1 **IFI16 directly senses viral RNA and enhances RIG-I transcription**

2 **and activation to restrict influenza virus infection**

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Abstract

The Retinoic acid-inducible gene I (RIG-I) receptor senses cytoplasmic viral RNA and activates type I interferons (IFN-I) and downstream antiviral immune responses. How RIG-I binds to viral RNA and how its activation is regulated remains unclear. Here, using IFI16 knockout cells and p204- deficient mice, we demonstrated that the DNA sensor IFI16 enhances IFN-I production to inhibit IAV replication. IFI16 positively upregulates RIG-I transcription through direct binding to and recruitment of RNA Pol II to the RIG-I promoter. IFI16 also binds to influenza viral RNA via its HINa domain, and to RIG-I protein with its PYRIN domain, thus promoting IAV-induced K63- linked polyubiquitination and RIG-I activation. Our work demonstrate that IFI16 is a positive regulator of RIG-I signaling during influenza virus infection, highlighting its role in RLR-mediated innate immune response to IAV and other RNA viruses, and suggesting its possible exploitation to modulate the antiviral response.

Keywords: Influenza virus; RNA recognition; IFI16; RIG-I; type I interferons

Introduction

Influenza virus is one of the most important causes of respiratory tract infection, resulting in at least 26 million influenza illnesses, 250,000 hospitalizations and 14,000 deaths during the 2019-2020 season in the United States (*https://www.cdc.gov/flu/season/index.html*). A(H1N1)/pdm2009, H3N2, and influenza B (B/Yamagata or B/Victoria lineage) viruses are mainly responsible for seasonal influenza epidemics each year. Moreover, avian influenza virus remains a significant additional threat to human health, in particular the H5, H7 and H9 subtypes. Although vaccination is the most effective way to control IAV, prediction of evolving immunogenic epitopes as well as challenges of vaccine production and distribution often limit vaccine efficacy and availability. Furthermore, antiviral resistant IAV strains continued to be identified; they include H274Y mutation in the neuraminidase (NA)[1,2], and three major mutations (L26F, V27A, and S31N) in the M2 protein[3,4]. Hence, the development of effective interventions against influenza virus infection remains an outstanding public health need. Targeting an essential host factor critical for influenza infection is a promising antiviral strategy[5].

Several families of pattern recognition receptors (PRRs) have been described: toll like receptors (TLRs), retinoic acid inducible gene-I (RIG-I) receptors (RLRs) and nucleotide oligomerization domain (NOD)-like receptors (NLRs) are involved in the recognition of influenza viruses[6-8]. Among them, RIG-I is thought to be the most important sensor of influenza virus[9]; it binds the 5'ppp-RNA of the virus, leading it to undergo conformational changes and exposing its caspase activation and recruitment

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67   domains (CARDs)[10], which is then ubiquitinated by the action of E3 ligases such as

68   tripartite motif 25 (TRIM25)[11] and RIPLET[12]. TRIM25 activates RIG-I through the

69   generation of unanchored K63-linked polyubiquitin chains interacting with the

70   CARDs[13], or through the generation of anchored K63-linked polyubiquitin chains

71   attached to lysine 172 of RIG-I[11]. This process results in the interaction of RIG-I with

72   the mitochondrial antiviral signaling (MAVS) adaptor, which leads to the subsequent

73   activation of IRF3/7 and NF-κB and thereby inducing the expression of IFN-I and

74   pro-inflammatory cytokines[14]. Therefore, RIG-I activation has to be tightly regulated

75   to ensure effective virus inhibition with minimal excessive inflammatory response.

76   Interferon-γ-inducible protein-16 (IFI16) is a member of the pyrin and HIN

77   domain (PYHIN) containing protein family, which encodes a class of homologous

78   proteins that share a 200-amino acid signature motif (HIN)[15]. IFI16 was first reported

79   as a sensor of transfected and viral DNA involved in innate signaling[16,17] and

80   functions as an innate immune sensors in eukaryotic cells[18-24]. IFI16 senses the

81   double-stranded DNA (dsDNA) from invading DNA viruses including herpes simplex

82   virus 1 (HSV-1), Kaposi sarcoma-associated herpesvirus (KSHV), vaccinia virus

83   (VACV)[21,24,25], the single-stranded DNA (ssDNA) from HIV-infected CD^{4+} T cells

84   and nuclear damaged DNA from etoposide-treated keratinocytes[22,26]. DNA

85   recognition by IFI16 induces the activation of the stimulator of interferon

86   genes-TANK-binding kinase 1-interferon regulatory factor 3 (STING-TBK1-IRF3)

87   pathway, leading to the induction of IFN-I or ASC-caspase 1-dependent

88   inflammasome to produce interleukin-1β (IL-1β)[21,24,26]. Additionally, a role for IFI16

89   in RNA virus infection has been identified; IFI16 transcriptionally regulates the IFN-I

90   gene expression in Sendai virus infection[27]. The murine ortholog of IFI16 (p204) is

91   highly induced in mouse hepatitis coronavirus infection and inhibits IRF7-mediated

92   IFN-I production[28]. Moreover, it was shown that IFI16 interacts with MAVS to

93   promote MAVS-mediated production of IFN-I that inhibits porcine reproductive and

94   respiratory syndrome virus 2 replication[29]. More recently, IFI16 was found to directly

95   bind Chikungunya virus (CHIKV) genome RNA and restrict viral replication and

96   maturation[30], which further suggests that IFI16 may play a crucial role in RNA virus

97   infection. However, the precise role of IFI16 in influenza virus infection has not been

98   elucidated.

99   Here, we showed that IFI16, which was identified as an influenza viral RBP, was

100   highly induced both *in vitro* and *in vivo* during IAV infection. We further

101   demonstrated that IFI16 upregulated *RIG-I* transcription by binding its HINa domain

102   to the *RIG-I* promoter, and interacted with both IAV vRNAs and RIG-I to promote

103   influenza virus-induced K63-linked polyubiquitination of RIG-I. Collectively, these

104   results indicate that IFI16 is a key positive regulator of RIG-I signaling in antiviral

105   innate immune responses to influenza virus infection.

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Results

IFI16 is a viral RBP involved in influenza virus infection

Owing to its RNA genome, influenza virus utilizes RNA-binding proteins (RBPs) of both viral and host origin for its replication. To uncover comprehensive viral RNA (vRNA)-host protein interactions, we performed affinity purification coupled with mass spectrometry (AP-MS) analysis of influenza vRNA complexes. The eight vRNA segments of H7N9 virus were individually transcribed and labeled with biotin *in vitro*, and incubated with lysates from IAV virus-infected THP-1 cells. vRNA complexes bound to streptavidin magnetic beads were analyzed by mass spectrometry (Fig.1A). Domain enrichment analysis of co-purified proteins for each RNA bait revealed that more than 90% of the isolated proteins harbored nucleic acid binding domains (Fig.1B). We identified 70 candidate vRNA-binding proteins that could bind to no less than 3 baits. A high number of co-purified proteins have been reported as RBPs, such as PTBP1[31], TRA2B[32], DDX5[33], DDX3X[34], and RBBP6[35], which indicates that our approach was effective in identifying vRNA-interacting proteins for influenza virus. Expression results demonstrated that *IFI16* was most highly expressed for the duration of IAV infection (Fig.1C) which suggests that IFI16 may play an important role in IAV infection.

To determine whether IFI16 is involved in IAV replication, we first evaluated *IFI16* expression in PR8-infected human cells. *IFI16* mRNA level was significantly upregulated in THP-1, A549 and HEK293 cells during PR8 infection (Extended Data Fig.1A-C). We also found that PR8 infection significantly elevated *p204* expression in

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133   mice lung tissues at 1 day post infection (dpi), 3 dpi and 5 dpi (Extended Data Fig.1D).

134   PR8 virus actively induced the expression of *IFI16* in THP-1 (Extended Data Fig.1E)

135   and A549 cells (Fig.1D) cells. Furthermore, UV-inactivated PR8 virus did not induce

136   *IFI16* expression in A549 cells compared with live virus infection (Extended Data

137   Fig.1F-G). Collectively, the findings suggest that *IFI16* is involved in the modulation

138   of influenza virus infection.

139   In uninfected cells, IFI16 was mainly localized in the nucleus; PR8 infection

140   induced accumulation of IFI16 in the nucleus and cytoplasm (Fig.1E-F). Consistent

141   with the immunostaining results, PR8 virus infection caused build-up of IFI16 protein

142   in the cytoplasm and nucleus (Extended Data Fig.1H). And treatment of A549 cells

143   with human IFN-γ or Poly(I:C) also induced IFI16 accumulation in the nucleus and

144   cytoplasm (Extended Data Fig.1I-K). It was reported that acetylation of IFI16

145   modulates its cellular distribution and cytoplasm translocation in DNA virus

146   infection[25,36]. During IAV infection, we also detected the acetylation of IFI16 in PR8

147   virus-infected A549 cells at 12 and 24 hpi (Fig.1G, Extended Data Fig.1L).

148   Additionally, low levels of nuclear acetylated IFI16 PLA dots were detected in

149   uninfected A549 cells (Fig.1H). In contrast, acetylated IFI16 dots were clearly

150   elevated in the nucleus and cytoplasm at 24 hpi (Fig.1H). We found that IAV-induced

151   accumulation of IFI16 into the cytoplasm was abolished in C646-treated cells

152   (Extended Data Fig.1M). Collectively, *IFI16* is highly upregulated during IAV

153   infection and whose associated acetylation could confer functional modification such

154   as stabilization of IFI16 protein.

IFI16 inhibits IAV infection *in vitro* and *in vivo*

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156   To determine the impact of IFI16 on influenza virus, *IFI16*-Flag vectors were

157   transfected into A549 cells followed by PR8 infection at 1.0 MOI. Overexpression of

158   IFI16 significantly reduced viral titers of PR8 at 18 hpi (Fig.2A) and reduced the

159   expression of viral NP and M1 proteins (Fig.2B). Increasing the amounts of

160   transfected *IFI16*-Flag vectors in HEK293T-Gluc cells resulted in impaired viral

161   replication in a dose-dependent manner (Fig.2C). In addition, overexpression of IFI16

162   inhibited the expression of mRNA and vRNA of NP and M1 genes in A549 cells

163   (Extended Data Fig.2A), and protein levels of NP at 6, 12 and 18 hpi in HEK293 cells

164   (Extended Data Fig.2B). Conversely, when *IFI16* was knockdown by siRNA in A549

165   cells, there were significant increases in viral protein and titer of progeny viruses

166   (Extended Data Fig.2C-D). To investigate the function of endogenous IFI16 during

167   IAV infection, *IFI16$^{+/+}$* and *IFI16$^{-/-}$* A549 cells were infected with PR8 virus. Infected

168   *IFI16$^{-/-}$* A549 cells produced higher viral titers at 12 and 24 hpi (Fig.2D) and protein

169   levels of viral NP and M1 at 12 and 18 hpi (Fig.2E). In a gain-of-function experiment,

170   exogenous expression of IFI16 in *IFI16$^{-/-}$* A549 cells effectively reduced the

171   expression of viral NP and M1 proteins at 12 and 18 hpi (Fig.2F). Accordingly, we

172   found that the replication of GFP-tagged PR8 virus was markedly increased in *IFI16$^{-/-}$*

173   A549 cells compared with *IFI16$^{+/+}$* A549 cells (Fig.2G). Collectively, these findings

174   indicate that IFI16 inhibits influenza virus replication in human cells.

175   Additionally, PR8-infected WT mice suffered significantly less weight loss than

176   *p204*-deficient (KO) mice, and started to regain body weight by 8 dpi (Fig.3A).

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177   Consequently, survival rate of PR8 infected *p204-/-* mice was significantly poorer than

178   infected WT mice (Fig.3B), which suggests that control of IAV infection *in vivo* also

179   requires *p204*. In lung tissues of KO mice, PR8 virus replication at 4 and 6 dpi was

180   higher (Fig.3C), with accompanying greater viral NP mRNA and vRNA levels

181   (Fig.3D), in lung tissues of KO mice than in corresponding WT mice. Moreover, viral

182   NP staining was more intense in lung sections of KO mice at 5 dpi than in

183   corresponding WT mice (Fig.3E). Notably, gross- and histo-pathology revealed that

184   lung tissues of PR8 virus-infected KO mice displayed more extensive damage at 3

185   and 5 dpi (Fig.3F), and more severe inflammatory damage at 3 dpi (Fig.3G), than in

186   corresponding WT mice. Accordingly, IL-6 and MCP-1 proteins in BALFs were also

187   significantly higher in PR8-infected WT mice than those found in corresponding

188   *p204-/-* mice (Fig.3H-I). Furthermore, p204 deficiency dramatically inhibited their

189   transcription: *IFN-β*, *viperin*, *OAS1*, *ISG15* and *IL-6* in PR8 virus-infected BMDMs

190   (Fig.3J-N). Therefore, IFI16 is required for host defense against influenza virus

191   infection *in vitro* and *in vivo*.

192   **IFI16 enhances the production of IFN-I in IAV infection**

193   DNA recognition by IFI16 induces the activation of STING-TBK1-IRF3

194   pathway, leading to the induction of IFN-I[21,24,26]. To investigate innate immune

195   activation by IFI16, we performed transcriptomic analysis in *IFI16+/+* and *IFI16-/-*

196   A549 cells infected with PR8 virus for 12 h. In GO-term enrichment analysis, 10

197   enriched terms identified were related to antiviral responses (Fig.4A). The number of

198   genes associated with individual terms as well as enrichment probability were lower

199   in *IFI16^{-/-}* A549 cells (Fig.4A). Sixteen ISGs were found to be exclusively

200   upregulated in *IFI16^{+/+}* cells during IAV infection, while only 4 ISGs were

201   upregulated in *IFI16^{-/-}* A549 cells (Fig.4B). Collectively, these results show that the

202   induction of ISGs was reduced in the absence of IFI16. We next evaluated the

203   function of IFI16 in the induction of IFN-I. RT-qPCR analysis showed that

204   overexpression of *IFI16* enhanced PR8 virus-induced expression of *IFN-β*, *ISG15* and

205   *IL-6* at 6 and 12 hpi (Extended Data Fig.3A-C). Conversely, IFI16 deficiency greatly

206   reduced the PR8 virus-induced expression of *IFN-β*, *RIG-I*, *ISG56*, and *viperin*

207   (Fig.4C-F) as well as *IFN-α4*, *IRF7* and *CXCL5* (Extended Data Fig.3D-F) in A549

208   cells. Furthermore, overexpression of IFI16 in A549 cells increased PR8

209   virus-induced IFN-β protein production at 12 and 18 hpi in a dose-dependent manner

210   (Fig.4G), while IFI16 deficiency markedly decreased IFN-β production (Fig.4H),

211   which suggest that IFI16 mediates antiviral effects through influenza virus-induced

212   IFN-I signaling.

213   To examine the contribution of IFI16 to the RIG-I signaling, we transfected

214   *IFI16*-Flag or control plasmids into A549 cells followed by infection with PR8 virus

215   at 1.0 MOI. Overexpression of IFI16 in infected cells enhanced protein detection of

216   RIG-I, phosphorylated (p)-TBK1, p-IRF3 and p-P65 relative to infected controls

217   (Fig.4I). In contrast, deficiency of IFI16 in infected cells led to reduced protein

218   detection of RIG-I, p-TBK1 and p-IRF3 compared with the *IFI16^{+/+}* group (Fig.4J).

219   Consistent with these results, overexpression of IFI16 in the presence of 5'ppp-RNA

220   also increased protein detection of RIG-I, p-TBK1 and p-IRF3 (Extended Data

221   Fig.3G). Furthermore, exogenous expression of IFI16 in infected *IFI16*^{-/-} A549 cells

222   restored the protein expression of RIG-I, p-TBK1 and p-IRF3 (Extended Data Fig.3H).

223   Immunofluorescence results showed that IFI16 deficiency reduced the level of p-IRF3

224   (Ser396) in nuclear in PR8 virus-infected A549 cells (Fig.4K-L). Taken together,

225   these findings indicate that IFI16 is a potent stimulator of the IFN-I response in

226   influenza virus infection.

227   **IFI16 transcriptionally upregulates the expression of *RIG-I***

228   Overexpression of IFI16 progressively upregulated RIG-I protein expression in

229   A549 cells in a time-dependent manner (Fig.5A) and in a dose-dependent manner

230   (Extended Data Fig.4A). HEK293 cells co-transfected with *IFI16*-Myc and

231   *RIG-I*-Flag followed by cycloheximide (CHX) treatment did not exhibit evidence of

232   RIG-I protein degradation (Extended Data Fig.4B). And overexpression of IFI16 also

233   increased protein and mRNA expression of RIG-I in *ifnar1*^{-/-}A549 cells in a

234   dose-dependent manner (Extended Data Fig.4C-4D). Furthermore, A549 cells

235   transfected with *IFI16*-Flag vectors showed rising levels of *RIG-I* mRNA in a

236   vector-dose dependent manner (Extended Data Fig.4E) and a progressive

237   time-dependent manner (Fig.5B). Consistent with the findings in A549 cells, mRNA

238   expression of *RIG-I* in PR8 virus infected lung tissues of *p204*-deficient (KO) mice

239   was significantly reduced at 1 and 3 dpi compared with corresponding WT mice

240   (Extended Data Fig.4F). Taken together, our results indicate that IFI16 upregulates

241   *RIG-I* from the transcriptional level.

242   To further assess the role of IFI16 in the regulation *RIG-I*, luciferase reporter

243 vector driven by a *RIG-I* promoter was used in transfections of HEK293 cells. The

244 *RIG-I* promoter was responsive to IFI16 overexpression in a vector-dose dependent

245 manner (Fig.5C), which indicates that IFI16 is capable of transactivating the *RIG-I*

246 promoter.

247 　　To determine the minimum promoter length of *RIG-I* that is responsive to the

248 IFI16 induction, an extensive series of *RIG-I* promoter deletion constructs, each

249 spliced to luciferase reporter gene, were generated for co-transfections with increasing

250 amounts of *IFI16*-Flag expression vectors (Fig.5D-I). The minimum *RIG-I* promoter

251 responsive to *IFI16*-Flag overexpression was found to be between -371 to -360 bp in

252 length (Fig.5I). Furthermore, Flag antibody pull down experiments, based on a series

253 biotinylated double-stranded DNA probes that spanned the minimum promoter

254 section (Extended Data Fig.4G), found that only probe p2-mut4 which harbored

255 mutations between the -371 to -360 region of the promoter failed to bind IFI16-Flag

256 (Extended Data Fig.4H). ChIP-qPCR assays, using two sets of primer pairs that target

257 the -500 to -250 bp region of *RIG-I* promoter, further demonstrated direct binding of

258 IFI16 to the *RIG-I* promoter (Fig.5J). Collectively, IFI16 is capable of binding to the

259 *RIG-I* promoter to promote *RIG-I* transcription.

260 　　Next, to determine whether IFI16 affects the recruitment of RNA Pol II to the

261 RIG-I promoter, chromatin immunoprecipitation was performed with Pol II antibody

262 on *IFI16*^{+/+} and *IFI16*^{-/-} A549 cells, infected with PR8 virus at 1.0 MOI for 0 and 12 h.

263 qPCR targeting the detection of the basal promoter site of *RIG-I* showed more RNA

264 Pol II binding to the basal promoter of infected *IFI16*^{+/+} than infected *IFI16*^{-/-} A549

265 cells (Fig.5K), but no significant difference on the GAPDH promoter (Extended Data

266 Fig.4I), suggesting that during infection IFI16 facilitates RNA Pol II recruitment to

267 the basal promoter of *RIG-I*.

268   To investigate the domain(s) in IFI16 responsible for activating *RIG-I*

269 transcription, we constructed *IFI16* expression mutants bearing different domain

270 deletions (Extended Data Fig.4J). Mutants without the HINa domain completely lost

271 the ability to activate the *RIG-I*-luc reporter gene (Fig.5L). In a separate experiment,

272 we also found that in the absence of the HINa domain there was no binding of IFI16

273 to biotinylated *RIG-I* promoter (Extended Data Fig.4K). Deletion of HINa domain in

274 IFI16 abrogated its antiviral activity in response to PR8 virus infection (Extended

275 Data Fig.4L). Taken together, IFI16 binds *RIG-I* promoter with its HINa domain

276 facilitating the recruitment of RNA Pol II to the site, thereby enhancing the

277 transcriptional activation of *RIG-I*.

278 **IFI16 binds vRNAs and interacts with RIG-I**

279   Since IFI16 is also a RBP, we determined if IFI16 binds vRNAs as with *RIG-I*

280 promoter. Pull down assays using cell lysates of HEK293 cells, separately

281 overexpressing Flag-tagged IFI16, RIG-I, MDA-5 and TBK1 proteins, incubated with

282 biotinylated NP vRNA showed binding of IFI16, RIG-I and MDA-5 to the vRNAs

283 (Extended Data Fig.5A). MST results showed that the HINa domain also exhibited

284 high affinity for full-length NS vRNA (Extended Data Fig.5B). To further

285 demonstrate binding between IFI16 and other IAV RNAs, purified GST-IFI16

286 proteins were incubated with fluorescein-labeled HA, NP, PA and PB2 vRNAs. MST

287   assays also showed IFI16 binding with the different vRNAs (Extended Data Fig.5C).

288   Furthermore, RNA FISH analysis revealed increasing co-localization of IFI16 with

289   NP vRNA in PR8 virus-infected A549 cells at 6 and 12 hpi (Fig.6A). To identify

290   bound vRNAs during infection, RNA eluted from co-precipitation of *IFI16*-Flag from

291   IAV-infected *RIG-I*$^{-/-}$ cells was subjected to deep sequencing analysis which detected

292   IFI16 binding to all eight IAV gene segments (Fig.6B). RIG-I has been found with

293   IAV genomic fragments[37]. IFI16 was also specifically enriched in genomic IAV

294   segments during infection (Fig.6B). Importantly, only wild-type IFI16 and IFI16

295   mutants bearing the HINa domain associated with vRNA fragments during infection

296   (Fig.6C, Extended Data Fig.5D-E). Taken together, these results indicate that during

297   infection IFI16 can directly bind viral genome RNAs via its HINa domain.

298   To determine if IFI16 is involved in the recognition of vRNAs by RIG-I during

299   influenza virus infection, co-IP assays were performed that demonstrated the

300   endogenous interaction of IFI16 and RIG-I during PR8 virus infection in A549 cells

301   (Fig.6D-E). Furthermore, *in situ* PLA microscopy showed co-localization of IFI16

302   and RIG-I in the cytoplasm of PR8 virus-infected A549 cells (Fig.6F). Consistent with

303   PLA results, confocal microscopy also found increasing interaction between IFI16

304   and RIG-I during IAV infection (Extended Data Fig.5F). The PYRIN domain can

305   mediate the interaction between IFI16 and host proteins[38,39]. The IFI16-ΔPYRIN-GFP

306   mutant was unable to co-localize with RIG-I in HEK293 cells, indicating that the

307   PYRIN domain is required in the protein-protein interaction (Extended Data Fig.5G).

308   Interestingly, *PYRIN*-GFP transfection into HEK293 cells resulted in its protein

309 co-localization with RIG-I that formed filamentous structures (Extended Data Fig.5H

310 -I). IFI16 filamentous structures are involved in antiviral responses by associating

311 with host restriction factors[40-42]. Collectively, these findings indicate that IFI16 binds

312 influenza viral RNAs and associates with RIG-I in influenza virus-infected cells.

313 **RIG-I is required for IFI16-mediated antiviral response in IAV infection**

314 K63-linked polyubiquitination of RIG-I by TRIM25 is essential for its

315 activation[11]. To explore the potential role of IFI16 in the promotion of RIG-I signaling,

316 we examined the interaction between TRIM25 and RIG-I in PR8 virus-infected

317 *IFI16*$^{+/+}$ and *IFI16*$^{-/-}$ A549 cells. Co-IP and *in situ*-PLA experiments indicated that

318 IFI16 deficiency impaired the interaction and co-localization between RIG-I and

319 TRIM25 due to their reduced expression in infected *IFI16*$^{-/-}$ cells, and inhibited

320 K63-linked ployubiquitination of RIG-I during virus infection (Extended Data

321 Fig.6A-B, 7A). To further determine the impact of IFI16 on RIG-I polyubiquitination,

322 we transfected HEK293 cells with Myc control or *IFI16*-Myc, *RIG-I*-Flag,

323 *TRIM25*-His and either HA-ubiquitin, HA-ubiquitin-K48 or HA-ubiquitin-K63

324 plasmids. Co-IP experiments showed that IFI16 remarkably enhanced the interaction

325 between TRIM25 and RIG-I and promoted RIG-I polyubiquitination in the presence

326 of HA-ubiquitin and HA-ubiqutin-K63, but not HA-ubiquitin-K48 (Extended Data

327 Fig.7B). Thus, IFI16 facilitates TRIM25 binding to RIG-I and consequently

328 promotes K63-linked polyubiquitination of RIG-I. RIP-RT-qPCR assays showed that

329 overexpression of IFI16 significantly enhanced the RIG-I binding to PA vRNA

330 (Extended Data Fig.7C). And correspondingly, IFI16 deficiency also markedly

331   reduced RIG-I binding to PA vRNA in PR8 virus-infected A549 cells (Extended Data

332   Fig.7D). RIP-EMSA and RNA pull-down experiments further demonstrated that the

333   binding of PA vRNA with RIG-I is sharply increased with increasing amount of IFI16

334   (Extended Data Fig.6C-6D). Furthermore, RNA co-purified with RIG-I by RIP

335   analysis from IAV-infected *IFI16*$^{+/+}$ cells could induce the production of IFN-β more

336   effectively than that from *IFI16*$^{-/-}$ cells (Extended Data Fig.7E-F), suggesting that

337   IFI16 facilitates the stable binding of RIG-I to vRNAs during IAV infection.

338   Importantly, only wild-type IFI16 and IFI16 mutants bearing HINa and PYRIN

339   domain, but not HINb, promoted vRNA binding to RIG-I (Extended Data Fig.6E) and

340   enhanced the production of IFN-β during IAV infection (Extended Data Fig.6F).

341   Consistent with these observations, IFN-β reporter assays showed that overexpression

342   of IFI16 significantly enhanced PR8 virus-induced expression of IFN-β-luciferase

343   reporter genes (Extended Data Fig.7G). Moreover, RIG-I-induced activity of

344   IFN-β-luciferase reporter rose with increasing amounts of IFI16 expression vectors

345   used in transfections (Extended Data Fig.7H). Taken together, these results suggest

346   that IFI16 promotes the activation of RIG-I signaling and in so doing, boosts the

347   production of IFN-I.

348   Finally, to investigate whether antiviral function of IFI16 in IAV infection is

349   dependent on RIG-I, A549 cells were transfected with the *RIG-I-* or *MAVS*-targeting

350   siRNAs and then infected with IAV. Western blotting showed that the level of RIG-I

351   or MAVS was significantly reduced by *RIG-I*-targeting siRNA#2835 (Extended Data

352   Fig.7I) or *MAVS*-targeting siRNA#571 transfection (Extended Data Fig.6G). IAV

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353   infection could not effectively stimulate the production of IFN-β in *RIG-I-* or

354   *MAVS*-silenced *IFI16$^{+/+}$* A549 cells (Extended Data Fig.6H, 7J). Overexpression of

355   IFI16 in *RIG-I-* or *MAVS*-silenced A549 cells could not induce the production of

356   IFN-β during IAV infection (Extended Data Fig.6I, 7K). Additionally, *RIG-I$^{-/-}$*

357   HEK293 cells were transfected with *IFI16*-Flag or Flag vectors and then infected with

358   PR8 virus at 1.0 MOI. Overexpression of IFI16 failed to inhibit viral NP and M1

359   replication in PR8 virus-infected *RIG-I$^{-/-}$* cells at 6 and 12 hpi (Extended Data Fig.7L),

360   whereas exogenous expression of RIG-I in *RIG-I$^{-/-}$* cells rescued the inhibitory effect

361   of IFI16 on viral titers at 24 hpi (Extended Data Fig.7M). Taken together, our results

362   indicate that the antiviral function of IFI16 is dependent on RIG-I.

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375   **Discussion**

376   Host cells possess critical sensors that can discriminate viral and host nucleic

377   acids. IFI16 has been demonstrated to be a sensor of viral DNA in innate immune

378   signaling[16,17]. However, its role in sensing viral RNA during infection, in particular

379   influenza virus infection, hitherto is unknown. In this study, we demonstrated that

380   IFI16 is a viral RBP and is induced during influenza virus infection. IFI16 inhibited

381   influenza virus replication in human cell lines and in mice through enhanced the

382   induction of IFN-I. IFI16 enhances the transcription of *RIG-I*, binds viral RNAs and

383   interact with RIG-I protein, thereby increasing the sensitivity of RIG-I signaling.

384   These findings establish a critical role for IFI16 in antiviral innate immune response

385   to influenza virus and possibly other RNA viruses (a proposed signaling model is

386   depicted in Extended Data Fig. 8).

387   We found that IFI16 was strongly induced by IAV, which is consistent with

388   previous findings that IFI16 could be induced by RNA viruses infection and be

389   upregulated by the type I and type III interferons as an ISG[27-29]. IFI16 is known to

390   boost sensing of intracellular DNA and subsequent IFN-I induction[21,24,26]; it functions

391   as a cytoplasmic immune sensor-mediating pyroptotic death of tissue CD^{4+} T cells

392   abortively infected with HIV-1[22]. Thus, the main role of IFI16 had been assumed to be

393   a cytoplasmic sensor of viral DNA[43]. However, this is at odds with the findings that

394   IFI16 is predominantly localized in the nucleus[36]. We found that IFI16 is induced in

395   the nucleus and acts as a positive transcriptional regulator of *RIG-I* during IAV

396   infection. These results confirm previous findings that IFI16 regulates RNA

397 virus-mediated IFN-I responses as a transcriptional factor [27]. We also found that IFI16

398 was induced in the cytoplasm, sensed and interacted with influenza viral RNAs,

399 which is consistent with recent findings that IFI16 directly binds to incoming

400 Chikungunya virus genome RNA and acts as a PRR[30]. Overexpression of IFI16 in

401 cells significantly increased IFN-β expression and reduced in viral titers, whereas

402 knockdown or knockout of *IFI16* had the opposite effects, suggesting IFI16 is an

403 important antiviral factor in IAV infection. Therefore we demonstrate an important

404 mechanism of IFI16 in RLR-mediated antiviral innate immune response to influenza

405 virus and further our understanding of the role of IFI16 in innate immunity.

406 Viral RNA sensor RIG-I is thought to be the most important sensor of influenza

407 virus infection[9] in the recognition of cytoplasmic dsRNA leading to the transcriptional

408 activation of IFN-I and downstream ISGs[14]. Thus, identification of positive regulators

409 of RIG-I could be important in the control of virus infection. DNA-dependent

410 activator of IFN-regulatory factor (DAI), a cytosolic DNA sensor, has already been

411 found to recognize genomic RNA and regulate virus-induced cell-death pathways and

412 thereby plays an important role in the pathogenesis of IAV infection[44,45]. Here, we

413 demonstrated that IFI16 binds to viral RNAs and enhances RIG-I-mediated

414 production of IFN-I during IAV infection. We further identified that IFI16 interacted

415 with RIG-I that involves the PYRIN domain of IFI16. An interaction of IFI16 with

416 RIG-I as a complex via siRNA was previously reported[46]. Notably, we found that

417 IFI16 facilitates RIG-I binding to IAV vRNAs during infection and promotes

418 virus-induced K63-linked polyubiquitination of RIG-I, indicating that IFI16 positively

419   contributes to RIG-I-dependent antiviral responses. Presently, it is not clear if the

420   filamentous structures of IFI16 are necessary for IFI16-vRNAs and IFI16-RIG-I

421   interactions, or about the types of viral RNA structures that are needed to interact with

422   IFI16.

423   Collectively, our study shows that IFI16 induced by influenza virus infection

424   positively regulates the RIG-I signaling by enhancing its transcriptional expression

425   through recruitment of RNA Pol II to the RIG-I promoter, and sensing of viral RNA to

426   promote virus-induced K63-linked polyubiquitination of RIG-I. This study highlights

427   an important mechanism of IFI16 in RLR-mediated innate antiviral immune response

428   to IAV and possibly other RNA viruses infection, and expands our understanding of

429   the functions of the innate immune system in intracellular virus recognition which

430   could help to develop new strategies to modulate antiviral responses.

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Materials and Methods

Ethics statement

All animal experiments were performed in accordance with institutional guidelines of China Agricultural University (CAU) (approval SKLAB-B-2010-003) and approved by the Beijing Association for Science and Technology of China (approval SYXK, Beijing, 2007-0023).

Cells

Madin-Darby canine kidney cells (MDCK), human embryonic kidney cells (HEK293), and human lung adenocarcinoma epithelial cells (A549) were maintained in-house. THP-1 cell line was kindly provided by Dr. Shijun Zheng (China Agricultural University). $IFI16^{-/-}$ A549 and $RIG-I^{-/-}$ HEK293 cell lines were generously given by Dr. Yu Chen (Sun Yat-sen University) and Dr. Wenjun Liu (Institute of Microbiology, Chinese Academy of Sciences), respectively. $Ifnar1^{-/-}$ A549 cell line was generously given by Dr. Ying Zhu (Wuhan University). And 2fTGH-ISRE cell line was kindly provided by Dr. Fuping You (Peking University)[47]. HEK293T-Gluc cells were kindly provided by Dr. Shan Cen (Chinese Academy of Medical Sciences and Peking Union Medical School)[48]. Primary bone marrow-derived macrophages (BMDMs) were produced as described[49]. Cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin.

Viruses

Influenza A/Puerto Rico/8/1934 (PR8; H1N1) strain was maintained in our

463 laboratory. PR8-GFP virus was generated by insertion of GFP CDS sequences at the

464 carboxyl terminal of NS1 as described previously[50]. The methods of cell culture, mice

465 infection, and virus titration in lung tissues were performed as previously described[49].

466 **Plasmids construction and transfection**

467 *IFI16*, *RIG-I*, *TBK1*, *TRIM25* and *MAVS* genes were amplified by PCR using

468 PR8 virus-infected THP-1 cells at 8 hours post-infection (hpi). Full-length and

469 mutated IFI16 expression constructs were generated using PRK5 containing different

470 tags or pCDNA3.1-GFP vectors by recombinase-mediated recombination. Full-length

471 RIG-I expression vectors were created using PRK5-Flag or pCDNA3.1-mCherry

472 vectors. Full promoter sequences or truncations of RIG-I were generated using

473 PGL3.0 luciferase reporter (Promega). Plasmids transfection experiments in HEK293

474 or A549 cells were performed using Lipofectamine 3000 reagents (Invitrogen).

475 *IFI16*$^{+/+}$ or *IFI16*$^{-/-}$ A549 cells were transfected with indicated siRNAs using

476 Lipofectamine RNAiMAX reagents (Invitrogen, Carlsbad, CA, USA).

477 ***In vivo* virus infection**

478 p204-deficient (*p204*$^{-/-}$) mice were kindly provided by Dr. Wei Tang (Shandong

479 University). Sex- and age-matched C57BL/6 mice were purchased from department

480 of Laboratory Animal of Charles River, Beijing. Seven-weeks-old mice were

481 inoculated intranasally with PR8 virus at a dose 50 $TCID_{50}$ in 50 μL of

482 phosphate-buffered saline (PBS). Body weight and survival were monitored daily

483 after infection. Lung tissue lysates were generated by homogenizing snap-frozen lung

484 tissues 2 times (20 second each time) in MEM medium, and centrifuging the lung

485 suspensions at 2000 rpm for 15 min. $TCID_{50}$ assays were performed on MDCK cells

486 and $TCID_{50}$ values were calculated as previously described[49]. WT and $p204^{-/-}$ mice

487 were inoculated intranasally with PR8 virus at a dose 100 $TCID_{50}$. The

488 bronchoalveolar fluid (BALF) was collected at 0 and 3 dpi. The BD™ CBA Mouse

489 Inflammation Kit (BD Biosciences, #552364) was used to quantitatively measure the

490 IL-6 and MCP-1 levels in BALF.

491 **Antibodies and reagents**

492 Anti-IFI16 (ab191211), anti-TBK1 (ab40676), anti-NP (ab104870), anti-TBK1

493 (ab40676), anti-Ubiquitin (linkage-specific K63) (ab271929) and anti-RNA

494 polymerase II (ab5095) antibodies were from Abcam. Anti-IFI16 (sc-8023) and

495 anti-TRIM25 (sc-166926) antibodies were from Santa Cruz. Rabbit anti-p65

496 (#10745-1-AP), anti-HA (#66006-2-Ig) and anti-RIG-I (#20566-1-AP) antibodies

497 were from Proteintech. Anti-IRF3 (YT2398) antibody was from ImmunoWay

498 Biotechnology Company. Anti-Phospho-TBK1 (Ser172; #5483); anti-Phospho-IRF3

499 (Ser396; #4947), anti-IFI16 (#14970S), anti-FLAG (#8146), anti-His (#9991)

500 anti-Myc (#2278); anti-Phospho-p65 (Ser536; #3033) antibodies were from Cell

501 Signaling Technology Inc.; Poly(I:C) (TLRL-PIC) was from InvivoGen. Recombinant

502 human interferon-gamma (IFN-γ) (HY-P7025) and C-646 (HY-13823, 10 mg) was

503 from MedChemExpress.

504 *In vitro* **transcription and biotin-labeling RNA purification**

505 Templates for T7 RNA transcription were linearized from H7N9 pSPT9 plasmids

506 coding for individual RNA segments of H7N9 virus. T7 transcription reactions were

507   carried out with T7 RNA polymerase in transcription buffer and biotin-dNTPs mix

508   according to manufacturer's instructions (Promega)[51]. Following DNase I treatment,

509   biotin-labeled vRNAs were extracted with phenol/chloroform, ethanol precipitated

510   and purified with RNAeasy columns (Aidlab Biotechnologies) and analyzed on

511   denaturing agarose gels for correct size.

512   **Affinity purification coupled with mass spectrometry (AP-MS)**

513   THP-1 cells were treated with phorbolmyristateacetate (PMA) for 12 h, and

514   infected with H7N9 virus (at 1 MOI). After 12 h infection, cells were lysed with lysis

515   buffer and incubated with *in vitro* transcribed 2 pM biotin-labeled viral RNAs for 4 h

516   followed by incubation with pre-washed Dynabeads M-280 Streptavidin (Sigma) for 3

517   h at 4°C. Beads were washed five times with NT2 buffer (50 mM Tris-HCl pH 7.0,

518   150 mM NaCl, 1 mM $MgCl_2$, 0.05% NP-40); protein complex bound to the beads was

519   then boiled with 20 μL PBS at 100°C for 10 min. Pulled-down proteins were

520   identified by LC-MS.

521   **Western blotting**

522   Western blotting was performed as previously described[49]. Briefly, protein

523   samples were mixed with loading buffer supplemented with 10% β-mercaptoethanol,

524   heated at 95°C for 5 min, and separated on a 10% SDS-PAGE under reducing

525   conditions. After electrophoresis, protein samples were electroblotted onto

526   polyvinylidene difluoride (PVDF) membranes (BioRad), and blocked for 2 h in

527   Tris-buffered saline (10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl) containing

528   5% (w/v) non-fat dry milk and 0.5‰ (v/v) Tween-20. The blots were incubated with

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529   the primary antibodies overnight at 4°C. The next day, the blots were incubated with

530   corresponding horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 h

531   at room temperature (RT). HRP antibody binding was detected using a standard

532   enhanced chemiluminescence (ECL) kit (Thermo Scientific).

533   **Quantitative real-time RT-PCR (RT-qPCR)**

534   Total RNA from virus-infected cells or lung tissues was extracted using an RNA

535   isolation kit (Thermo Scientific). First-strand cDNA was synthesized from 1 μg of

536   total RNA using TransScript RT reagent Kit (TransGen). Uni-12 primer was used for

537   the detection of influenza viral RNA and oligo dT and random primers were used for

538   detecting host and viral genes. Generated cDNA was subjected to qPCR in a 25 μL

539   reaction volume using FastStart Universal SYBR Green Master mix (Roche, China).

540   Human *β-actin* and mouse *GAPDH* genes were amplified for normalization of the

541   cDNA amount used in qPCR. Reactions were conducted in triplicates, and the data

542   were analyzed using the $2^{-\Delta\Delta Ct}$ method. qPCR primers used in this study are listed in

543   Table S1.

544   **Type I Interferons bioassay**

545   During PR8 virus infection, IFN-I released in human cell culture media was

546   quantified in2fTGH-ISRE cells as previously described[52]. In brief, 200 mL of cell

547   culture supernatants were incubated with confluent 2fGTH-ISRE-Luci cells in

548   24-well plate for 6 h. Cells were then lysed in passive lysis buffer and subjected to

549   luciferase quantification (Promega). A serial dilution of recombinant human IFN-β

550   (Invitrogen) was used as reference.

Luciferase reporter assays

551   **Luciferase reporter assays**

552   HEK293 cells seeded on 24-well plates were co-transfected with 125 ng of the

553   luciferase reporter plasmids and equal amount of various expression plasmids or

554   empty controls. Ten ng of pRL-TK plasmids were used as an internal control. After 24

555   h of transfection, Rluc and Fluc expression were measured in the presence or absence

556   of virus stimulation for 12 h using the Dual-Luciferase Reporter Assay kit (Promega)

557   in a TD-20/20 Luminometer according to the manufacturers' recommendations.

558   Results were normalized to corresponding control reporter constructs.

559   Gaussia Luciferase reporter assays were performed according to standard

560   procedures described in detail elsewhere[48]. Briefly, HEK293T-Gluc cells were

561   transfected with either *IFI16*-Flag or Flag control plasmids, followed by infection

562   with PR8 virus (MOI = 1) for 24 h, and viral infectivity was evaluated by the Gluc

563   activity. After a further incubation of 24 h, cell supernatants were collected and

564   measured for Gluc activity. Dimethyl sulfoxide (DMSO) was used as negative

565   controls. The inhibition rate of the tested compounds was calculated with the

566   following equation, where RLU indicates relative light unit:

567   Inhibition rate $= (\text{RLU}_{\text{infected cells}} - \text{RLU}_{\text{tested compound}}) / (\text{RLU}_{\text{infected cells}} -$

568    $\text{RLU}_{\text{mock-infected cells}}) \, 100\%$

569   **Histology**

570   WT and *p204*^{-/-} mice were euthanized and sacrificed at the indicated time points

571   after PR8 virus infection. Lung tissues were harvested and fixed with 4%

572   formaldehyde, followed by paraffin embedding. For histopathological analysis, 5- to

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573   7-µm sections were sectioned longitudinally through the left and right lung and

574   stained using a standard hematoxylin and eosin (H&E) protocol.

575   **Immunohistochemistry**

576   Lung sections were deparaffinized with xylene and rehydrated with ethanol

577   gradations and water. Endogenous peroxidase activity was blocked using 3%

578   hydrogen peroxide in methanol. PBS containing 0.05% Tween-20 was used to wash

579   lung tissue sections in between steps. Lung sections were incubated with the primary

580   anti-NP antibody (ab20343) at a 1:100 dilution or with the isotype control at the same

581   concentration at 4°C overnight in a humidified chamber. Sections were subsequently

582   incubated with the horseradish peroxidase-conjugated secondary antibody for 60 min

583   at RT. Immunodetection was performed using the Vector Elite ABC Kit (Vectastain,

584   Vector).

585   **Co-immunoprecipitation (Co-IP)**

586   For Co-IP assays, A549 cells were infected with PR8 virus at an MOI of 5. After

587   infection, cell samples were collected and lysed in 800 µL of IP lysis buffer (Thermo

588   Scientific) containing protease and phosphatase inhibitors. A portion of each whole

589   cell lysate sample was kept to confirm protein expression levels, and 500 µg of cell

590   lysates were used for Co-IP assays. Lysates were incubated with anti-IFI16,

591   anti-RIG-I or IgG antibodies overnight at 4°C under constant rotation, and then 40 µL

592   of protein A/G beads (Santa Cruz) were added and incubated for 2 h at 4°C under

593   gentle rotation. The beads were then washed four times with cold lysis buffer, and

594   analyzed by SDS-PAGE and Western blotting.

RNA immunoprecipitation-qPCR (RIP-qPCR)

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596   Two 10-cm^2 dishes (10^7 cells per dish) of A549 cells were infected with PR8

597 virus for 12 h. Cells were lysed with RIP lysis buffer (50 mM HEPES, 150 mM KCl,

598 2 mM EDTA, 1 mM NaF, 0.5% NP40, 0.5 mM DTT, 1× protease inhibitor cocktail,

599 25 units RNasin) for 30 min at 4°C. Cell lysates were centrifuged at 12,000 rpm for

600 15 min at 4°C and the supernatants were subjected to RNA immunoprecipitation. A 50

601 μL aliquot of cell supernatant was saved as input, and the remaining samples were

602 each incubated with 5 μg anti-IFI16 antibody or IgG antibody and 40 μL protein A/G

603 beads for 10 h at 4°C under gentle shaking. After IP, the beads were pelleted and

604 washed four times with RIP wash buffer (50 mM Tris pH 7.4, 150 mM NaCl, 1 mM

605 MgCl$_2$, 0.05% NP40), resuspended in 250 mL of DNase digestion buffer (40 mM Tris

606 pH 8.0, 10 mM MgSO$_4$, 1 mM CaCl$_2$), and treated with 25U RNasin (Promega) and

607 2U DNase I (NEB) at 37°C for 20 min. Beads were then washed and resuspended in

608 100 μLL RIP wash buffer, 10% of each sample was removed for immunoblot analysis.

609 And samples were treated with 4U proteinase K at 55°C for 30 min. The input and

610 immunoprecipitated RNAs were isolated by 1 mL of TRIzol reagent (Sigma), and

611 viral RNAs were analyzed by RT-qPCR or RNA-seq.

Chromatin Immunoprecipitation-qPCR (ChIP-qPCR)

612

613   Approximately 10^7 treated cells were cross-linked with 1% formaldehyde at RT

614 for 10 min, and the cross-linking was quenched with 0.125 M glycine for 5 min. Cells

615 were then collected by centrifugation, lysed with SDS lysis buffer containing protease

616 inhibitor cocktail, and sonicated to shear the DNA. The sonicated DNA-Protein

617   complexes were incubated with anti-IFI16 (Cell Signaling Technology, #D8B5T),

618   RNA pol-II (abcam, ab5095), or control IgG (Beyotime, #A7016) antibodies. The

619   immuno complexes were collected using Protein G Dynabeads (10004d, Invitrogen).

620   The Dynabeads were washed one time with wash buffer A (20 mM Tris-HCl (pH 8.0),

621   500 mM NaCl, 2 mM EDTA, 0.1% SDS, 1% Triton X-100), one time with wash

622   buffer B (10 mM Tris-HCl (pH 8.0), 250 mM LiCl, 1 mM EDTA, 1%NP-40), and

623   three times with wash buffer C (1 mM EDTA, 10 mM Tris-HCl, pH 8.0). The beads

624   were eluted with 100 mL elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1%

625   SDS), followed by incubation at 65°C overnight to reverse cross-linking. The next day,

626   the DNA was purified with QIAquick PCR purification kit (Magen, D211102) and

627   analyzed using FastStart Universal SYBR Green Master mix (Roche, China).

628   **Confocal microscopy**

629   A549 or HEK293 cells on coverslips were washed twice with pre-warmed PBS

630   and fixed with 4% paraformaldehyde for 15 min at RT. Cells were subsequently

631   permeablized with immunostaining permeabilization buffer containing Triton X-100

632   (Beyotime Biotechnology) for 10 min and blocked in QuickBlock™ Blocking Buffer

633   for 20 min at RT. Fixed cells were incubated with indicated antibodies diluted in

634   immunostaining primary antibody dilution buffer at 4°C overnight. Coverslips were

635   then washed three times with PBS and incubated with Alexa Fluor 488-conjugated

636   secondary antibodies or Alexa Fluor 555-conjugated secondary antibodies for 1 h at

637   37°C. Coverslips were finally washed three times and mounted onto microscope

638   slides with DAPI Staining Solution (Beyotime Biotechnology) for 8 min and

639   examined by confocal microscopy. Immunostained cells were visualized using the

640   Nikon Super-resolution laser scanning confocal microscope under a 100-time oil

641   objective and analyzed by the Imaris 9.2 platform.

642   ***In situ* proximity ligation assay (PLA) microscopy**

643   A DuoLink PLA kit (#DUO92105-1KT, Sigma) was used to test protein-protein

644   interactions as described in the protocol. WT or *IFI16$^{-/-}$* A549 cells were infected with

645   PR8 virus at 1.0 MOI for 12 h, fixed and permeabilized as described in the confocal

646   microscopy section and blocked with DuoLink blocking buffer for 30 min at 37°C.

647   Cells were incubated with corresponding primary antibodies diluted in DuoLink

648   dilution buffer. After washing, cells were incubated with species-specific PLA probes

649   (Plus and Minus) for 1 h at 37°C under hybridization conditions and in the presence

650   of 2 additional oligonucleotides to facilitate hybridization of PLA probes if they were

651   in close proximity (<40 nm). Ligase was then added and incubated for 30 min at 37°C

652   to join hybridized oligonucleotides. Amplification polymerase was added to generate

653   a concatemeric product extending from the oligonucleotide arm of the PLA probe.

654   Finally, a detection solution consisting of fluorescence-labeled oligonucleotides was

655   added, and the labeled oligonucleotides were hybridized to the concatemeric products.

656   Nuclei was stained with Duolink *in situ* mounting medium containing DAPI.

657   **Ubiquitination assay**

658   HEK293 cells were transfected with plasmids encoding *RIG-I*-Flag, *TRIM25*-His

659   with or without coexpression of *IFI16*-Myc or Myc empty vectors, and HA-Ubiquitin

660   (WT) or HA-Ubiquitin mutants (K48 or K63). After 24 h transfection, cells were

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661 harvested and lysed in RIPA buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1%

662 NP-40, 0.1% SDS, and 1 mM EDTA) containing protease inhibitor cocktail and

663 10 μM deubiquitinase inhibitor N-ethylmaleimide (NEM, Sigma). The cell extracts

664 were immunoprecipitated with anti-Flag antibody overnight at 4°C and then beads

665 were added to the samples for 1-1.5 h at 4°C. The beads were washed three times with

666 RIPA buffer and analyzed by immunoblotting with anti-HA antibody.

667 **RNA fluorescence *in situ* hybridization (RNA FISH)**

668 A549 cells were grown in 24-well slide chambers and infected with PR8 at an

669 MOI of 1. At 12 hpi, cells were fixed for 15 min in 4% PFA, then permeablized and

670 dehydrated by sequential 3 min incubations as follows: once with 70% ethanol, once

671 with 85% ethanol, and three times with 100% ethanol. Cells were then hybridized

672 with the Alexa Fluor® 488-conjugated NP RNA target probes (NP-probes,

673 GenePharma) of PR8 virus in hybridization buffer for 10 min at 75°C. Cells were

674 further incubated for 12-16 h at 37°C. Finally, cells were stained with anti-IFI16 and

675 secondary antibodies, and the nuclei was stained with DAPI as previously described.

676 **Microscale thermophoresis technology (MST)**

677 HEK293 cells were separately transfected with the *HINa*-GFP, *HINb*-GFP, and

678 *PYRIN*-GFP expression vectors. After 24 h of transfection, cell lysates were collected

679 and incubated with two-fold serial dilutions of indicated viral RNAs in

680 MST-optimized buffer at a constant concentration (20-100 nM). Equal volumes of

681 binding reactions were mixed by pipetting and incubated for 15 min at RT. Mixtures

682 were enclosed in standard-treated glass capillaries and loaded into the instrument

683 (Monolith NT.115, NanoTemper, Germany). To identify whether IFI16 directly binds

684 to the IAV RNAs, the transcribed RNAs were labeled with fluorescein RNA labeling

685 mix (#41027920, Sigma) and purified as previously described. Purified GST-IFI16

686 proteins (Abcam, ab158724) were incubated with different amounts of IAV full-length

687 fluorescein labeled RNAs, followed by MST assays. For all the measurements,

688 200-1000 counts were obtained for the fluorescence intensity. Kd values were

689 determined with the NanoTemper analysis tool.

690 **RNA pull down assay**

691 HEK293 cells were transfected with Flag-tagged *RIG-I*, *IFI16*, *TBK1* or *MAVS*

692 vectors. After 24 h transfection, cells were collected and lysed with lysis buffer (50

693 mM Tris-HCl pH 7.0, 150 mM NaCl, 1 mM MgCl$_2$, 0.05% NP-40). Cell lysates were

694 mixed with transcribed biotin-labeled viral NP RNA for 4 h at 4°C and incubated with

695 pre-washed Dynabeads M-280 Streptavidin (Sigma) for another 3 h at 4°C. The

696 protein samples bound to the beads were boiled and analyzed by SDS-PAGE and

697 Western blotting.

698 **DNA pull down assay**

699 IFI16 proteins were purified from *IFI16*-Flag overexpressing HEK293 cells by

700 immunoprecipitation using M2 beads (Sigma). Biotinylated mutant DNA probes were

701 synthesized by an EMSA Probe Biotin Labeling Kit (Beyotime, #GS008) and were

702 annealed and incubated with the purified Flag-tagged IFI16 proteins for 30 min in

703 binding buffer (10 mM Tris, 1 mM KCl, 1%NP-40, 1 mM EDTA, 5% glycerol) at RT.

704 Then, 40 µL prewashed Dynabeads M-280 Streptavidin (Sigma) were added for

705 incubation at 4°C for 1 h. The mutant probe-binding proteins were eluted by boiling

706 and analyzed by immunoblotting.

707 **Statistical analysis**

708 For all the bar graphs, data were shown as means ± SEM. All statistical analyses

709 were performed using GraphPad Prism software version 7.00 (GraphPad Software

710 Inc., USA). Kaplan-Meier method was employed for survival analysis. Differences in

711 means were considered statistically significant at $p < 0.05$. And significance levels are

712 as follow: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; NS., non-significant.

713 **Reporting Summary**

714 Further information on research design is available in the Nature Research

715 Reporting Summary linked to this article.

716 **Data Availability**

717 Mass spectrometry proteomics data have been deposited with the

718 ProteomeXchange Consortium via the PRIDE[53] partner repository

719 (https://www.ebi.ac.uk/pride/) with the dataset identifiers PXD020723 and

720 10.6019/PXD020723. The accession number for RNA-seq data: GSE157609,

721 GSE158122.

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727 **Author contributions**

728 Z.M.J and F.H.W performed and analyzed most of experiments; Y.Y.Z, T.W, and

729 W.H.G performed affinity purification coupled with mass spectrometry analysis; S.F.Y,

730 H.L.S, J.P, Y.P.S, M.Y.W, and Q.T, generated biochemical reagents; C.J.G and K.C.C

731 guided and analyzed the data; F.H.W and J.H.L conceived and supervised the study.

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749   **Competing interest declaration**

750   The authors declare no competing interests.

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Figure legends

794 **Figure 1. IFI16 is induced by IAV infection and involved in the pathogenesis of**

795 **virus infection.**

796 (A) Schematic representation of the viral RNA pull down-MS approach to identify

797 viral RNA-binding proteins in THP-1 cells. (B) Protein domain types significantly

798 enriched among the human interacting proteins with indicated vRNA baits. The

799 value range of Q value is [0,1]. The closer it is to zero, the more significant the

800 enrichment is. Domains with $Q \leq 0.05$ are defined as domains that are significantly

801 enriched in differentially genes. (C) mRNA expression levels, determined by

802 RT-qPCR, of 70 candidate viral RNA-binding proteins in THP-1 cells infected with

803 PR8 virus at 2.0 MOI. RNA samplings were taken at 0, 6, 12 and 24 hpi. (D) IFI16

804 protein expression in PR8 virus-infected (1.0 MOI) A549 cells was evaluated by

805 Western blotting at 0, 6, 12, 18 and 24 hpi. β-Actin detection used as loading control.

806 (E) Intracellular localization of IFI16 was assessed in PR8 virus-infected A549 cells at

807 0, 6, 12 and 18 hpi by confocal microscopy. Scale bars, 5 μm. (F) Quantification of

808 intracellular localization of IFI16 in cells as in E. The data represent means ± SD.

809 (n= 3 independent experiments). (G) A549 cells were infected with PR8 virus at 0, 12

810 and 24 hpi. Cell lysates were then immunoprecipitated with anti-IFI16. Bound

811 proteins were analyzed by immunoblots with anti-acetylated lysine. (H) A549 cells

812 were pre-incubated with C-646 for 2 h, infected with PR8 virus for 1 h, washed with

813 PBS and incubated in complete medium with or without C-646. *In situ* PLA assay

814 used to assess acetylation of IFI16 with anti-IFI16 and anti-acetylated lysine

815 antibodies on 24 hpi. Scale bars, 28.7 μm (top) and 14.6 μm (bottom). (B): the results

816 were assessed using a parametric paired t-test (Student's one-tailed t-test). (D) to (H):

817 Data presented are representative of three independent experiments. Statistical

818   significance in (F) was determined by unpaired two-tailed Student's t-test.

819   **Figure 2. IFI16 expression inhibits IAV infection *in vitro*.**

820   (A) A549 cells were transfected with *IFI16*-Flag plasmids or control for 24 h and then

821   infected with PR8 virus at 1.0 MOI for 18 h. Viral titers were measured by $TCID_{50}$

822   assay. Data presented as means ± SEMs and are representative of three independent

823   experiments. (B) A549 cells were transfected with *IFI16*-Flag plasmids or control for

824   24 h and then infected with PR8 virus at 1.0 MOI. NP and M1 proteins were detected.

825   Data were quantified and shown as the ratio of NP to β-actin and M1 to β-actin (right),

826   the data represent means ± SEMs (n = 3). (C) HEK293T-Gluc cells were transfected

827   with different amounts of *IFI16*-Flag or control, followed by infection with PR8 virus

828   (MOI = 1) for 24 h. Viral infectivity was determined. Data presented as means ±

829   SEMs and are representative of three independent experiments. (D) Viral titers in PR8

830   virus-infected (1.0 MOI) $IFI16^{+/+}$ and $IFI16^{-/-}$ A549 cells were measured by $TCID_{50}$

831   assay. Data presented as means ± SEMs and are representative of three independent

832   experiments. (E) The NP and M1 protein expression in 1 MOI of PR8 virus-infected

833    $IFI16^{+/+}$ and $IFI16^{-/-}$ A549 cells was analyzed. Data were quantified and shown as the

834   ratio of NP to β-actin and M1 to β-actin (right), the data represent means ± SEMs (n

835   = 3). (F) $IFI16^{+/+}$ and $IFI16^{-/-}$ A549 cells were transfected with *IFI16*-Flag plasmids or

836   empty control for 24 h and then infected with PR8 virus at 1.0 MOI. NP and M1

837   proteins in virus-infected A549 cells was detected. Data were quantified and shown as

838   the ratio of NP to β-actin and M1 to β-actin (right) the data represent means ± SEMs

839   (n = 3). (G) Fluorescence microscopy images of viral replication (green) in $IFI16^{+/+}$

840   and $IFI16^{-/-}$ A549 cells after infection with GFP-PR8 virus for 12 h. Scale bar,

841   200 μm. (B) and (D) to (G): data are representative of three independent experiments.

842   Statistical significance in (A) to (F) was determined by unpaired two-tailed Student's

843   t-test.

844   **Figure 3. *p204*-deficient mice are susceptible to IAV infection.**

845   (A) WT and *p204*$^{-/-}$ mice (n = 6) were infected with 50 TCID$_{50}$ of PR8 virus. Changes

846   in body weight were monitored daily. Data presented as means ± SD. (B) Survival of

847   WT and *p204*$^{-/-}$ mice infected with 50 TCID$_{50}$ of PR8 virus. Data presented were

848   pooled from three independent experiments. Kaplan-Meier Survival Curves were

849   compared using the log-rank (Mantel-Cox) analysis. (C) Viral titers in lung tissues

850   from 50 TCID$_{50}$ of PR8 virus-infected WT and KO mice at 4 and 6 dpi were

851   determined by EID$_{50}$ assay. Data are from three independent experiments with n = 6

852   mice per group run in triplicate. Error bars indicate SEM. (D) Viral NP mRNA and

853   vRNA in lung tissues of PR8 virus-infected WT and KO mice at 3 dpi were

854   determined by RT-qPCR. Data presented as means ± SEMs and are representative of

855   three independent experiments. (E) Viral NP protein expression in lung tissue sections

856   from virus-infected WT and KO mice (n =3) mice was examined by

857   immunohistochemistry. Representative sections of one mouse out of three are shown.

858   Scale bars, 800 μm (top) and 80 μm (bottom). (F) Gross lesion of lung tissues from

859   virus-infected WT and *p204*$^{-/-}$ mice at 3 and 5 dpi. Representative sections of one

860   mouse out of three are shown. (G) H&E staining of lung tissues from virus-infected

861   WT and *p204*$^{-/-}$ mice at 3 dpi. Scale bars, 600 μm (left) and 120 μm (right).

862   Representative of H&E staining images from 6 mice per group of three independent

863   experiments. (H) to (I): IL-6 and MCP-1 levels in BALF from virus-infected WT and

864   KO mice (n = 4) were quantified. Data are presented as means ± SD. (J) to (N) mRNA

865   expression of *IFN-β*, *viperin*, *OAS1*, *ISG15*, and *IL-6* in BMDMs of virus-infected

866   WT and KO mice was determined by RT-PCR. Data presented as means ± SEMs and

867   are representative of three independent experiments. Statistical significance in (A), (C)

868   to (D) and (H) to (N) was determined by unpaired two-tailed Student's t-test. ns =

869   non-significant.

870   **Figure 4. IFI16 enhances RIG-I-mediated production of IFN-I during IAV**

871   **infection.**

872   (A) GO-term analysis of up-regulated host proteins in IAV-infected $IFI16^{+/+}$ and

873   $IFI16^{-/-}$ A549 cells. Top 10 GO-terms ordered by enrichment p-values are shown.

874   p-values as indicated in legend and indices in cells correspond to number of protein

875   groups associated to individual GO-terms in respective comparison. (B) Differential

876   expression of host genes between IAV-infected and mock conditions in $IFI16^{+/+}$ (X

877   axis) and $IFI16^{-/-}$ (Y axis). Proteins exhibiting significant differential response are

878   highlighted in green (ISGs) or yellow (non-ISGs). (C) to (F): Gene expression of

879   $IFN\text{-}\beta$ (C), $RIG\text{-}I$ (D), $ISG56$ (E), $viperin$ (F) in PR8 virus-infected $IFI16^{+/+}$ and

880   $IFI16^{-/-}$ A549 cells at 0, 6, and 12 hpi was determined by qPCR. (G) A549 cells were

881   transfected with $IFI16$-Flag expression vectors or empty vector for 24 h and then

882   infected with PR8 virus. IFN-I in supernatants were quantified IFN-bioassay at 2, 12,

883   18, 24 and 36 hpi from 1.0 MOI infection. (H) $IFI16^{+/+}$ and $IFI16^{-/-}$ A549 cells were

884   infected with PR8 virus at a 1.0 MOI. IFN-I in supernatants were then measured by

885   IFN-bioassay at 2, 12, 18, 24 and 36 hpi. (I) A549 cells were transfected with

886   $IFI16$-Flag expression vectors or empty control for 24 h and then infected with 1.0

887   MOI of PR8 virus for 0, 2, 4, 8 and 12 h. RIG-I triggered downstream signaling

888   molecules were evaluated with indicated antibodies. (J) RIG-I triggered downstream

889   signaling molecules in PR8 virus-infected $IFI16^{+/+}$ and $IFI16^{-/-}$ A549 cells at 0, 4, 8,

890   and 12 hpi were analyzed with indicated antibodies. (K) $IFI16^{+/+}$ and $IFI16^{-/-}$ A549

891   cells were infected with PR8 virus at 5.0 MOI. Nuclear localization of p-IRF3 was

892   then determined by p-IRF3 (Ser396) intracellular immunostaining for confocal

893   microscopy. Scale bar represents 10 μm. (L) Quantification of nuclear localization of

894   p-IRF3 (Ser396).(I) to (K): Data are representative of three independent experiments.

895   (A): the results were assessed using a parametric paired t-test (Student's one-tailed

896   t-test). (C) to (H) and (L): Data presented as means ± SD from three independent

897   experiments, and the significance of the results was assessed using a parametric

898   paired t-test (Student's two-tailed t-test). ns = non-significant

899   **Figure 5. IFI16 upregulates RIG-I expression.**

900   (A) RIG-I expression in *IFI16*-Flag overexpressing A549 cells at 0, 18, 24, and 30 h

901   post-transfection was detected by Western blotting. β-Actin detection was used as

902   loading control. (B) RIG-I mRNA expression in A549 cells transfected with

903   *IFI16*-Flag plasmids for 0, 12, 18 and 24 h was quantified by RT-qPCR. (C)

904   Luciferase activity of RIG-I reporter in HEK293 cells transfected with increasing

905   amounts of *FI16*-Flag expression vectors for 24 h. Luciferase levels were normalized

906   to Renilla levels. Values shown are fold changes over empty vector control. (D)

907   Schematic diagram of 2000 bp promoter sequence of RIG-I gene and corresponding

908   mutants. (E) HEK293 cells were co-transfected with the RIG-I promoter mutant

909   constructs and increasing amounts of *IFI16*-Flag as indicated. Luciferase levels were

910   normalized to Renilla values. Values shown as fold change over empty vector control.

911   (F) to (G): Schematic diagram of part of the RIG-I promoter sequence and every 50

912   bp-deletion mutants, and luciferase reporter assay on the truncated mutants was

913   performed as the described in (E). (H) to (I): Schematic diagram of part of the RIG-I

914   promoter sequence and every 10 bp-deletion mutants, and luciferase reporter assay of

915   these truncated mutants was performed as the described in (E). (J) A549 cells were

916   infected with PR8 virus for 12 h followed by ChIP assay. The promoter sequence

917   binding to the IFI16 was determined by RT-qPCR. (K) *IFI16*^{+/+} and *IFI16*^{-/-} A549

918   cells were infected with PR8 virus for 0 and 12 h, followed by ChIP assay. RNA Pol II

919   recruitment to RIG-I promoter was assessed by RT-qPCR. Data presented as percent

920   input minus IgG background. (L) Luciferase activity of RIG-I promoter-luciferase

921   reporter in HEK293 cells transfected with full-length IFI16 vectors and truncated

922   mutant plasmids. Luciferase levels were normalized to Renilla values. Values shown

923   as fold change over empty vector control. (A): Data are representative of three

924   independent experiments. (B) to (L): Data presented as means ± SD from three

925   independent experiments. Statistical significance in (B) to (C) and (E) to (L) was

926   determined by unpaired two-tailed Student's t-test. ns = non-significant.

927   **Figure 6. IFI16 binds viral RNA and associates with RIG-I protein**

928   (A) Co-localization of endogenous IFI16 (green) and viral NP RNA (red) in PR8

929   virus-infected A549 cells at 0, 6, and 12 hpi was detected by RNA FISH. Nuclei were

930   stained with DAPI (blue). Scale bars, 10 μm. Quantification of the co-localization of

931   IFI16 and NP RNA in cells (bar plots). Means ±SD from 3 biological samples. (B)

932   Integrated Genome Viewer representation of captured IAV genomic (negative polarity)

933   reads from Flag-immunoprecipitations of PR8-infected $RIG\text{-}I^{-/-}$ cells overexpressing

934   *IFI16*-Flag (top) or *RIG-I*-Flag (bottom). Each horizontal green bar represents a single

935   150 nt read and the position where it aligns relative to an IAV gene segment. (C) PCR

936   detection of PB2 vRNAs in eluted RNA from *RIG-I*-Flag, *IFI16*-Flag and indicated

937   Flag-tagged IFI16 deletions. (D) A549 cells were infected with PR8 virus at 5.0 MOI

938   for indicated duration, followed by co-immunoprecipitation with anti-IFI16 or IgG

939   and immunoblotting analysis with anti-RIG-I antibody. (E) A549 cells were infected

940   with   PR8   virus   at   5.0   MOI   for   indicated   duration,   followed   by

941   co-immunoprecipitation with anti-RIG-I or IgG and immunoblotting analysis with

942   anti-IFI16 antibody. (F) A549 cells were infected with 1.0 MOI of PR8 virus and then

943   analyzed by PLA with anti-IFI16 and anti-RIG-I antibodies at 0, 12 and 24 hpi. The

944   right panels are enlarged. Red point indicates IFI16 plus RIG-I complexes, green

945   point indicates the viral M1 protein. Scale bars, 60 μm (left), 10 μm (right). Data

946   presented are representative of three independent experiments.

947   **Table 1. List of primer pairs used for real-time PCR in this study.**

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A

Influenza A virus

↓ 12hpi

THP-1

↓ Lysis

Strep — Viral RNA

↓ Elute & Digest

Mass Spectrometry Analysis

B

Protein Domain Enrichment

RNA-binding S4 domain

Helicase, superfamily 1/2, ATP-binding domain

Domain associated with zinc fingers

Carbamoyl-phosphate synthetase large subunit-like, ATP-binding domain

K Homology domain

Biotin-binding site

Double-stranded RNA-binding-like domain

RNA recognition motif domain

HA M NP NS PA PB1 PB2 NA

Count
● 10
● 20
● 30
● 40
● 50

Q value
0.06
0.04
0.02

C

Transcription level

RBBP6, DDX5, DHX9, DDX59, ZC3HAV1, DDX3X, ADAR, ATP5B, ATP5F, PTBP1, PTBP3, ZNF90, ILF2, ILF3, SF1, U2AF1, U2AF2, SRRM1, ZFR, NXF1, SRSF5, EEF1A1, EIF2AK2, RPS3, RPS4X, RPS7, HMGA1, TAF1C, CPSF7, PCBP2, HNRNPC, FUBP1, FUBP3, RBBP4, SRSF1, SRSF2, SRSF3, SRSF6, SRSF7, SFPQ, RBM25, RBM39, ATP5A, PHB2, MCCC1, VDAC1, OAS1, TFAM, VDAC2, IFI27, IFI16, DDX17, TRIM25, IMMT, DHX30, HNRNPD, SNRPD1, SYNE1, HNRNPA1, SNRPF, ZNF385A, HNRNPR, RPS9, G3BP2, IGF2BP2, QKI, NONO, KHSRP, PCCA, TRA2B

0   6   12   24   (hpi)

Fold difference (log₂)
0 4 8 12

D

A549 cells

PR8 virus 0 6 12 18 24 (hpi)

IFI16 ——— 100 (kDa) / 70

NP ——— 55

β-actin ——— 40

E

0hpi   6hpi   Enlarged (DAPI / IFI16)

12hpi   18hpi   Enlarged

5 μm

F

IFI16 relative fluorescence intensity

Nucleus / Cytoplasm

p=0.0001
p<0.0001
p<0.0001
p=0.0038

0 12 18 (hpi)

G

PR8 virus

IP: IFI16   0 12 24 (hpi)
IB: Acet. Lys ——— 100 (kDa) / 70

IFI16 ——— 100

WCL
NP ——— 55

β-actin ——— 40

H

Uninfected   PR8 virus 24h

DMSO   C646   DMSO   C646

PLA[IFI16+ Acet.Lys.]+DAPI

28.7μm

Enlarged

14.6μm

A
Body weight (%)

d6: *p*=0.0446
d7: *p*=0.0219
d8: p=0.0328
d9: p=0.0487

WT
*p*204⁻/⁻

(dpi)

B
Percent survival (%)

p=0.0039**

WT
*p*204⁻/⁻

(dpi)

C
Viral titer (log₁₀EID50/ml)

WT
*p*204⁻/⁻

p=0.0188
p=0.0132

4   6   (dpi)

D
Lung
NP mRNA (fold)
p=0.0001

Lung
NP vRNA (fold)
p=0.0046

WT
*p*204⁻/⁻

E
WT   *p*204⁻/⁻
5dpi

F
WT   *p*204⁻/⁻
3dpi
5dpi

G
3dpi
WT
*p*204⁻/⁻

H
IL-6 (pg/ml)
p=0.0285
WT
*p*204⁻/⁻
0   3   (dpi)

I
MCP-1 (pg/ml)
p=0.0451
WT
*p*204⁻/⁻
0   3   (dpi)

J
BMDM
IFN-β mRNA (fold)
p=0.0006
ns
WT
*p*204⁻/⁻
0   6   (hpi)

K
BMDM
Viperin mRNA (fold)
p=0.0003
ns
WT
*p*204⁻/⁻
0   6   (hpi)

L
BMDM
OAS mRNA (fold)
p=0.0111
ns
WT
*p*204⁻/⁻
0   6   (hpi)

M
BMDM
ISG15 mRNA (fold)
p=0.0018
ns
WT
*p*204⁻/⁻
0   6   (hpi)

N
BMDM
IL-6 mRNA(fold)
p=0.0418
ns
WT
*p*204⁻/⁻
0   6   (hpi)

A

	#wt	#ko
type I interferon signaling pathway	18	11
defense response to virus	35	18
response to virus	23	14
immune response	58	13
immune system process	41	13
innate immune response	49	22
response to interferon-beta	4	3
negative regulation of viral genome replication	22	13
positive regulation of immune response	4	3
cellular response to interferon-alpha	3	2

0.004
0.003
0.002
0.001
8.060e-020

B

non-significant significant ISGs
significant non-significant ISGs

log2 FoldChange IFI16 ko (IAV vs Mock)
log2 FoldChange IFI16 wt (IAV vs Mock)

IFI44 OASL ISG15 IFIT2 RTP4
MX1 RIG-I HELZ2
ISG20 CRABP2 DDX60 PSMB9
LIPA MOV10 TRIM25 TSPO

C IFN-β mRNA (fold) — IFI16(+/+), IFI16(-/-); ns; $p<0.0001$; $p=0.0115$; (hpi) 0 6 12

D RIG-I mRNA (fold) — IFI16(+/+), IFI16(-/-); ns; $p=0.0026$; $p=0.0007$; (hpi) 0 6 12

E ISG56 mRNA (fold) — IFI16(+/+), IFI16(-/-); ns; $p=0.0060$; $p<0.0001$; (hpi) 0 6 12

F Viperin mRNA (fold) — IFI16(+/+), IFI16(-/-); ns; $p=0.0075$; $p=0.0040$; (hpi) 0 6 12

G IFN (U/ml) — EV, IFI16; $p=0.0107$ *; $p=0.0228$ *; PR8 virus 2 12 18 24 36 (hpi)

2h 12h 18h 24h 36h; Flag IFI16-Flag; Flag; β-actin; 100 (kDa); 40

H IFN (U/ml) — IFI16(+/+), IFI16(-/-); $p=0.0211$ *; $p=0.0025$ **; $p=0.0116$ *; 2 12 18 24 36 (hpi)

I

PR8 virus:	Flag	IFI16-flag	
	0 2 4 8 12	0 2 4 8 12	(hpi) (kDa)
IFI16			100 / 70
RIG-I			130 / 100
p-TBK1			100 / 70
TBK1			100 / 70
p-IRF3			55
IRF3			55
p-P65			65
P65			65
β-actin			40

J

PR8 virus:	IFI16 (+/+)	IFI16 (-/-)	
	0 4 8 12	0 4 8 12	(hpi)
IFI16			100(kDa) / 70
RIG-I			130 / 100
p-TBK1			100 / 70
TBK1			100 / 70
P-IRF3			55
IRF3			55
β-actin			40

K IFI16(+/+); IFI16(-/-); DAPI p-IRF3(Ser396) Merge; 0hpi 6hpi 12hpi; 10 μm

L %p-IRF3 translocation — IFI16(+/+), IFI16(-/-); $p=0.0014$; $p=0.0007$; 6 12 (hpi)

A

DAPI IFI16
0hpi
NP vRNA Merge
10 μm

DAPI IFI16
6hpi
NP vRNA Merge
10 μm
Colocalization

DAPI IFI16
12hpi
NP vRNA Merge
10 μm
Colocalization

B

IFI16 Reads
RIG-I Reads
Flag Reads

HA M NA NP NS PA PB1 PB2

C

RIP-PCR

Maker RIG-I IFI16 ΔHINa ΔPYRIN IgG Input

(bp)
5000
3000
2000
1500
1000
800
500
300

Full length PB2 vRNA

DI subgenomic PB2 vRNA

D

0hpi 12hpi
IP: IgG IFI16 IgG IFI16 (kDa)

IB
RIG-I — 130 — 100
IFI16 — 100 — 70

WCL
RIG-I — 130 — 100
IFI16 — 100 — 70
M1 — 25
β-actin — 40

E

0hpi 12hpi
IP: IgG RIG-I IgG RIG-I (kDa)

IB
RIG-I — 130 — 100
IFI16 — 100 — 70

WCL
RIG-I — 130 — 100
IFI16 — 100 — 70
M1 — 25
β-actin — 40

F

PLA[IFI16+RIG-I]+DAPI + M1 Enlarged

0hpi 60 μm 10 μm
12hpi 60 μm 10 μm
24hpi 60 μm 10 μm

A

6hpi

NP — IFI16, ● Empty vector ■

6hpi: $p=0.0226$ (mRNA), $p=0.0129$ (vRNA)

12hpi: $p<0.0001$ (mRNA), $p<0.0004$ (vRNA)

18hpi: $p=0.0003$ (mRNA), $p=0.0004$ (vRNA)

M

6hpi: $p=0.0010$ (mRNA), $p=0.0118$ (vRNA)

12hpi: $p=0.0050$ (mRNA), ns (vRNA)

18hpi: $p=0.0121$ (mRNA), $p=0.0028$ (vRNA)

RNA transcription(fold)

B

HEK293 cells — Flag / IFI16-Flag — 0 6 12 18 (hpi)

IFI16 — 100 (kDa), 70

NP — 55

β-actin — 40

C

IAV: siRNA NC / IFI16-siRNA — 0 12 18 24 Marker 0 12 18 24 (hpi)

IFI16 — 100 (kDa), 70

NP — 55

M1 — 25

β-actin — 40

D

$p=0.0175$

Viral titer (log$_{10}$TCID$_{50}$/ml)

siRNA NC ▲, IFI16-siRNA ■

24 (hpi)

A

IFI16-Flag

| | 0 | 10 | 50 | 100 | 500 | (ng) |

IFI16 —— 100 (kDa)
RIG-I —— 130 / 100
β-actin —— 40

B

RIG-I-Flag

Myc / IFI16-Myc

CHX: 0 12 24 / 0 12 24 (h)

Myc —— 100 (kDa) / 70
Flag —— 130 / 100
β-actin —— 40

C

Ifnar1^-/- A549 cells

IFI16-Flag 0 100 400 800 (ng)

RIG-I —— 130 (kDa) / 100
Flag —— 100 / 70
β-actin —— 40

D

RIG-I mRNA (fold)

p=0.0015
p=0.0161

0 50 500 (ng)

E

RIG-I mRNA (fold)

p=0.0001
p=0.0016
ns

0 10ng 100ng 1000ng (IFI16)

F

RIG-I mRNA (fold)

WT / p204(-/-)

p<0.0001
p=0.0011
ns

0 1 3 (dpi)

G

p1: (-450bp) AAAGCTAAACATAGACTTACCCTGGCACCCAGCCGACGTGGGAGAACTG (-400bp)
p2: (-400bp) GTACACAGGTGTACACTTTACAAAACTCATCGAATTGCACACTCAGATTGT (-350bp)
p2-mut1: **ACGCCACGAC**GTACACTTTACAAAACTCATCGAATTGCACACTCAGATTGT
p2-mut2: GTACACAGGT**ACGCCATGTC**CAAAACTCATCGAATTGCACACTCAGATTGT
p2-mut3: GTACACAGGTGTACACTTT**AAGCAAGAGC**CGAATTGCACACTCAGATTGT
p2-mut4: GTACACAGGTGTACACTTTACAAAACTCATA**GCACTATGAA**CTCAGATTGT
p2-mut5: GTACACAGGTGTACACTTTACAAAACTCATCGAATTGCACG**ATAGAACGTC**

Biotinylated probe

H

+IFI16-Flag

p1 p2 p2-mut1 p2-mut2 p2-mut3 p2-mut4 p2-mut5 Biotinylated probe

(kDa)
100 / 70
DNA pulldown IB: Flag

100 / 70
Input IB: Flag

J

| | 1 | 87 | 192 | 393 | 515 | 729 |
IFI16-WT PYRIN HINa HINb
ΔPYRIN HINa HINb
ΔHINa PYRIN HINb
ΔHINb PYRIN HINa
PYRIN PYRIN

L

EV IFI16-WT ΔPYRIN ΔHINa ΔHINb PYRIN

(kDa)
Flag —— 100 / 70 / 55 / 15

NP —— 55
1.08 0.61 0.70 0.92 0.50 0.73

β-actin —— 40

I

RNA PolII recruitment Input (%)

GAPDH promoter

ns

IgG Pol II / IgG Pol II (12hpi)
IFI16(+/+) / IFI16(-/-)

K

IFI16-WT IFI16-WT ΔPYRIN ΔHINa ΔHINb PYRIN

IP: non Bio / Bio-RIG-I promoter

IB: Flag

10% Input IB: Flag

(kDa)
100 / 70 / 55 / 15

A

Biotin-NP RNA

Flag-IFI16 Flag-RIG-I Flag-MDA5 Flag-TBK1

(kDa)

IP: Streptavidin

Bound protein
IB: Flag

130
100
70

Cell lysates
IB: Flag

130
100
70

B

HINa-GFP

EC50=1.4142E-07

PYRIN-GFP

NO binding

HINb-GFP

NO binding

C

IFI16   HA vRNA   EC50=1.0775E-07

IFI16   PA vRNA   EC50=9.2785E-08

IFI16   NP vRNA   EC50=2.33E-07

IFI16   PB2 vRNA   EC50=6.88E-08

D NP

Fold diff. Eluted vRNA (relative to RIG-I)

RIG-I IFI16 ΔPYD ΔHINa ΔHINb PYD

E NA

Fold diff. Eluted vRNA (relative to RIG-I)

RIG-I IFI16 ΔPYD ΔHINa ΔHINb PYD

F

RIG-I   DAPI   IFI16   Merge   Enlarged   Colocalization

18h   10 µm 10 µm 10 µm

6h   10 µm 10 µm 10 µm

0h   10 µm 10 µm 10 µm

G

IFI16 mutants   mCherry-RIG-I   Merge   Enlarged   Colocalization

IFI16-GFP   20 µm 20 µm 20 µm 20 µm

PYRIN-GFP   20 µm 20 µm 20 µm 20 µm

ΔPYRIN-GFP   20 µm 20 µm 20 µm 20 µm

ΔHINa-GFP   20 µm 20 µm 20 µm 20 µm

H

IFI16-Flag ΔPYD ΔHINa ΔHINb PYD   (kDa)

IP: Myc
IB: Flag

10% Input
IB: Flag

100
70
55

15

IP: Myc
IB: Myc

10% Input
IB: Myc

100
70

I

DAPI   PYRIN-GFP   Volume View

5 µm   5 µm

RIG-I-mCherry   Merge

5 µm   5 µm   5 µm

Merge

A IFI16(+/+) PLA[RIG-I+TRIM25]+DAPI Enlarged   IFI16(-/-) PLA[RIG-I+TRIM25]+DAPI Enlarged

Mock
23.8 μm   7.45 μm   23.8 μm   7.45 μm

IAV
23.8 μm   7.45 μm   23.8 μm   7.45 μm

B IFI16(+/+) PLA[RIG-I+K63Ub]+DAPI Enlarged   IFI16(-/-) PLA[RIG-I+K63Ub]+DAPI Enlarged

Mock
40.3 μm   14.3 μm   40.3 μm   14.3 μm

IAV
40.3 μm   14.3 μm   40.3 μm   14.3 μm

C
IP: Flag
GST-IFI16
—   50ng 200ng Beads

Biotinylated
PA RNA

D
PA RNA pulldown(5pmol)
GST-IFI16
Beads — 30ng 60ng 120ng

IB：Flag   130 (kDa)
100

IB: GST   130
100

E
RIG-I IP PA vRNA (fold)
p=0.0024
p=0.0020
EV IFI16 ΔPYRIN ΔHINa ΔHINb PYRIN
ns   ns   ns
Virus + + + + + +

F
IFN (U/ml)
p=0.0007
p=0.0119
p=0.0001
EV EV IFI16 ΔHINa ΔPYRIN ΔHINb PYRIN
ns   ns
Virus - + + + + + +

G
Negative ctrl siRNA#206 siRNA#403 siRNA#571 (kDa)
MAVs   70
β-actin   40

H
IFN-β (U/ml)
■ IFI16⁺/⁺+siRNAnegative
□ IFI16⁻/⁻+siRNAnegative
■ IFI16⁺/⁺+siRNA#571
■ IFI16⁻/⁻+siRNA#571
DMEM   PR8 virus

I
IFN-β (U/ml)
p=0.0011
ns
PRK5-Flag + + - + + -
IFI16-Flag - + + - + +
Virus - + + - + +
siRNA negative   siRNA#571