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Dual targeting of RIG-I and MAVS by MARCH5 mitochondria ubiquitin ligase in innate immunity

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ABSTRACT

The mitochondrial antiviral signaling (MAVS) protein on the mitochondrial outer membrane acts as a central signaling molecule in the RIG-I-like receptor (RLR) signaling pathway by linking upstream viral RNA recognition to downstream signal activation. We previously reported that mitochondrial E3 ubiquitin ligase, MARCH5, degrades the MAVS protein aggregate and prevents persistent downstream signaling. Since the activated RIG-I oligomer interacts and nucleates the MAVS aggregate, MARCH5 might also target this oligomer. Here, we report that MARCH5 targets and degrades RIG-I, but not its inactive phosphomimetic form (RIG-I^{S8E}). The MARCH5-mediated reduction of RIG-I is restored in the presence of MG132, a proteasome inhibitor. Upon poly(I:C) stimulation, RIG-I forms an oligomer and co-expression of MARCH5 reduces the expression of this oligomer. The RING domain of MARCH5 is necessary for binding to the CARD domain of RIG-I. In an *in vivo* ubiquitination assay, MARCH5 transfers the Lys 48-linked polyubiquitin to Lys 193 and 203 residues of RIG-I. Thus, dual targeting of active RIG-I and MAVS protein oligomers by MARCH5 is an efficient way to switch-off RLR signaling. We propose that modulation of MARCH5 activity might be beneficial for the treatment of chronic immune diseases.

1. Introduction

Upon RNA virus infection, the host utilizes retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), one of the pattern recognition receptors (PRRs), to recognize specific viral products called pathogen-associated molecular patterns (PAMPs). PRRs trigger signals leading to antiviral immune responses, resulting in the production of type I interferons (IFNs), proinflammatory cytokines, and chemokines. At each step of immune response, there are stimulatory and inhibitory signals that control the immune response; ubiquitination is one such signal. RIG-I and melanoma differentiation-associated gene 5 (MDA5) belong to the RLR family and share common functional motifs although they recognize distinct non-self RNA signatures. Both RIG-I and MDA5 contain the following: an N-terminal tandem caspase recruitment domain (CARDs) that is highly conserved, a central DExD/H box RNA helicase domain with an ATP-binding motif, and a C-terminal domain (CTD) [1–3]. Upon binding to cytoplasmic viral RNA, RIG-I gets activated and is translocated to the mitochondria. RIG-I then interacts with its adaptor protein, the mitochondrial antiviral signaling protein (MAVS, also known as IPS-1, VISA, or Cardiff) on the mitochondrial membrane. Upon interacting with RIG-I, MAVS forms prion-like functional aggregates and recruits downstream molecules such as TNF receptor-associated factors (TRAFs), NF-κB essential modulator, and interferon (IFN) regulatory factors; thereby, promoting the production of IFNs and cytokines [4]. Subsequent type I IFN signaling rapidly induces the expression of several IFN stimulated genes (ISGs), which restrict virus infection and replication.

CARD belongs to the death domain superfamily [5] and is often

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involved in protein-protein interactions. N-terminal tandem CARDs of RIG-I form oligomeric filaments upon activation signals [6] and these RIG-I filaments are required to induce functional MAVS protein aggregates on the mitochondria membrane [7,8]. These MAVS protein aggregates are detergent-insoluble prion-like aggregates and appear to be highly ordered filament formed by MAVS CARD [4]. Thus, protein-protein interactions involving CARDs in RLRs are central to antiviral signaling. However, spontaneous MAVS oligomerization that is correlated with increased secretion of type I IFN [9] has been reported in systemic lupus erythematosus (SLE) patients. Accordingly, activation of RLR signaling is essential to restrain RNA-virus infection. However, excessive activation of this pathway can be detrimental to the host. Hence, RLR signaling needs to be tightly controlled at each step [10].

We previously showed that MARCH5 E3 ubiquitin ligase on the mitochondrial outer membrane degraded the MAVS and prevented persistent downstream signaling. MARCH5 did not interact with monomeric MAVS but specifically degraded the MAVS aggregate [11]. Thus, it appears that MARCH5-mediated degradation of MAVS does not interfere with RLR signaling, but it switches off the persistent activation of MAVS. This is different from other known regulators that disrupt MAVS signaling. UBX domain protein 1 (UBXN1) interferes with MAVS oligomerization and Poly(I:C)-binding protein 2 (PCBP2) [12] and Nedd4 family interacting protein 1 (Ndfip1) [13] facilitate the degradation of MAVS by recruiting cytosolic E3 ligases. It is noteworthy that MARCH5, also known as MITOL, plays a crucial role in protein quality control. It specifically recognizes and binds to mutated superoxide dismutase 1 and expanded polyglutamine aggregates that accumulate in the mitochondria [14,15]. Likewise, MARCH5/MITOL protects neuronal cells from mitochondrial damage caused by accumulation of S-nitrosylated microtubule-associated protein 1B-light chain 1 (LC1) [16]. In addition, a recent report showed that MARCH5 degrades FUN14 Domain Containing 1 (FUNDC1) involved in mitochondrial quality control [17]. The underlying mechanism of MARCH5 in recognizing protein aggregates is still unknown, but its characteristics undeniably show that it is a crucial quality control factor which makes an essential contribution to mitochondrial homeostasis.

In this study, we examined whether MARCH5 might also target the RIG-I oligomer in addition to MAVS aggregates in the mitochondria. We found that MARCH5 targets and degrades the functional RIG-I in a proteasome dependent manner. Thus, dual targeting of active RIG-I and MAVS oligomers by MARCH5 is an efficient way to turn off RLR signaling. We propose that modulation of MARCH5 activity might be beneficial for the treatment of chronic immune diseases such as SLE.

2. Materials and methods

2.1. Plasmids

Myc-tagged MARCH5-truncated mutants (MARCH5 C (ΔRING), MARCH5 N (ΔT234)) were generated using Myc-MARCH5^{WT} as a template. Flag-RIG-I and Flag-N-RIG-I were kindly provided by Dr. Hui Zhong. Flag-RIG-I lysine mutants (K99R, K154R, K164R, K169R, K181R, K193R, K203R, K284R, K292R, K644R, K796R) were generated with PCR, using a site-directed mutagenesis kit (Intron). Reporter gene constructs were provided by Dr. Jae U. Jung (University of Southern California, Los Angeles, CA, USA). The MARCH5 shRNA plasmid targeting 3′-UTR sequences previously established was used in this study [18].

2.2. Generation of MARCH5 KO cells

MARCH5 knockout HEK293T cells were previously generated using transcription activator-like effector nuclease (TALEN) technology. The MARCH5-specific TALEN plasmids were obtained from ToolGen, Inc. [11].

2.3. Cell culture and transfections

HEK293T (ATCC, ACS-450), Raw264.7 (ATCC, TIB-71) cells were cultured in DMEM (Invitrogen) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (GIBCO BRL) in a 5% CO_2 incubator at 37 °C. Plasmid DNA constructs were transfected using polyethylenimine (Polysciences). Poly(I:C) (InvivoGen) was transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

2.4. Luciferase reporter assay

HEK293T cells were transfected with a NF-κB of luciferase reporter plasmid, renilla luciferase plasmid, an indicated variety expression plasmids or control (pcDNA) plasmid. Transfected cells were stimulated with poly(I:C) (1 μg/ml) for 48 h. Luciferase activity was measured at 60 h after transfection using a luminometer (BioTek) with a dual-luciferase reporter assay system according to the manufacturer's instructions (Promega). Data represent relative firefly luciferase activity, normalized to renilla luciferase activity.

2.5. Ubiquitination assay

Cells were treated with 10 μM of MG132 for 12 h before harvest. Whole cells were lysed with RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.1% sodium deoxycholate, 5 mM EDTA and 5 mM EGTA) containing complete protease and phosphatase inhibitors. The same amount of protein lysates (700–1000 μg) was immunoprecipitated with anti-FLAG-M2 affinity agarose beads at 4 °C for 2 h ~ overnight. The immune complex was washed extensively four times with RIPA buffer and boiled for 5 min with 2 X sample buffer. Analysis of ubiquitination was performed by immunoblotting using anti-Ub antibody or anti-HA antibody.

2.6. Immunoblot analysis and immunoprecipitation assay

For immunoblotting analysis, cell lysates lysed by RIPA buffer supplemented with protease and phosphatase inhibitors. The cell lysates were homogenized and centrifuge to remove cell debris. Protein concentrations were determined using Bradford assay and separated on gradient SDS-PAGE and transferred to the nitrocellulose membrane (GE healthcare). Membranes were blocked with 5% Bovine serum albumin (BSA) for 1 h at room temperature and incubated with primary antibody overnight at 4 °C. And thereafter with the peroxidase-linked secondary antibodies for 1 h at room temperature. The blots were visualized using the ECL system (GE healthcare). The following antibodies were used in immunoblotting analysis: anti-MARCH5 (1:1000) are from Novus, anti-GAPDH (1:1000), anti-MAVS (1:1000), anti-IRF3 (1:1000), anti-phospho-IRF3 (Ser396; 1:1000) and antibodies were purchased from Cell Signaling Technology. Anti-FLAG (M2; 1:2000) was from Sigma. Antibodies for anti-HA (1:1000), anti-Ub (1:1000), anti-Tubulin (1:1000), anti-c-Myc (1:1000) were purchased from Santa Cruz Biotechnology.

2.7. Semi-denaturing detergent agarose gel electrophoresis

Semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) was performed according to a published protocol with minor modifications [19]. In brief, whole cell pellets were resuspended in 1 X sample buffer (0.5 X TBE, 10% glycerol, 2% SDS, and 0.0025% bromophenol blue) and loaded onto a vertical 1.5% agarose gel (Bio-Rad). After electrophoresis in the running buffer (1 X TBE and 0.1% SDS) for 35 min with a constant voltage of 100 V at 4 °C, the blots were transferred to PVDF membrane (Millipore) for immunoblotting [4].

2.8. Immunofluorescence

For visualization of subcellular localization, HEK293T cells were seeded on coverslips in six-well plates and transfected with indicated plasmids. 12 h after transfection, cells were trasfected with poly(I:C). At 48 h post-transfection, the cells were pre-treated with 125 nM of MitoTracker-Red (Molecular Probes) for 30 min before harvest. Cells fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, rinsed with PBS, and permeabilized with methanol for 20 min. For immunofluorescence staining, cells were blocked with 1% bovine serum albumin in PBS for 1 h at room temperature. The cells were then rinsed with PBS and incubated with mouse anti-Myc or anti-Flag monoclonal antibody (Sigma, USA, 1:500 dilution) overnight at 4 °C, followed by incubation with goat α-mouse IgG secondary antibody (1:500 dilution) for 1 h at room temperature. The nuclei of the cells were stained with 50 μg/mL DAPI. After final washing, coverslips were mounted in VECTASHIELD mounting medium (Vector lab). Images were captured and analyzed using the Nikon (A1R) confocal microscopy.

2.9. CHX chase assay

After transfection of plasmids, cells were treated with CHX (100 μg/ml) for various times ranging from 0 to 12 h. Lysates were lysed and analyzed by immunoblotting with indicated antibodies.

3. Results

3.1. MARCH5 targets and degrades the RIG-I oligomer

We previously reported that the MARCH5 ubiquitin ligase present on the mitochondrial outer membrane specifically degrades the functional MAVS aggregates and prevents persistent activation of MAVS protein [11]. Since the activated RIG-I tetramers recruit MAVS through CARD-CARD interaction and might form the RIG-I/MAVS complex on the mitochondrial membrane, we addressed whether MARCH5 also targets RIG-I on the mitochondria. To test this, we transfected HEK293T cells with the activated form of RIG-I (N-terminal RIG-I with 2 caspase recruitment domains (CARDs: N-RIG-I) which nucleates the MAVS oligomer and increasing the concentration of MARCH5 [20,21]. We found that Myc-MARCH5 significantly reduced the expression levels of Flag-N-RIG-I in a dose-dependent manner. The reduction in N-RIG-I levels appeared to be mediated by the proteasome pathway since a proteasome inhibitor, MG132, restored the N-RIG-I levels (Fig. 1A). Likewise, a ubiquitin ligase defective mutant of MARCH5^{H43W} was not able to reduce the N-RIG-I levels (Fig. 1B), suggesting that the ubiquitin-proteasome pathway plays a role in MARCH5-mediated degradation of RIG-I. It has been shown that phosphorylation of Ser 8 on the first CARD of RIG-I negatively regulates the RIG-I signaling pathway, reducing RIG-I activation through TRIM25-dependent ubiquitination and MAVS binding [3]. Here, we utilized a phospho-dead mutant of RIG-I (RIG-I^{S8A}) and a phosphomimetic RIG-I (RIG-I^{S8E}) in which Ser was switched to Ala and Glu, respectively and examined whether MARCH5 regulated these RIG-I mutant proteins. Interestingly, co-immunoprecipitation assay revealed that MARCH5 bound to RIG-I wild-type (RIG-I^{WT}) and RIG-I^{S8A} but not to RIG-I^{S8E} (Fig. 1C). Likewise, MARCH5 was able to reduce the expression levels of RIG-I^{S8A} but not RIG-I^{S8E}. The reduction in the levels of RIG-I^{S8A} requires MARCH5 E3 ligase activity (Fig. 1D). Immunofluorescence staining analysis verifies that after poly(I:C) stimuli, both N-RIG-I and RIG-I^{S8A} co-localized with mitochondria whereas RIG-I^{S8E} was excluded from the mitochondria (Fig. 1E). These results indicate that MARCH5 targets the activated RIG-I oligomer. Next, we determined the oligomerization patterns of endogenous RIG-I upon poly(I:C) transfection in Raw264.7 cells. In a native gel, RIG-I showed the oligomeric patterns in a poly(I:C) dose-dependent manner accompanied with the phosphorylation of TBK1, a downstream regulator (Fig. 1F). We found that the overexpression of MARCH5 not only decreased the N-RIG-I levels in SDS-PAGE, but also significantly reduced the oligomeric form of N-RIG-I (Fig. 1G). Together, our data showed that MARCH5 targets and degrades the activated RIG-I in addition to MAVS aggregates.

3.2. CARD domain of RIG-I and RING domain of MARCH5 are required for MARCH5-RIG-I interaction

Next, we investigated the interaction between MARCH5 and RIG-I. Flag-RIG-I and Flag-N-RIG-I were overexpressed in HEK293T cells along with Myc-MARCH5 and co- immunoprecipitation assay was carried out using anti-Flag antibody. We found that MARCH5 interacted with both RIG-I and N-RIG-I, but MARCH5 interaction with N-RIG-I was stronger than that with RIG-I (Fig. 2A). Next, we utilized deletion mutants of RIG-I to examine its interaction with MARCH5. We found that the RIG-I with the 2 CARD domains (N-RIG-I) showed strong binding to MARCH5 whereas RIG-I without the 2 CARD domains (C-RIG-I) lost its binding ability to MARCH5 (Fig. 2B). Meanwhile, MARCH5 having a RING domain deletion (MARCH5 C) lost its binding ability to RIG-I, but a MARCH5 mutant with the deletion of transmembrane domains 2–4 (MARCH5 N) showed binding to RIG-I (Fig. 2C), indicating that RING domain of MARCH5 is necessary for its interaction with RIG-I. In immunofluorescence staining analysis, we observed that MARCH5 N showed more diffused pattern in the cytoplasm although part of them still co-localized with mitochondria (Supplementary Fig. 1B). In addition, we found that Flag-C-RIG-I appears to lose the ability to locate to mitochondria (Supplementary Fig. 1A). Thus, we could not exclude the possibility that Flag-C-RIG-I did not bind MARCH5 because they could not localize to mitochondria. Together, our data showed that CARD domains of RIG-I and RING domain of MARCH5 are required for the interaction between MARCH5 and RIG-I.

3.3. MARCH5 ubiquitinates RIG-I using Lys 48-linked polyubiquitin chain

MARCH5 is an E3 ubiquitin ligase that conjugates the ubiquitin molecule to the target protein. We thus examined whether MARCH5 ubiquitinates RIG-I molecules. To test this, HEK293T cells were transfected with Flag-RIG-I and Myc-MARCH5 and precipitants with anti-Flag antibody-conjugated beads were analyzed using anti-ubiquitin (Ub) antibody. We found a strong dose-dependent increase in polyubiquitination of RIG-I by MARCH5 (Fig. 3A). In contrast, no ubiquitination pattern of RIG-I was observed in cells transfected with MARCH5^{H43W}. We next compared RIG-I ubiquitination in MARCH5$^{+/+}$ and MARCH5$^{-/-}$ cells. Polyubiquitination pattern of RIG-I in MARCH5$^{+/+}$ cells was observed whereas it was scarcely seen MARCH5$^{-/-}$ cells (Fig. 3B). Polyubiquitination formed each ubiquitin molecules through 7 lysine residues. The Lys (K) 48 dependent ubiquitination mainly leads to the proteasome-dependent degradation of the target protein; and K63 dependent ubiquitination is involved in signaling pathways [9]. To determine whether MARCH5 uses K48 ubiquitin-dependent degradation for RIG-I, we generated the K48R and K63R ubiquitin mutants in which K48 and K63 ubiquitin proteins were substituted with Arg (R), respectively. Wild type ubiquitin and ubiquitin mutants (K48R, K63R and 2KR double mutants) were introduced into cells along with RIG-I and MARCH5, and ubiquitination of RIG-I was determined using anti-HA antibody. Immunoblot analysis revealed that cells expressing K48R or 2KR ubiquitin mutants did not show polyubiquitinated RIG-I (Fig. 3C). However, cells expressing K63R ubiquitin still retained the polyubiquitinated RIG-I as those in wild type ubiquitin. We also verified the MARCH5-dependent ubiquitylation of RIG-I after poly(I:C) stimuli and found that endogenous RIG-I ubiquitylation was significantly reduced in MARCH5 knockout cells (Fig. 3D). Protein stability of RIG-I was also examined after cycloheximide (CHX) treatment (Supplementary Fig. 2) and the data indicated that protein stability of RIG-I maintained better in cells treated with shRNA for MARCH5. Together, the data here showed that MARCH5 ubiquitinates

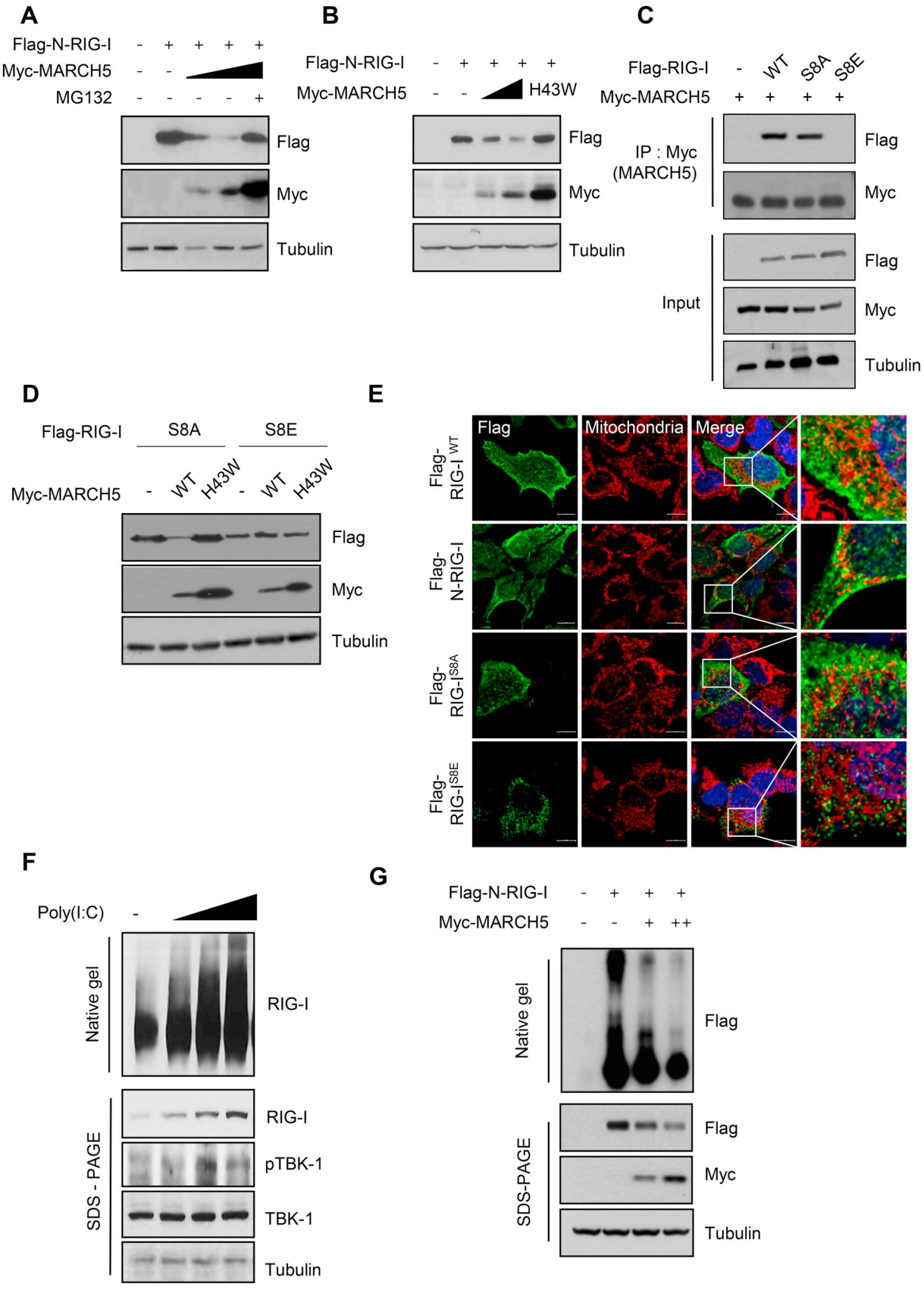

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Fig. 1. MARCH5 degrades the RIG-I oligomer. (A, B) Immunoblotting of Flag-N-RIG-I and Myc-MARCH5^{WT} or Myc-MARCH5^{H43W} in HEK293T cells. Cells were co-transfected with indicated plasmids for 36 h and treated with MG132 for 12 h before harvest. Expression levels were analyzed by immunoblotting with indicated antibodies. (C) Immunoprecipitation analysis of Myc-MARCH5 and Flag-RIG-I (WT: full length, S8A, S8E). HEK293T cells co-transfected with indicated plasmids and interaction was analyzed by immunoblotting with indicated antibodies. (D) Cells were co-transfected with indicated plasmids for 36 h and expression levels were analyzed by immunoblotting with indicated antibodies. (E) HEK293T cells were transfected with plasmids expressing Flag-N-RIG-I or Flag-RIG-I^{WT} and mutants (S8A, S8E). 12 h after transfection, cells were trasfected with poly(I:C) for 24 h. The subcellular localization of RIG-I by an immunofluorescence staining. Mitochondria was co-stained using MitoTracker–Red. (F, G) The oligomerization patterns of endogenous RIG-I or Flag-N-RIG-I. The cells were stimulated by transfection with poly(I:C) for dose-dependent manner in Raw264.7 cells (F) or indicated plasmids in HEK293T cells (G) for 36 h. Cell pellets were mixed with 2 X native sample buffer, followed by native-PAGE and SDS-PAGE.

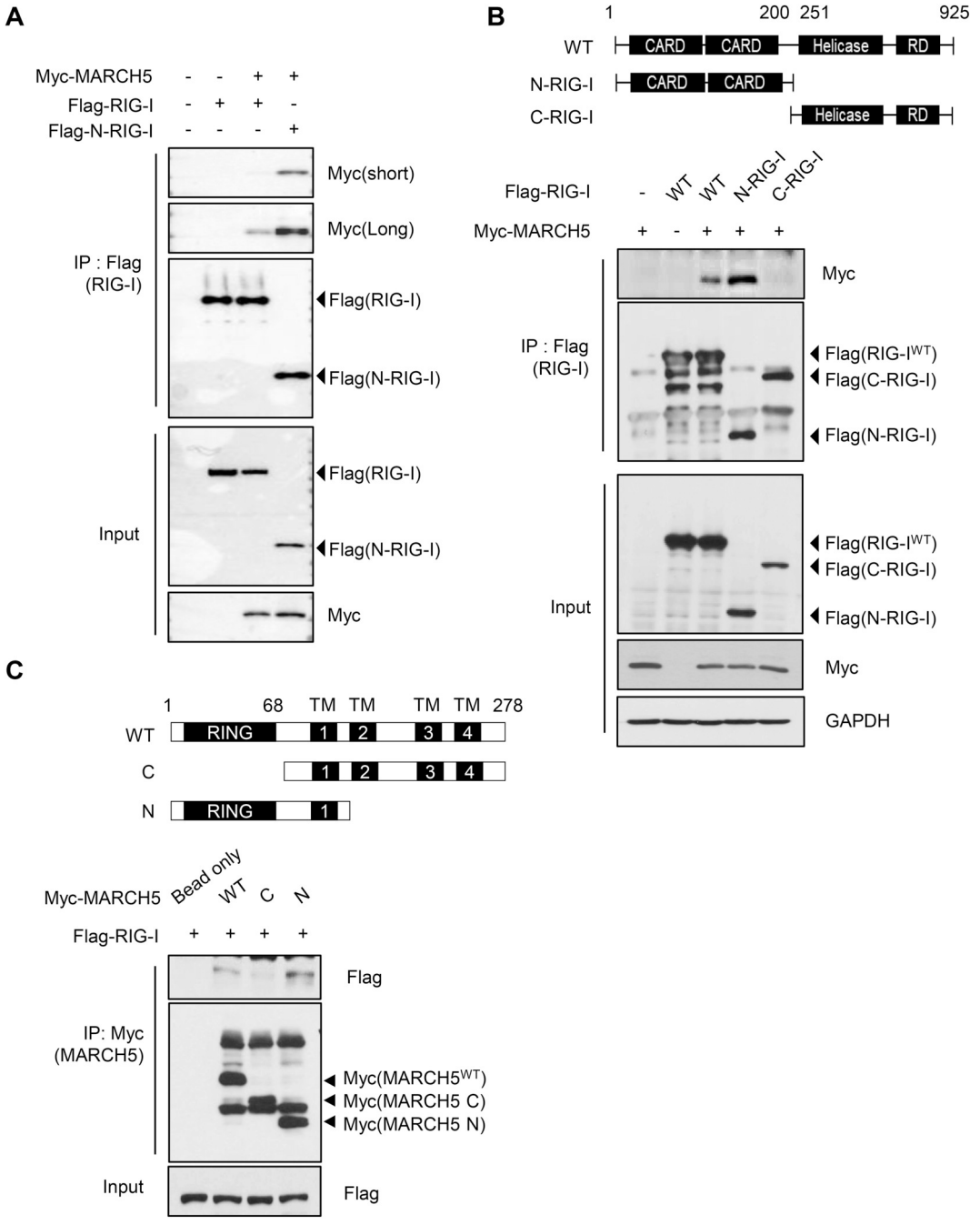

Fig. 2. MARCH5 interacts with RIG-I. (A) Interaction between MARCH5 and RIG-I in HEK293T cells. Cells were transfected with Flag-RIG-I or Flag-N-RIG-I along with Myc-MARCH5. Interaction was determined by immunoblotting with anti-Myc antibody after lysates were immunoprecipitated with anti-Flag antibody. (B, C) Immunoprecipitation analysis of Flag-RIG-I or Myc-MARCH5-truncated mutants. HEK293T cells were co-transfected with indicated plasmids and interaction was analyzed by immunoblotting with indicated antibodies. Flag-RIG-I fragments (RIG-I^{WT} (full length), N-RIG-I, C-RIG-I) (B). Semi-*in vivo* pulldown assay using Myc-MARCH5 fragments (MARCH5^{WT} (full length), MARCH5 C (ΔRING), MARCH5 N (ΔT234)) (C).

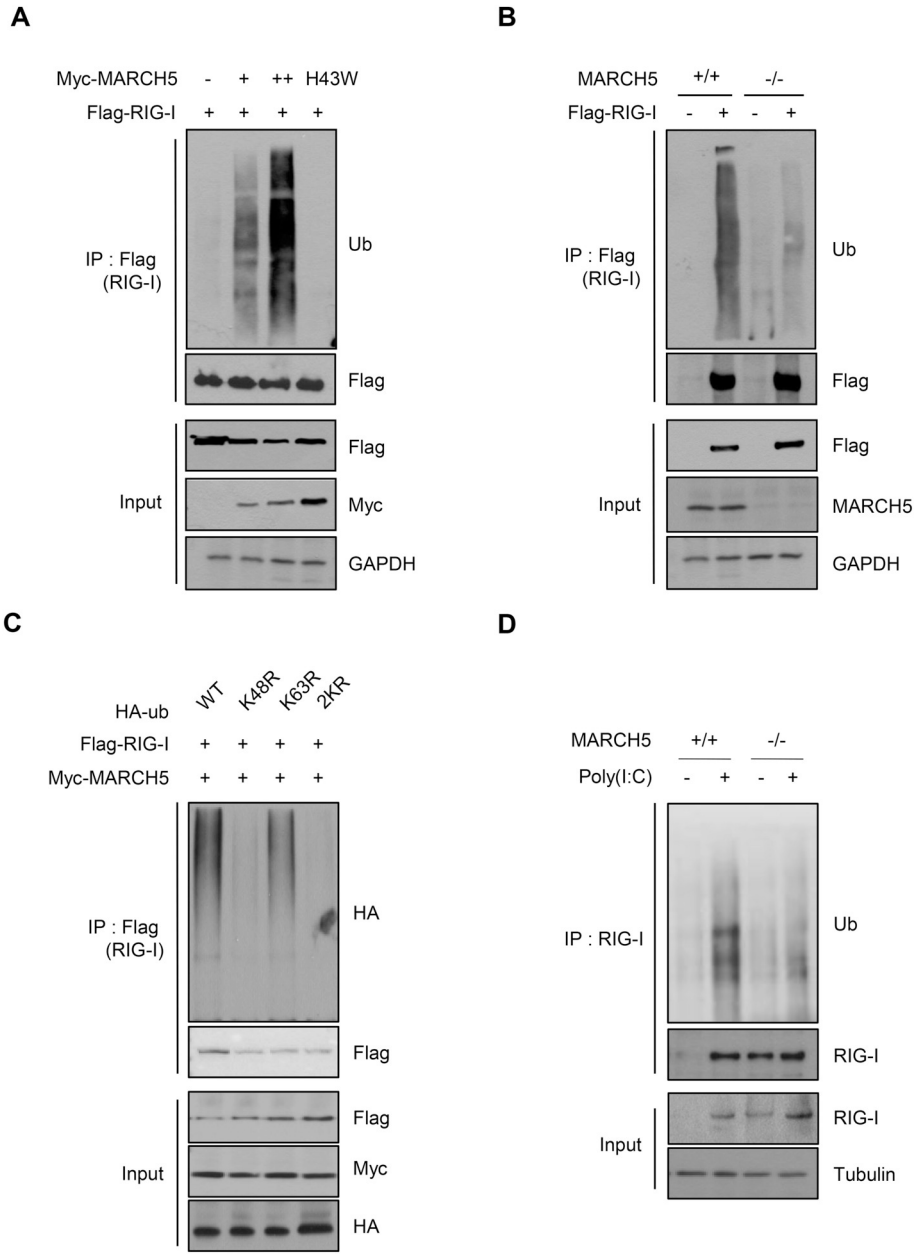

Fig. 3. RIG-I is ubiquitinated by MARCH5. (A) Ubiquitination assay of Flag-RIG-I by using anti-Flag antibody in transfected HEK293T cells along with Myc-MARCH5 WT or H43W mutant. Levels of ubiquitination were determined by anti-Ub antibody. (B) Ubiquitination of Flag-RIG-I in MARCH5$^{+/+}$ or MARCH5$^{-/-}$ HEK293T cells transfected with Flag-RIG-I. (C) Ubiquitination assay of Flag-RIG-I along with Myc-MARCH5 and HA-Ub lysine mutants. Ubiquitination of Flag-RIG-I by ubiquitin mutants were assessed by immunoblotting with anti-HA antibody. (D) The cells were transfected with poly(I:C) for 48 h and analyzed for ubiquitination of endogenous RIG-I in MARCH5$^{+/+}$ or MARCH5$^{-/-}$ HEK293T cells.

RIG-I using Lys 48-linked polyubiquitin chain and degrades through the proteasome pathway.

3.4. MARCH5 attaches ubiquitin on the Lys 193 and 203 residues of RIG-I

Next, we examined which lysine of RIG-I was ubiquitinated by MARCH5 and searched for putative ubiquitination sites using Ub Pred website (www.ubpred.org). This analysis predicted 12 putative Lys sites and we substituted each of them to Arg. We transfected HEK293T cells with Myc-MARCH5 and each of these RIG-I mutants and analyzed the RIG-I expression levels. Among the 12 Lys substitutes of RIG-I, we found that Lys 193 and 203 residues of RIG-I appeared to be targets of MARCH5 since expression levels of K193R and K203R mutants were not affected by MARCH5 (Fig. 4A). Expression of all other Lys substitutes of RIG-I was reduced by MARCH5 (Fig. 4A and Supplementary Fig. 3). *In vivo* ubiquitination assay revealed that polyubiquitin chains on RIG-I K193R and K203R mutants were significantly reduced by MARCH5, whereas the K284R mutant still retained the polyubiquitin chains (Fig. 4B). To address functional role of these RIG-I mutants in RLR signaling, we next examined protein expressions of RIG-I mutants and MAVS after poly(I:C) transfection for 48 h. We found that MARCH5 significantly reduced the RIG-I^{WT} whereas RIG-I K193R and K203R retained their protein stability (Fig. 4C). Interestingly, endogenous MAVS expression levels in these cells were all reduced. In the NF-kB luciferase reporter assay after poly(I:C) stimuli, we also found that MARCH5 not only reduced the luciferase activities in cells expressing RIG-I^{WT} and RIG-I K284R, but also those in cells introduced with RIG-I K193R or K203R (Fig. 4D). This is also verified by p-IRF3 levels in these cells (Fig. 4E). The p-IRF3 levels were reduced by MARCH5 in cells with RIG-I K284R as well as with RIG-I K193R/K203R. These data suggest that MARCH5 dual-targets the activated RIG-I as well as MAVS and MAVS is a major determinant of downstream signaling. Together, our data suggest that dual targeting by MARCH5 may be an efficient way to switch-off RLR signaling.

4. Discussion

In the present study, we propose that the mitochondrial E3 ligase,

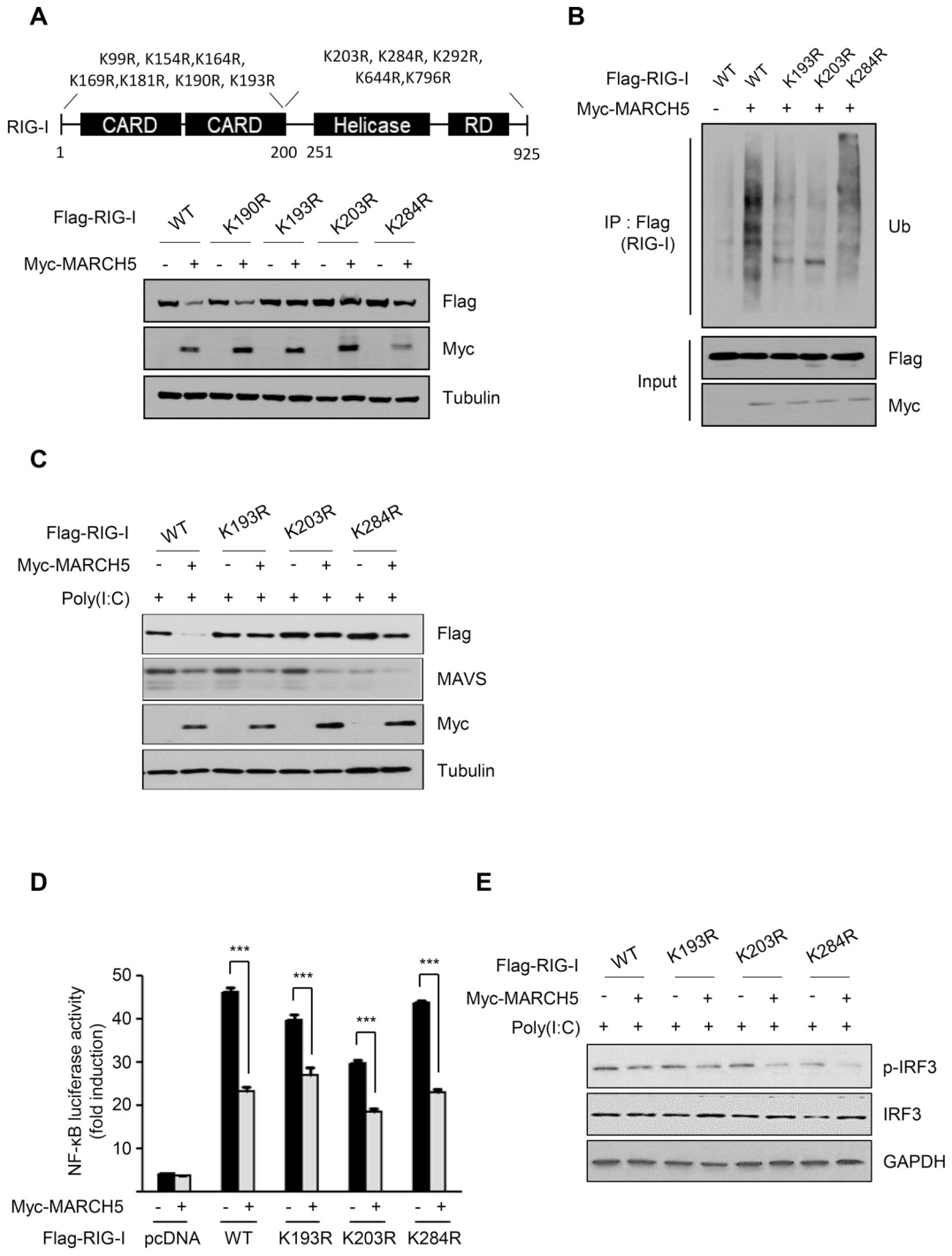

Fig. 4. MARCH5 transfers ubiquitin to Lys193 and Lys203 residues of RIG-I. (A) Expression of Flag-RIG-I lysine mutants by Myc-MARCH5 in HEK293T cells. (B) Ubiquitination of Flag-RIG-I lysine mutants by Myc-MARCH5. Ubiquitination of Flag-RIG-I lysine mutants was assessed by immunoblotting with anti-Ub antibody. (C, E) Immunoblotting of Flag-RIG-I lysine mutants and Myc-MARCH5 in HEK293T cells for 12 h and transfected with poly(I:C) for 48 h before harvest. Expression levels were analyzed by immunoblotting with indicated antibodies. (D) Promoter assay of NF-κB. Graphs represent fold-induction relative to the luciferase activity in control cells. Error bars mean ± SEM. (n = 4). All data are representative of at least three independent experiments. Statistical significance was determined using two-way ANOVA followed by Bonferroni's *post hoc* tests. ($^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$).

MARCH5 is an intriguing negative regulator of RLR signaling. MARCH5 prevents persistent antiviral immune response at the mitochondria by dual targeting of the activated RIG-I and MAVS oligomers and promoting their degradation. To the best of our knowledge, this is the first report of an E3 ligase targeting two different molecules of the RLR signaling pathway. Both RIG-I and MAVS contain CARD domain. Two CARDs of RIG-I are unmasked in the presence of viral RNAs which then interacts with the CARD of MAVS in the mitochondria. MARCH5 binds to the activated RIG-I and MAVS oligomers at the mitochondria and degrades these active protein complexes.

Ubiquitination is one of the major post-translational modifications that regulates innate immune signaling [22]. Lys63-linked polyubiquitin chains on RIG-I mediated by TRIM25 and Riplet are shown to be important for RIG-I activation [23–25] although a recent report demonstrated that Riplet and not TRIM25, is the obligatory ubiquitin E3 ligase for RIG-I activation [6]. In contrast, RIG-I with Lys 48-linked

polyubiquitin chains is transferred to proteasome and subjected to degradation. RING finger E3 ligases of RNF125 and RNF122 have been implicated in inhibiting RIG-I signaling. RNF125 and RNF122 interact with and ubiquitinate the N-terminal CARDs of RIG-I [26,27]. RNF122 has been shown to specifically promote K48-ubiquitination of RIG-I Lys 115 and 146. Here we showed that MARCH5 also interacted with and ubiquitinated the CARDs of RIG-I on Lys 193 and 203 residues. Thus, CARDs of RIG-I appeared to be the major interacting domains with E3 ubiquitin ligases as well as sites for ubiquitination. The protein, A20, an ubiquitin-editing protein with both ubiquitin ligase and de-ubiquitinase activities, has also been shown to inhibit RIG-I-mediated antiviral signaling [28].

A unique feature of MARCH5 E3 ligase in the signaling of RLRs is the dual targeting of RIG-I and MAVS. In addition, MARCH5 tends to have a selectivity towards protein oligomers or aggregates since it did not bind to monomeric MAVS in unstimulated cells [11]. Transfection of poly(I:C) appeared to trigger the RIG-I oligomer in a dose-dependent manner in a native polyacrylamide gel (Fig. 1F). Interestingly, we also found that MARCH5 did not interact with the inactive phosphomimetic RIG-I^{S8E} (Fig. 1C). This Ser 8 phosphorylation on the CARD of RIG-I is shown to suppress RIG-I activation by interfering with TRIM25-dependent ubiquitination and MAVS binding [3]. Thus, inactive RIG-I^{S8E} mutant is resistant to MARCH5 (Fig. 1D), since MARCH5 might not bind to cytosolic RIG-I monomer. This is also supported by a previous finding that MARCH5/MITOL degraded misfolded or protein aggregates but not normal SOD protein [14,15]. All these findings strongly suggest that MARCH5 plays a protective role in mitochondrial homeostasis, preventing prolonged accumulation of protein oligomers or aggregates in the mitochondria.

It is likely that aberrant and sustained activation of RLR signaling is detrimental to the host. SLE is a complex autoimmune disease and elevated IFN activity was observed in blood samples of lupus patients with active disease [29]. Interestingly, recent studies showed that some SLE patients exhibited a higher molecular weight MAVS aggregate and these patients showed significantly higher serum concentrations of IFN-γ. In addition, polymorphism of MAVS and MDA5, another RNA helicase, showed differences with respect to IFN production and susceptibility to autoimmune disease [30,31]. Thus, modulation of MARCH5 activity by targeting RIG-I/MDA5 and MAVS oligomers could be used as a potential diagnostic and therapeutic strategy for treating autoimmune diseases.

Declaration of Competing Interest

The authors have declared that no conflict of interest exists.

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Appendix A. Supplementary data

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