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The Ubiquitin E3 Ligase RAUL Negatively Regulates Type I Interferon through Ubiquitination of the Transcription Factors IRF7 and IRF3

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SUMMARY

In the course of combating infectious agents, type I interferon (IFN) needs a timely downregulation mechanism to avoid detrimental overreaction. Here we showed a mechanism for restraining type I IFN responses, which relied on a HECT domain ubiquitin (Ub) E3 ligase, RAUL. RAUL limited type I IFN production by directly catalyzing lysine 48-linked polyubiquitination of both interferon regulatory factor 7 (IRF7) and IRF3 followed by proteasome-dependent degradation. Suppression of RAUL by dominant-negative RAUL or siRNA augmented both basal and virus-induced production of type I IFN, which resulted in reduced viral replication. The Kaposi's sarcoma-associated herpes virus immediate-early lytic cycle trigger protein RTA recruited this mechanism to augment its countermeasures against the host antiviral response. These results unveil a previously unrecognized "brake mechanism" for type I IFN that maintains proper low amounts of type I IFN under physiological conditions and restrains its magnitude when the antiviral response intensifies.

INTRODUCTION

Interferon regulatory factor 7 (IRF7) and IRF3 are master transcriptional factors that regulate type I interferon (IFN) gene (IFN-α and IFN-β) induction and innate immune defenses after virus infection (Honda et al., 2005). IRF7, the master regulator for type I IFN, is induced by toll-like receptor 7 (TLR7) and TLR9, whereas IRF3 is activated through TLR3 and TLR4 (Doyle et al., 2002). These factors need to be destroyed in a timely manner after their activation, and this task is fulfilled by the ubiquitin (Ub)-proteasome system (Kodadek et al., 2006). Recent studies indicate that both IRF7 and IRF3 proteins undergo ubiquitination-mediated proteolysis: prolyl isomerase Pin1, E3 ligase Ro52, Ub ligase (E3) RBCC protein interacting with PKC1 (RBCK1), and human immunodeficiency virus (HIV)1 accessory proteins Vpr and Vif have been implicated indirectly for IRF3

(Barrington et al., 1998; Hemmi et al., 2004; Higgs et al., 2008; Kawai et al., 2004; Nakagawa and Yokosawa, 2000; Okumura et al., 2008; Saitoh et al., 2006; Yu et al., 2005; Zhang et al., 2008), whereas the Kaposi's sarcoma-associated virus (KSHV) immediate-early lytic cycle trigger protein, RNA transcriptional activator (RTA, also known as ORF50) and the rotavirus nonstructural protein NSP1 have been shown to target IRF7 for proteolysis (Barro and Patton, 2007; Yu et al., 2005). Yet the endogenous Ub E3 ligase for IRF7 is still unknown (Kaisho and Tanaka, 2008). Although Pin1 and RBCK1 have been implicated in the ubiquitination of activated IRF3, an endogenous E3 ligase that directly ubiquitinates IRF3 has also not been reported. The fact that the usually low expression of short half-lived IRF7 in normal cells can be boosted by treatment with proteasome inhibitors (Yu et al., 2005) clearly implies that there exists a cellular ubiquitination mechanism for controlling the abundance of the IRF7 protein, and this remains a key missing component in understanding regulation of the type I IFN pathway.

Ubiquitination requires the sequential actions of three enzymes: Ub-activating enzyme (E1), Ub-conjugating enzyme (E2), and Ub ligase (E3), and the E3 dictates which target protein gets ubiquitinated. Different Ub chain structures tend to direct distinctive functional consequences, which is best manifested by lysine 48- versus lysine 63-linked Ub chains, resulting in proteolysis or functional activation, respectively (Chen, 2005; Hershko and Ciechanover, 1998). There are two types of E3 ligase, HECT (homologous to E6-associated protein C terminus) domain and the really interesting new gene (RING) finger domain E3 Ub ligases. Similar to phosphorylation, ubiquitination is a reversible process, and this is accomplished by deubiquitinases (DUBs; also known as deubiquitinating enzymes), which remove Ub moieties from Ub-protein conjugates, leading to reduced ubiquitination signaling (Komander et al., 2009).

The ubiquitous nature of pathogens and stress proteins are such that if left unchecked, the host will be overwhelmed by immune activation. Therefore, the immune system needs to constantly create (or maintain) a balance between activation and inhibition to avoid detrimental overreaction. Although immunologists have long been focusing on activation machinery, a picture of how immune balance is achieved is beginning to emerge (Liew et al., 2005). The present work set out to investigate the other side of the coin, the mechanisms that restrain type I IFN responses. RAUL (RTA-associated ubiquitin ligase)

(also known as KIAA10 or UBE3C) is an established HECT domain E3 Ub ligase that is able to catalyze a variety of unanchored poly-Ub chain types in vitro and targets the putative transcriptional factor TIP120B for proteolysis, yet its physiological functions are largely unknown (Garcia-Gonzalo and Rosa, 2005; Wang et al., 2006; You and Pickart, 2001; You et al., 2003).

The first implication that this particular ligase might play a role in the IFN pathway came when RAUL, together with IRF7, were identified in our yeast two-hybrid screening as binding partners for the Kaposi's sarcoma-associated virus (KSHV) immediate-early lytic cycle trigger protein, RTA. We also observed that RTA, as a viral E3 Ub ligase, promotes direct Ub-proteasome-dependent proteolysis of IRF7, the master regulator of type I IFN (Yu et al., 2005). In addition, we observed that the viral E3 RTA can cooperate with cellular E3 RAUL to enhance proteolysis of both IRF7 and IRF3. These observations, together with the fact that IRF7 and IRF3 share structural and functional similarities as well as phosphorylation pathways (Sharma et al., 2003), prompted us to hypothesize that RAUL itself might regulate both the IRF7 and IRF3 proteins, thus negatively regulating type I IFN. We further hypothesized that KSHV RTA not only copies but also recruits this cellular negative regulatory mechanism for type I IFN to mute antiviral innate responses.

Here we showed that RAUL regulates type I IFN by targeting both IRF7 and IRF3 for lysine 48-linked ubiquitination and proteolysis, which represents an important pathway in maintaining proper low amounts of type I IFN under physiological conditions, and for restraining its magnitude when the antiviral innate response intensifies. We also showed that RAUL was positively regulated by a deubiquitinating enzyme ubiquitin-specific processing protease 7 (Usp7 also known as HAUSP) and that KSHV RTA utilized and enhanced this mechanism to augment its countermeasures against the host antiviral response, which may represent a common strategy adopted by viruses to counteract innate immunity.

RESULTS

RAUL Associates with IRF7 and IRF3

A necessary step for a HECT domain E3 ligase to initiate ubiquitination is physical binding to its target protein. To determine whether and how RAUL binds to either the IRF7 or IRF3 proteins under more physiological conditions in mammalian cells, we first used immunoprecipitation to confirm that endogenous binding between RAUL and IRF7 or IRF3 occurred in the mouse bone marrow dendritic cell line DC 2.4 (Figure 1A). Next, reciprocal immunoprecipitation and immunoblot experiments indicated that RAUL indeed interacted with IRF7 and IRF3 (but not IRF1) in human cells after exogenous coexpression (Figures S1A and S1B available online). Third, we verified the associations in vitro by glutathione-S-transferase (GST) pull-down assays and showed that both IRF7 and IRF3 interacted with RAUL through their N-terminal regions (Figures S1C and S1D). Fourth, further deletion analysis showed that the amino-terminal regions of IRF7 (amino acids 103–151) (Figure S1E) and IRF3 (amino acids 1–128) (Figure S1F) were required for mediating the interaction with full-length RAUL protein, and that RAUL associated with IRF7 and IRF3 via its N-terminal region (1–655) (Figure S1G). Fifth, we tested whether the binding is dependent upon IRF7

phosphorylation status and found that all three IRF7 phosphorylation-deficient mutants (IRF7S483A, IRF7Δ477-479, and IRF7D490A) retained their ability to bind RAUL (Figure S1H, lanes 1, 2, and 4). A similar pattern was observed with IRF3 phosphorylation mutants (data not shown), indicating that the binding between IRF7 or IRF3 and RAUL are independent of their phosphorylation status. Finally, we examined whether RAUL catalytic activity affects the binding and found that its catalytically inactive mutant (C1051A, designated as Myc-RAUL-M) was unchanged in its binding ability for IRF7 (Figure S1H, lane 3). Collectively, these results show that RAUL binds specifically to both IRF7 and IRF3 both inside cultured cells and in cell-free extracts.

RAUL Directs IRF7 and IRF3 for Proteolysis

We next sought to determine whether RAUL, like KSHV E3 RTA, might promote IRF7 or IRF3 proteasome-dependent proteolysis. Indeed, coexpression of wild-type (WT) RAUL but not the catalytic domain point mutant of RAUL, with IRF7 or IRF3 led to a loss of both proteins in a dose-dependent manner, which was prevented by treatment with the proteasome inhibitor MG132 (Figures 1B–1E). We also observed that the N-terminal deletion mutant (RAULΔ132, designated as RAUL-DN in this article) as well as its catalytically inactive mutant (Myc-RAUL-M) functionally acted in a dominant-negative fashion to inhibit RAUL function (data not shown). These results indicate that RAUL mediates IRF7 and IRF3 proteolysis in a ubiquitination- and proteasome-dependent manner. To determine whether RAUL also regulates endogenous IRF7 and IRF3, we transfected increasing amounts of RAUL into 293T cells, which caused a dose-dependent loss of the endogenous IRF7 and IRF3 proteins (Figure 1F). To determine which domains of RAUL are involved in this proteolysis, we cotransfected RAUL mutants, which lack either the substrate binding domain (RAULΔ132) or the catalytic cysteine (RAUL-M), together with IRF3 plasmids into 293T cells. Both the substrate-binding and intact catalytic C-terminal domains of RAUL proved to be required (Figure 1G), suggesting that the degradation involves both specific-target recognition and the enzymatic activity of RAUL. We also tested whether RAUL-mediated proteolysis depends on IRF3's phosphorylation status. However, Figure 1H shows that RAUL promoted degradation of both WT IRF3 and its phosphorylation-deficient mutant (IRF3S339A) in a cotransfection system, indicating that the proteolysis is independent of IRF3 phosphorylation status. A similar pattern was observed for IRF7 phosphorylation mutants (data not shown).

We also examined whether the proteolysis occurs in the nucleus or cytoplasm. A subcellular localization experiment showed that when expressed alone, IRF7 and IRF3 were largely localized in the cytoplasm, but that when coexpressed with RAUL, a large proportion of both forms of IRF7 and IRF3 could no longer be detected (Figure 1I, lane 4). RAUL exhibited a similar subcellular expression pattern as IRF3 and IRF7 (Figure 1I, lane 3). Therefore, RAUL is capable of regulating both the resting (cytoplasmic) and activated (nuclear) forms of both IRF7 and IRF3, the key transcription factors mediating innate type I IFN responses to cell stress, and RAUL directs them for proteasome-dependent proteolysis in a manner that is dependent on both the substrate-binding and catalytic domains of RAUL.
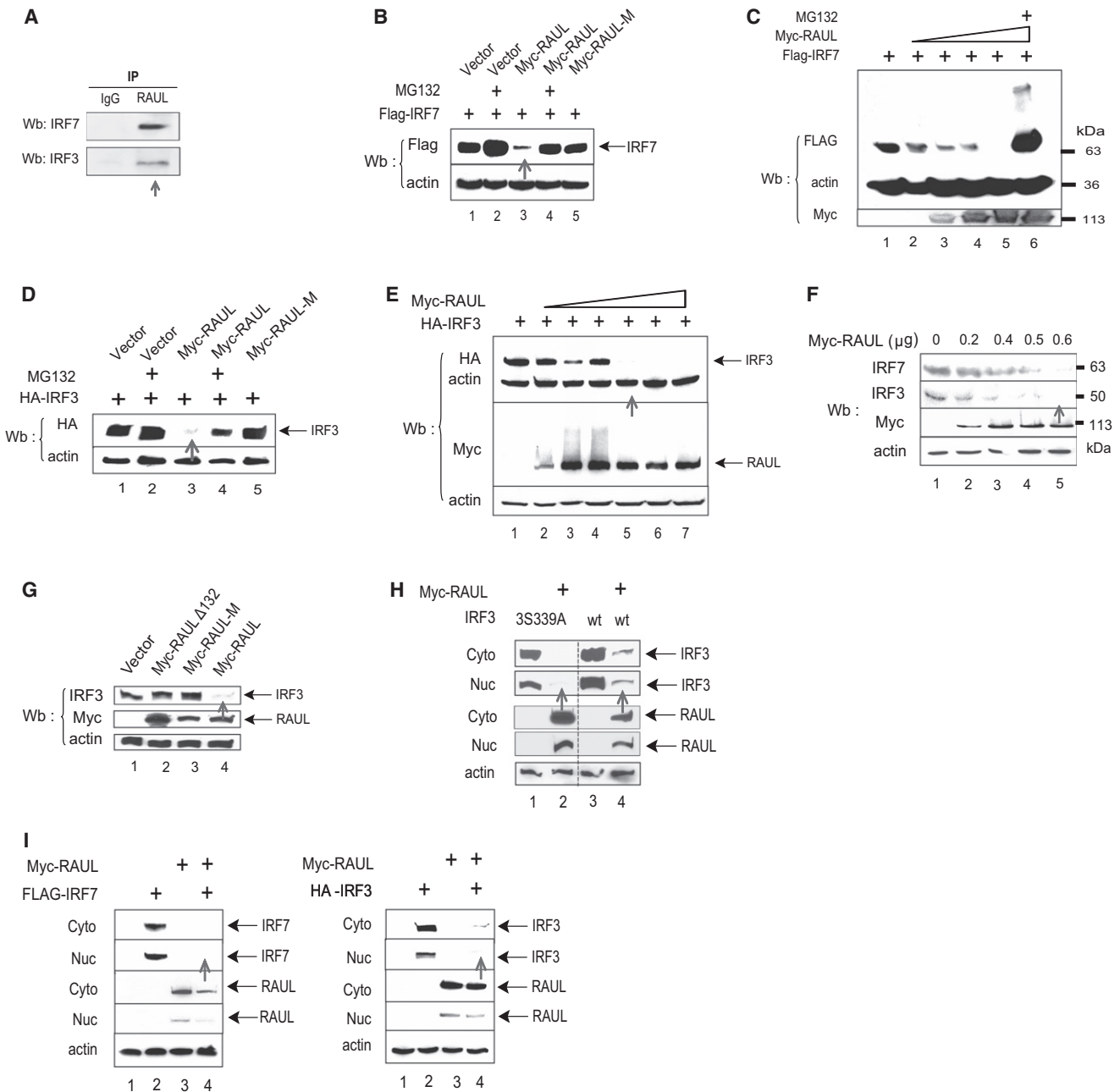
Figure 1. RAUL Directs IRF7 and IRF3 for Proteolysis

(A) Interaction between endogenous RAUL and IRF7 or IRF3. Lysates of DC 2.4 mouse bone marrow dendritic cells treated with the proteasome inhibitor MG132 were immunoprecipitated with anti-RAUL and immunoblotted with anti-IRF7 or anti-IRF3.

(B–E) RAUL, but not its catalytically inactive mutant, causes loss of IRF7 and IRF3 proteins. Flag-IRF7 or HA-IRF3 was cotransfected with Myc-RAUL or Myc-RAUL-M (point mutant C1051A) into 293T cells in the presence of MG132 (0.5 μM for 12 hr). The amounts of IRF7 and IRF3 proteins were evaluated by immunoblot. (C and E) Dose response with increasing amounts of Myc-RAUL.

(F) Enforced RAUL expression causes loss of endogenous IRF7 and IRF3 proteins. The indicated increasing amounts of Myc-RAUL plasmid were transfected into 293T cells, and the endogenous IRF7 or IRF3 protein were assessed by IRF7 or IRF3 immunoblotting.

(G) RAUL-mediated IRF3 proteolysis is dependent on its substrate binding and catalytic domains. WT RAUL or its substrate binding domain deletion mutant (Myc-RAULΔ132, also designated as Myc-RAUL-DN) or its catalytically inactive mutant (Myc-RAUL-M) were transfected into 293T cells and endogenous IRF3 protein was evaluated by IRF3 immunoblotting.

(H) RAUL-mediated loss of IRF3 occurs independently of IRF3 phosphorylation status. Myc-RAUL and WT IRF3 or its phosphorylation-deficient mutant IRF3S339A were cotransfected into 293T cells, cytoplasmic and nuclear portions were isolated, and amounts of IRF3 and RAUL were determined by immunoblotting.

(I) Subcellular distribution pattern. 293T cells were transfected with Myc-RAUL together with Flag-IRF7 or HA-IRF3 plasmids. Cytoplasmic and nuclear fractions were isolated and IRF7, IRF3, and RAUL proteins were detected by immunoblot with Flag, HA, or RAUL antibodies, respectively.
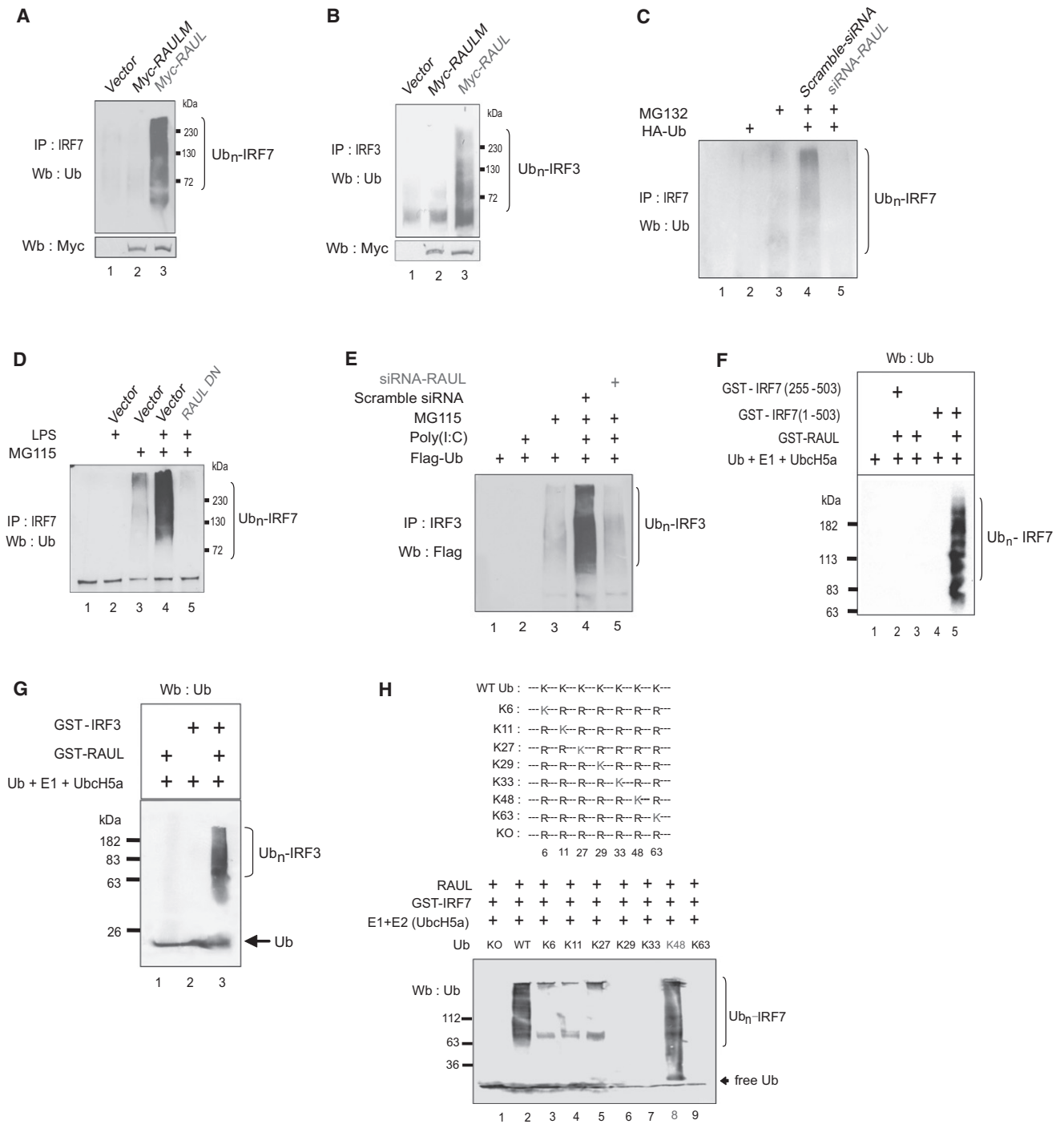
Figure 2. RAUL Ubiquitinates IRF7 and IRF3 In Vivo and In Vitro

(A and B) RAUL mediates ubiquitination of endogenous IRF7 and IRF3. DG75 B-lymphocyte cells were electroporated with WT Myc-RAUL or its catalytically inactive mutant (Myc-RAUL-M) plasmids in the presence of proteasome inhibitor MG115, lysates were immunoprecipitated with anti-IRF7 and IRF3, and ubiquitinated forms of IRF7 and IRF3 were determined by Ub immunoblotting.

(C) RAUL is required for endogenous IRF7 ubiquitination. Mouse bone marrow dendritic cells DC 2.4 were electroporated with siRNA-RAUL or scramble siRNA together with HA-Ub plasmid and the indicated cells were treated with MG132. The cell lysates were immunoprecipitated with anti-IRF7 followed by Ub immunoblotting.

(D) RAUL is required for ubiquitination of endogenous IRF7 after TLR4 activation. Mouse primary bone marrow dendritic cells were isolated (Tseng et al., 2001) and electroporated with dominant-negative (Myc-RAUL-DN) plasmid or control vector and the indicated cells were treated with LPS (100 ng/ml for 9 hr) and proteasome inhibitor MG115. Conjugated IRF7 was determined by immunoprecipitation with anti-IRF7 followed by Ub immunoblotting.

RAUL Ubiquitinates IRF7 and IRF3 In Vivo and In Vitro

We then investigated whether RAUL-mediated IRF7 and IRF3 proteolysis occurs through ubiquitination. Initially, we asked whether RAUL can induce the addition of ubiquitin chains on endogenous IRF7 and IRF3 proteins and observed that ectopic expression of WT RAUL, but not of its catalytically inactive mutant, produced polyubiquitinated forms of both proteins in the human DG7 lymphoma cell line (Figures 2A and 2B). These results indicate that RAUL overexpression induced endogenous IRF7 and IRF3 ubiquitination. To determine whether endogenous IRF7 undergoes ubiquitination in resting conditions, dendritic cells DC 2.4 were electroporated with hemagglutinin (HA)-Ub and treated with MG132. IRF7 was readily polyubiquitinated (Figure 2C), but this was substantially reduced in the absence of either HA-Ub or MG132. Notably, depletion of RAUL by specific small interfering RNA (siRNA) (see below) in the same cells abolished IRF7 ubiquitination, suggesting that IRF7 undergoes ubiquitination and proteasome-mediated degradation events, even in the absence of pathogen induction stresses, in which RAUL appears to be essential. We proceeded to determine whether RAUL is required for IRF7 and IRF3 polyubiquitination under stress conditions. Primary mouse bone marrow dendritic cells were isolated (Tseng et al., 2001) and electroporated with a dominant-negative form of RAUL (RAUL-DN) to suppress RAUL functions and the cells were treated with lipopolysaccharide (LPS) to mimic TLR4 activation. As shown in Figure 2D, LPS readily stimulated IRF7 ubiquitination, whereas suppression of RAUL by this approach abolished the induced IRF7 ubiquitination. Likewise, when a stable 293T cell line ectopically expressing toll-like receptor 3 (293T-TLR3), which constitutively express TLR3, was stimulated with poly(I:C) to mimic TLR3 activation, polyubiquitinated forms of IRF3 were induced, but silencing RAUL abrogated the effect (Figure 2E). Therefore, these results suggest that RAUL is also required for polyubiquitination of the much higher stress-induced amounts of IRF7 and IRF3.

To determine whether RAUL is able to directly catalyze the conjugation of ubiquitination on both proteins, we reconstituted the ubiquitination of IRF7 and IRF3 in a cell-free system by using purified glutathione-S-transferase (GST)-IRF7 or GST-IRF3, E1, UbcH5a (E2), and Ub proteins. RAUL efficiently catalyzed the conjugation of poly-Ub chains on both IRF7 and IRF3 in cooperation with the E2 enzyme UbcH5a. Omission of any one of these ingredients or by using an IRF7 N-terminal deletion mutant as substrate abolished ubiquitination (Figures 2F and 2G). Collectively, these results provide strong evidence that RAUL catalyzes polyubiquitination on IRF7 and IRF3 both in vitro and in cells under both physiologically normal and induced stress settings.

Therefore, we conclude that RAUL represents a bona fide endogenous Ub E3 ligase that directly catalyzes ubiquitination of both IRF3 and IRF7.

RAUL Catalyzes Lysine 48-Linked Poly-Ub Chains on IRF7 and IRF3

To determine what type of Ub chain structure was formed on the IRF7 and IRF3 proteins, first we employed a set of mutants in which six out of the seven lysine residues (lysine 6, −11, −27, −29, −33, −48, and −63) in the Ub molecule were mutated to arginine, leaving just one lysine for polymerization. These recombinant Ub point mutants plus the Ub mutant with all seven lysines mutated to arginine (KO) were tested in a cell-free Ub assay to determine which lysine is utilized (Figure 2H, top). As shown in Figure 2H (bottom) and Figure S2A, RAUL catalyzed the conjugation of poly-Ub chains on both proteins only with the Ub mutant retaining a lysine at position 48 (K48), indicating that RAUL catalyzes lysine 48-linked Ub chains on both proteins in vitro. We then tested these Ub single lysine mutants plus a lysine 48 point mutation (K48R) in transfected cells. In accord with the in vitro cell-free data, IRF7 and IRF3 polyubiquitination was observed only with Ub mutants containing lysine at position 48 (K48), but not with any mutants where lysine 48 was eliminated (K48R) (Figures S2B–S2D). Therefore, these findings provide evidence that the poly-Ub chains on IRF7 and IRF3 by RAUL are linked primarily via lysine 48 of Ub.

RAUL Is Important for Expression of IRF7 and IRF3 as well as Restraining Type I IFN Responses

To determine whether RAUL is a physiologically relevant regulator for IRF7 and IRF3, we generated a plasmid-based RAUL siRNA. Figure 3A shows that the RAUL-specific siRNA caused a very effective reduction of RAUL protein compared to a randomly scrambled negative control siRNA. Furthermore, silencing RAUL caused a dramatic accumulation of endogenous IRF7 and IRF3 proteins in 293T, HeLa, and primary mouse spleen cells ($Rag2^{-/-}$ c.c7 TCR transgenic T lymphocyte cells) (Figures 3B and 3C). To eliminate the possibility that the accumulation of IRF7 and IRF3 here was caused by nonspecific double-stranded RNA-mediated activation of IFN responses, IRF1 and IRF2 were included as controls and their amounts were not affected (Figure 3B), confirming the specificity of the siRNA. RAUL silencing also led to accumulation of endogenous IRF3 and IRF7 protein by more than 10-fold in a dose-dependent manner (Figure 3D) and extended their half-lives by several-fold in the presence of cycloheximide (CHX) (Figure 3E), indicating a crucial role for RAUL in negative regulation of the resting forms of both proteins.

(E) RAUL is required for ubiquitination of IRF3 after TLR3 activation. 293T-TLR3 cells were transfected with Flag-Ub plasmids and indicated cells were treated with poly(I:C) (25 μg/ml for 9 hr) or MG115 (0.5 μM for 12 hr). Conjugated IRF3 was evaluated by immunoprecipitation with anti-IRF3 followed by immunoblot with anti-Flag.

(F and G) RAUL catalyzes the conjugation of poly-Ub chains on IRF7 and IRF3 in vitro. Purified GST-RAUL protein was incubated with various combinations of WT or truncated GST-IRF7 or GST-IRF3 plus E1, UbcH5a (E2), and Ub at 37°C for 1 hr. After the ubiquitination reaction, the samples were subjected to immunoblot with Ub antibody for detection of polyubiquitinated forms of IRF7 and IRF3. Only a low catalytic concentration of GST-RAUL (20 nM, as an E3 source) was used compared to 400 nM of target proteins to minimize interference from RAUL autoubiquitination (lane 3).

(H) Determining IRF7 and IRF3 Ub chain structure by single lysine mutant assay in vitro. Illustration of Ub lysine mutants (top). Purified WT Ub or its single lysine mutants, plus E1, E2 (Ubc5a), GST-IRF7, and RAUL were employed in in vitro cell-free Ub assays and ubiquitinated forms of the proteins were evaluated by Ub immunoblotting (bottom).
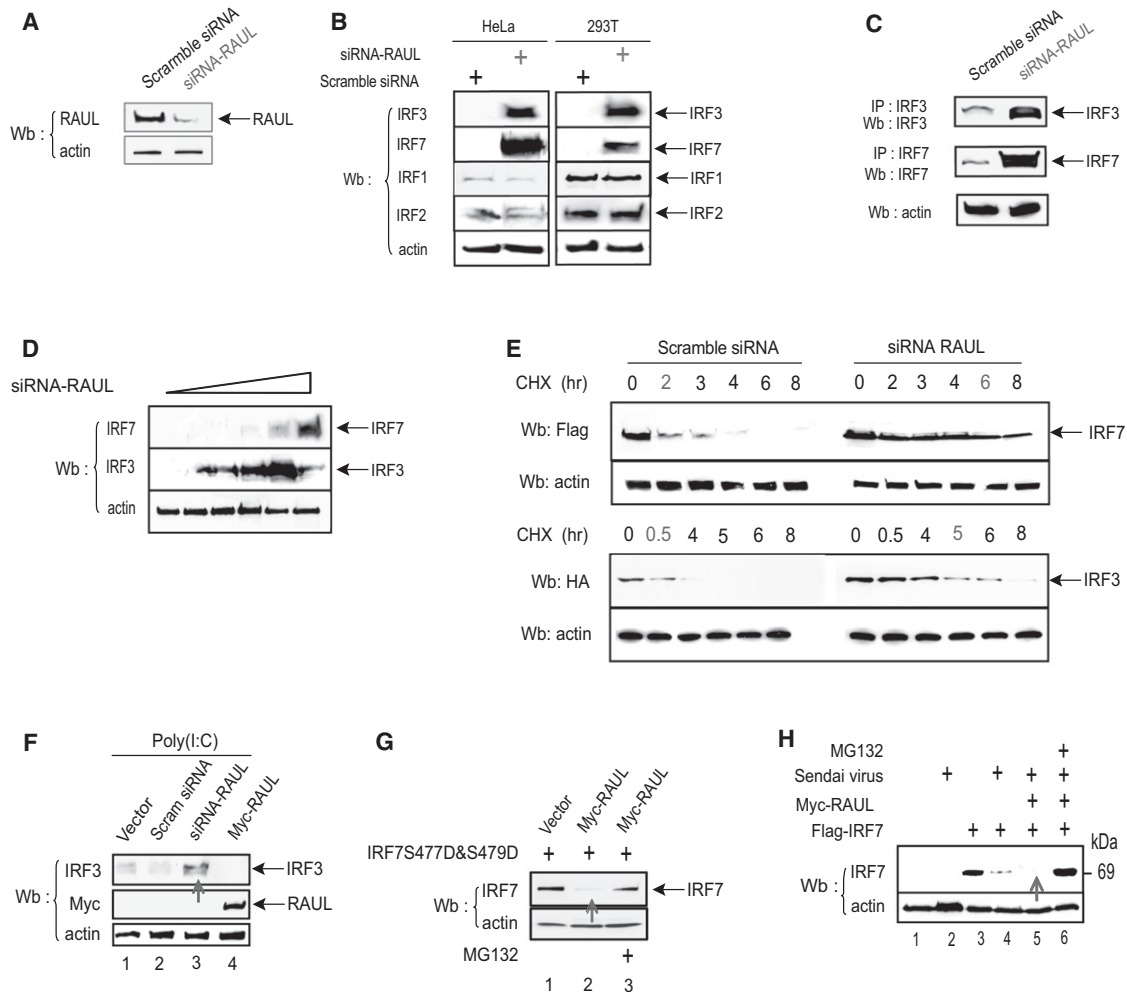
Figure 3. Silencing RAUL Increases IRF7 and IRF3 Expression
(A) Immunoblot of lysates with RAUL-specific antibody from 293T cells transiently expressing siRNA-RAUL or scramble siRNA.
(B and C) Reducing RAUL specifically induces high amounts of endogenous IRF7 and IRF3 proteins in both cell lines and primary cells.
(B) Immunoblot with anti-IRF7 or anti-IRF3 of lysates from cells transiently expressing siRNA-RAUL or scramble siRNA in HeLa or 293T cells.
(C) Isolated primary T lymphocytes from 5-week-old $Rag2^{-/-}$ 5c.c7 TCR transgenic knockout mice were activated with pigeon cytochrome C (PCC) and expanded for 4 days. After activation, more than 90% of the cell population was TCR-specific transgenic T lymphocyte cells, which were then transfected with siRNA RAUL or scrambled siRNA. Endogenous IRF3 and IRF7 proteins were determined by IRF3 or IRF7 immunoprecipitation followed by immunoblot.
(D) siRNA-mediated reduction of RAUL increases accumulation of both the IRF7 and IRF3 proteins in a dose-dependent manner. 293T cells were transfected with 2-fold increasing amounts of siRNA-RAUL, and endogenous IRF7 or IRF3 protein was determined by IRF7 or IRF3 immunoblot.
(E) Silencing RAUL extends the half-lives of IRF7 and IRF3 proteins. Flag-IRF7 or HA-IRF3 were cotransfected with siRNA-RAUL or scrambled siRNA into 293T cells, and the protein synthesis inhibitor cyclohexamide (CHX) was added at a concentration of 30 μg/ml at 36 hr for the indicated times. The IRF7 and IRF3 proteins were determined by Flag or HA immunoblot.
(F) Silencing RAUL in 293T-TLR3 cells increases the amount of IRF3 protein. Immunoblot analysis of IRF3 protein from 293T-TLR3 cells transiently expressing siRNA-RAUL or Myc-RAUL. poly(I:C) was used to stimulate TLR3 activation.
(G) RAUL also regulates a constitutively active form of IRF7. Immunoblot analysis of lysates with IRF7 antibody from 293T cells transiently coexpressing Myc-RAUL and IRF7S477D and S479D in the presence and absence of MG132.
(H) Enforced RAUL expression augments the Sendai virus-mediated loss of IRF7 protein. Myc-RAUL and Flag-IRF7 plasmids as indicated were transfected into 293T cells infected with Sendai virus (16 hr) with or without treatment with MG132. IRF7 protein was evaluated by immunoblot with a Flag antibody.

To address whether RAUL also regulates the active forms of both proteins, we examined how silencing of RAUL affected the amounts of IRF7 and IRF3 under stress conditions. First, in 293T-TLR3 cells treated with poly(I:C) to activate IRF3, the reduction of RAUL by siRNA resulted in an accumulation of the endogenous active form of IRF3 protein over the control (Figure 3F). Similarly, coexpression of RAUL with a constitutively active form of IRF7 (S477D and S479D) (Lin et al., 2000) in 293T cells led to the loss of these proteins, which was rescued by treatment with the proteasome inhibitor MG132 (Figure 3G). In Sendai virus-infected cells, coexpression of RAUL with IRF7 caused a further loss of the expected to be fully active form of

IRF7, which could be prevented by adding MG132 (Figure 3H). These results demonstrate a role for RAUL in the negative regulation of both TLR3-activated IRF3 and Sendai virus-activated forms of IRF7 proteins. Collectively, these data indicate that RAUL downregulates both resting and active forms of both proteins.

We next investigated whether suppression of RAUL by either siRNA or dominant-negative approaches would translate into an increase of type I IFN production under resting and stress conditions. Indeed, depletion of RAUL by siRNA strongly activated IFN-α1 and IFN-β reporter activities, and Sendai virus infection further increased their activities (Figure 4A). An identical pattern was also observed in poly(I:C)-stimulated 293T-TLR3 cells (Figure 4B). Likewise, silencing RAUL in DC 2.4 cells augmented both basal and LPS-stimulated IFN-α1 mRNA (Figure 4C). These siRNA and dominant-negative studies provide confirmation of the importance of RAUL in regulating the expression of type I IFN.

We also examined whether ectopic expression of RAUL would affect IRF7- and IRF3-dependent type I IFN activation. Coexpression of RAUL together with IRF7 or IRF3 in 293T cells completely abolished the IRF7- and IRF3-mediated type I IFN reporter gene activities in both the absence and presence of Sendai virus or of poly(I:C) or LPS (Figures 4D–4F). These results imply that RAUL exerts a powerful restrictive function on IRF7 and IRF3-dependent type I IFN responses via TLR signaling in a variety of pathogenic settings. Evidently, with the IRF7 and IRF3 proteins degraded, the cells' ability to launch a type I IFN response was eliminated. As indicated in Figure 4G, coexpression of RAUL with IRF7 or IRF3 completely inhibited their induction of endogenous IFN-α and IFN-β mRNA measured by real-time reverse transcription-polymerase chain reaction (RT-PCR). Notably, ectopic expression of RAUL alone decreased IFN-α and IFN-β to 20%–50% of the basal amounts in mock-treated cells as well. The results for IFN-α and IFN-β mRNA measurements were essentially the same as for IFN-promoter driven luciferase (IFN-LUC) reporter assays for all of the above experiments with poly(I:C) and LPS induction (not shown, except for Figure 4C).

Collectively, these data demonstrate that RAUL regulates the stability and amount of both the inactive and activated forms of IRF7 and IRF3 proteins and points to a critical role for RAUL both in maintaining proper low amounts of type I IFN in normal conditions, as well as for restraining the magnitude of induced antiviral responses. A previous study demonstrated that Pin1 also indirectly targets IRF3 for destabilization through polyubiquitination and suggested that additional posttranslational mechanisms may be involved (Saitoh et al., 2006). RAUL could represent one of them, although our further investigation has revealed that the E3 activity of RAUL toward IRF7 and IRF3 is independent of Pin1 activity and vice versa (data not shown).

The Role of RAUL in Antiviral Innate Immunity

To address the role of RAUL in the innate antiviral response, we infected mouse embryonic fibroblasts (MEFs) with KSHV and monitored alterations in overall viral load in the infected cells via a real-time PCR-based method, which is able to quantify the copy number of KSHV genomes (Figure 5A). The amount of KSHV DNA generated in these the infected cells was measured

as 51 copies per 100 ng of cell DNA. Ectopic expression of RAUL, which should suppress IRF3- and IRF7-mediated type I IFN production, boosted the production of KSHV by nearly 5-fold (240 versus 51 copies). In contrast, in the cells expressing dominant-negative or siRNA RAUL, the viral copy number was instead decreased by 68%–77% (12 and 16 versus 51 copies) (Figure 5B). A parallel type I IFN assay by real-time PCR confirmed a reduction of type I IFN mRNA production subsequent to ectopic expression of RAUL and an increase after expression of siRNA RAUL (Figure 5C).

To test the generality of this effect, we examined whether the expression of RAUL also affects the replication of Sendai virus in infected MEFs. A similar pattern was obtained: Sendai virus infection readily induced IFN-β production, while concurrent ectopic expression of RAUL abolished it (Figure 5D, lane 8 versus 5). As a result, the virus proliferated more efficiently (Figure 5E, lane 8 versus 5). In contrast, suppression of RAUL by dominant-negative or siRNA boosted IFN-β production (Figure 5D, lanes 6 and 7 versus 5) and caused a reduction of viral proliferation (Figure 5E, lanes 6 and 7 versus 5). We also examined whether RAUL affected KSHV DNA concentration in latently infected primary effusion lymphoma (PEL) BCBL1 cell lines selected to stably express either RAUL or siRNA-RAUL. Ectopic expression of RAUL increased the KSHV viral load over the control by 716% (9837 versus 1373 copies), and depletion of RAUL by siRNA reduced the viral DNA yield by 73% (364 versus 1373 copies) (Figure 5F).

Previous studies have suggested that two other Ub E3 ligases, RBCK1 and Ro52, also regulate IRF3 ubiquitination and type I IFN, although it is unknown whether the IRF3 ubiquitination mediated by these E3s is direct or whether it occurs with the endogenous forms of these proteins. Therefore, a question arose about their contributions relative to RAUL to IRF3 ubiquitination, to IFN-β production, and more importantly to viral replication. To compare their E3 activity toward catalyzing IRF3 conjugation, we first silenced all three E3s individually via RNA interference in 293T-TLR3 cells and examined the conjugation of endogenous IRF3 in response to poly(I:C). Figures 5G and 5H depict the efficacy of siRNA-RBCK1 and siRNA-Ro52 in silencing RBCK1 and Ro52 genes in 293T cells. As shown in Figure 5I, this resulted in different degrees of reduction in IRF3 conjugation with the largest reduction being observed after silencing RAUL. Next, we evaluated the effects of these siRNAs on IFN-β production. Silencing RBCK1 or RAUL or Ro52 in Sendai virus-infected 293T cells caused an increase of IFN-β mRNA production by 3-, 12-, and 4-fold, respectively, with lower effects on mock-infected cells (Figure 5J). Again, silencing RAUL produced the greatest increase both with and without Sendai virus infection. Last, we compared the impact of silencing these E3s on Sendai virus replication. Compared to the control, depletion of RBCK1 or RAUL or Ro52 led to a reduction in Sendai virus yield by 24%, 76%, and 56%, respectively (Figure 5K). Collectively, these data suggest that all three E3s play some role in the negative regulation of IFN-β production through directly or indirectly catalyzing proteolytic IRF3 ubiquitination. Nevertheless, RAUL evidently has by far the more predominant role, possibly because of its unique capability to directly ubiquitinate both IRF3 and IRF7. Overall, our findings offer biological evidence that RAUL, through negatively controlling IRF7 and IRF3 protein
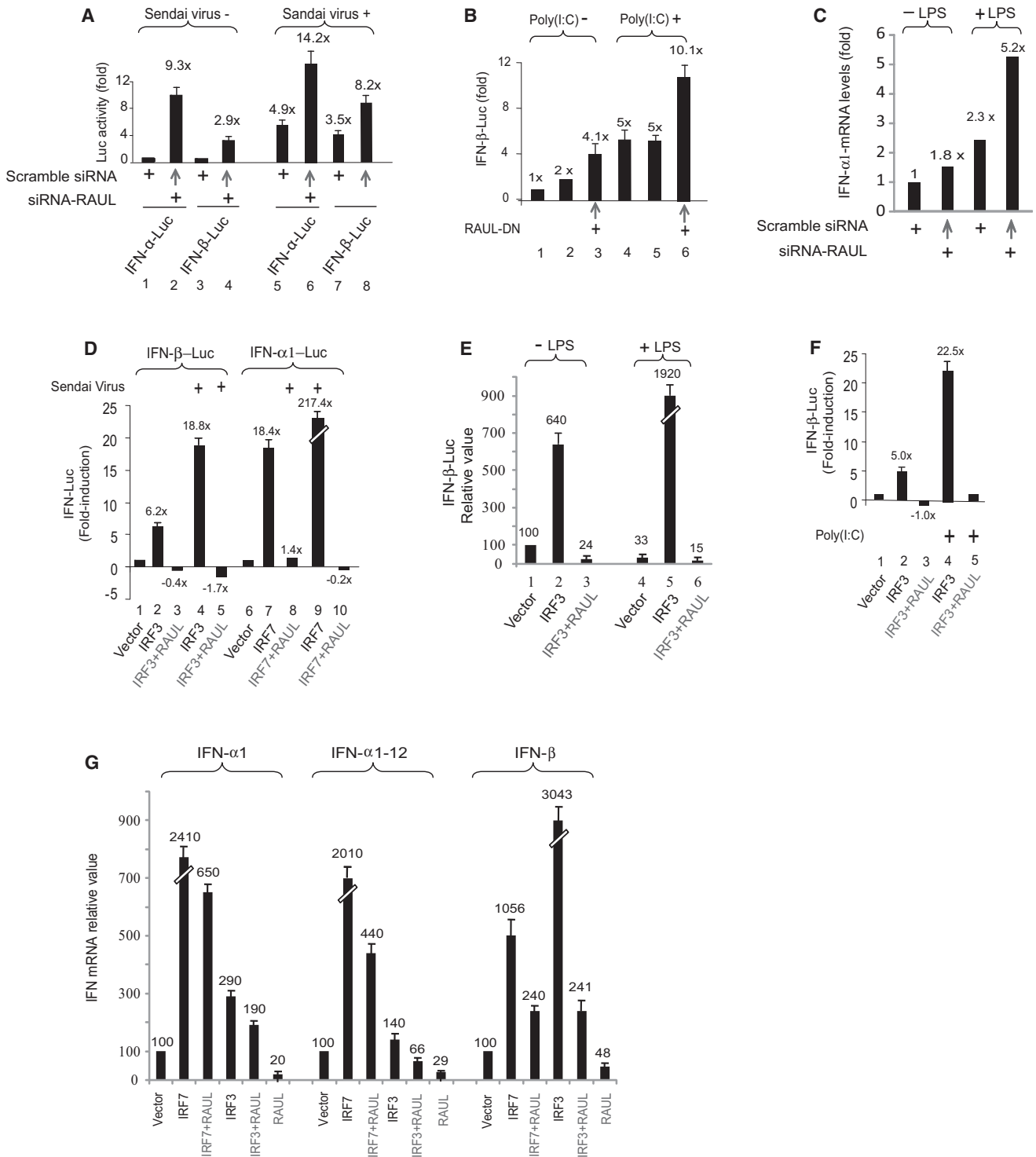
Figure 4. RAUL Negatively Regulates IRF7- and IRF3-Mediated Type 1 IFN Production

(A–C) Suppression of RAUL by siRNA or dominant-negative augments type 1 IFN activity.

(A) Luciferase assay of 293T cells transiently expressing siRNA-RAUL together with IFN-α1 or IFN-β luciferase reporter plasmids. The cells were infected with Sendai virus for 16 hr before harvest. The basal IFN-α and IFN-β activities were arbitrarily set to 1.0.

(B) Luciferase assay of IFN-β promoter activity from 293T-TLR3 cells transiently expressing dominant-negative RAUL in the presence or absence of poly(I:C) (25 μg/ml) for 9 hr to activate IRF3.

(C) Silencing of RAUL increases IFN-α1 mRNA in dendritic cells. DC 2.4 cells were electroporated with siRNA-RAUL or scrambled siRNA in the presence or absence of LPS. Total RNA was isolated and subjected to SYBR green real-time RT-PCR with primers for IFN-α1.

stability, strongly limits the magnitude of the innate antiviral response.

KSHV Recruits and Enhances the Function of RAUL

Both IRF7 and RAUL are binding partners for KSHV RTA, and we also observed that RTA can enhance the ability of RAUL to degrade both IRF7 and IRF3 in a cotransfection system (Figure 6A). These observations prompted us to investigate the possibility that RTA utilizes or modifies the RAUL regulatory mechanism for type I IFN to augment its own direct effects on blocking the antiviral response. We first confirmed the interaction between RTA and RAUL in vitro by a cell-free GST pull-down assay (Figure 6B). By using specific antibodies to both proteins, we also detected this binding with the endogenous protein in KSHV-infected BCBL1 cells, especially after TPA treatment, which induced the expression of RTA (Figure 6C). To address the functional consequences of this binding, we examined whether RTA affected the stabilization of RAUL in both TRExRTA BCBL1 and standard BCBL1 PEL cell lines. Induction of RTA expression by either doxycycline (Dox) or TPA, respectively, substantially increased the steady-state amount of endogenous RAUL (Figures 6D and 6E), which in turn decreased the abundance of IRF3 and IRF7 (Figure 6E), but it had no effect on the amounts of IRF1 (Figure 6D), whose stability is also regulated independently by a ubiquitination-mediated pathway (Nakagawa and Yokosawa, 2000). To address whether this change in the abundance of RAUL might involve posttranslational modification, we coexpressed RTA with RAUL in 293T cells. RTA dramatically reduced the degree of RAUL self-ubiquitination, with a concomitant increase in unconjugated RAUL (Figure 6F), implying that RTA-mediated deubiquitination of RAUL rescues it from self-induced protein degradation.

KSHV RTA Mediates RAUL Deubiquitination via Usp7

RAUL, as an E3 ligase, mediates the ubiquitination of not only its substrates but also itself (self-ubiquitination), leading to proteolysis of RAUL. We set out to investigate the mechanism underlying KSHV RTA-mediated RAUL deubiquitination and found that RTA promotes RAUL deubiquitination through a deubiquitinating enzyme Usp7 (formerly known as HAUSP) (Everett et al., 1997), which is best known for stabilizing p53 through deubiquitination (Li et al., 2002).

First, we investigated whether Usp7 can directly interact with RAUL. By using specific Usp7 and RAUL antibodies, we detected an interaction between endogenous RAUL and endogenous Usp7 proteins in BCBL1 cells (Figure 7A). To test the specific deubiquitination activity of Usp7 on RAUL, we examined whether Usp7 regulated RAUL ubiquitination in transfected cells. As shown in Figure 7B, a large amount of conjugated RAUL was detected in cells transfected with RAUL and HA-Ub; however, RAUL ubiquitination was completely abolished by Usp7 expression, and the Usp7 catalytically inactive mutant Usp7-cs failed to

deubiquitinate RAUL. We then examined whether Usp7 can directly deubiquitinate RAUL in a purified protein system. A poly-ubiquitinated form of RAUL was generated in an in vitro Ub-conjugation system in the presence of E1, E2 (UbcH5a), and Ub. The resulting purified product was then subjected to in vitro deubiquitination in the presence of the WT or mutant Usp7 proteins purified from insect cells (Everett et al., 1997). RAUL alone generated high-molecular-weight moieties in the presence of E1, E2, and Ub, but including the Usp7 protein efficiently eliminated the poly-Ub moieties attached to RAUL, whereas its catalytically inactive point mutant Usp7-cs was functionally defective in deubiquitinating RAUL (Figure 7C, lanes 2 and 3). These data collectively indicate that Usp7 can deubiquitinate RAUL both inside cultured cells and in cell-free systems.

We then investigated the functional consequences of the deubiquitination of RAUL. To determine the effect of Usp7 on RAUL protein, we examined the endogenous RAUL protein in Usp7 WT and silenced cells. Ablation of Usp7 gave rise to a decrease in RAUL protein and a concomitant increase in its substrate IRF3 (Figure 7D). This result indicates that Usp7 expression plays an important role in maintaining the stability of RAUL and presumably also of its cellular functions. To assess the effect of Usp7 toward the E3 activity of RAUL, we purified RAUL protein by immunoprecipitation from cells that either expressed RAUL alone or coexpressed RAUL together with Usp7, and incubated them in vitro with purified GST-IRF3, E1, UbcH5a, and Ub to evaluate the activity of RAUL toward IRF3. Inclusion of RAUL from RAUL + Usp7-expressing cells resulted in a large increase of the ubiquitinated forms of IRF3 compared to in the absence of Usp7 (Figure 7E). This result indicates that Usp7 augments the E3 enzymatic activity of RAUL.

We next investigated whether KSHV RTA mediates RAUL deubiquitination through Usp7. To determine whether RTA assists in recruiting Usp7 to RAUL, we used doxycyclin (DOX) to induce RTA expression in TREx-BCBL1 cells. By using specific antibodies, an increase in the association of endogenous Usp7 with RAUL was observed in the presence of RTA (Figure 7F), suggesting that RTA recruits Usp7 to the RAUL protein to form RTA-Usp7-RAUL complexes and that this inducible Usp7-RAUL association can be enhanced upon RTA activation. To determine whether Usp7 is required for RTA-mediated RAUL deubiquitination, we transfected RTA into both parental colon cancer HCT116 $Usp7^{+/+}$ cells and into a derived cell line with the $Usp7$ gene homozygously disrupted (HCT116 $Usp7^{-/-}$). As shown in Figure 7G, ectopic expression of RTA in WT $Usp7$ cells resulted in deubiquitination of endogenous RAUL, whereas added RTA failed to deubiquitinate RAUL in $Usp7$ null cells ($Usp7^{-/-}$). Therefore, this result provides strong evidence that the deubiquitination and stabilization of RAUL by RTA occur via Usp7.

Finally, we sought to explore the functional consequence of RTA-promoted, Usp7-mediated RAUL deubiquitination by

(D–F) Enforced RAUL expression abolishes IRF7- and IRF3-mediated type I IFN induction under Sendai virus or LPS or poly(I:C) stimulation. The indicated cells were cotransfected with IFN-α1 or IFN-β luciferase reporters, IRF7 or IRF3, and RAUL and the indicated cells were infected with Sendai virus (16 hr) or stimulated by LPS (100 ng/ml, for 9 hr) or poly(I:C). IFN promoter activation was determined by luciferase assay.

(G) RAUL inhibits IRF7- or IRF3-mediated type I IFN mRNA production. 293T cells were cotransfected with IRF7, IRF3, and RAUL plasmids as indicated. Total RNA was isolated and subjected to SYBR green real-time RT-PCR with primers for IFN-α1, IFNα-1-12, and IFN-β, respectively, to assess type 1 IFN mRNA output.
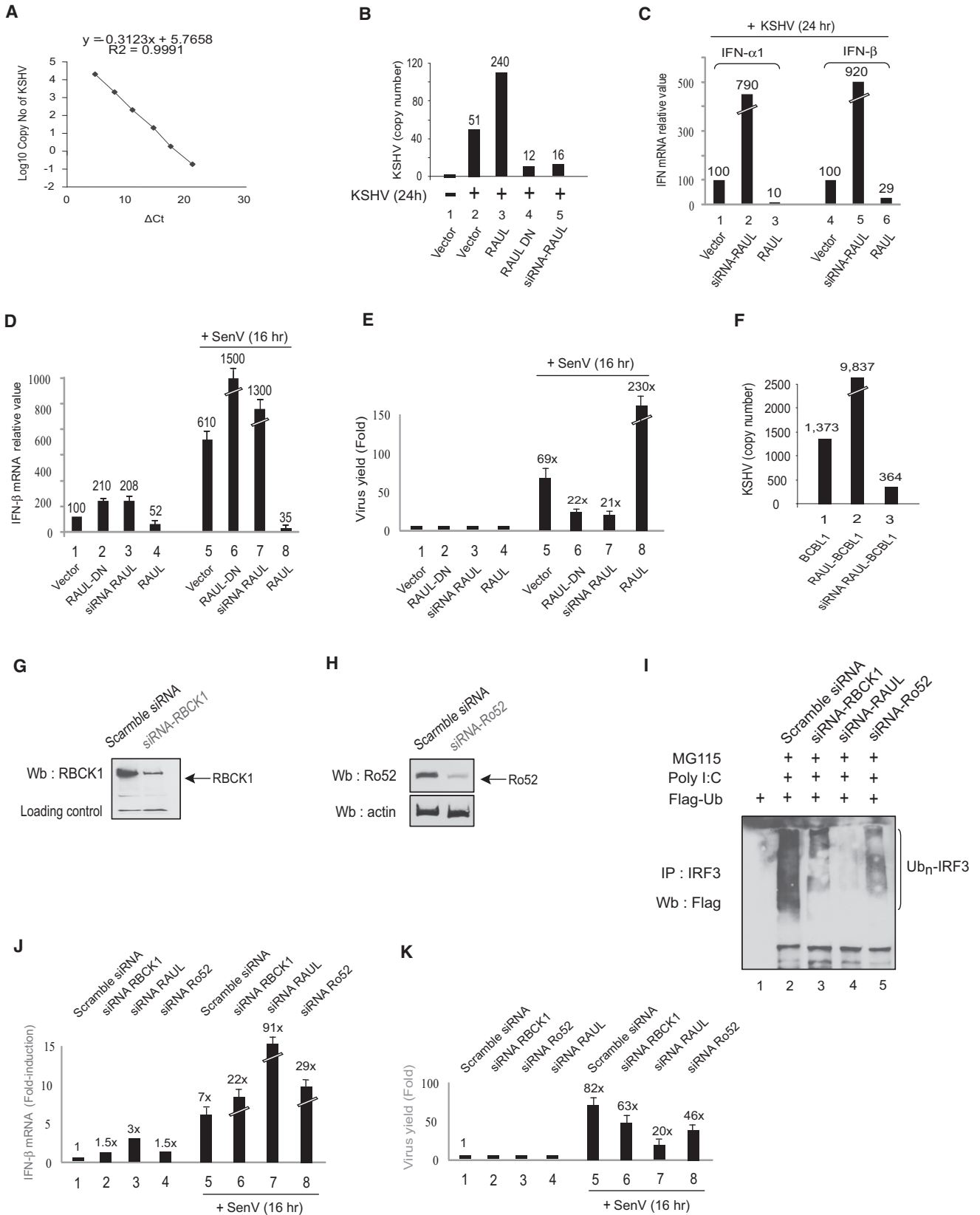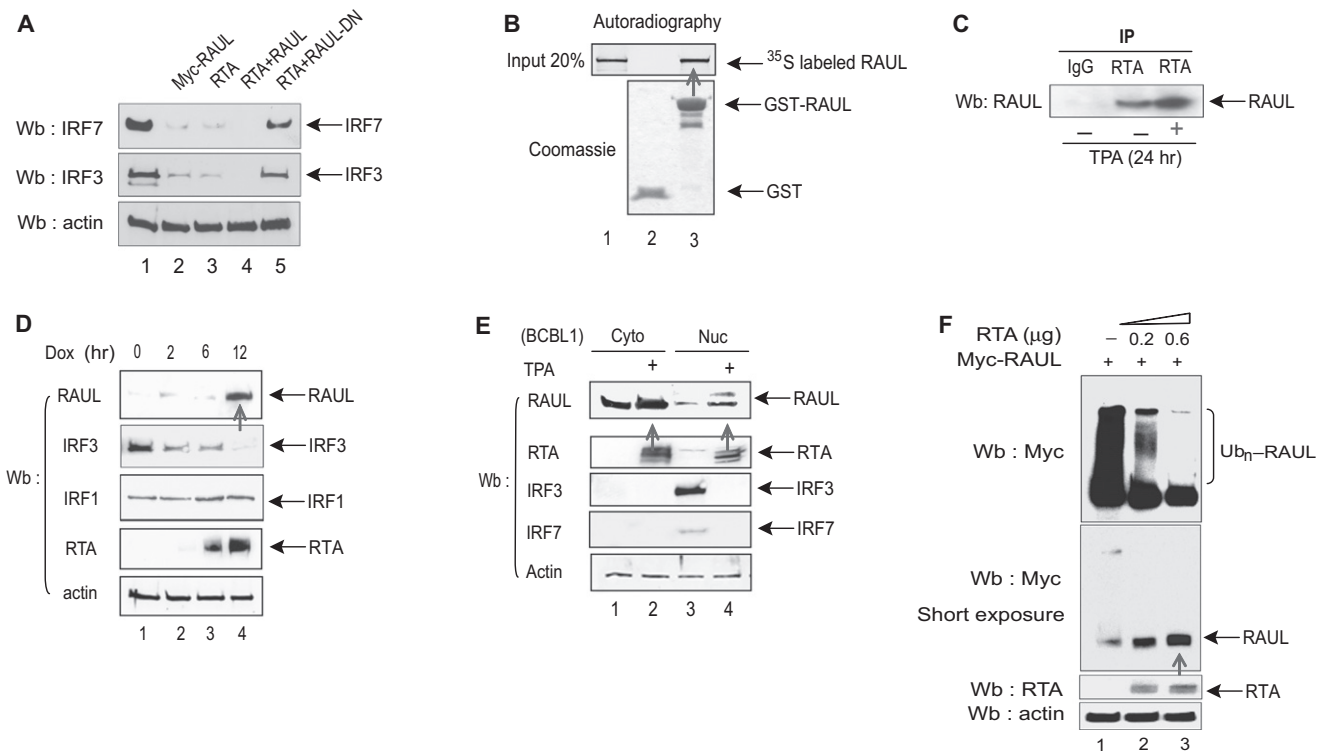
Figure 6. RTA Recruits and Enhances RAUL

(A) RTA enhances the ability of RAUL to degrade IRF7 and IRF3. 293T cells were cotransfected with RTA, Myc-RAUL, or Myc-RAUL-DN, IRF7, and IRF3 as indicated and the amounts of IRF7 and IRF3 proteins were detected by IRF7 or IRF3 immunoblot.

(B and C) RTA binds to RAUL in vitro and in vivo.

(B) A GST-pull down assay was carried out with ^{35}S-labeled in vitro translated RAUL and GST-RTA proteins.

(C) Cell lysates from BCBL1 PEL cells were immunoprecipitated with anti-RTA followed by RAUL immunoblot to detect endogenous RTA-RAUL interaction with TPA.

(D and E) Induction of RTA stabilizes RAUL protein.

(D) TREx BCBL1 cells treated with doxycycline (Dox) for the indicated times to induce RTA expression, and RAUL, IRF3, IRF1, and RTA proteins were monitored by immunoblot with the indicated antibodies.

(E) BCBL1 PEL cells were treated with TPA to induce RTA expression and cytoplasmic and nuclear fractions were isolated. The RAUL, IRF7, IRF3, and RTA proteins were evaluated by immunoblot with the indicated antibodies.

(F) RTA mediates RAUL deubiquitination. RTA and Myc-RAUL plasmids were cotransfected into 293T cells and ubiquitinated forms of RAUL were determined by immunoblot with anti-Myc. To show both ubiquitinated and nonubiquitinated forms of RAUL, a short-exposure film was included (bottom).

coexpressing RTA, RAUL, and IRF7 in 293T cells. As before, expression of RTA cooperated with RAUL to enhance degradation of the IRF7 protein (Figure 7H); however, concurrently silencing Usp7 severely impaired this effect. Likewise, RTA increased the ability of RAUL to suppress IRF3-mediated IFN-β reporter activation in 293T-TLR3 cells, whereas RTA failed to

Figure 5. RAUL Regulates the IFN-Mediated Antiviral Response

(A) A SYBR real-time PCR-based quantitative approach for determining KSHV viral load. The standard curve was constructed with KSHV RAP DNA as target by the comparative Ct method. The viral load value, normalized to reference (β-actin), is given by $2^{-\Delta\Delta Ct}$ in the Applied Biosystems 7500 Real Time PCR system expressed as copy number per 100 ng of infected cell genomic DNA.

(B–E) Role of RAUL in KSHV and Sendai virus-infected mouse embryonic fibroblast cells (MEFs). MEF cells were electroporated with WT RAUL, dominant RAUL-DN, or siRNA-RAUL and the cells were infected with KSHV or Sendai virus for the indicated times. The cell lysates were assayed for viral DNA copy number (KSHV) per 100 ng of genomic DNA or relative viral RNA load (Sendai virus) (B and E) as well as for type 1 IFN mRNA production by real-time RT-PCR (C and D).

(F) Role of RAUL in KSHV-infected BCBL1 cells. Lysates from BCBL1-derived cell lines stably expressing either RAUL or siRNA-RAUL were assayed for KSHV viral DNA copy number per 100 ng genomic DNA.

(G and H) Comparison of the contribution of RBCK1, RAUL, and Ro52 to endogenous IRF3 ubiquitination. Immunoblot of lysates with RBCK1- (G) or Ro52- (H) specific antibody from 293T cells transiently expressing siRNA-RBCK1 or siRNA-Ro52 or scramble siRNA.

(I) Comparison of siRNA silencing to reduce polyubiquitination of IRF3 in activated cells. 293T-TLR3 cells were transfected with siRNA against RBCK1 or RAUL or Ro52 together with Flag-Ub and the cells were treated with poly(I:C) to induce poly-Ub conjugation of IRF3 in the presence of the proteasome inhibitor MG115. The conjugation of endogenous IRF3 was detected by immunoprecipitation with IRF3 antibody and immunoblot with Flag antibody.

(J and K) Relative roles of RBCK1, RAUL, and Ro52 in viral replication. 293T cells were transfected with siRNA against RBCK1 or RAUL or Ro52, and then infected with Sendai virus for the indicated times. The cell lysates were assayed for IFN-β mRNA production (J) and for viral RNA load of Sendai virus by real-time RT-PCR (K).
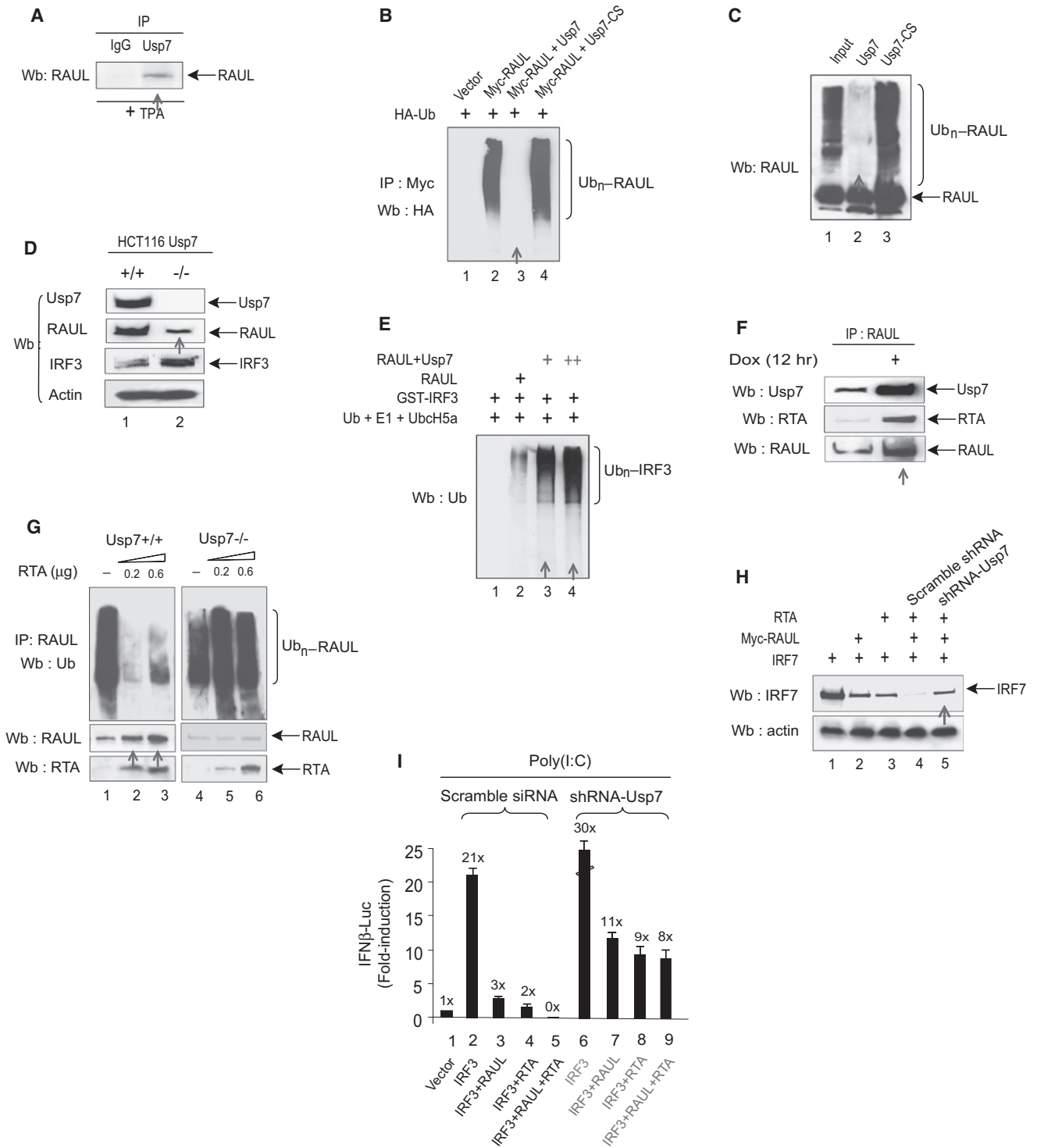
Figure 7. RTA Mediates RAUL Deubiquitination through Usp7

(A) Endogenous RAUL and Usp7 interact. BCBL1 cells were treated with TPA for 24 hr and cell lysates were immunoprecipitated with anti-Usp7 followed by RAUL immunoblot to detect bound protein.

(B and C) Usp7 deubiquitinates RAUL in vivo and in vitro. 293T cells were transfected with Myc-RAUL, WT Usp7, Usp7-cs, and HA-Ub. The cells were treated with MG132 for 6 hr before harvest. Polyubiquitinated forms of RAUL were detected by immunoprecipitation with anti-Myc followed by HA immunoblot.

(C) Purified RAUL protein (400 nM) plus E1, E2 (UbcH5a), and Ub were incubated at 37°C for 1 hr to catalyze self-ubiquitination. The Ub-RAUL protein was then purified by immunoprecipitation with RAUL antibody and incubated with purified Usp7 protein or its catalytically inactive mutant (Usp7-cs) in deubiquitination buffer at 37°C for 2 hr. The resulting reactions were subjected to immunoblot with RAUL.

do so in the same cells after Usp7 was silenced by shRNA (Figure 7I). The Usp7 shRNA also led to reduced inhibition of IRF3-mediated IFN-LUC activity by both RAUL and RTA alone. These results demonstrate that RTA augments RAUL's activity toward its substrates IRF7 and IRF3 through Usp7 and reinforces the notion that Usp7 acts as a positive regulator for RAUL.

Taken together, these findings reveal an important posttranslational regulatory mechanism for RAUL, in which the DUB enzyme Usp7 positively regulates RAUL by preventing it from proteolytic self-ubiquitination and that KSHV RTA exploits this mechanism while amplifying RAUL activity to mute antiviral responses.

DISCUSSION

In this report, we have identified an important negative regulatory mechanism acting on the type I IFN pathway that is probably required for both appropriate cellular homeostasis and for resetting the basal state after induction of antiviral innate immunity. This mechanism relies on the E3 ligase activity of RAUL, which exerts its inhibitory action on type I IFN by directly catalyzing lysine 48-linked polyubiquitination on both IRF7 and IRF3, the two most crucial transcriptional factors for mounting type I IFN responses. In all circumstances tested, increased or decreased amounts of RAUL negatively correlated with either strong negative or positive effects, respectively, on endogenous type I IFN production.

The implications of this mechanism are 3-fold. (1) In physiological conditions, it helps to keep type I IFN production at proper low basal amounts through targeting IRF7 and IRF3 ubiquitination and degradation. (2) Under stress conditions, in which type I IFN amounts rise dramatically, it may act as a "speed brake" to prevent detrimental overproduction. (3) Given its crucial role in type I IFN regulation, control of RAUL function may also become a battleground in the war between viruses and the host during infection. High interferon doses administered to patients can cause toxic effects, indicating a need for timely and spatially coordinated downregulation mechanisms for type I IFN and for effective combat against infectious agents. We propose that RAUL, operating upstream of IRF7 and IRF3, is a key factor for maintaining appropriate cellular homeostasis and for antiviral innate immunity through controlling both the basal amount and intensity of induced type I IFN responses. We speculate that some of the other previously reported viral and cellular proteins that influence the ubiquitination status of both proteins might also exert their actions through the Ub E3 ligase RAUL.

IRF7 and IRF3 comply with all four of the criteria that should be met by specific HECT domain E3 ligase substrates (You et al., 2003). (1) They both bind to the N-terminal substrate binding domain of RAUL. (2) Purified RAUL specifically catalyzes the conjugation of ubiquitin on IRF7 and IRF3 in vitro. (3) Their protein amounts are reduced by coexpression with RAUL, but not by its catalytically inactive mutant, and this destabilization is proteasome dependent. (4) Depletion of RAUL increases the stability and hence overall amount of both proteins.

Our investigation into the role of KSHV RTA in RAUL signaling led us to uncover a critical role for deubiquitination in the regulation of RAUL function. Ubiquitination is a dynamic process that involves constantly adding and removing Ub moieties from the substrate. Self-ubiquitination plays an important role in the regulation of RING finger domain ligases TRAF2, TRAF3, and TRAF6, and deubiquitination-mediated protein stabilization has emerged recently as an accompanying regulatory step (Chomvarin et al., 2004; Friedman et al., 2008; Kayagaki et al., 2007; Wang et al., 2001; Yang et al., 2006). HECT domain Ub ligases, once thought to be constitutively active and to recognize their substrates independently of their posttranslational modification, are also subjected to posttranslational regulation (Gao et al., 2004; Wiesner et al., 2007). It appears that, on one hand, RAUL is capable of catalyzing polyubiquitination constitutively, an ability that is required for regulating the resting forms of the IRF7 and IRF3 proteins; whereas on the other hand, RAUL activity can also itself be regulated through posttranslational mechanisms under pathogenic settings, which is important for regulating the active forms of both IRF proteins.

Several recent studies have indicated that some Ub E3 ligases are physically associated with DUBs that directly reverse self-ubiquitination of the ligase, thus regulating its activity. However, only one HECT domain ligase, Rsp5, has been previously shown to be regulated by a DUB Ubp2, and no effect on its E3 ligase activity was observed (Kee and Huibregtse, 2007; Shembade et al., 2010). Our findings suggest that RAUL can be rescued from undergoing self-ubiquitination-mediated degradation by Usp7 via direct deubiquitination, which represents an important posttranslational regulatory mechanism for RAUL. Its activity is increased through deceleration of the self-induced degradation and decreased via acceleration of the degradation. Thus it appears that Usp7 is capable of cleaving Ub chains with both lysine 48- and 63-linkage from both RING finger and HECT domain E3 Ub ligases.

(D) Usp7 upregulates RAUL protein amounts. Colon cancer cell lines HCT116 $Usp7^{+/+}$ or $Usp7^{-/-}$ cells were immunblotted with the indicated antibodies to evaluate both RAUL and its target substrate IRF3.

(E) Usp7 enhances the E3 activity of RAUL toward IRF3 in vitro. Immunoprecipitated purified RAUL protein from either RAUL-expressing 293T cells or RAUL+Usp7-expressing 293T cells were incubated with its substrate GST-IRF3, plus E1, UbcH5a, and Ub in vitro at 37°C for 1 hr. Conjugated IRF3 was determined by Ub immunoblot.

(F) RTA increases the recruitment of Usp7 to RAUL complexes. TRExBCBL1 cells were treated with Doxycyclin (Dox) for 12 hr to activate RTA expression. Endogenous RTA-Usp7-RAUL interaction was evaluated by immunoblot with the indicated antibodies.

(G) RTA promotes RAUL deubiquitination. HCT116 ($Usp7^{+/+}$ or $Usp7^{-/-}$) cells were electroporated with the indicated amounts of RTA plasmid and RAUL self-ubiquitination was determined by Ub immunoblotting. Unconjugated RAUL and RTA were measured by RAUL or RTA immunoblotting.

(H) RTA enhances the ability of RAUL to degrade IRF7 via Usp7. 293T cells were cotransfected with IRF7, Myc-RAUL, RTA, and shRNA-Usp7 as indicated, and IRF7 protein amounts were evaluated by IRF7 immunoblotting.

(I) RTA and RAUL cooperate in suppressing IRF3-mediated IFN-β reporter activity. 293T-TLR3 cells were transfected with the indicated combinations of IRF3, RAUL, RTA, shRNA-Usp7, and IFN-β-LUC plasmids and the cells were treated with poly(I:C) for 9 hr to mimic TLR3 activation. IRF3-mediated IFN-β reporter activity in the cells that were pretreated shRNA-RAUL or scrambled IFN-β activation was determined by luciferase assays.

Much of our knowledge with regard to immunity comes from understanding how cells deal with viral infection, from smallpox to human immunodeficiency virus (HIV). The observation that KSHV RTA activates RAUL activity through Usp7 embodies a striking example of virus-mediated manipulation of a cellular ubiquitination pathway. This is reminiscent of another HECT domain Ub E3 ligase, E6AP, which mediates the ubiquitination of p53 in cells that express the human papillomavirus (HPV) E6 oncoprotein. Evidently Sendai virus is also able to take advantage of RAUL signaling during infection, suggesting that this scheme may be generally relevant to other viral infections. A common strategy adopted by viruses to counteract innate immunity is to mimic or seize existing cellular pathways and to utilize them in their favor (McCormick and Ganem, 2005; Scheffner et al., 1990). Therefore exploring these "commandeered pathways" unveils not only the pathogenesis involved but also uncharted cellular pathways.

EXPERIMENTAL PROCEDURES

Reagents

pCMV-MyC-RAUL (WT, 1083 amino acids) and the pCMV-MyC-RAUL HECT domain point mutant (C1051A), as well as pET3a-RAULWT, pET3a-RAUL-ND (1–655), and pET3a-RAUL-CD (655–1083) for in vitro translation were provided by C.M. Pickart (Bloomberg School of Hygiene, Johns Hopkins University). RAULΔ132 was provided M. Wang. pIRF7A (1–503), pFLAG-IRF7A (1–503), pGST-IRF7 (1–503), and pGST-IRF7 (255–503) and reporter plasmids encoding firefly luciferase (LUC) under the control of human IFN-α1 (–140 to +9) and IFN-β (–280 to +20) promoter elements were provided by Y. Yuan (Zhu et al., 2002). pIRF7DN (aa 1–12 and 103–503) was provided by T. Maniatis (Wathelet et al., 1998). pIRF7C was provided by J.S. Pagano (University of North Carolina) (Zhang and Pagano, 1997). IRF7S477D and S479D was provided by J. Hiscott. pHA-Ub was provided by K. Watanabe (Sasaki et al., 2002). pFLAG-Ub, pHA-IRF3, pHA-IRF3 (1–128), pHA-IRF3 (1–374), pGST-IRF3 (58–427), and pGST-IRF3 (175–427) were provided by T. Fuita (Wathelet et al., 1998). pGST-IRF3 was provided by M.E. Hardy (Veterinary Molecular Biology Laboratory) (Graff et al., 2002), 293T-TLR3 cell lines was provided by K. Fitzgerald (Fitzgerald et al., 2003), and HCT116 $Usp7^{+/+}$ and $Usp7^{-/-}$ cell lines were provided by B. Vogelstein (Cummins et al., 2004). Purified Usp7 protein was provided by R. Everett (Everett et al., 1997); pcDNA3-Pin1 was provided by A. Ryo (Saitoh et al., 2006); and pSR-Pin1 (siRNA-Pin1), pSR-Rli (control siRNA), and pEFHA-IRF3S339A were provided by S. Yamaoka (Saitoh et al., 2006). $Rag2^{-/-}$ 5c.c7 TCR transgenic cells were provided by Y. Zheng and J.D. Powell (Zheng et al., 2007).

Monoclonal antibodies against β-actin, ubiquitin, FLAG, HA, and Myc-epitopes were purchased from Sigma. Rabbit polyclonal antibody against GST, Usp7, Ro52, RBCK1, IRF7, and IRF3 were purchased from Santa Cruz. Control nonimmune antibodies are indicated as IgG. RAUL antibody was generated in rabbits by using a peptide corresponding to the near N-terminal sequence of RAUL (Y-KTRPKVSLGGASRKEEK-SC). shRNA-Usp7 was provided by R. Everett.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and two figures and can be found with this article online at doi:10.1016/j.immuni.2010.11.027.

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