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.

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.

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

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,

alveolar cells, neuronal/glial 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$^{-/-}$) 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$^{-/-}$ 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$^{-/-}$ 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

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

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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

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

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

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

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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

(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%

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

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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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

32

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-I$^{-/-}$). (B and C) The effect of

852 hHB-overexpression on the replications of SeV, VSV, NDV, and EMCV in

853 MDA5$^{-/-}$ and RIG-I$^{-/-}$ cells. MDA5$^{-/-}$ (B) or RIG-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-I$^{-/-}$ and MDA5$^{-/-}$ cells. MDA5$^{-/-}$/hHB$^{-/-}$ cells

860 and MDA5$^{-/-}$ (E) or RIG-I$^{-/-}$/hHB$^{-/-}$ and RIG-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

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℃. 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 ($P > 0.05$).

934

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 *** ($P < 0.001$).

952

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)

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.