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

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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/MDA5mediated signaling pathways; MDA5-dsRNA interaction; ubiquitination; reactive oxygen species

Importance

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.

65 **Introduction**

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

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

84 RIG-I and MDA5 share high structural homologies and signaling features
85 (11). However, they sense different species and natures of viral RNAs (12). It
86 is now well established that RIG-I primarily senses the 5′-triphosphate
87 (5′ppp)-containing viral RNAs and some specific sequence motifs in the viral

88 RNA, such as poly(U/UC) (13-15). In contrast to RIG-I, the characteristics of
89 viral PAMPs sensed by MDA5 activation remain elusive. It has been
90 proposed that MDA5 can recognize long dsRNA as well as web-like RNA
91 aggregates (16). As countermeasures, it has been shown that RIG-I- and
92 MDA5-mediated signaling pathways are differentially regulated (5). Some
93 viral proteins exert different effects on the RIG-I- and MDA5-mediated
94 pathways. For instance, the paramyxovirus V protein regulates the MDA5- but
95 not RIG-I-mediated signaling (17). Besides viral proteins, numerous host
96 molecules have been identified to regulate the RIG-I-mediated signaling. The
97 ARF-like protein 16 (Arl16) and the anti-apoptotic protein A20 interact with
98 RIG-I to inhibit antiviral responses (18, 19); cylindromatosis (CYLD) and
99 ubiquitin specific peptidase 21 (USP21) remove K63-linked polyubiquitin
100 chains to suppress the RIG-I-mediated signaling (20, 21), and ring-finger
101 protein 125 (RNF125) can trigger the proteasome-mediated degradation of
102 RIG-I (22). Several MDA5-associated host proteins have also been identified,
103 such as ADP-ribosylation factor-like protein 5B (Arl5B) and dihydroacetone
104 kinase (DAK) (23, 24). However, the regulatory mechanisms of these two
105 RLRs are insufficiently understood.

106 Hemoglobin is the main oxygen-carrying protein in vertebrates and many
107 invertebrates, and in adult humans, it exists as a tetramer composed of two
108 α-chains and two β-chains (25). The old belief of hemoglobin expression
109 indicates that hemoglobin is expressed only by the erythroid cells. However,
110 this belief has been challenged by the recent findings that hemoglobin is
111 expressed in a wide variety of non-erythrocytes including hepatocytes,

112 alveolar cells, neuronal/glial cells, and endometrial cells (26-29). Hemoglobin
113 exerts multiple functions and plays important roles in resistance to the
114 invasion of pathogens and the regulation of the innate immunity (30, 31).
115 Peptides derived from hemoglobin have a great potential as therapeutic drug
116 candidates (32, 33). However, the antiviral activities and the underlying
117 mechanisms of hemoglobin are poorly explored. Previously, for the first time,
118 we have corroborated that the porcine hemoglobin subunit beta (pHB) is able
119 to suppress the growth of classical swine fever virus (CSFV) through the
120 regulation of the RIG-I-mediated type I IFN responses (34). However, the
121 roles of the HBs of other species in innate immunity have not yet been
122 determined. Due to the significant amino acid homology (84.4%) between
123 human HB (hHB) and pHB, we speculated that they have functional
124 homologies in regulating antiviral innate immunity.

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

132

133 **Results**

134 **hHB is involved in the defense responses against RNA viruses.**

135 To verify whether hHB modulates host antiviral responses, we first

136 evaluated the content of hHB in different non-erythroid cell lines. The
137 expression analysis showed that hHB was expressed in the cell lines derived
138 from various tissue cells, including kidney cells (HEK293T), liver cells
139 (HepG2), lung cells (A549), cervix cells (HeLa), and peripheral blood
140 mononuclear cells (THP-1) (Fig. 1A). To investigate the effect of hHB on the
141 infection of RNA viruses, we generated hHB-deficient HEK293T ($hHB^{-/-}$) cells
142 using the CRISPR/Cas9 system, resulting in efficient knockout of hHB, and
143 overexpressed hHB in HEK293T cells (Fig. 1B). Higher viral replication was
144 observed in $hHB^{-/-}$ cells when infected with Sendai virus (SeV), vesicular
145 stomatitis virus (VSV), and Newcastle disease virus (NDV), but lower
146 replication of encephalomyocarditis virus (EMCV) was exhibited in $hHB^{-/-}$ cells
147 (Fig. 1C). Accordingly, overexpression of hHB led to the resistance to SeV,
148 VSV, or NDV infection, but enhancement of the growth of EMCV (Fig. 1D).
149 Taken together, these results indicate that hHB can regulate the replication of
150 RNA viruses.
151

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

153 RIG-I and MDA5 function as key viral RNA sensors through recognizing
154 different viral RNA species (12). While many negative-stranded RNA viruses
155 including SeV, VSV, and NDV are mainly sensed by RIG-I, the viral RNA of
156 picornaviruses such as EMCV is almost exclusively recognized by MDA5 (12).
157 In conjunction to aforesaid results, hHB exerted an antiviral function against
158 SeV, VSV, and NDV. However, hHB appeared to be beneficial for the
159 replication of EMCV. Therefore, we speculated that hHB may differentially

160 regulate the RIG-I- and MDA5-mediated signaling pathways to modulate
161 antiviral immune responses. We first explored the contribution of hHB in the
162 RIG-I-mediated signaling and noticed that overexpression of hHB significantly
163 enhanced the RIG-I-induced activations of the IFN-β promoter and ISRE,
164 which was saturated at high concentrations (0.7-0.8 μg) (Fig. 2A and B).
165 Consistently, hHB also promoted the IFN-β promoter activation in the cells
166 stimulated with cytoplasmic short poly(I:C), which is a specific ligand of RIG-I
167 (Fig. 2C). In addition, overexpression of hHB upregulated the RIG-I- or short
168 poly(I:C)-induced transcriptions of IFN-β, and IFN-stimulated genes including
169 GBP1 and ISG56 (Fig. 2D–F).

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

181

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

183 We next investigated the contribution of hHB in MDA5-mediated type I

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

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

201

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

204 We further verified the contribution of hHB-mediated regulation of the
205 RIG-I- and MDA5-mediated signaling to the replication of RNA viruses. We
206 generated MDA5-deficient HEK293T (MDA5$^{-/-}$) and RIG-I-deficient HEK293T
207 (RIG-I$^{-/-}$) cells and overexpressed hHB in the MDA5$^{-/-}$ and RIG-I$^{-/-}$ cells (Fig.

208 4A) to investigate the relevance of hHB and the replication of RNA viruses in
209 these cells. The results showed that overexpression of hHB led to the
210 resistance against SeV, VSV, or NDV in the MDA5$^{-/-}$ cells but enhanced the
211 replication of these viruses in the RIG-I$^{-/-}$ cells (Fig. 4B and C). Moreover,
212 although hHB also promoted the replication of EMCV in the RIG-I$^{-/-}$ cells, it
213 displayed little impact on the replication of EMCV in the MDA5$^{-/-}$ cells (Fig. 4B
214 and C).

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

225

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

227 After establishing the relationship between hHB and the MDA5/RIG-I
228 pathways in regulating IFN-β, we explored whether hHB affects the
229 expression of RIG-I or MDA5. HEK293T cells cotransfected with hHB and
230 either RIG-I or MDA5 were used to monitor the expression of RIG-I or MDA5.
231 The results showed that overexpression of hHB failed to alter the exogenous

232 protein expression of RIG-I and MDA5 at all the tested gradient doses (Fig.
233 5A and B). In addition, the mRNA levels of RIG-I and MDA5 remained
234 unchanged upon overexpression of hHB (Fig. 5C and D). There was also no
235 difference between the mRNA levels of RIG-I or MDA5 in the $hHB^{-/-}$ cells and
236 the WT cells (Fig. 5E). Finally, we demonstrated that the endogenous
237 expression of RIG-I and MDA5 remained unaffected in hHB-overexpressing
238 cells (Fig. 5F). Correspondingly, there are no differences observed in the
239 endogenous expression of RIG-I or MDA5 in the $hHB^{-/-}$ cells and the WT cells
240 (Fig. 5G).

241

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

244 The results presented so far clearly articulate the involvement of
245 RIG-I/MDA5 in mediating the hHB-dependent regulation of type I IFN
246 pathway without affecting the RIG-I/MDA5 protein expression. We next
247 mechanistically investigated whether hHB affects the functions of these RLRs.
248 Previous studies have demonstrated that sensing different types of viral RNA
249 is required for the activation of MDA5 and RIG-I to initiate their signal
250 transductions (35-37). RIG-I and MDA5 also recognize short poly(I:C) and
251 long poly(I:C) as the synthetic dsRNA analogues respectively (38). Thus, we
252 examined whether hHB affected the interaction between RIG-I or MDA5 and
253 dsRNA using short or long poly(I:C)-binding assay. The results showed that
254 RIG-I interacted with short poly(I:C) independent of hHB (Fig. 6A). However,
255 overexpression of hHB significantly inhibited the interaction of MDA5 with

256 long poly(I:C) (Fig. 6B), and long poly(I:C) interacted with MDA5 more
257 effectively in the $hHB^{-/-}$ cells than in the WT cells (Fig. 6C), indicating that hHB
258 acts as a repressor of MDA5 activation by inhibiting the MDA5-dsRNA
259 interaction. Since RLRs interact with dsRNA and hHB interferes with this
260 activity of MDA5, we next determined the interaction between hHB and
261 dsRNA. Interestingly, hHB was precipitated with long poly(I:C) but not with
262 short poly(I:C) (Fig. 6D). Due to the association of hHB with dsRNA and the
263 involvement of hHB in MDA5-dsRNA interaction, we were interested to
264 investigate if the interaction with the dsRNA occurs at the interface of hHB
265 and MDA5. Co-immunoprecipitation analysis indicated no identifiable
266 interaction of hHB with either MDA5 or RIG-I (Fig. 6E). These results imply
267 the possibility that the hHB binding to dsRNA may compete for the interaction
268 of MDA5 with the dsRNA ligand and this competition may result in the
269 reduction of IFN-β induction.

270 Upon interacting with the dsRNA ligand, the ubiquitination of RIG-I or
271 MDA5 occurs before recruitment to the mitochondria-associated membrane
272 and binding to MAVS (39). It suggested that the ubiquitination of RIG-I and
273 MDA5 is crucial for the activation of RLRs signaling. Thus, we evaluated the
274 ubiquitination of RIG-I and MDA5 upon hHB overexpression. Based on the
275 disruption of MDA5-dsRNA interaction by hHB, the ubiquitination of MDA5
276 was certainly suppressed by hHB in a dose-dependent manner (Fig. 6F).
277 However, the ubiquitination of RIG-I was potentiated by increasing hHB
278 protein expression (Fig. 6G). It has been shown that RIG-I has different
279 ubiquitination forms and the K63-linked ubiquitination of RIG-I is positively

280 required for RIG-I activation, whereas the K48-linked ubiquitination will result
281 in the destabilization of RIG-I (22, 40). To verify if hHB promotes RIG-I
282 activation through potentiating RIG-I ubiquitination, we constructed two
283 ubiquitin mutants in which all lysine residues were replaced with arginine
284 except K48 or K63 (HA-K48Ub or HA-K63Ub). The results showed that hHB
285 enhanced the K63-linked but not the K48-linked RIG-I ubiquitination (Fig. 6H).
286 Moreover, the total ubiquitination of RIG-I and K63-linked RIG-I ubiquitination
287 were lower in $hHB^{-/-}$ cells than that in WT cells (Fig. 6I). In addition, to uncover
288 whether the action of hHB is characteristic or non-characteristic, we also
289 examined whether the alpha subunit of hemoglobin (hHA) has similar effects
290 on the activation of RIG-I or MDA5. The results showed that both the
291 RIG-I-short poly(I:C) and the MDA5-long poly(I:C) interactions were
292 independent of hHA (Fig. 6J). Furthermore, the ubiquitination of MDA5
293 remained unchanged by hHA (Fig. 6K), but the ubiquitination of RIG-I was
294 enhanced by hHA in a dose-dependent manner (Fig. 6L). Moreover, hHA
295 could promote the short poly(I:C)-induced transcription of IFN-β but not the
296 long poly(I:C)-induced transcription of IFN-β (Fig. 6M). These data imply that
297 the inhibition of the MDA5-dsRNA interaction is a characteristic of hHB.

298

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

301 Considering that hemoglobin is able to regulate the production of ROS,
302 which is a key factor for the host cell to trigger an efficient activation of
303 immunity (41-43), we evaluated if ROS was involved in the hHB-mediated

304 regulation of RIG-I or MDA5 signaling pathway. So, we first explored the links
305 among hHB, ROS, and virus infections. hHB was overexpressed in HEK293T
306 cells and ROS production was monitored by using the oxidant-sensitive
307 fluorescent detection probe DCFH-DA. The results demonstrated that the
308 hHB increased intracellular ROS accumulation in a dose dependent manner
309 (Fig. 7A). In addition, the intracellular ROS accumulation was also
310 upregulated by SeV in a dose-dependent manner (Fig. 7B). Thus, we also
311 monitored the effect of hHB on the ROS accumulation in SeV-infected cells.
312 The overexpression of hHB promoted the ROS production in a dose
313 dependent manner at the early time of SeV infection (Fig. 7C). However, the
314 hHB-overexpressed cells showed lower intracellular ROS accumulation at the
315 later time of SeV infection as the replication of SeV was suppressed (Fig. 7D).
316 Consistently, SeV induced lower ROS production in $hHB^{-/-}$ cells at the earlier
317 time compared with the WT cells, but the $hHB^{-/-}$ cells accumulated more
318 intracellular ROS at the later time of SeV infection (Fig. 7E).

319 Next, we investigated if ROS is required for the hHB-mediated regulation
320 of the MDA5 signaling pathway. As shown in Fig. 8A and B, MDA5- or
321 RIG-induced IFN-β transcription was significantly reduced by tempol (a ROS
322 inhibitor). Moreover, hHB still suppressed long poly(I:C)-induced activation of
323 IFN-β promoter in the presence of tempol (Fig. 8C). Accordingly, tempol
324 treatment could not counteract the inhibition of the MDA5- or long
325 poly(I:C)-induced transcription of IFN-β by hHB overexpression (Fig. 8D). To
326 exclude the unspecific effects of tempol and the disturbance of the
327 RIG-I-mediated signaling pathway, two other ROS inhibitors,

328 diphenyleneiodonium chloride (DPI) and N-acetyl-L-cysteine (NAC) were also
329 tested in the RIG-I$^{-/-}$ cells. DPI or NAC treatment also had no effect on the
330 hHB-mediated inhibition of the MDA5- or long poly(I:C)-induced transcription
331 of IFN-β in the RIG-I$^{-/-}$ cells (Fig. 8E). However, hHB-induced upregulation of
332 the IFN-β promoter activation in response to short poly(I:C) was inhibited by
333 tempol (Fig. 8F). Tempol also suppressed the hHB-induced upregulation of
334 the RIG-I- or short poly(I:C)-induced transcription of IFN-β (Fig. 8G).
335 Moreover, DPI or NAC also obviously counteracted the hHB-mediated
336 facilitation of RIG-I- or short poly(I:C)-induced IFN-β transcription in the
337 MDA5$^{-/-}$ cells (Fig. 8H). Thus, we evaluated the effects of hHB on the
338 ubiquitination of RIG-I upon tempol treatment. Consistent with previous
339 findings, when ROS was suppressed, the ubiquitination of RIG-I was no
340 longer enhanced by hHB (Fig. 8I). To further verify the role of ROS in the
341 hHB-mediated regulation of RIG-I activation, we investigated the effects of
342 hHB on the K63-linked RIG-I ubiquitination when the ROS was suppressed.
343 The results showed that the K63-linked ubiquitination of RIG-I was no longer
344 enhanced by hHB when the ROS accumulation was inhibited (Fig. 8J). These
345 implicate that the upregulation of the RIG-I signaling by hHB is probably
346 related to the hHB-induced ROS, whereas hHB regulates the MDA5 signaling
347 in an alternative way.
348

349 ## Discussion

350 Generally, RIG-I and MDA5, the cytoplasmic RNA helicase proteins, are
351 the main sensors of RNA viruses in triggering type I IFNs in eukaryotes (3, 4).

352 Exploring the molecular events of the RIG-I/MDA5 signaling pathway is
353 critical for understanding the complex innate immune responses against RNA
354 viruses. In this study, we identified hHB as a novel innate immune regulator of
355 the RIG-I/MDA5-mediated antiviral signaling pathways, which further
356 advances our understanding of the regulatory mechanisms involved in the
357 RLRs-mediated signaling pathways.

358 The activation of RIG-I or MDA5 is a complex regulatory process,
359 including viral RNA binding, structural rearrangement, dephosphorylation,
360 ubiquitination, and binding to MAVS to activate the downstream antiviral
361 signaling (44-46). Despite functional overlaps between the RIG-I and MDA5
362 pathways, our results showed that hHB could differently regulate these
363 signaling pathways. RIG-I and MDA5 recognize differential viral RNAs (12). In
364 our study, hHB significantly inhibited the replication of SeV, VSV, and NDV,
365 which are mainly sensed by RIG-I, but enhanced the growth of EMCV which
366 almost only activate MDA5-mediated signaling (Figs. 1 and 4). Moreover, we
367 showed that hHB promoted RIG-I signaling and remarkably inhibited
368 MDA5-mediated type I IFN production (Figs. 2 and 3). Therefore, it is
369 plausible that hHB differently regulates RIG-I and MDA5 activation in the
370 upstream of the MAVS-mediated signaling. RIG-I and MDA5 share a similar
371 structural framework implicated in the viral dsRNA recognition and detection
372 of short and long poly(I:C) as the synthetic dsRNA analogues, respectively
373 (38, 47). Although the RIG-I recognition of viral RNA has been mostly clarified,
374 how MDA5 recognizes viral RNA is yet to be determined. Our results
375 demonstrated that hHB had no obvious influence on the binding of short

376 poly(I:C) to RIG-I, but hHB acted as a direct repressor of MDA5 by interfering
377 with the interaction between MDA5 and long poly(I:C) (Fig. 6B and C). In
378 addition, hHB could bind to the long poly(I:C) but it failed to interact with short
379 poly(I:C) or MDA5 and RIG-I (Fig. 6D–F). These data imply that hHB-dsRNA
380 may compete with the MDA5-dsRNA interaction and thus negatively
381 regulates the MDA5-mediated IFN pathway. Moreover, ubiquitination plays a
382 critical role in the regulation of RIG-I and MDA5 activation (3). The E3
383 ubiquitin ligases TRIM25-catalyzed K63-linked ubiquitination of RIG-I and the
384 TRIM65-catalyzed K63-linked ubiquitination of MDA5 positively regulate
385 RIG-I- and MDA5-mediated signaling pathways, respectively (40, 48). In the
386 present study, we found that hHB promoted the K63-linked ubiquitination of
387 RIG-I, whereas it inhibited the MDA5 ubiquitination (Fig. 6G–I). Interestingly,
388 our results showed that hHA had a similar effect on the activation of RIG-I that
389 it promotes the RIG-I ubiquitination after RNA virus infection. These findings
390 suggest that the action of hHB on the MDA5-dsRNA interaction is
391 characteristic, but the ability of hHB to facilitate the activation of RIG-I may
392 owe to the general characteristic of hemoglobin.

393 Hemoglobin is the main respiratory protein in vertebrates and many
394 invertebrates. It exerts multiple functions and plays an important role in
395 resistance to pathogens invasion (31). Besides functioning as a major host
396 respiratory protein, hemoglobin also can be specifically activated by
397 pathogens to produce ROS to constitute a part of the host defense strategy
398 (41, 49, 50). For example, human hemoglobin significantly enhances the
399 ROS production under microbial proteases stimulation but not the host

400 proteases stimulation (51). ROS plays a key role in immunity and
401 pathogen-killing (52-54). The host respiratory proteins directly exploit the
402 invasion of microbes to produce ROS, resulting in localized cytotoxicity to
403 rapidly kill the neighboring pathogens (51, 55). Recently, the association of
404 ROS with the RLRs signaling has been reported. The host cell requires ROS
405 to efficiently trigger the RIG-I-mediated IRF3 activation and IFN-β expression
406 (56). This implicates that ROS may provide a mediator for hHB to be involved
407 the regulation of the RIG-I signaling. Here, we demonstrated that hHB
408 increases the ROS level in cells and the antioxidant inhibitors including
409 tempol, DPI, and NAC could counteract the hHB-mediated upregulation of the
410 RIG-I-mediated signaling pathway, but could not eliminate the effects of hHB
411 on MDA5 signaling (Figs. 8). Moreover, inhibition of ROS by tempol
412 suppresses the hHB-mediated facilitation of RIG-I ubiquitination, especially
413 the K63-linked ubiquitination of RIG-I. These certify that ROS is required for
414 the hHB-mediated regulation of RIG-I ubiquitination, which indirectly
415 promotes the activation of the RIG-I signaling.

416 The innate immune system has evolved various strategies to prevent
417 harmful overproduction of type I IFNs during viral infection. Thus, several host
418 molecules are capable of regulating the type I IFN production via multiple
419 pathways, including the RIG-I/MDA5 signaling pathway. For example, DAK is
420 a specific repressor of the MDA5-mediated signaling and the deubiquitinating
421 activity of A20 inhibits the RIG-I-mediated signaling (19, 23). It is also worth
422 noticing that several host factors adopted multiple ways to regulate the
423 RIG-I/MDA5 signaling. For example, IFN-β levels are increased following

424 stimulation with activators of the RIG-I signaling in protein kinase R
425 (PKR)-null cells and the absence of PKR severely impairs the
426 MDA5-mediated IFN induction (57). In this study, we identified that hHB was a
427 pleiotropic regulator of the RIG-I/MDA5-mediated signaling pathway.
428 Moreover, hHB could affect RIG-I/MDA5 signaling in a direct or indirect
429 manner. hHB directly inhibits the binding of MDA5 to dsRNA and negatively
430 regulates the MDA5-mediated IFN production (Fig. 9). On the other hand,
431 hHB is involved in the regulation of cellular oxidative stress to enhance the
432 RIG-I ubiquitination, which indirectly promotes the RIG-I-mediated IFN
433 production (Fig. 9). These findings imply that hHB contributes to the
434 safeguard mechanisms needed for controlling the RLR signaling pathway.

435 Currently, many regulators have been identified to have direct effects on
436 a single point or a single pathway of antiviral innate immunity (58-60). For
437 these regulators, the direct effects are appreciated and emphatically studied.
438 However, their indirect impacts on intercellular microenvironment, such as
439 redox state, pH, and ion leakage, are relatively ignored. In the present study,
440 although hHB can directly inhibit type I IFN production through interfering with
441 the MDA5-mediated signaling pathway, hHB-induced change of the
442 intercellular redox state will concurrently impede this inhibition through
443 promoting the RIG-I-mediated signaling pathway. This reveals the importance
444 of intercellular microenvironment in the regulation of antiviral innate immunity
445 and implies the reason why the effects of some regulators are always
446 fluctuating in response to the nature of stimuli. Owing to these indirect effects
447 of hHB through influencing cellular microenvironment, the hHB-mediated

448 innate immune regulation may be dependent on the cellular state and
449 stimulus types and so on, rather than only dependent on its expression level.
450 Therefore, future work is required to further understand the regulation
451 mechanisms of antiviral innate immunity and to improve the effectiveness of
452 some regulators.

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

464

465 **Materials and methods**

466 **Cells, viruses, and plasmids.**

467 HEK293T (ATCC® CRL-3216™) cells, a human embryonic kidney cell
468 line, and BHK-21 (ATCC® CCL-10), a baby hamster Syrian kidney cell line,
469 obtained from the American Type Culture Collection (ATCC), were cultured in
470 Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum
471 (FBS). SeV and NDV-GFP were propagated in SPF chicken embryos and

472 titrated in chicken red blood cells by hemagglutination assay. EMCV and
473 VSV-GFP were propagated in BHK-21 cells. The p3×Flag-hHB plasmid
474 encoding the hHB protein (GenBank accession no. NM_000518.4) with the
475 3×Flag tag at its N-terminus was constructed by cloning the hHB cDNA into
476 the p3×Flag-CMV-10 vector (Sigma-Aldrich). The plasmids pEGFP-RIG-I and
477 pEGFP-MDA5 encoding the RIG-I and MDA5 proteins, respectively, with a
478 GFP tag at the C-terminus were constructed by cloning the human RIG-I,
479 MDA5, and MAVS cDNA into the pEGFP-C1 vector (Clontech Laboratories).
480 pEF-Myc-RIG-I and pEF-Myc-MDA5 encode the human RIG-I and MDA5,
481 respectively.

482

483 **Generation of hHB$^{-/-}$, -RIG-I$^{-/-}$, and -MDA5$^{-/-}$ cells using HEK293T cells.**

484 We generate hHB$^{-/-}$, -RIG-I$^{-/-}$, and -MDA5$^{-/-}$ cells using the lentiviral
485 expressing CRISPR-Cas9 vector (lentiCRISPRv2, Addgene). The hHB-,
486 RIG-I-, and MDA5-specific sgRNA sequences were: hHB (forward, 5′- GTA
487 ACG GCA GAC TTC TCC TC-3′; reverse, 5′-GAG GAG AAG TCT GCC GTT
488 ACC-3′), RIG-I (forward, 5′-GGG TCT TCC GGA TAT AAT CC-3′; reverse,
489 5′-GGA TTA TAT CCG GAA GAC CCC-3′), and MDA5 (forward, 5′-CGA ATT
490 CCC GAG TCC AAC CA-3′; reverse, 5′-TGG TTG GAC TCG GGA ATT
491 CGC-3′), respectively. Lenti-CRISPR virions were packaged in HEK293T
492 cells by transfecting the psPAX2 plasmid (Addgene), the pMD2.G plasmid
493 (Addgene) and either the lentiCRISPRv2 vector containing hHB, RIG-I, or
494 MDA5-specific sgRNA, or empty lentiCRISPRv2 plasmid as a control.
495 The suspensions were harvested at 72 hours posttransfection (hpt).

496 HEK293T cells were infected with the suspensions and treated with 1.5 μg/ml
497 puromycin for 5 d. The cells were lysed and the hHB, RIG-I, or MDA5
498 expression was analyzed by Western blotting.
499

500 **Dual-luciferase reporter assay.**

501 HEK293T cells were cotransfected with the IFN-β promoter or ISRE
502 firefly luciferase (FLuc) reporter plasmid (pIFN-β-FLuc or pISRE-FLuc), the
503 indicated amounts of p3×Flag-hHB, and internal reference reporter
504 TK-*Renilla* luciferase (RLuc) as an internal control (pRLuc-TK). The total
505 amounts of the plasmid DNAs were equalized with the empty control vector
506 p3×Flag-CMV-10 (p3×Flag-EV). At 24 hpt, the cells were infected with SeV or
507 PBS for another 24 h. Then cells were lysed and the activities of the reporter
508 genes were determined using a Dual-Luciferase® Reporter Assay System
509 10-Pack (Promega). The luciferase induction mediated by IFN-β promoter
510 (IFN-β-Luc) or ISRE (ISRE-Luc) was presented as relative expression level of
511 FLuc/RLuc. For the RIG-I- or MDA5-mediated response, HEK293T cells were
512 cotransfected with pIFN-β-FLuc/pISRE-FLuc and pRLuc-TK as well as
513 pMyc-RIG-I, pMyc-MDA5, short poly(I:C) (catalog no. tlrl-picw; InvivoGen), or
514 long poly(I:C) (catalog no. tlrl-pic; InvivoGen). The luciferase activities were
515 measured at 24 hpt and relative expressions were calculated as described
516 above.
517

518 **Real-time RT-PCR.**

519 Total RNA was extracted from HEK293T cells using the TRIzol reagent

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

527

528 **ROS production assay.**

529 Total ROS production was measured by probing with the
530 2’,7’-dichlorodihydrofluorescein diacetate (DCHF-DA) probe (catalog no.
531 S0033; Beyotime Biotechnology) and was evaluated using Enspire
532 Multimode Plate Reader (Perkin Elmer). The HEK293T cells were transfected
533 with p3×Flag-hHB at the indicated amounts. The total amount of the plasmid
534 DNA was equalized with the p3×Flag-EV. At 24 hpt, the cells were infected
535 with SeV or treated with PBS for 24 h. Then the cells were washed with PBS
536 and incubated with the DCFH-DA probes for 30 min at 37°C. After incubation,
537 the cells were washed twice with PBS and the 2’,7’-dichlorofluorescein (DCF)
538 fluorescence was measured.

539

540 **Western blotting.**

541 Equivalent amount of each sample was resolved by SDS-PAGE and
542 transferred to nitrocellulose membranes (Hybond-C Super; GE Healthcare).
543 The membranes were blocked with 5% skim milk in PBS containing 0.1%

544 Tween (PBST) and incubated for 2 h at room temperature with the primary
545 antibodies at an appropriate dilution (anti-Flag, -Myc, -MDA5, and -RIG-I
546 MAbs at 1:1,000 and anti-hHB MAb at 1:500) (catalog nos. F1804 and M4439;
547 Sigma-Aldrich and catalog nos. sc-134513, sc-48932, and sc-22718; Santa
548 Cruz). The membranes were washed by PBST and then incubated with
549 IRDye® 800CW goat anti-mouse IgG (H+L), donkey anti-goat IgG (H+L), and
550 goat anti-rabbit (H+L) (catalog nos. 926-32210, 926-32214, and 926-32211;
551 LiCor BioSciences) or goat anti-mouse IgG-peroxidase (catalog no. A5278;
552 Sigma) at 1:10,000 for 1 h at 37°C, and the blots were scanned using the
553 Odyssey infrared imaging system (LiCor BioSciences) or
554 Fluorescence/Chemiluminescence imaging system (Clinx Science
555 instruments).

556

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

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

567

568 **Poly(I:C) binding assay.**

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

578

579 **Statistical analysis.**

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

583

584

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591

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772

773 **Figure legends**

774 **Fig. 1. hHB is involved in the antiviral responses to RNA viruses.** (A) The
775 expression of hHB in different non-erythroid cell lines including HEK293T,
776 HepG2, A549, HeLa, and THP-1. (B) The knockout efficiency of hHB in
777 hHB-deficient HEK293T ($hHB^{-/-}$) cells compared with the expression level of
778 hHB in the wild-type HEK293T (WT) and the overexpression of hHB in
779 HEK293T cells. (C) $hHB^{-/-}$ cells were more sensitive to Sendai virus (SeV),
780 vesicular stomatitis virus (VSV), and Newcastle disease virus (NDV) but more
781 resistant to encephalomyocarditis virus (EMCV). $hHB^{-/-}$ and WT cells were
782 infected with SeV or EMCV for 24 h and the RNA level of the SeV N protein or
783 the EMCV 3D protein was determined by real-time RT-PCR. In addition,
784 $hHB^{-/-}$ and WT cells were infected with VSV-GFP or NDV-GFP for 48 h. The
785 GFP expression level was analysed by Western blotting. (D) Overexpression
786 of hHB inhibited the replications of SeV, VSV, and NDV but enhanced EMCV
787 growth. HEK293T cells were transfected with p3×Flag empty vector
788 (p3×Flag-EV) or p3×Flag-hHB for 24 h and then infected with SeV or EMCV
789 for 24 h or infected with VSV-GFP or NDV-GFP for 48 h. The RNA level of the

790 SeV N protein or the EMCV 3D protein or the GFP expression of VSV-GFP or
791 NDV-GFP was tested. The data represent the mean ± standard deviation
792 from three independent experiments. Significant differences are denoted by *
793 ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).
794

795 **Fig. 2. hHB enhances the RIG-I-mediated antiviral signaling.** (A–C)
796 Overexpression of hHB upregulated RIG-I-mediated activation of the IFN-β
797 promoter and ISRE. HEK293T cells were cotransfected with the indicated
798 amounts of p3×Flag-hHB (hHB), pMyc-RIG-I, TK-*Renilla* luciferase (RLuc)
799 internal reference reporter plasmid (pRLuc-TK), and firefly luciferase (FLuc)
800 reporter plasmid (pIFN-β-FLuc) (A) or ISRE firefly luciferase reporter plasmid
801 (pISRE-FLuc) (B) for 24 h. Additionally, HEK293T cells were cotransfected
802 with pRLuc-TK, pIFN-β-FLuc, short poly(I:C), and the indicated amounts of
803 p3×Flag-hHB for 24 h (C). The activation of the IFN-β promoter or ISRE was
804 presented as the activities of the luciferase reporter genes induction mediated
805 by the IFN-β promoter (IFN-β-Luc induction) or ISRE (ISRE-Luc induction)
806 and calculated as the relative levels of FLuc/RLuc. (D–F) Overexpression of
807 hHB enhanced the transcription of IFN-β, GBP1, and ISG56. HEK293T cells
808 were cotransfected with p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-RIG-I or
809 short poly(I:C) for 24 h and the IFN-β (D), GBP1 (E), and ISG56 (F) mRNA
810 levels in cells were analyzed using real-time RT-PCR. (G and H) The RIG-I
811 mediated lower activation of the IFN-β promoter and ISRE in hHB$^{-/-}$ cells.
812 hHB$^{-/-}$ and WT cells were cotransfected with pMyc-RIG-I or short poly(I:C) as
813 well as pRLuc-TK and pIFN-β-FLuc (G) or pISRE-FLuc (H). At 24 hpt, the

814 activation of IFN-β promoter or ISRE was tested. (I–K) The RIG-I mediated
815 lower transcription of IFN-β, GBP1, and ISG56 in hHB$^{-/-}$ cells. hHB$^{-/-}$, and WT
816 cells were transfected with pMyc-RIG-I or short poly(I:C). At 24 hpt, the IFN-β
817 (I), GBP1 (J), and ISG56 (K) mRNA abundances were tested. (L) The effect
818 of hHB on the IFN-β promoter activation. HEK293T cells were cotransfected
819 with the indicated amounts of p3×Flag-hHB, pRLuc-TK, and pIFN-β-FLuc for
820 24 h. The IFN-β promoter activation was tested as described above. The data
821 represent the mean ± standard deviation from three independent experiments.
822 Significant differences are denoted by * ($P < 0.05$), ** ($P < 0.01$), or *** ($P <$
823 0.001). NS, not significant ($P > 0.05$).

824

825 **Fig. 3. hHB inhibits the MDA5-mediated antiviral signaling.** (A–C)
826 Overexpression of hHB suppressed MDA5-mediated activation of the IFN-β
827 promoter and ISRE. HEK293T cells were cotransfected with the indicated
828 amounts of p3×Flag-hHB, pMyc-MDA5, pRLuc-TK, and pIFN-β-FLuc (A) or
829 pISRE-FLuc (B) for 24 h. Additionally, HEK293T cells were cotransfected with
830 pRLuc-TK, pIFN-β-FLuc, long poly(I:C), and the indicated amounts of
831 p3×Flag-hHB for 24 h (C). The effect of hHB on the activation of IFN-β
832 promoter or ISRE was examined as described above. (D–F) Overexpression
833 of hHB decreased the transcription of IFN-β, GBP1, and ISG56. HEK293T
834 cells were transfected with the p3×Flag-EV (EV) or p3×Flag-hHB and
835 pMyc-MDA5 or long poly(I:C) for 24 h and the IFN-β (D), GBP1 (E), and
836 ISG56 (F) mRNA levels in cells were analyzed. (G and H) The MDA5
837 mediated higher activation of the IFN-β promoter and ISRE in hHB$^{-/-}$ cells.

838 $hHB^{-/-}$ and WT cells were cotransfected with pMyc-MDA5 or long poly(I:C) as
839 well as pRLuc-TK and pIFN-β-FLuc (G) or pISRE-FLuc (H). At 24 hpt, the
840 activation of IFN-β promoter or ISRE was analyzed. (I–K) The MDA5
841 mediated higher transcription of IFN-β, GBP1, and ISG56 in $hHB^{-/-}$ cells.
842 $hHB^{-/-}$ and WT cells were transfected with pMyc-MDA5 or long poly(I:C). At 24
843 hpt, the IFN-β (I), GBP1 (J), and ISG56 (K) mRNA abundances were tested.
844 The data represent the mean ± standard deviation from three independent
845 experiments. Significant differences are denoted by * ($P < 0.05$), ** ($P < 0.01$),
846 or *** ($P < 0.001$). NS, not significant ($P > 0.05$).
847

848 **Fig. 4. hHB regulates the RIG-I- and MDA5-mediated antiviral response**
849 **to RNA viruses.** (A) The knockout efficiency of RIG-I and MDA5 in HEK293T
850 cells, and overexpression of hHB in MDA5-deficient HEK293T ($MDA5^{-/-}$) and
851 RIG-I-deficient HEK293T ($RIG\text{-}I^{-/-}$). (B and C) The effect of
852 hHB-overexpression on the replications of SeV, VSV, NDV, and EMCV in
853 $MDA5^{-/-}$ and $RIG\text{-}I^{-/-}$ cells. $MDA5^{-/-}$ (B) or $RIG\text{-}I^{-/-}$ (C) cells were transfected
854 with p3×Flag-hHB for 24 h and then were infected with SeV or EMCV for 24 h
855 or infected with VSV-GFP or NDV-GFP for 48 h. The RNA level of the SeV N
856 protein or the EMCV 3D protein or the GFP expression level of VSV-GFP or
857 NDV-GFP was tested. (D) The knockout efficiency of RIG-I and MDA5 in
858 $hHB^{-/-}$ cells. (E and F) Deficiency of hHB differently affected the replication of
859 SeV, VSV, NDV, and EMCV in $RIG\text{-}I^{-/-}$ and $MDA5^{-/-}$ cells. $MDA5^{-/-}/hHB^{-/-}$ cells
860 and $MDA5^{-/-}$ (E) or $RIG\text{-}I^{-/-}/hHB^{-/-}$ and $RIG\text{-}I^{-/-}$ cells (F) were infected with SeV
861 or EMCV for 24 h or infected with VSV-GFP or NDV-GFP for 48 h. Then the

862 RNA level of the SeV N protein or the EMCV 3D protein or the GFP
863 expression level of VSV-GFP or NDV-GFP was measured as above. The data
864 represent the mean ± standard deviation from three independent experiments.
865 Significant differences are denoted by * ($P < 0.05$), ** ($P < 0.01$), or *** ($P <$
866 0.001). NS, not significant ($P > 0.05$).

867

868 **Fig. 5. hHB does not affect RIG-I or MDA5 expression.** (A and B)
869 Overexpression of hHB had no effects on the exogenous protein expression
870 of RIG-I or MDA5. HEK293T cells were cotransfected with the indicated
871 amounts of p3×Flag-hHB and pMyc-RIG-I or pMyc-MDA5 for 24 h and the
872 RIG-I (A) and MDA5 (B) expression was analyzed by Western blotting. (C and
873 D) Overexpression of hHB did not affect the RIG-I and MDA5 transcription.
874 HEK293T cells were transfected with the indicated amounts of p3×Flag-hHB
875 (hHB) for 24 h and then RIG-I (C) and MDA5 (D) mRNA levels in cells were
876 analyzed using real-time RT-PCR. (E) The mRNA levels of RIG-I and MDA5
877 exhibited no difference between the hHB$^{-/-}$ and WT cells. The mRNA levels of
878 RIG-I and MDA5 in the cells were analyzed using real-time RT-PCR. (F) The
879 endogenous protein expression of RIG-I and MDA5 had no change in the
880 hHB-overexpression cells. HEK293T cells were transfected with the indicated
881 amounts of p3×Flag-hHB for 48 h and the RIG-I and MDA5 expression was
882 analyzed by Western blotting analysis. (G) The endogenous protein
883 expression of RIG-I and MDA5 in the hHB$^{-/-}$ and WT cells was examined by
884 Western blotting analysis. NS, not significant ($P > 0.05$).

885

886 **Fig. 6. hHB inhibits the binding of dsRNA to MDA5 but not to RIG-I and**
887 **enhances the ubiquitination of RIG-I.** (A) hHB has no influence on the
888 interaction of RIG-I with short poly(I:C). HEK293T cells were cotransfected
889 with pMyc-RIG-I and p3×Flag-hHB for 48 h. Then the cells were lysed and the
890 cell lysates were incubated with the photobiotin-labeled short poly(I:C) for 4 h
891 at 4°C. Then the biotinylated RNA-protein compounds were precipitated with
892 Dynabeads® Streptavidin for 1 h at room temperature. The bound proteins
893 were applied to SDS-PAGE, and then analyzed by Western blotting. (B and C)
894 hHB inhibits the binding of MDA5 to the long poly(I:C). HEK293T cells were
895 transfected with pMyc-MDA5 and p3×Flag-hHB (B) or the $hHB^{-/-}$ and the WT
896 cells were transfected with pMyc-MDA5 (C). The cell lysates were collected at
897 48 hpt and incubated with the photobiotin-labeled long poly(I:C) for 4 h at 4°C.
898 Then the biotinylated RNA-protein compounds were precipitated with
899 Dynabeads® Streptavidin for 1 h at room temperature. The bound proteins
900 were analyzed as described above. (D) hHB interacted with long poly(I:C) but
901 not short poly(I:C). HEK293T cells were transfected with p3×Flag-hHB. At 48
902 hpt, the cell lysates were collected and incubated with the photobiotin-labeled
903 short poly(I:C) or long poly(I:C) for 4 h at 4°C. Then the biotinylated
904 RNA-protein compounds were precipitated with Dynabeads® Streptavidin for
905 1 h at room temperature. The bound proteins were analyzed by Western
906 blotting. (E) Co-IP analysis of interaction between hHB and RIG-I or MDA5 as
907 described in Materials and Method. (F–H) The effects of hHB on the
908 ubiquitinations of RIG-I and MDA5. HEK293T cells were cotransfected with
909 the indicated plasmids for 12 h and then infected with SeV for 12 h. The

910 prepared cell extracts were analyzed by IP analysis using an anti-Myc
911 monoclonal antibody followed by Western blotting with the indicated
912 antibodies. (I) The ubiquitination of RIG-I in the $hHB^{-/-}$ cells. The $hHB^{-/-}$ and
913 the WT cells were cotransfected with the indicated plasmids for 12 h and then
914 infected with SeV for 12 h. The prepared cell extracts were analyzed as
915 described above followed by Western blotting with the indicated antibodies. (J)
916 hHA has no influence on the interactions of RIG-I-short poly(I:C) and
917 MDA5-long poly(I:C). HEK293T cells were cotransfected with pMyc-RIG-I or
918 pMyc-MDA5 and p3×Flag-hHA for 48 h. Then the cells were lysed and the cell
919 lysates were incubated with the photobiotin-labeled short poly(I:C) or long
920 poly(I:C) for 4 h at 4°C. Then the biotinylated RNA-protein compounds were
921 precipitated with Dynabeads® Streptavidin for 1 h at room temperature. The
922 bound proteins were analyzed by Western blotting. (K and L) The effect of
923 hHA on the ubiquitination of RIG-I and MDA5. HEK293T cells were
924 cotransfected with the indicated plasmids for 12 h and then infected with SeV
925 for 12 h. The prepared cell extracts were analyzed as described above
926 followed by Western blotting with the indicated antibodies. (M)
927 Overexpression of hHA promoted the RIG-I-mediated activation of the IFN-β
928 transcription but not the MDA5-mediated activation of the IFN-β transcription.
929 HEK293T cells were transfected with p3×Flag-EV (EV) or p3×Flag-hHA and
930 short poly(I:C) or long poly(I:C) for 24 h and the IFN-β mRNA level in cells
931 was analyzed using real-time RT-PCR. The data represent the mean ±
932 standard deviation from three independent experiments. Significant
933 differences are denoted by ** ($P < 0.01$). NS, not significant ($P > 0.05$).

934

935 **Fig. 7. hHB and viral infections promote the intracellular ROS**
936 **accumulation.** (A) hHB increased intracellular ROS accumulation. HEK293T
937 cells were transfected with p3×Flag-hHB at the indicated concentrations for
938 24 h and then the cytoplasmic ROS formations were monitored by using the
939 oxidant-sensitive fluorescent probe DCFH-DA as described in Materials and
940 methods. (B) SeV infection promoted intracellular ROS accumulation.
941 HEK293T cells were infected with 10, 20, 30, 40, or 50 hemagglutinin units
942 (HAUs)/ml SeV for 24 h. Then the cytoplasmic ROS formations were tested.
943 (C and D) The effect of hHB on the ROS accumulation in the SeV-infected
944 cells. HEK293T cells were transfected with p3×Flag-hHB at the indicated
945 concentrations for 24 h and then were treated with 20 HAUs/ml SeV. Then the
946 cytoplasmic ROS formations were measured at 12 h (C) and 48 h (D) after
947 infection. (E) The SeV-induced ROS accumulation in $hHB^{-/-}$ cells. WT cells
948 and $hHB^{-/-}$ cells were infected with SeV and the cytoplasmic ROS formations
949 were measured at 12 h and 48 h after infection. The data represent the mean
950 ± standard deviation from three independent experiments. Significant
951 differences are denoted by * ($P < 0.05$), ** ($P < 0.01$), or *** ($P < 0.001$).

952

953 **Fig. 8. Tempol inhibits hHB-induced facilitation of the RIG-I signaling**
954 **pathway, but has no effect on the hHB-induced inhibition of the MDA5**
955 **signaling pathway.** (A) The effects of tempol on long poly(I:C)- or short
956 poly(I:C)-induced activation of the IFN-β promoter. HEK293T cells were
957 transfected with pRLuc-TK, pIFN-β-FLuc, and long poly(I:C) or short poly(I:C)

958 for 12 h and then treated with 3 mM tempol or PBS for 12 h. The effects of
959 hHB on the IFN-β promoter activation were tested as described above. (B)
960 The effects of tempol on long poly(I:C)- or short poly(I:C)-induced IFN-β
961 mRNA transcription. HEK293T cells were transfected with long poly(I:C) or
962 short poly(I:C) for 12 h and then treated with 3 mM tempol or PBS for 12 h.
963 The IFN-β mRNA level in cells was analyzed using real-time RT-PCR. (C)
964 Tempol suppressed hHB-induced inhibition of the activation of the IFN-β
965 promoter in response to long poly(I:C). HEK293T cells were transfected with
966 the 3×Flag-hHB (hHB) at the indicated concentrations, in addition to
967 pRLuc-TK, pIFN-β-FLuc, and long poly(I:C) for 12 h. Then the cells were
968 treated with 3 mM tempol or PBS for 24 h. The effects of hHB on the IFN-β
969 promoter activation were tested. (D) The effect of tempol on the
970 hHB-regulation of MDA5-mediated mRNA transcription of IFN-β. HEK293T
971 cells were transfected with the p3×Flag-EV (EV) or p3×Flag-hHB and
972 pMyc-MDA5 or long poly(I:C) for 12 h. Then the cells were treated with 3 mM
973 tempol or PBS for 12 h and the IFN-β mRNA levels in cells were analyzed
974 using real-time RT-PCR. (E) The effect of diphenyleneiodonium chloride (DPI)
975 and N-acetyl-L-cysteine (NAC) on the hHB-regulation of MDA5-mediated
976 mRNA transcription of IFN-β. RIG-I$^{-/-}$ cells were transfected with the
977 p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-MDA5 or long poly(I:C) for 12 h.
978 Then the cells were treated with 3 μM DPI, 10 mM NAC or PBS for 12 h and
979 the IFN-β mRNA level in cells was tested. (F) Tempol suppressed
980 hHB-induced upregulation of the activation of the IFN-β promoter in response
981 to short poly(I:C). HEK293T cells were transfected with the p3×Flag-hHB

982 (hHB) at the indicated concentrations, as well as pRLuc-TK, pIFN-β-FLuc,
983 and short poly(I:C) for 12 h. Then cells were treated with 3 mM tempol or PBS
984 for 24 h. The effects of hHB on the IFN-β promoter activation were analyzed
985 as described above. (G) The effect of tempol on the regulation of
986 RIG-I-mediated IFN-βmRNA transcription by hHB. HEK293T cells were
987 transfected with the p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-RIG-I or
988 short poly(I:C) for 12 h. Then the cells were treated with 3 mM tempol or PBS
989 for 12 h and the IFN-β mRNA levels in cells were analyzed using real-time
990 RT-PCR. (H) The effect of DPI and NAC on the hHB-regulation of
991 RIG-I-mediated mRNA transcription of IFN-β. MDA5$^{-/-}$ cells were transfected
992 with the p3×Flag-EV (EV) or p3×Flag-hHB and pMyc-RIG-I or short poly(I:C)
993 for 12 h. Then the cells were treated with 3 μM DPI, 10 mM NAC or PBS for
994 12 h and the IFN-β mRNA level in cells was tested. (I and J) The effect of
995 tempol on the hHB-induced RIG-I ubiquitination and the K63-linked RIG-I
996 ubiquitination. HEK293T cells were cotransfected with the indicated plasmids.
997 At 12 h after transfection, the cells were infected with SeV and 3 mM tempol
998 for 12 h. The prepared cell extracts were analyzed as described above. (K)
999 The effect of hHA on the ROS accumulation in the SeV-infected cells.
1000 HEK293T cells were transfected with p3×Flag-hHA at the indicated
1001 concentrations for 24 h and then were infected with SeV. Then the
1002 cytoplasmic ROS formations were measured at 12 h after infection. The data
1003 represent the mean ± standard deviation from three independent experiments.
1004 Significant differences are denoted by * (P < 0.05), ** (P < 0.01) or *** (P <
1005 0.001). NS, not significant (P > 0.05).

1006

1007 **Fig. 9. Schematic model of the hHB-mediated regulation of RIG-I/MDA5**

1008 **signaling pathways.** The specific details of the model were described in the

1009 text. Ub, ubiquitin; Ⓟ, phosphate group; Mito, mitochondrion.