Human hemoglobin subunit beta functions as a pleiotropic regulator of the RIG-I/MDA5-mediated antiviral innate immune responses

Qian Yang¹#, Si-Yu Bai¹#, Lian-Feng Li¹, Su Li¹, Yuexiu Zhang¹, Muhammad Munir², Hua-Ji Qiu¹*

¹State Key Laboratory of Veterinary Biotechnology, Harbin Veterinary Research Institute, Chinese Academy of Agricultural Sciences, Harbin, China

²Division of Biomedical and Life Sciences, Faculty of Health and Medicine, Lancaster University, United Kingdom

*Corresponding author
E-mail: qiuhuaji@caas.cn or qiuhuaji@163.com (HJQ)

#These authors contributed equally to this article.
Abstract

Hemoglobin is an important oxygen-carrying protein and plays crucial roles in establishing host resistance against pathogens and regulating innate immune responses. The hemoglobin subunit beta (HB) is an essential component of hemoglobin, and we have previously demonstrated that the antiviral role of the porcine HB (pHB) is mediated by promoting the type I interferon pathways. Thus, considering the high homology between human HB (hHB) and pHB, we hypothesized that hHB also play an important role in the antiviral innate immunity. In this study, we characterized hHB as a regulatory factor for the replication of RNA viruses by differentially regulating the RIG-I- and MDA5-mediated antiviral signaling pathways. Furthermore, we showed that hHB directly inhibited the MDA5-mediated signaling through reducing the MDA5-dsRNA affinity. Additionally, hHB required hHB-induced reactive oxygen species to promote the RIG-I-mediated signaling through enhancing the K63-linked RIG-I ubiquitination. Taken together, our findings suggest that hHB is a pleiotropic regulator of the RIG-I/MDA5-mediated antiviral responses and further highlight the importance of intercellular microenvironment including redox state in regulating the antiviral innate immune responses.

Keywords: human hemoglobin subunit beta; RNA viruses; a pleiotropic regulator; RIG-I/MDA5-mediated signaling pathways; MDA5-dsRNA interaction; ubiquitination; reactive oxygen species
Hemoglobin, the most important oxygen-carrying protein, is involved in the regulation of innate immune responses. We have previously reported that the porcine hemoglobin subunit beta (HB) exerts an antiviral ability through regulating the type I interferon production. However, the antiviral activities and the underlying mechanisms of HBs originated from other animals have been poorly understood. Here, we identified human HB (hHB) as a pleiotropic regulator of the replication of RNA viruses through regulating the RIG-I/MDA5-mediated signaling pathways. hHB enhances the RIG-I mediated antiviral responses through promoting the RIG-I ubiquitination depending on the hHB-induced reactive oxygen species (ROS), while it blocks the MDA5-mediated antiviral signaling through suppressing the MDA5-dsRNA interaction. Our results contribute to understand the crucial roles of hHB in the regulation of the RIG-I/MDA5-mediated signaling pathways. We also provide a novel facet to the correlation of the intercellular redox state with the regulation of antiviral innate immunity.
Introduction

Type I interferons (IFNs) establish the first line of defense against viruses (1). They are activated in response to signaling cascades initiated by the effective interactions between the genetically encoded host pattern recognition receptors (PRRs) and pathogen-associated molecular patterns (PAMPs) (2). Viral nucleic acid is one of the well-characterized PAMPs. Depending on the genetic nature of viruses, the retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) function as the key viral RNA sensors and mediators of IFN-production (3, 4).

RIG-I and melanoma differentiation-associated gene 5 (MDA5) are major members of RLRs and contain a central DExD/H box helicase domain, which is responsible for recognizing viral RNA, and two caspase recruitment domains (CARD) at their N-terminal regions (5). Upon sensing viral RNA, RIG-I and MDA5 undergo conformational alterations and interact with the adaptor mitochondrial antiviral signaling protein (MAVS, also called IPS-1, VISA, or Cardif) through the CARD domains (6, 7). Acting as a central adaptor, MAVS initiates downstream antiviral signaling through activating the downstream IKK-α/β/γ and TBK1/IKKi kinases, resulting in the activation of NF-κB and IRF3/IRF7 to transcriptionally induce the type I IFNs (8-10).

RIG-I and MDA5 share high structural homologies and signaling features (11). However, they sense different species and natures of viral RNAs (12). It is now well established that RIG-I primarily senses the 5′-triphosphate (5′ppp)-containing viral RNAs and some specific sequence motifs in the viral
RNA, such as poly(U/UC) (13-15). In contrast to RIG-I, the characteristics of viral PAMPs sensed by MDA5 activation remain elusive. It has been proposed that MDA5 can recognize long dsRNA as well as web-like RNA aggregates (16). As countermeasures, it has been shown that RIG-I- and MDA5-mediated signaling pathways are differentially regulated (5). Some viral proteins exert different effects on the RIG-I- and MDA5-mediated pathways. For instance, the paramyxovirus V protein regulates the MDA5- but not RIG-I-mediated signaling (17). Besides viral proteins, numerous host molecules have been identified to regulate the RIG-I-mediated signaling. The ARF-like protein 16 (Arl16) and the anti-apoptotic protein A20 interact with RIG-I to inhibit antiviral responses (18, 19); cylindromatosis (CYLD) and ubiquitin specific peptidase 21 (USP21) remove K63-linked polyubiquitin chains to suppress the RIG-I-mediated signaling (20, 21), and ring-finger protein 125 (RNF125) can trigger the proteasome-mediated degradation of RIG-I (22). Several MDA5-associated host proteins have also been identified, such as ADP-ribosylation factor-like protein 5B (Arl5B) and dihydroacetone kinase (DAK) (23, 24). However, the regulatory mechanisms of these two RLRs are insufficiently understood.

Hemoglobin is the main oxygen-carrying protein in vertebrates and many invertebrates, and in adult humans, it exists as a tetramer composed of two α-chains and two β-chains (25). The old belief of hemoglobin expression indicates that hemoglobin is expressed only by the erythroid cells. However, this belief has been challenged by the recent findings that hemoglobin is expressed in a wide variety of non-erythrocytes including hepatocytes,
alveolar cells, neuronal/glia cells, and endometrial cells (26-29). Hemoglobin exerts multiple functions and plays important roles in resistance to the invasion of pathogens and the regulation of the innate immunity (30, 31). Peptides derived from hemoglobin have a great potential as therapeutic drug candidates (32, 33). However, the antiviral activities and the underlying mechanisms of hemoglobin are poorly explored. Previously, for the first time, we have corroborated that the porcine hemoglobin subunit beta (pHB) is able to suppress the growth of classical swine fever virus (CSFV) through the regulation of the RIG-I-mediated type I IFN responses (34). However, the roles of the HBs of other species in innate immunity have not yet been determined. Due to the significant amino acid homology (84.4%) between human HB (hHB) and pHB, we speculated that they have functional homologies in regulating antiviral innate immunity.

In the present study, we identified hHB as a pleiotropic regulator of the innate antiviral immunity through regulating the RIG-I/MDA5-mediated signaling pathways. We investigated the molecular mechanisms underlying the hHB-induced differential regulation of the RIG-I- and MDA5-mediated type I IFN responses in humans. Our results illustrate the importance of hHB in regulating antiviral responses and provide novel insights into functional differences in the RIG-I- and MDA5-mediated antiviral innate immunity.

Results

hHB is involved in the defense responses against RNA viruses.

To verify whether hHB modulates host antiviral responses, we first
evaluated the content of hHB in different non-erythroid cell lines. The expression analysis showed that hHB was expressed in the cell lines derived from various tissue cells, including kidney cells (HEK293T), liver cells (HepG2), lung cells (A549), cervix cells (HeLa), and peripheral blood mononuclear cells (THP-1) (Fig. 1A). To investigate the effect of hHB on the infection of RNA viruses, we generated hHB-deficient HEK293T (hHB\textsuperscript{-/-}) cells using the CRISPR/Cas9 system, resulting in efficient knockout of hHB, and overexpressed hHB in HEK293T cells (Fig. 1B). Higher viral replication was observed in hHB\textsuperscript{-/-} cells when infected with Sendai virus (SeV), vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV), but lower replication of encephalomyocarditis virus (EMCV) was exhibited in hHB\textsuperscript{-/-} cells (Fig. 1C). Accordingly, overexpression of hHB led to the resistance to SeV, VSV, or NDV infection, but enhancement of the growth of EMCV (Fig. 1D).

Taken together, these results indicate that hHB can regulate the replication of RNA viruses.

**hHB promotes the RIG-I-mediated antiviral signaling.**

RIG-I and MDA5 function as key viral RNA sensors through recognizing different viral RNA species (12). While many negative-stranded RNA viruses including SeV, VSV, and NDV are mainly sensed by RIG-I, the viral RNA of picornaviruses such as EMCV is almost exclusively recognized by MDA5 (12). In conjunction to aforesaid results, hHB exerted an antiviral function against SeV, VSV, and NDV. However, hHB appeared to be beneficial for the replication of EMCV. Therefore, we speculated that hHB may differentially
regulate the RIG-I- and MDA5-mediated signaling pathways to modulate antiviral immune responses. We first explored the contribution of hHB in the RIG-I-mediated signaling and noticed that overexpression of hHB significantly enhanced the RIG-I-induced activations of the IFN-β promoter and ISRE, which was saturated at high concentrations (0.7-0.8 μg) (Fig. 2A and B). Consistently, hHB also promoted the IFN-β promoter activation in the cells stimulated with cytoplasmic short poly(I:C), which is a specific ligand of RIG-I (Fig. 2C). In addition, overexpression of hHB upregulated the RIG-I- or short poly(I:C)-induced transcriptions of IFN-β, and IFN-stimulated genes including GBP1 and ISG56 (Fig. 2D–F).

We also determined the impact of hHB knockout on the RIG-I-mediated signaling pathway. Dual-luciferase reporter assay indicated that RIG-I- and short poly(I:C)-induced activations of the IFN-β promoter and ISRE were significantly impaired in hHB−/− cells (Fig. 2G and H). Correspondingly, lower mRNA levels of IFN-β, GBP1, and ISG56 were observed in the RIG-I- or short poly(I:C)-treated hHB−/− cells (Fig. 2I–K). These results highlight the involvements of RIG-I in hHB-mediated regulation of type I IFNs. To exclude the possibility that hHB can activate the IFN-β transcription by itself, we investigated the effect of the hHB-overexpression on the activation of the IFN-β promoter. The luciferase measurements showed that hHB was unable to activate the IFN-β promoter in HEK293T cells by itself (Fig. 2L).

**hHB inhibits the MDA5-mediated antiviral signaling.**

We next investigated the contribution of hHB in MDA5-mediated type I
IFN signaling. We examined the activation of IFN-β promoter and ISRE in hHB and MDA5 cotransfected HEK293T cells. The results demonstrated that overexpression of hHB significantly decreased the MDA5-induced IFN-β promoter and ISRE activation in a dose-dependent manner (Fig. 3A and B). The suppressive effects of hHB on IFN-β promoter activation were also evident in the cells stimulated with the long poly(I:C), which is a specific ligand of MDA5 (Fig. 3C). Overexpression of hHB also inhibited the transcriptions of IFN-β, GBP1, and ISG56 induced by MDA5- or long poly(I:C) (Fig. 3D–F).

To further verify the contribution of hHB in MDA5-mediated signaling, we examined the impact of hHB-knockout on the IFN-β transcription. We observed that the activations of IFN-β promoter and ISRE and the transcription of IFN-β, GBP1, and ISG56 in response to long poly(I:C) or MDA5 stimulation were significantly higher in the hHB−/− cells compared with that in the wild-type HEK293T (WT) cells (Fig. 3G–K). Based on these findings, it is plausible that hHB can distinctly regulate IFN-β production through the MDA5- and RIG-I-mediated signaling pathways.

**hHB regulates the replication of RNA viruses through the RIG-I- and MDA5-mediated signaling pathways.**

We further verified the contribution of hHB-mediated regulation of the RIG-I- and MDA5-mediated signaling to the replication of RNA viruses. We generated MDA5-deficient HEK293T (MDA5−/−) and RIG-I-deficient HEK293T (RIG-I−/−) cells and overexpressed hHB in the MDA5−/− and RIG-I−/− cells (Fig.
to investigate the relevance of hHB and the replication of RNA viruses in these cells. The results showed that overexpression of hHB led to the resistance against SeV, VSV, or NDV in the MDA5−/− cells but enhanced the replication of these viruses in the RIG-I−/− cells (Fig. 4B and C). Moreover, although hHB also promoted the replication of EMCV in the RIG-I−/− cells, it displayed little impact on the replication of EMCV in the MDA5−/− cells (Fig. 4B and C).

In order to further assess the combinatorial impacts of RLR and hHB on viruses, we next generated double knockout cells deficient in MDA5 and hHB (MDA5−/−/hHB−/−) or RIG-I and hHB (RIG-I−/−/hHB−/−) (Fig. 4D). MDA5−/−/hHB−/− cells showed higher viral replication when infected with SeV, VSV, or NDV compared with MDA5−/− cells, but exhibited similar replication level of EMCV with MDA5−/− cells (Fig. 4E). Moreover, deficiency of hHB in RIG-I−/− cells led to the resistance to SeV, VSV, NDV, or EMCV infection (Fig. 4F). Taken together, these results indicate that hHB can differently regulate the defense response of host cells to RNA viruses through the RIG-I- and MDA5-mediated antiviral signaling pathway.

hHB has no effect on the expression of RIG-I or MDA5.

After establishing the relationship between hHB and the MDA5/RIG-I pathways in regulating IFN-β, we explored whether hHB affects the expression of RIG-I or MDA5. HEK293T cells cotransfected with hHB and either RIG-I or MDA5 were used to monitor the expression of RIG-I or MDA5. The results showed that overexpression of hHB failed to alter the exogenous
protein expression of RIG-I and MDA5 at all the tested gradient doses (Fig. 5A and B). In addition, the mRNA levels of RIG-I and MDA5 remained unchanged upon overexpression of hHB (Fig. 5C and D). There was also no difference between the mRNA levels of RIG-I or MDA5 in the hHB⁻/⁻ cells and the WT cells (Fig. 5E). Finally, we demonstrated that the endogenous expression of RIG-I and MDA5 remained unaffected in hHB-overexpressing cells (Fig. 5F). Correspondingly, there are no differences observed in the endogenous expression of RIG-I or MDA5 in the hHB⁻/⁻ cells and the WT cells (Fig. 5G).

**hHB interferes with the MDA5-dsRNA interaction and enhances the ubiquitination of RIG-I.**

The results presented so far clearly articulate the involvement of RIG-I/MDA5 in mediating the hHB-dependent regulation of type I IFN pathway without affecting the RIG-I/MDA5 protein expression. We next mechanistically investigated whether hHB affects the functions of these RLRs. Previous studies have demonstrated that sensing different types of viral RNA is required for the activation of MDA5 and RIG-I to initiate their signal transductions (35-37). RIG-I and MDA5 also recognize short poly(I:C) and long poly(I:C) as the synthetic dsRNA analogues respectively (38). Thus, we examined whether hHB affected the interaction between RIG-I or MDA5 and dsRNA using short or long poly(I:C)-binding assay. The results showed that RIG-I interacted with short poly(I:C) independent of hHB (Fig. 6A). However, overexpression of hHB significantly inhibited the interaction of MDA5 with
long poly(I:C) (Fig. 6B), and long poly(I:C) interacted with MDA5 more effectively in the hHB+ cells than in the WT cells (Fig. 6C), indicating that hHB acts as a repressor of MDA5 activation by inhibiting the MDA5-dsRNA interaction. Since RLRs interact with dsRNA and hHB interferes with this activity of MDA5, we next determined the interaction between hHB and dsRNA. Interestingly, hHB was precipitated with long poly(I:C) but not with short poly(I:C) (Fig. 6D). Due to the association of hHB with dsRNA and the involvement of hHB in MDA5-dsRNA interaction, we were interested to investigate if the interaction with the dsRNA occurs at the interface of hHB and MDA5. Co-immunoprecipitation analysis indicated no identifiable interaction of hHB with either MDA5 or RIG-I (Fig. 6E). These results imply the possibility that the hHB binding to dsRNA may compete for the interaction of MDA5 with the dsRNA ligand and this competition may result in the reduction of IFN-β induction.

Upon interacting with the dsRNA ligand, the ubiquitination of RIG-I or MDA5 occurs before recruitment to the mitochondria-associated membrane and binding to MAVS (39). It suggested that the ubiquitination of RIG-I and MDA5 is crucial for the activation of RLRs signaling. Thus, we evaluated the ubiquitination of RIG-I and MDA5 upon hHB overexpression. Based on the disruption of MDA5-dsRNA interaction by hHB, the ubiquitination of MDA5 was certainly suppressed by hHB in a dose-dependent manner (Fig. 6F). However, the ubiquitination of RIG-I was potentiated by increasing hHB protein expression (Fig. 6G). It has been shown that RIG-I has different ubiquitination forms and the K63-linked ubiquitination of RIG-I is positively
required for RIG-I activation, whereas the K48-linked ubiquitination will result in the destabilization of RIG-I (22, 40). To verify if hHB promotes RIG-I activation through potentiating RIG-I ubiquitination, we constructed two ubiquitin mutants in which all lysine residues were replaced with arginine except K48 or K63 (HA-K48Ub or HA-K63Ub). The results showed that hHB enhanced the K63-linked but not the K48-linked RIG-I ubiquitination (Fig. 6H). Moreover, the total ubiquitination of RIG-I and K63-linked RIG-I ubiquitination were lower in hHB\(^{-}\) cells than that in WT cells (Fig. 6I). In addition, to uncover whether the action of hHB is characteristic or non-characteristic, we also examined whether the alpha subunit of hemoglobin (hHA) has similar effects on the activation of RIG-I or MDA5. The results showed that both the RIG-I-short poly(I:C) and the MDA5-long poly(I:C) interactions were independent of hHA (Fig. 6J). Furthermore, the ubiquitination of MDA5 remained unchanged by hHA (Fig. 6K), but the ubiquitination of RIG-I was enhanced by hHA in a dose-dependent manner (Fig. 6L). Moreover, hHA could promote the short poly(I:C)-induced transcription of IFN-β but not the long poly(I:C)-induced transcription of IFN-β (Fig. 6M). These data imply that the inhibition of the MDA5-dsRNA interaction is a characteristic of hHB.

Reactive oxygen species (ROS) is required for the hHB-induced upregulation of the RIG-I signaling pathway.

Considering that hemoglobin is able to regulate the production of ROS, which is a key factor for the host cell to trigger an efficient activation of immunity (41-43), we evaluated if ROS was involved in the hHB-mediated
regulation of RIG-I or MDA5 signaling pathway. So, we first explored the links among hHB, ROS, and virus infections. hHB was overexpressed in HEK293T cells and ROS production was monitored by using the oxidant-sensitive fluorescent detection probe DCFH-DA. The results demonstrated that the hHB increased intracellular ROS accumulation in a dose dependent manner (Fig. 7A). In addition, the intracellular ROS accumulation was also upregulated by SeV in a dose-dependent manner (Fig. 7B). Thus, we also monitored the effect of hHB on the ROS accumulation in SeV-infected cells. The overexpression of hHB promoted the ROS production in a dose dependent manner at the early time of SeV infection (Fig. 7C). However, the hHB-overexpressed cells showed lower intracellular ROS accumulation at the later time of SeV infection as the replication of SeV was suppressed (Fig. 7D). Consistently, SeV induced lower ROS production in hHB−/− cells at the earlier time compared with the WT cells, but the hHB−/− cells accumulated more intracellular ROS at the later time of SeV infection (Fig. 7E).

Next, we investigated if ROS is required for the hHB-mediated regulation of the MDA5 signaling pathway. As shown in Fig. 8A and B, MDA5- or RIG-induced IFN-β transcription was significantly reduced by tempol (a ROS inhibitor). Moreover, hHB still suppressed long poly(I:C)-induced activation of IFN-β promoter in the presence of tempol (Fig. 8C). Accordingly, tempol treatment could not counteract the inhibition of the MDA5- or long poly(I:C)-induced transcription of IFN-β by hHB overexpression (Fig. 8D). To exclude the unspecific effects of tempol and the disturbance of the RIG-I-mediated signaling pathway, two other ROS inhibitors,
diphenyleneiodonium chloride (DPI) and N-acetyl-L-cysteine (NAC) were also tested in the RIG-I<sup>−/−</sup> cells. DPI or NAC treatment also had no effect on the hHB-mediated inhibition of the MDA5- or long poly(I:C)-induced transcription of IFN-β in the RIG-I<sup>−/−</sup> cells (Fig. 8E). However, hHB-induced upregulation of the IFN-β promoter activation in response to short poly(I:C) was inhibited by tempol (Fig. 8F). Tempol also suppressed the hHB-induced upregulation of the RIG-I- or short poly(I:C)-induced transcription of IFN-β (Fig. 8G). Moreover, DPI or NAC also obviously counteracted the hHB-mediated facilitation of RIG-I- or short poly(I:C)-induced IFN-β transcription in the MDA5<sup>−/−</sup> cells (Fig. 8H). Thus, we evaluated the effects of hHB on the ubiquitination of RIG-I upon tempol treatment. Consistent with previous findings, when ROS was suppressed, the ubiquitination of RIG-I was no longer enhanced by hHB (Fig. 8I). To further verify the role of ROS in the hHB-mediated regulation of RIG-I activation, we investigated the effects of hHB on the K63-linked RIG-I ubiquitination when the ROS was suppressed. The results showed that the K63-linked ubiquitination of RIG-I was no longer enhanced by hHB when the ROS accumulation was inhibited (Fig. 8J). These implicate that the upregulation of the RIG-I signaling by hHB is probably related to the hHB-induced ROS, whereas hHB regulates the MDA5 signaling in an alternative way.

**Discussion**

Generally, RIG-I and MDA5, the cytoplasmic RNA helicase proteins, are the main sensors of RNA viruses in triggering type I IFNs in eukaryotes (3, 4).
Exploring the molecular events of the RIG-I/MDA5 signaling pathway is critical for understanding the complex innate immune responses against RNA viruses. In this study, we identified hHB as a novel innate immune regulator of the RIG-I/MDA5-mediated antiviral signaling pathways, which further advances our understanding of the regulatory mechanisms involved in the RLRs-mediated signaling pathways.

The activation of RIG-I or MDA5 is a complex regulatory process, including viral RNA binding, structural rearrangement, dephosphorylation, ubiquitination, and binding to MAVS to activate the downstream antiviral signaling (44-46). Despite functional overlaps between the RIG-I and MDA5 pathways, our results showed that hHB could differently regulate these signaling pathways. RIG-I and MDA5 recognize differential viral RNAs (12). In our study, hHB significantly inhibited the replication of SeV, VSV, and NDV, which are mainly sensed by RIG-I, but enhanced the growth of EMCV which almost only activate MDA5-mediated signaling (Figs. 1 and 4). Moreover, we showed that hHB promoted RIG-I signaling and remarkably inhibited MDA5-mediated type I IFN production (Figs. 2 and 3). Therefore, it is plausible that hHB differently regulates RIG-I and MDA5 activation in the upstream of the MAVS-mediated signaling. RIG-I and MDA5 share a similar structural framework implicated in the viral dsRNA recognition and detection of short and long poly(I:C) as the synthetic dsRNA analogues, respectively (38, 47). Although the RIG-I recognition of viral RNA has been mostly clarified, how MDA5 recognizes viral RNA is yet to be determined. Our results demonstrated that hHB had no obvious influence on the binding of short
poly(I:C) to RIG-I, but hHB acted as a direct repressor of MDA5 by interfering with the interaction between MDA5 and long poly(I:C) (Fig. 6B and C). In addition, hHB could bind to the long poly(I:C) but it failed to interact with short poly(I:C) or MDA5 and RIG-I (Fig. 6D–F). These data imply that hHB-dsRNA may compete with the MDA5-dsRNA interaction and thus negatively regulates the MDA5-mediated IFN pathway. Moreover, ubiquitination plays a critical role in the regulation of RIG-I and MDA5 activation (3). The E3 ubiquitin ligases TRIM25-catalyzed K63-linked ubiquitination of RIG-I and the TRIM65-catalyzed K63-linked ubiquitination of MDA5 positively regulate RIG-I- and MDA5-mediated signaling pathways, respectively (40, 48). In the present study, we found that hHB promoted the K63-linked ubiquitination of RIG-I, whereas it inhibited the MDA5 ubiquitination (Fig. 6G–I). Interestingly, our results showed that hHA had a similar effect on the activation of RIG-I that it promotes the RIG-I ubiquitination after RNA virus infection. These findings suggest that the action of hHB on the MDA5-dsRNA interaction is characteristic, but the ability of hHB to facilitate the activation of RIG-I may owe to the general characteristic of hemoglobin.

Hemoglobin is the main respiratory protein in vertebrates and many invertebrates. It exerts multiple functions and plays an important role in resistance to pathogens invasion (31). Besides functioning as a major host respiratory protein, hemoglobin also can be specifically activated by pathogens to produce ROS to constitute a part of the host defense strategy (41, 49, 50). For example, human hemoglobin significantly enhances the ROS production under microbial proteases stimulation but not the host
proteases stimulation (51). ROS plays a key role in immunity and pathogen-killing (52-54). The host respiratory proteins directly exploit the invasion of microbes to produce ROS, resulting in localized cytotoxicity to rapidly kill the neighboring pathogens (51, 55). Recently, the association of ROS with the RLRs signaling has been reported. The host cell requires ROS to efficiently trigger the RIG-I-mediated IRF3 activation and IFN-β expression (56). This implicates that ROS may provide a mediator for hHB to be involved the regulation of the RIG-I signaling. Here, we demonstrated that hHB increases the ROS level in cells and the antioxidant inhibitors including tempol, DPI, and NAC could counteract the hHB-mediated upregulation of the RIG-I-mediated signaling pathway, but could not eliminate the effects of hHB on MDA5 signaling (Figs. 8). Moreover, inhibition of ROS by tempol suppresses the hHB-mediated facilitation of RIG-I ubiquitination, especially the K63-linked ubiquitination of RIG-I. These certify that ROS is required for the hHB-mediated regulation of RIG-I ubiquitination, which indirectly promotes the activation of the RIG-I signaling.

The innate immune system has evolved various strategies to prevent harmful overproduction of type I IFNs during viral infection. Thus, several host molecules are capable of regulating the type I IFN production via multiple pathways, including the RIG-I/MDA5 signaling pathway. For example, DAK is a specific repressor of the MDA5-mediated signaling and the deubiquitinating activity of A20 inhibits the RIG-I-mediated signaling (19, 23). It is also worth noticing that several host factors adopted multiple ways to regulate the RIG-I/MDA5 signaling. For example, IFN-β levels are increased following
stimulation with activators of the RIG-I signaling in protein kinase R (PKR)-null cells and the absence of PKR severely impairs the MDA5-mediated IFN induction (57). In this study, we identified that hHB was a pleiotropic regulator of the RIG-I/MDA5-mediated signaling pathway. Moreover, hHB could affect RIG-I/MDA5 signaling in a direct or indirect manner. hHB directly inhibits the binding of MDA5 to dsRNA and negatively regulates the MDA5-mediated IFN production (Fig. 9). On the other hand, hHB is involved in the regulation of cellular oxidative stress to enhance the RIG-I ubiquitination, which indirectly promotes the RIG-I-mediated IFN production (Fig. 9). These findings imply that hHB contributes to the safeguard mechanisms needed for controlling the RLR signaling pathway.

Currently, many regulators have been identified to have direct effects on a single point or a single pathway of antiviral innate immunity (58-60). For these regulators, the direct effects are appreciated and emphatically studied. However, their indirect impacts on intercellular microenvironment, such as redox state, pH, and ion leakage, are relatively ignored. In the present study, although hHB can directly inhibit type I IFN production through interfering with the MDA5-mediated signaling pathway, hHB-induced change of the intercellular redox state will concurrently impede this inhibition through promoting the RIG-I-mediated signaling pathway. This reveals the importance of intercellular microenvironment in the regulation of antiviral innate immunity and implies the reason why the effects of some regulators are always fluctuating in response to the nature of stimuli. Owing to these indirect effects of hHB through influencing cellular microenvironment, the hHB-mediated
innate immune regulation may be dependent on the cellular state and stimulus types and so on, rather than only dependent on its expression level. Therefore, future work is required to further understand the regulation mechanisms of antiviral innate immunity and to improve the effectiveness of some regulators.

In summary, we identified hHB as a novel innate immune regulator of RNA viruses through multifunctional and pleiotropic regulation of the RIG-I/MDA5 signaling pathways. On one hand, hHB promoted the RIG-I-mediated signaling pathway by enhancing the RIG-I ubiquitination. On the other hand, hHB remarkably inhibited MDA5-mediated type I IFN production through interfering with the MDA5-dsRNA interaction. We mechanistically illustrated the crucial roles of hHB in regulation and safeguard needed for antiviral innate immunity. Our findings also highlight the importance of the intercellular microenvironment such as redox state in the regulation of antiviral innate immunity and thus warrant future studies to fully explore the complex induction of innate immunity.

Materials and methods

Cells, viruses, and plasmids.

HEK293T (ATCC® CRL-3216™) cells, a human embryonic kidney cell line, and BHK-21 (ATCC® CCL-10), a baby hamster Syrian kidney cell line, obtained from the American Type Culture Collection (ATCC), were cultured in Dulbecco’s modified Eagle’s medium (DMEM) with 10% fetal bovine serum (FBS). SeV and NDV-GFP were propagated in SPF chicken embryos and
titrated in chicken red blood cells by hemagglutination assay. EMCV and VSV-GFP were propagated in BHK-21 cells. The p3\times\text{Flag}-hHB plasmid encoding the hHB protein (GenBank accession no. NM_000518.4) with the 3\times\text{Flag} tag at its N-terminus was constructed by cloning the hHB cDNA into the p3\times\text{Flag}-CMV-10 vector (Sigma-Aldrich). The plasmids pEGFP-RIG-I and pEGFP-MDA5 encoding the RIG-I and MDA5 proteins, respectively, with a GFP tag at the C-terminus were constructed by cloning the human RIG-I, MDA5, and MAVS cDNA into the pEGFP-C1 vector (Clontech Laboratories).

pEF-Myc-RIG-I and pEF-Myc-MDA5 encode the human RIG-I and MDA5, respectively.

**Generation of hHB\textsuperscript{−/−}, -RIG-I\textsuperscript{−/−}, and -MDA5\textsuperscript{−/−} cells using HEK293T cells.**

We generate hHB\textsuperscript{−/−}, -RIG-I\textsuperscript{−/−}, and -MDA5\textsuperscript{−/−} cells using the lentiviral expressing CRISPR-Cas9 vector (lentiCRISPRv2, Addgene). The hHB-, RIG-I-, and MDA5-specific sgRNA sequences were: hHB (forward, 5′-GTA ACG GCA GAC TTC TCC TC-3′; reverse, 5′-GAG GAG AAG TCT GCC GTT ACC-3′), RIG-I (forward, 5′-GGG TCT TCC GGA TAT AAT CC-3′; reverse, 5′-GGA TTA TAT CCG GAA GAC CCC-3′), and MDA5 (forward, 5′-CGA ATT CCC GAG TCC AAC CA-3′; reverse, 5′-TGG TTG GAC TCG GGA ATT CGC-3′), respectively. Lenti-CRISPR virions were packaged in HEK293T cells by transfecting the psPAX2 plasmid (Addgene), the pMD2.G plasmid (Addgene) and either the lentiCRISPRv2 vector containing hHB, RIG-I, or MDA5-specific sgRNA, or empty lentiCRISPRv2 plasmid as a control. The suspensions were harvested at 72 hours posttransfection (hpt).
HEK293T cells were infected with the suspensions and treated with 1.5 μg/ml puromycin for 5 d. The cells were lysed and the hHB, RIG-I, or MDA5 expression was analyzed by Western blotting.

**Dual-luciferase reporter assay.**

HEK293T cells were cotransfected with the IFN-β promoter or ISRE firefly luciferase (FLuc) reporter plasmid (pIFN-β-FLuc or pISRE-FLuc), the indicated amounts of p3×Flag-hHB, and internal reference reporter TK-Renilla luciferase (RLuc) as an internal control (pRLuc-TK). The total amounts of the plasmid DNAs were equalized with the empty control vector p3×Flag-CMV-10 (p3×Flag-EV). At 24 hpt, the cells were infected with SeV or PBS for another 24 h. Then cells were lysed and the activities of the reporter genes were determined using a Dual-Luciferase® Reporter Assay System 10-Pack (Promega). The luciferase induction mediated by IFN-β promoter (IFN-β-Luc) or ISRE (ISRE-Luc) was presented as relative expression level of FLuc/RLuc. For the RIG-I- or MDA5-mediated response, HEK293T cells were cotransfected with pIFN-β-FLuc/pISRE-FLuc and pRLuc-TK as well as pMyc-RIG-I, pMyc-MDA5, short poly(I:C) (catalog no. tlrl-picw; InvivoGen), or long poly(I:C) (catalog no. tlrl-pic; InvivoGen). The luciferase activities were measured at 24 hpt and relative expressions were calculated as described above.

**Real-time RT-PCR.**

Total RNA was extracted from HEK293T cells using the TRIzol reagent
(catalog no. 15596026; Invitrogen). RNAs were converted to cDNA using Reverse Transcriptase XL (catalog no. 2621; TaKaRa). The transcription levels of IFN-β, RIG-I, and MDA5 in hHB-treated or untreated HEK293T cells with or without SeV infection were quantified by the $2^{-\Delta\Delta C_T}$ Method (61). The mRNA level of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was set as an internal loading control. Primers used for the real-time RT-PCR are listed in Table 1.

**ROS production assay.**

Total ROS production was measured by probing with the 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA) probe (catalog no. S0033; Beyotime Biotechnology) and was evaluated using Enspire Multimode Plate Reader (Perkin Elmer). The HEK293T cells were transfected with p3×Flag-hHB at the indicated amounts. The total amount of the plasmid DNA was equalized with the p3×Flag-EV. At 24 hpt, the cells were infected with SeV or treated with PBS for 24 h. Then the cells were washed with PBS and incubated with the DCFH-DA probes for 30 min at 37°C. After incubation, the cells were washed twice with PBS and the 2',7'-dichlorofluorescein (DCF) fluorescence was measured.

**Western blotting.**

Equivalent amount of each sample was resolved by SDS-PAGE and transferred to nitrocellulose membranes (Hybond-C Super; GE Healthcare). The membranes were blocked with 5% skim milk in PBS containing 0.1%
Tween (PBST) and incubated for 2 h at room temperature with the primary antibodies at an appropriate dilution (anti-Flag, -Myc, -MDA5, and -RIG-I MAbs at 1:1,000 and anti-hHB MAb at 1:500) (catalog nos. F1804 and M4439; Sigma-Aldrich and catalog nos. sc-134513, sc-48932, and sc-22718; Santa Cruz). The membranes were washed by PBST and then incubated with IRDye® 800CW goat anti-mouse IgG (H+L), donkey anti-goat IgG (H+L), and goat anti-rabbit (H+L) (catalog nos. 926-32210, 926-32214, and 926-32211; LiCor BioSciences) or goat anti-mouse IgG-peroxidase (catalog no. A5278; Sigma) at 1:10,000 for 1 h at 37°C, and the blots were scanned using the Odyssey infrared imaging system (LiCor BioSciences) or Fluorescence/Chemiluminescence imaging system (Clinx Science instruments).

**Coimmunoprecipitation (Co-IP) assay.**

HEK293T cells were transfected with p3×Flag-hHB together with pMyc-RIG-I or pMyc-MDA5. At 48 hpt, the cells were lysed with NP-40 buffer (catalog no. P0013F; Beyotime) with 1 mM phenylmethanesulfonyl fluoride (PMSF) at 4°C for 30 min. The supernatants were collected and precleared with protein G-agarose (catalog no. 11243233001; Roche) for 2 h at 4°C. Then the mixtures were centrifuged and the supernatants were incubated with an anti-Flag M2 affinity gel (catalog no. A2220; Sigma-Aldrich) overnight at 4°C. The gels were washed with the NP-40 buffer and the precipitated proteins were tested by Western blotting analysis.
Poly(I:C) binding assay.

HEK293T cells were transiently transfected with pMyc-MDA5 or pMyc-RIG-I and p3×Flag-hHB plasmids and lysed with NP-40 buffer containing Recombinant RNase Inhibitor (RRI) (catalog no. 2313A; TaKaRa). Poly(I:C) was labeled with photobiotin (catalog no. A14216; Baomanbio) using a mercury vapor lamp. The cell lysates were incubated with the labeled poly(I:C) for 4 h at 4°C. Then the biotinylated RNA-protein compounds were precipitated with Dynabeads® Streptavidin (catalog no. 11205D; Invitrogen) for 1 h at room temperature using gentle rotation. After washing with PBS, the bound proteins were analyzed by Western blotting analysis.

Statistical analysis.

All experiments were performed with at least three independent replicates. Results were analyzed by SPSS 18.0 software using Student’s t test. P < 0.05 was considered to be statistically significant.

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References


**Figure legends**

**Fig. 1. hHB is involved in the antiviral responses to RNA viruses.** (A) The expression of hHB in different non-erythroid cell lines including HEK293T, HepG2, A549, HeLa, and THP-1. (B) The knockout efficiency of hHB in hHB-deficient HEK293T (hHB−/−) cells compared with the expression level of hHB in the wild-type HEK293T (WT) and the overexpression of hHB in HEK293T cells. (C) hHB−/− cells were more sensitive to Sendai virus (SeV), vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) but more resistant to encephalomyocarditis virus (EMCV). hHB−/− and WT cells were infected with SeV or EMCV for 24 h and the RNA level of the SeV N protein or the EMCV 3D protein was determined by real-time RT-PCR. In addition, hHB−/− and WT cells were infected with VSV-GFP or NDV-GFP for 48 h. The GFP expression level was analysed by Western blotting. (D) Overexpression of hHB inhibited the replications of SeV, VSV, and NDV but enhanced EMCV growth. HEK293T cells were transfected with p3×Flag empty vector (p3×Flag-EV) or p3×Flag-hHB for 24 h and then infected with SeV or EMCV for 24 h or infected with VSV-GFP or NDV-GFP for 48 h. The RNA level of the
SeV N protein or the EMCV 3D protein or the GFP expression of VSV-GFP or NDV-GFP was tested. The data represent the mean ± standard deviation from three independent experiments. Significant differences are denoted by * (P < 0.05), ** (P < 0.01), or *** (P < 0.001).

Fig. 2. hHB enhances the RIG-I-mediated antiviral signaling. (A–C) Overexpression of hHB upregulated RIG-I-mediated activation of the IFN-β promoter and ISRE. HEK293T cells were cotransfected with the indicated amounts of p3×Flag-hHB (hHB), pMyc-RIG-I, TK-Renilla luciferase (RLuc) internal reference reporter plasmid (pRLuc-TK), and firefly luciferase (FLuc) reporter plasmid (pIFN-β-FLuc) (A) or ISRE firefly luciferase reporter plasmid (pISRE-FLuc) (B) for 24 h. Additionally, HEK293T cells were cotransfected with pRLuc-TK, pIFN-β-FLuc, short poly(I:C), and the indicated amounts of p3×Flag-hHB for 24 h (C). The activation of the IFN-β promoter or ISRE was presented as the activities of the luciferase reporter genes induction mediated by the IFN-β promoter (IFN-β-Luc induction) or ISRE (ISRE-Luc induction) and calculated as the relative levels of FLuc/RLuc. (D–F) Overexpression of hHB enhanced the transcription of IFN-β, GBP1, and ISG56. HEK293T cells were cotransfected with p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-RIG-I or short poly(I:C) for 24 h and the IFN-β (D), GBP1 (E), and ISG56 (F) mRNA levels in cells were analyzed using real-time RT-PCR. (G and H) The RIG-I mediated lower activation of the IFN-β promoter and ISRE in hHB−/− cells. hHB−/− and WT cells were cotransfected with pMyc-RIG-I or short poly(I:C) as well as pRLuc-TK and pIFN-β-FLuc (G) or pISRE-FLuc (H). At 24 hpt, the
activation of IFN-β promoter or ISRE was tested. (I–K) The RIG-I mediated lower transcription of IFN-β, GBP1, and ISG56 in hHB+/ cells. hHB−/−, and WT cells were transfected with pMyc-RIG-I or short poly(I:C). At 24 hpt, the IFN-β (I), GBP1 (J), and ISG56 (K) mRNA abundances were tested. (L) The effect of hHB on the IFN-β promoter activation. HEK293T cells were cotransfected with the indicated amounts of p3×Flag-hHB, pRLuc-TK, and pIFN-β-FLuc for 24 h. The IFN-β promoter activation was tested as described above. The data represent the mean ± standard deviation from three independent experiments. Significant differences are denoted by * (P < 0.05), ** (P < 0.01), or *** (P < 0.001). NS, not significant (P > 0.05).

Fig. 3. hHB inhibits the MDA5-mediated antiviral signaling. (A–C) Overexpression of hHB suppressed MDA5-mediated activation of the IFN-β promoter and ISRE. HEK293T cells were cotransfected with the indicated amounts of p3×Flag-hHB, pMyc-MDA5, pRLuc-TK, and pIFN-β-FLuc (A) or pISRE-FLuc (B) for 24 h. Additionally, HEK293T cells were cotransfected with pRLuc-TK, pIFN-β-FLuc, long poly(I:C), and the indicated amounts of p3×Flag-hHB for 24 h (C). The effect of hHB on the activation of IFN-β promoter or ISRE was examined as described above. (D–F) Overexpression of hHB decreased the transcription of IFN-β, GBP1, and ISG56. HEK293T cells were transfected with the p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-MDA5 or long poly(I:C) for 24 h and the IFN-β (D), GBP1 (E), and ISG56 (F) mRNA levels in cells were analyzed. (G and H) The MDA5 mediated higher activation of the IFN-β promoter and ISRE in hHB+/ cells.
hHB\textsuperscript{-} and WT cells were cotransfected with pMyc-MDA5 or long poly(I:C) as well as pRLuc-TK and pIFN-\(\beta\)-FLuc (G) or pISRE-FLuc (H). At 24 hpt, the activation of IFN-\(\beta\) promoter or ISRE was analyzed. (I–K) The MDA5 mediated higher transcription of IFN-\(\beta\), GBP1, and ISG56 in hHB\textsuperscript{-} cells. hHB\textsuperscript{-} and WT cells were transfected with pMyc-MDA5 or long poly(I:C). At 24 hpt, the IFN-\(\beta\) (I), GBP1 (J), and ISG56 (K) mRNA abundances were tested. The data represent the mean ± standard deviation from three independent experiments. Significant differences are denoted by * (\(P \leq 0.05\)), ** (\(P \leq 0.01\)), or *** (\(P \leq 0.001\)). NS, not significant (\(P > 0.05\)).

**Fig. 4.** hHB regulates the RIG-I- and MDA5-mediated antiviral response to RNA viruses. (A) The knockout efficiency of RIG-I and MDA5 in HEK293T cells, and overexpression of hHB in MDA5-deficient HEK293T (MDA5\textsuperscript{-}/-) and RIG-I-deficient HEK293T (RIG-I\textsuperscript{-}/-). (B and C) The effect of hHB-overexpression on the replications of SeV, VSV, NDV, and EMCV in MDA5\textsuperscript{-}/- and RIG-I\textsuperscript{-}/- cells. MDA5\textsuperscript{-}/- (B) or RIG-I\textsuperscript{-}/- (C) cells were transfected with p3\times\text{Flag}\text{-}hHB for 24 h and then were infected with SeV or EMCV for 24 h or infected with VSV-GFP or NDV-GFP for 48 h. The RNA level of the SeV N protein or the EMCV 3D protein or the GFP expression level of VSV-GFP or NDV-GFP was tested. (D) The knockout efficiency of RIG-I and MDA5 in hHB\textsuperscript{-}/- cells. (E and F) Deficiency of hHB differently affected the replication of SeV, VSV, NDV, and EMCV in RIG-I\textsuperscript{-}/- and MDA5\textsuperscript{-}/- cells. MDA5\textsuperscript{-}/-/hHB\textsuperscript{-}/- cells and MDA5\textsuperscript{-}/- (E) or RIG-I\textsuperscript{-}/-/hHB\textsuperscript{-}/- and RIG-I\textsuperscript{-}/- cells (F) were infected with SeV or EMCV for 24 h or infected with VSV-GFP or NDV-GFP for 48 h. Then the
RNA level of the SeV N protein or the EMCV 3D protein or the GFP expression level of VSV-GFP or NDV-GFP was measured as above. The data represent the mean ± standard deviation from three independent experiments. Significant differences are denoted by * (P < 0.05), ** (P < 0.01), or *** (P < 0.001). NS, not significant (P > 0.05).

Fig. 5. hHB does not affect RIG-I or MDA5 expression. (A and B) Overexpression of hHB had no effects on the exogenous protein expression of RIG-I or MDA5. HEK293T cells were cotransfected with the indicated amounts of p3×Flag-hHB and pMyc-RIG-I or pMyc-MDA5 for 24 h and the RIG-I (A) and MDA5 (B) expression was analyzed by Western blotting. (C and D) Overexpression of hHB did not affect the RIG-I and MDA5 transcription. HEK293T cells were transfected with the indicated amounts of p3×Flag-hHB (hHB) for 24 h and then RIG-I (C) and MDA5 (D) mRNA levels in cells were analyzed using real-time RT-PCR. (E) The mRNA levels of RIG-I and MDA5 exhibited no difference between the hHB−/− and WT cells. The mRNA levels of RIG-I and MDA5 in the cells were analyzed using real-time RT-PCR. (F) The endogenous protein expression of RIG-I and MDA5 had no change in the hHB-overexpression cells. HEK293T cells were transfected with the indicated amounts of p3×Flag-hHB for 48 h and the RIG-I and MDA5 expression was analyzed by Western blotting analysis. (G) The endogenous protein expression of RIG-I and MDA5 in the hHB−/− and WT cells was examined by Western blotting analysis. NS, not significant (P > 0.05).
Fig. 6. hHB inhibits the binding of dsRNA to MDA5 but not to RIG-I and enhances the ubiquitination of RIG-I. (A) hHB has no influence on the interaction of RIG-I with short poly(I:C). HEK293T cells were cotransfected with pMyc-RIG-I and p3×Flag-hHB for 48 h. Then the cells were lysed and the cell lysates were incubated with the photobiotin-labeled short poly(I:C) for 4 h at 4°C. Then the biotinylated RNA-protein compounds were precipitated with Dynabeads® Streptavidin for 1 h at room temperature. The bound proteins were applied to SDS-PAGE, and then analyzed by Western blotting. (B and C) hHB inhibits the binding of MDA5 to the long poly(I:C). HEK293T cells were transfected with pMyc-MDA5 and p3×Flag-hHB (B) or the hHB−/− and the WT cells were transfected with pMyc-MDA5 (C). The cell lysates were collected at 48 hpt and incubated with the photobiotin-labeled long poly(I:C) for 4 h at 4°C. Then the biotinylated RNA-protein compounds were precipitated with Dynabeads® Streptavidin for 1 h at room temperature. The bound proteins were analyzed as described above. (D) hHB interacted with long poly(I:C) but not short poly(I:C). HEK293T cells were transfected with p3×Flag-hHB. At 48 hpt, the cell lysates were collected and incubated with the photobiotin-labeled short poly(I:C) or long poly(I:C) for 4 h at 4°C. Then the biotinylated RNA-protein compounds were precipitated with Dynabeads® Streptavidin for 1 h at room temperature. The bound proteins were analyzed by Western blotting. (E) Co-IP analysis of interaction between hHB and RIG-I or MDA5 as described in Materials and Method. (F–H) The effects of hHB on the ubiquitinations of RIG-I and MDA5. HEK293T cells were cotransfected with the indicated plasmids for 12 h and then infected with SeV for 12 h. The
prepared cell extracts were analyzed by IP analysis using an anti-Myc monoclonal antibody followed by Western blotting with the indicated antibodies. (I) The ubiquitination of RIG-I in the hHB−/− cells. The hHB−/− and the WT cells were cotransfected with the indicated plasmids for 12 h and then infected with SeV for 12 h. The prepared cell extracts were analyzed as described above followed by Western blotting with the indicated antibodies. (J) hHA has no influence on the interactions of RIG-I-short poly(I:C) and MDA5-long poly(I:C). HEK293T cells were cotransfected with pMyc-RIG-I or pMyc-MDA5 and p3×Flag-hHA for 48 h. Then the cells were lysed and the cell lysates were incubated with the photobiotin-labeled short poly(I:C) or long poly(I:C) for 4 h at 4°C. Then the biotinylated RNA-protein compounds were precipitated with Dynabeads® Streptavidin for 1 h at room temperature. The bound proteins were analyzed by Western blotting. (K and L) The effect of hHA on the ubiquitination of RIG-I and MDA5. HEK293T cells were cotransfected with the indicated plasmids for 12 h and then infected with SeV for 12 h. The prepared cell extracts were analyzed as described above followed by Western blotting with the indicated antibodies. (M) Overexpression of hHA promoted the RIG-I-mediated activation of the IFN-β transcription but not the MDA5-mediated activation of the IFN-β transcription. HEK293T cells were transfected with p3×Flag-EV (EV) or p3×Flag-hHA and short poly(I:C) or long poly(I:C) for 24 h and the IFN-β mRNA level in cells was analyzed using real-time RT-PCR. The data represent the mean ± standard deviation from three independent experiments. Significant differences are denoted by ** (P < 0.01). NS, not significant (P > 0.05).
Fig. 7. hHB and viral infections promote the intracellular ROS accumulation. (A) hHB increased intracellular ROS accumulation. HEK293T cells were transfected with p3×Flag-hHB at the indicated concentrations for 24 h and then the cytoplasmic ROS formations were monitored by using the oxidant-sensitive fluorescent probe DCFH-DA as described in Materials and methods. (B) SeV infection promoted intracellular ROS accumulation. HEK293T cells were infected with 10, 20, 30, 40, or 50 hemagglutinin units (HAUs)/ml SeV for 24 h. Then the cytoplasmic ROS formations were tested. (C and D) The effect of hHB on the ROS accumulation in the SeV-infected cells. HEK293T cells were transfected with p3×Flag-hHB at the indicated concentrations for 24 h and then were treated with 20 HAUs/ml SeV. Then the cytoplasmic ROS formations were measured at 12 h (C) and 48 h (D) after infection. (E) The SeV-induced ROS accumulation in hHB−/− cells. WT cells and hHB−/− cells were infected with SeV and the cytoplasmic ROS formations were measured at 12 h and 48 h after infection. The data represent the mean ± standard deviation from three independent experiments. Significant differences are denoted by * (P < 0.05), ** (P < 0.01), or *** (P < 0.001).

Fig. 8. Tempol inhibits hHB-induced facilitation of the RIG-I signaling pathway, but has no effect on the hHB-induced inhibition of the MDA5 signaling pathway. (A) The effects of tempol on long poly(I:C)- or short poly(I:C)-induced activation of the IFN-β promoter. HEK293T cells were transfected with pRLuc-TK, pIFN-β-FLuc, and long poly(I:C) or short poly(I:C)
for 12 h and then treated with 3 mM tempol or PBS for 12 h. The effects of hHB on the IFN-β promoter activation were tested as described above. (B) The effects of tempol on long poly(I:C)- or short poly(I:C)-induced IFN-β mRNA transcription. HEK293T cells were transfected with long poly(I:C) or short poly(I:C) for 12 h and then treated with 3 mM tempol or PBS for 12 h. The IFN-β mRNA level in cells was analyzed using real-time RT-PCR. (C) Tempol suppressed hHB-induced inhibition of the activation of the IFN-β promoter in response to long poly(I:C). HEK293T cells were transfected with the 3×Flag-hHB (hHB) at the indicated concentrations, in addition to pRLuc-TK, pIFN-β-FLuc, and long poly(I:C) for 12 h. Then the cells were treated with 3 mM tempol or PBS for 24 h. The effects of hHB on the IFN-β promoter activation were tested. (D) The effect of tempol on the hHB-regulation of MDA5-mediated mRNA transcription of IFN-β. HEK293T cells were transfected with the p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-MDA5 or long poly(I:C) for 12 h. Then the cells were treated with 3 mM tempol or PBS for 12 h and the IFN-β mRNA levels in cells were analyzed using real-time RT-PCR. (E) The effect of diphenyleneiodonium chloride (DPI) and N-acetyl-L-cysteine (NAC) on the hHB-regulation of MDA5-mediated mRNA transcription of IFN-β. RIG-I−/− cells were transfected with the p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-MDA5 or long poly(I:C) for 12 h. Then the cells were treated with 3 μM DPI, 10 mM NAC or PBS for 12 h and the IFN-β mRNA level in cells was tested. (F) Tempol suppressed hHB-induced upregulation of the activation of the IFN-β promoter in response to short poly(I:C). HEK293T cells were transfected with the p3×Flag-hHB
(hHB) at the indicated concentrations, as well as pRLuc-TK, pIFN-β-Fluc, and short poly(I:C) for 12 h. Then cells were treated with 3 mM tempol or PBS for 24 h. The effects of hHB on the IFN-β promoter activation were analyzed as described above. (G) The effect of tempol on the regulation of RIG-I-mediated IFN-β mRNA transcription by hHB. HEK293T cells were transfected with the p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-RIG-I or short poly(I:C) for 12 h. Then the cells were treated with 3 mM tempol or PBS for 12 h and the IFN-β mRNA levels in cells were analyzed using real-time RT-PCR. (H) The effect of DPI and NAC on the hHB-regulation of RIG-I-mediated mRNA transcription of IFN-β. MDA5−/− cells were transfected with the p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-RIG-I or short poly(I:C) for 12 h. Then the cells were treated with 3 μM DPI, 10 mM NAC or PBS for 12 h and the IFN-β mRNA level in cells was tested. (I and J) The effect of tempol on the hHB-induced RIG-I ubiquitination and the K63-linked RIG-I ubiquitination. HEK293T cells were cotransfected with the indicated plasmids. At 12 h after transfection, the cells were infected with SeV and 3 mM tempol for 12 h. The prepared cell extracts were analyzed as described above. (K) The effect of hHA on the ROS accumulation in the SeV-infected cells. HEK293T cells were transfected with p3×Flag-hHA at the indicated concentrations for 24 h and then were infected with SeV. Then the cytoplasmic ROS formations were measured at 12 h after infection. The data represent the mean ± standard deviation from three independent experiments. Significant differences are denoted by * (P < 0.05), ** (P < 0.01) or *** (P < 0.001). NS, not significant (P > 0.05).
Fig. 9. Schematic model of the hHB-mediated regulation of RIG-I/MDA5 signaling pathways. The specific details of the model were described in the text. Ub, ubiquitin; $\mathcal{P}$, phosphate group; Mito, mitochondrion.