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RNF213 modulates γ-herpesvirus infection and reactivation via targeting the viral Replication and Transcription Activator

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Interferons (IFNs) and the products of interferon-stimulated genes (ISGs) play crucial roles in host defense against virus infections. Although many ISGs have been characterized with respect to their antiviral activity, their target specificities and mechanisms of action remain largely unknown. Kaposi's sarcoma-associated herpesvirus (KSHV) is a gammaherpesvirus that is linked to several human malignancies. Here, we used the genetically and biologically related virus, murine gammaherpesvirus 68 (MHV-68) and screened for ISGs with anti-gammaherpesvirus activities. We found that overexpression of RNF213 dramatically inhibited MHV-68 infection, whereas knockdown of endogenous RNF213 significantly promoted MHV-68 proliferation. Importantly, RNF213 also inhibited KSHV de novo infection, and depletion of RNF213 in the latently KSHV-infected iSLK-219 cell line significantly enhanced lytic reactivation. Mechanistically, we demonstrated that RNF213 targeted the Replication and Transcription Activator (RTA) of both KSHV and MHV-68, and promoted the degradation of RTA protein through the proteasome-dependent pathway. RNF213 directly interacted with RTA and functioned as an E3 ligase to ubiquitinate RTA via K48 linkage. Taken together, we conclude that RNF213 serves as an E3 ligase and inhibits the de novo infection and lytic reactivation of gammaherpesviruses by degrading RTA through the ubiquitin–proteasome pathway.

γ-herpesvirus | interferon-stimulated genes | RNF213 | RTA

As the first line of host defense, innate immunity is rapidly activated upon detection of invading pathogens and plays an important role in limiting their spread. Type I interferons (IFNs) are multifunctional cytokines that act as key components of the innate immune response to viral infections. The cellular factors that mediate this defense are the products of interferon-stimulated genes (ISGs). Although hundreds of ISGs have been identified (1), only a limited number of them have been characterized in detail with respect to their antiviral activities. These ISGs have been reported to interfere with various key steps of virus life cycle via different mechanisms. For example, cholesterol-25-hydroxylase (CH25H) blocks cellular entry of several enveloped viruses (2–4), and in the case of hepatitis C virus, also inhibits viral replication via targeting the viral NS5A protein (5); IFI16 restricts viral genes transcription through epigenetic modifications (6–8). Zinc-finger antiviral protein and protein kinase R inhibit virus translation and replication (9–12); Viperin and tetherin prevent viral egress from the cell (13–16). However, for most ISG products, little is known about their target specificities and their mechanisms of actions.

Kaposi's sarcoma-associated herpesvirus (KSHV) is a human γ-herpesviruses and is etiologically associated with Kaposi's sarcoma, primary effusion lymphoma, and multicentric Castleman's disease (17–20). Studies on the function of KSHV lytic proteins had been severely hampered until recent years, due to the lack of an effective de novo lytic infection system and more importantly the lack of suitable small animal models. Murine gammaherpesvirus-68 (MHV-68) is genetically and biologically related to KSHV and offers an experimental system that overcomes some of the challenges associated with studies of human γ-herpesviruses infection (21, 22). Replication and Transcription Activator (RTA), mainly encoded by ORF50, is a key mediator that controls the switch of viral life cycle from latency to lytic replication for KSHV and MHV-68 (23–27). As an immediate-early gene product, RTA activates the expression of many viral and cellular genes that have been implicated in the replication and pathogenesis of γ-herpesviruses (28–30). Besides serving as a transcriptional activator, RTA also modulates the activities of cellular factors. It blocks interferon-regulatory factor (IRF7)-mediated interferon-alpha (IFN-α) and interferon-beta (IFN-β) messenger RNA (mRNA) production and promotes the ubiquitination and degradation of IRF7 protein in a proteasome-dependent fashion (31). RTA may also degrade the adaptor MyD88 through the ubiquitin–proteasome pathway and impair Toll-like receptor signaling to evade innate immunity (32, 33).

Significance

Kaposi's sarcoma-associated herpesvirus (KSHV) is the etiologic agent for Kaposi's sarcoma, primary effusion lymphoma and multicentric Castleman's disease. Interferons play important roles in controlling KSHV replication. To explore the underlying mechanism, we screened a library of interferon-stimulated genes (ISGs) we previously built and identified RNF213, which suppresses gammaherpesvirus replication. RNF213 is the susceptibility gene for Moyamoya Disease in East Asians. Here, we found that RNF213 inhibits KSHV de novo infection and reactivation by down-regulating the expression of a virally encoded "molecular switch" protein named RTA. RNF213 interacts with RTA directly and promotes RTA ubiquitination and degradation through the ubiquitin–proteasome pathway. Our study uncovers the antiviral function of RNF213 and provides insights into understanding interferon-mediated host defense against herpesvirus infections.

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Given the high prevalence of γ-herpesvirus infection, it is important to identify host factors that modulate γ-herpesvirus replication. Interferons play important roles in restricting γ-herpesvirus lytic replication and reactivation from latency in vitro and in vivo (34–37). Although the antiviral role of IFNs in γ-herpesviruses infection is firmly established, only a limited number of ISG products have been ascribed anti-γ-herpesvirus functions. For example, ISG15 was reported to inhibit MHV-68 replication in vivo, and consistently $ISG15^{-/-}$ mice exhibited increased susceptibility to MHV-68 infection (38). CH25H, a broadly antiviral ISG, also inhibits MHV-68 infection by blocking membrane fusion between the virus and the cell (2, 5, 39). IFIT proteins restrict KSHV replication by positively regulating IFN-β production and inhibiting KSHV mRNA abundance (40). However, the functions of many other ISGs during γ-herpesvirus infection remain to be elucidated.

In this study, we used a recombinant MHV-68 that expresses a luciferase as a reporter (41) and screened an ISG library that we built (42) for activities that modulate γ-herpesvirus replication. The screen identified RNF213 as an ISG product that inhibits MHV-68 replication. RNF213 is the susceptibility gene for Moyamoya Disease (MMD, a cerebrovascular disorder) in East Asians (43, 44). We further showed that RNF213 also inhibits KSHV de novo infection and reactivation from latency. Mechanistically, we demonstrated that RNF213 functions as a ubiquitin E3 ligase, interacts with RTA from both MHV-68 and KSHV, and promotes RTA polyubiquitination and degradation via the proteasome pathway. Taken together, these results revealed a previously undiscovered host strategy to modulate γ-herpesvirus lytic replication and latency.

Results

Inhibition of MHV-68 Replication by RNF213 Expression. Although interferons are critical in limiting γ-herpesviruses infection and pathogenesis (36, 37), anti-γ-herpesvirus functions have been studied for only a few ISGs so far (38–40). To identify additional ISGs that modulate γ-herpesvirus infection, we first identified and generated a small library of 138 ISGs that had not been reported by the time of study (42). We then took advantage of MHV-68-M3FL, a recombinant MHV-68 that expresses a luciferase reporter (41), to screen for anti-MHV-68 ISGs (Fig. 1*A*). MHV-68 luciferase activity was measured and normalized to infected cells that transfected with empty vector (EV). The screen revealed dozens of ISGs that were capable of inhibiting MHV-68 replication. These include several known anti-MHV-68 ISGs, such as CH25H and MX2, validating our screen. Interestingly, RNF213, whose gene mutations were associated with MMD (44), exhibited marked inhibitory effect on MHV-68 replication (Fig. 1*B* and *SI Appendix,* Table S1). To confirm that RNF213 is an antiviral ISG, we first verified that MHV-68 infection induced up-regulation of RNF213 in different cell lines (Fig. 1*C* and *SI Appendix,* Fig. S1 *A–C*). To further investigate the effect of RNF213 on viral lytic replication, we performed overexpression or knockdown experiment. Overexpression of RNF213 markedly reduced the replication of MHV-68-M3FL, as indicated by firefly luciferase activities, and this inhibition was dose dependent (Fig. 1*D*). In contrast, when RNF213 expression was down-regulated by specific shRNAs (Fig. 1*E*), viral replication was significantly increased over that in shCtrl-transfected control cells, demonstrating that RNF213 knock-down enhanced MHV-68-M3FL replication (Fig. 1*F*). Taken together, these results indicated that RNF213 is an antiviral ISG that functionally suppressed MHV-68 infection.

Suppression of Intracellular Events of MHV-68 Infection by RNF213. Several studies have reported that some ISGs exert antiviral activities via affecting the functions of various cellular signaling pathways. For instance, USP18, a negative regulator of IFN response, inhibits JAK-STAT signaling pathway (45). Cyclic GMP-AMP synthase (cGAS), an IFN-stimulated gene, mediates the subsequent positive feedback regulation of DNA-triggered IFN-I production (46). GBP1 exerts inhibitory effects on dengue virus infection by regulating the activity of NF-κB (47). To determine whether RNF213 affects the functions of various cellular signaling pathways, luciferase activities of reporters driven by IFN-β, interferon-stimulated response element, or NF-κB promoters were measured after stimulation by Sendai virus infection (*SI Appendix*, Fig. S2 *A–C*) or poly(dA:dT) (*SI Appendix,* Fig. S2 *D–F*). The results revealed that RNF213 overexpression did not have an effect on the activities of these promoters under either treatment, suggesting that RNF213 did not up-regulate the IFN pathway and that RNF213 most likely inhibits MHV-68 replication through a direct mechanism.

We next determined the specific step(s) within MHV-68 lytic replication cycle that is affected by RNF213. We first performed a virus entry assay and the results showed that RNF213 expression did not affect the virus binding/entry process (Fig. 2*A*). Next, we examined the inhibitory effect of RNF213 on MHV-68 genomic DNA replication using a de novo infection-replication assay, as previously described (48). As shown in Fig. 2*B*, pMOL, which contains the *ori*Lyt fragment, replicated and yielded an expected *Dpn-I* resistant band in the presence of MHV-68 infection, whereas the control plasmid pGEM-T failed to replicate. However, the replication efficiency of pMOL decreased remarkably after RNF213 overexpression, suggesting that RNF213 inhibited viral DNA replication. We further measured the mRNA levels of viral immediate-early (IE) and early (E) genes in wild-type MHV-68 Bacterial Artificial Chromosome (BAC)-transfected cells. Remarkable decrease in the mRNA levels of MHV-68 E genes (*ORF6*, *ORF40*, and *ORF59*), as measured by qRT-PCR, were detected in RNF213-expressing cells at both 24 and 48 hpt. The level of IE gene transcripts, *rta*, was also significantly down-regulated in the presence of RNF213 at 48 hpt (Fig. 2 *C* and *D*). Consistently, the levels of viral late proteins also decreased significantly in RNF213-expressing cells (Fig. 2*E*). Taken together, these results indicated that the RNF213 acts mainly on the immediate-early phase of MHV-68 replication to inhibit viral proliferation.

RNF213 Suppresses Transcriptional Activation by MHV-68 RTA and KSHV RTA. RTA serves as a "molecular switch" in the life cycle of MHV-68 and initiates viral lytic replication by activating the transcription of many downstream genes (25, 28). Since transcription of viral IE/E genes and genomic DNA replication were inhibited by RNF213 (Fig. 2 *B–D*), we next sought to examine whether RNF213 inhibits the transcriptional activation of those genes by RTA, using a luciferase reporter system (49). As expected, activation of the *RTA* and *ORF48* promoters by MHV-68 RTA (hereafter called mRTA) was remarkably inhibited in the presence of RNF213 (Fig. 3*A*). Two possible explanations may account for how RNF213 suppresses the transcriptional activation of mRTA: RNF213 inhibits the transcriptional activation function of mRTA, and/or RNF213 negatively affects the level of mRTA protein. The result of cotransfection experiment showed that the level of ectopically expressed mRTA protein was reduced in the presence of RNF213 (Fig. 3*B*). During MHV-68 infection, RNF213 also decreased the level of endogenous mRTA protein (Fig. 3*C*). Furthermore, qRT-PCR results demonstrated that RNF213 did not affect the mRNA level of mRTA (Fig. 3*D*).

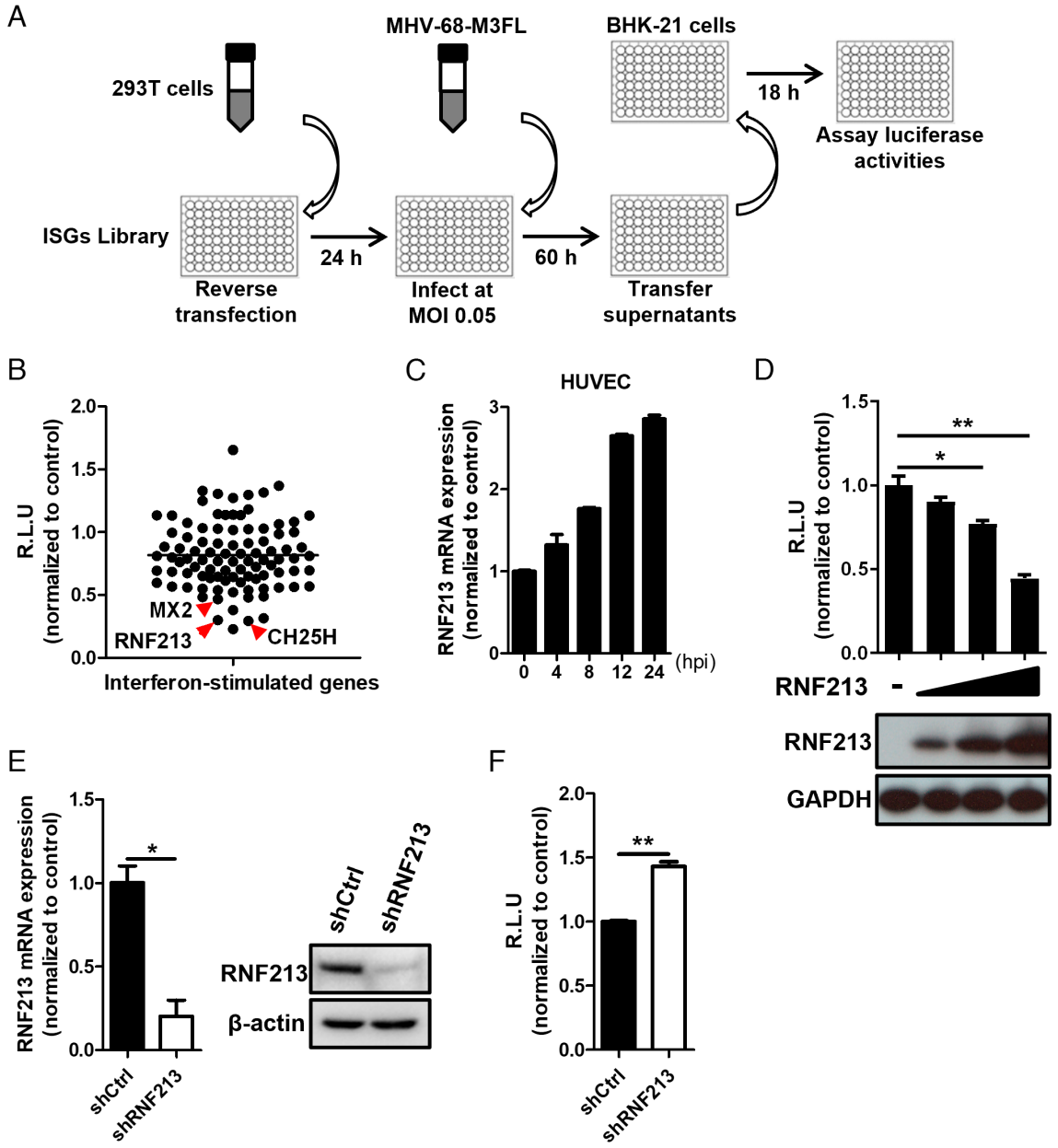

Fig. 1. RNF213 inhibits MHV-68 replication. (*A* and *B*) Screen of an ISG library for anti-MHV-68 activities. HEK293T cells were individually reverse transfected with ISG expression plasmid and infected with a recombinant MHV-68 (M3FL) at a MOI of 0.05 in triplicates. At 60 hpi, 10 μL supernatants was transferred to naïve BHK21 cells, and the luciferase activities of BHK21 cells were measured 18 h later (*A*). Each dot represents an ISG, and its effect on MHV-68 replication is calculated by normalizing the RLU to that of the control sample (i.e., cells transfected with an empty vector followed by infection with M3FL). R.L.U.: Relative Luciferase Unit. (*B*). (*C*) Expression of RNF213 was induced by MHV-68 infection. HUVEC cells were infected with MHV-68. The mRNA levels of RNF213 were examined by qRT-PCR at the indicated time points. Error bars represent SDs. (*D*–*F*) The effect of RNF213 overexpression or knockdown on MHV-68 replication. Plasmid expressing RNF213 (*D*) or shRNF213 (*E* and *F*) was transfected into HEK293 cells. After 24 h, HEK293 cells were infected with M3FL at a MOI of 0.05. At 60 hpi, supernatants were collected to infect naïve BHK21 cells, and luciferase activities were measured at 18 hpi. (*C*–*F*) Data presented are mean ± SEM of three independent measurements, representative of three independent experiments (*$P < 0.05$, **$P < 0.01$; two-tailed Student's *t* test, n = 3).

Since the homologous RTA protein in KSHV (hereafter called kRTA) also serves as a molecular switch for KSHV life cycle, we next tested whether RNF213 can also inhibit the function and expression of kRTA. Similarly, RNF213 decreased activation of the *kRTA* and *vIL-6* promoters by kRTA (Fig. 3*E*). In cotransfection experiment, RNF213 also reduced the expression level of kRTA protein (Fig. 3*F*). Moreover, when iSLK cells were transfected with pMyc-RNF213 for 24 h and then treated with Doxycycline (DOX) to induce kRTA expression, immunoblotting revealed that the kRTA protein level was also reduced in the presence of RNF213 in a dose-dependent manner (Fig. 3*G*). qRT-PCR results demonstrated that RNF213 did not change the mRNA levels of kRTA (Fig. 3*H*). Collectively, these results indicated that RNF213 down-regulates both mRTA and kRTA expression at the protein level.

Inhibition of KSHV De Novo Infection by RNF213 Expression. Like mRTA, kRTA is expressed during KSHV both de novo infection and reactivation from latency, plays an essential role in the regulation of KSHV life cycle. Therefore, we tested the effect of RNF213 on KSHV de novo infection. First, the kinetics of RNF213 expression was examined in human umbilical vein endothelial cells (HUVEC) after KSHV infection. We observed that the mRNA and protein levels of RNF213 started to increase after 4 h of KSHV de novo infection (Fig. 4 *A* and *B*), indicating that KSHV infection induced RNF213 expression. Next,

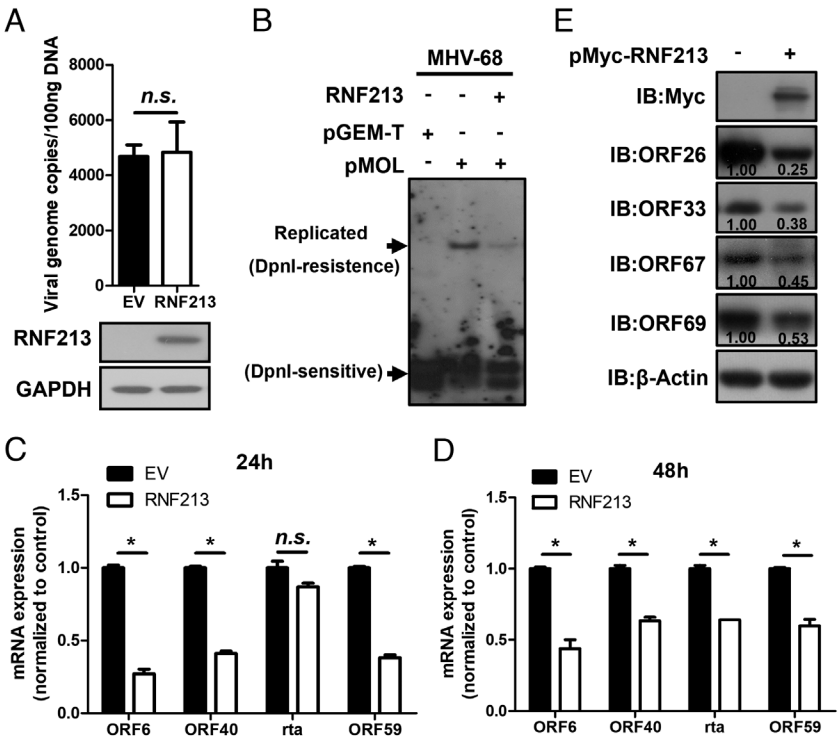

Fig. 2. Suppression of intracellular events of MHV-68 infection by RNF213. (*A*) Effect of RNF213 expression on MHV-68 entry. HEK293A cells were transfected with pFlag-RNF213 or an empty vector as a control. At 24 hpi, cells were infected with MHV-68 at a MOI of 50 for 1 h. MHV-68 genomic DNA copy numbers were measured by qPCR and RNF213 expression measured by western blotting. (*B*) Effect of RNF213 expression on viral DNA replication. Cells were cotransfected with a plasmid harboring the MHV-68 origin of lytic replication (pMOL) and pFLAG-RNF213 or an empty vector, as indicated. After 24 h, cells were infected with MHV-68, and at 24 hpi, total cellular DNA was extracted, digested with *Dpn I* and *Pst I* overnight and analyzed by Southern blotting. (*C* and *D*) Effect of RNF213 expression on transcription of viral genes. pFLAG-RNF213 and wild-type MHV-68 BAC were cotransfected into 293T cells. At the indicated time points, total RNA was extracted and transcription of viral genes analyzed by qRT-PCR and normalized to that of GAPDH. (*E*) Effect of RNF213 expression on viral protein levels. The 293T cells were transfected with pFLAG-RNF213 or an empty vector, and cells were infected with MHV-68 at 24 hpt. At 24 hpi, cells were collected for western blotting to examine viral protein levels using specific antibodies as indicated. (*C* and *D*) Data presented are mean ± SEM of three independent measurements, representative of three independent experiments. (*$P < 0.05$, n.s.: not significant; two-tailed Student's *t* test, n = 3).

iSLK cells were transfected with pMyc-RNF213 for 24 h and then infected by GFP-KSHV produced from iSLK-BAC16, in which GFP expression is under the control of the Elongation factor 1 alpha (EF-1α)promoter. The results of flow cytometry clearly showed that RNF213 inhibited KSHV de novo infection significantly (Fig. 4 *C* and *D*). Consistently, when the expression level of endogenous RNF213 was down-regulated by shRNA (shRNF213) (Fig. 4 *E* and *F*), the infection efficiency of GFP-KSHV was markedly increased, as demonstrated by the higher number of GFP-positive cells compared with that of shCtrl-treated cells (Fig. 4 *G* and *H*). These results demonstrated that KSHV de novo infection induces RNF213 expression, which then inhibits KSHV de novo infection.

RNF213 Restricts KSHV Reactivation. We next examined the effect of RNF213 on KSHV reactivation from latency by utilizing a KSHV latently infected cell line, iSLK-219 (50). The iSLK-219 cells harbor a latent KSHV genome which expresses a GFP marker driven by the constitutively active EF-1α promoter, an RFP marker driven by the promoter of viral gene *PAN*, and a doxycycline (DOX)-inducible RTA expression cassette. Therefore, GFP serves as a marker for infected cells while RFP serves as a marker for KSHV reactivation. As shown in Fig. 5*A*, after DOX induction of KSHV reactivation, the number of RFP-positive cells decreased with increasing amounts of transfected RNF213 plasmid (Fig. 5*B*), indicating lower reactivation efficiencies compared with empty vector control. The supernatants from these cell cultures were collected to infect fresh iSLK to determine viral titers, as reflected by the percentage of GPF-positive cells analyzed by flow cytometry. Consistently, the viral titers in supernatants from cells overexpressing RNF213 were also reduced significantly in a dose-dependent manner (Fig. 5 *C* and *D*). We further examined the impact of RNF213 knockdown on KSHV reactivation. Down-regulation of RNF213 by shRNA (Fig. 5*E*) markedly enhanced KSHV reactivation, as indicated by higher percentage of RFP-positive cells (Fig. 5*F*). Accordingly, the viral titers in supernatants from cells silenced for RNF213 expression were much higher compared with those treated with shCtrl (Fig. 5 *G* and *H*). These results demonstrated that RNF213 restricts reactivation of KSHV from latency in iSLK-219 cells.

RNF213 Alters the Stability of RTA Proteins and Degrades RTA Through the Proteasome Pathway. Since RNF213 did not affect the expression of either mRTA or kRTA at the transcriptional level but reduced mRTA and kRTA protein levels (Fig. 3), we reasoned that RNF213 may alter the stability of RTA proteins. 293T cells were cotransfected with expression plasmids for kRTA or mRTA plus RNF213 or vector control, and 24 h later, treated with cycloheximide (CHX) to block de novo synthesis of proteins. Western blotting showed that the stability of kRTA and mRTA proteins was significantly reduced in the presence of RNF213 as compared with that in control cells (*SI Appendix,* Fig. S3 *A* and *B*).

Protein degradation in eukaryotic cells mainly takes place through the proteasome pathway or the lysosome pathway (51, 52). The proteasome pathway only recognizes ubiquitinated substrates for degradation, whereas cytosolic components or extracellular materials can be delivered into the lysosome for degradation. We therefore cotransfected cells with expression plasmids for kRTA or mRTA plus RNF213 or vector control, and treated cells with either MG132, an inhibitor of the proteasome pathway, or NH_4Cl, an inhibitor of the lysosome pathway. Reduction of kRTA or mRTA protein level by RNF213 was not affected by treatment with NH_4Cl (Fig. 6 *A* and *B*); in contrast, such reduction was blocked by treatment with MG132 (Fig. 6 *C* and *D*). Furthermore, prolonged treatment with MG132 restored the levels of both RTA proteins in RNF213-expressing cells (*SI Appendix,* Fig. S3 *C–F*). These data indicated that the reduction of RTA protein levels by RNF213 is not mediated through the lysosome pathway, but through the proteasome pathway.

RNF213 Interacts with RTA Directly. We next investigated whether RNF213 directly interacts with these two RTA proteins and mediates their degradation through the proteasome pathway. We

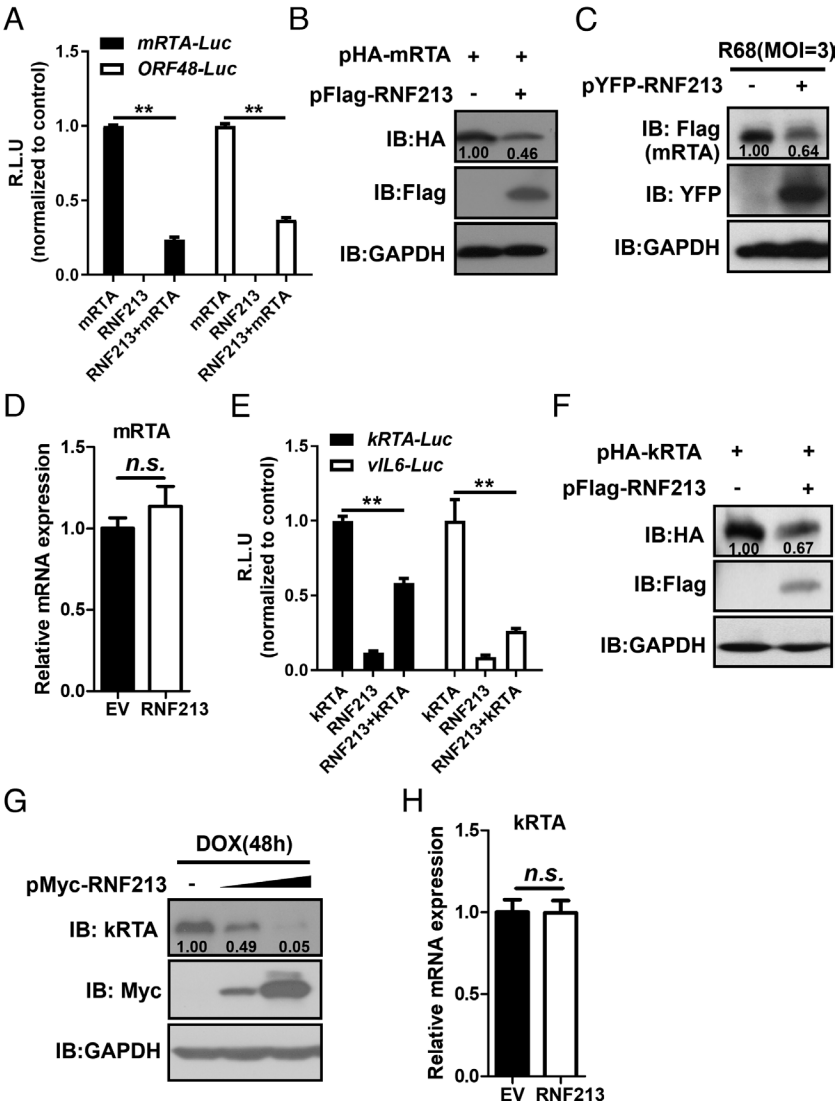

Fig. 3. RNF213 suppresses transcriptional activation by MHV-68 and KSHV RTA proteins through reducing RTA expression. (*A*) HEK293T cells were individually cotransfected with expression plasmid(s) (pMyc-RNF213 and/or pFlag-mRTA) plus the reporter plasmid driven by the MHV-68 promoter (pmRTAp-luc or pORF48p-luc). pTK-Rluc was also included in each sample as an internal control. After 36 h, luciferase activities were measured by the dual-luciferase reporter assay. (*B*) pFlag-RNF213 and pHA-mRTA were cotransfected into HEK293T cells. After 36 h, protein levels of RTA, RNF213, and GAPDH were determined by western blotting. (*C*) HEK293T cells were transfected with pYFP-RNF213 or empty vector as a control. After 24 h, cells were infected with a recombinant MHV-68 expressing FLAG-tagged mRTA (R68), and protein levels of mRTA, RNF213, and GAPDH were determined by western blotting at 18 hpi. (*D*) Effect of RNF213 on stability of mRTA transcripts. pFlag-RNF213 or empty vector was cotransfected with pHA-mRTA into HEK293T cells. After 24 h, total RNA was extracted, and the transcript level of mRTA was determined by qRT-PCR and normalized to that of GAPDH in the same sample. (*E*) HEK293T cells were individually cotransfected with expression plasmid(s) (pMyc-RNF213 and/or pFlag-kRTA) plus the reporter plasmid driven by the KSHV promoter (pkRTAp-luc or pvIL-6p-luc). pTK-Rluc was also included in each sample as an internal control. After 36 h, luciferase activities were measured by the dual-luciferase reporter assay. (*F*) HEK293T cells were transfected with HA-tagged kRTA with or without Flag-tagged RNF213. Anti-HA and anti-flag antibodies were used for western blotting. (*G*) iSLK cells were transfected with vector or different amounts of pMyc- RNF213, and 24 h posttransfection, cells were treated with DOX for 24 h, and kRTA expression was analyzed by western blotting. (*H*) Effect of RNF213 on stability of kRTA transcripts. pFlag-RNF213 or empty vector was cotransfected with pHA-kRTA into HEK293T cells. After 24 h, total RNA was extracted and the transcript level of kRTA was determined by qRT-PCR and normalized to that of GAPDH in the same sample. (*A*, *D*, and *E*) Data presented are mean ± SEM of three independent measurements, representative of three independent experiments. (***P* < 0.01, n.s.: no significance; two-tailed Student's *t* test, n = 3).

cotransfected plasmids expressing RNF213 and mRTA/kRTA and performed coimmunoprecipitation experiments. The results showed that RNF213 interacts with mRTA and kRTA (Fig. 6 *E* and *F*). Importantly, RNF213 also interacts with endogenous mRTA in the context of MHV-68 infection (Fig. 6*G*). To test whether RNF213 also interacts with kRTA expressed from viral genome, we utilized 293T-219 cells, which harbor latent KSHV genome and can be induced with 12-O-tetradecanoylphorbol-13-acetate (TPA) and valproate (VPA) for KSHV reactivation. The results showed that RNF213 pulls down endogenous kRTA during KSHV replication (Fig. 6*H*). Moreover, we expressed and purified recombinant GST-RNF213 protein in bacteria, and performed GST pull-down assays with mRTA/kRTA protein, which were synthesized using rabbit reticulocyte lysates. The result demonstrated that RNF213 directly interacts with mRTA/kRTA (Fig. 6*I*).

RNF213 Is a Ubiquitin E3 Ligase and Polyubiquitinates RTA via K48 Linkage. Since protein degradation via the proteasome pathway requires protein ubiquitination and RNF213 interacts with RTA, we further investigated whether RNF213 serves as an E3 ligase for RTA ubiquitination. Plasmids expressing RNF213, RTA, and Ub were cotransfected into 293T cells for intracellular ubiquitination assays. As shown in Fig. 7*A*, RNF213 overexpression indeed led to much enhanced polyubiquitination of mRTA/kRTA in vivo, suggesting that RNF213 may be a ubiquitin E3 ligase for RTA. In order to directly confirm that RNF213 is an E3 ligase for RTA, we expressed and purified from *Escherichia coli* recombinant GST-RNF213 protein as well as components of the ubiquitin-proteasome system, i.e., E1 (His-UbA1), E2 (His-UbcH5c), and His-ubiquitin. We also generated in vitro-translated mRTA/kRTA and performed an in vitro ubiquitination reconstitution assay. As expected, mRTA and kRTA were both polyubiquitinated in the presence of RNF213, but not in the absence of RNF213 (Fig. 7 *B* and *C*). In addition, RNF213 overexpression with WT-Ub and K63R-Ub, but not K48R-Ub, led to polyubiquitination of mRTA and kRTA (Fig. 7 *D* and *E*). Taken together, these results demonstrated that RNF213 is an E3 ligase for RTA proteins and catalyzes K48-linked polyubiquitination for their degradation through the proteasome pathway.

Discussion

Viral infection induces host immune response, and interferons play critical roles in host innate immunity against viral infection. The antiviral effect of IFNs is known to be mediated by ISGs, which disrupt various steps of virus replication (1). Although many ISGs have been identified to restrict diverse families of viruses (53, 54), only a few ISGs have been identified as having antiviral functions against γ-herpesviruses. In this study, we performed functional screens of ISGs for their antiviral activities against MHV-68, and identified RNF213 as one of the ISGs that

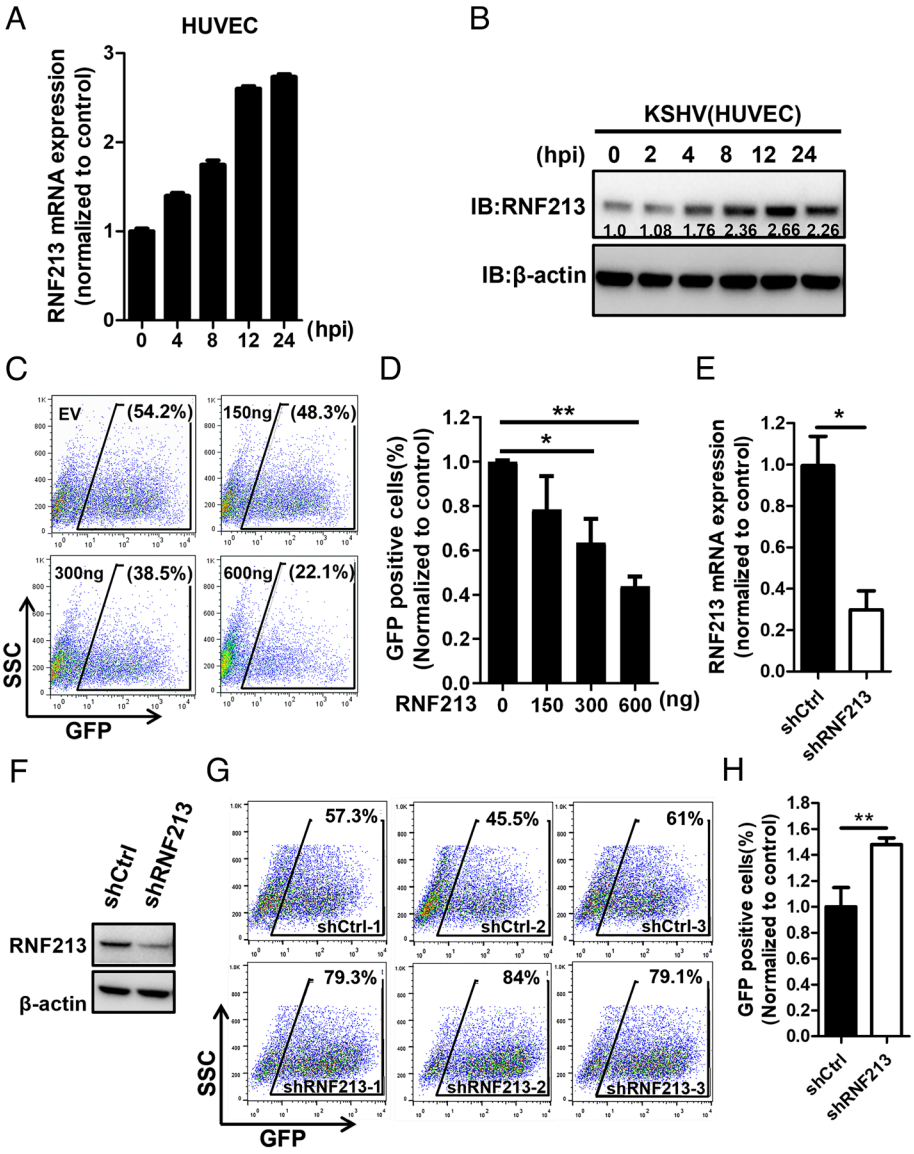

Fig. 4. Inhibition of KSHV de novo infection by RNF213 expression. (*A* and *B*) HUVEC cells were infected with KSHV at MOI = 5, and cells were collected at the indicated time points. The endogenous RNF213 mRNA and protein levels were analyzed by qRT-PCR and western blotting, respectively. (*C* and *D*) iSLK cells were transfected with pMyc-RNF213 for 24 h, and then infected by GFP-KSHV. GFP-positive cells were analyzed by flow cytometry (*C*), and values calculated and presented in (*D*). The amount of plasmids in each sample was adjusted with empty vector, if necessary, to a total of 600 ng. (*E*–*H*) iSLK cells were transfected with shRNF213 or shCtrl for 36 h (*E* and *F*), and then infected by GFP-KSHV. The GFP-positive cells were analyzed by flow cytometry (*G*), and values calculated and presented in (*H*). (*D*, *E*, and *H*) Data presented are mean ± SEM of three independent measurements, representative of three independent experiments. (**P* < 0.05, ***P* < 0.01; two-tailed Student's *t* test, n = 3).

modulate MHV-68 replication. We validated the antiviral function of RNF213 on both MHV-68 and KSHV and showed that RNF213 modulates γ-herpesviruses lytic replication and latency by serving as a ubiquitin E3 ligase to degrade RTA through the ubiquitin–proteasome pathway (Fig. 7*F*).

Several in vitro studies have reported that establishment of γ-herpesvirus infection is capable of antagonizing IFN signaling cascades (55–60). However, type I interferons and IRF1 have been shown to limit the replication of γ-herpesviruses (36, 39). Interferons also play a critical role in restricting γ-herpesviruses' reactivation (34, 35). In addition, mice deficient in interferon receptors or an IFN signaling component, signal transducer, and activator of transcription 2 were reported to be highly susceptible to γ-herpesvirus infection (37). Thus, IFN response is likely to play a key role in controlling herpesvirus lytic replication and reactivation in vivo. One possible scenario for interferons' regulatory roles in herpesvirus life cycle is that some interferon-induced products inhibit the functions of KSHV proteins, such as RTA and Latency-associated Nuclear Antigen (LANA), which are critical regulators of viral life cycle. However, the roles of individual ISGs in restricting γ-herpesviruses' infection remains poorly understood and warrants further investigation.

Various studies have reported that a comprehensive screen for antiviral ISGs is an effective method to discover host factors against a diverse panel of viruses (53, 54). In our study, we detected the anti-MHV68 activity of several ISGs candidates and found that RNF213 is sufficient and required for host to inhibit MHV-68 infection (Fig. 1). Moreover, MHV-68 infection up-regulated RNF213 expression, suggesting that RNF213 may play a role in modulating virus infection (Fig. 1 and *SI Appendix*, Fig. S1). Further studies revealed that RNF213 has no effect on viral entry but inhibits viral genomic DNA replication and subsequent proteins expression (Fig. 2). During lytic replication of MHV-68, the viral genome is expressed in a temporally regulated cascade, including immediate early (IE), early (E), and late (L) gene expression, which are accompanied by viral

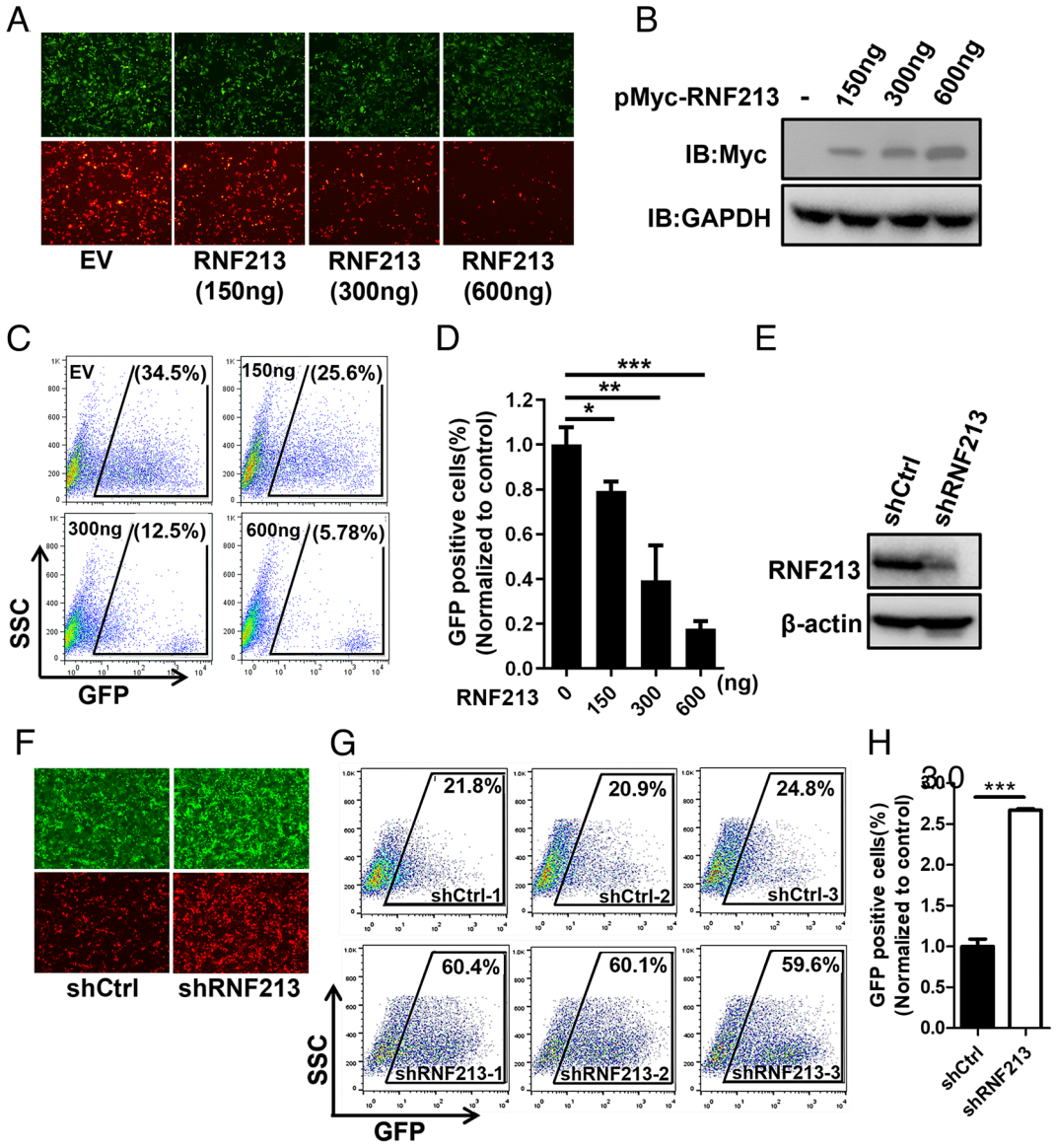

Fig. 5. RNF213 restricts KSHV reactivation. (*A*–*D*) iSLK-219 cells were transfected with empty vector or increasing amounts of RNF213 expression plasmid, and 24 h later, treated with DOX for 48 h. GFP and RFP expression was analyzed by fluorescent microscopy. (*A*), and cell lysates examined by western blotting as indicated (*B*). The amount of plasmids in each sample was adjusted with empty vector, if necessary, to a total of 600 ng. Cell culture supernatants were collected to infect fresh iSLK cells, and GFP-positive iSLK cells were analyzed by flow cytometry (*C*). The percentages of GFP-positive cells were normalized to that in empty vector-transfected sample (control), with that in control sample set as 1 (*D*). (*E*–*H*) iSLK-219 cells were transfected with shCtrl or shRNF213, and 36 h later, treated with DOX for 48 h. Knockdown of RNF213 expression was confirmed by western blotting (*E*), and GFP and RFP expression was analyzed by fluorescent microscopy (*F*). Cell culture supernatants were transferred to fresh iSLK cells, and GFP-positive cells were analyzed by flow cytometry (*G*). The percentages of GFP-positive cells in shRNF213-transfected samples were normalized to those in shCtrl-transfected samples, with shCtrl samples set as 1 (*H*). Values are calculated from the results shown in panel *E*. (*D* and *H*) Data presented are mean ± SEM of three independent measurements, representative of three independent experiments. (**P* < 0.05, ***P* < 0.01, ****P* < 0.001; two-tailed Student's *t* test, n = 3).

DNA replication, protein expression, and virus production. Since viral proteins encoded by IE gene (such as RTA encoded by ORF50) and E genes (such as ORF6, ORF40 and ORF59) are required for viral DNA replication, we examined their expression. Our results indeed showed that RNF213 decreased the mRNA levels of ORF6, ORF40, and ORF59 at 24 h and 48 h after MHV-68 infection (Fig. 2 *C* and *D*). While the mRNA level of RTA only decreased slightly at 24 hpi, it was down-regulated significantly at 48 hpi (Fig. 2 *C* and *D*), consistent with the autoactivation activity of RTA (61). Moreover, we found that RNF213 inhibited transactivation activity of RTA through reducing mRTA expression (Fig. 3 *A*–*C*). Importantly, RNF213 also decreased RTA expression of KSHV and inhibited transactivation activity of kRTA (Fig. 3 *E*–*G*). Consequently, RNF213 inhibited KSHV de novo infection (Fig. 4).

KSHV reactivation from latency is important for virus dissemination and the pathogenesis of KSHV-associated diseases. Thus, a better understanding of the regulation of viral life cycle could provide insights into developing promising treatments for KSHV persistent infection and KSHV-related diseases. RTA is the molecular switch and plays a key role in regulating the reactivation of KSHV from latency to the lytic replication. In addition to viral proteins and microRNAs that were reported to regulate RTA functions (62, 63), many host factors were also found to modulate RTA expression or function. Toth Z et al. reported that EZH2, a H3K27me3 histone methyltransferase, inhibits RTA expression and lytic replication (64). Sirt6 also attenuates KSHV reactivation by decreasing RTA expression (65). MDM2, a ubiquitin E3 ligase, was reported to interact with PARS (protein abundance regulatory signal) domain of RTA and promote RTA degradation (66). The

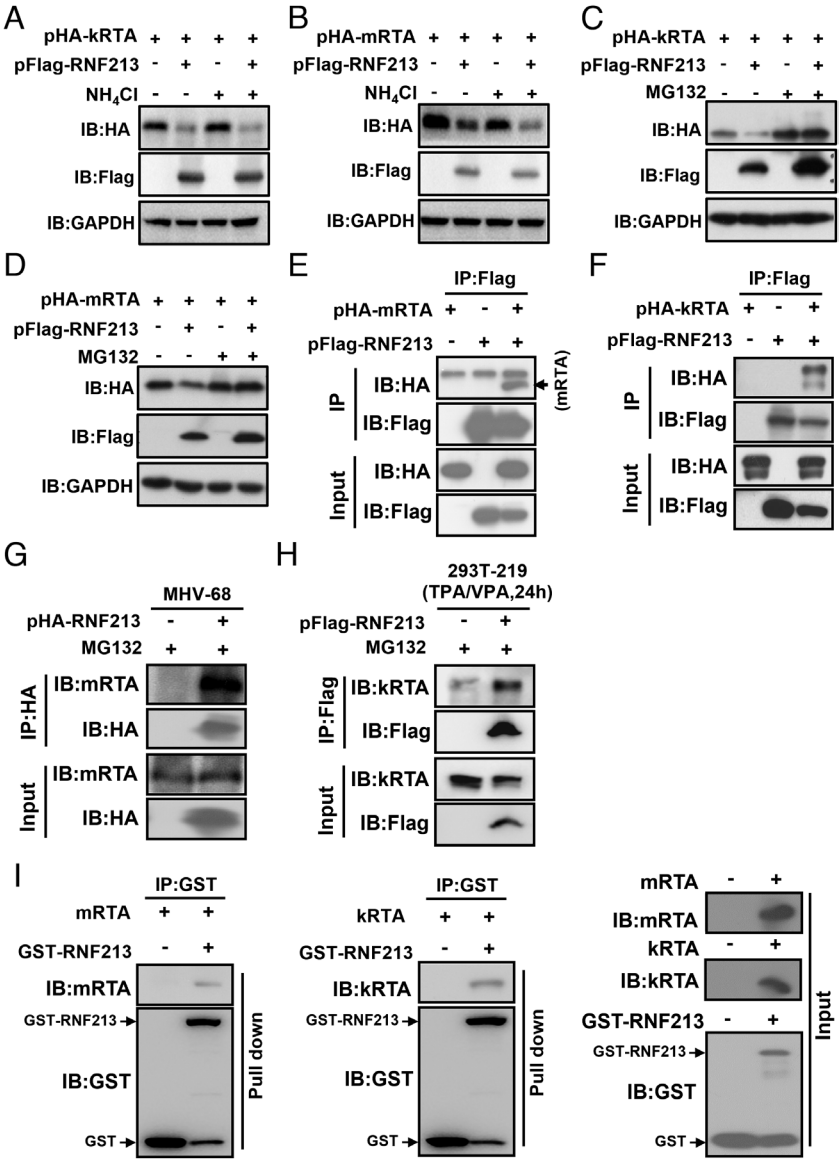

Fig. 6. RNF213 interacts with RTA directly and degrades RTA through the proteasome pathway. (*A* and *B*) HEK293T cells were transfected with pHA-kRTA (*A*)/pHA-mRTA (*B*) and pFLAG-RNF213 or vector control, and treated with 20 μM NH_4Cl as indicated. Anti-Flag and anti-HA antibodies were used for western blotting. (*C* and *D*) HEK293T cells were transfected with pHA-kRTA (*C*)/pHA-mRTA (*D*) and pFLAG-RNF213 or vector control, and treated with 10 μM MG132 as indicated. Anti-Flag and anti-HA antibodies were used for western blotting. (*E*) 293T cells were cotransfected with pHA-mRTA and pFLAG-RNF213 or vector control, treated with MG132 and harvested for co-IP with Flag antibody-conjugated agarose beads. The precipitated proteins were analyzed by western blotting. (*F*) 293T cells were cotransfected with pHA-kRTA and pFLAG-RNF213 or vector control, treated with MG132 and harvested for co-IP with FLAG antibody-conjugated agarose beads. (*G*) HEK293A cells were transfected with pHA-RNF213 or empty vector as a control, infected with MHV-68 for 24 h, and co-IP was performed with HA antibody-conjugated agarose beads. (*H*) 293T-219 cells were transfected with pFlag-RNF213 or empty vector as a control, and 24 h posttransfection, treated with TPA and VPA for 24 h, and co-IP was performed with FLAG antibody-conjugated agarose beads. (*I*) In vitro GST pull-down assay. Bacterially expressed GST or GST-RNF213 attached to beads were incubated with in vitro-translated mRTA or kRTA. RTA binding to beads was detected by western blotting.

PARS domain is also targeted by NCOA2, which competes with MDM2 to enhances the stability of RTA (67). In this study, we found RNF213 affects transcriptional activation function of RTA by down-regulating RTA protein expression (Fig. 3). Further studies showed that RNF213 interacted with RTA directly, and served as a ubiquitin E3 ligase to degrade RTA through the ubiquitin–proteasome pathway (Figs. 6 and 7).

A hallmark of the herpesvirus life cycle is the establishment of long-term latency in infected cells. During primary infection or latency, RTA mRNA could be detected at low levels in infected cells (68). Once a threshold level of RTA expression is achieved, the lytic replication may proceed to completion. Along this line, our results demonstrated that overexpression of RNF213 restricted KSHV lytic reactivation induced by DOX, whereas depletion of RNF213 promoted KSHV lytic reactivation (Fig. 5). Since RTA is a key molecular switch for KSHV life cycle, our results strongly indicated that, by serving as a negative regulator or RTA, RNF213 is a host factor that regulates the balance between latency and lytic replication for KSHV.

RNF213 was previously identified as the susceptibility gene for Moyamoya Disease via a genome-wide linkage and association study (43, 44). Allelic variations in RNF213 confer the risk of Moyamoya Disease; however, the exact mechanism by which the RNF213 abnormality relates to MMD remains unknown. While this study was ongoing, several groups also examined the role of RNF213 in lipid metabolism and antimicrobial activity (69). Otten et al. reported that RNF213 acts as an E3 ligase that can promote ubiquitination of LPS directly, and this is required for antibacterial autophagy (70). Other studies showed that RNF213 is required for host defense against *Listeria monocytogenes* and *Chlamydia trachomatis* which lack LPS, suggesting different substrates ubiquitylated by RNF213 (71, 72). Besides bacterial pathogens, RNF213 also exhibits antiviral activity against several viruses including HSV-1; however, the molecular mechanisms remain elusive (71, 73). In this work, we not only revealed the anti-herpesvirus function of RNF213, but also identified its viral protein target.

In summary, we discovered found the antiviral mechanism of RNF213 and demonstrated that RNF213 serves as a ubiquitin E3 ligase and inhibits the de novo infection and lytic reactivation of γ-herpesviruses by degrading RTA through the ubiquitin–proteasome pathway.

Materials and Methods

Cell Lines, Viruses, and Plasmids. All cells were cultured at 37 °C in the presence of 5% CO_2. HUVEC, 293T, 293A cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Gibco), and THP-1 cells were grown in RPMI 1640 (Hyclone) with

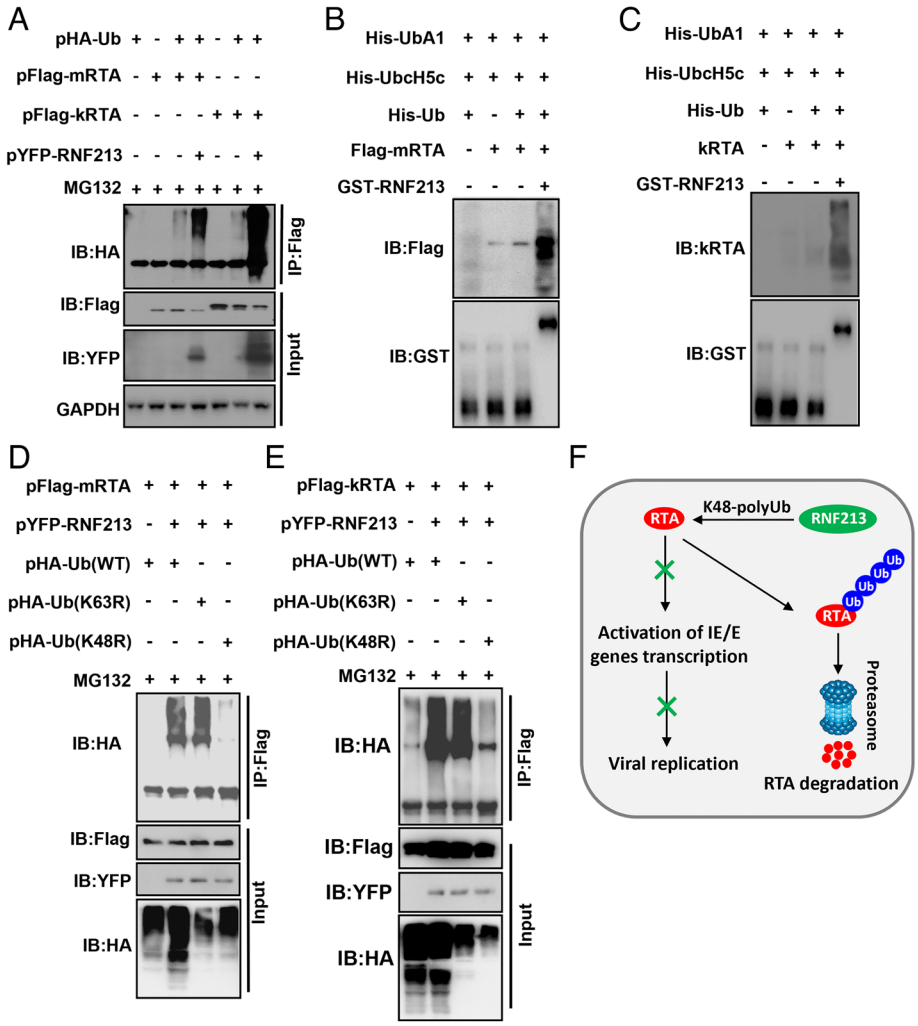

Fig. 7. RNF213 promotes polyubiquitination of RTA via K48 linkage. (*A*) HEK293T cells were cotransfected with the plasmids as indicated. After 24 h, cells were treated with MG132 for 18 h. Whole-cell extracts were dissociated with 1% SDS and immunoprecipitated with anti-Flag antibody-conjugated beads and followed by western blotting using antibodies as indicated. (*B* and *C*) RNF213 ubiquitinates RTA in an in vitro ubiquitination assay. Bacterially expressed GST-RNF213 was incubated with in vitro-translated mRTA or kRTA in the presence of recombinant E1, E2, and ubiquitin at 30℃ for 1 h, followed by western blotting with the indicated antibodies. (*D* and *E*) RNF213 enhanced K48-linked ubiquitination of RTA. HEK293T cells were cotransfected with the indicated plasmids in the presence of MG132. Whole-cell extracts were dissociated with 1% SDS and immunoprecipitated with M2 Flag beads and followed by western blotting using antibodies as indicated. (*F*) Schematic model for RNF213-mediated RTA degradation in response to MHV-68 and KSHV infection. RNF213 interacts with RTA encoded by viral IE gene, and ubiquitinates RTA. RTA tagged with ubiquitin is degraded through the proteasome pathway and viral early gene expression, and hence viral lytic replication is inhibited.

10% fetal bovine serum (FBS) and penicillin-streptomycin. The iSLK, iSLK-219, 293T-219, and iSLK-BAC16 cells were kindly provided by Ren Sun (University of California, Los Angeles), Jae U Jung (University of Southern California), and Don Ganem (University of California, San Francisco). The iSLK-219 and iSLK-BAC16 were cultured in DMEM containing 10% FBS, puromycin (1 μg/mL), G418 (250 μg/mL), hygromycin (500 μg/mL), and penicillin–streptomycin. MHV-68 and MHV-68-M3FL were originally obtained from Ren Sun. R68, in which RTA is expressed as a Flag-tagged fusion protein, was described previously (49). The complementary DNA (cDNA) sequence of RNF213 was individually cloned into expression vectors to generate HA-, Flag-, Myc-, GST-, or YFP-tagged expression constructs. Plasmids pHA-RTA, pFlag-RTA and pORF48p-Luc, pRTAp-luc and pMOL were described previously (48, 49). Primer pairs used for PCR amplification of the genes are listed in *SI Appendix*, Table S2. Purified UbA1, UbcH5c and Ub proteins were kind gifts from Zusen Fan (Institute of Biophysics, Beijing, China) and were described previously (74).

Antibodies and Reagents. Rabbit polyclonal antibodies against MHV-68 RTA, MHV-68 ORF67, MHV-68 ORF69, KSHV RTA and mouse antibody against ORF33 were prepared by the Experimental Animal Center, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences. Rabbit polyclonal antibody anti-ORF26 was a kind gift from Ren Sun. A goat polyclonal antibody against RNF213 (ab58580) was purchased from Abcam. Rabbit antibodies against HA (HX1820) and Flag (HX1819) were purchased from HUAXINGBIO. Mouse antibodies against FLAG (F1804), HA (9653), MYC (M4439), YFP (MAB3580), GST (05-782), beta-actin (A5441), GAPDH (G8795), mouse M2 (FLAG)-conjugated agarose beads (A2220) and HA-conjugated agarose beads (A2095) were purchased from Sigma. MG132 and cycloheximide (CHX) were also from Sigma and were diluted in dimethyl sulfoxide. Consensus interferon (Alfacon-1) was purchased from Amgen and used at 1,000 IU/mL.

RNA and DNA Extraction and Real-Time PCR. Total RNA was extracted with TRIzol reagent (Life Technologies) according to the manufacturer's instructions. One microgram of RNA was used for reverse transcription with Genomic DNA Eraser reverse transcription kits (Takara). Quantitative real-time PCR was performed with SYBR select master mix (Life Technologies) according to the manufacturer's instructions. Total DNA was extracted by phenol–chloroform extraction, and the copy number of viral genomes was measured as described previously (49).

RNA Interference. The short hairpin RNA (shRNA) expression plasmid pSUPER-retro was used for gene expression knockdown (Oligoengine). The targeting shRNA sequence was RNF213 (BC032220.1). The scrambled shRNA was used as a negative control. The 293A cells were transfected with shRNA expression plasmids and qRT-PCR was performed to determine the effect of knockdown on RNF213.

Dual-Luciferase Reporter Assay. The Dual-luciferase reporter assay system (Promega) was used to measure promoter activities. Reporter plasmids and an RTA expression plasmid were cotransfected into 293T cells with or without an RNF213 expression plasmid. pRL-TK plasmid expressing Renilla luciferase was also included to normalize for firefly luciferase activity. The 293T cells were harvested at 36 h posttransfection, and cells were incubated with 1×passive lysis buffer provided by the manufacturer. Luciferase activities were measured according to the manufacturer's instructions.

Virus Entry Assay. 293A cells were transfected with an expression plasmid for RNF213 or the empty vector, exposed to MHV-68 at an multiplicity of infection (MOI) of 50 at 37 ℃ for 1 h to allow for virion binding and internalization, and then washed with phosphate buffered saline (PBS) for 10 times, followed by trypsin treatment for 30 min to remove uninternalized viruses. Real-time PCR using specific primers against viral genome was performed to measure the amount of internalized viruses.

Southern Blotting. Southern blotting was performed as described previously (48). Briefly, 293T cells were transfected with plasmids as indicated, and 24 h posttransfection, cells were infected with MHV-68 at a MOI of three. At 24 hpi, cells were harvested, and total cellular DNA was extracted. The DNA was digested with *Dpn* I and *Pst* I to examine the replicated plasmid DNA, and then run on a 0.8% agarose gel in 1×TAE buffer. The gel was treated with 0.25 M HCl, followed by alkaline denaturation and neutralization. DNAs were transferred onto Hybond-N+ membrane (Amersham Pharmacia) via capillary transfer in 10×SSC buffer and immobilized by UV-crosslinking. Southern blotting was carried out with DIG high prime DNA labeling and detection starter kit II (Roche) with a probe against the pGEM-T vector.

Immunoprecipitation and Western Blotting. 293T cells in 6-cm dishes were transfected with the indicated plasmids. After 24 to 36 h, cells were lysed in cell lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100 plus cocktail and phenylmethylsulfonyl fluoride) for 30 min at 4 ℃. Five percent of the cell lysate was taken as input, and the remaining was immunoprecipitated with appropriate antibodies for 4 h at 4 ℃, followed by precipitation with FLAG or HA-conjugated agarose beads. Immunoprecipitates were washed with cell lysis buffer five times and boiled in SDS-PAGE loading buffer for western blotting. Samples were separated with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore). After blocking in PBS containing 0.1% Tween-20 and 5% skim milk, the blots were probed with the indicated antibodies.

GST Pull-Down Assay. The GST pull-down assay was performed with a MagneGST pull-down system (Promega, V8870) according to the manufacturer's instructions. Briefly, plasmid pGST-RNF213 or pGST was transformed into BL21 *E. coli*, and expression of GST-RNF213 or GST was induced by the addition of 0.2 mM isopropylthiogalactoside for 16 h with continuous shaking at 16 ℃. Cells were harvested, and lysed in MagneGST Cell Lysis Reagent containing RNase-free DNase. Solubilized proteins were incubated with MagneGST particles for binding and purification. RTA protein was translated in vitro from pcDNA3.1-RTA with a TNT-coupled transcription/translation system (Promega, L1170). For GST pull-down assays, GST-RNF213 fusion protein or GST protein bound to MagneGST particles was incubated with RTA with rotation for 1 h at room temperature, followed by five washes with MagneGST Binding/Washing buffer. Bound proteins were eluted in SDS lysis buffer for western blotting.

Ubiquitination Assay. Ubiquitination assays were performed in 293T cells as described previously (75). After 24 h, cells were treated with 5 μM MG132 for 18 to 24 h and lysed with lysis buffer containing 1% SDS, incubated at 95 ℃ for 30 min, and then diluted to 0.1% SDS with lysis buffer. To detect ubiquitinated form of RTA, RTA was immunoprecipitated from diluted lysates using an anti-Flag antibody at 4 ℃ for 2 h, followed by incubation with protein G Sepharose (Santa Cruz). Immunoprecipitates were washed five times with cell lysis buffer and boiled in SDS-PAGE loading buffer for western blotting. For in vitro ubiquitination assays, E1 (His-UbA1), E2 (His-UbcH5c), His-ubiquitin, and GST-RNF213 proteins were purified from *E. coli* as described previously (74). RTA protein was expressed in vitro with a TNT-coupled transcription/translation system (Promega, L1170). The in vitro ubiquitination assays were performed by mixing E1 (His-UbA1), E2 (His-UbcH5c), E3 (GST-RNF213), His-ubiquitin, and RTA in the ubiquitination buffer (50 mM Tris-HCl, 5 mM $MgCl_2$, 2 mM dithiothreitol, and 2 mM adenosine triphosphate, pH 7.4) at 30 ℃ for 90 min.

Statistical Analysis. Unless otherwise indicated, data are presented as mean ± SEM of three independent experiments. All statistical analyses were performed with GraphPad Prism 7.0, and the statistics were analyzed by unpaired Student's *t* test. *P* values were provided as $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$.

Data, Materials, and Software Availability. All study data are included in the article and/or *SI Appendix*.

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