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RAD23A negatively regulates RIG-I/MDA5 signaling through promoting TRAF2 polyubiquitination and degradation

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ABSTRACT

RIG-I/MDA5 plays a pivotal role in innate immunity by detecting intracellular double-stranded RNA (dsRNA) and activating the transcription of type I interferons and proinflammatory factors, but the exactly regulating mechanism of RIG-I/MDA5 signaling remains elusive. In this study, UbL-UBA domain containing protein RAD23A was identified as a negative regulator of RIG-I/MDA5-mediated signaling activation through a small interfering RNA (siRNA)-based screening. Knockdown of RAD23A augmented the expression of RIG-I/MDA5-mediated expression of proinflammatory cytokines and IFN-β whereas ectopic expression of RAD23A showed the converse effect. Moreover, we confirmed the interaction between RAD23A and tumor necrosis factor receptor-associated factor 2 (TRAF2), an essential mediator of RIG-I/MDA5 signaling, and found that RAD23A down-regulated TRAF2 protein level through ubiquitin–proteasome system. Therefore, this study identified RAD23A as a novel negative regulator of RIG-I/MDA5 mediated anti-virus response.

1. Introduction

Innate immunity detects microbial pathogen invasion through germline-encoded pattern-recognition receptors (PRRs) which recognize pathogen associated molecular patterns (PAMPs), including exogenous DNA, single-stranded (ss) RNA, double-stranded (ds) RNA and glycoproteins, *etc.* Viral dsRNA is recognized through two distinct pathways. Toll-like receptor 3 (TLR3) detects dsRNA phagocytosed in endosomes; helicases retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene-5 (MDA-5) detect cytoplasmic dsRNA generated during viral replication [1,2]. Activation of RIG-I/MDA5 leads to the assembly of a signaling complex composed of MAVS, tumor necrosis factor (TNF) receptor-associated factors (TRAFs), TANK-binding kinase 1 (TBK1), inhibitor of nuclear factor κB kinase ε (IKKε), *etc.* [3–6]. Then, transcription factors NF-κB, IRF3/IRF7, and ATF/c-Jun will be activated and promote the expression of proinflammatory cytokines such as TNF-α, interleukin-6 (IL-6) and type I interferons (IFN-I) [7].

Immune signaling pathways have to be tightly regulated to insure a successful immune response against viral infections; otherwise, aberrant IFNs or proinflammatory cytokines expression would be destructive rather than protective. Recent works have shown that RIG-I/MDA5 mediated NF-κB activation is transducted by TRADDs, TRAFs, RIP1, and FADD, which are also essential mediators of other signaling pathways such as TNF receptor signaling and TLRs signaling. The RIG-I/MDA5 signaling to IRFs has been reported to proceed through TRADD, TRAFs, TANK, NAP1, NEMO, TBK1, and IKKε [6]. However, many of the details in the regulation of RIG-I/MDA5 signaling need to be clarified. The mitochondrial protein MAVS [3,8], which directly associates with RIG-I/MDA5, is believed to be the central component of RIG-I/MDA5 signaling complex. MAVS contains TRAF2 and TRAF6 binding motifs and is reported to interact with both proteins [3,5]. Mikkelsen et al., reported that TRAF2 is essential for phosphorylation of the IκB and p38 and IFN-stimulated genes expression [9]. Yoshida et al., found that TRAF6 and MEKK1 play important roles in RLR signaling to NF-κB and MAPKs. TRAF3 is indispensable for RIG-I/MDA5-mediated activation of IFN regulatory factors (IRFs) [10,11]. Therefore, the fine-tuning of TRAFs is an important step for RIG-I/MDA5 signaling regulation.

Ubiquitination plays a key regulatory role in diverse cellular events, including innate immunity signaling [12,13]. The best known function of ubiquitination is to target protein degradation by the proteasome through covalent attachment of polyubiquitin

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chains (Lys-48 linked), which is a major mechanism by which cells regulate the abundance of particular proteins [14]. Whereas K63-linked polyubiquitination can direct other outcomes, such as DNA repair, protein recruitment, protein kinase activation and chromatin dynamics [15]. UBDs (ubiquitin binding domains) are a collection of modular protein domains that non-covalently bind to ubiquitin [16,17]. UBDs, such as the ubiquitin-associated domain (UBA), ubiquitin-interacting motif (UIM), A20 zinc finger (ZnF) domain influence various cellular events through binding to and regulating ubiquitinated proteins. Increasing evidences showed that UBD containing proteins play essential roles in regulating signaling in immune system [18,19].

To identify potential roles of UBD containing proteins in RIG-I/MDA5-mediated NF-κB and IRFs signaling, we used siRNAs to knockdown various UBD containing proteins in human embryonic kidney (HEK) 293 cells. Further, to mimic the viral RNA infection, polyinosine:polycytidine acid [poly(I:C)], the RIG-I/MDA5 ligand, was transfected into cells. The NF-κB activation and IFN-β production were determined by luciferase reporter assay. We identified RAD23A but not its homolog RAD23B, as a negative regulator of RIG-I/MDA5 signaling, and found that RAD23A was involved in RIG-I/MDA5 signaling through binding to TRAF2. Furthermore, we found that RAD23A down-regulate TRAF2 by promoting its degradation through ubiquitin–proteasome system. Therefore, our finding indicated that RAD23A could be a novel regulator of antivirus immunity.

2. Materials and methods

2.1. siRNA based screening

For screening assay, HEK293 cells were transfected with NF-κB and IFN-β luciferase reporter for 6 h and re-plated in 96-well format. Cell density should be 50–60% confluent after 12 h and the indicated siRNA (20 nM) smart pool (Thermo Scientific DharmaFECT) was transfected. 48 h later, cells were treated with Lipofectamine 2000 (Invitrogen) packed poly(I:C) (5 mg/ml, Invivogen) for 10 h, then NF-κB and IFN-β reporter activity were measured with Dual-Luciferase Reporter Assay System (Promega).

2.2. Transfection

HEK293 and MEF cells were cultured in DMEM containing 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO2. Transfection of plasmids was performed by using Vigofect (Vigorous) or Lipofectamine™ LTX Reagent (Invitrogen) following the manufacture's instruction. For HEK293 and MEF cells, siRNAs were performed by using Lipofectamine RNAiMAX (Invitrogen). RAW264.7 (Mouse Abelson murine leukemia virus-transformed macrophage; monocyte/macrophage cells) cells were maintained in DMEM containing 5% FBS and transfection of siRNAs is performed by using Amaxa® Cell Line Nucleofector® Kit V(LONZA, DCV-1031) and Nucleofector® Device (LONZA). Transfection of poly(I:C) was performed by using Lipofectamine 2000 (Invitrogen).

2.3. Cells harvest and Immunoprecipitation

The total cell lysates were prepared in RIPA buffer containing 20 mM Tris–HCl, pH 7.4, 150 mM NaCl, 10 mM EDTA, 1% (vol/vol) Triton X-100, 1% deoxycholate and cocktail (Roche). For immunoprecipitation experiments, HEK293 cells were lysed in E1A buffer (50 mM Hepes, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40, 5 mM EDTA) and cocktail (Roche). The whole cell homogenates were centrifuged and the supernatant was incubated with indicated antibodies and rotated at 4 °C overnight, then, incubated with protein A/G-Sepharose (GE) for another 2 h. Immunoprecipitates were washed five times, and then they were boiled in loading buffer and subjected to SDS–PAGE.

2.4. Real-time PCR analysis and ELISA

MEF cells were transfected with siRNA and treated with Lipofectamine 2000 packed poly(I:C) for 4–6 h. Cells were collected and the total RNA was extracted by Trizol® (Invitrogen). The diluted RNA was reverse transcripted and analysis with SYBR® Premix Ex Taq™ II (Perfect Real Time) (TAKARA, DRR081A) and Applied Biosystems 7300 real-time PCR System. The primers listed here were used to amplify a specific fragment of the following genes: IFN-β: F-ATGAGTGGTGGTTGCAGGC, R-TGACCTTTCAAATGCAGTAGATTCA; IL-6: F-CTGCAAGAGACTTCCATCCA, R-TGACCTTTCAAATGCAGTAGATTCA; TNF-α: F-CATCTTCTCAAAATTCGAGTGACAA, R-TGGGAGTAGACAAGGTACAACCC; GAPDH: F-AAGAGCCCATCAGAGGAATCC, R-CCTGCTTCACCACCTTCTTGA; RAD23A: F-AAGAGCCCATCAGAGGAATCC, R-CATGATCTCCGTCAGCATCGT; RAD23B: F- TGGAAGTGGGCACATGAATTAC, R-TCTTCATCAAAGTTCTGCTGTA.

For mouse IL-6 and IFN-β ELISAs, commercial kits (DAKEWE Biotech and Biotechnology Systems, respectively) were used following the manufacture's instruction.

2.5. Ubiquitylation assay

HEK293 cells were co-transfected with HA-TRAF2 and Myc-ubiquitin in the presence or absence of Flag-RAD23A for 36 h in DMEM with 10% FBS. Cells were treated with MG132 (10 uM) for 2 h, then the total cell lysates were prepared in RIPA buffer. Proteins were boiled at 95 °C for 5 min in the presence of 1% SDS to remove non-covalently associated proteins and diluted to 0.1% SDS. Samples were then immunoprecipitated overnight with 2 μg/ml HA antibody (Santa Cruz Biotechnology, Sc-7392) at 4 °C and protein A-agarose beads for another 2 h. After washing with RIPA buffer for five times, the immunocomplexes were boiled in loading buffer and subjected to SDS–PAGE, followed by Western blot with anti-Myc antibody. The light chain specific HRP goat anti-mouse IgG (Jackson ImmunoResearch, 115-035-174) was used to avoid heavy chain of antibody.

3. Results and discussion

3.1. Knockdown of RAD23A augments RIG-I/MDA5 mediated signaling activation

RIG-I/MDA5 recognizes viral RNAs in cytoplasm and triggers signal transduction cascades that finally lead to the production of type I IFNs and proinflammatory cytokines to suppress viral replication and assembly. This signaling is closely regulated, but the exact mechanism of this regulation, however, remains elusive. To explore potential roles of UBD containing proteins in dsRNA sensor RIG-I/MDA5 mediated signaling, we used a siRNA library to knockdown human genes encoding proteins containing UBD domain. The impact on NF-κB activation or IFN-β production in response to poly(I:C) was determined by luciferase reporter assay. We found that knockdown of RAD23A leads to higher NF-κB and IFN-β luciferase reporter activity. Some of the data were showed in Fig. 1(A). RAD23B and RAD23A are homologs and play redundant roles in some biologic processes [20], however, knockdown of RAD23B causes no significant changes in poly(I:C) induced activation of both reporters (Fig. 1A). To confirm this result, we depleted RAD23A with individual siRNA instead of siRNA pool, and observed similar result (Fig. 1B). In addition, we also found that ectopic

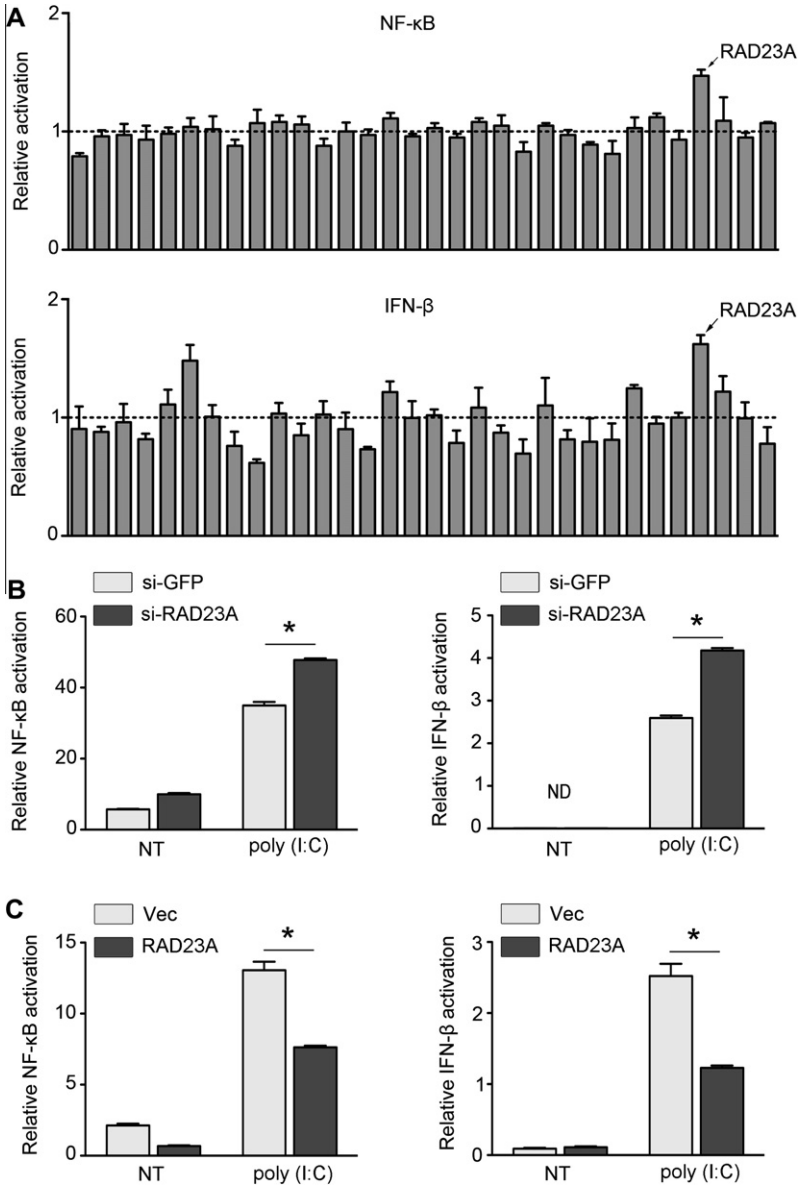

Fig. 1. An siRNA-based screening for UBD domain containing family members. (A) HEK293 cells were transfected with NF-κB and IFN-β luciferase reporter for 6 h and replated in 96-well format. Then the cells were transfected with indicated siRNA (20 nM) smart pool. 48 hours later, cells were treated with poly(I:C) for 10 h and NF-κB and IFN-β activity was measured. (B) HEK293 cells were manipulated as (A), except for the siRNA was single target. (C) HEK293 cells transfected with Flag-RAD23A or Flag-vector were treated with poly(I:C) and NF-κB and IFN-β reporter activity were measured. Data represented as the mean ± SD of triplicate samples. $^{*}p < 0.05$.

expression of RAD23A has the converse effect (Fig. 1C). These results suggest that RAD23A acts as a potential negative regulator of RIG-I/MDA5 signaling. RAD23B shares 50% homology with RAD23A. Knockout mice revealed that RAD23A and RAD23B have a fully redundant role in nucleotide excision repair (NER), and a partially redundant function in embryonic development [20]. Interestingly, studies showed RAD23A and RAD23B act differently when they are binding to the proteasome and polyubiquitinated proteins [21], suggesting that they have functions distinctive from each other in specific biological processes. This hypothesis is supported by our finding that RAD23A but not RAD23B is involved in RIG-I/MDA5-mediated signaling.

3.2. RAD23A negatively regulates RIG-I/MDA5 mediated cytokine expression

Anti-virus response elicited by RIG-I/MDA5 signaling is executed through type I IFNs and proinflammatory cytokines. Type I IFNs are essential in inducing an antiviral state and contribute to the subsequent antigen specific adaptive immune response system, and proinflammatory cytokines are crucial for eliminating virus infection by provoking inflammation and recruiting innate and acquired immune cells. To determine the roles of RAD23A in the regulation of poly(I:C) induced expression of type I IFNs and proinflammatory cytokines, we first examined the mRNA expression of IFN-β, TNF-α and IL-6 in poly(I:C) treated MEF cells with RT-PCR. The results showed that poly(I:C) induced significant higher levels of these genes in RAD23A knockdown cells compared with control cells (Fig. 2A). Consistent with the result from siRNA screening, knockdown of RAD23B did not affect the expression of TNF-α, IL-6, and IFN-β (Fig. 2B), suggesting that RAD23A functions distinctly from RAD23B in regulating RIG-I/MDA5 signaling. We next tested whether RAD23A regulates RIG-I/MDA5 mediated cytokine secretion in monocyte/macrophage cell line RAW264.7 using ELISA assay. We found that RAD23A knockdown cells secreted significantly higher level of IL-6 and IFN-β (Fig. 2C). To-

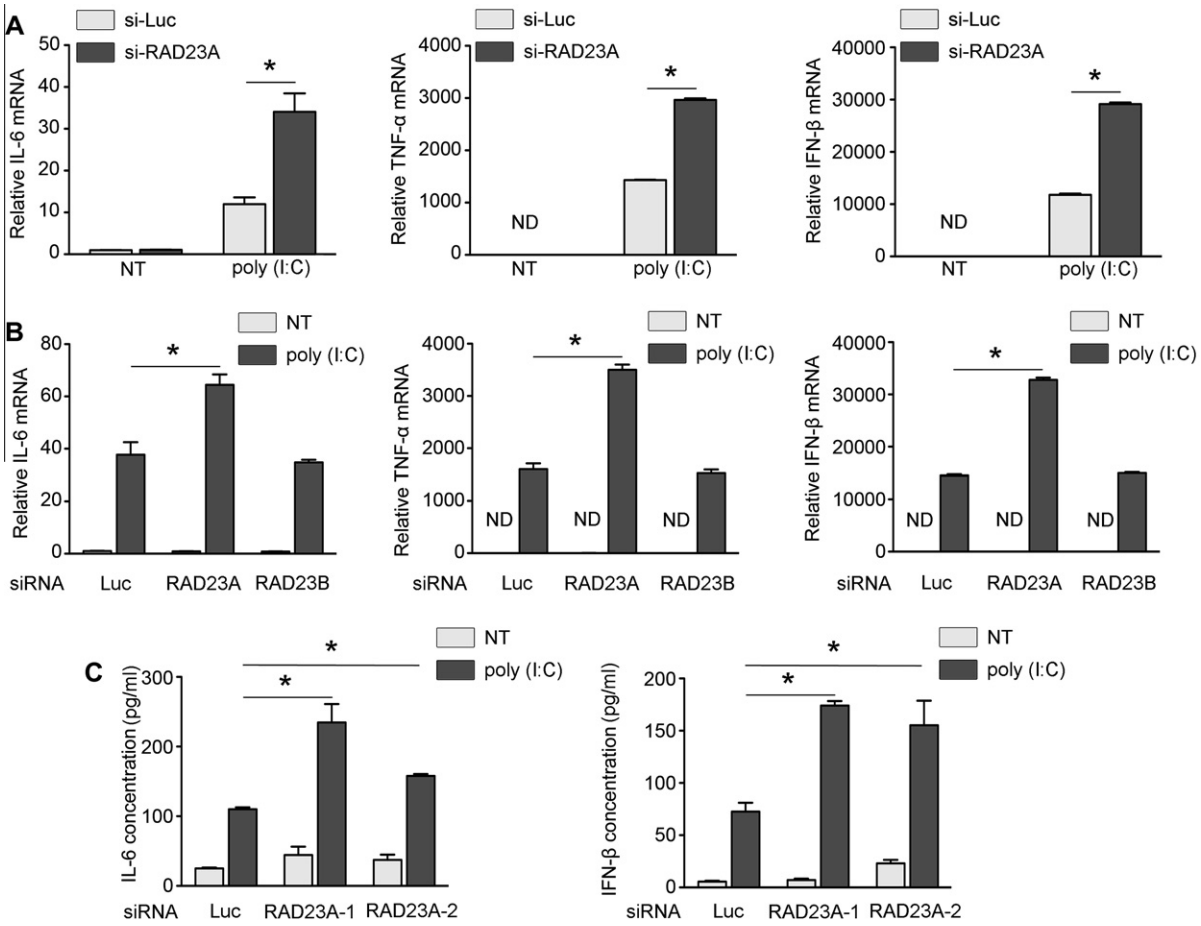

Fig. 2. Regulation of RIG-I/MDA5 signaling by RAD23A. (A) The control and RAD23A knockdown MEF cell lines were untreated or treated with poly(I:C) for 4–6 h. Total RNAs from these cells were harvested. IL-6, TNF-α, and IFN-β transcript levels were measured using RT-PCR normalized to GAPDH. (B) The control, RAD23A and RAD23B knockdown MEF cell lines were harvested as (A). IL-6, TNF-α, and IFN-βRNA were measured. (C) The control and RAD23A knockdown RAW264.7 cell lines were either untreated or treated with poly(I:C) for 18 h. The supernatants from these cell cultures were collected and subjected to the mouse IL-6 and IFN-β ELISA analysis according to the manufacturer's instructions. Data represented as the mean ± SD of triplicate samples. ND means non-detected. $^{*}p < 0.05$.

gether, these data suggest that RAD23A negatively regulates the expression of cytokines triggered by RIG-I/MDA signaling, and may function to prevent cells from overly activated anti-virus response.

3.3. RAD23A interacts with TRAF2 through UBA1 domain

RIG-I/MDA5 contains a DExD/H box helicase domain and two caspase recruiting domain (CARD)-like domains required for eliciting downstream signaling pathways. MAVS interacts with RIG-I/MDA5 through CARD domain, which provides essential link between RIG-I/MDA5 and downstream signaling molecules, such as TRAFs. TRAF2 binds directly to the TRAF-interacting motifs in the proline-rich region of MAVS to facilitate NF-κB, IRF3/7 activation [6]. RAD23A has been found interacting with TRAF2 previously using a stringent high-throughput yeast two hybrid system [22]. To verify whether RAD23A is involved in RIG-I/MDA5 signaling regulation through binding to TRAF2, we first tested the effect of RAD23A on NF-κB and INF-β activity mediated by different mediators of RIG-I/MDA5 signaling. We found that overexpression of Flag-RAD23A dramatically inhibited RIG-I/MDA5 and TRAF2 but not IKK-ε induced reporter activity (Fig. 3A), suggesting that RAD23A might target TRAF2 or a step downstream of TRAF2. We next confirmed the interaction between RAD23A and TRAF2 in mammalian cells (Fig. 3B). With regard to the homology between TRAF2 and TRAF3, interaction between TRAF3 and RAD23A was also tested. HEK293 cells were co-transfected with Flag-RAD23A together with HA-TRAF2 or HA-TRAF3. As indicated in (Fig. 3B), HA-TRAF2 but not HA-TRAF3 could be co-immunoprecipitated in the presence of Flag-RAD23A. Vice versa, Flag-RAD23A could also be co-immunoprecipitated with HA-TRAF2 when Flag antibodies were used to perform immunoprecipitation (Fig. 3C). The proteasome-interacting protein RAD23A contains an N-terminal ubiquitin-like (UbL) domain and two ubiquitin-associated (UBA) domains: an internal UBA1 domain and a C-terminal UBA2 domain. The UbL domain of RAD23A is important for its interaction with the 26S proteasome and participate participation in the proteasomal degradation pathway [23]. The UBA domain was originally identified as a sequence motif present in proteins linked to the ubiquitination system and non-covalently binding to ubiquitin. To define which domain of RAD23A is required for interacting with TRAF2, the four RAD23A-GFP mutants [1–120 (UbL), 121–215 (UBA1), 216–305 (XPC), and 306–363 (UBA2)] were created. The four mutants and full length plasmids were co-transfected with HA-TRAF2 plasmid into HEK293 cells and Co-IP experiments were performed. Only UBA1 domain of RAD23A interacted with TRAF2 (Fig. 3D). These results indicated that the interaction of RAD23A with TRAF2 is mediated by UBA1 domain. Taken together, these evidences suggested that RAD23A may be involved in RIG-I/MDA5 signaling by interacting with TRAF2. Considering TRAF2 also plays essential roles in NF-κB activation in signalings such as TNFR and TLRs, we also tested the effect of RAD23A on TLR3 mediated

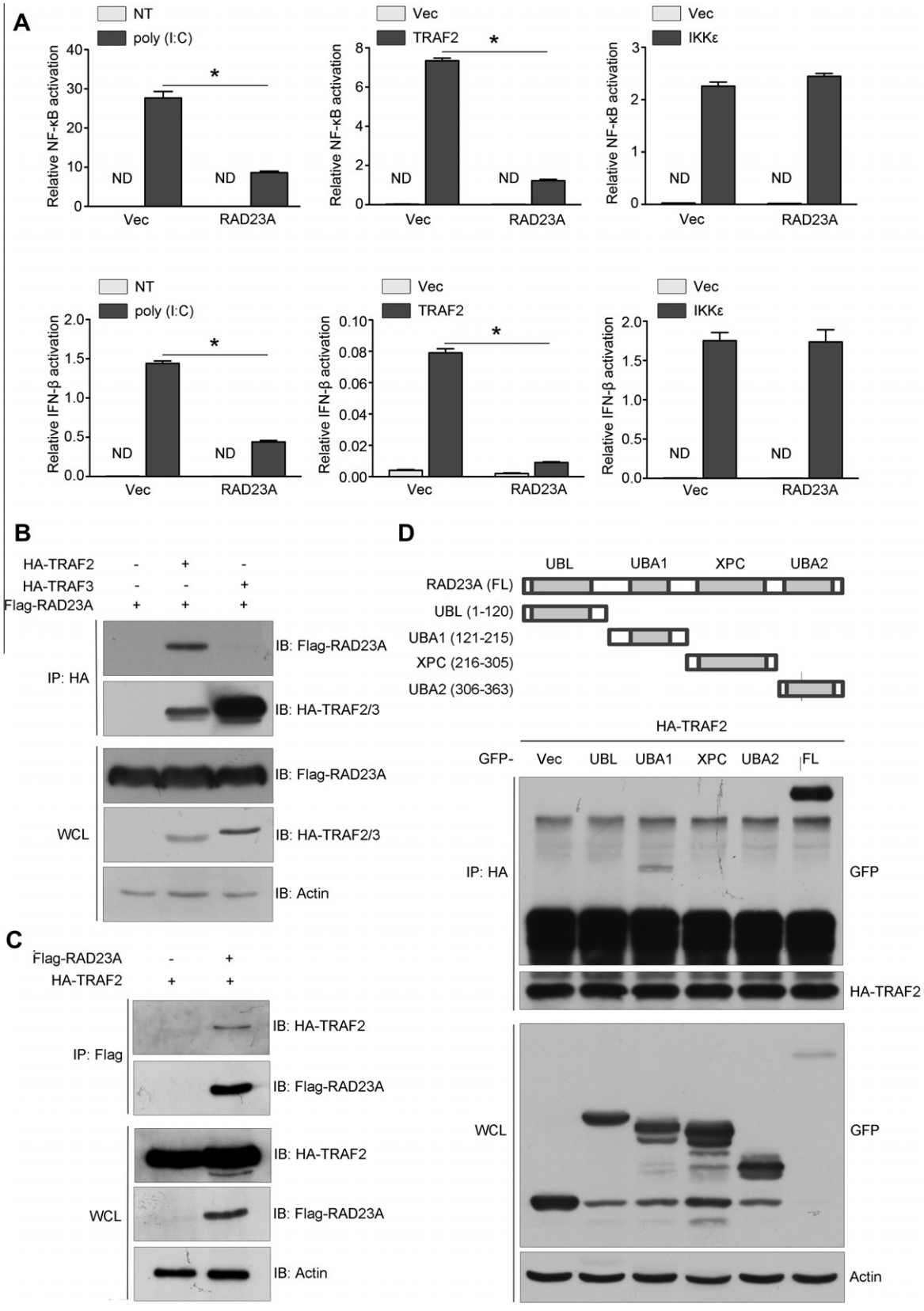

Fig. 3. Regulation of RIG-I/MDA5 signaling by RAD23A and its interaction with TRAF2. (A) HEK293 cells were co-transfected with the indicated reporters and activators, together with Flag-RAD23A or Flag-vector. NF-κB or IFN-β reporter activity was measured after 36 h of transfection. Data represented as the mean ± SD of triplicate samples. $^{*}p < 0.05$. (B and C) Interactions between RAD23A and TRAF2. HEK293 cells were transfected as indicated. Cell lysates were immunoprecipitated using HA or Flag antibody, and the immunoprecipitates were probed with anti-Flag or anti-HA. (D) Schematic diagram illustrate the structural domains of RAD23A and the four RAD23A-GFP truncations [1–120 (UbL), 121–215 (UBA1), 216–305 (XPC), and 306–363 (UBA2)] were created. The truncations and full length plasmids were co-transfected with HA-TRAF2 plasmid into HEK293 cells as indicated and Co-IP experiments were performed.

NF-κB and IFN-β activation, and found that RAD23A exhibited similar function (data not shown). However, the exact roles that RAD23A plays in specific signaling pathways need further investigation.

3.4. RAD23A promotes ubiquitin-proteasome dependent TRAF2 degradation

As an UbL-UBA domain containing protein, RAD23A was reported to promote substrate recognition by proteasome [24]. To explore the molecular mechanisms of RAD23A regulating RIG-I/MDA5 signaling through TRAF2, we first examined whether RAD23A regulates the protein level of TRAF2. As showed in (Fig. 4A), increasing expression of RAD23A resulted in a significant decrease in the protein level of TRAF2, but nearly no change of TRAF2 mRNA level (Fig. 4B), suggesting that RAD23A regulates TRAF2 expression at post-transcriptional level. To examine the effect of RAD23A overexpression on TRAF2 degradation, protein translation in cells were inhibited by cycloheximide treatment, and TRAF2 protein levels were detected at the indicated times of treatment. As shown in (Fig. 4C), RAD23A overexpression caused a decrease of TRAF2 half-life, which indicates that RAD23A accelerates TRAF2 degradation. We further found that the effect of RAD23A on TRAF2 protein level was blocked by proteasome inhibitor MG132 (Fig. 4D), suggesting that the ubiquitin-proteasome pathway is required for RAD23A mediated downregulation of TRAF2. Indeed, TRAF2 poly-ubiquitination was significantly enhanced upon transfection of RAD23A (Fig. 4E). In conclusion, RAD23A regulates TRAF2 level by promoting its ubiquitination and proteasome mediated degradation. Some studies suggested that RAD23A inhibits ubiquitination and ubiquitin dependent degradation, whereas other studies demonstrated that RAD23A could

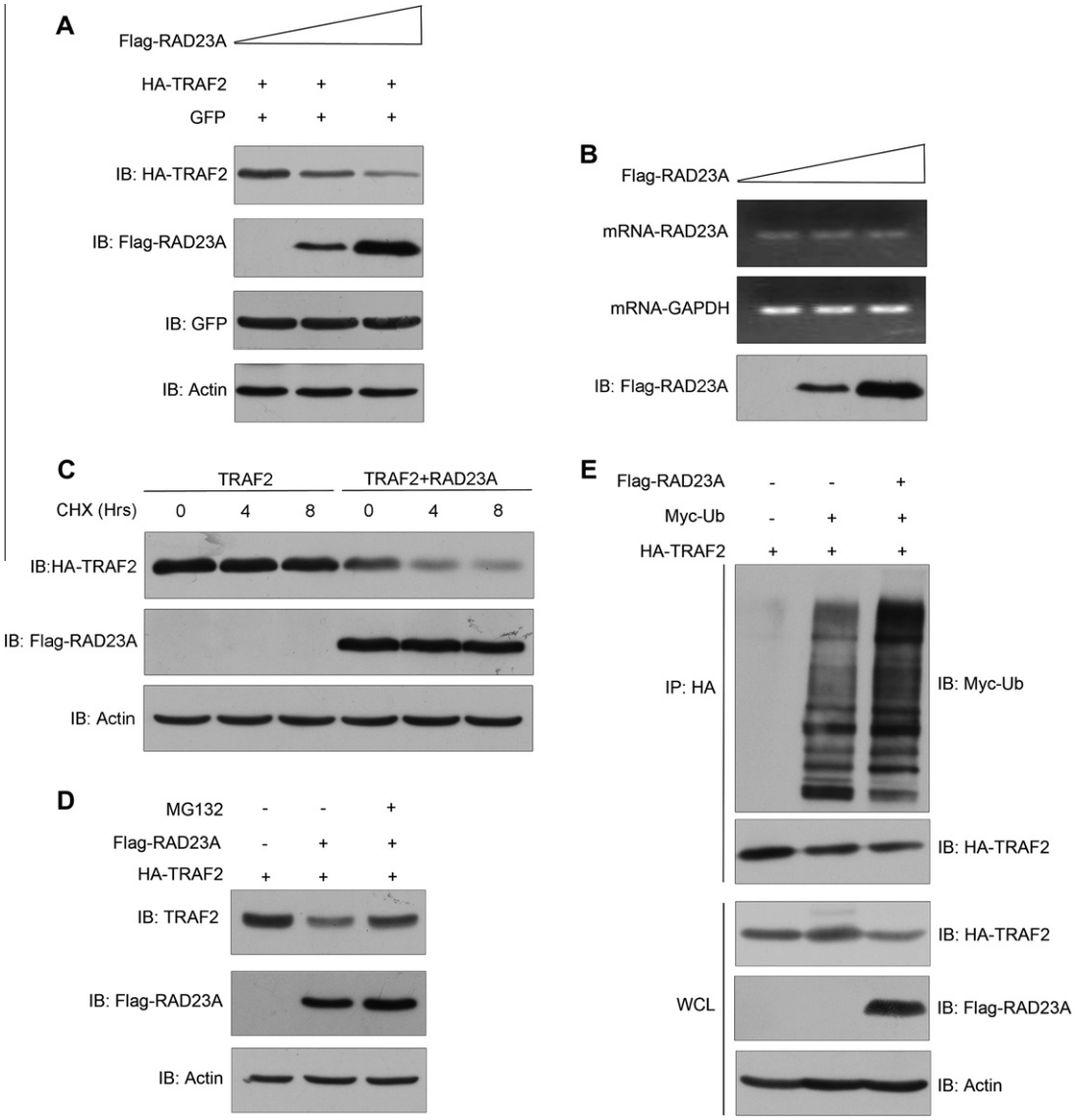

Fig. 4. RAD23A promotes TRAF2 degradation through proteasome. (A and B) HEK293 cells were co-transfected with HA-TRAF2, GFP and different dose of Flag-RAD23A plasmids. The cells were cultured for additional 24 h and Western blot was performed with anti-HA, anti-Flag, and anti-GFP. At the same time, cells were harvested with Trizol® and TRAF2 mRNA levels were assessed by RT-PCR. (C) HEK293 cells were co-transfected with HA-TRAF2 and control or Flag-RAD23A plasmid. 24 hours later, 75 μg/ml of cycloheximide was added, and the treatment was terminated at 0, 4, and 8 h time points. (D) As indicated, HEK293 cells were co-transfected with HA-TRAF2 and control or Flag-RAD23A plasmid. After 24 h, the cells were treated with or without 10 μM MG132 for 6 h. Then the cells were lysed and HA-TRAF2 protein levels were detected by immunoblotting. (E) HEK293 cells were transfected with HA-TRAF2, Myc-ubiquitin (Myc-Ub), and either Flag-vector or Flag-RAD23A. Thirty six hours later, cells were treated with MG132 for 2 h and cell lysates were harvested. HA-TRAF2 was immunoprecipitated with HA antibody, and Western blotting was done with anti-Myc.

promote protein degradation by targeting proteins to the proteasome [24,25]. These seems contradictory studies suggested that ubiquitylated proteins bound to RAD23A are protected from ubiquitin chain elongation or deubiquitylation. Our data implicated that RAD23A might increase TRAF2 ubiquitination by inhibiting its deubiquitylation.

4. Conclusions

RIG-I/MDA5 recognizes viral RNAs in cytoplasm and triggers signal transduction cascades that finally lead to the production of type I IFNs and proinflammatory cytokines to suppress viral replication and assembly. This signaling is closely regulated, but the exact mechanism of this regulation, however, remains elusive. In our study, RAD23A, an UBD domain containing protein, was identified as a novel regulator of RIG-I/MDA5 signaling in a siRNA-based screening. RAD23A negatively regulates RIG-I/MDA5-mediated expression of proinflammatory cytokines and IFNs. Moreover, RAD23A inhibits RIG-I/MDA5 signaling by interacting with TRAF2 and down-regulates its protein level. Therefore our study identified a novel regulatory molecule, RAD23A, which targets TRAF2 for ubiquitin-proteasome degradation, may play a role in precise control of cellular virus-triggered type I IFNs and proinflammatory cytokines production.

Acknowledgments

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