

1 **DDX50 is a viral restriction factor that enhances TRIF-dependent IRF3 activation**

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12 **Abstract**

13 The transcription factors IRF3 and NF- κ B are crucial in innate immune signalling in response
14 to many viral and bacterial pathogens. However, mechanisms leading to their activation
15 remain incompletely understood. Canonical RLR signalling and detection of viral RNA is
16 dependent upon the receptors RIG-I, MDA5 and TLR3. Alternatively, the DExD-Box RNA
17 helicases DDX1-DDX21-DHX36 activate IRF3/NF- κ B in a TRIF-dependent manner
18 independent of RIG-I, MDA5 or TLR3. Here we describe DDX50, which shares 55.6% amino
19 acid identity with DDX21, as a component of the dsRNA sensing machinery and signalling
20 pathway. Deletion of DDX50 in mouse and human cells impaired activation of the IFN β
21 promoter, IRF3-dependent endogenous gene expression and cytokine/chemokine
22 production in response to cytoplasmic dsRNA (polyIC transfection), and infection by RNA
23 and DNA viruses. Mechanistically, DDX50 co-immunoprecipitated with TRIF and DDX1,
24 promoting complex formation upon stimulation. Furthermore, whilst MAVs/TBK1 induced
25 signalling is intact in *Ddx50* KO cells, TRIF-dependent signalling was impaired suggesting
26 DDX50 drives TRIF-dependent *Ifn β* transcription. Importantly, loss of DDX50 resulted in
27 increased replication and dissemination of vaccinia virus, herpes simplex virus and Zika
28 virus highlighting its important role as a viral restriction factor.

29 **Author summary**

30 The detection of viral RNA or DNA by host RNA or DNA sensors and the subsequent
31 antiviral immune response are crucial for the outcome of infection and host survival in
32 response to a multitude of viral pathogens. Detection of viral RNA or DNA culminates in the
33 upregulation of inflammatory cytokines, chemokines and pathogen restriction factors that
34 augment the host innate immune response, restrict viral replication and clear infection. The
35 canonical RNA sensor RIG-I is a member of the large family of DExD/H-box helicases,
36 however the biological role of many DExD/H-box helicases remain unknown. In this report,
37 we describe the DExD-Box helicase DDX50 as a new component of the RNA sensing
38 machinery. In response to DNA and RNA virus infection, DDX50 functions to enhance
39 activation of the transcription factor IRF3, which enhances antiviral signalling. The biological
40 importance of DDX50 is illustrated by its ability to restrict the establishment of viral infection
41 and to diminish the yields of vaccinia virus, herpes simplex virus and Zika virus. These
42 findings increase knowledge of the poorly characterised host protein DDX50 and add
43 another factor to the intricate network of proteins involved in regulating antiviral signalling in
44 response to infection.

45 Introduction

46 Interferon regulatory factor 3 (IRF3)-dependent signalling leading to type I interferon (IFN)
47 expression is crucial for pathogen clearance and host survival in response to infection by
48 many viral and bacterial pathogens (1,2). IRF3 signalling is tightly regulated and is triggered
49 by intracellular cytoplasmic/endoplasmic detection of viral RNA (dsRNA/5'-ppp/pp-RNA) (3)
50 and DNA by pattern recognition receptors (PRRs) (4). The retinoic acid-inducible gene I
51 (RIG-I)-like receptors (RLRs), which include DDX58 (RIG-I), DDX21, DDX1, DHX36, DDX60,
52 DDX3, DHX9, DHX33, MDA5, and LPG2, as well as Toll-like receptor (TLR) -3, bind directly
53 to, or form complexes with, viral dsRNA or 5'-ppp-RNA to activate downstream kinases and
54 induce expression of type I IFN (5). RIG-I/MDA5 activation leads to mitochondrial antiviral
55 signalling protein (MAVS)-dependent autophosphorylation of TANK-binding protein-1
56 (TBK1). In turn, TBK1 phosphorylates IRF3, leading to its dimerisation and nuclear
57 translocation. In parallel, the transcription factor nuclear factor kappa-light-chain-enhancer of
58 activated B cells (NF- κ B) is activated in an inhibitor of nuclear factor kappa-B kinase subunit
59 beta (I κ K β) dependent-manner (6). IRF3 and NF- κ B trigger co-transcriptional upregulation of
60 IFNs and inflammatory cytokines and chemokines, including IFN β and C-X-C motif
61 chemokine 10 (CXCL10/IP-10), as well as IRF3-dependent viral restriction factors (7,8). This
62 establishes the host antiviral innate immune response, restricting viral replication and
63 clearing infection.

64 The prototypical RLR, RIG-I, comprises a DExD-box ATPase-dependent RNA helicase with
65 an N-terminal caspase activation and recruitment domain (CARD) and a C-terminal auto-
66 inhibitory regulatory domain (RD). Briefly, under resting conditions, RIG-I is held in an
67 autoinhibitory conformation. Upon agonist (5'-ppp/pp-RNA or short dsRNA) binding to the
68 RD, RIG-I undergoes conformational change, dimerisation and activation (9). Through
69 interaction with the E3 ligase tripartite-motif containing protein 25 (TRIM25) and subsequent
70 K63-linked ubiquitylation of RIG-I, signalling is transduced via complex formation with the
71 adaptor MAVS (10). Similarly, MDA5 signalling converges at MAVS activation, but differs

72 from RIG-I receptor signalling due to alterations in ligand specificity. Alternatively, TLR3
73 differs in cellular compartmentalisation/localisation and signals in a TIR domain-containing
74 adapter molecule 1 (TICAM-1 or TRIF) and TBK-dependent manner independent of MAVS.
75 MDA5 recognises high molecular weight dsRNA (11) or mRNA lacking 2'-O-methylation at
76 the 5' cap (12), whereas TLR3 detects dsRNA in the endosomal or extracellular
77 compartments. These subtle differences mean that during infection numerous RLRs and
78 RNA sensors are activated in parallel or independent of one another and this is dependent
79 upon cell type, the pathogen and/or the specific ligands present.

80 Other DExD-Box RNA helicase family members play an essential role in IRF3 signalling in
81 response to viral PAMPs, including DDX60 (13,14), DDX1, DHX36, DDX21 (15), DHX33
82 (16) and DDX41 (17). Miyashita and colleagues identified DDX60 as a component of RLR-
83 dependent signalling, acting through RIG-I and MDA5 to trigger optimal IRF3-dependent
84 gene expression (13,14). However, a role for DDX60 in RLR signalling is contentious and
85 recently it was reported to be dispensable for IFN production in response to several RLR
86 agonists (18). Additionally, DDX1, DHX36 and DDX21 form a cytoplasmic complex with
87 TRIF upon detection of 5'-ppp-RNA or dsRNA (PolyIC). Upon complex formation, DDX1 and
88 DHX36 interaction, and TRIF recruitment, are DDX21-dependent, whereas DDX1 acts as
89 the complex RNA sensor. Interestingly, this complex acts independently of TLR3, RIG-I and
90 MDA5 in mouse dendritic cells (DCs) and mouse embryonic fibroblasts (MEFs) (15).

91 A recent RNAi screen, implicated the relatively uncharacterised DExD-Box RNA helicase
92 proteins DDX17 and DDX50 as putative positive regulators of IFN β promoter activity in
93 response to cytoplasmic 5'-ppp-RNA (19). DDX50 is a paralogue of DDX21 sharing 55.6 %
94 amino acid identity (20). DDX21 (Gu α ; nucleolar protein 2) and DDX50 (Gu β ; nucleolar
95 protein 1) are the only members of the Gu family of nucleolar RNA helicases and contain a
96 highly homologous GUCT (Gu C-terminal) domain, which is followed by an arginine-serine-
97 rich C-terminal tail in DDX50 (21). DDX50, as the name suggests, is localised to the nucleoli
98 and *in vitro* assays have demonstrated that both DDX21 and DDX50 have ATPase and

99 helicase activity, however DDX50 lacks RNA folding activity (21). Although DDX21 and
100 DDX50 may have arisen by gene duplication on chromosome 10, these proteins have non-
101 redundant roles. DDX21 targets RNA substrates with a 21- or 34-nt duplex and 5'-
102 overhangs, whereas DDX50 targets only 21-nt duplex RNA for unwinding (21). On the other
103 hand, DDX50 is required for optimal DDX21 unwinding activity, suggesting some co-
104 dependence (21). Little is known about the biological function of DDX50, with one study
105 suggesting it may be involved in MAP-kinase signalling through interaction with c-Jun (22).

106 By using CRISPR technology to knockout *Ddx50* in MEFs and *DDX50* in human embryonic
107 kidney 293T cells (HEK293Ts), DDX50 was identified as a restriction factor for the DNA
108 viruses vaccinia virus (VACV) and herpes simplex virus type 1 (HSV-1) and the RNA virus
109 Zika (ZIKV). Mechanistically, DDX50 enhances IRF3-dependent gene transcription and
110 cytokine synthesis and secretion in response to cytoplasmic dsRNA, and RNA or DNA virus
111 infection.

112 **Results**

113 **DDX50 is a novel factor required for nucleic acid sensing**

114 To investigate the putative role of DDX50 in cytoplasmic RNA sensing, CRISPR-mediated
115 knockouts (KO) were generated in MEFs and HEK293Ts. Successful KO was confirmed by
116 immunoblotting (Fig. S1B and E) and genomic sequencing of individual alleles (Fig. S1A, C,
117 D, F). Sequencing indicated frameshifts in exon 1 (Fig. S1C) and exon 4 (Fig. S1F)
118 producing nonsense mutations and introduction of an early stop codon. No differences in
119 morphology or growth properties between the wild type (WT) and KO cells were observed
120 (data not shown). Initially, the contribution of DDX50 to IRF3 signalling in response to RLR
121 agonists was investigated. Cells were co-transfected with a Firefly Luciferase reporter
122 plasmid under the control of the IFN β promoter (pIFN β -Luc) and an internal control plasmid
123 constitutively expressing Renilla Luciferase (pTK-RL). These cells were further mock-
124 transfected or transfected with PolyIC (dsRNA analogue), infected with an RNA virus
125 (Sendai virus (SeV)) or treated with extracellular PolyIC. IFN β promoter activity was then
126 measured relative to Renilla luminescence and non-stimulated controls. Promoter activity
127 was significantly diminished in KO cells in comparison to WT cells in response to all stimuli
128 (Fig. 1A), validating the results observed in the initial RNAi screen (19). Consistent with this
129 observation, knockout of *Ddx50* reduced the expression of endogenous NF- κ B and/or IRF3-
130 dependent genes (*Isg56*, *Cxcl10* and *Ifnb*) in response to PolyIC and SeV as measured by
131 RT-qPCR (Fig. 1B) and ELISA (CXCL10 and IL-6; Fig. 1C-D). Collectively, this indicates that
132 DDX50 affects both the IRF3 or IRF3 and NF- κ B branch of signalling in response to
133 cytoplasmic dsRNA. Importantly, the defect in NF- κ B/IRF3-dependent gene expression in
134 response to PolyIC was rescued by transduction and complementation of *Ddx50* KO cells
135 with a lentiviral vector encoding *Ddx50* (Fig. 2A-C). This ruled out CRISPR off target effects
136 for the observed defect. Furthermore, overexpression of DDX50 augmented IFN β promoter
137 activity (Fig. S2A-B) and secretion of CXCL10 and IL-6 in response to PolyIC transfection
138 (Fig. S2C-D). Interestingly, overexpression of DDX50 alone induced secretion of CXCL10

139 (Fig. S2C), indicating pathway activation above basal level even in the absence of
140 stimulation. Concomitant experiments in HEK293T *DDX50* KO lines confirmed a
141 dependence on DDX50 for optimal pathway activation in response to SeV infection in a
142 human cell line (Fig. 1E-F).

143 **DDX50 is required for IRF3/NF- κ B driven gene expression during DNA virus infection**

144 To investigate the biological relevance of DDX50, WT or KO MEFs were infected with two
145 large dsDNA viruses, VACV and HSV-1. The modified vaccinia Ankara (MVA) strain and the
146 HSV-1 Δ ICPO strain each elicit strong innate immune responses in tissue culture and were
147 therefore used to increase pathway activation and sensitivity, as described previously
148 (23,24). Following infection with either MVA or HSV-1 Δ ICP0, the expression of IRF3 and/or
149 NF- κ B-dependent *Isg56* and *Ifnb* were significantly diminished in the KO cells in comparison
150 to infected WT cells (Fig. 3A-B). This correlated with decreased secretion of CXCL10 and IL-
151 6 as determined by ELISA (Fig. 3C-D). Although significant, the effect of *Ddx50* KO on
152 *Cxcl10* expression by RT-qPCR following infection with both viruses was less pronounced
153 (Fig. 3A-B). Overall, this highlights the importance of DDX50 in innate immune signalling
154 during viral infection.

155 **Loss of *Ddx50* does not alter IL-1 α or TNF α -mediated NF- κ B activation**

156 Deletion of *Ddx50* impaired the induction of NF- κ B/IRF3-co-transcribed genes in response to
157 dsRNA transfection, ssRNA virus infection (Fig. 1) and dsDNA virus infection (Fig. 2) and
158 previously was reported to modulate MAP kinase signalling (22). Therefore, alternative
159 pathways were tested to determine if the observed defect was specific to RLR signalling. WT
160 or KO MEFs were treated with IL-1 α or TNF α and activation of the NF- κ B promoter or
161 expression of *Il-6* and *Nfkb1a* were measured by Luciferase reporter gene assay or RT-
162 qPCR, respectively. No differences in NF- κ B promoter activity or NF- κ B-dependent gene
163 expression were observed (Fig. 4A-C), indicating that DDX50 does not play a role in
164 canonical IL-1 receptor- or TNF receptor-induced NF- κ B signalling. Collectively, these data

165 suggest DDX50 acts at the stage of IRF3 activation specifically or at or upstream of MAVS
166 and/or TRIF activation before the RNA sensing pathways diverge to activate IRF3 and NF-
167 κ B.

168 **DDX50 accumulates in the cytoplasm to activate signalling upstream to MAVS**

169 To investigate where DDX50 acts in the pathway and determine how it facilitates activation
170 of IRF3/NF- κ B in response to dsRNA, the phosphorylation of IRF3 was examined. This is a
171 key step in IRF3-dependent signalling and leads to IRF3 dimerisation, nuclear translocation
172 and IRF3-dependent gene transcription. Interestingly, DDX50 was observed to be important
173 for IRF3 phosphorylation at Ser386/396 following PolyIC transfection of both MEFs and
174 HEK293Ts (Fig. 5A-B), mapping DDX50 function to upstream of IRF3 phosphorylation.
175 DDX50 is reported to reside in the nucleolus. However, to act upstream of IRF3
176 phosphorylation in the canonical cascade one would expect DDX50 to be cytosolic. To
177 explore this further biochemical fractionation of MEFs and anti-DDX50 immunoblotting with
178 or without prior pathway stimulation was used to assess the subcellular localisation of
179 DDX50 in response to cytoplasmic dsRNA. LaminA/C and α -tubulin served as nuclear and
180 cytoplasmic fraction controls, respectively. As described, under resting conditions, the
181 majority of DDX50 was in the nuclear fraction (21) (Fig. 5C). However, DDX50 accumulated
182 in the cytoplasm 1 h post-stimulation (Fig. 5C). At 2 h post-stimulation the level of DDX50 in
183 the cytoplasm returned to basal levels (Fig. 5C). To support this finding the assay was
184 repeated and the localisation of HA-tagged DDX50 was analysed by immunofluorescence.
185 Under resting conditions DDX50 was restricted to the nucleolus, with weak nuclear staining.
186 In agreement with the biochemical fractionation assay, accumulation of DDX50 in distinct
187 cytoplasmic puncta was observed 1 h post infection with SeV (Fig. 5D).

188 The nucleocytoplasmic shuttling of DDX50 upon stimulation led us to investigate at which
189 stage in the activation of the IRF3/NF- κ B pathway DDX50 might function. The IRF3/NF- κ B
190 pathway can be activated by transfection and overexpression of key proteins acting at

191 specific stages of the pathway. Therefore, to map in more detail where DDX50 acts,
192 plasmids encoding TBK1, MAVS or TRIF were co-transfected into WT or KO MEFs along
193 with pLuc-IFN β and pTK-RL. Activation of the pathway was measured by Firefly and Renilla
194 Luciferase activation as before. No differences in fold activation were observed between the
195 WT and KO cells upon expression of TBK1 or MAVS (Fig. 5E). However, activation was
196 significantly impaired in the KO cell line upon expression of TRIF, mapping DDX50 upstream
197 to or independent of MAVS, but at or downstream of TRIF activation. Notably, DDX50
198 shares 55.6 % amino acid identity with DDX21, which is essential for TRIF recruitment to
199 MAVS via complex formation with DDX1 and DHX36 in response to cytoplasmic dsRNA
200 (15).

201 **DDX50 augments TRIF recruitment to activate signal transduction**

202 An essential TRIF-binding domain of DDX21 has been mapped to residues 467-487 within
203 the RNA helicase C domain (15). Strikingly, this motif shares 86% amino acid identity with
204 DDX50 (Fig. 6A). This level of homology was specific for DDX50 and not due to the helicase
205 C domain consensus sequence, because it was not detected within other DExD-box family
206 members such as DHX36 (Fig. 6A). Due to the high level of identity between DDX21 and
207 DDX50 we investigated whether DDX50 can co-immunoprecipitate the DDX1-DDX21-
208 DHX36-TRIF complex. To this end, co-immunoprecipitation assays were performed using
209 extracts of MEF cell lines that stably expressed DDX50-HA and that were transfected with
210 TRIF-cTAP or GFP-Flag. Following stimulation, DDX50-HA specifically co-
211 immunoprecipitated TRIF-cTAP (Fig. 6B). This was confirmed by reciprocal
212 immunoprecipitation in HeLa cells, where hDDX50-HA specifically co-immunoprecipitated
213 with TRIF-cTAP (Fig. 6C). Due to the quality of available anti-TRIF antibodies, co-
214 immunoprecipitation of endogenous TRIF could not be tested. However, endogenous DDX1
215 did co-immunoprecipitate with DDX50-HA, albeit at low levels (Fig. 6D). This led to the
216 hypothesis that DDX50 may form a cytoplasmic RNA sensing complex with TRIF, to activate
217 TRIF-dependent NF- κ B and IRF3 activation. To test this hypothesis, the ability of TRIF to

218 form a complex with DDX1 in WT or DDX50 KO cells was investigated. Interestingly, in the
219 absence of DDX50 the co-immunoprecipitation of endogenous DDX1 with TRIF was
220 diminished, indicating DDX50 may facilitate optimal TRIF recruitment to the RIG-I/MDA5
221 independent RNA sensing complex (Fig. 6E).

222 **DDX50 is a viral restriction factor**

223 IRF3 is a crucial viral restriction factor that controls the transcriptional upregulation of
224 cytokines, chemokines, viral restriction factors and type I IFNs and thereafter IFN-stimulated
225 genes (ISGs) downstream of IFN-induced signalling. Given the role of DDX50 in IRF3/NF-
226 κ B-dependent type I IFN production and the synthesis of cytokines during viral infection, its
227 potential as a viral restriction factor was investigated. WT and DDX50 KO cells were infected
228 at either high MOI or low MOI with the dsDNA viruses VACV (MEF, MOI 5 or 0.0001;
229 HEK293T, MOI 5 or 0.0003) and HSV-1 (MOI 0.01) or ZIKV (MOI 1 or 0.1), a ssRNA virus,
230 and virus replication and dissemination were analysed by virus titration and plaque
231 formation. VACV infection produces both single enveloped intracellular mature virus (IMV)
232 and double enveloped cell associate enveloped virus (CEV) and extracellular enveloped
233 virus (EEV) (25). CEVs induce the formation of actin tails to propel virions towards
234 uninfected neighbouring cells. Alternatively, EEVs are released from infected cells and
235 mediate long range dissemination (26). To investigate if loss of *Ddx50* alters viral replication
236 or release, VACV strain Western Reserve (WR) encoding GFP fused to the virus capsid
237 protein A5 (A5-GFP VACV) was used to infect WT or KO MEFs/HEK293Ts at MOI 5 and the
238 total virus or extracellular virus titres 24 h p.i. were determined by plaque assay. No
239 differences in titres of cell associated virus (IMV plus CEV) or released virus (EEV) were
240 observed (Fig. 7A-B) and equal amounts of EEV were produced (approximately 2 % of the
241 total titre; Fig. 7A). Next, virus dissemination and replication were assessed at low MOI.
242 Monolayers of WT or KO MEFs/HEK293Ts were infected at MOI 0.0001 or 0.0003 with A5-
243 GFP-VACV or at MOI 0.01 with HSV-1 strain 17 (S17) encoding GFP fused to Vp26 (Vp26-
244 GFP) and viral titres were determined. Loss of DDX50 in MEFs and HEKs conferred an

245 approximate 6- and 3.5-fold increase in the yield of VACV at 24 and 48 h p.i., respectively
246 (Fig. 8A-B). This difference was not restricted to VACV, and loss of DDX50 resulted in an
247 increase in yield of HSV-1 following low MOI (Fig. 8C). In line with higher viral titres,
248 synthesis of the VACV specific late gene product D8 was enhanced in KO MEFs (Fig. 8F).
249 Notably, the number of plaques formed by VACV was increased on the KO MEFs and
250 HEK293Ts compared to control cells (Fig. 8E and G; Supp Fig. S3A). This suggests that
251 DDX50 restricts plaque formation when cells are infected at low MOI and without DDX50 a
252 greater proportion of virus particles entering cells escape host defences and establish a
253 plaque. Consistent with this observation, complementation of KO MEFs with pCW57-*Ddx50*-
254 HA but not the empty vector (EV) reduced viral yields and plaque formation efficiency to WT
255 levels (Fig. 8G-H). Furthermore, overexpression of hDDX50-HA but not hDDX28-HA in WT
256 human fibroblasts restricted VACV, resulting in significantly lower viral titres (Fig. S3B).

257 Given that the activation of IRF3 restricts RNA virus infection as well, a ZIKV replication
258 assay was performed in the absence of DDX50. Parental HEK293T and derived DDX50-/-
259 cells were infected with ZIKV at MOI 1 or 0.1. Three days p.i., supernatants of infected cells
260 were collected and infectious virus was titrated by plaque assay on Vero E6 cells. The
261 absence of DDX50 increased ZIKV replication following low MOI (Fig. 8D), however in
262 concordance with dsDNA viral infection, no difference was observed at high MOI (Fig. 7C).
263 This suggests that the role of DDX50 in promoting activation of the IRF3 pathway contributes
264 to the restriction on ZIKV infection. Together, these results provide evidence that DDX50
265 promotes antiviral signalling during infection and is a restriction factor for both DNA and RNA
266 viruses, with its loss resulting in increased viral spread and subsequent replication in tissue
267 culture.

268 Discussion

269 Type I IFNs are critical regulators of antiviral immunity and infection control and therefore,
270 understanding the mechanisms leading to their production during infection is important.
271 During the last decade, much research has studied the canonical RLRs and RNA sensors
272 RIG-I, MDA5 and TLR3, and has investigated their activation, expression and mechanisms
273 of regulation in response to RLR agonists and infection. Zhang and colleagues described a
274 TLR3, RIG-I and MDA5-independent pathway in mouse dendritic cells in which cytoplasmic
275 RNA was sensed by a complex consisting of DDX1-DDX21-DHX36, leading to recruitment of
276 TRIF (15). Here, DDX50 is described as a new component of the RNA sensing signalling
277 pathway. DDX50 is a TRIF-binding RNA helicase that is an integral member of TRIF-
278 dependent IRF3/NF- κ B activation in fibroblast/epithelial cells. Aside from the initial *in vitro*
279 characterisation of the RNA helicase functional domains of DDX50, little is understood about
280 its cellular role. A previous study concluded that DDX50 is required for MAP kinase
281 activation through c-Jun binding (22). However, whilst a defect in RNA sensing and
282 signalling was observed here, no differences in TNFR/IL-1R-dependent NF- κ B signalling
283 were detected. Differences in the signalling cascade that were observed are independent of
284 MAP kinase and activator protein 1 (AP-1) activation, indicating that this is an independent
285 role for DDX50.

286 DDX50 is needed for optimal IRF3/NF- κ B-dependent gene expression, and cytokine
287 synthesis and secretion following stimulation with dsRNA analogue PolyIC, SeV infection or
288 infection with the dsDNA viruses HSV-1 and VACV. Further investigation found that without
289 DDX50, IRF3 phosphorylation was impaired downstream of these stimuli, but that signalling
290 was intact following activation via MAVS overexpression. This mapped the activity of
291 DDX50, a nucleolar protein, to early in the signalling cascade upstream or independent of
292 MAVS activation. DDX50 shuttling and cytoplasmic accumulation in distinct puncta upon
293 stimulation is reminiscent of DDX1/TRIF staining in response to PolyIC treatment and is
294 consistent with a role for DDX50 in cytoplasmic regulation of IRF3 signalling (15). The RNA

295 sensing complex consisting of DDX1, DHX36 and DDX21 identified by Zhang and
296 colleagues did not report on DDX50. However, this was performed in mouse dendritic cells
297 and the expression of DDX50 varies from cell type to cell type (human atlas data). It may be
298 that DDX50 plays a more significant role in non-haematopoietic cells or that it was below
299 detection in the initial screen. Alternatively, the high sequence identity between DDX50 and
300 DDX21 may have masked the role of DDX50 following knockdown of DDX21. In the DDX1-
301 DHX36-DDX21 complex, DDX21 acts as a scaffold to recruit TRIF upon DDX1 agonist
302 binding. Therefore, we hypothesise that DDX50 may act in a similar fashion. Notably, whilst
303 DDX21 and DDX50 have non-redundant roles, DDX50 is essential for DDX21 helicase
304 activity *in vitro* (21). So, it is possible that DDX50 may function to support DDX21 or even
305 DDX1 (the RNA binding protein) activity in this complex, which may explain why DDX50 is
306 not functionally redundant. Whilst diminished binding of TRIF and DDX1 was observed in the
307 absence of DDX50, it was not abolished, and the role of the functional domains of DDX50,
308 DDX21 and DDX1 in RNA sensing warrants future experimentation.

309 Consistent with a role for DDX50 in innate immune signalling, DDX50 is shown to be a viral
310 restriction factor. Loss of DDX50 resulted in an attenuated immune response to infection
311 with VACV or HSV-1 and enhanced replication of VACV, ZIKV and HSV-1 in tissue culture
312 after low MOI infection. Notably, a greater number of VACV plaques were formed on KO cell
313 lines suggesting that DDX50 acts to restrict viral infection and in its absence a greater
314 proportion of infecting virus particles escape host defences and lead to plaque formation. At
315 high MOI, there were no differences in virus yield suggesting that infection of a single cell by
316 many incoming virus particles can overcome DDX50-mediated restriction. This is
317 reminiscent of cellular restriction factors involved in innate immune signalling. These data
318 provide evidence of the biological relevance of DDX50 for antiviral signalling during infection.
319 Interestingly, DDX50 is reported to co-immunoprecipitate with the positive sense RNA virus
320 Dengue (DENV) RNA (27) and recent publications using siRNA to knockdown DDX50,
321 suggest that it may inhibit DENV replication (28). Following knockdown, the authors reported

322 a reduction in IFN β promoter activity and therefore hypothesised that DDX50 may regulate
323 type I IFN production during DENV infection (29). This is consistent with our findings that the
324 ZIKV titre is increased in the absence of DDX50, and together provides evidence that
325 DDX50 is a viral restriction factor in response to multiple RNA and DNA viruses. Therefore,
326 DDX50 as a restriction factor may extend beyond the viruses tested in this study and act
327 broadly to detect viral RNA and restrict viral replication through activation of IRF3-dependent
328 gene transcription.

329 Although DDX50 was required for optimal signal transduction its absence did not abolish
330 signalling in response to viral infection or stimulation. Given that DDX50 binds TRIF, a
331 protein that is non-essential for RIG-I/MDA5 signalling, and that DDX1 acts independent of
332 canonical RIG-I signalling, we propose that DDX50 acts in concert with other receptors for
333 optimal antiviral signalling and restriction. Whilst this study identifies a role for DDX50 in
334 RNA sensing, both HSV-1 and VACV are DNA viruses. HSV-1 is reported to be restricted
335 mostly by the cGAS-STING pathway (30). However, DNA sensing and antiviral signalling is
336 positively regulated by both TRIF and RNA sensing during HSV-1 infection (31–33),
337 highlighting the essential role of RNA sensors during DNA virus infection. Cells infected with
338 VACV contain large amounts of dsRNA late during infection (34,35). This is due to the virus
339 intermediate and late genes lacking specific transcriptional termination sequences and so
340 lengthy overlapping transcripts are produced that hybridise to form dsRNA (36). These
341 transcripts can be sensed and activate innate immune signalling pathways (37). In addition,
342 such dsRNA can bind to and activate IFN-induced proteins such as PKR and 2'-5'
343 oligoadenylate synthetase (OAS) to mediate translational shutoff. The importance of dsRNA
344 in activating host defences is illustrated by the fact that VACV, despite being a dsDNA virus,
345 encodes a dsRNA binding protein called E3 (38), that contributes to virulence (39). It is
346 important to note that TRIF is also an essential component of the STING pathway (31).
347 Therefore, the level to which DDX50 restricts DNA viruses in an RNA-sensing dependent
348 manner, or whether it can further influence TRIF signalling in the cGAS-STING pathway,

349 warrants future investigation. Furthermore, the importance of DDX50 in RNA sensing and its
350 contribution in antiviral immunity requires validation *in vivo*. Unfortunately, to date there are
351 no KO mice or models available, however with the recent success in generating *Ddx21* KO
352 mice, it may soon be a plausible avenue for investigation.

353 In conclusion, the DExD-Box RNA helicase DDX50 is identified as a crucial component in
354 the host cell RNA sensing machinery, acting to facilitate IRF3 activation and inhibit viral
355 dissemination. It is proposed that DDX50 may act through the recruitment of TRIF to the
356 DDX1 RNA sensing complex.

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361 from Adam Karpf (Addgene plasmid #71783).

362 **Material and Methods**

363 **Cells, plasmids, reagents and viruses**

364 All reagents were purchased from Sigma unless stated otherwise. BSC-1 (ATCC CCL-26),
365 U2OS (ATCC HTB-96), HEK293T (ATCC CRL-11268) and immortalised mouse embryonic
366 fibroblasts (MEF) were all grown in Dulbecco's modified Eagle's medium (DMEM) high
367 glucose (Gibco), supplemented with 10 % foetal bovine serum (FBS; Pan Biotech), 50 µg/ml
368 penicillin/streptomycin (P/S), non-essential amino acids (NEAA). HeLa (ATCC CCL-2) and
369 human fibroblasts (HF) clone EF-1-F (sourced from Doorbar lab, University of Cambridge)
370 were grown in MEM (Gibco) supplemented with 10 % FBS, 50 µg/ml P/S and non-essential
371 amino acids (NEAA). All cells were grown at 37 °C in a 5 % CO₂ atmosphere and were
372 routinely screened for mycoplasma contamination. All plasmids constructed in this study are
373 listed in Table S1. Vaccinia virus (VACV) strain Western Reserve (WR) recombinant vA5-
374 GFP (40), modified vaccinia virus Ankara (MVA) (41), HSV-1 S17 GFP-Vp26 (42) and HSV-
375 1 ΔICPO (43) were described. The titre of infectious viral particles (plaque-forming units per
376 ml, p.f.u./ml) was determined by plaque assay on BSC-1 cells for VACV WR and on U2OS
377 for HSV-1. Sendai virus Cantell strain (Licence No. ITIMP17.0612A) was a gift from Steve
378 Goodbourn, St George's Hospital Medical School, University of London. ZIKV engineered to
379 express a mCherry marker (44) was a kind gift from Dr. Trevor Sweeney, Department of
380 Pathology, University of Cambridge.

381 **CRISPR-cas9 generation of knockout cell lines**

382 Guide RNA design and synthesis, and pX459 plasmid construction was performed following
383 the Zhang lab protocol (45). Specific guide RNAs are described in Table S1. To generate
384 KOs, MEFs were transfected with pX459 plasmids using LT1 following the manufacturer's
385 protocol. Twenty-four h post transfection MEFs and HEK293Ts were treated with 4 µg/ml
386 and 1 µg/ml puromycin (Invitrogen) for 48 h, respectively. Single cell clones were selected
387 by limiting dilution, expanded, and screened for DDX50 protein levels by immunoblot. To

388 confirm successful knockouts, the genomic DNA of selected clones was purified following
389 the manufacturer's protocol (Qiagen, QIAamp DNA mini kit). *Ddx50* was amplified using the
390 primer pair gagcgtccttctctggagattg / ctcaagtctgcccattctctcg and *DDX50* was amplified using
391 the primer pair ctgtgtcaccaggtggcatg / gactcgtgtaactttcttccc. Single allele PCR amplicons
392 were then cloned into pCR2.1-TOPO by blunt end ligation (Thermofisher) and 10 clones
393 were sequenced for each KO cell line clone. Single allele sequencing results were compared
394 to the sequence results of the gDNA PCR amplicon to check all alleles had been identified
395 and that all mutations resulted in frameshift truncations.

396

397 **pLDT and pCW57 cell line generation**

398 WT and *Ddx50*^{-/-} MEF and WT HF cell lines inducibly overexpressing DDX50 were obtained
399 by transduction using lentivirus vectors. pLDT and pCW57 cell lines were generated as
400 described (46) with the following alterations. MEFs and HFs were selected in 4 µg/ml
401 puromycin (Invitrogen), followed by single cell selection. For HF pLDT-TetR-GFP was co-
402 packaged along with the pLDT-MCS plasmids and selected for with 500 µg/ml neomycin
403 (Gibco).

404 **Luciferase reporter assay**

405 HEK293T, HF and MEF cell lines were transfected with 10 ng of the internal control plasmid
406 pTK-Renilla (pRL-TK, Promega) or 60 ng of the reporter plasmid pLUC-NF-κB (R.
407 Hofmeister, University of Regensburg, Germany) or pLUC-IFNβ (T. Taniguchi, University of
408 Tokyo) using LT1 transfection reagent and following the manufacturer's instructions
409 (MirusBio Ltd). Where stated, plasmids encoding TRIF, MAVS or TBK-1 (K.A. Fitzgerald,
410 University of Massachusetts Medical School) were co-transfected. Twenty-four h post-
411 transfection, cells were stimulated with IL-1α (Invivogen) or TNFα (Invivogen) at 100 ng/ml or
412 transfected with 5 µg/ml high molecular weight (HMW) PolyIC (Invivogen) using
413 Liopfectamine 2000 (Invitrogen), or mock-transfected with lipofectamine only, or treated
414 exogenously with 5 µg/ml PolyIC, or left unstimulated for 6 h in DMEM or MEM with 2 %

415 FBS. Alternatively, cells were stimulated by SeV infection at 1:100 dilution of stock for 24 h.
416 Following stimulation cells were lysed in 1 x Passive lysis buffer (Promega) and Firefly
417 luciferase and Renilla luminescence were measured using the MARS data analysis software
418 on the FLUOstar Omega Luminometer (BMG Labtech). Relative luminescence levels were
419 calculated by normalising Firefly luminescence to Renilla and data are presented as relative
420 to the non-stimulated untreated condition, or EV where relevant, for each cell line. Each
421 condition was performed with quadruplicate technical replicates and is representative of two
422 biological repeats.

423 **ELISAs and RT-qPCR**

424 MEFs were seeded in DMEM with 2 % FBS and HEK293Ts were seeded in DMEM with 10
425 % FBS. After 18 h cells were mock-transfected or transfected with 5 µg/ml HMW PolyIC
426 (Invivogen) using Lipofectamine 2000 (Thermofisher) for 7 h or infected with SeV (Cantell
427 Strain) for 4.5 or 24 h where stated. The culture medium was cleared by centrifugation at 17,
428 000 x g and stored at -20 °C before analysis by ELISA. The level of human or mouse
429 CXCL10/IP-10 was determined using a DuoSet ELISA kit (R&D Systems) and the level of
430 mouse IL-6 was determined using a DuoSet ELISA kit (R&D systems) following the
431 manufacturer's instructions. Data were collected and analysed using the MARS data
432 analysis software on the FLUOstar Omega Luminometer (BMG Labtech). Experiments were
433 carried out in triplicate and measured with technical repeats, unless stated otherwise. RNA
434 extraction, cDNA synthesis and RT-qPCR were carried out as described previously using
435 first strand synthesis (Invitrogen) (47). qPCR was performed using the primers indicated in
436 Table S2.

437 **Immunoprecipitations**

438 HeLa cells were transfected with pLDT-hDDX50-HA and co-transfected with pCDNA3-GFP-
439 Flag or pCDNA3-TRIF-cTAP where stated. For MEFs, DDX50-HA pCW57 cell lines were
440 induced with 2 µg/ml doxycycline 24 h prior to transfection with pCDNA3-GFP-Flag or

441 pCDNA3-TRIF-cTAP. WT and *DDX50*^{-/-} HEK293Ts were transfected with pCDNA3-TRIF-
442 cTAP. Twenty-four h post transfection cells were stimulated by transfection with 5 µg/ml
443 PolyIC or infected with SeV (1:200) where stated. Following stimulation, cells were washed
444 and lysed in 50 mM Tris pH 7.6, 150 mM NaCl, 1 % NP40 (IGEPAL CA-630), 1 mM EDTA,
445 10 % glycerol and supplemented with protease inhibitor. Proteins were immunoprecipitated
446 as described (48) with M2 Flag-beads or HA-beads. After the final wash, beads were
447 incubated in 4 x sample buffer (Tris 0.5 M pH 6.8, 40 % glycerol, 6 % SDS, 1 %
448 bromophenol blue and 0.8 % β-mercaptoethanol), boiled and analysed by immunoblotting.

449 **Immunoblotting**

450 Samples were prepared by the addition of 4 x sample buffer, boiled and separated by gel
451 electrophoresis in Tris-glycine SDS (TGS) buffer (20 mM Tris, 192 mM glycine, 1 % (w/v)
452 SDS) and transferred to a nitrocellulose membrane (GE Healthcare) in Tris glycine (TG)
453 buffer (20 mM Tris-HCl pH 8.3, 150 mM glycine) using the Turboblot system (BioRAD).
454 Membranes were blocked in 5 % milk in Tris-buffered saline (10 mM Tris, 150 mM NaCl) pH
455 7.4 with 0.1% (v/v) Tween-20 (TBS-T) for 1 h before incubating with the primary antibody
456 overnight at 4 °C. Primary antibodies: rabbit monoclonal anti-Flag (F7425), anti-DDX50
457 (Abcam; ab109515), anti-IRF3 Ser386 (Abcam, ab76493), rabbit polyclonal anti-HA (H6908),
458 mouse monoclonal anti-Flag (F1804), anti-α-tubulin (Millipore; 05-829), anti-DDX50 (Santa
459 cruz, sc-81077), anti-DDX1 (Santa cruz; sc-271438), anti-LaminA/C (Abcam; ab8984),
460 mouse polyclonal anti-IRF-3 S396 (CST; #4947S) or mouse monoclonal anti-D8 clone AB1.1
461 (49). Membranes were washed 3 times in TBS-T before incubating with secondary
462 antibodies for 1 h. Secondary antibodies were goat anti-rabbit IRDye 800CW (926-
463 68032211; LiCOR) and goat anti-mouse IRDye 680LT (926-68020; LiCOR) or, for
464 immunoprecipitated samples, biotin-anti-mouse light chain followed by streptavidin IRDye
465 680LT (926-68031; LiCOR) was used. Finally, membranes were washed 3 times in TBS-T,
466 dried and imaged using the LiCOR system and Odyssey software. For protein level
467 comparisons, densitometry was calculated using ImageJ.

468 **Virus growth analysis**

469 To measure viral spread, confluent monolayers of WT or KO MEFs were infected with 80
470 p.f.u of vA5-GFP or 200 p.f.u of HSV-1 S17 Vp26-GFP in DMEM with 2 % FBS.
471 Alternatively, for the single step virus replication analysis, cells were infected with 5 p.f.u/cell
472 of vA5-GFP. Plates were rocked regularly at 37 °C for 2 h before incubating at 37 °C for the
473 indicated times. Plaques were imaged using an Axiovert.A1 inverted fluorescence
474 microscope connected to a Zeiss MRc colour camera and processed using Axiovision Rel.
475 4.8 imaging software. To determine the viral titre, the medium and cells were collected,
476 freeze-thawed three times, sonicated at 2.0 for 20 s three times (for VACV only) and titrated
477 on BSC-1 or U2OS for VACV and HSV-1, respectively. For ZIKV infection and titration, 3 ×
478 10⁶ parental HEK293T or DDX50^{-/-} cells were seeded on poly-D-lysine pre-coated 6-well
479 plates. Cells were infected with ZIKV at MOI 0.1 the next day. Three infection p.i.,
480 supernatants of the infected cells were collected and virus infectivity was titrated by plaque
481 assay on Vero E6 cells. To titrate ZIKV samples, Vero E6 cells on 6-well plates (90%
482 confluence) were infected for 2 h, the inoculum was removed and cells were incubated in
483 MEM with 1.5% carboxymethyl cellulose for 5 d. Cells were then fixed with 4%
484 paraformaldehyde (PFA) and stained with toluidine blue.

485 **Cell sub-fractionation**

486 Following stimulation for the times indicated, cells were washed in PBS and fractionated
487 using the NE-PER™ Nuclear and Cytoplasmic Extraction Kit following the manufacturer's
488 protocol (ThermoFisher).

489 **Immunofluorescence**

490 Briefly, cells were fixed in 4 % PFA/PBS for 20 min, washed in PBS, quenched in 150 mM
491 NH₄Cl/PBS for 10 min and permeabilised in 0.1 % Triton X-100/PBS for 10 min, before a
492 final wash and block in 5 % FBS/PBS. Cells were stained by inverted incubation in 5 %
493 FBS/PBS with anti-rabbit HA (dilution 1:100) antibody for 1 h, washed in 5 % FBS/PBS and

494 incubated for a further 30 min with the secondary goat anti-rabbit IgG Alexa-Fluor 488
495 (Jackson immunoresearch; 111-545-003). Coverslips were mounted in Mowiol (10 % w/v
496 Mowiol4–88 (CalBiochem), 25 % v/v glycerol, 100 mM Tris-HCl pH 8.5, 0.5 µg/ml DAPI (4',6-
497 diamidino-2-phenylindole, Sigma) and images were acquired using a Zeiss LSM780 confocal
498 laser scanning microscopy system and processed using the Zeiss Zen microscope and
499 Axiovision 4.8 software.

500 **Statistics**

501 All experiments are presented as technical or biological averages where stated. Data
502 presented are the mean +/- SD. All assays were analysed by unpaired T-test with GraphPad
503 Prism 8 Software where $p < 0.05 = *$, $p < 0.01 = **$, $p < 0.001 = ***$ and $p < 0.0001 = ****$.

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- 646

647 **Fig. 1 DDX50 (RH-II/Guβ) is required for intracellular nucleic acid sensing.** Firefly
648 luciferase activity of WT or *Ddx50*^{-/-} MEFs transfected with plasmids encoding Firefly
649 Luciferase under the *Ifnβ* promoter and Renilla. **(A)** Cells were left untreated or treated with
650 5 µg/ml extracellular PolyIC (epIC), transfected with 5 µg/ml PolyIC (pIC) for 6 h or infected
651 with Sendai virus (SeV) for 24 h. **(B)** WT or *Ddx50*^{-/-} MEFs were transfected with
652 lipofectamine only or 5 µg/ml pIC for 7 h, and the fold induction of *Isg56*, *Cxcl10* or *Ifnb*
653 mRNA levels, relative to *Gapdh*, were analysed by RT-qPCR. **(C)** Secreted levels of
654 CXCL10 and IL-6 in the medium at 7 h post transfection with PolyIC or **(D)** 4.5 h post
655 infection with SeV were analysed by ELISA. **(E)** Firefly Luciferase activity of WT or *DDX50*^{-/-}
656 HEK293Ts transfected with plasmids encoding Firefly Luciferase under the *Ifnβ* promoter
657 and Renilla. Cells were infected for 24 h with SeV or left untreated. Data are representative
658 of at least three independent experiments. **(F)** Secreted levels of CXCL10 in the medium at
659 24 h post infection of WT or *DDX50*^{-/-} HEK293Ts with SeV, were analysed by ELISA. Data
660 are representative of at least three independent experiments. Statistical significance shown
661 for WT stimulated vs KO stimulated.

662 **Fig. 2 DDX50 rescues nucleic acid sensing in *Ddx50*^{-/-} MEFs.** **(A-C)** WT MEFs
663 transduced with pLDT-EV and *Ddx50*^{-/-} MEFs transduced with pLDT-EV or pLDT-*Ddx50*
664 were transfected with lipofectamine only or 5 µg/ml PolyIC for 7 h. **(A)** *Isg56*, *Cxcl10* or *Ifnb*
665 mRNA levels, relative to *Gapdh*, were analysed by RT-qPCR and **(B)** secreted CXCL10 was
666 measured by ELISA. Representative of at least two independent experiments. Statistical
667 significance shown for WT stimulated vs KO stimulated. **(C)** Expression of DDX50 was
668 confirmed by SDS-PAGE and immunoblotting.

669 **Fig. 3 DDX50 is required for IRF3-dependent signalling in response to the dsDNA**
670 **viruses HSV-1 and VACV.** WT MEFs or *Ddx50*^{-/-} MEFs were infected for 3 or 6 h at 10
671 p.f.u./cell with HSV-1 S17 ΔICPO **(A and C)** or MVA **(B and D)** or left uninfected. **(A-B)**
672 mRNA was extracted and *ifnb*, *Isg56* and *Cxcl10* levels were analysed by RT-qPCR relative
673 to *Gapdh*. Representative of at least two independent experiments. **(C-D)** Secretion of

674 CXCL10 and IL-6 were measured at 3 and 6 h post infection by ELISA. Representative of
675 three independent experiments performed in quadruplicate. Statistical significance shown for
676 WT infected vs KO infected.

677 **Fig. 4 DDX50 (RH-II/Gu β) is not required for NF- κ B-dependent gene transcription. (A)**
678 WT or *Ddx50*^{-/-} MEFs were transfected with pNF- κ B-Luc or pTK-RL, as an internal control.
679 Cells were left untreated or stimulated for 7 h with 100 ng/ml IL-1 α and Firefly Luciferase
680 activity was measured. **(B and C)** WT or *Ddx50*^{-/-} MEFs were left untreated or stimulated for
681 1 h with 100 ng/ml TNF α **(B)** or IL-1 α **(C)**. Following mRNA extraction, the fold induction of
682 *Nfkb1a* and *Il-6* mRNA levels relative to *Gapdh* were analysed by RT-qPCR. Representative
683 of 3 independent experiments. Statistical significance shown for WT infected vs KO infected.

684 **Fig. 5 DDX50 accumulates in the cytoplasm in response to cytoplasmic dsRNA and**
685 **acts upstream to, or independent of, MAVS activation. (A and B)** Representative
686 immunoblot of phosphorylated IRF3 at **(A)** Ser396 or **(B)** Ser386 (pIRF3) for **(A)** WT or
687 *Ddx50*^{-/-} MEFs transfected with lipofectamine only or 5 μ g/ml PolyIC for 3 and 6 h or **(B)** WT
688 and DDX50^{-/-} HEK293Ts untreated or infected with SeV for 18 h. Level of IRF3
689 phosphorylation was calculated by densitometry, relative to α -tubulin **(A)** or actin **(B)** and is
690 representative of at least two independent experiments. **(C)** Representative immunoblot
691 following transfection of WT MEFs with 2.5 μ g/ml PolyIC for the indicated times and isolation
692 of the cytoplasmic (cyt) and nuclear fractions (nuc). Immunoblots were stained for DDX50 or
693 α -tubulin and laminA/C as cytoplasmic and nuclear fraction controls, respectively.
694 Representative of three independent experiments. **(D)** Immunofluorescence staining for
695 DDX50 localisation. HeLa cells were transfected with pLDT-DDX50-HA and left uninfected
696 (NI) or infected for 1.5 h with SeV. DDX50 localisation was visualised using an anti-HA
697 antibody. DAPI was used to stain the nucleus. Representative of three independent
698 experiments. **(E)** Luciferase activity of WT or *Ddx50*^{-/-} MEFs co-transfected with EV or
699 indicated plasmids along with plasmids encoding Firefly Luciferase under the *Irfn β* promoter
700 and Renilla as an internal control. Experiments shown are representative of at least three

701 independent experiments. Statistical significance shown for WT stimulated vs KO stimulated.

702 **Fig. 6 DDX50 co-IPs TRIF and DDX1 and facilitates complex formation (A)** Schematic
703 depicting the essential TRIF-binding domain of human DDX21 and the corresponding
704 homologous region in DDX50 or DDX1 and DHX36 as a comparison. **(B)** Immunoblots from
705 co-IP experiments of MEF DDX50-HA cell lines transiently transfected with GFP-Flag and
706 TRIF-cTAP. **(C)** Immunoblots from co-IP experiments of HeLa cell lines transiently
707 transfected with DDX50-HA along with GFP-Flag or TRIF-cTAP. Representative of two
708 independent experiments. *, non-specific band. **(D)** Immunoblots from co-IP experiments of
709 HeLa cell lines transiently transfected with DDX50-HA or DDX28-HA and blotting for
710 endogenous DDX1. **(E)** Immunoblots from co-IP experiments of HEK293T WT or DDX50 KO
711 cells transiently transfected with TRIF-cTAP and blotting for endogenous DDX1.
712 Experiments are representative of three independent experiments. IB, immunoblot; IP,
713 immunoprecipitation.

714 **Fig. 7 DDX50 does not impact viral replication after high MOI. (A)** WT or *Ddx50*^{-/-} MEFs
715 were infected with A5-GFP VACV WR at 5 p.f.u./cell for 24 h. Viral titres were determined by
716 plaque assay of the medium only (EEV) or total (medium plus cells) on BSC-1 cells. Average
717 of three independent experiments. **(B and C)** WT or *Ddx50*^{-/-} HEK293Ts were infected with
718 **(B)** A5-GFP VACV WR at 5 p.f.u./cell for 16 h or **(C)** ZIKV at MOI 1 for 72 h. Viral titres were
719 calculated by titration of cell lysates on Vero E6. Average of two independent experiments.
720 Statistical significance shown for WT vs KO.

721

722 **Fig. 8 DDX50 is a viral restriction factor. (A-B)** Monolayers of WT or *Ddx50*^{-/-} MEFs were
723 infected with A5-GFP VACV WR at 80 p.f.u. (MOI = 0.0001). Viral titres at 24 h p.i. were
724 determined by plaque assay on BS-C-1 cells and are represented as p.f.u./ml (left panel) or
725 fold increase in replication relative to WT cells (right panel). **(B)** As in **(A)** but using WT or
726 *DDX50*^{-/-} HEK293Ts infected at MOI 0.0003 for 48 h. Results shown are representative of 2
727 independent experiments. **(C)** Monolayers of WT or *Ddx50*^{-/-} MEFs were infected with HSV-1
728 S17 Vp26-GFP at 6600 p.f.u. (MOI = 0.01). Viral titres at 48 h p.i. were determined by
729 plaque assay on U2OS cells and are represented as p.f.u./ml (left panel) and fold increase in
730 replication relative to WT cells (right panel). **(D)** WT or *DDX50*^{-/-} HEK293Ts were infected
731 with ZIKV at MOI 0.1 for 72 h. Titres were determined by plaque assay on Vero E6 cells and

732 data are shown as for A-C. Titres shown are an average of 2 independent experiments. **(E)**
733 Cells were infected at low MOI as in **(A)** and **(D)** and plaque numbers were enumerated 24 h
734 p.i.. Data are expressed as the plaque formation efficiency on KO cells compared to WT
735 cells. Representative of two independent experiments. **(F)** WT or *Ddx50*^{-/-} MEFs were
736 infected with A5-GFP VACV WR at MOI = 0.0001 and expression of the VACV late protein
737 D8 was analysed by immunoblot at 24 h p.i. Representative of two independent
738 experiments. **(G-H)** WT or *Ddx50*^{-/-} MEFs transduced with pCW57-EV or pCW57-*Ddx50*-HA
739 were infected as in **(G)**. Representative fluorescence images of plaque morphology following
740 infection. Scale bar, 500 μM. **(H)** Viral titres at 24 h p.i. from cells infected as in **(G)** For all
741 panels unless stated otherwise titres shown are representative of at least 3 independent
742 experiments and fold changes shown are an average of at least 2 independent experiments.
743 Statistical significance is shown for WT EV vs KO EV.

744 **Supporting information**

745 **Table S1. Constructs and primers used in the study**

746 **Table S2. Primers for qPCR**

747 **Fig. S1. CRISPR-Cas9 mediated knockout of *Ddx50/DDX50* (*RH-II/Guβ*) in fibroblasts.**

748 **Fig. S2. DDX50 overexpression augments nucleic acid sensing.**

749 **Fig. S3. Overexpression of DDX50 inhibits VACV dissemination and replication.**

○ WT □ KO

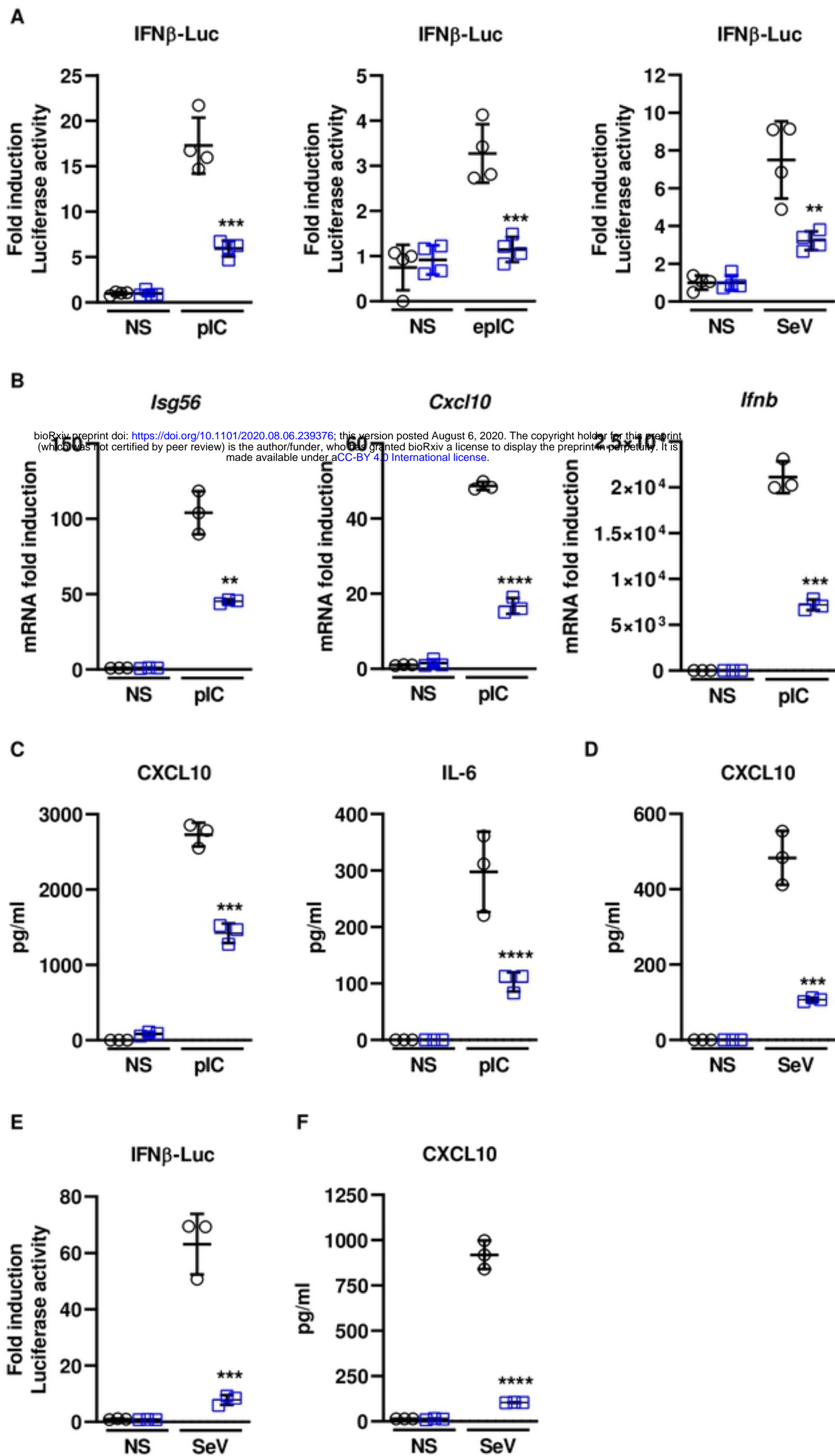


Fig. 1

○ WT □ KO △ KO + *Ddx50*

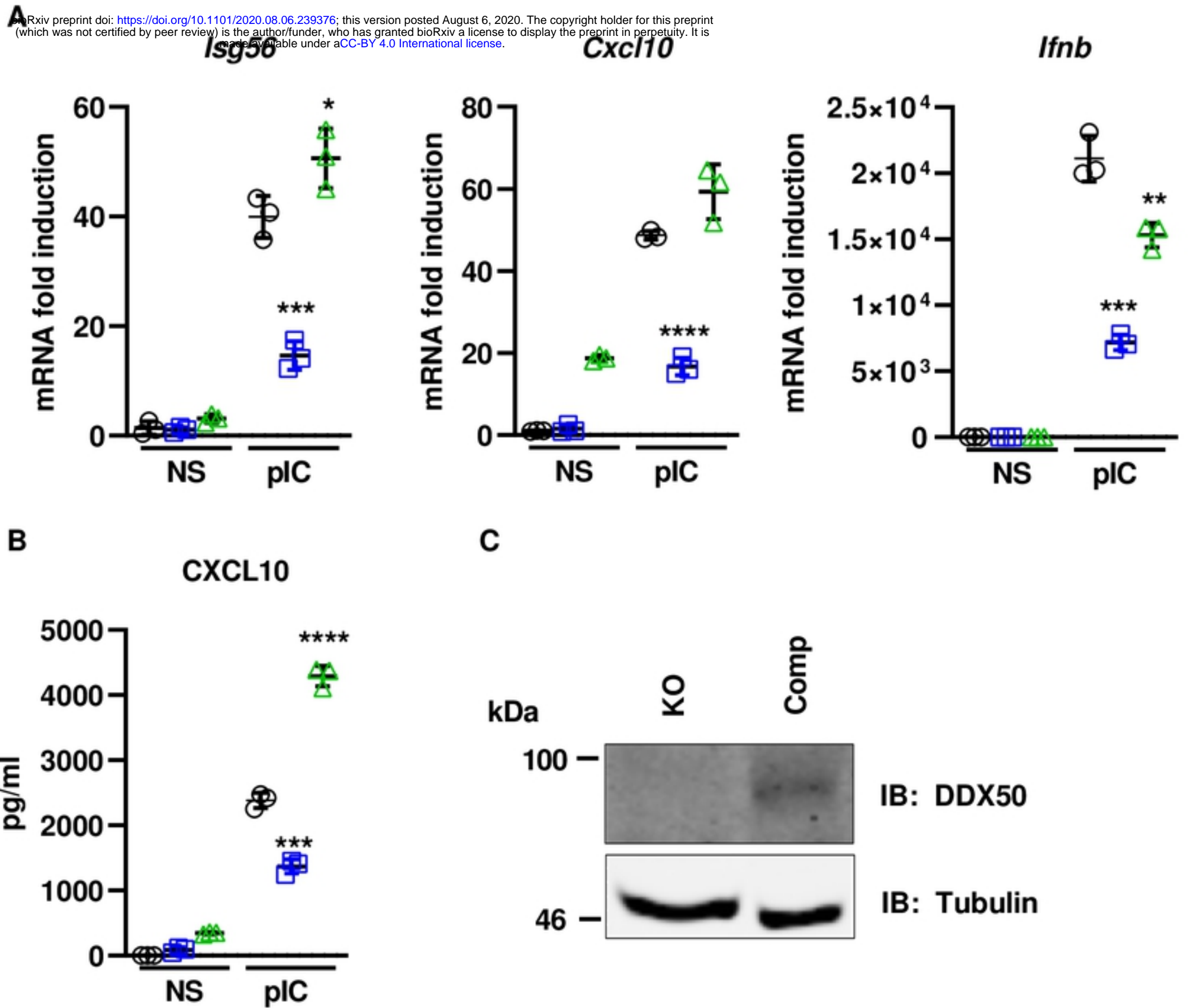


Fig. 2

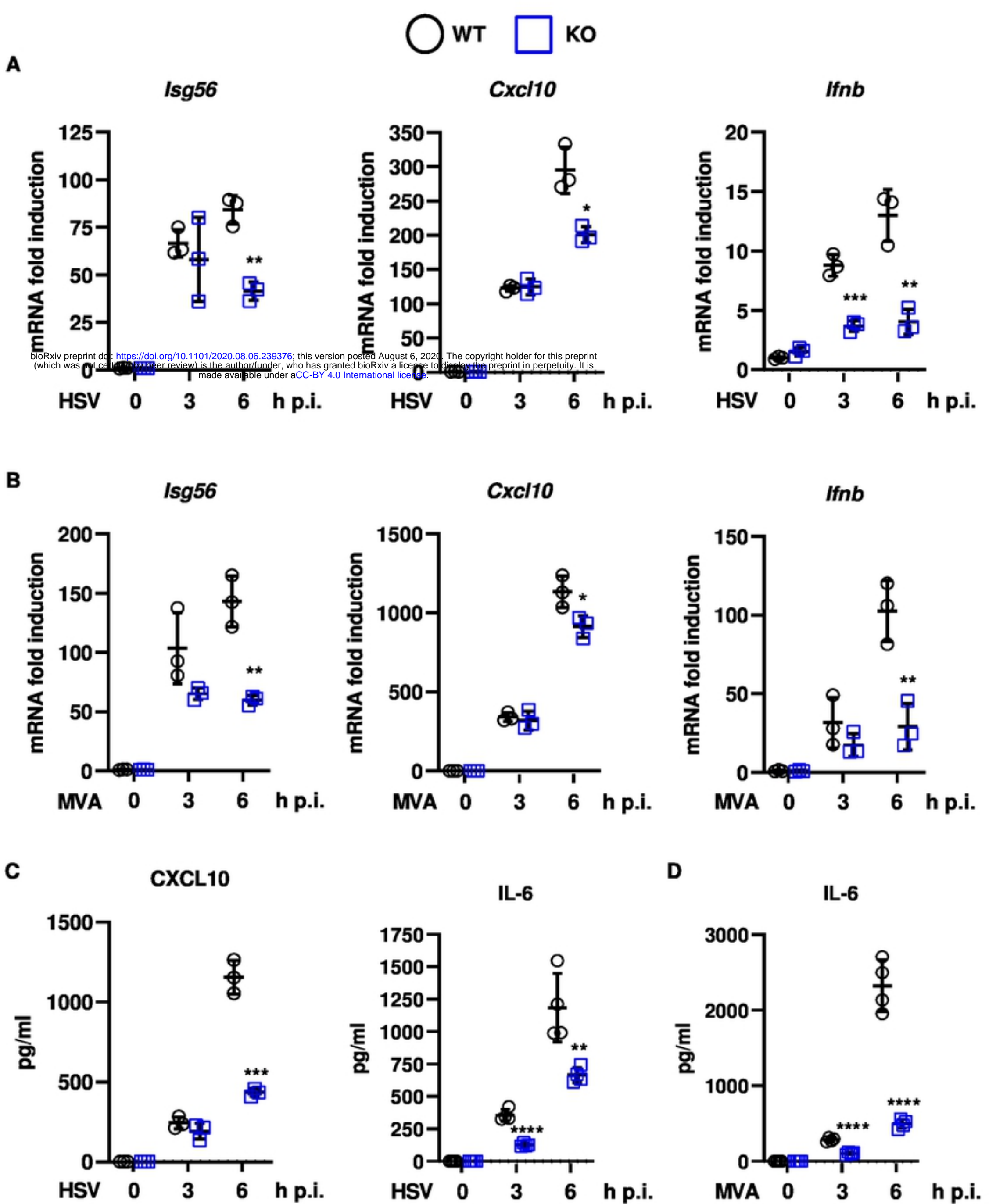


Fig. 3

○ WT □ KO

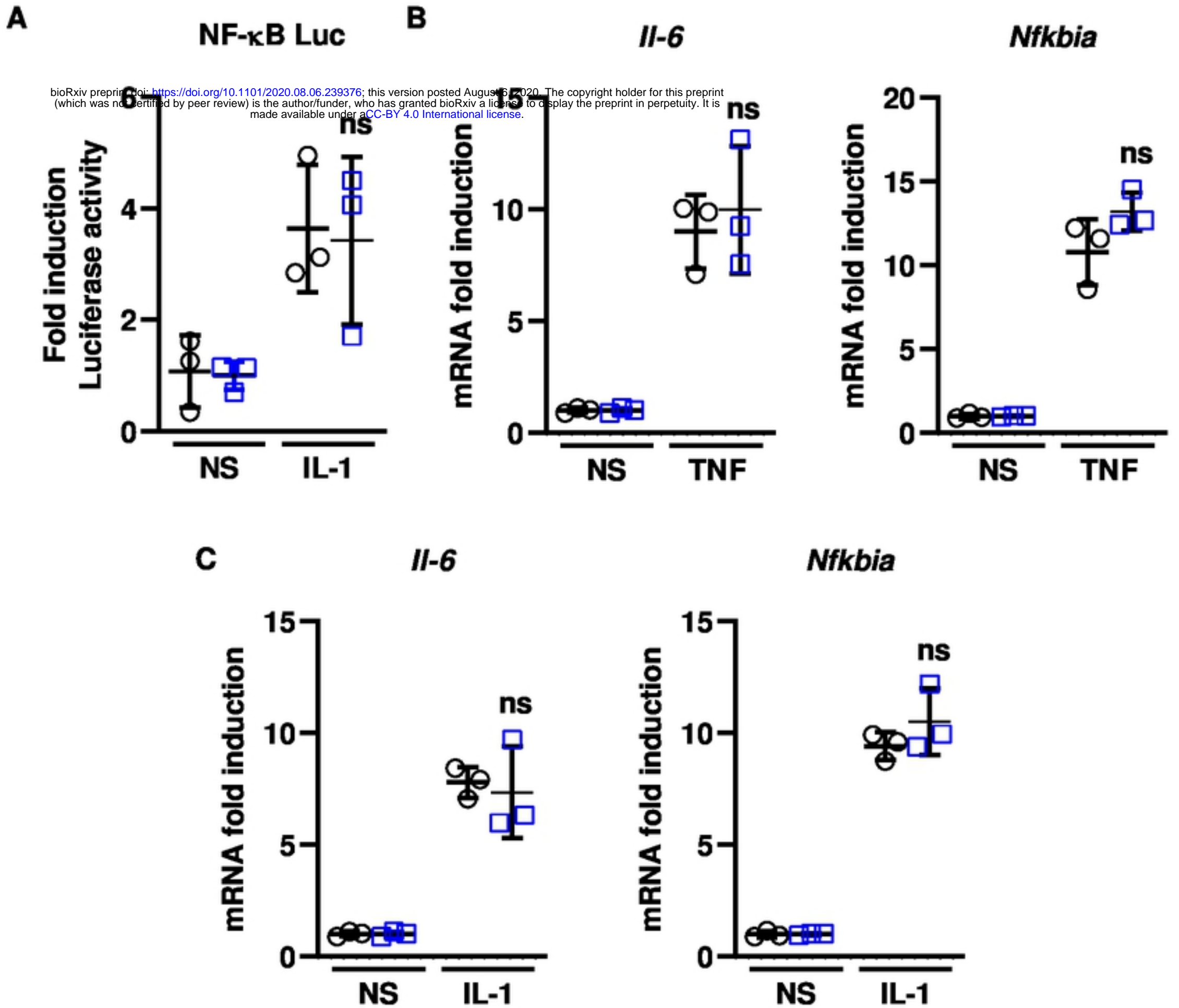
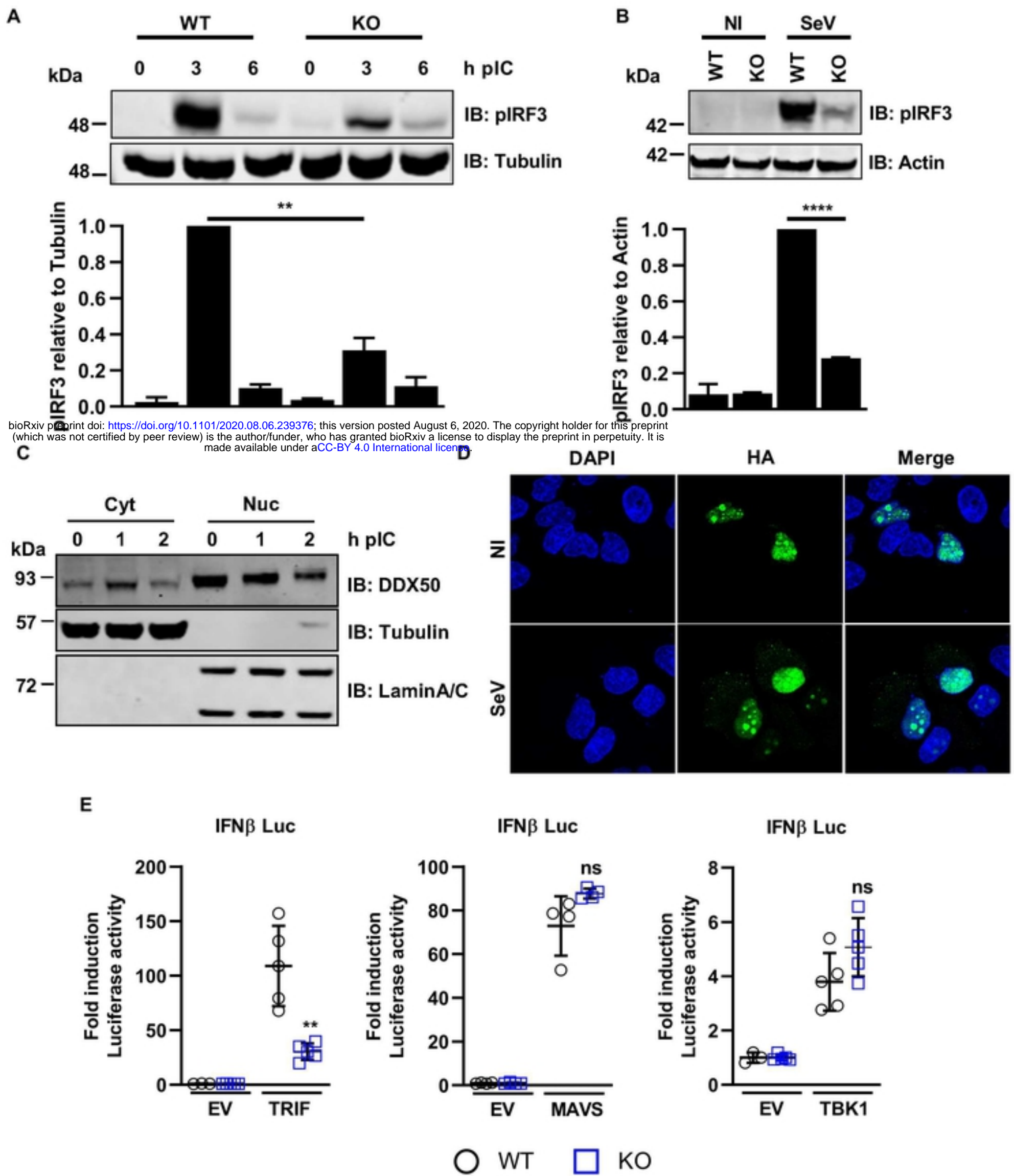
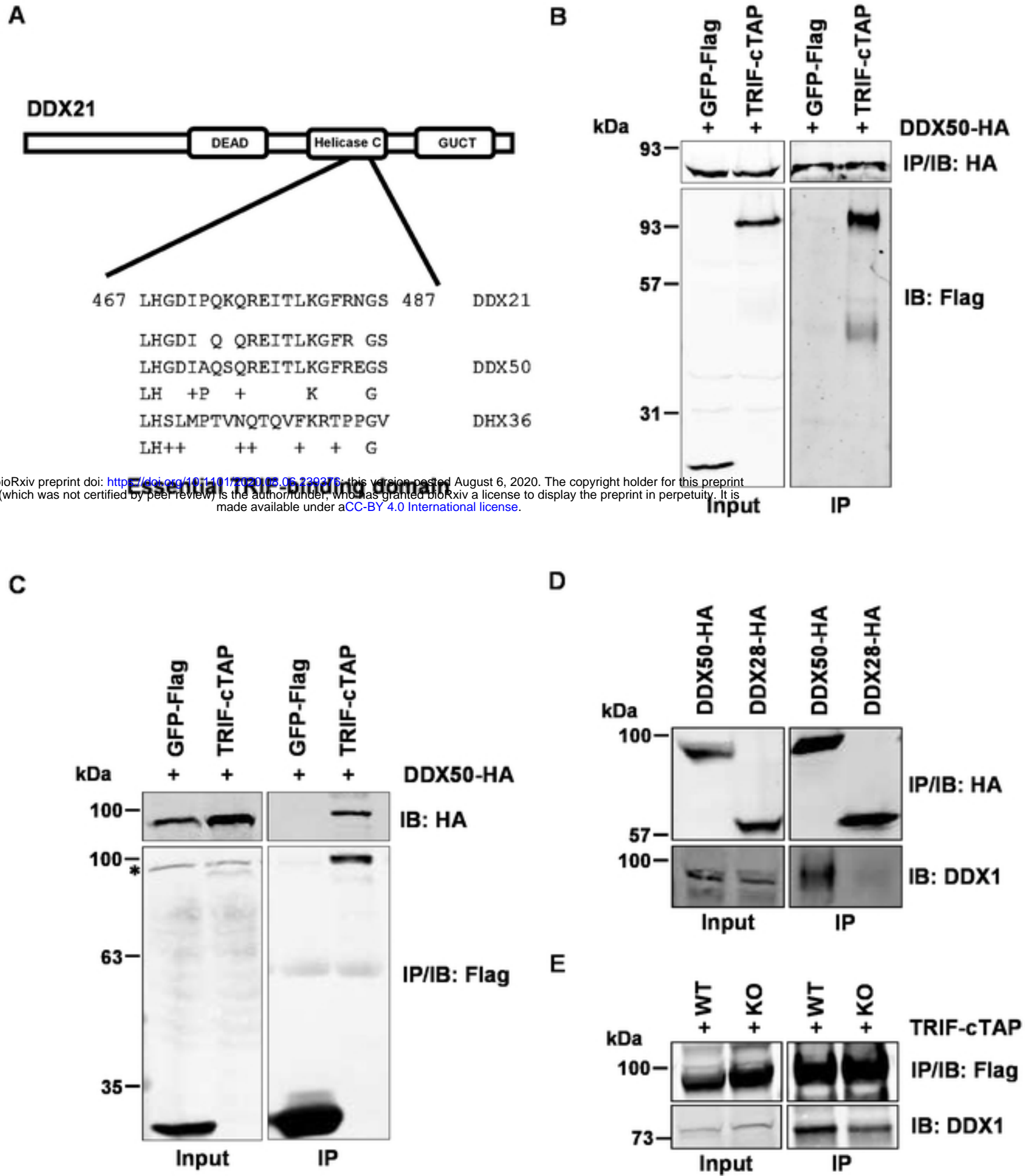


Fig. 4



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Fig. 5



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Fig. 6

○ WT □ KO

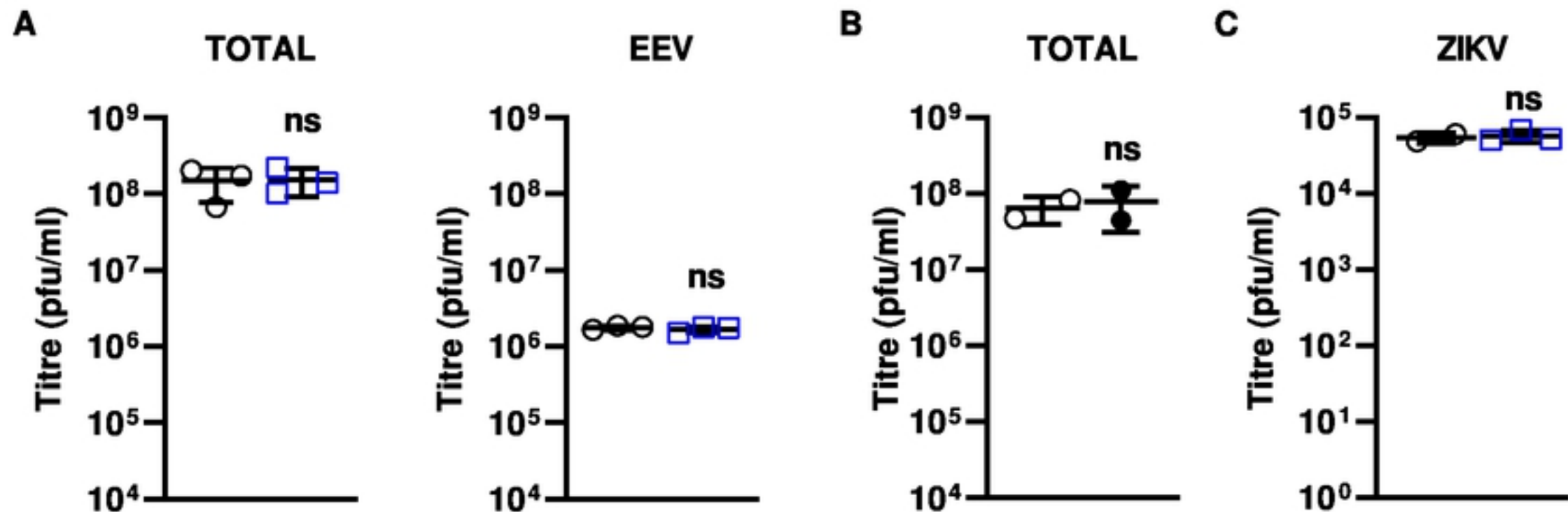


Fig. 7

○ WT □ KO

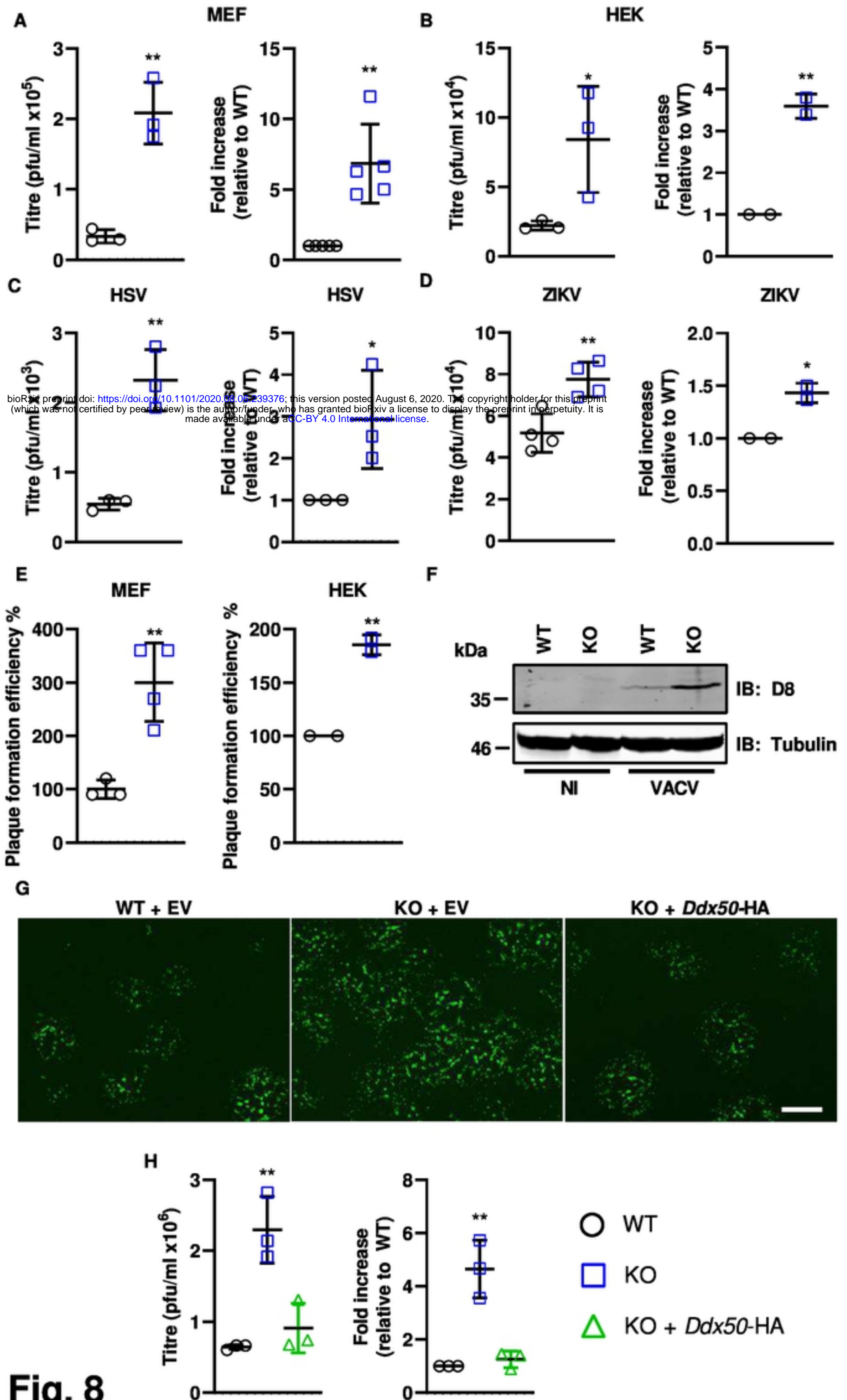


Fig. 8