DDX60 Is Involved in RIG-I-Dependent and Independent Antiviral Responses, and Its Function Is Attenuated by Virus-Induced EGFR Activation

Highlights

- Human DDX60 functions as a ligand-specific sentinel for RIG-I activation
- DDX60 plays a role in RIG-I-mediated innate immune response in vivo
- DDX60 is involved in a viral RNA degradation pathway
- Virus-mediated EGF receptor activation attenuates DDX60 antiviral activities

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In Brief

Type I IFN production and viral RNA degradation are important for antiviral innate immune responses. Oshiumi et al. show that DDX60 is involved in both RIG-I-dependent type I IFN production and RIG-I-independent viral RNA decay pathways and that DDX60 functions as a sentinel for cytoplasmic antiviral innate immune response.
DDX60 Is Involved in RIG-I-Dependent and Independent Antiviral Responses, and Its Function Is Attenuated by Virus-Induced EGFR Activation

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http://dx.doi.org/10.1016/j.celrep.2015.04.047
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SUMMARY

RIG-I-mediated type I interferon (IFN) production and nuclease-mediated viral RNA degradation are essential for antiviral innate immune responses. DDX60 is an IFN-inducible cytoplasmic helicase. Here, we report that DDX60 is a sentinel for both RIG-I activation and viral RNA degradation. We show that DDX60 is an upstream factor of RIG-I that activates RIG-I signaling in a ligand-specific manner. DDX60 knockout attenuates RIG-I signaling and significantly reduces virus-induced type I IFN production in vivo. In addition, we show that DDX60 is involved in RIG-I-independent viral RNA degradation. DDX60 and RIG-I adaptor MAVS double-knockout mice reveal a role for DDX60-dependent RNA degradation in antiviral responses. Several viruses induced DDX60 phosphorylation via epidermal growth factor receptor (EGFR), leading to attenuation of the DDX60 antiviral activities. Our results define DDX60 as a sentinel for cytoplasmic antiviral response, which is counteracted by virus-mediated EGFR receptor activation.

INTRODUCTION

The innate immune system is essential for controlling viral infection. Cytoplasmic viral RNA and polyI:C are recognized by RIG-I-like receptors including RIG-I and MDA5, which trigger the induction of type I and III interferons (IFNs) and other inflammatory cytokines via the MAVS adaptor molecule (also called IPS-1, Cardif, and VISA) (Loo and Gale, 2011). MAVS activates TBK1 protein kinase, leading to autophosphorylation of TBK1 (Soulat et al., 2008). Autophosphorylated TBK1 is essential for IRF-3 phosphorylation, which results in type I IFN expression (Soulat et al., 2008). The RIG-I protein comprises two N-terminal caspase-activation and recruitment domains (CARDs), a DExD/H box (DDX) helicase domain, and a C-terminal regulatory domain (RD) (Saito et al., 2007). The RD and DDX helicase domains bind double-stranded RNA (dsRNA) (Takahasi et al., 2008), leading to K63-linked polyubiquitination of the RD by the Riplet ubiquitin ligase (Oshiumi et al., 2013). Riplet-mediated ubiquitination allows TRIM25 to access RIG-I CARDs, which is essential for triggering the induction of type I IFN production via the MAVS adaptor (Chan and Gack, 2015; Oshiumi et al., 2013). In contrast, cytoplasmic viral DNA is recognized by DNA sensors, including cyclic GMP-AMP synthase (cGAS), DAI, IFI16, DDX41, and Mre11 (Desmet and Ishii, 2012; Kondo et al., 2013). Chen and colleagues have recently shown that cGAS is essential for type I IFN production in response to viral DNA (Gao et al., 2013; Li et al., 2013a; Sun et al., 2013). Those cytoplasmic nucleic acids sensors require sentinels for recognition of viral RNA and DNA. The high mobility group box proteins, HMGB1, 2, and 3, bind to cytoplasmic immunogenic nucleic acids and function as universal sentinels for RNA and DNA sensors (Yanai et al., 2009).

Approximately 60 genes encode DDX helicases in the human genome. Bowie and colleagues first reported that a member of the DDX superfamily is involved in RIG-I signaling (Schröder et al., 2008; Soulat et al., 2008). Subsequently, other studies have shown that DDX superfamily members such as DDX3, DHX29, DHX36, and DDX60 are involved in RIG-I-dependent type I IFN production in response to viral RNA and DNA (Desmet and Ishii, 2012; Sugimoto et al., 2014; Yoo et al., 2014). However, the in vivo roles of these non-RIG-I-like helicases remain unclear.

DDX60 (also called FLJ20035) is an IFN-inducible gene that has been identified via a microarray analysis of genes induced by viral infection in human dendritic cells (DCs) (Miyashita et al., 2011). The ectopically expressed DDX60 protein associates with RIG-I and promotes RIG-I RNA-binding activity, leading to RIG-I-mediated type I IFN expression (Miyashita et al., 2011). The DDX60 helicase domain binds to viral RNA and DNA, and the DDX60 ATP-binding site is essential for DDX60-mediated
RIG-I activation (Miyashita et al., 2011). Genetic studies indicated that DDX60 acts upstream of RIG-I in the innate immune response (Miyashita et al., 2011). In contrast, Rice and colleagues have shown that ectopically expressed DDX60 suppresses hepatitis C virus (HCV) replication in a RIG-I-independent manner (Schoggins et al., 2011); however, the underlying mechanism remains unknown. A DDX60 paralog, DDX60L, is located upstream of DDX60, and the DDX60L protein shows similarity to DDX60; however, the function of DDX60L remains unknown.

Cytoplasmic viral RNA degradation pathways are important for the suppression of viral replication. The Xm1 RNA 5’–3’ exonuclease plays a crucial role in HCV RNA degradation and suppresses HCV replication (Li et al., 2013b). The zinc-finger antiviral protein (ZAP) is a host antiviral factor required for the degradation of viral RNA (Chen et al., 2008; Guo et al., 2007). ZAP associates with components of the RNA exosome, which is a protein complex essential for processing the 3’ end of host and viral RNAs (Houseley et al., 2006). Several DDX helicases are essential for RNA exosome function (Schmid and Jensen, 2008). A phylogenetic analysis indicated that the DDX60 RNA helicase domain is similar to a component of the RNA exosome, Ski2, and DDX60 associates with RNA exosome core components (Miyashita et al., 2011); however, the role of DDX60 in RNA degradation by RNA exosome remains unclear.

HCV, the influenza A virus, and other viruses are known to activate the epidermal growth factor (EGF) receptor, which is a Tyr kinase receptor (Diao et al., 2012; Ueki et al., 2013). Influenza A virus- and respiratory-virus-induced EGF receptor activation suppresses IRF1-induced type II IFN production (Ueki et al., 2013). The HCV-induced EGF receptor activation reduces STAT3 phosphorylation and STAT1 dimerization and attenuates antiviral activities (Lupberger et al., 2008); however, the physiological role of DDX60 phosphorylation remains unknown. In this study, we demonstrated that DDX60 plays a crucial role in the antiviral innate immune response in vivo and that the DDX60 antiviral activities are regulated by EGF signaling.

RESULTS

DDX60 Associates with the RIG-I RNA Sensor
First, we investigated the physical interaction between endogenous DDX60 and RIG-I. Endogenous DDX60 protein levels increased after transfection with a RIG-I ligand, short poly(C) (Figure 1A), and endogenous RIG-I was co-immunoprecipitated with endogenous DDX60 protein after short poly(C) transfection (Figure 1B), suggesting a physical interaction between endogenous RIG-I and DDX60. Second, we investigated subcellular localization of DDX60. Endogenous DDX60 protein exhibited a cytoplasmic localization in resting cells, and the DDX60 staining became strong after vesicular stomatitis virus (VSV) infection or transfection with short poly(C) (Figure 1C). Co-localization of DDX60 with RIG-I was detected after VSV infection or stimulation with short poly(C) transfection (Figure 1C). RIG-I localizes at stress granule after stimulation with short poly(C) transfection (Onomoto et al., 2012). As observed for RIG-I, DDX60 colocalized with transfected rhodamine-conjugated short poly(C) (R-poly(C)) and the stress-granule marker G3BP after transfection with short poly(C) or another RIG-I ligand, HCV 3’-UTR dsRNA (Saito et al., 2008) (Figure 1D). Taken together, these data indicate that DDX60 binds to RIG-I after stimulation.

To evaluate the role of DDX60 in RIG-I-mediated signaling, we next investigated the effect of DDX60 knockdown on RIG-I signaling. DDX60 knockdown reduced IFN-β promoter activation in response to short poly(C) or HCV 3’ UTR dsRNA transfection (Figure 1E). In contrast, DDX60 knockdown failed to reduce this promoter activation in response to other RIG-I ligands, in-vitro-transcribed HCV JFH1 single-stranded RNA (ssRNA) (Saito et al., 2008) or a 63-nt 5’-triphasophated ssRNA (5’-ppp ssRNA) (Takahasi et al., 2008) (Figure 1E). Unlike DDX60, knockdown of other DDX superfamily members (DDX60L or DDX30) reduced the promoter activation induced by the four RIG-I ligands (Figures 1E and S1A). DDX3, but not DDX60 or DDX60L, knockdown reduced the promoter activation in response to salmon genomic DNA transfection (Figure 1F). These data suggest a ligand-specific involvement of DDX60 in RIG-I signaling.

DDX60, DDX60L, and DDX3 knockdown did not reduce the promoter activation induced by RIG-I and Riplet ectopic expression (Figure 1G). A microscopic analysis showed that RIG-I colocalized with transfected R-poly(C), whereas colocalization was barely detected in DDX60-, DDX60L-, and DDX3-knockdown cells (Figures 1H and S1B). HMGB1 universal sentinel localization on transfected R-poly(C) was detected even in DDX60-, DDX60L-, and DDX3-knockdown cells (Figures 1I and S1B). Taken together, these data suggest that DDX60 acts upstream of RIG-I.

Figure 1. DDX60 Is a Sentinel Helicase for Cytoplasmic Viral RNA Sensor RIG-I
(A and B) HEK293FT cells were stimulated with short poly(C) transfection. Whole-cell extract (WCE) was prepared at the indicated time points. Immunoprecipitation (IP) was performed with antibody to DDX60 or control immunoglobulin G (IgG). The samples were analyzed by immunoblot assay using anti-RIG-I, anti-β-actin antibodies, and p125Luc and internal control vectors for 24 hr, and luciferase activity was determined.
(E and F) siRNAs for negative control, DDX3, DDX60, and DDX60L were transfected into HEK293 cells for 48–72 hr. Short poly(C), HCV 3’ UTR dsRNA, HCV JFH1 ssRNA, 63 bp of 5’-triphasophated ssRNA (5’-ppp ssRNA), or salmon sperm genomic DNA was transfected into HEK293 cells with p125Luc and Renilla luciferase (internal control) vectors for 24 hr, and luciferase activity was determined.
(G) siRNAs as indicated were transfected into HEK293 cells for 48 hr. RIG-I and Riplet expression vectors were transfected into HEK293 cells together with p125Luc and Renilla luciferase vectors for 24 hr, and luciferase activity was determined.
(H and I) siRNAs as indicated were transfected into HeLa cells for 48 hr. R-poly(C) was transfected into HeLa cells for 6 hr (H) or 16 hr (I), and the cells were fixed and stained with anti-RIG-I (H) or anti-HMGB1 (I) antibody. The localizations of R-poly(C) and the endogenous proteins were observed with confocal microscopy. The scale bar represents 10 μm. Data are presented as mean ± SD. See also Figure S1.
Generation of DDX60-Deficient Mice

Human DDX60 and DDX60L are located in tandem on chromosome IV (Figure 2A). DDX60L mRNA was expressed in lymph node, prostate, stomach, thyroid, tongue, and trachea and was induced by type I IFN stimulation in HeLa cells as DDX60 (Figures S2A and S2B). The phylogenetic tree constructed with DDX60 and DDX60L protein sequences indicated that mouse DDX60 is an ortholog of human DDX60, but not DDX60L (Figure S2C).

To further clarify the role of DDX60 in the RIG-I-dependent pathway, we generated a DDX60-deficient mouse. Exon 10 of mouse DDX60 was removed by a gene-targeting method (Figure 2B). RNA expression of exon 10 was not detected in the DDX60-homozygous mutants, and expression of exon 30–31 regions was lower than 5% of the expression level detected in wild-type (WT) mice (Figure S2D). RIG-I, MDA5, MAVS, TICAM-1, and TLR3 expression was not affected by the DDX60 mutation (Figure S2E). DDX60-knockout (DDX60-KO) mice were born at the expected Mendelian ratio and exhibited normal development and breeding (Figure S2F).

Mouse DDX60 Is Involved in the RIG-I-Dependent Type I IFN Expression Pathway

WT and DDX60-KO mouse embryonic fibroblast (MEF) cells were stimulated with HCV JFH1 ssRNA, HCV 3' UTR dsRNA, or short poly:C transfection, and the activation of the IFN-β promoter was determined by reporter-gene assay. Unlike human DDX60, mouse DDX60 was required for promoter activation in response to all three RIG-I ligands (Figure 2C). We determined the IFN-α, IFN-β, and interleukin-6 (IL-6) protein levels in culture medium by ELISA. DDX60 KO reduced IFN-α, IFN-β, and IL-6 production from MEFs in response to the three RIG-I ligands (Figures 2D–2F), whereas residual type I IFN and IL-6 production from MEFs in response to the three RIG-I ligands was still detected in DDX60 KO MEFs (Figures 2D–2F). These data indicate the existence of DDX60-dependent and DDX60-independent pathways during RIG-I activation. Unlike HEK293 cells, 5'-ppp ssRNA did not induce IFN-β and IL-6 production from MEFs (Figures 2D and 2F).

DDX60 KO reduced the endogenous IFN-β and IFN-α2 mRNA expression induced by short poly:C, HCV 3' UTR dsRNA, or VSV infection (Figures 2G–2I). VSV-induced IFN-α/β/3 mRNA expression was also reduced by DDX60 KO (Figures 2D–2F). TBK1 phosphorylation was induced by short poly:C transfection, and DDX60 KO reduced the phosphorylation of TBK1 (Figure 2J). In contrast, IFN-β and IFN-α2 mRNA expression induced by lipopolysaccharide (LPS) stimulation were comparable between WT and DDX60 KO MEFs (Figure 2K). DDX60 KO reduced dsDNA- or HSV-1-induced IFN-α2 mRNA expression only at early time points and failed to reduce IFN-β mRNA expression (Figures 2L and 2M). Taken together, our data demonstrate that DDX60 plays a crucial role for RIG-I activation in fibroblast cells.

Although DDX60 was ubiquitously expressed in mouse tissues, its expression level was different among different tissues (Figure 3A). Bone-marrow-derived dendritic cells (BM-DCs) robustly expressed DDX3 and DHX36, which are involved in the RIG-I activation pathway (Oshiumi et al., 2010b; Yoo et al., 2014), whereas DDX60 was exclusively expressed in peritoneal macrophages (Figure 3A).

Next, we examined the role of DDX60 in DCs and macrophages. Interestingly, DDX60 KO reduced virus- or RIG-I-ligand-induced IFN-α and IFN-β production in peritoneal macrophages, but not in BM-DCs (Figure 3B–3E). Next, we investigated the role of DDX60 in splenic CD11c+ cells. KO of DDX60 also reduced type I IFN production from splenic CD11c+ cells after VSV infection (Figure S3). These data indicated that DDX60 is required for RIG-I-mediated type I IFN production in peritoneal macrophages and splenic CD11c+ cells, and our data also indicate a cell-type-specific role of DDX60 in RIG-I activation.

DDX60 KO reduced HSV-1-induced IFN-α production in peritoneal macrophages (Figure 3F), whereas DDX60 was dispensable for HSV-1-induced IFN-β production in BM-DCs or peritoneal macrophages (Figure 3F and 3G). These data suggest that DDX60 plays only a minor role in the DNA-sensing pathway.

DDX60 Plays a Crucial Role in Type I IFN Production In Vivo

Next, we investigated the in vivo role of DDX60. First, we observed type I IFN levels in sera after intraperitoneal injection of short poly:C. Serum IFN-β levels in DDX60-KO mice were comparable to those of WT mice (Figure 4A).

Second, we observed hepatic type I IFN expression in response to hydrodynamically injected HCV RNA as described previously (Okamoto et al., 2014; Saito et al., 2008). RIG-I KO abolishes hepatic type I IFN expression in response to hydrodynamically injected HCV JFH1 ssRNA (Saito et al., 2008). Although HCV RNA levels in DDX60-KO mouse livers were slightly higher than those detected in WT mouse livers (Figure 4B), DDX60 KO markedly reduced hepatic IFN-β and IFN-α2 mRNA levels and serum IFN-β protein levels after hydrodynamic injection (Figures 4C and 4D).

Third, we investigated in vivo the antiviral innate immune response after intraperitoneal VSV infection. DDX60 KO reduced
serum IFN-α protein levels after VSV infection, and VSV-induced splenic IP-10, CCL5, and IFN-α2/3 mRNA expression was also reduced by DDX60 KO (Figures 4E and 4F). DDX60 KO significantly increased viral titers in mouse brain after intranasal VSV infection (Figure 4G). VSV RNA levels in mouse brain and lung were also increased by DDX60 KO (Figure S4). These data indicate that DDX60 plays a crucial role in the RIG-I-dependent innate immune response in vivo.

EGF Attenuates RIG-I Signaling via DDX60 Phosphorylation

Next, we focused on regulatory mechanism of DDX60 function. Several viruses activate the EGF receptor to facilitate viral infection (Ueki et al., 2013). A recent mass-spectrometry analysis has shown that two tyrosine residues of DDX60 (Tyr-793 and Tyr-796) are phosphorylated by the EGF receptor (Tong et al., 2008). The two residues are located in the vicinity of the Lys-791 residue, which is important for DDX60 ATP-binding activity (Figure 5A); moreover, the K791A mutation is known to reduce DDX60-dependent RIG-I activation (Miyashita et al., 2011). Thus, we investigated whether EGF receptor activation attenuates the antiviral innate immune response via DDX60 phosphorylation.

First, we examined whether the activation of the EGF receptor induces DDX60 Tyr phosphorylation. For this purpose, we constructed a DDX60-YF mutant, in which Tyr-793 and Tyr-796...
Figure 5. EGF-Induced DDX60 Phosphorylation Impairs DDX60 ATP-Binding Activity
(A and B) Schematic representation of the DDX60 ATP-binding site (A) and a DDX60-YF mutant (B).
(C and D) HEK293 cells were cultured in medium supplemented with or without 10 ng/ml EGF and transfected with a vector carrying HA-tagged DDX60 for 24 hr. Lysates were immunoprecipitated with anti-phospho-tyrosine (p-Y) antibody. The proteins were detected with anti-HA and β-actin antibodies (C). The band intensities were normalized to β-actin intensity (D).
(E and F) HEK293 cells were cultured in medium supplemented with 10 ng/ml EGF and transfected with a vector carrying HA-tagged DDX60 or DDX60-YF for 24 hr. Lysates were immunoprecipitated with anti-p-Y antibody. The proteins were detected with anti-HA and β-actin antibodies (E). The band intensities were normalized to β-actin intensity (F).

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were replaced with Phe residues (Figure 5B). HEK293 cells expressing hemagglutinin (HA)-tagged DDX60 were cultured in medium supplemented with or without 10 ng/ml EGF for 24 hr. A cell lysate was prepared, and phospho-Tyr (p-Y) proteins were subsequently immunoprecipitated using an anti-p-Y antibody. The precipitated phospho-DDX60 (p-DDX60) proteins were detected by western blotting. We found that p-DDX60 was significantly increased by EGF treatment (Figure 5C and 5D) and that the DDX60-YF mutation reduced EGF-induced DDX60 Tyr-phosphorylation (Figures 5E and 5F). This indicated that the phosphorylation of DDX60 Tyr-793 and Tyr-796 was induced by EGF treatment.

Second, we investigated the effect of Tyr phosphorylation on DDX60 function. To test the ATP-binding activity, we performed a pull-down assay with ATP-analog-conjugated agarose beads. Interestingly, EGF treatment reduced DDX60-ATP binding activity (Figures 5G and 5H). Next, we investigated the RNA-binding activity of DDX60 by a pull-down assay with biotin-conjugated dsRNA. EGF treatment did not reduce DDX60 RNA-binding activity (Figures 5I and 5J). Microscopic observation showed that ectopically expressed DDX60 was partially colocalized with the EGF receptor before and after EGF treatment, and DDX60 aggregates appeared after EGF treatment (Figures 5K and 5S).

Third, we investigated whether endogenous DDX60 Tyr phosphorylation is induced by the EGF treatment. To test this possibility, we used a proximity ligation assay (PLA), which enables the visualization of endogenous protein modification (Jarvius et al., 2007). We used anti-DDX60 and anti-p-Y antibodies. If endogenous DDX60 Tyr residues are phosphorylated after EGF treatment, it is expected that the PLA-positive (p-Y-DDX60) signals will increase. As expected, the p-Y-DDX60 signals were increased by EGF treatment in HeLa and HuH-7 cells (Figures 6A–6D).

Fourth, we investigated the effect of EGF on endogenous DDX60 function. If EGF treatment impairs DDX60-dependent RIG-I activation, it will reduce RIG-I signaling. As expected, EGF treatment reduced IFN-β and IFN-α promoter activation induced by short poly(I:C) transfection in HEK293, HuH-7, and MEF cells (Figures 6E and 6F). Long poly(I:C)-mediated IFN-β promoter activation was moderately reduced by EGF treatment (Figure 6G). Conversely, knockdown of the EGF receptor enhanced short poly(I:C)-induced IFN-β promoter activation (Figure 6H). VSV-induced IRF3 phosphorylation and IFN-β expression was also enhanced by EGF receptor knockdown (Figures 6I and 6J). In contrast, MAVS-induced IFN-β promoter activation was not reduced by EGF treatment (Figure 6K). Overexpression of DDX60-YF, which is resistant to EGF, canceled the EGF effect on IFN-β promoter activation (Figure 6L), suggesting that EGF treatment reduces IFN-β promoter activation via DDX60. Moreover, EGF treatment reduced poly(I:C)-induced IFN-β promoter activation in WT MEFs, but not in DDX60-KO MEFs (Figure 6M). Taken together, these data indicate that EGF treatment attenuates RIG-I-mediated IFN-β promoter activation via DDX60 phosphorylation.

**Viruses Modulate DDX60 Function via EGF Signaling**

Influenza A virus and HCV activate the EGF receptor, and inhibitors of the EGF receptor augment innate immune responses during viral infections (Lupberger et al., 2013; Ueki et al., 2013). We performed a PLA and found that influenza A virus infection increased p-Y-DDX60 signals and HCV infection induced p-Y-DDX60 signals (Figures 6N, 6O, and S6A), suggesting that influenza A virus and HCV induce endogenous DDX60 phosphorylation. We found that colocalization of endogenous DDX60 and EGF receptor increased after influenza A virus infection (Figures 6P and 6Q). EGF treatment reduced VSV- or influenza A virus-induced IFN-β, IP10, and tumor necrosis factor α (TNF-α) mRNA expression in HeLa cells, which were cultured in serum-free medium (Figures S6B and S6C), and an EGF receptor inhibitor, erlotinib, increased influenza A virus-induced IFN-β and IP10 mRNA expression (Figure S6D). To investigate the role of DDX60 in the EGF-mediated suppression during viral infection, WT and DDX60 KO MEFs were infected with VSV in the presence or absence of EGF. Although DDX60 KO reduced VSV-induced IFN-β and IFN-λ2/3 expression in MEFs cultured in serum-free medium, DDX60 KO failed to reduce IFN expression in cells cultured in serum-free medium supplemented with 10 ng/ml EGF (Figure 6R). Taken together, these data indicate that virus induces DDX60 phosphorylation via EGF receptor activation, which results in attenuation of DDX60-dependent RIG-I activation.

**DDX60 Is Involved in a Viral RNA Degradation Pathway**

Previously, Rice and colleagues have reported that ectopically expressed DDX60 suppresses HCV replication in a RIG-I-independent manner (Schoggins et al., 2011). Although Riplet and MAVS were inactivated in a hepatocyte cell line (O cells) that contained HCV 1b full-length replicons (Oshiuni et al., 2013), DDX60 knockdown increased HCV RNA levels in O cells (Figure 7A), suggesting the existence of RIG-I-independent DDX60 antiviral activity. Thus, we next investigated the RIG-I-independent antiviral activity. DDX60 is similar to SKI2, a component of the RNA exosome, which degrades host and viral RNAs. Small interfering RNA (siRNA) for EXOSC4, a core component of the RNA exosome, also increased HCV RNA levels in O cells (Figures 7B and 7C, 1193–1207, May 26, 2015).
To test the role of DDX60 in viral RNA degradation, we carried out electroporation assay as described before (Li et al., 2013b). A replication defective HCV JFH1-GND ssRNA was electroporated into HuH-7.5 cells, and viral RNA levels were subsequently determined by qRT-PCR. As expected, DDX60 knockdown delayed HCV RNA degradation in HuH-7.5 cells or RIG-I knockdown HuH-7 cells (Figures 7C and 7D). Next, we used an inhibitor for HCV polymerase, MK-0608, and investigated viral RNA decay as described previously (Sedano and Sarnow, 2014). siRNA for DDX60 delayed the HCV RNA decay after addition of the inhibitor (Figure 6E). As seen with DDX60 knockdown, EXOSC4 knockdown also delayed the degradation of electroporated HCV RNA in HuH-7.5 cells (Figure 7F), and co-localization of endogenous DDX60 with EXOSC4 was detected in O cells (Figure 7G). These data indicate that DDX60 is involved in HCV RNA degradation. To further corroborate the RIG-I-independent DDX60 function, we investigated the DDX60 antiviral activity in RIG-I-deficient HuH7.5 cells (Saito et al., 2007). We found that siRNA for DDX60 augmented cytopathic effect of VSV on HuH-7.5 cells and increased VSV titer in culture medium (Figure 7H), and siRNA for DDX60 delayed VSV dsRNA degradation (Figure 7I). The degradation of VSV dsRNA or HCV JFH1 GND ssRNA was also delayed in DDX60 KO MEFs (Figures 7J and 7K). Collectively, our data indicate that DDX60 is involved in a RIG-I-independent viral RNA degradation pathway. We also observed that erlotinib treatment increased HCV RNA degradation and reduced HCV RNA levels in O cells (Figures 7L and 7M), indicating that EGF receptor activation attenuates not only DDX60-dependent RIG-I signaling but also DDX60-dependent viral RNA degradation.

To investigate the role of DDX60 in antiviral response in vivo, WT and DDX60-KO mice were intranasally infected with VSV, and survival was monitored. DDX60 KO increased mortality after viral infection (Figure 7N). Next, we assessed the in vivo role of RIG-I-independent DDX60 antiviral activity. We compared the survival of MAVS and DDX60 double-KO mice with MAVS single-KO mice after VSV infection. Interestingly, the double-KO mice were more susceptible to VSV than MAVS single-KO mice (Figure 7O), suggesting that RIG-I-independent DDX60 antiviral activity plays a crucial role in vivo antiviral response.

**DISCUSSION**

DDX60 is an IFN-inducible gene, and the ectopically expressed DDX60 protein associates with RIG-I (Miyashita et al., 2011). Here, we demonstrated that DDX60 co-localized with the RIG-I protein, RIG-I ligand, and a stress granule marker, G3BP, where RIG-I recognizes viral RNA (Onomoto et al., 2012). We also showed that DDX60 bound to RIG-I after stimulation. Considering that DDX60 acts upstream of RIG-I, our data support a model in which DDX60 functions as a sentinel for RIG-I activation. Cytoplasmic HMGBs bind to all immunogenic nucleic acids and function as universal sentinels for RIG-I activation (Yanai et al., 2009). In contrast, our data indicate the ligand-specific involvement of DDX60 in RIG-I activation. These observations suggest that RIG-I requires not only universal sentinels but also ligand-specific sentinels for its activation. It is notable that other DDX helicases, such as DDX3 and DDX60L, exhibited different specificities to nucleic acids. Helicase-related Snf2 family enzymes are known to translocate DNA-associated proteins to regulate nucleic acid metabolism, such as transcription or homologous recombination (Kingston and Narlikar, 1999; Solinger et al., 2002). Considering that DDX3 and DDX60 increase RIG-I binding to dsRNA (Miyashita et al., 2011; Oshiumi et al., 2010b), these DDX helicases might be required to translocate RNA-associated proteins to promote RIG-I recognition of viral RNA.
Figure 7. DDX60 Promotes Viral RNA Degradation

(A and B) siRNA for negative control, DDX60 (A), or EXOSC4 (B) was transfected into O cells with HCV replicons for 48 hr (A) or 72 hr (B), then HCV RNA levels were determined by qRT-PCR. The results are expressed as mean ± SD (n = 3).

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MEFs, peritoneal macrophages, and splenic CD11c⁺ cells required DDX60 for efficient type I IFN production, whereas BM-DCs did not. These data indicate a cell-type-specific role of DDX60. Because other DDX helicases are also involved in RIG-I-mediated type I IFN production and highly expressed in BM-DCs, those helicases might function as sentinels for RIG-I and compensate for the defect of DDX60. Interestingly, Liu and colleagues reported a cell-type-specific role of DHX29 (Sugimoto et al., 2014). These data indicate that there are several cell-type-specific sentinel helicases for RIG-I activation.

Rice and colleagues showed that DDX60 antiviral activity is specific for HCV and does not require RIG-I (Schoggins et al., 2011). They used STAT-1-deficient cells, in which type I IFN induced by RIG-I signaling cannot activate the antiviral response (Schoggins et al., 2011). Here, we demonstrated that DDX60 was involved not only in RIG-I-mediated type I IFN signaling but also in the RIG-I-independent HCV RNA degradation pathway. This finding is consistent with the results of the previous study by Rice and colleagues (Schoggins et al., 2011). DDX60 was involved in VSV RNA degradation, and DDX60 and MAVS double-KO mice were more susceptible to VSV infection than MAVS single-KO mice. These data indicate that DDX60-mediated viral RNA degradation plays a crucial role in antiviral response in vivo.

Here, we revealed a regulatory mechanism of EGF-receptor-mediated suppression of DDX60 antiviral activity. EGF signaling induced DDX60 Tyr phosphorylation, resulting in malfunction of its ATP binding activity required for antiviral activity. We also found that EGF treatment induced DDX60 aggregation in the cytoplasm. The aggregates might sequestrate or inhibit virus-induced DDX60 and/or RIG-I at a later phase. Further study is required to clarify the physiological significance of the cytoplasmic DDX60 aggregates induced by EGF signaling.

A recent study revealed that EGF signaling inhibited IRF1-mediated type III IFN production (Ueki et al., 2013). Considering that RIG-I activates IRF1 to induce type III IFN (Oendall et al., 2014), EGF-signaling-mediated suppression of IRF1 might be caused by DDX60 phosphorylation. On the other hand, it has been reported that EGF signaling reduced STAT1 dimerization (Lupberger et al., 2013). These observations imply that EGF receptor activation inhibits several steps of antiviral innate immune responses. A previous study demonstrates that inhibitors for EGF receptor suppress HCV infection in a human liver chimeric mouse model in vivo (Lupberger et al., 2011). Therefore, virus-mediated EGF receptor activation is important for virus to escape the host innate immune response in vivo. Because various types of viruses activate EGF receptor to facilitate their replication (Ueki et al., 2013), our findings provide an insight into the mechanism underlying the promotion of viral infection and replication by virus-mediated EGF receptor activation.

Unlike what was observed for DDX60, EGF-receptor-mediated Tyr phosphorylation is essential for the activation of TLR3, which recognizes viral RNA (Yamashita et al., 2012). Thus, a high EGF concentration is expected to promote the TLR3-mediated, but not the DDX60-mediated, innate immune response. TLR3 promotes cross-priming and activates cytotoxic T lymphocytes (Schulz et al., 2005). Conversely, the RIG-I pathway is required for initial antiviral responses (Koyama et al., 2007). Serum EGF and EGF receptor expression levels on the cell surface are reduced during aging (Shurin et al., 2007). Thus, EGF-mediated control of the innate immune responses may be a cause of age-dependent functional changes in innate immunity.

**EXPERIMENTAL PROCEDURES**

**Cells, Viruses, and Reagents**

Cell culture media for HEK293, HeLa, O, HuH-7, and HuH-7.5 cells were described previously (Oshiumi et al., 2009, 2013). Preparation of mouse BM-DCs and peritoneal macrophages was described previously (Oshiumi et al., 2010a). WT and DDX60⁻/⁻ MEFs were prepared from day 12.5 to day 14.5 embryos. MEFs were immortalized using large T antigen of SV40. Preparation of VSV Indiana strain, SeV (HVJ strain), and HSV-1 was described previously (Miyashita et al., 2011). Influenza A virus (PR8) was provided by Y. Sakoda (Hokkaido University). We used salmon sperm dsDNA (Invitrogen), erlotinib (Funakoshi), mouse recombinant EGF, (Wako), anti-DDX60 polyclonal antibody (SIGMA), anti-HA polyclonal antibody (SIGMA), anti–ha-actin monoclonal antibody (AC-15) (SIGMA), anti-RIG-I monoclonal antibody (Alme-1) (Axxora Platform), anti-G3BP antibody (Abcam), anti-NK (TBIK1) antibody (Ep6111) (Abcam), anti-p–TBIK1 rabbit monoclonal antibody (CST), anti-p–IRF3 (S396) rabbit monoclonal antibody (CST), and anti–IRF3 antibody (Invitrogen and Zymed). Preparation of viral RNA is described in Supplemental Experimental Procedures.

**Electroporation**

1 µg viral RNA (HCV JFH-1 GND ssRNA or VSV dsRNA) and 1 x 10⁵ cells were suspended in 400 µl of OPTI-MEM I in a 4-mm cuvette and pulsed once atCell Reports, Volume 11, Pages 1193–1207, May 26, 2015 ©2015 The Authors 1205
Pull-Down Assay
An ATP affinity test was purchased from JENA BIOSCIENCE. 6A-H-ATP-agarose, 8A-H-ATP-agarose, AP-ATP-agarase, and EDA-ATP-agarase were mixed. An ATP affinity test was conducted according to the manufacturer’s instructions. A pull-down assay using biotin-conjugated dsRNA was described previously (Oshiumi et al., 2013).

Treatment with EGF
To examine the effect of EGF on short poly:C-induced IFN-β promoter activation, EGF was added to medium for HEK293 and HuH-7 cells 24 hr before transfection. Cells were transfected with p125lac reporter, Renilla luciferase vector (internal control), and short poly:C for 24 hr. To investigate the effect of DDX60-YF mutation, EGF was added to media of HEK293 cells 24 hr before transfection. Cells were transfected with p125lac reporter, Renilla luciferase vector, and WT DDX60 or DDX60-YF encoding vector for 24 hr and transfected with short poly:C for 8 hr. To investigate the effect of EGF on IFN-β, IP10, and TNF-α mRNA expression after VSV or Flu infection, HeLa cells were cultured in serum-free media with or without EGF for 24 hr, and infected with VSV.

SUPPLEMENTAL INFORMATION
Supplemental Information includes Supplemental Experimental Procedures and six figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.04.047.

AUTHOR CONTRIBUTIONS
Y.M. and M. Okabe generated DDX60-deficient mice. H.O., M. Miyashita, and M. Okamoto performed all other experiments. H.O. designed the experiments and wrote the manuscript. M. Matsumoto and T.S. supervised the research.

ACKNOWLEDGMENTS
We thank Dr. A. Chan (Genentech), Dr. M. Kohara (Tokyo Metropolitan Institute of Medical and Science), and Dr. K. Shimotohno (National Center for Global Health and Medicine) for critical comments, and we thank all colleagues in our laboratory. A plasmid encoding HCV JFH-1-GND (pSGR-JFH-1-GND) and O cells were kindly gifted from Dr. T. Wakita (National Institute of Infectious Diseases) and Dr. N. Kato (Okayama University), respectively. This work was supported in part by Kato Memorial Bioscience Foundation and Grants-in-Aid from Ministry of Education, Science, and Culture and Ministry of Health, Labor, and Welfare of Japan and by the GI-CoRE project.

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