcare).

Reagents and antibodies
Lipofectamine 2000 (Invitrogen) was used to transfect cells with poly(I:C), which was purchased from Sigma-Aldrich. zVAD-fmk was purchased

MATERIALS AND METHODS
Fly stocks
Flies were raised on standard Drosophila medium at 25°C. The following strains were used in this study: GS11266 (Drosophila Genetic Research Center at Kyoto Institute of Technology), pw-GAL4 (41), UAS-Dp38aDN (42), UAS-DASK1-IR (25), UAS-DmelDhx15-IR (11107R-2; NIG-Fly), UAS-GFP65T, and hsGAL4 (Bloomington Drosophila Stock Center).

Western blotting
Cells were lysed and subjected to Western blotting analysis as previously described

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Immunoprecipitation
HEK 293 cells were lysed with IP lysis buffer. After centrifugation, the supernatants were subjected to IP with anti-FLAG affinity 1E6 beads (Sigma). The beads were washed with IP lysis buffer and then subjected to SDS-PAGE and Western blotting analysis.

Subcellular fractionation
HELa cells were homogenized in homogenizing buffer [10 mM Hepes-KOH (pH 7.4), 0.22 M mannitol, 0.07 M sucrose, 1 mM PMSF, and leupeptin (5 mg/ml)], followed by 20 strokes with a Dounce homogenizer. The homogenates were separated by centrifugation (Fig. 4A). Each fraction was dissolved in IP lysis buffer and analyzed by Western blotting.

Reporter assays
HEK 293 cells seeded in 24-well plates were transiently cotransfected with 50 ng each of the reporters p125-Luc and p55C1B-RLuc [provided by T. Fujita, Kyoto University, Japan (43)] and IgxB-Luc [provided by K. Matsumoto, Nagoya University, Japan (43)] together with the indicated siRNAs. As an internal control, cells were simultaneously transfected with 5 ng of Renilla (pRL-TK) or firefly luciferase reporter plasmids (pGL4.10). The luciferase activity was measured with the PicaGene Dual Sea Pansy Luminescence Kit (Wako).

Cell death assays
LDH activity was measured with the LDH Cytotoxic Test (Wako), and the LDH activity released into the culture medium was quantified as a percentage of the total LDH activity of the cell lysates and the culture medium. Cellular ATP amounts were measured with the CellTiter-Glo Luminescent Cell Viability Assay (Promega) and were quantified as a percentage compared to the amounts in cells that were not transfected with poly(I:C).

Caspase-3 activity assay
HELa cells were lysed in RIPA buffer and clarified by centrifugation. The supernatants were mixed with Caspase-3 Substrate II, Fluorogenic (Calbiochem), and the fluorescence intensity was then measured. The DEVDase activity of caspase-3 in each sample was normalized to the total amount of protein present.

Measurement of ATPase activity
HEK 293 cells transiently transfected with plasmids encoding FLAG-tagged DHX15 proteins were lysed and subjected to IP with anti-FLAG affinity 1E6 beads. The beads were washed twice each with wash buffer 1 [500 mM NaCl, 20 mM tris (pH 7.5), 5 mM EGTA, 1% Triton X-100] and wash buffer 2 [150 mM NaCl, 20 mM tris (pH 7.5), 5 mM EGTA], and then 100 μl of reaction mixture, which contains 45 mM tris-HCl (pH 8.0), 25 mM NaCl, 2.2 mM dithiothreitol, 1 mM MgCl2, 1 mM ATP, 0.6 mM poly(A) [measured as AMP (adenosine 5'-monophosphate) concentration], 0.01% Triton X-100, 0.1 mM EDTA, was added to the beads, and the beads were incubated at 30°C. After 15, 30, 45, or 60 min, the amount of ADP (adenosine 5'-diphosphate) in 20 μl of the reaction mixture was measured as described previously (44). Briefly, the reactants were added to 2× ADP detection reagent [2 mM glucose, 200 μM NADP+, 100 μM resazurin, ADP-oxidase (2 U/ml), glucose-6-phosphate dehydrogenase (2 U/ml), and diaphorase I (2 U/ml)] in buffer A [100 mM tris-HCl, 10 mM MgCl2 (pH 7.5)] in 96-well plates and then were incubated at room temperature for 60 min. Fluorescence intensity as a result of the formation of resorufin was measured on a microplate reader with an excitation wavelength of 530 nm and an emission wavelength of 600 nm.

RNA binding analysis
To generate pol(I:C)-coated agarose beads, pol(C)-coated beads (Sigma-Aldrich) were resuspended in two volumes of pol(I) (2 mg/ml, Sigma-Aldrich)
that was prepared in 50 mM tris (pH 7.0), 150 mM NaCl. The mixture was then rocked gently overnight at 4°C, collected by centrifugation at 1000g; washed with 50 mM tris (pH 7.0), 150 mM NaCl, resuspended in the same buffer as a 10% final slurry, and stored at 4°C until required for use. For poly(C) and poly(I:C) pull-down assays, poly(C)- or poly(I:C)-coated beads were equilibrated in binding buffer [50 mM tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40] as a 10% slurry and were combined with an equal volume of whole-cell lysates prepared from HEK 293 cells transiently transfected with plasmids encoding FLAG-DHX15 or FLAG-RIG-1 that were treated with lysis buffer [50 mM tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% NP-40, 10% glycerol, 1 mM PMSF, and leupeptin (5 mg/ml)]. The cell extracts were supplemented with protease and phosphatase inhibitors. The mixtures were incubated with gentle agitation for 2 hours at 4°C. Beads were then washed three times with lysis buffer and subjected to Western blotting analysis.

Expression plasmids
Complementary DNAs (cDNAs) encoding DHX15 orthologs in human and Drosophila (CG11107) were cloned by RT-PCR with total RNA isolated from HeLa cells and Drosophila embryos, respectively. Expression plasmids for FLAG-RIG-1 and its N-terminal CARD domain (RIG-I-N) and for FLAG-MAVS were gifts from T. Fujita (Kyoto University, Japan) (5, 45). To generate the HA-MAVS expression plasmid, FLAG-MAVS was used as a template cdNA. The following oligonucleotides were used for the amplification of the indicated cDNAs. human DHX15 (sense: 5′-ggtctagaCTAGTGCAGACGCCGCCGGTA-3′; antisense: 5′-ggcctcgagTCAGTACTGTGAATATTC-3′); DmelDHX15 (CG11107) (sense: 5′-ggtctagaATGTCCAAGCGTCGAATCG-3′; antisense: 5′-gcggccgcATGTCCAAGCGGCAC-3′); Aedes aegypti DHX15 (sense: 5′-gcctcgagATGTCCAAGCGTCGAATCG-3′; antisense: 5′-ggtctagaCTAGTGCAGACGCCGCCGGTA-3′). Nucleotides in lowercase include sequences for digestion with restriction enzymes (Eco RI or Xho I). Digested fragments were inserted into the mammalian expression plasmid pCAGGS, digested with Xho I and Xba I, inserted into FLAG-tagged pcDNA3, and supplemented with protease and phosphatase inhibitors. The mixtures were incubated with gentle agitation for 2 hours at 4°C. Beads were then washed three times with lysis buffer and subjected to Western blotting analysis.

Statistical analysis
Data were analyzed with the Student’s t test or by one-way analysis of variance (ANOVA) followed by the Dunnett’s multicomparison test. P values were calculated, and P < 0.05 was considered to be statistically significant. Statistical analyses were performed with GraphPad Prism version 5 for Macintosh (GraphPad Software).

SUPPLEMENTARY MATERIALS
www.sciencemag.org/cgi/content/full/7323/ra40/DC1

REFERENCES AND NOTES


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Selecting the Antiviral Response with an RNA Helicase

The RIG-I–like receptor family of RNA helicases detect viral RNA, activate the mitochondrial adaptor protein MAVS, and stimulate mitogen-activated protein kinase (MAPK) and nuclear factor κB (NF-κB) signaling, as well as interferon production. Mosallanejad et al. found that another RNA helicase, DHX15, physically associated with MAVS through its RNA helicase domain to facilitate the activation of MAPK and NF-κB signaling, but not interferon production, in cells treated with an RNA mimic or infected with RNA viruses. *Drosophila* DHX15 also activated MAPKs, suggesting the DHX15 may play a more general role in stress responses.