

1 **ISG15 Monomer Promotes IFN $\alpha$ -mediated Antiviral Activity**  
2 **against Pseudorabies Virus by Facilitating phosphorylation**  
3 **of STAT1/STAT2**

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11 **ABSTRACT**

12 Pseudorabies virus (PRV), which presently lacks both an antiviral drug and a viable  
13 therapeutic option, is a major viral pathogen that poses a danger to the pig industry  
14 worldwide. Interferon-stimulated gene 15 (ISG15) is strongly upregulated during viral  
15 infections and has been reported to have proviral or antiviral properties, depending on  
16 the virus and host species. Our previous studies demonstrated ISG15 was remarkably  
17 upregulated during PRV infection, and the overexpression of ISG15 inhibited PRV  
18 replication. Nevertheless, the exact mechanism through which ISG15 influences PRV  
19 replication poorly understood unclear. Here, we demonstrate that ISG15 accumulation  
20 induced by PRV infection requires viral gene expression and viral growth.  
21 Conjugation inhibition assays showed that ISG15 imposes its antiviral effects via  
22 unconjugated (free) ISG15 and affects the viral release. In addition, we found ISG15  
23 promoted IFN $\alpha$ -mediated antiviral activity against PRV by facilitating the  
24 phosphorylation of STAT1 and STAT2, along with an increase of ISGF3-induced  
25 ISRE promoter activity. Furthermore, we evaluated the role of ISG15 in host defense  
26 to control PRV infection by using ISG15<sup>-/-</sup> mice model. When challenged with PRV,  
27 ISG15<sup>-/-</sup> mice exhibited increased morbidity and mortality, as well as viral load  
28 compared to WT mice. Pathological examination revealed increased lesions,

29 mononuclear cellular infiltration and neuronal death in the brains of ISG15<sup>-/-</sup> mice,  
30 along with the upregulation of the cytokines. Our findings establish the importance of  
31 ISG15 in IFN $\alpha$ -induced antiviral response and in the control of PRV infection.

32 **KEYWORDS** Interferon-stimulated gene 15, pseudorabies virus, monomer, antiviral  
33 response, IFN $\alpha$ , STAT1/STAT2

## 34 **Introduction**

35 Pseudorabies virus (PRV), an alphaherpesviruses, is a significant viral pathogen of  
36 pigs, wreaking havoc on the global pig industry [1]. PRV is able to establish persistent  
37 infection in peripheral neurons of the host with no specific clinical symptoms, which  
38 is usually as a useful model for understanding alpha herpes virus biology and host's  
39 innate immune response [2, 3]. Recent evidence indicates that PRV can induce severe  
40 clinical symptoms in people, such as acute encephalitis, under specific conditions [4-  
41 8]. Despite intensive research, neither specific antiviral therapy nor effective vaccines  
42 against PRV are currently available [4, 9]. Therefore, a better understanding of the  
43 interactions between PRV infection and the host responses that inhibit PRV infection  
44 is of great importance.

45 In response to the viral invasion, the host evolves various defense mechanisms.  
46 Among these, the type I interferon (IFN-I) plays a central role in host defense against  
47 viral infections. IFN-I, represented by IFN $\alpha$  and IFN $\beta$ , binds to their respective  
48 receptors and activates the JAKs, which subsequently phosphorylate STAT1 and  
49 STAT2. The phosphorylated (p-) STAT1 and p-STAT2 complex with IRF9, resulting  
50 in the formation of ISG factor 3 (ISGF3). ISGF3 shuttles to the nucleus, where it  
51 binds to the IFN-stimulated response element (ISRE) in DNA and stimulates the  
52 transcription of hundreds of interferon-stimulated genes (ISGs) involved in the host  
53 antiviral response [10, 11]. There is increasing evidence that PRV encodes proteins to  
54 antagonize the IFN response by suppressing IFN production, blocking IFN  
55 downstream signaling, or regulating specific ISGs [12-14]. ISG15, a ubiquitin-like  
56 modifier, is among the most frequently induced proteins by IFN-I and has been  
57 reported to have proviral or antiviral activities, depending on the virus and host

58 species [15]. Like ubiquitination, ISG15 may be covalently attached to substrates via  
59 a conserved C-terminal Gly-Gly motif. This process is termed ISGylation through a  
60 cascading reaction catalyzed by E1 activating (Ube1L), E2 conjugating (UbcH8), and  
61 E3 ligase enzymes (Herc5), which are also induced by IFN-I, which has been  
62 demonstrated to cause either a gain or a loss of function of target proteins [16]. ISG15  
63 can be removed from its target proteins by the ubiquitin-specific protease USP18,  
64 making the ISGylation process reversible [17-20]. In addition to its conjugated form,  
65 unconjugated (free) ISG15 plays cytokine activity when released to the extracellular

66 With respect to PRV, our previous study showed that ISG15 is greatly upregulated  
67 during PRV infection, and ISG15 overexpression inhibits PRV replication [21].  
68 However, the mechanism underlying the anti-PRV effect of ISG15 *in vitro* and *in vivo*  
69 remains unexplored. Here, we characterized ISG15 expression profiles during PRV  
70 infection, and found that ISG15 inhibits PRV replication via the ISG15 monomer.  
71 Significantly, ISG15 silencing impairs IFN $\alpha$ -mediated anti-PRV effect by blocking  
72 phosphorylation of STAT1 and STAT2. Furthermore, ISG15 knockout mice exhibited  
73 enhanced PRV infection, as evidenced by high mortality, increased viral titer and  
74 severe inflammatory. These results reveal a critical role for ISG15 in IFN $\alpha$ -induced  
75 host antiviral activity and will provide a potential cellular therapeutic strategy.

## 76 **RESULTS**

### 77 **ISG15 monomer and conjugated protein accumulation during PRV infection**

78 Although ISG15 expression has reportedly increased during PRV infection in our  
79 previous study, how free and conjugated ISG15 proteins impact PRV infection and  
80 their roles in host defenses against PRV infection remain largely unknown. We sought  
81 to dissect the ISG15 behavior during PRV infection by analyzing the mRNA levels of  
82 ISG15 and PRV glycoprotein E (gE), a late viral gene, at different times post  
83 infection. As shown in Figure 1A, PRV infection induces a progressive increase in  
84 ISG15 RNA in a time-dependent manner. The increase started at 6 hours post-  
85 infection (hpi), reaching the peak level at 24 hpi. PRV-gE RNA increase trend was  
86 consistent with ISG15 RNA (Fig.1A).

87 To confirm the results mentioned above, immunoblotting was used to determine if  
88 ISG15 and PRV-gE RNA levels were translated into protein levels. Accumulation of  
89 unconjugated ISG15 and elevated high-molecular-weight ISG15-conjugated proteins  
90 were detected from the above parallel infection samples. Unconjugated ISG15  
91 monomer were detected beginning at 12 hpi, while the abundance of the ISG15-  
92 conjugated protein was observed at 6 hpi (Fig.1B). Meanwhile, PRV-gE expression  
93 started to be detected at 12 hpi and reached the maximum level at 24 hpi (Fig.1B).  
94 Compared to mock-infected cells, increased levels of ISG15-conjugated and free  
95 ISG15 proteins persisted over a 48-hour time period, reaching maximum level at 24  
96 hpi. This finding suggested that the increase of ISG15 monomer/conjugates was  
97 related to an increase in PRV-gE abundance. To generate additional support for this  
98 point, we conducted a dose-response investigation by infecting PRV at different  
99 multiplicities of infection (MOI). We found that the overall ISG15 monomer and  
100 conjugation levels induced by PRV infection in a dose-dependent manner (Fig. 1C to  
101 1D).

102 Finally, the ISG15 expression patterns induced by PRV infection or IFN stimulation  
103 were compared by Western blotting. The obtained data suggested that PRV-induced  
104 ISGylation differs to some extent from that of IFN $\alpha$ , as well as some specific bands  
105 were apparent in PRV-infected cells (Fig.1E).

106 These results suggested that ISG15 accumulation was induced early in the PRV  
107 replication cycle and began to enhance with the expression of late viral gene.  
108 Therefore, we speculate the ISG15 abundance may be associated with viral DNA  
109 synthesis or late viral gene expression.

#### 110 **ISG15 accumulation are triggered by PRV gene expression**

111 To analyze whether viral DNA synthesis impacts free and conjugated ISG15  
112 accumulation, PK15-infected cell cultures were treated with phosphonoacetic acid  
113 (PAA) to suppress viral DNA synthesis and late gene expression. As expected, PAA  
114 effectively reduced the transcript and expression of ISG15, along with the inhibition  
115 of PRV-gE protein expression (Fig. 2A). Similar levels of unconjugated and  
116 conjugated ISG15 proteins were observed in PAA-treated cultures following mock- or



117 infected with PRV. Thus, blocking late gene expression with PAA significantly  
118 decreased ISG15 accumulation.

119 For further evidence, PRV virions were inactivated by UV irradiation [22], and  
120 viral inactivation was verified by a lack of viral growth after 24h post-inoculation.  
121 Infection of PK15 cells with UV-inactivated PRV resulted in greatly reduce in the  
122 transcription of ISG15 as well as free and conjugated ISG15 proteins levels, in  
123 comparison with that in cultures infected with active, unirradiated PRV (Fig. 2B). In  
124 addition, the conjugated ISG15 abundance is most likely via promoting an E1-E2-E3  
125 enzymatic cascade of ISGylation. Overall mRNA levels of the Ube1L, UbcH8, Herc5  
126 and USP18 progressively rised, demonstrating that the PRV replication process  
127 activates the ISGylation pathway (Fig. 2C). These results indicated that active PRV  
128 replication was needed for the free ISG15 and conjugate expression.

### 129 **ISG15 silencing enhances PRV replication**

130 To analyze how ISG15 impacts PRV replication, ISG15 was deleted (ISG15<sup>-/-</sup>) in  
131 PK15 cells using CRISPER/Cas9 editing technology in our previous study [23].  
132 ISG15 wild-type (WT) and ISG15<sup>-/-</sup> cells were either mock-infected or infected with  
133 PRV at an MOI of 1. A significantly higher of amount of PRV-gE protein was  
134 observed in ISG15<sup>-/-</sup> cells compared to WT cells at various time points post infection  
135 (Fig. 3A). Additionally, with the increase of MOI, the magnitude of this effect was  
136 also increased (Fig. 3A). Similar changes in PRV replication were found between WT  
137 and ISG15<sup>-/-</sup> cells infected with PRV for 24h, as evidenced by fluorescence  
138 microscopy (Fig. 3B). Furthermore, a significant growth in PRV titer was detected in  
139 ISG15<sup>-/-</sup> cells in comparison with WT cells at the same time point following infection  
140 (Fig.3C). Our findings established that ISG15 silencing accelerated PRV replication.

141 It has been reported that ISG15 may affect virus entry [24] or release [25, 26], so  
142 we next sought to determine the role of ISG15 in those steps of PRV replication. We  
143 first determined whether ISG15 disrupts PRV replication by impacting virus entry into  
144 the cells. PK15 cells were transfected with a plasmid expressing ISG15 or a control  
145 plasmid before being infected with PRV, meanwhile, ISG15<sup>-/-</sup> cells were also infected  
146 with PRV. Then, the PRV-gE RNA was quantified by RT-qPCR at various times post-

147 infection. PRV-gE, a protein encoded by late gene, participates in virion assembly and  
148 release [27]. The results identified an obvious reduction of the PRV-gE RNA in the  
149 ISG15-overexpressing cells compared to control cells starting 6 hpi, while loss of  
150 ISG15 significantly enhance PRV-gE RNA level (Fig. 3D). This shows that ISG15  
151 restricts PRV growth at a post-entry stage of infection. Additionally, the virus  
152 associated with cells and released to the supernatant were also detected by plaque  
153 assay. As illustrated in Figure 3E, a significant decrease was observed in virus titers  
154 from cell-associated fraction of WT cells expressing ISG15 compared to the same  
155 fraction of WT cells, while an increase in ISG15 knockout cells (Fig.3E, Cell-  
156 associated). Similarly, more than 3-fold reduction in virus titer was observed in the  
157 supernatant of WT cells overexpressing ISG15 with respect to WT and ISG15<sup>-/-</sup> cells.  
158 This indicates that ISG15 limits PRV replication occurring before virus release.

159 Combinedly, these findings suggest that ISG15 generated by host cells inhibits PRV  
160 replication, possibly due to the virus release inhibition.

#### 161 **Antiviral activity of ISG5 against PRV is due to ISG15 monomer**

162 To further determine whether ISG15 achieves its antiviral effect against PRV via  
163 free or conjugated form, three different experiments were carried out.

164 First, due to the fact that exposure of the C-terminal Gly-Gly motif is essential for  
165 conjugation of ISG15 to substrates [28], these two residues were replaced with Ala  
166 using site-directed mutagenesis to generate a mutate plasmid ISG15AA employed as a  
167 control. PK15 cells were transfected with an empty control vector, a ISG15-  
168 expressing plasmid, or an ISG15AA expressing plasmid. After 24 hours, the cells  
169 were infected with PRV and then viral protein expression and viral titer were  
170 measured. As expected, we found a significant decrease in PRV-gE expression level,  
171 as well as PRV titer (3.2-fold) (Fig. 4A), compared to cells transfected with empty  
172 vector. Notably, there wasn't any significant differences detected in PRV titer and  
173 protein levels between these cells transfected with ISG15 and ISG15AA plasmid (Fig.  
174 4A). Similar results were obtained from a parallel transfection / infection in ISG15<sup>-/-</sup>  
175 cells (Fig. 4B). These data suggest that ISG15 exerts its antiviral activity against PRV  
176 through ISG15 monomer-dependent or conjugation-independent mechanisms.

177 To verify these results and assess the influence of free ISG15 on PRV infection, a  
178 second approach was adopted by knocking down Ube1L to prevent the synthesis of  
179 ISG15 conjugates [29]. Prior to infection with PRV, PK15 cells were transfected with  
180 either a control siRNA or a siRNA targeting Ube1L, and ISG15 un-conjugated and  
181 conjugated proteins abundances were measured by immunoblotting. We observed that  
182 Ube1L-silenced cells displayed higher free ISG15 than control cells, nonetheless, a  
183 decrease in the expression levels of ISG15 conjugates (Fig. 4C). Furthermore, when  
184 Ube1L-silenced cells compared with the control cells, a significant drop in viral titer  
185 was found (Fig. 4C).

186 The third strategy involved silencing exogenous USP18, a deconjugating protease  
187 specific for ISG15 that removes ISG15 from targets [30]. This result is quite the  
188 opposite of the results from Ube1L-silencing experiments. Knockdown of USP18  
189 resulted in an increased induction of the protein ISGylation, obvious enhancement in  
190 PRV-gE protein expression and viral production (Fig. 4D). This indicated that ISG15  
191 inhibits PRV replication via free ISG15.

192 Collectively, these results indicate that free ISG15, rather than ISGylation,  
193 possesses an important antiviral activity against PRV.

#### 194 **ISG15 contributes to IFN $\alpha$ antiviral activity against PRV**

195 Previous reports have indicated that IFN $\alpha$  treatment of ISG15-deficient patient cells  
196 increased their resistance to several viral infection by viruses [31]. PRV is capable of  
197 establishing persistent infections, partly due to its ability to circumvent the host's  
198 antiviral defenses, notably the type I IFN [13, 27]. Thus, to investigate whether ISG15  
199 is involved in IFN-I mediated antiviral effect, WT and ISG15<sup>-/-</sup> cells infected with  
200 PRV with or without IFN $\alpha$  treatment. Compared with their respective controls, the  
201 expression of PRV-gE and viral productions in ISG15<sup>-/-</sup> cells were obviously  
202 increased when treated with IFN $\alpha$  (Fig.5A to 5C). This finding implies that complete  
203 loss of ISG15 impairs IFN $\alpha$  antiviral response against PRV.

204 To confirm this effect was caused by free ISG15, we performed gene rescue of  
205 ISG15 by transfecting a plasmid expressing ISG15AA in ISG15<sup>-/-</sup> cells. A similar  
206 level of PRV-gE expression was detected in ISG15<sup>-/-</sup> cells transfected with ISG15AA-

207 expressing plasmid and WT cells, indicating the ISG15 gene rescue is successful. As  
208 shown in Figure 5D, PRV-gE expression was much lower in ISG15<sup>-/-</sup> cells transfected  
209 with ISG15AA-expressing plasmid than that of ISG15<sup>-/-</sup> cells with empty vector. In  
210 line with the above results, ISG15 silencing impairs anti-PRV response of IFN $\alpha$  via a  
211 monomer form.

212 Altogether, our data supports that free ISG15 promotes IFN $\alpha$ -mediated antiviral  
213 activity against PRV.

#### 214 **ISG15 facilitates the phosphorylation of STAT1 and STAT2**

215 To understand the mechanism by which ISG15 participates in IFN $\alpha$ -mediated  
216 antiviral activity, we monitored the phosphorylation levels of STAT1 and STAT2 in  
217 WT versus ISG15<sup>-/-</sup> cells following PRV infection with or without IFN- $\alpha$  treatment.  
218 As illustrated in Figure 6A and B, IFN $\alpha$  stimulation accelerated pSTAT1 and pSTAT2,  
219 while ISG15 silencing significantly inhibited the expression of pSTAT1 (Fig. 6A) and  
220 pSTAT2 (Fig. 6B) regardless of the inclusion or exclusion of IFN $\alpha$  treatment.  
221 Subsequently, subcellular fractionation and Western blot analysis show that the IFN $\alpha$ -  
222 induced nuclear translocation of STAT1/STAT2 in the ISG15<sup>-/-</sup> cells was reduced  
223 accordingly (Fig. 6C). This observation was consistent with the data from  
224 immunofluorescence images (Fig. 6D), indicating that ISG15 silencing blocks the  
225 nuclear translocation of the STAT1 and STAT2. This provides strong evidence that  
226 ISG15 silencing inhibits STAT1 and STAT2 nuclear accumulation by blocking nuclear  
227 import.

228 ISG15 was found to be predominantly localized in the cytoplasm (Fig. 6C), the  
229 mechanism for ISG15 to influence this step is likely through altering the expression  
230 levels of the components essential for IFN signaling pathway. Since ISG15 silencing  
231 inhibited the phosphorylation of STAT1 and STAT2, we hypothesized that ISG15  
232 deletion blocked the interaction of the STAT1 and STAT2. Thus, a co-  
233 immunoprecipitation assay (Co-IP) was performed to detect whether ISG15 impacted  
234 the interactions of STAT1 and STAT2. First, WT and ISG15<sup>-/-</sup> cells were co-  
235 transfected with HA-STAT1 and Myc-STAT2. Twenty-four hours later, the cell lysates  
236 were immunoprecipitated with an anti-HA antibody and then immunoblotting with

237 anti-Myc antibody, and the results showed the expression level of STAT1 in the  
238 presence of STAT2 in the samples from the two group cells (Fig.6E). In parallel, the  
239 presence of STAT1 in both samples was verified by IP with a Myc-tag antibody  
240 followed by blotting with an anti-HA-tag antibody. Meanwhile, the interaction  
241 between endogenous STAT1 and STAT2 was also analyzed by Co-IP assay, and  
242 similar results were observed (Fig. 6F). This indicated that ISG15 silencing attenuated  
243 the interaction between STAT1 and STAT2.

244 Collectively, these results suggest that ISG15 promotes the IFN $\alpha$ -induced  
245 phosphorylation of STAT1 and STAT2.

#### 246 **ISG15 silencing inhibits ISGF3-induced ISRE reporter activity**

247 The heterodimerization of phosphorylated STAT1 and STAT2 associates with IRF9,  
248 forming the ISGF3 complex that subsequently enters the nucleus to activate ISRE-  
249 dependent transcription [32]. We have found ISG15 silencing suppresses the  
250 phosphorylation of STAT1 and STAT2, so we speculate ISG15 may affect the  
251 formation of ISGF3 complex, thereby inhibiting the expression of ISGs. To dissect the  
252 idea that ISG15 is involved in the ISGF3 formation, WT or ISG15<sup>-/-</sup> cells were treated  
253 with IFN $\alpha$  or left untreated following PRV infection. Subcellular fractionation and  
254 subsequent Western blot analysis showed that ISG15 silencing downregulated the  
255 phosphorylation level of STAT1 and STAT2 induced by IFN $\alpha$ , while had only slight  
256 impact on the expression of IRF9 (Fig.7A). This suggests a complete loss of ISG15  
257 attenuates the form of ISGF3 complex, thereby inhibiting the ISGF3 translocation into  
258 the nucleus.

259 To study the effect of ISG15 on ISGF3-induced ISRE promoter activation, WT and  
260 ISG15<sup>-/-</sup> cells were co-transfected with the plasmid-encoded STAT1, STAT2 and/or  
261 IRF9, together with ISRE-luciferase and Renilla luciferase reporters. Twelve hours  
262 later, the cells were infected with PRV and then collected for analysis of luciferase  
263 activity induced by ISRE. The result showed that ISRE promoter activity markedly  
264 diminished compared to the other control groups ( $p < 0.05$ ; Fig.7B), suggesting that  
265 ISG15 promoted to the ISRE promoter activation. Additionally, activated ISGF3  
266 drives the transcription of ISGs, which are important for the control of viral infections

267 [33]. We also detected the expression of IFIT1, 2',5'-oligoadenylate synthetase 1  
268 (OAS1) and myxovirus resistance protein A (MxA), the downstream transcription  
269 factors of IFN signaling. Results indicated that ISG15 deletion significantly down-  
270 regulated the transcription levels of IFIT1, MxA and OAS1 (Fig. 7C).

271 Taken together, these data provided evidence that a complete loss of ISG15 blocked  
272 the ISGF3 formation, attenuated ISRE promoter activity and downregulated the  
273 transcription levels of ISGs.

#### 274 **ISG15<sup>-/-</sup> mice are more sensitive to PRV infection**

275 Further, we examined the role of ISG15 in host defense to control PRV infection  
276 using genetically knockout ISG15 (ISG15<sup>-/-</sup>) mice. A survival analysis was first  
277 performed to confirm the role of ISG15 in host survival during PRV infection.  
278 C57BL/6N (B6, WT) and ISG15<sup>-/-</sup> mice were infected with PRV-QXX via  
279 subcutaneous injection and monitored for 6 days post infection (dpi). The infected  
280 ISG15<sup>-/-</sup> mice began to die at 3 dpi with severe itchiness symptoms such as. The  
281 survival rate of ISG15<sup>-/-</sup> mice reached 100% until 6 dpi, while the survival rate of WT  
282 mice was only 46.7% (Fig. 8A), suggesting that the occurrence of increased  
283 susceptibility to PRV infection in the complete loss of ISG15. We also observed a  
284 marked increase in ISG15 protein in brains of infected WT mice (Fig. 8B), which is  
285 consistent with the results obtained in the cell model. PRV-gE gene copies and viral  
286 titers of brains were examined by RT-qPCR and plaque assay individually for each  
287 mouse. As expected, PRV-gE gene copies and viral titers in ISG15<sup>-/-</sup> mice were  
288 significantly higher than those in WT mice (Fig. 8C to D).

289 Encephalitis caused by PRV infection in the central neuron system is a pivotal  
290 factor leading to animal death [34]. Therefore, we detect the degree of encephalitis in  
291 the brain tissues of infected WT and ISG15<sup>-/-</sup> mice by histopathological observation.  
292 Hematoxylin-and-eosin staining showed greater inflammatory damage, necrotic  
293 neurons, and more glial cells in ISG15<sup>-/-</sup> mice than those in WT mice (Fig. 8E). We  
294 further determined IL-6, TNF- $\alpha$  and IL-1 $\beta$  protein levels in the brains of the WT and  
295 ISG15<sup>-/-</sup> mice by ELISA. Increased protein levels of IL-6, TNF- $\alpha$  and IL-1 $\beta$  were  
296 observed in ISG15<sup>-/-</sup> mice compared to WT mice (Fig. 8F). This suggests that ISG15

297 may have a direct or indirect role in encephalitis.

298 Taken together, our observations imply that ISG15 plays a critical role in host anti-  
299 PRV response.

### 300 **Discussion**

301 To date, the PRV-host interactions that induces ISG15 expression and the impact of  
302 the ISG15 monomer and conjugates on PRV replication remained unexplored. Here,  
303 we establish that although the ISG15 abundance are triggered by PRV infection, they  
304 are subsequently tempered, but not completely abrogated by viral gene expression.  
305 Preventing PRV gene expression and viral growth greatly reduced ISG15  
306 accumulation. Therefore, both free and conjugated ISG15 accumulation in response to  
307 PRV infection was dependent on viral gene expression and viral growth. Moreover,  
308 deletion of ISG15 remarkably promoted PRV replication, indicating that ISG15 has a  
309 significant anti-PRV function. Additionally, we noticed that ISG15 had to accumulate  
310 in large amounts before virus infection to carry out its anti-PRV role. ISG15 seems to  
311 affect a stage in the PRV cycle before virus release, since PRV titers increased in both  
312 cell-associated and released virus following ISG15 silencing (Fig. 3). In contrast, it  
313 has been recently reported that ISG15 blocks the entry and/or uncoating phase of the  
314 murine norovirus life cycle [24]. Furthermore, ISG15-deficient mice display more  
315 sensitive to PRV infection, indicating ISG15 exerts a critical role for restricting PRV  
316 infection against PRV *in vitro* and *in vivo*.

317 We found that ISG15 exerts its anti-PRV effect relying on ISG15 monomer, as  
318 demonstrated by the effect of an unconjugated form of ISG15, the inhibition of  
319 ISGylation by UBE1L silencing, or the enhancement of ISGylation by USP18  
320 silencing. It is possible that extracellular ISG15 monomer directly interferes with PRV  
321 replication to prevent viral infection, as evidenced by the high levels of ISG15  
322 expression before PRV-gE expression (Fig.1). In other words, when ISG15 is  
323 expressed at high levels before virus infection, as in cells stimulated by IFN $\alpha$ , no viral  
324 proteins are present to counteract the ISG15 antiviral activity. Another possibility is  
325 that ISG15 accumulation may promote type I IFN signaling and/or the expressions of  
326 ISGs induced by viral infection. The positive correlation between PRV infection and



327 ISG15 expression was also observed from the infected mice (Fig.8B and 8D),  
328 pointing to the antiviral role of ISG15 in PRV infection *in vivo*. Our result contrast  
329 with the previous studies that ISG15-deficient patients who display no enhanced  
330 susceptibility to viruses *in vivo*. This reflects the ISG15 function may vary depending  
331 on the virus and host species.

332 Type I IFN is critical for controlling PRV infection *in vitro* and *in vivo*. Recent  
333 investigations demonstrated that ISG15 acted as a negative regulator of type I IFN  
334 signaling exerted antiviral response during viral infection [31, 33]. However, our  
335 results provided some evidence supporting ISG15 as a positive regulator of IFN $\alpha$ -  
336 mediated antiviral response against PRV as following: 1) ISG15 silencing impairs the  
337 antiviral activity of IFN $\alpha$  against PRV; 2) ISG15 deletion blocks STAT1 and STAT2  
338 phosphorylation through inhibition of the interaction between STAT1 and STAT2; 3)  
339 ISG15 facilitates the ISGF3 complex formation and ISRE promoter activity; and 4)  
340 The transcription level of ISGs genes induced by IFN $\alpha$  greatly reduced in ISG15  
341 knockout cells. These data demonstrate ISG15 as a key positive regulator in IFN  
342 signaling and confirm its importance in host defense response against PRV infection,  
343 which may be more broadly against other viruses as well.

344 We found that ISG15 was involved in two crucial steps in IFN signaling, including  
345 the active pSTAT1 and pSTAT2 and the ISGF3 formation (Fig. 7 to 8). This may be  
346 partly because ISG15 knockout reduced interactions of STAT1 and STAT2 (Fig. 6E to  
347 F). Since ISG15 mainly localizes in the cytoplasm, we suppose the mechanism for  
348 ISG15 to impact this step is likely direct. The result that the lack of ISG15 decreases  
349 STAT1 and STAT2 phosphorylation with hindering interactions between STAT1 and  
350 STAT2 suggests that ISG15 may be involved in the formation of the STAT1-STAT2  
351 heterodimer. Although the regulation of the ISGF3-mediated transcription of ISGs in  
352 the nucleus is not well understood, we demonstrate that ISG15 carries out a critical  
353 positive regulator in this process. ISG15 seems to function by enhancing the ISGF3  
354 recruitment to the promoter of ISGs and promoting the transcription of ISGs, this is  
355 complex. ISGF3 complex is essential for ISRE activation. Thus, we speculate that  
356 ISG15 may act as a regulator promoting ISGF3 to its ISGs promoters for efficient



357 gene transcription. A similar mode of action is also observed in Bclaf1 that regulated  
358 the type I interferon responses and was degraded by alphaherpesvirus US3 [35].

359 We further studied the effects of ISG15 on PRV infection *in vivo* by using ISG15<sup>-/-</sup>  
360 mice model. The results identified that ISG15<sup>-/-</sup> mice displayed increased morbidity  
361 and mortality rates, viral replication, as well as promotes development of viral  
362 encephalitis in the brains of mice. This finding confirms that ISG15 positively  
363 regulates host anti-PRV effect *in vivo*, which may be broadly for other viruses as well.  
364 Others and our studies have highlighted a critical role of ISG15 during viral  
365 infections, and it could be a viable option for developing therapeutic target for  
366 controlling PRV.

## 367 MATERIALS AND METHODS

368 **Cell culture and virus.** Porcine kidney epithelial cells (PK15) were cultured at  
369 37°C in 5% CO<sub>2</sub> in Dulbecco's modified Eagle medium (DMEM; Gibco, Grand  
370 Island, NY, USA) supplemented with 10% fetal bovine serum (FBS; Gibco) and 1%  
371 penicillin- streptomycin (DingGuo, Beijing, China). The PRV-QXX virus was  
372 preserved in our laboratory. For experiments, PRV was amplified in PK15 cells, and  
373 virus titers were determined using a plaque assay, as previously described [21]. PRV  
374 infections was performed at an MOI of 1 PFU/cell.

375 **Chemicals and chemical treatments.** IFN $\alpha$  (Pbl, NJ, USA) was dissolved in 0.1%  
376 BSA and used at a final concentration of 1000 U/mL. The viral DNA polymerase  
377 inhibitor phosphonoacetic acid (PAA) was dissolved in deionized water and utilized at  
378 a concentration of 300  $\mu$ g/mL (GlpBio, Montclair, CA, USA). The chemicals was  
379 added to the cultures at the indicated concentrations.

380 **Quantitative RT-PCR and Western blots.** According to the protocol of the  
381 manufacturer, RNA was extracted from cells using the TRIzol reagent (Takara, Shiga,  
382 Japan) and reverse transcribed using the PrimeScript<sup>TM</sup> RT reagent Kit (Takara).  
383 Quantitative RT-PCR was used to determine gene expression using the SYBR Green  
384 Realtime Master Mix (Takara, DaLian, China). Table 1 contains a list of all primers  
385 used in this study. All values were normalized to the level of  $\beta$ -actin mRNA, and

386 relative expression was calculated using the comparative cycle threshold ( $2^{-\Delta\Delta CT}$ )  
387 method.

388 The cells were harvested and washed twice with PBS before being lysed with  
389 RIPA. After 15 minutes of centrifugation at 13,000 rpm, the supernatant fraction was  
390 collected. The BCA Protein Assay Kit was used to assess the protein concentration in  
391 supernatants (Beyotime Biotechnology, Shanghai, China). Equivalent quantities of  
392 each protein sample were electrophoresed on SDS-PAGE gels and transferred to  
393 PVDF membranes (Pall Corporation, Ann Arbor, MI, USA). The primary antibodies  
394 directed against the following proteins were: anti-ISG15 (1:3000 dilution; Abcam);  
395 anti- $\beta$ -actin, anti-HA, anti-myc (1:3000 dilution; Proteintech, Wuhan, China); anti-  
396 PRV-glycoprotein E (gE); anti-phospho-Tyr701 STAT1; anti-STAT1 (1:3000 dilution;  
397 Cell Signaling); anti-phospho-Tyr690 STAT2; anti-STAT2 (1:3000 dilution; Cell  
398 Signaling); anti-IRF9 (1:3000 dilution; Cell Signaling); anti-USP18 (1:3000 dilution;  
399 Cusabio Wuhan, China). Secondary antibodies conjugated with horseradish  
400 peroxidase against rabbit or mouse (1:5000 dilution; Santa Cruz) were used. The ECL  
401 Western blotting Analysis System was used to reveal protein bands (Millipore, United  
402 States). Densitometry was performed with ImageJ software and standardized against  
403  $\beta$ -actin.

404 **Immunofluorescence assay.** PK15 cells were plated into a confocal dish and  
405 transfected with HA-STAT1 or myc-STAT2 plasmid. 4% paraformaldehyde was used  
406 to fix the monolayer cells, and 0.5% Triton X-100 were used to permeabilized at 4°C  
407 with (Solarbio Life Science, Beijing, China). Following a wash with PBS, cells were  
408 permeabilized in blocking solution (5% bovine serum albumin in PBS) for 1 h. Fixed  
409 cells were treated with a primary antibody specific for PRV-gE followed by an Alexa  
410 Fluor 488-conjugated secondary antibody against mouse (Proteintech, Wuhan, China).  
411 4', 6-diamidino-2-phenylindole (DAPI) was used to stain the cell nuclei (Solarbio).  
412 Fluorescence pictures were acquired by confocal laser scanning microscopy  
413 (Olympus, Tokyo, Japan).

414 **ISG15 mutant plasmid.** The nonconjugative ISG15 plasmid pCAGGS-ISG15AA  
415 was constructed from pCAGGS-ISG15 using the site-directed mutagenesis kit

416 (Beyotime), with the following primer pair: forward, 5'- TATA  
417 TGAATCTGCGCCTGCGGGCGGCCGGACAGGG-3', and reverse, 5'-  
418 CCCTGTCCCGGCCGCCCGCAGGCGCAGATTCATATA-3'.

419 **siRNA silencing.** Twenty-four hours prior to transfection, PK15 cells were plated  
420 in 24-well plates. The cells were transfected with control small interfering RNAs  
421 (siRNAs), or specific siRNAs against Ube1L or USP18 using with 1  $\mu$ L  
422 Lipofectamine RNAiMAX reagent (Invitrogen) per well. At 24 hpt, the cells were  
423 infected with PRV (MOI=1). At four hpt, the culture media was changed with fresh  
424 medium containing 1000 U/mL IFN $\alpha$ , which was maintained throughout the infection  
425 duration. At 24 hpi, supernatants were collected for the viral titration, and cells were  
426 extracted for Western blotting and RT-qPCR analysis. The siRNAs sequences  
427 employed in this study were as follows: Ube1L no. 1:  
428 GCACUCCCACCUGAUAAA; Ube1L no. 2: CAGCC UCACUCUUCAUGAU;  
429 USP18 no. 1: GUCUCCAGAAGUACAAUAUTT; USP18 no. 2:  
430 CCAGUGUACUUAUGGAAAU; NC: UUCUCCGAACGUGUCACGU.

431 **ISRE-luciferase reporter assay.** Co-transfection of PK15 and ISG15<sup>-/-</sup>-PK15 cells  
432 with the identified plasmid and the ISRE-Luc reporter plasmid (100 ng) plus the  
433 internal control pRL-TK reporter plasmid was performed (5 ng). Cells were treated  
434 with IFN $\alpha$  (1000 U/mL) for 12 h and were harvested to conduct dual-luciferase  
435 reporter assay (Promega, Madison, WI). Firefly luciferase activity values were  
436 normalized to Renilla luciferase activity, and the relative fold changes in IFN-treated  
437 samples compared to IFN-untreated control were calculated.

438 **Co-immunoprecipitation (Co-IP) assays.** PK15 cells were co-transfected with  
439 HA-STAT1 and myc-STAT2 plasmids and then lysed with ice-cold lysis buffer (25  
440 mM Tris-HCl, pH 7.4, 1% NP-40, 150 mM NaCl, 1 mM EDTA) supplemented with  
441 protease inhibitors (Sigma). Cell lysate was cleared by centrifugation at 14,000 $\times$ g for  
442 5 min at 4°C. Primary antibodies against HA, STAT1 or STAT2 (dilution 1:1000;  
443 Proteintech) were added to the supernatants. After three washes with TBS, SDS-  
444 PAGE sample buffer was added, and proteins were separated by SDS-PAGE and  
445 immunoblotted to determine STAT1 and STAT2 interaction.

446 **PRV challenge assay *in vivo*.** The ISG15 knockout mice were generated from the  
447 Cyagen Biosciences (Cyagen, China). The seven-week-old male ISG15<sup>-/-</sup> mice and  
448 WT mice were randomly divided into two groups consisting of 15 mice each,  
449 respectively. The mice had free access to pelleted food and water during the  
450 experimental period. Each mouse was challenged by the subcutaneous infection with  
451 50µl of DMEM containing 5×10<sup>3</sup> TCID<sub>50</sub> of PRV-QXX. All the mice were monitored  
452 daily, and the mortality was recorded from 1 to 6 days post-infection. The brain  
453 samples were excised to detect the viral titer and the gE gene copies by plaque and  
454 RT-qPCR, respectively. Blood serum were also collected and kept at 4 °C to detect  
455 inflammatory factor through specific antibodies by enzyme-linked immunosorbent  
456 assay (ELISA). In parallel, the brain tissues were fixed in neutral-buffered formalin  
457 for histological analysis. All the animal experiments used in this study were approved  
458 by the Animal Ethics Committee of Henan Agricultural University.

459 **Statistical analysis.** GraphPad Prism 8 software was used to conduct statistical  
460 comparisons. The difference between groups was determined using Student's *t*-tests,  
461 and *P* values less than 0.05 were considered statistically significant (*p* < 0.05). The  
462 standard errors of the mean (SEM) of at least three independent experiments are  
463 shown for each data.

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593

## 594 **Figure Legends**

595 **FIG 1** Upregulation of ISG15 and ISGylation expression during PRV infection. (A) PK15  
596 cells were infected with PRV (MOI=1), and supernatants were collected at various time points  
597 following infection. PRV-gE and ISG15 mRNA levels were detected by RT-qPCR. The data  
598 represent the fold increase in gE and ISG15 RNA levels in PRV-infected cells relative to  
599 mock-infected cells. (B) Total protein extracts from panel A were tested for immunoblot  
600 analysis using antibodies specific for ISG15, PRV-gE, or  $\beta$ -actin. (C) PK15 cells were either  
601 mock-infected or infected with PRV at indicated MOI, and total RNA was harvested 24 hpi.  
602 RT-qPCR was used to quantify PRV-gE and ISG15 mRNA level. (D) As in panel C, Western  
603 blot analysis was used to determine ISG15 expression and protein ISGylation accumulation.  
604 (E) ISG15 expression patterns induced by IFN $\alpha$  (1000 U/ml) or PRV infection were compared  
605 by Western blotting. \*\*  $p < 0.01$  by Student's test.

606 **FIG 2** Regulation of free ISG15 and ISGylation accumulation in PRV-infected PK15 cells. (A  
607 and B) PK15 cells were mock-infected and PRV-infected left untreated or treated with PAA.  
608 Total RNA was collected 24 hpi and ISG15 mRNA was detected by RT-qPCR (A). Total  
609 proteins were harvested 24 hpi and the protein levels of ISG15 and PRV-gE were analyzed by  
610 Western blot (B). (C and D) The transcript and expression levels of ISG15 were detected in  
611 PK15 cells infected with PRV or UV-inactivated PRV 24 hpi, by RT-qPCR and Western blot  
612 respectively. (E) Total RNA was collected from PK15 cells mock-infected or PRV-infected  
613 for 24 h, and RT-qPCR was used to determine the mRNA levels of ISGylation enzymes

614 (Ube1L, UbcH8, Herc5 and USP18). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  (*t*-test).

615 **FIG 3** ISG15 inhibits PRV replication. (A and B) PRV-gE protein levels were detected in WT  
616 and ISG15<sup>-/-</sup> cells at identified time points and MOI by Western blot. (C) WT and ISG15<sup>-/-</sup>  
617 cells were infected with PRV (MOI=1) for 24 h, and the PRV-gE protein was measured using  
618 immunofluorescence microscopy assay. (D) PK15 cells were transfected with ISG15-  
619 overexpressing plasmid and then infected with PRV (MOI=1) 12 h later. PRV-gE RNA was  
620 quantified by RT-qPCR at different times post-infection. The data represent the percentages of  
621 expression of PRV-gE in ISG15-transfected cells or ISG15 knockout cells compared with  
622 cells transfected with a control plasmid (100%). (E) The PRV titer in the culture supernatant  
623 or associated with cells was determined by plaque assay at 24 hpi. Each experiment was  
624 repeated at least three times separately. \*,  $p < 0.05$ ; \*\*  $p < 0.01$  (*t*-test).

625 **FIG 4** Antiviral activity of ISG15 against PRV relies on free ISG15. (A) PK15 cells were  
626 transfected with either an empty control plasmid, an ISG15-expressing plasmid, or an plasmid  
627 expressing ISG15 mutant (ISG15AA). After 24 hours, the cells were infected with PRV  
628 (MOI=1). Total proteins were collected 24 hpi, and Western blot was used to determine the  
629 expression of ISG15 unconjugated and conjugated protein. The PRV titer was detected by  
630 plaque assay. (B) As in panel A, ISG15<sup>-/-</sup> cells were transfected and infected, and the ISG15  
631 protein expression and virus titer were determined. (C to D) PK15 cells were transfected with  
632 either control siRNA or Ube1L siRNAs (C), USP18 siRNAs (D), and infected 24 h later with  
633 PRV (MOI=1). Protein extracts were harvested at 24 hpi and determined by Western blot with  
634 anti-ISG15 and anti-gE antibodies. Total RNA was collected to determine PRV titer by plaque  
635 assay. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  (*t*-test).

636 **FIG 5** ISG15 promotes IFN $\alpha$  antiviral activity against PRV. (A to C) Prior to infection with  
637 PRV at an MOI of 1, WT and ISG15<sup>-/-</sup> cells were either treated with IFN $\alpha$  (1000 U/ml) or left  
638 untreated. Protein extracts from the cells were harvested 24 hpi and analyzed by Western blot.  
639 Relative fold changes in PRV-gE expression levels are indicated between WT and ISG15<sup>-/-</sup>  
640 cells in the absence and presence of IFN $\alpha$  (B to C). Total RNA and supernatants were  
641 harvested 24 hpi to determine the PRV-gE mRNA level and virus titers by RT-qPCR and  
642 plaque assay, respectively. (D) ISG15<sup>-/-</sup> cells were transfected with either a plasmid  
643 expressing ISG15 mutant or an empty plasmid. After 12 h, WT and ISG15<sup>-/-</sup> cells were treated  
644 with or without IFN $\alpha$  (1000 U/ml) before infecting with PRV. Western blot was used to  
645 analyze the protein expression of ISG15 and gE, The data are presented as means standard  
646 error of the mean  $\pm$  (SEM) of at least three independent experiments. \*,  $p \leq 0.05$  (*t*-test).

647 **FIG 6** ISG15 facilitates the phosphorylation of STAT1/STAT2. (A to B) WT and ISG15<sup>-/-</sup>  
648 PK15 cells were treated or un-treated with IFN $\alpha$  (1000 U /ml) and then infected 12 h later  
649 with PRV (MOI=1). At 24 hpi, the relative pSTAT1 and pSTAT2 levels were analyzed by

650 Western blotting with the specified antibodies. The ratio of pSTAT1 (pSTAT2) and STAT1-tot  
651 (pSTAT2-tot) protein levels relative to control was calculated by ImageJ software. (C)  
652 Subcellular fractionation and Western blotting were used to identify phosphorylated STAT1  
653 and STAT2 in nuclear and cytoplasmic fractions of WT and ISG15<sup>-/-</sup> cells. The same blot was  
654 incubated with antibodies against  $\beta$ -actin and histone H3 as controls for loading and  
655 fractionation. (D) Immunofluorescence of WT and ISG15<sup>-/-</sup> cells transfected with HA-STAT1  
656 or Myc-STAT2 and stimulated with IFN $\alpha$ . Nuclei (blue) and pSTAT1/pSTAT2 (red) were  
657 detected. (E) WT cells and ISG15<sup>-/-</sup> PK15 cells were co-transfected with HA-STAT1 and  
658 Myc-STAT2. At 24 hpt, the cells were treated with IFN $\alpha$  and infected with PRV (MOI=1), and  
659 the STAT1 and STAT2 expression were analyzed by anti-HA and anti-Myc antibodies. (F)  
660 Endogenous STAT1 and STAT2 expression were detected from cells treated with IFN $\alpha$  and  
661 infected with PRV, with anti-STAT1 (Tyr 701) and anti-STAT2 (Tyr 690) specific antibodies.  
662 \*,  $p \leq 0.05$  (*t*-test).

663 **FIG 7** ISG15 silencing facilitates the formation of ISGF3. (A) WT and ISG15<sup>-/-</sup> cells were  
664 treated or un-treated with IFN $\alpha$  and then infected with PRV. Subcellular fractionation and  
665 sequent Western blotting were used to detect p-STAT1, p-STAT2, total STAT1, total STAT2,  
666 IRF9 in the cytoplasmic and nuclear fractions of WT and ISG15<sup>-/-</sup> cells. The same blot was  
667 incubated with antibodies against  $\beta$ -actin and histone H3 as controls for loading and  
668 fractionation. (B) WT and ISG15<sup>-/-</sup> cells were co-transfected with STAT1, and/or STAT2,  
669 and/or IRF9, pGL4.17-ISRE-Luc (firefly luciferase) and pRL-TK (Renilla luciferase). ISRE  
670 promoter activity was measured at 24 hpi for Dual-luciferase reporter gene assay. (C) IFIT1,  
671 MxA and OAS1 mRNA levels were quantified the samples from WT or ISG15<sup>-/-</sup> cells with or  
672 without IFN $\alpha$  treatment. Fold change in mRNA levels relative to the untreated group was  
673 calculated using the  $2^{\Delta\Delta CT}$  method, and the  $\beta$ -actin gene was used as the housekeeping gene.  
674 \*\*,  $p \leq 0.001$ ; \*\*\*  $p \leq 0.0001$  (*t*-test).

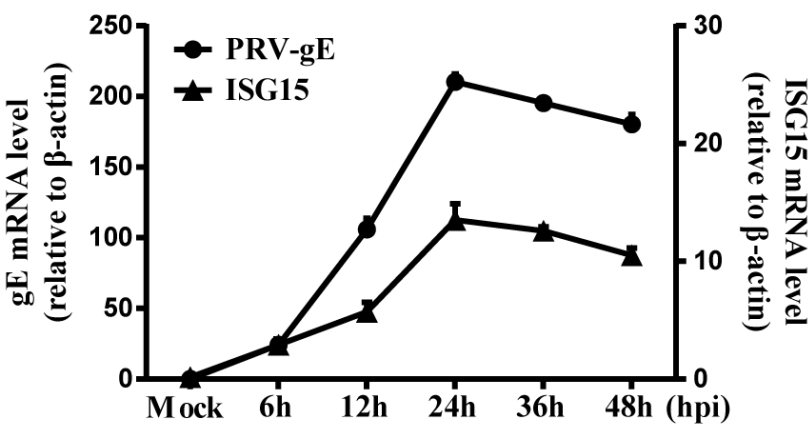
675 **FIG 8 PRV challenge assay in vivo.** Seven-week-old male ISG15<sup>-/-</sup> mice (n=15) and WT  
676 mice (n=15) were inoculated with  $5 \times 10^3$  TCID<sub>50</sub> of PRV-QXX subcutaneous. (A) Survival of  
677 the infected WT and ISG15<sup>-/-</sup> mice was monitored until day 6 after infection. Statistical  
678 significance was determined by the log-rank test. (B to D) Brain tissues of the infected mice  
679 were collected at different days post-infection. The ISG15 expression, PRV-gE copies and  
680 viral titers were detected by Western blotting, RT-qPCR, and plaque assay respectively. (E)  
681 The histopathological features of brains of the infected WT and ISG15<sup>-/-</sup> mice. The brain  
682 tissues were sectioned and stained with hematoxylin-eosin. Magnified images of the regions  
683 with black rectangles in infected WT and ISG15<sup>-/-</sup> mice, respectively. Representative images  
684 are shown: ND: nuclear disintegration of Purkinje cell; N: neurons; NN: necrotic neurons; G:  
685 glial cells; PS: shrinkage of Purkinje cells; P: Purkinje cells; PN: necrotic Purkinje cells; MI:

686 mononuclear cellular infiltration. (F) The concentrations of IL-6, TNF- $\alpha$  and IL-1 $\beta$  in serum  
687 of infected WT and ISG15<sup>-/-</sup> mice were determined by ELISA.

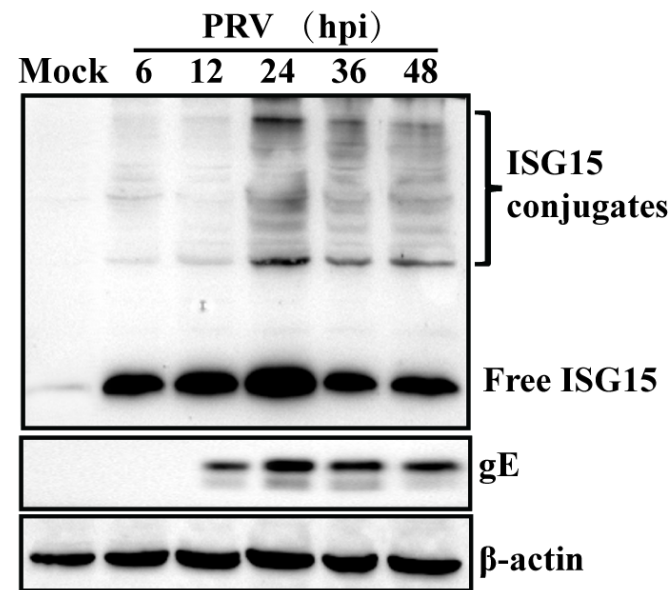
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689 **FIG 9** Mechanism that has been provided. Type I IFN induced by PRV infection mediates  
690 antiviral response by upregulating ISG15. Our work demonstrates that increased ISG15  
691 positively regulates IFN $\alpha$ -induced antiviral activity against PRV, by facilitating  
692 phosphorylation of STAT1 and STAT2. This regulation results in increased ISG15 inhibiting  
693 PRV replication (red line).

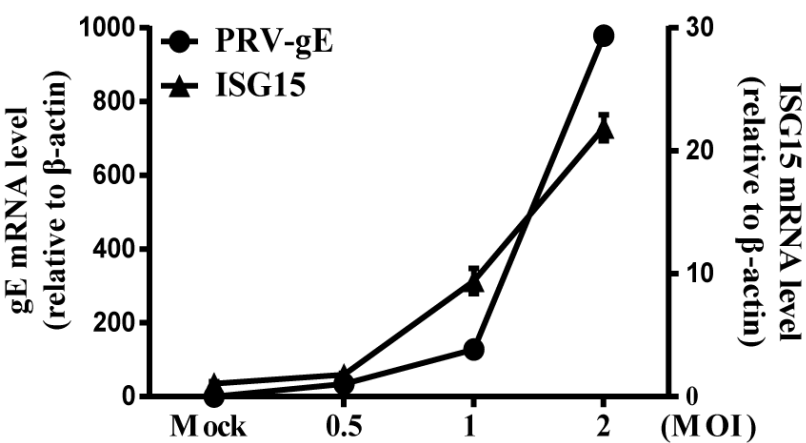
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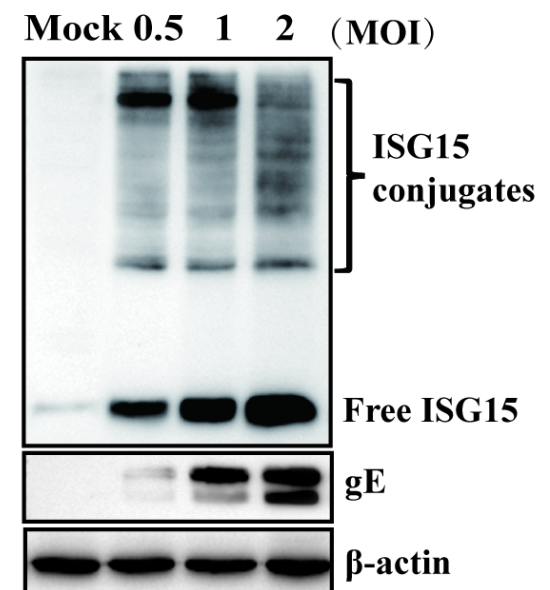
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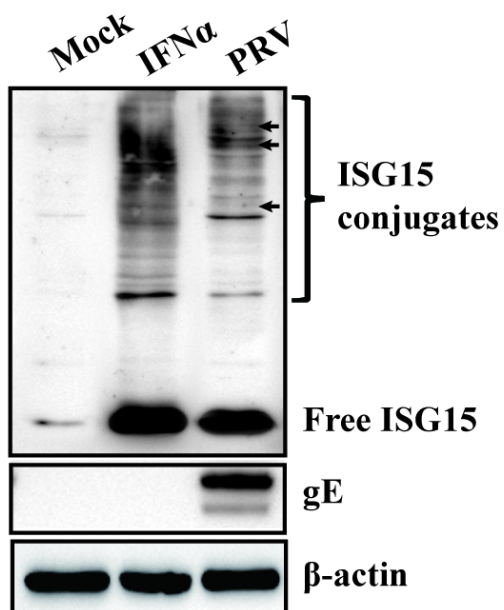
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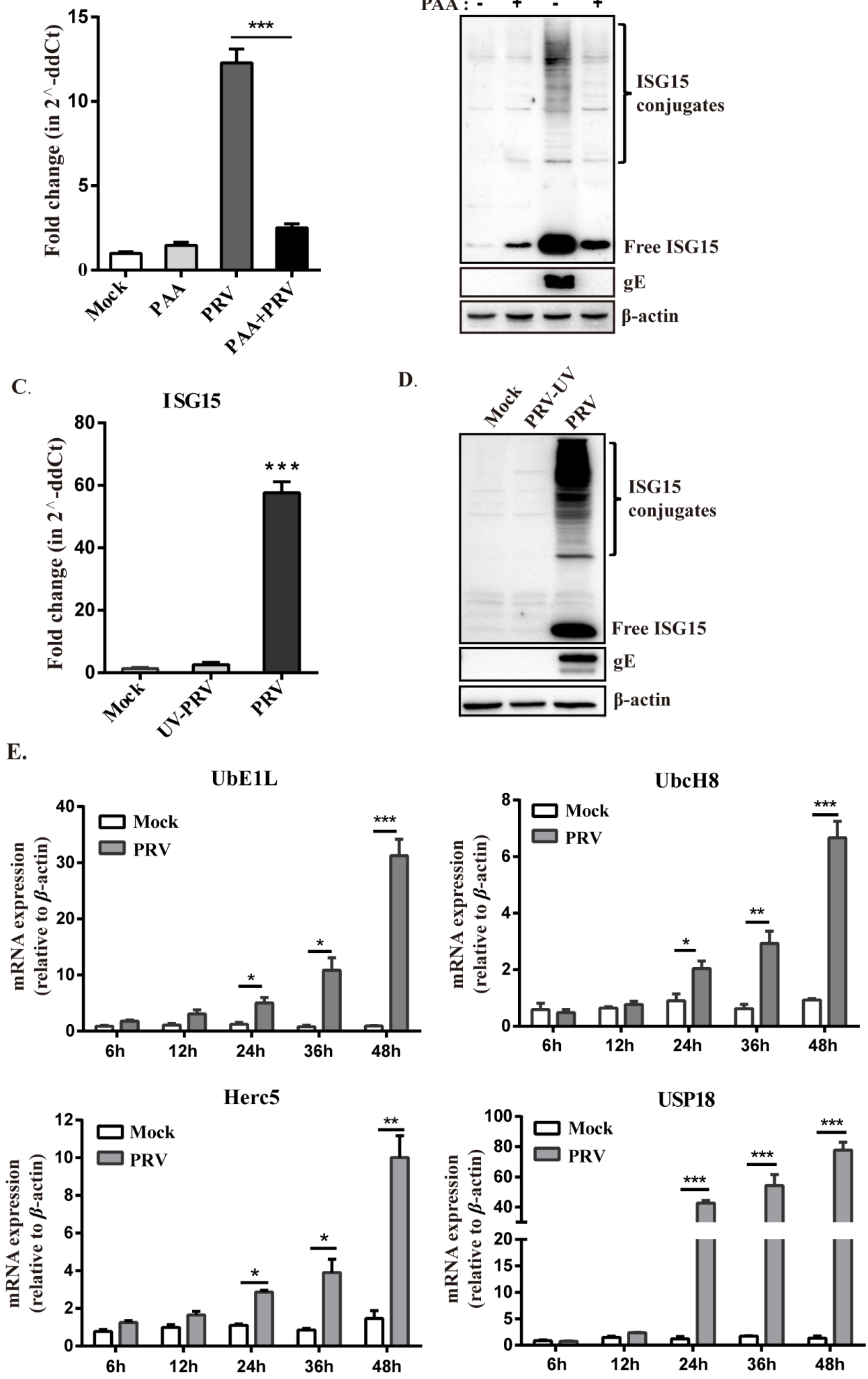


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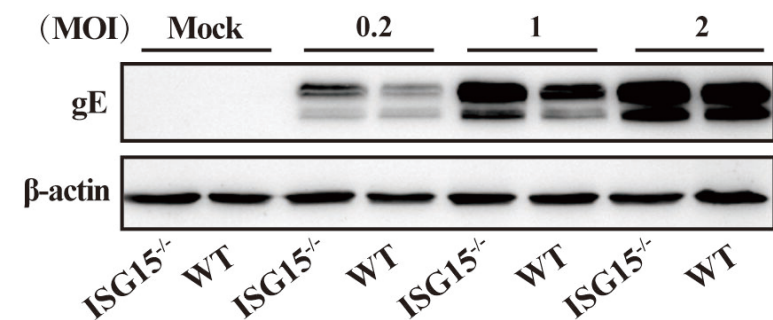
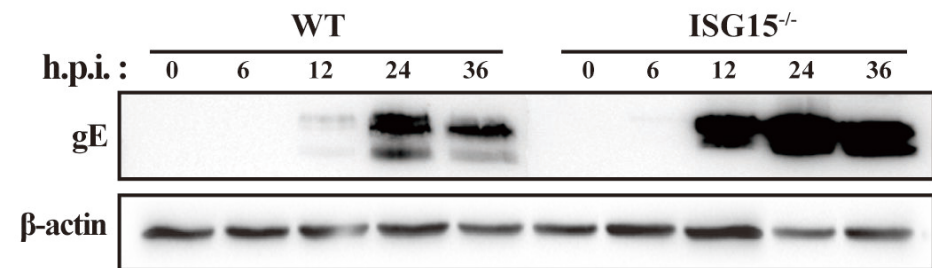
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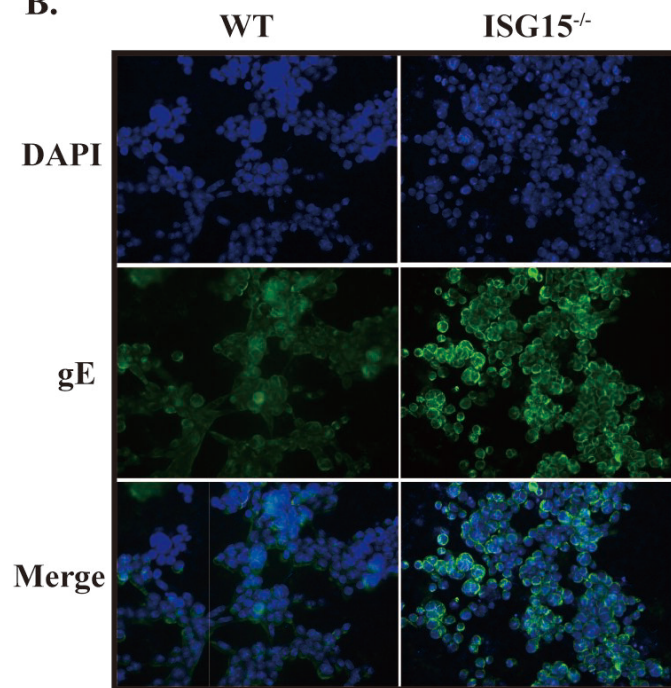




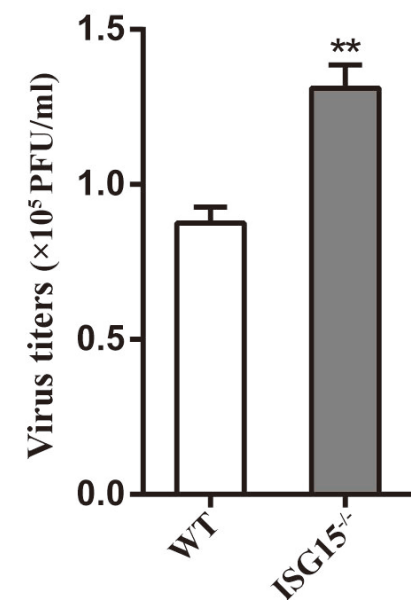
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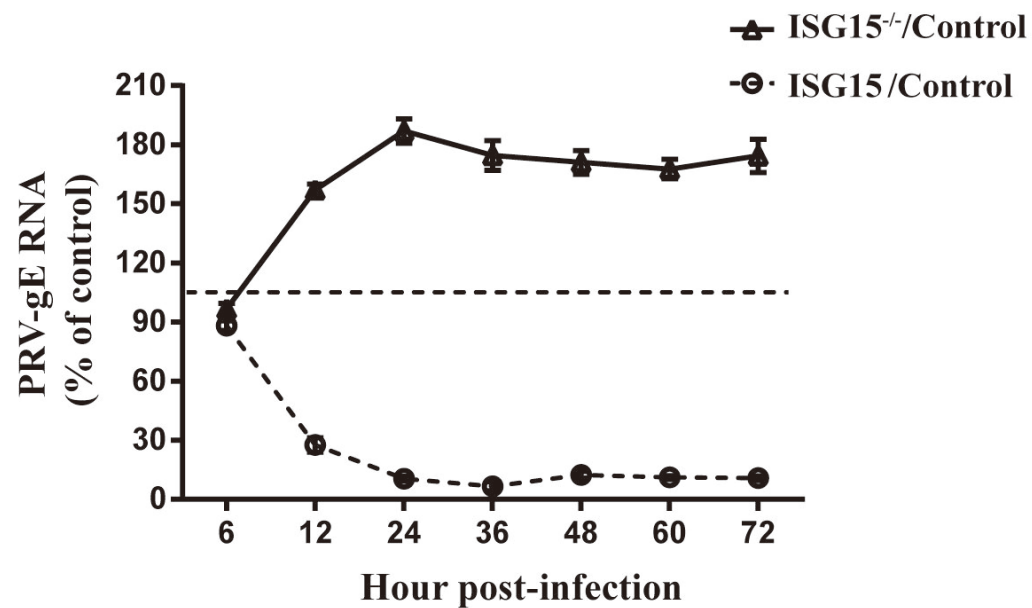
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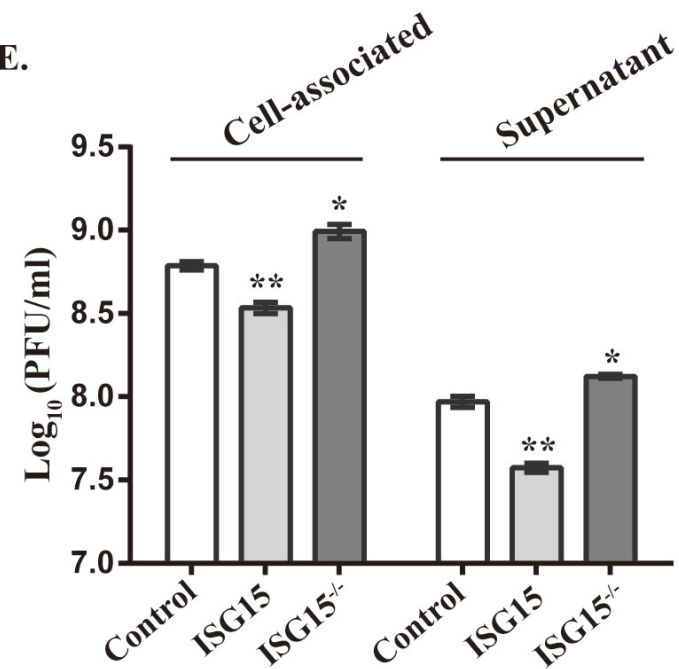
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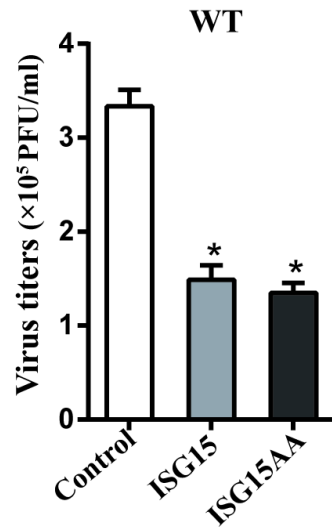
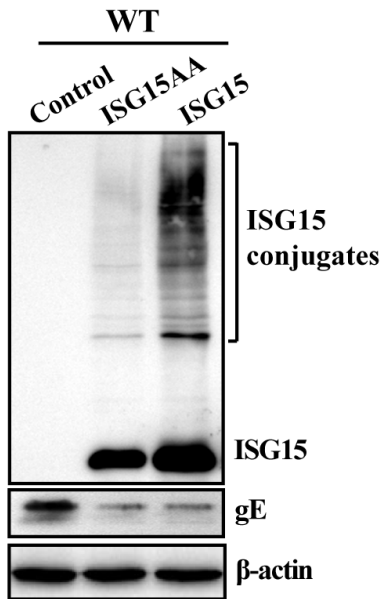


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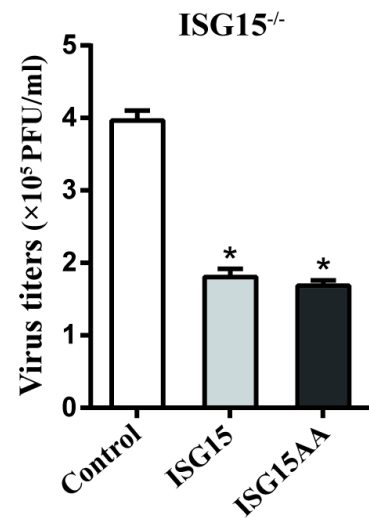
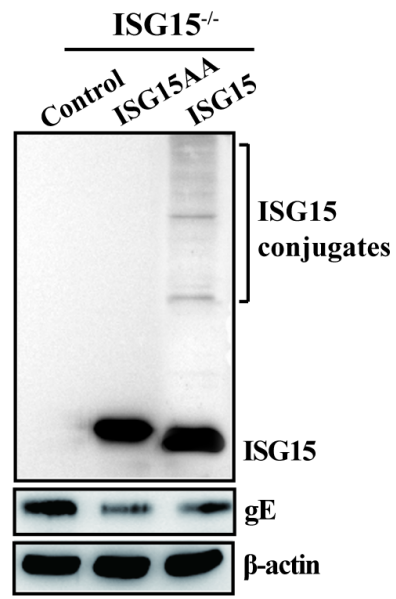




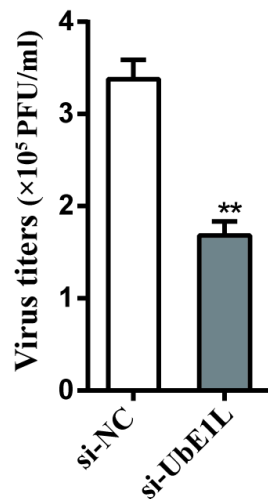
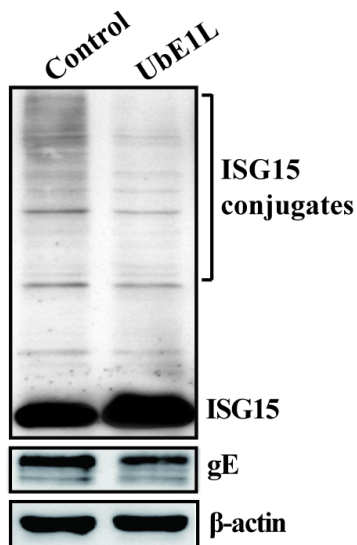
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