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► **To cite this version:**

Ségolène Gracias, Maxime Chazal, Alice Decombe, Yves Unterfinger, Adrià Sogues, et al.. Tick-borne flavivirus NS5 antagonizes interferon signaling by inhibiting the catalytic activity of TYK2. *EMBO Reports*, 2023, 24, pp.e57424. 10.15252/embr.202357424 . hal-04251902

HAL Id: hal-04251902

<https://hal.science/hal-04251902>

Submitted on 27 Oct 2023

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1 **Tick-borne flavivirus NS5 antagonizes interferon signaling by inhibiting the catalytic**
2 **activity of TYK2**

3
4 **Short title**

5 Antagonism of JAK-STAT signaling by Tick-borne flaviviruses
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7

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28 **Abstract**

29 The mechanisms utilized by different flaviviruses to evade antiviral functions of interferons are
30 varied and incompletely understood. Using virological approaches, biochemical assays and mass
31 spectrometry analysis, we report here that the NS5 protein of tick-borne encephalitis virus (TBEV)
32 and Louping Ill virus (LIV), two related tick-borne flaviviruses, antagonize JAK-STAT signaling
33 through interactions with tyrosine kinase 2 (TYK2). Co-immunoprecipitation (co-IP) experiments,
34 yeast gap-repair assays, computational protein-protein docking and functional studies identified a
35 stretch of 10 residues of the RNA dependent RNA polymerase domain of tick-borne flavivirus NS5,
36 but not mosquito-borne NS5, that is critical for interaction with the TYK2 kinase domain. Additional
37 co-IP assays performed with several TYK2 orthologs revealed that the interaction was conserved
38 across mammal species. *In vitro* kinase assays showed that TBEV and LIV NS5 reduced the catalytic
39 activity of TYK2. Our results thus illustrate a novel mechanism by which viruses suppress the
40 interferon response.
41

42 **Teaser**

43 Inhibition of the catalytic activity of a key kinase of the JAK/STAT pathway by a viral protein

44 **Introduction**

45 While mosquitoes are the major vectors of pathogens in tropical regions, ticks are the leading
46 vectors in temperate climates (1). In Europe, *Ixodes ricinus* is the major tick species and the most
47 significant vector of pathogens (2), including tick-borne encephalitis virus (TBEV) and Louping Ill
48 virus (LIV), two flaviviruses that display 95% identity at the amino acid level. These viruses cause
49 severe central nervous system disease mostly in humans and sheep, respectively. Besides Central and
50 Eastern Europe, TBEV circulates in Russia, China and Japan (3). It causes several thousands of human
51 cases per year, with recent increases attributed to climate changes, population dynamics, the range of
52 permissive ticks and shifts in land usage (3, 4). Human cases of LIV, though rare, have been reported
53 in the United Kingdom, Ireland, southwestern Norway and northwestern Spain (5). Ticks can serve as
54 reservoirs and/or vectors, while the vertebrate hosts provide a major mechanism for ticks to acquire
55 infection. TBEV circulates in large woodland animals and rodents, such as bank voles and yellow-
56 necked mice (6). LIV is mainly detected in sheep, mountain hares and red grouse (5, 7). Other large
57 mammals, such as cattle, goats, dogs, pigs and horses also serve as hosts for LIV (5, 7).

58 Members of the flavivirus genus are enveloped viruses containing a positive-stranded RNA
59 genome of ~11-kb (8). Upon viral entry into the host cell, the viral genome is released and translated
60 by the cellular machinery into a large polyprotein precursor. The latter is processed by host and viral
61 proteases into three structural proteins (the capsid protein (C), the precursor of the M protein (prM)
62 and the envelope (E) glycoprotein) and seven non-structural proteins (NS) called NS1, NS2A, NS2B,
63 NS3, NS4A, NS4B and NS5 (8). The structural proteins form the virus particles whereas the NS
64 proteins play a central role in viral replication, transcription and assembly, as well as modulation of
65 innate responses (8).

66 The replication of viruses, including flaviviruses, activates a rapid innate immune response that
67 controls viral replication and spread. This response is initiated by the recognition of viral nucleic acids
68 by pathogen recognition receptors (PRRs) (9), leading to the expression of type I interferon
69 (IFN)(IFN- α and - β) and type III IFN (IFN- λ). Secreted IFN- α/β and IFN- λ s (IL-28a, IL-28b and IL-
70 29) will then bind to their heterodimeric receptors, IFNAR1/IFNAR2 and IFN- λ R1/IL-10R2,
71 respectively (10), at the surface of infected and surrounding cells. Signal transduction downstream of
72 these receptors is initiated by the transphosphorylation of the associated JAK tyrosine kinases (JAK1,
73 JAK2, TYK2)(11, 12). In turn, the activated JAK tyrosine kinase phosphorylate the bound receptor,
74 forming a docking site for STAT1 and STAT2. At this docking site, JAKs phosphorylate the STAT
75 proteins. The phosphorylation of the STAT proteins triggers the formation of the interferon-stimulated
76 factor gene 3 (ISGF3) complex (STAT1p, STAT2p and IRF9), which migrates to the nucleus where it
77 binds to the IFN stimulation response element (ISRE), a motif lying within the promoter region of
78 approximately 2 000 genes (13). Transcriptional activation of these IFN-stimulated genes (ISGs)
79 establishes the antiviral state (14), whereby the induced effector proteins target specific stages of viral
80 replication, such as entry into host cells, protein synthesis, replication or assembly of new virus

81 particles. Some of these effectors are specific to a virus or a viral family, while others have broad-
82 spectrum antiviral functions.

83 Flavivirus-encoded strategies to evade IFN signaling contributes to efficient replication in
84 mammalian hosts. The NS5 of several mosquito-borne flaviviruses displays functional convergence in
85 antagonizing the JAK-STAT signaling pathway, albeit by virus-specific mechanisms (15). Dengue
86 virus (DENV) NS5 degrades STAT2 via the recruitment of the ubiquitin ligase UBR4 (16). NS5 of
87 Zika virus (ZIKV) also binds and degrades STAT2 in a proteosomal-dependent, but UBR4-
88 independent, fashion (17). Yellow fever virus (YFV) NS5 also interacts with STAT2, which blocks
89 the binding of ISGF3 to ISRE promoter elements in IFN-stimulated cells (18). Much remains to be
90 learned on evasion of the JAK/STAT cascade by tick-borne flaviviruses, though some regulators of
91 the pathway have been identified as NS5 targets (15). For instance, IFNAR1 surface expression is
92 reduced in human cells infected with either TBEV or the closely related Langkat virus (LGTV), via the
93 recruitment of prolidase by NS5 (19). TBEV NS5 has also been implicated in JAK/STAT antagonism
94 via an interaction with the protein scribble (20) that may target NS5 to the plasma membrane. Whether
95 a LIV protein mediates IFN evasion is entirely unknown.

96 We showed here that the RNA-dependent RNA polymerase domain (RdRp) of NS5 of tick-borne
97 flaviviruses interacts with a stretch of 10 residues of TYK2, a key element of the JAK/STAT pathway.
98 Consequently, the ability of TYK2 to phosphorylate its substrates is impaired and the IFN response is
99 inhibited.

100

101 **Results**

102

103 **TBEV and LIV infection antagonize IFN γ 2 signaling in Huh7 and 293T cells.**

104 We first examined the effect of IFN treatment on TBEV and LIV replication in human
105 hepatocarcinoma Huh7 cells, which are extensively used in *Flaviviridae* research. When infected with
106 TBEV (Hypr strain) or LIV at an MOI of 0.5 or 0.05, respectively, approximately 80% of Huh7 cells
107 were positive for the viral protein E 24 hours post-infection (Fig. 1A). When Huh7 cells were infected
108 under the same conditions and then treated with IFN γ 2 for 8 h, the viral RNA yield was unaffected, as
109 measured by RT-qPCR analysis (Fig. 1B). This suggests that, when replication is well established
110 (Fig. 1A and B), these two viruses are insensitive to IFN γ 2 treatment (Fig. 1B). Moreover, IFN γ 2
111 increased *ISG15* and *ISG56* mRNA levels in non-infected Huh7 cells (Fig. 1C, D), but not in infected
112 cells (Fig. 1C, D), further suggesting that viral replication inhibited the effect of IFN γ 2. Of note,
113 *ISG15* and *ISG56* mRNA levels were higher in TBEV-infected cells than in LIV-infected cells,
114 independently of IFN γ 2 treatment (Fig. 1A and B).

115 We then assessed the level of expression and the activation of STAT1 and STAT2 in infected
116 Huh7 cells by Western blot analysis. As expected, 30 min of IFN γ 2 treatment induced tyrosine
117 phosphorylation of STAT1 and STAT2 in non-infected cells (Fig. 1E). By contrast, in cells infected

118 with TBEV or LIV for 24 hours and then treated with IFN α 2 for 30 min, STAT1 and STAT2 were not
119 tyrosine phosphorylated (Fig. 1E). Infection did not alter STAT1 and STAT2 protein levels. TYK2
120 abundance was also similar in all conditions (Fig. 1E). These data suggest that none of these three key
121 components of the JAK/STAT pathway were degraded during infection. We further investigated the
122 ability of TBEV and LIV replication to antagonize IFN signaling in another cellular model (293T
123 cells) by following the subcellular localization of the phosphorylated form of STAT1 using fluorescent
124 microscopic assays. In cells treated with IFN α 2, pSTAT1 localized in the nucleus (Fig. 1F). By
125 contrast, no STAT1p was detected in 293T cells infected with TBEV or LIV and treated with IFN α 2
126 30 minutes prior to fixation (Fig. 1F). These results confirm the Western blot analysis performed in
127 Huh7 cells (Fig. 1E) showing that tick-borne flaviviruses limit STAT1 and STAT2 phosphorylation.

128 Together, these data show that infection by both TBEV and LIV antagonizes STAT1/2 activation
129 in response to IFN α 2 stimulation in Huh7 and 293T cells.

130

131 **NS5 of TBEV and LIV dampen IFN signaling in 293T cells.**

132 To identify TBEV and LIV proteins that antagonize IFN signaling in human cells, the open
133 reading frames (ORFs) corresponding to each of the 10 viral proteins of TBEV and LIV were cloned
134 downstream of a N-terminal 3X-FLAG tag. Additional constructs coding for TBEV and LIV precursor
135 M proteins (prM) were generated. We also included in the analysis a FLAG-tagged version of the NS5
136 of YFV, which serves as a positive control since it precludes STAT2 incorporation into the ISGF3
137 complex in IFN-I-stimulated human cells (18). These experiments were conducted in 293T cells,
138 which are easy to transfect. Western blot analysis confirmed that all TBEV and LIV proteins, as well
139 as YFV NS5, were expressed (Fig. EV1). We analyzed the ability of TBEV and LIV proteins to block
140 IFN α 2-stimulated signal transduction by using a firefly luciferase reporter gene under the control of
141 an ISRE promoter. A plasmid expressing *Renilla* luciferase was used to assess transfection efficiency.
142 Both TBEV and LIV NS5 diminished the activity of the ISRE promoter as significantly as YFV NS5
143 in 293T cells stimulated with IFN α 2 for 16 hours (Fig. 2A). The expression of the other viral proteins
144 did not affect IFN α 2-induced ISRE activity (Fig. 2A). The activity of ISRE was inhibited by TBEV
145 and LIV NS5 in a dose-dependent manner (Fig. 2B). Similar experiments were performed with YFV
146 NS5 (Fig. EV2). These experiments revealed that TBEV and LIV NS5 were inhibiting ISRE
147 activation in IFN-stimulated 293T cells more potently than YFV NS5.

148 To investigate the ability of TBEV or LIV NS5 to inhibit STAT1 activation in individual cells,
149 we expressed the FLAG-tagged version of the viral proteins and analyzed the localization of
150 endogenous pSTAT1 in IFN α 2-treated 293T cells (Fig. 2D and 2E). Mock-transfected cells or cells
151 expressing the C protein of TBEV, which does not interfere with IFN signaling (Fig. 2A), were used
152 as negative controls. In about 85% of stimulated cells expressing TBEV C proteins, pSTAT1 localized
153 to the nucleus (Fig. 2D and 2E). In contrast, pSTAT1 was nuclear in fewer than 1% of cells expressing
154 TBEV or LIV NS5. By comparison, pSTAT1 localized to the nucleus in around 45% of cells

155 expressing YFV NS5 (Fig. 2D and 2E). These results further suggest that LIV and TBEV NS5
156 proteins are antagonists of the JAK/STAT pathway. Consistently, Western blot analysis revealed that
157 STAT1 and STAT2 were less phosphorylated in IFN α 2-stimulated 293T cells expressing TBEV or
158 LIV NS5 than in cells expressing a control plasmid (Fig. 2F). As expected based on previous
159 experiment performed on U6A and Hela cells (18), STATs phosphorylation remained intact in cells
160 expressing YFV NS5 (Fig. 2F), confirming that YFV NS5 acts downstream of STAT1 and STAT2
161 activation. In line with these data, *ISG56* and *ISG15* mRNA levels were ~75% lower in IFN α 2-
162 stimulated 293T cells expressing TBEV or LIV NS5 than in cells transfected with a control plasmid
163 (Fig. 2G and 2H). As expected (18), the expression of YFV NS5 also reduced the abundance of *ISG56*
164 and *ISG15* mRNAs in cells stimulated for 16 hours, as compared to control cells (Fig. 2G and 2H).
165 Similarly, the expression of the three NS5 proteins significantly diminished the *ISG56* and *ISG15*
166 mRNA levels in cells treated with IL-29 (Fig. 2G and 2H).

167 Thus, TBEV and LIV NS5 proteins antagonize the induction of at least two ISGs by IFN-I or
168 IFN-III stimulation in 293T cells, possibly by acting on protein(s) upstream of STAT1/2
169 phosphorylation and common to both signaling pathways.

170

171 **TYK2 is a cellular partner of TBEV and LIV NS5.**

172 To identify the cellular partner(s) of NS5 that may be involved in IFN signaling antagonism, the
173 FLAG-tagged versions of TBEV and LIV NS5 proteins were expressed and affinity purified from
174 human 293T cells (Fig. 3A). Cells transfected with empty vectors were used as controls. NS5
175 interacting partners were analyzed by mass spectrometry (MS). The first analysis, based on the peptide
176 intensities, identified ~2,000 host proteins co-purifying with the two NS5 proteins (Table EV1). When
177 the data were filtered (p-value greater than 0.02, fold change > 3 and unique peptide count >2), 244
178 cellular partners of LIV NS5 were identified and 256 for TBEV NS5. We also analyzed the data with
179 two other MS scoring algorithms: MiST (21) and SAINTexpress (22). A total of 153 proteins for LIV
180 NS5 and 130 proteins for TBEV NS5, which were identified in two out of three analyses, were
181 considered high confidence partners of TBEV and LIV NS5 (Fig. 3B). Among these high-confidence
182 partners, 107 proteins were common to the two NS5 (Fig. 3B, light purple circles). Several of these
183 common hits, such as ATRX and CTR9, were previously identified by MS analyses as partners of
184 ZIKV, DENV and West Nile virus (WNV) NS5 (23, 24), validating our approach. Of note, the TBEV
185 and LIV NS5 interactomes were enriched in nuclear proteins, such as ATRX, CTR9 and SMARCA4.
186 Consistently, immunofluorescence analysis revealed that the NS5 proteins of TBEV and LIV localized
187 both in the cytoplasm and nucleus of transfected Huh7 cells, treated or not with IFN α 2 (Fig. EV3). In
188 infected Huh7 cells, the two NS5 proteins also localized in both in the cytoplasm and nucleus (Fig.
189 EV4), as do the NS5 proteins of mosquito-borne flaviviruses (25–29). TYK2 was the sole component
190 of the JAK/STAT pathway that was found among the 107 common high-confidence partners of the
191 two tick-borne NS5 (Fig. 3B). Since the MS analysis was performed in unstimulated cells, the

192 interaction between NS5 and TYK2 is expected to take place independently of the TYK2 activation
193 state.

194 To confirm the interaction between TYK2 and NS5, co-immunoprecipitation assays were
195 performed in Huh7 cells infected for 24 hours. NS5 proteins of TBEV and LIV were
196 immunoprecipitated using antibodies raised against LGTV NS5 (19). These experiments revealed that
197 endogenous TYK2 co-precipitated with TBEV and LIV NS5 in infected cells (Fig. 3C), confirming
198 the interaction identified by MS analysis in NS5-transfected cells (Fig. 3C). The ability of TBEV and
199 LIV NS5 to antagonize IFN signaling may thus be mediated by their interaction with TYK2.

200

201 **The RdRp domain, but not the MTase domain, of TBEV and LIV NS5 physically interact**
202 **with TYK2 and antagonize the JAK/STAT pathway.**

203 Flavivirus NS5 contains two domains, an N-terminal methyltransferase (MTase) domain and an
204 RNA-dependent RNA polymerase (RdRp) domain (Fig. 4A). To identify which of these two domains
205 interacted with TYK2, FLAG-tagged version of the MTase and RdRp domains of TBEV and LIV NS5
206 were expressed in 293T cells together with a V5-tagged version of TYK2. Cells expressing FLAG-
207 tagged versions of the full-length NS5 proteins of TBEV and LIV were used as positive controls while
208 cells expressing YFV FLAG-NS5 were used as negative controls. The NS5 proteins were concentrated
209 with anti-FLAG antibodies. Analysis of the immunoprecipitates with anti-V5 and anti-FLAG
210 antibodies revealed that TYK2 co-precipitated with full-length TBEV and LIV NS5 but not with YFV
211 NS5 (Fig. 4B), thus confirming previous data obtained in infected cells (Fig. 3C). The RdRp domain,
212 but not the MTase domain, was found sufficient to immunoprecipitate TYK2 (Fig. 4B). Whether the
213 interaction between tick-borne NS5 and TYK2 is direct was assessed by Gap repair assays in yeast. In
214 these assays, yeast clones are first selected based on protein expression and then on protein-protein
215 interaction (30). Yeast colonies grew in the presence of TYK2 and full-length NS5 of both TBEV and
216 LIV but not in the presence of full-length YFV NS5 (Fig. 4C). Moreover, yeast growth occurred when
217 TYK2 was expressed together with NS5 RdRp domains, but not with MTase domains (Fig. 4C),
218 validating an interaction between the NS5 RdRp domain and TYK2 (Fig. 4B). Consistently,
219 expression of either of the two RdRp domains considerably reduced the activity of the ISRE promoter
220 in IFN γ -stimulated 293T cells (Fig. 4D). In agreement with these data, tyrosine phosphorylation of
221 STAT1 and STAT2 was reduced in IFN γ -stimulated 293T cells expressing TBEV or LIV RdRp as
222 compared to cells expressing the MTase domains or the control plasmid (Fig. 4E).

223 Together, these data show that the RdRp domain, but not the MTase domain, of TBEV and LIV
224 NS5 physically interacts with TYK2 and antagonizes the JAK/STAT pathway.

225

226 **A variable region of the RdRp domain of tick-borne flaviviruses NS5 is involved in TYK2**
227 **binding and IFN antagonism.**

228 To determine whether TYK2 targeting is a common feature of flavivirus NS5, 293T cells were
229 co-transfected with TYK2-V5 and a collection of FLAG-NS5 proteins. NS5 proteins derived from
230 LGTV, three *Culex*-borne flaviviruses (WNV, Usutu virus (USUV) and Japanese encephalitis virus
231 (JEV)) and three *Aedes*-borne flaviviruses (ZIKV, DENV and YFV) were included in the analysis
232 (Fig. EV5). These co-immunoprecipitation experiments revealed an interaction between LGTV NS5
233 and TYK2 (Fig. 5A) and further confirmed the interaction between TYK2 and NS5 from TBEV and
234 LIV (Fig. 5A). By contrast, none of the NS5 proteins of the six mosquito-borne flaviviruses interacted
235 with TYK2 (Fig. 5A). This suggests that the interaction of NS5 with TYK2 is unique to tick-borne
236 flaviviruses. Flavivirus NS5 proteins display conserved sequences and their overall structures are
237 largely overlapping (31). However, one exposed region located between the RdRp catalytic motifs B
238 and C shows some sequence diversity (Fig. 5B) and could account for the specificity of the binding of
239 NS5 of tick-borne flaviviruses to TYK2. The protein structures of YFV (Protein Data Bank (PDB)
240 6QSN) and TBEV (PDB 7D6N) NS5 are very similar with a calculated structural root-mean-square
241 deviation (rmsd) of 1.63Å after aligning all atoms (Fig. 5C). An important structural feature is the
242 presence of the so-called B-C loop (31) with the insertion of a DES sequence in the NS5 of YFV
243 which is absent in tick-borne flaviviruses (Fig. 5B and C). The DES residues, which provide a
244 negatively charged motif in a surface-accessible domain of the NS5-YFV (Fig. 5C), could modulate
245 electrostatic protein-protein interactions. To test whether the variable region (VR) of the NS5 B-C
246 loop is involved in TYK2 binding, residues 631-641 of TBEV NS5 were replaced by residues 631-644
247 of YFV NS5 in FLAG-tagged versions of full length NS5 (TBEV-NS5-VR_{YFV}) or the RdRp domain
248 (TBEV-RdRp-VR_{YFV}) (Fig. 5D). The expression of NS5-VR_{YFV} did not affect the activity of the ISRE
249 promoter in IFN β -stimulated 293T cells (Fig. 5E). Consistently, co-immunoprecipitation
250 experiments performed in 293T cells revealed that, by contrast to wild-type NS5 and RdRp domain,
251 NS5-VR_{YFV} and the RdRp-VR_{YFV} did not bind to TYK2 (Fig. 5F). These data show that the residues
252 631-641 of TBEV NS5 are necessary for its antagonist effect on IFN β -response and for TYK2
253 binding.

254 To explore the relevance of the NS5-TYK2 interaction for TBEV replication, we took advantage
255 of a replicon derived from the strain Neudoerfl (32), in which we replaced the NS5 BC region by the
256 BC region of the Hypr strain (rTBEV) or by the VR mutated NS5 (rTBEV-VR_{YFV}) that is unable to
257 bind TYK2. We also generated, as a negative control, a replication-defective replicon bearing double
258 D-to-A mutations in the RdRp catalytic sequence GDD (rTBEV-GAA mutant, corresponding to
259 residues 663-665 in TBEV NS5)(31). RT-qPCR analysis performed on Huh7 cells electroporated with
260 the three *in vitro*-synthesized rTBEV RNAs revealed that RNAs derived from rTBEV-VR_{YFV}
261 replicated similarly to RNAs derived from rTBEV (Fig. 5G). Viral RNA detected in cells
262 electroporated with *in-vitro* transcribed rTBEV-GAA RNAs represented the amount of non-replicative
263 viral RNA that penetrated the cells (Fig. 5G). Huh7 electroporated with RNAs derived from rTBEV,
264 rTBEV-VR_{YFV} and rTBEV-GAA were stimulated 3 days later with IFN β for 30 min. In agreement

265 with experiments performed in TBEV-infected cells (Fig. 1E), STAT1 and STAT2 were not tyrosine
266 phosphorylated in cells expressing RNAs from rTBEV replicon for 3 days and then stimulated with
267 IFN α 2 (Fig. 5H). By contrast, both STATs were activated in cells expressing the VR_{YFV} replicon (Fig.
268 5H). Thus, NS5 interaction with TYK2 is critical to antagonize the IFN response during viral
269 replication.

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The interaction between NS5 and TYK2 is conserved across several mammalian species.

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To test whether TBEV and LIV NS5 proteins were able to interact with TYK2 from diverse mammalian species, the two viral proteins were co-expressed with full-length V5-tagged versions of cow, sheep, goat, cat or dog TYK2. With the exception of cats, all these animals have been reported to be permissive hosts for TBEV and LIV (5, 7). Western blotting analysis showed that all the TYK2 orthologs migrated similarly and were comparably expressed (Fig. 6A). Cow, goat, dog and cat TYK2 co-precipitated with full-length TBEV and LIV NS5, albeit with different efficiencies (Fig. 6A). A faint signal was also observed for sheep TYK2 in the NS5 eluate (Fig. 6A). To investigate further the potential difference in affinity of NS5 to TYK2 from ruminant species, the interactions were assessed by yeast gap repair assays. Colonies were observed when yeast expressed NS5 and TYK2 from all the tested ruminant species (Fig. 6B), suggesting that interaction between TYK2 and the NS5 of tick-borne flaviviruses is conserved in ruminant host species. Both TBEV and LIV may thus be able to counteract IFN signaling in a variety of mammalian hosts. However, the different levels of NS5-bound TYK2 orthologs may reflect genuine difference in binding efficacy.

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TBEV and LIV NS5 proteins interact with the tyrosine kinase domain of TYK2 and affect its catalytic activity.

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As all four members of the JAK family, TYK2 is made of an N-terminal FERM domain, an SH2-like domain, a kinase-like (KL) or pseudokinase domain and a C-terminal tyrosine kinase (TK) domain (33)(Fig. 6C). The KL domain contains the subdomains shared by protein kinases, but lacks several residues that are essential for enzymatic activity, while the TK domain contains all the conserved residues associated with tyrosine kinases (33). To determine which domain of TYK2 is targeted by tick-borne NS5, V5-tagged versions of TYK2 mutants lacking one, two or three of the four domains (34) (Fig. 6C) were examined for NS5 binding by co-immunoprecipitation in 293T cells. TYK2 deletion mutants were all expressed at the predicted size (Fig. 6D). Interestingly, only the proteins that retained the TK domain (FL, C, Δ KL and TK) co-precipitated with full-length TBEV and LIV NS5 (Fig. 6D). Mutants lacking the TK domain (N, Δ TK, C Δ TK) did not bind NS5. These results suggest that TBEV and LIV NS5 proteins directly target the catalytic TK domain of TYK2. The molecular details of the interaction between the variable part of the NS5 B-C loop and the TYK2 TK domain were further characterized by computational docking (see materials and methods). The most favorable model predicts an interface area of 677.3 Å² between the two proteins with a binding energy

302 of -2.9 kcal/mol (Fig. 6E). According to this docked model, the NS5 of TBEV and the TYK2 TK
303 domain would interact via five hydrogens bonds and three salt bridges (Fig. 6E). Six out of seven
304 TYK2 residues that may be targeted by NS5 (Q939, Q946, D949, K1046, V1048 and E1050) are
305 conserved in TYK2 orthologs (Fig. S4), further suggesting that TBEV and LIV may be able to
306 counteract JAK/STAT signaling in several mammalian hosts. At the exception of K1046, none of
307 these residues are shared by the three other members of the JAK family (EV6, boxed in red).

308 Most of these interactions involve a central alpha helix located close to the active site of the TK
309 domain (Fig. 6E), suggesting that NS5 may restrict TYK2 enzymatic activity, including auto-
310 phosphorylation. To investigate this possibility, we analyzed the level of expression and activation of
311 endogenous TYK2 in 293T cells expressing the viral proteins and stimulated with IFN α 2 for 15 min.
312 In cells expressing TBEV or LIV NS5, the level of phosphorylated TYK2 was significantly less than
313 in cells expressing either an empty vector or YFV NS5 (Fig. 6F and EV7A). The level of total TYK2
314 was similar under all conditions (Fig. 6F), confirming that TYK2 is not degraded in the presence of
315 flavivirus NS5 (Fig. 1E). These data suggest that expression of TBEV and LIV NS5 affects IFN-
316 induced TYK2 activation.

317 To assess whether TBEV and LIV NS5 proteins inhibit TYK2 catalytic activity, luminescence-
318 based *in vitro* kinase assays were performed using purified full-length NS5 and RdRp domains, the
319 TYK2 tyrosine kinase domain (aa871-1187) and a peptide derived from the Insulin Receptor
320 Substrate-1 (IRS-1). IRS-1 is known to be phosphorylated by TYK2 in human cells (35). YFV NS5
321 and the MTase domain of NS5 LIV were included in the analysis as negative controls. The expected
322 molecular mass of the purified viral proteins was verified by Coomassie staining (Fig. EV7B). TYK2
323 kinase activity, which was assessed by measuring ATP levels, was significantly reduced in the
324 presence of TBEV and LIV NS5 (full-length or RdRp domains), but not in the presence of the LIV
325 MTase domain nor YFV NS5 (Fig. 6G). Similar experiments were performed by replacing IRS-1-
326 derived peptides by purified STAT1. Western blot analysis showed that STAT1 was phosphorylated
327 by TYK2 when the two proteins were incubated by themselves or with YFV NS5 (Fig. 6H). By
328 contrast, no phosphorylated STAT1 was detected when TBEV and LIV NS5 were added in the
329 reaction (Fig. 6H and EV7C). These experiments confirm that the interaction between NS5 proteins of
330 tick-borne flaviviruses and TYK2 is direct. They also revealed that NS5 proteins of tick-borne
331 flaviviruses affect the ability of TYK2 to phosphorylate one of its natural substrates.

332 Together, these data suggest that TBEV and LIV NS5 impair the ability of TYK2 to
333 autophosphorylate and phosphorylate its substrates, such as the juxtaposed JAK1, IFNAR1, STAT1
334 and STAT2.

335

336 Discussion

337 We found that TBEV and LIV replication antagonizes STAT1/2 activation in response to IFN α 2
338 stimulation in Huh7 and 293T cells. Modest quantities of ISG15 and ISG56 transcripts were however

339 detected in TBEV-infected cell, but not in LIV-infected cells. Some so-called ISGs are expressed in an
340 IFN-independent, RIG-I/IRF3 dependent manner (36). ISG56 is, indeed, a prototypical IRF3-driven
341 gene (37). TBEV RNA could be more accessible to RIG-I than LIV RNA, and thus activate the
342 expression of a subset of ISGs, including ISG56 and ISG15, in an RIG-I/IRF3-dependent, IFN-
343 independent manner. One could also envisage that LIV has evolved a more efficient strategy than
344 TBEV to antagonize the RIG-I/IRF3 axis. Alternatively, TBEV, and not LIV, may activate the UPR
345 response, which leads to a rapid IRF3-dependent response (38).

346 The non-structural protein NS5 of *Aedes*-borne (DENV, ZIK and YFV), *Culex*-borne (WNV and
347 JEV) and tick-borne (TBEV and LGTV) flaviviruses are known to dampen the activation of the
348 JAK/STAT pathway in human cells (15, 39). We show here that ectopic expression of TBEV and LIV
349 NS5 is sufficient to reduce the activation of STAT1 and STAT2, as well as ISRE activity, in Huh7 and
350 293T cells. Expression of the two NS5 proteins also reduced the upregulation of *ISG56* and *ISG15*
351 upon IFN-I and -III stimulation in 293T cells. This is in agreement with previous findings showing
352 that expression of NS5 TBEV suppressed STAT1 phosphorylation in IFN α -stimulated Hela and 293
353 cells (20). Thus, we confirmed here that TBEV NS5 is an IFN signaling antagonist and identified LIV
354 NS5 as a novel one. Our data also suggest that TBEV and LIV NS5 act upstream of STAT1 and
355 STAT2 activation and target a protein common to IFN-I and -III signaling pathways, which would
356 therefore exclude IFN-I and -III receptors.

357 Three proteins of the JAK/STAT pathway have been identified as cellular partners of flavivirus
358 NS5: IFNAR2, IFNGR1 and STAT2. NS5 of DENV, ZIKV and YFV, which are *Aedes*-borne
359 flaviviruses, interacted with human STAT2 in immunoprecipitation experiments in 293T cells (16–18,
360 40). Once bound to ZIKV or DENV NS5, STAT2 is degraded by the proteasome (16, 17). Binding of
361 YFV NS5 to STAT2 likely precludes the assembly of the ISGF3 complex in IFN-stimulated human
362 cells (18). NS5 from Spondweni virus (SPOV), which is closely related to ZIKV, also bound STAT2
363 in immunoprecipitation experiments in 293T cells, albeit less efficiently than DENV or YFV NS5
364 (17). LGTV NS5 bound IFNAR2 and IFNGR1 in immunoprecipitation experiments performed in
365 VERO cells expressing NS5 (41). Surprisingly, IFNAR2 expression was not affected during LGTV
366 infection (19, 41), thus the role of NS5/IFNAR2 interaction in flavivirus IFN antagonism remains to
367 be elucidated. Similarly, the relevance of the NS5/IFNGR1 interaction in LGTV replication requires
368 further investigation.

369 Our MS analysis identified TYK2, a key player of the JAK/STAT pathway that transduces both
370 IFN-I and -III signals (42), as an interacting partner of TBEV and LIV NS5. We showed that
371 endogenous TYK2 co-purified with NS5 from Huh7 cells infected with TBEV or LIV. Yeast-based
372 assays revealed that the interaction between the kinase and LIV/TBEV NS5 was direct. This is in
373 agreement with the identification of TYK2 among the interacting partners of NS5 TBEV in
374 independent yeast two-hybrid screens (43, 44). In these previous studies (43, 44), the interaction was
375 not validated in human cells and its functionality was not investigated. We found that LGTV NS5 also

376 interacted with TYK2 in co-IP experiments performed in transfected 293T cells. By contrast, co-IP
377 experiments performed in primary human monocyte-derived dendritic cells infected with LGTV failed
378 to identify an interaction between NS5 and endogenous TYK2 (41). Immunoprecipitated TYK2 was
379 probed using an uncharacterized anti-TBEV ascitic fluid (41). The limit of detection of the TBEV
380 ascitic fluid may explain why the interaction with TYK2 was missed in primary dendritic cells.

381 Our data showing an interaction between TYK2 and tick-borne NS5 do not contradict previous
382 results showing that LGTV NS5 binds IFNAR2 (41), especially since these two cellular proteins are
383 part of the same complex (42). Both interactions are mediated via the RdRp domain but different
384 residues of the polymerase may be involved. Our co-IP experiments, which were performed in cells
385 expressing mutant NS5, identified a variable region of the RdRp domain (aa 631-641) as necessary for
386 TYK2 binding. The IFNAR2 domain targeted by TBEV NS5 has not been identified yet.

387 An interaction between flavivirus NS5 and IFNAR1 has not been demonstrated yet. However,
388 IFNAR1 surface expression was reduced in 293 cells expressing LGTV NS5 (19). This was also the
389 case in 293 cells infected with LGTV, TBEV or WNV (19), but not in A549 cells infected with JEV
390 (45). This effect on IFNAR surface expression is mediated by a direct interaction between NS5 and
391 prolidase (peptidase D; PEPD). PEPD was identified as a partner of LGTV NS5 by yeast-2-hybrid
392 analysis and the interaction was confirmed by co-IP performed in 293 cells (19). Further co-IP
393 experiments revealed that WNV and TBEV NS5 also bound PEPD (19). Both IFNAR1 cell surface
394 expression and maturation, which were monitored by assessing glycosylation patterns in Western blot
395 analysis, were compromised in 293 cells expressing reduced levels of PEPD (19). NS5 from TBEV,
396 LGTV and WNV thus reduces IFNAR cell-surface levels by targeting a regulator of its maturation and
397 trafficking. The residue D380 of the NS5 RdRp is important for PEPD binding (19). Of note, TYK2,
398 which interacts with IFNAR1 via its FERM domain (46), is important for stabilizing IFNAR1 at the
399 cell surface in human cells (47). In the absence of TYK2, IFNAR1 localized in a perinuclear
400 compartment while TYK2 overexpression enhanced surface IFNAR1 localization by inhibiting its
401 endocytosis (47). Thus, one can envisage that NS5 binding to TYK2 alters the TYK2/IFNAR1
402 interaction and thus also contributes to reduction in IFNAR1 surface expression in infected cells.

403 The protein scribble, which is another binding partner of TBEV NS5, may also play a role in
404 flavivirus JAK/STAT antagonism. Scribble was recovered from yeast-two-hybrid screens performed
405 with TBEV NS5 (20, 43, 44). We also identified scribble as a partner of NS5 LIV by MS analysis, but
406 surprisingly, not of TBEV NS5. The interaction between NS5 and endogenous scribble was validated
407 by co-immunoprecipitation experiments in HeLa cells expressing TBEV NS5 (20). The MTase domain
408 mediates scribble binding (20). A mutant NS5 defective in scribble binding failed to accumulate at the
409 plasma membrane and lost its ability to inhibit JAK-STAT signaling in HeLa cells (20). Scribble may
410 thus help tick-borne NS5 to traffic to the cell surface where it antagonizes IFN signaling. However, we
411 and others (48) have found that the expression of the tick-borne RdRp domain alone reduced the
412 activity of ISRE and STATs' activation as efficiently as full-length NS5, suggesting that the MTase

413 domain is dispensable for JAK/STAT antagonism. Thus, NS5 may reach the plasma membrane in a
414 scribble-independent manner.

415 None of the 6 mosquito-borne NS5 proteins that we tested co-immunoprecipitated with TYK2 in
416 293T cells. These results agree with yeast-two-hybrid screens that recovered TYK2 as a partner of
417 NS5 TBEV but not as a partner of four mosquito-borne NS5 (DENV, WNV, JEV and Kunjin virus)
418 (43). Similarly, a mammalian two-hybrid analysis failed to show a direct interaction between TYK2
419 and JEV NS5 (49). Thus, targeting TYK2 to antagonize IFN signaling may be a mechanism specific to
420 tick-borne flaviviruses. It would be of interest to determine whether NS5 of other tick-borne
421 flaviviruses, such as Powassan virus and Omsk hemorrhagic fever virus, also bind TYK2.

422 We found that the interaction between TYK2 and NS5 was conserved across several mammalian
423 species that are relevant for tick-borne flavivirus ecology. Computational protein-protein docking
424 identified seven residues potentially involved in the interaction between NS5 and TYK2. Six of these
425 are conserved in TYK2 orthologs, which further suggest that NS5 could antagonize the JAK-STAT
426 pathway in a panel of mammalian hosts. However, our co-IP analysis, which were performed under
427 stringent conditions, suggest that differences may exist in the binding affinity between NS5 and TYK2
428 orthologs. Antagonism of IFN signaling by tick-borne NS5 may thus contribute to the delineation of
429 the host range of tick-borne flaviviruses. Of note, a single ortholog of STAT, with strong homology to
430 vertebrate STAT5, and a single ortholog of JAK are represented in the genome of the tick *Ixodes*
431 *scapularis*, but no TYK2 ortholog has as yet been identified (50, 51).

432 Yeast-based assays showed that the interaction between TYK2 and TBEV/LIV NS5 is direct. Co-
433 IP experiments revealed that the two NS5 proteins bind the TK domain of TYK2, which was
434 supported by our docking model. Out of seven TYK2 residues identified by the model as potentially
435 critical for NS5 binding, six are not shared by other JAKs. This suggests that NS5 proteins of tick-
436 borne flaviviruses specifically bind TYK2 and not the other member of the JAK family. *In vitro* kinase
437 assays demonstrated that the ability of TYK2 to phosphorylate either synthetic substrates derived from
438 IRS-1 or full-length STAT1 was significantly reduced in the presence of TBEV or LIV NS5. Thus,
439 tick-borne NS5 proteins inhibit TYK2 catalytic activity *via* a direct interaction. Since TYK2 activity is
440 critical for downstream STAT-mediated signaling, NS5 expression probably results in impaired
441 induction of ISGs, thus favoring viral replication. Two other unrelated viral proteins were previously
442 known to interact with TYK2. The E6 protein of human papilloma virus 18 (HPV-18), which is a
443 double-stranded DNA virus belonging to the *Papillomaviridae* family, is known to interact with the
444 first 287 aa of TYK2 in co-IP experiments performed in HeLa cells (52). These TYK2 residues lie
445 within the FERM domain, which is important for the interaction with the cytoplasmic tails of
446 IFNAR1/2 and IFNLR (46). Thus, by competing for the FERM domain, the E6 protein prevents
447 binding of TYK2 to IFNAR in stimulated cells. Thus, the mechanism by which E6 restricts TYK2
448 function is different from that used by NS5 of tick-borne flaviviruses. The LMP-1 protein of Epstein-
449 Barr virus (EBV), which is a gamma-herpesvirus, is also interacting with TYK2 (53). The interaction

450 was identified by an affinity purification and MS approaches in lymphoblastoid cells and validated by
451 co-IP experiments in DG75 lymphoma cells (53). Western blot analyses showed that TYK2
452 phosphorylation was reduced in IFN γ stimulated B cells expressing LMP-1 (53). However, the exact
453 mechanism by which LMP-1 abolishes TYK2 function remains to be established. Mapping the TYK2
454 domain targeted by LMP-1 should give a first hint at the mechanism at play.

455 In sum, by binding TYK2 and inhibiting its catalytic activity, NS5 proteins of tick-borne
456 flaviviruses are employing a unique mechanism to antagonize IFN signaling. Our work highlights the
457 variety of strategies used by ecologically diverse flavivirus NS5 to counteract the IFN-induced
458 JAK/STAT pathway. It also highlights the pleiotropic function of the flavivirus NS5 polymerase
459 domain. How these functions are regulated remains to be investigated.

460

461 **Methods**

462

463 **Cell lines, viruses and infections**

464 Huh7 human hepatocellular carcinoma cells (kindly provided by A. Martin, Institut Pasteur Paris) and
465 Human embryonic kidney (HEK) 293T cells (American Type Culture Collection [ATCC] CRL-3216)
466 herein called 293T were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco)
467 containing GlutaMAX I and sodium pyruvate (Invitrogen) supplemented with 10% heat-inactivated
468 fetal bovine serum (FBS) (Dutscher) and 1% penicillin and streptomycin (10 000 IU/ml; Thermo
469 Fisher Scientific). African green monkey kidney epithelial VERO cells (ATCC) were maintained in
470 DMEM with 10% FBS.

471 Experiments with TBEV and LIV were performed in a BSL-3 laboratory, following safety and
472 security protocols approved by the risk prevention service of Institut Pasteur. The TBEV strain (Hypr
473 strain, isolated in Czech Republic in 1953) was obtained from the European Virus Archive (EVAg;
474 <https://www.european-virus-archive.com/>). Louping Ill virus (strain LI 3/1; APHA reference Arb126)
475 was kindly provided by Nick Johnson (Animal and Plant Health Agency, Addlestone, Surrey, UK).
476 Virus stocks were produced on VERO cells. Titration of infectious virions was performed by plaque
477 assays on VERO cells, as previously described for other flaviviruses (54). Huh7 and 293T were
478 infected at the MOIs indicated in the figure legends, followed by a 2-hour incubation in DMEM
479 medium containing 2% FBS. Infected cells were analyzed at the indicated time.

480

481 **Plasmids and cloning**

482 To clone TBEV and LIV viral ORFs downstream of the 3×FLAG sequence, we used a previously
483 described collection of viral open reading frames (ORF) cloned into pDONR207 (55). All ORF
484 sequences were then transferred by *in vitro* recombination (Gateway™ LR Clonase™ II Enzyme mix,
485 Invitrogen) into a Gateway™ compatible pCI-neo-3×FLAG final destination vector (kind gift from
486 Yves Jacob, Institut Pasteur, Paris). NS5 coding sequences from various flaviviruses and derivatives
487 (mutants and domains) were amplified from various sources using primers designed to add a 5' Not-I
488 restriction site and 3' XbaI / NheI / SpeI restriction sites (Appendix Table S1). They were cloned in
489 frame in NotI/XbaI digested p3×FLAG-CMV10 (Sigma-Aldrich) expression vector. For protein
490 purification, codon optimized NS5 sequences encoding full-length NS5 proteins or RdRp domains
491 thereof from TBEV, LIV and YFV, as well as the MTase domain of LIV, were synthesized and cloned
492 in the pET28 plasmid by the TWIST company. For yeast experiments, NS5 sequences were amplified
493 from pDONR207 (full-length and individual domains) and cloned into Gal4-BD-fused pPC97
494 plasmids (Invitrogen) with the Q5 Hot Start High-Fidelity DNA Polymerase (NEB) using specific
495 primers (Appendix Table S2).

496 Human TYK2 sequences were amplified from pRc-CMV-TYK2 or pRc-H9-TYK2ΔKL (47, 56) using
497 primers designed to add attb1 and attb2 recombination cassettes (Appendix Table S2). Amplicons

498 were purified and cloned into a pDONR221 entry vector using Gateway BP clonase II Enzyme Mix
499 (Thermo Fisher Scientific). pDONR221 containing human TYK2 coding sequence and pTwist-ENTR
500 containing TYK2 orthologue cDNAs (Appendix Table S3), which were kindly provided by Damien
501 Vitour (ANSES, Maisons-Alfort), were cloned into a pcDNA-DEST40-V5 expression vector using
502 Gateway LR clonase II Enzyme Mix (Thermo Fisher Scientific). TBEV replicons harboring NS5
503 variable region (VR) substitutions were generated by cloning PCR amplified sequences from
504 3xFLAG-NS5 TBEV and 3xFLAG-NS5 TBEV VR_{YFV} using primers listed in Appendix Table S1 into
505 KpnI/XbaI digested pTND/ Δ ME (kindly provided by Franz X. Heinz via Karin Stiasny and described
506 in (32)). A replication defective TBEV replicon was generated by PCR introducing GDD to GAA
507 substitutions in position 663-665 of NS5 by PCR using primers listed in Appendix Table S2.

508 pISRE-Luc, pGAS-Luc (kindly provided by Eliane Meurs, Institut Pasteur) and pRL-TK Renilla
509 Luciferase (Promega) plasmids were used for luciferase assays. pCi-Neo plasmids (Promega) were
510 used as “empty vector” (EV) controls in transfection experiments. All plasmids were grown in TOP10
511 cells (Thermo Fisher Scientific) and verified by sequencing.

512

513 **Yeast Gap Repair assays**

514 Physical interactions between TYK2 (human and orthologues) and NS5 (full-length or individual
515 domains) were tested by gap repair assays (30). Briefly, Gold strain yeasts (Clontech) carrying
516 plasmids expressing DB-fused NS5 sequences were co-transformed using a standard lithium/acetate
517 procedure with 10 ng of linearized pPC86 empty vector (Invitrogen) and 3 μ L of PCR products
518 generated from cDNA encoding TYK2. These PCR products were generated using pRC-CMV-TYK2,
519 Q5 and Hot Start High-Fidelity DNA Polymerase (NEB) and primers designed to add recombination
520 sites (Appendix Table S1) allowing recombination in yeasts. Since expression of full-length TYK2
521 was toxic in yeast, constructs corresponding to the nucleotides 2287 to 3564 (aa 763-1187) of TYK2
522 were used in these assays. Homologous recombination between pPC86 and the PCR product in yeast
523 cells allowed fusion of TYK2 cDNA downstream of the activation domain of Gal4 (Gal4-AD) and
524 growth on medium lacking leucine, tryptophan and histidine. Physical interaction between the BD-
525 fused viral bait and the AD-fused Tyk2 orthologue preys were selected by addition of 5mM 3-
526 aminotriazole (3-AT).

527

528 **Antibodies and cytokines**

529 TYK2 T10-2 mouse monoclonal antibodies were raised against a GST-fusion protein containing
530 amino acids 289-451 of human TYK2 (56). Affinity-purified chicken antibodies specific for LGTV
531 NS5 peptides (57) were used for co-immunoprecipitation experiments at 2 μ g per sample or for
532 Western blot analysis at a 1:1000 dilution. Rabbit Phospho-TYK2 (Y1054/1055) 9321S, rabbit
533 STAT2 4594S, rabbit Phospho-STAT2 (Y690) 4441S, rabbit STAT1 (42H3) 9175S and rabbit
534 Phospho-STAT1 (Y701) 7649S were obtained from Cell Signaling and used at a 1:1 000 dilution for

535 Western blot analysis. Mouse β -actin (Clone AC-74) and mouse FLAG M2 F3165-1MG were
536 obtained from Sigma-Aldrich and used at 1:10 000 and 1:2 000 dilutions in western blot, respectively.
537 FLAG M2 was used at 1:2 000 dilution for immunofluorescence staining. Mouse V5 (46-0705;
538 Thermo Fisher Scientific) was used at 1:5 000 in Western blot and 1:200 for immunofluorescence
539 staining. Phospho-STAT1 (TYR701) monoclonal antibody (15H13L67) from Invitrogen was used at
540 1:1000 dilution for immunofluorescence staining. Anti-Envelope MAb 4G2 antibody was used at 1:1
541 000 in flow cytometry and immunofluorescence assays. Anti-TBEV NS5 antibodies were generate
542 immunizing a rabbit at days 0, 17, and 24 with 150 μ g of full-length recombinant TBEV NS5. The
543 immunogen was mixed with Freund complete adjuvant for the first immunization and with Freund
544 incomplete adjuvant for the following immunizations. The rabbit was bled and the immune response
545 was monitored by titration of serum samples by ELISA on coated Protein. Immunoglobulins (IgG)
546 were purified from the expression medium by affinity chromatography on a 1 ml protein G column
547 (Cytiva). After sample application, the column was washed with 20 column volumes of PBS and the
548 protein was subsequently eluted with 10 column volumes of PBS supplemented with 0.1 M Glycine
549 (pH=2.3). Affinity-eluted IgG were finally polished on a HiLoad 16/600 Superdex 200 pg Pre-packed
550 column (Cytiva) using PBS buffer. Animal procedures were performed according to the French
551 legislation and in compliance with the European Communities Council Directives (2010/63/UE,
552 French Law 2013-118, February 6, 2013). The Animal Experimentation Ethics Committee of Pasteur
553 Institute (CETEA 89) approved this study. Anti-TBEV NS5 rabbit antibody was used 1:1 000 for
554 Western blot and immunofluorescence.

555 The following secondary antibodies were used: Alexa Fluor 488 goat anti-mouse IgG (H+L;
556 A11001); Alexa Fluor 647 goat anti-rabbit IgG (H+L) (Life Technologies); Alexa Fluor 680 goat anti-
557 mouse IgG (H+L) (Invitrogen); goat anti-chicken IgY (H+L) Dylight 800 (Invitrogen) and goat anti-
558 rabbit IgG (H+L) Dylight 800 (SA5-35571; Invitrogen). All the secondary antibodies were used at 1:1
559 000 for cytometry and immunofluorescence assays and 1:10 000 for Western blot analysis.

560 IFN- α 2b (34.829864) and human IL-29 (IFN lambda-1; 34-8298-64) were from PBL Biosciences
561 and Invitrogen, respectively. Infected cells were stimulated with IFN α 2b at a final concentration of 2
562 000 IU/ml, for 8 hours when analyzing ISG mRNA abundance or for 30 min when analyzing STAT1p
563 presence by immunofluorescence assays. For RT-qPCR analysis performed in NS5 expressing cells,
564 cells were stimulated overnight with IFN- α 2b (200 IU/ml) or IL-29 (100 ng/ μ l). For western blot
565 analysis, IFN treatment was performed 24 hours after infection with 400 IU/ml of IFN α 2 for a
566 duration indicated in the figure legends.

567

568 **Transfections**

569 293T cells were transfected using Trans IT[®]-293 (Mirus) following the manufacturer's protocol. For
570 measuring luciferase activity, 293T cells were transfected with a mixture of 80 ng of pISRE-Luc, 20

571 ng of pRL-TK-Renilla and 70 ng of the plasmid of interest. For immunofluorescence experiments,
572 293T cells were grown on coverslips in 24-well plates, transfected with 1 µg of the plasmid of interest
573 and fixed 24 hours later. For co-immunoprecipitation analysis, cells in 6-well plates were transfected
574 with 280 ng of each plasmid (560 ng of total DNA per well).

575

576 **Immunoblot and immunoprecipitation analysis**

577 Cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Sigma-Aldrich) supplemented
578 with a protease and phosphatase inhibitor cocktail (Roche). Samples were denatured in 4X Protein
579 Sample Loading Buffer (Li-Cor Bioscience) under reducing conditions (NuPAGE reducing agent,
580 Thermo Fisher Scientific), with the exception of cells expressing TBEV and LIV NS2A, which were
581 lysed in Mem-PER plus membrane protein extraction kit (Thermo Fisher scientific) and sonicated 20
582 min at 100% amplitude and 2/2 pulse. Proteins were separated by SDS-PAGE (NuPAGE 4 to 12%
583 Bis-Tris gel; Invitrogen) and transferred to nitrocellulose membranes (Bio-Rad) using a Trans-Blot
584 Turbo Transfer system (Bio-Rad). Alternatively, when analyzing TYK2 expression levels, proteins
585 were separated by SDS-PAGE (Bolt NuPAGE 8% Bis-Tris plus gel; Invitrogen) and transferred using
586 1X liquid transfer buffer (Invitrogen). Membranes were blocked with PBS-0.1% Tween 20 (PBS-T)
587 containing 5% milk. Alternatively, they were blocked with bovine serum albumin (BSA) when
588 analyzing levels of phosphorylated proteins or with BlokHen® Blocking Reagent when analyzing NS5
589 abundance. After blocking, the membranes were incubated overnight at 4°C with primary antibodies
590 diluted in blocking buffer or PBS-0.1% Tween 20 (PBS-T) for NS5 analysis. Finally, the membranes
591 were washed and incubated for 45 minutes at room temperature with secondary antibodies (anti-
592 rabbit/mouse IgG [H+L] DyLight 800/680 or anti-chicken IgY (H+L) DyLight 800) diluted in
593 blocking buffer or PBS-0.1% Tween 20 (PBS-T) for NS5 analysis and washed. Images were acquired
594 using an Odyssey CLx infrared imaging system (Li-Cor Bioscience).

595 For co-immunoprecipitation analysis, a fraction of the cell lysates was incubated overnight with
596 magnetic beads coupled with anti-FLAG M2 (M8823; Sigma-Aldrich) or PrecipHen
597 immunoprecipitation Reagent (P-1010; AVESLABS) following the manufacturer's protocol.
598 Following incubation, immunoprecipitates were washed four times with washing buffer and analyzed
599 by immunoblot as described above.

600

601 **RNA extraction and RT-qPCR analysis**

602 Total RNAs from cell lysates were extracted using the NucleoSpin RNA II kit (Macherey-Nagel)
603 following the manufacturer's protocol and were eluted in nuclease-free water. First-strand cDNA
604 synthesis was performed on 1 µg of total RNA with the RevertAid H Minus Moloney murine
605 leukemia virus (M-MuLV) reverse transcriptase (Thermo Fisher Scientific) using random primers
606 p(dN)₆ (Roche). Quantitative real-time PCR was performed on a real-time PCR system (Quant Studio
607 6 Flex; Applied Biosystems) with SYBR green PCR master mix (Life Technologies). Data were

608 analyzed with the $\Delta\Delta$ CT method, with all samples normalized to GAPDH (glyceraldehyde-3-
609 phosphate dehydrogenase). All experiments were performed in technical triplicate. The primers used
610 for RT-qPCR are listed in Appendix Table S4. Quantification of TBEV and LIV genomes were
611 determined by extrapolation from a standard curve generated from serial dilutions of the plasmid
612 encoding TBEV NS5 (pCi-Neo-NS5 TBEV).

613

614 **Luciferase assays**

615 Eight hours post-transfection, 293T cells were stimulated with 200 IU/ml of IFN α 2. Twenty-four
616 hours post-transfection, cells were lysed using Passive Lysis buffer (Promega) for at least 15 minutes
617 and luciferase activity was measured with Dual-Glo Luciferase Assay System (Promega) following
618 the manufacturer's protocol.

619

620 **Immunofluorescence assays**

621 Cells were fixed with 4% paraformaldehyde (PFA) (Sigma-Aldrich) for 30 minutes at room
622 temperature, permeabilized with methanol/ethanol (Sigma-Aldrich) V/V for 15 minutes, and then
623 blocked for 30 minutes with PBS containing 0.05% Tween and 5% BSA before incubation with the
624 indicated primary antibodies for 1 hour. After incubation, cells were washed three times with PBS
625 containing 0.05% Tween and 5% BSA. Secondary Alexa Fluor 488 or 647-conjugated antibodies were
626 added for 1 hour. After incubation, cells were washed twice with PBS containing 0.05% Tween and
627 5% BSA and once with PBS. Nuclei were stained 15 min using PBS/NucBlue (Life Technologies,
628 R37606). After washing, slides were mounted with Prolong gold (Life Technologies, P36930) imaging
629 medium. Images were acquired using a Leica SP8 confocal microscope.

630

631 **Flow cytometry**

632 Infected cells were fixed with cytofix/cytoperm kit (BD Pharmingen). Cells were washed three times
633 with wash buffer. They were then stained using the anti-E MAb 4G2 primary antibody and diluted in
634 wash buffer 1X for 1 hour at 4°C. Cells were again washed three times with wash buffer and stained
635 with secondary Alexa 488 antibody for 45 minutes in the dark at 4°C. Data were acquired using
636 Attune NxT Acoustic Focusing Cytometer (Life Technologies) and analyzed using FlowJo software.

637

638 **Mass spectrometry (MS) and data analysis**

639 Lysates of 293T cells transfected with plasmids expressing FLAG-NS5 TBEV, FLAG-NS5 LIV or
640 empty vectors (EV) were subjected to immunoprecipitation analysis using anti-FLAG magnetic beads,
641 as described above. Beads were incubated overnight at 37°C with 20 μ L of 25 mM NH $_4$ HCO $_3$ buffer
642 containing 0.2 μ g of sequencing-grade trypsin (Promega, Madison, WI, USA). The resulting peptides
643 were loaded and desalted on evotips provided by Evosep (Odense, Denmark) according to
644 manufacturer's procedure. Samples were analyzed on an Orbitrap Fusion mass spectrometer (Thermo

645 Fisher Scientific) coupled with an Evosep one system (Evosep) operating with the 30SPD method
646 developed by the manufacturer. Briefly, the method is based on a 44-min gradient and a total cycle
647 time of 48 min with a C18 analytical column (0.15 x 150 mm, 1.9 μ m beads, ref EV-1106) equilibrated
648 at room temperature and operated at a flow rate of 500 nl/min. MS grade H₂O/0.1 % MS grade formic
649 acid (FA) was used as solvent A and MS grade Acetonitrile (ACN)/0.1 % FA as solvent B. MS grade
650 H₂O, FA and ACN were from Thermo Fisher Scientific (Waltham, MA, USA).

651 The mass spectrometer was operated by data-dependent MS/MS mode. Peptide masses were
652 analyzed in the Orbitrap cell in full ion scan mode, at a resolution of 120,000, a mass range of m/z
653 350-1550 and an AGC target of $4 \cdot 10^5$. MS/MS were performed in the top speed 3s mode. Peptides
654 were selected for fragmentation by Higher-energy C-trap Dissociation (HCD) with a Normalized
655 Collisional Energy of 27% and a dynamic exclusion of 60 seconds. Fragment masses were measured
656 in an Ion trap in the rapid mode, with an AGC target of $1 \cdot 10^4$. Monocharged peptides and unassigned
657 charge states were excluded from the MS/MS acquisition. The maximum ion accumulation times were
658 set to 100 ms for MS and 35 ms for MS/MS acquisitions respectively. Label Free quantitation was
659 performed using Progenesis QI for proteomics software version 4.2 (Waters). The software was
660 allowed to automatically align data to a common reference chromatogram to minimize missing values.
661 Then, the default peak-picking settings were used to detect features in the raw MS files and a most
662 suitable reference was chosen by the software for normalization of data following the normalization to
663 all proteins method. A between-subject experiment design was chosen to create groups of four
664 biological replicates. MS/MS spectra were exported and searched for protein identification using
665 PEAKS STUDIO Xpro software (Bioinformatics Solutions Inc.). De Novo was run with the following
666 parameters: trypsin as enzyme (specific), half of a disulfide bridge (C) as fixed and deamidation
667 (NQ)/oxidation (M) as variable modifications. Precursor and fragment mass tolerances were set to
668 respectively 15 ppm and 0.5 Da. Database research was conducted against a Swissprot human
669 reference proteome FASTA file modified by the addition the NS5 sequences (release 2021_02, 20380
670 entries) and a maximum of 1 missed cleavage. The maximum of variable PTM per peptide was set to
671 4. Spectra were filtered using a 1% FDR. Identification results were then imported into Progenesis to
672 convert peptide-based data to protein expression data using the Hi-3 based protein quantification
673 method.

674 Multivariate statistics on protein measurements were performed using Qlucore Omics Explorer
675 3.7 (Qlucore AB, Lund, SWEDEN). A positive threshold value of 1 was specified to enable a log₂
676 transformation of abundance data for normalization *i.e.* all abundance data values below the threshold
677 will be replaced by 1 before transformation. The transformed data were finally used for statistical
678 analysis *i.e.* evaluation of differentially present proteins between two groups using a Student's
679 bilateral t-test and assuming equal variance between groups. Differential candidates were selected
680 using the following filters: p-value better than 0.02, fold change > 3 and unique peptide count >2,
681 identifying 244 proteins for LIV NS5, 256 proteins for TBEV NS5.

682 Generated AP-MS data were also analyzed using MiST (21) and SAINTExpress (22) algorithms.
683 Abundance data values of the four replicates for each NS5 and empty vector (EV) control were used to
684 run the MiST analysis using ‘HIV analysis’ mode to generate MiST scores normalized on protein size.
685 Cellular partners with a MiST Score > 0.70 were selected, identifying 142 proteins for LIV NS5 and
686 104 proteins for TBEV NS5. AP-MS dataset was analyzed in parallel with SAINTExpress (22) and
687 interactions with a SP score > 0.70 were selected, identifying 165 proteins for LIV NS5 and 162
688 proteins for TBEV NS5. Proteins candidates found enriched in 2 out of the 3 analysis (Student’s
689 bilateral t-test, MiST and SAINTExpress) were considered to be high-confidence partners. A total of
690 153 proteins for LIV NS5 and 130 for TBEV NS5 have been identified in this way, 107 of which are
691 common to LIV and TBEV. Interaction networks of NS5 proteins from TBEV and LIV were
692 represented using Cytoscape software (v3.9.1) (58).

693

694 ***In vitro* transcription and electroporation of TBEV replicons**

695 TBEV pTND/ Δ ME replicon plasmids (32) were linearized by NheI digestion and blunt ended using
696 Quick Blunting Kit (New England Biolab). Five μ g of purified DNA template were used for T7 *in*
697 *vitro* transcription using RiboMAX large-scale RNA production system T7 (Promega) in presence of
698 40 mM cap analog (Ribo m7G Cap, Promega) following the manufacturer’s instructions. After RQ1
699 DNase treatment (Promega), RNA was purified with RNA clean-up kit (Macherey-Nagel).

700 *In vitro* synthesized TBEV replicon RNA was introduced into Huh7 cells by electroporation.
701 Briefly, 8×10^6 trypsinized Huh7 cells were washed three times in cold PBS, resuspended in 400 μ L cold
702 PBS and electroporated with 6 μ g of RNA in 0.4-cm electroporation cuvettes (Biorad) with a 950 μ F
703 and 260 V pulse using Genepulser system (Biorad). After electroporation, cells were collected in 3.6
704 ml of warm medium, cell suspension were transferred to 24 well plates (5×10^5 cells per well) and
705 incubated at 37 °C under standard conditions.

706

707 **Recombinant NS5 production and purification**

708 Codon optimized sequences encoding full-length and RdRp domains of NS5 proteins from TBEV,
709 LIV and YFV, as well as the MTase domain of LIV were purchased from Twist Biosciences and
710 cloned into pET28 plasmids. Resulting plasmids encode fusion proteins with an N-term 6xHis
711 purification tag. The different constructs were expressed in *E. coli* C2566 pRARE2 cells (New
712 England Biolabs). Proteins were expressed overnight at 17°C in TB (with 25 μ g/mL Kanamycin and
713 17 μ g/mL Chloramphenicol), after induction with 250 μ M IPTG at OD₆₀₀ = 0.6. The cells were
714 resuspended on ice in Lysis Buffer 1 (50 mM NaPO₄ pH 7.5, 20% glycerol), then diluted 1:2 in Lysis
715 Buffer 2 (50 mM NaPO₄ pH 7.5, NaCl 1 M, 20% glycerol, 5 mM TCEP, 0.1% Triton X-100)
716 supplemented with 1 mg/mL Lysozyme, 10 μ g/mL DNase, and one anti-protease tablet (cOmplete™
717 ULTRA Tablets, Roche) for full-length NS5 and MTases, while RdRp were only resuspended in 50
718 mM NaPO₄ pH 7.5, NaCl 1 M, 20% glycerol, 5 mM TCEP, 1.6% IGEPAL, 10 mM b-

719 mercaptoethanol, 1 mM PMSF, 1 mg/mL Lysozyme, 22 µg/mL DNase. After sonication on ice and
720 clarification, the proteins from the soluble fraction were loaded onto TALON® Superflow™ cobalt-
721 based IMAC resin (Cytiva) after equilibration with 50 mM NaPO₄ pH 7.5, NaCl 0.5 M, 20% glycerol,
722 5 mM TCEP, 0.1% Triton X-100. The resin was washed with 5 column volume of Wash Buffer 1 (50
723 mM NaPO₄ pH 7.5, NaCl 1.5 M, 20% glycerol, 5 mM TCEP, 0.1% Triton X-100, 10 mM Imidazole)
724 then Wash Buffer 2 (50 mM NaPO₄ pH 7.5, NaCl 1.5 M, 20% glycerol, 5 mM TCEP, 10 mM
725 Imidazole), prior to elution with 50 mM NaPO₄ pH 7.5, NaCl 0.5 M, 20% glycerol, 5 mM TCEP, 250
726 mM Imidazole and 250 mM Glycine. Purified full-length and MTases underwent an extra step of size
727 exclusion chromatography on a gel filtration column (HiLoad® 16/600 Superdex® 200 pg, GE
728 Healthcare) against 20 mM Hepes pH 7.5, NaCl 0.75 M, 10% glycerol, 5 mM DTT. RdRp domains
729 however were loaded on a GE Hi-trap Heparin column after the cobalt resin, and were eluted by a
730 linear gradient of NaCl from 150 to 1000 mM in the elution buffer (50 mM NaPO₄ pH 7.5, 20 %
731 glycerol, 250 mM glycine and 0.5 mM TCEP) buffer. All proteins were buffer exchanged by dialysis
732 in 20 mM Hepes pH 7.5, NaCl 0.5 M, 40% glycerol, 5 mM TCEP. Eventually, proteins were
733 concentrated, ranging from 5 to 15 mg/mL and stored at -80°C. Staining of purified proteins after SDS
734 Page was performed using Coomassie Brilliant Blue R-250 reagents (Biorad) and imaged on an
735 Odyssey CLx infrared imaging system (Li-Cor Bioscience).

736

737 ***In vitro* TYK2 kinase assays**

738 TYK2 kinase activity was assessed using TYK2 Assay Kit (BPS Bioscience). The kinase domain
739 (aa871-1187) of TYK2 (10 ng) was incubated with a kinase substrate derived from the insulin receptor
740 substrate-1 (IRS1-peptide) in the presence of 10 µM of ATP accordingly to manufacturer's protocol.
741 Kinase reaction was carried out for one hour at 30°C, in the presence of 13 µmole of recombinant NS5
742 proteins diluted in 1X kinase buffer. After incubation, 50 µL of Kinase-Glo® MAX reagent was added
743 to 50 µL of the reaction, and luminescence was measured using a microplate reader. As TYK2
744 converts ATP to ADP during the kinase reaction, the level of ATP decreases after phosphorylation and
745 the kinase activity is inversely correlated to the luciferase activity. The activity of TYK2 alone in the
746 1X kinase buffer was set at 100%. We then replaced IRS1-peptides by about 100 ng of purified
747 STAT1 (Origene) and performed the assays under the same conditions and then submitted the samples
748 to Western blot analyses.

749

750 **Protein modelling and protein-protein docking**

751 X-ray structures of the NS5-TBEV and the TYK2 kinase domain exist (PDB codes 7D6N and
752 respectively 7K7O). However, both are incomplete due to unstructured and/or flexible regions. Since
753 these regions may be involved in protein-protein interaction, we used AlphaFold (59) to obtain gap-
754 free computed models of their three-dimensional structure. To model the interaction interface between
755 NS5-TBEV with the human TYK2 kinase domain, regions comprising residues 276 to 975 and 897 to

756 1176 were chosen, respectively. After ensuring that the AlphaFold predicted structures did not deviate
757 from the experimental X-ray published structures (rmsd < 1Å), an initial guess to obtain the starting
758 interaction position was performed. Rigid-body docking of NS5-TBEV and TYK2 was calculated
759 using the software HADDOCK (version 2.4)(60) restricting the interaction area of the NS5-TBEV to
760 the B-C loop (residues 616 to 658). In order to optimize and relax the docked model, we submitted the
761 top solution to the RosettaDock software (version 4.0)(61) to perform flexible backbone protein
762 docking with the centroid score function enabled. The top result with the lowest I_{sc} score (interface
763 energy) was selected as the most likely region of interaction. Finally, predicted interface between NS5
764 TBEV and TYK2 was analyzed using the software PISA (62) from the CCP4 suite (63). All structural
765 figures were generated with ChimeraX (version 1.4)(64).

766

767 **Data representation and statistical analysis**

768 Data are presented and analyzed using GraphPad Prism 9. Alignments and trees were generated using
769 CLC Genomics Workbench 22. Immunoblot analysis and relative quantification of protein abundance
770 was performed using ImageJ and pixel count was used to calculate the Densitometric ratio (DR).

771

772 **Data availability**

773 Imaging datasets produced in this study are available in the BioStudies database
774 (<https://www.ebi.ac.uk/biostudies/>) under accession number S-BSST1179.

775

776

777 **References**

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983
984

985 Acknowledgements

986 We thank Pierre Lafaye (Antibody Engineering Platform, Institut Pasteur, Paris) for the

987 production of the anti-NS5 antibodies, Gregory Caignard and Damien Vitour (both at UMR1161

988 Virologie Laboratoire de Santé Animale, ANSES, Maisons-Alfort, France) for the collection of

989 plasmids coding for TYK2 orthologs, Nick Johnson (Animal and Plant Health Agency, UK) for LIV,

990 Annette Martin (Institut Pasteur, Paris) for Huh7 cells, Yves Jacob (Institut Pasteur, Paris) for the

991 Gateway compatible pCI-neo-FLAG vector, Pierre-Olivier Vidalain (ENS Lyon) and Angeliki Anna

992 Beka (Institut Pasteur, Paris) for discussions and advice on MiST and SAINTexpress analyses. We

993 also thank Sonja Best (Rocky Mountain Laboratories, NIAID, NIH, Hamilton) for the antibody
994 against LGTV NS5, discussions and critical reading. We are very grateful to the Institut Monod MS
995 platform, in particular Guillaume Chevreux, Véronique Legros and Laurent Lignières, for performing
996 the MS analysis. We also thank our team members and Muriel Couplier for scientific inputs and
997 discussions.

998

999 **Funding.** This work is supported by the Institut Pasteur, CNRS and Agence Nationale de la
1000 recherche (grant numbers ANR-19-CE35-0015 and ANR-20-CE11-0024).

1001

1002 **Author contributions:**

1003 Conceptualization: SG, VC, NJ

1004 Methodology: SG, MC, AD, AS, ZL, JR, SP, ED, VC, NJ

1005 Investigation: SG, MC, AD, YU, AD, LP, VR, SAL, ML, MS

1006 Visualization: SG, MC, AD, YU, AS, LP, VR, SAL, ML, MS, ZL, JR, SP, ED, VC, NJ

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1010

1011 **Competing interests.** The authors declare that they have no competing interests.

1012

1013

1014

1015

1016 **Figure legends**

1017

1018 **Figure 1. TBEV and LIV infection antagonize IFN γ signaling in Huh7 and 293T cells.**

1019 (A) Huh7 cells were left uninfected (NI) or were infected with TBEV or LIV at a multiplicity of
1020 infection (MOI) of 0.5 and 0.05 respectively. Twenty-four h later, the percentages of cells expressing
1021 the viral E protein were determined by flow cytometry analysis. Data are the means \pm SD of three
1022 independent biological replicates.

1023

1024 (B-D) Huh7 cells were left uninfected (NI) or infected for 24 h with TBEV or LIV, at a MOI of 0.5
1025 and 0.05 respectively, and then treated or not with IFN γ at 2000 IU/ml for 8 h. The relative amounts
1026 of cell-associated viral RNA (B) were measured by RT-qPCR analysis and were expressed as genome
1027 equivalents (GE) per μ g of total cellular RNAs. The relative amounts of *ISG15* (C) and *ISG56* (D)
1028 mRNAs were determined by RT-qPCR analysis. Results were first normalized to *GAPDH* mRNA and
1029 then to non-treated uninfected mRNA levels, which were set at 1. Data are the means \pm SD of four
1030 independent biological replicates. One-way ANOVA tests were performed. ns: non-significant, *:

1031 $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

1032

1033 (E) Huh7 cells were left uninfected (NI) or were infected as in (A) for 24 h and stimulated or not with
1034 IFN γ 2 at 2 000 IU/ml for 30 min. Whole-cell lysates were analysed by Western blotting with
1035 antibodies against the indicated proteins. Data are representative of three independent biological
1036 replicates.

1037

1038 (F) 293T cells were left uninfected (NI) or were infected with TBEV or LIV at an MOI 0.02
1039 for 48 h. They were stimulated or not with IFN γ 2 at 2 000 IU/ml for 30 min before fixation. They
1040 were stained with antibodies recognizing the viral E protein (green), STAT1p (red) and NucBlue ®
1041 (blue). Images are representative of two independent biological replicates. Scale bars, 40 μm .

1042

1043

1044 **Figure 2. TBEV and LIV NS5 dampen IFN signaling in 293T cells.**

1045 (A) 293T cells were co-transfected with the Firefly luciferase reporter plasmid p-ISRE-luc, TK Renilla
1046 luciferase control plasmid pRLuc-TK, and plasmids encoding individual viral proteins. Empty vectors
1047 (EV) and plasmids encoding the NS5 of YFV protein were used as negative and positive controls,
1048 respectively. Cells were stimulated 7 hours post-transfection with IFN γ 2 at 200 IU/ml and assayed for
1049 luciferase activity 16 h later. The data were analyzed by first normalizing the Firefly luciferase activity
1050 to the Renilla luciferase (Rluc) activity and then to EV samples, which were set at 100%. Data are
1051 means \pm SD of three independent biological replicates. One-way ANOVA tests with Dunnett's
1052 correction were performed, **** $p < 0.0001$.

1053

1054 (B-C) 293T cells were co-transfected with Firefly luciferase reporter plasmid p-ISRE-luc, TK Renilla
1055 luciferase control plasmid pRLuc-TK and increasing amounts (ranging from 0.1 ng to 5 ng) of
1056 plasmids encoding the NS5 of TBEV (B) or LIV (C). Cells were stimulated 7 h post-transfection with
1057 IFN γ 2 at 200 IU/ml and assayed for luciferase activity at 24 hours post-transfection. Cells transfected
1058 with empty vector (EV) were used as negative controls. The data were analyzed by first normalizing
1059 the Firefly luciferase activity to the Renilla luciferase (Rluc) activity and then to EV samples, which
1060 were set at 100%. Data are mean \pm SD of three independent biological replicates. One-way ANOVA
1061 tests with Dunnett's correction were performed, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$). Western
1062 blot analyses were performed with anti-FLAG and anti-actin antibodies on the same samples. Non-
1063 transfected (NT) cells added in the Western blotting analysis for comparison. Data are representative
1064 of at least three independent biological replicates.

1065

1066 (D-E) 293T cells were mock-transfected (NT), transfected with empty plasmid (EV) or plasmids
1067 expressing FLAG-tagged NS5 from TBEV, LIV or YFV. Cells transfected with plasmids expressing

1068 FLAG-tagged C protein from LIV were used as negative controls. Twenty-four hours later, they were
1069 left untreated (NT) or treated with IFN γ 2 at 2000 IU/ml for 30 min before fixation. Cells were then
1070 stained with antibodies recognizing the FLAG tag of viral proteins (green), STAT1p (red) and
1071 NucBlue (blue). Images are representative of three independent biological replicates. Scale bars,
1072 40 μ m. (E) Percentages of STAT1p-positive nuclei among cells expressing viral proteins were
1073 estimated by analysing at least 15 fields (~150 cells) per condition. One-way ANOVA tests with
1074 Dunnett's correction were performed. ****p < 0.0001.

1075

1076 (F) 293T cells were mock-transfected (NT), transfected with empty plasmid (EV) or plasmids
1077 expressing FLAG-NS5 from TBEV, LIV or YFV for 24h. Cells were left untreated (NT) or stimulated
1078 with IFN γ 2 (400 IU/ml) 30 min before harvest. Whole-cell lysates were analysed by Western blotting
1079 with antibodies against the indicated proteins. Data are representative of three independent biological
1080 replicates.

1081

1082 (G-H) 293T cells were mock-transfected (NT), transfected with empty vectors (EV) or plasmids
1083 expressing FLAG-NS5 from TBEV, LIV or YFV. Twenty-four h later, they were left untreated (NT)
1084 or treated with IFN γ 2 (2000 IU/ml) or IL29 (100ng/ μ l) overnight. The relative amounts of *ISG15* (G)
1085 and *ISG56* (H) mRNAs were determined by RT-qPCR analysis. Results were first normalized to
1086 *GAPDH* mRNA and then to mRNA levels of treated cells transfected with EV, which were set at
1087 100%. Data are means \pm SD of three independent biological replicates. One-way ANOVA tests with
1088 Dunnett's correction were performed. ****p < 0.0001.

1089

1090

1091 **Figure 3. TBEV and LIV NS5 interact with TYK2.**

1092

1093 (A) 293T cells were transfected with empty plasmids (EV) or with plasmids expressing FLAG-tagged
1094 versions of TBEV or LIV NS5. Twenty-four hours later, cell lysates were immunoprecipitated with
1095 anti-FLAG magnetic beads. Immunoprecipitates were analysed by Western blotting with antibodies
1096 against the FLAG tag. Data are representative of four independent biological replicates.

1097

1098 (B) Mass spectrometry (MS) analysis was performed with four biological replicates per bait. Empty
1099 vector (EV) conditions were used as negative controls. Data were analyzed with three different MS
1100 scoring algorithms (see Results and Experimental procedures sections). Only proteins that were
1101 identified in 2 out of the 3 analyses were considered high confidence partners of NS5 and are
1102 represented here. Cellular partners common to TBEV and LIV NS5 are depicted in purple, while
1103 partners specific for TBEV or LIV NS5 are represented in darker purple and lighter purple,
1104 respectively. TYK2 is highlighted in yellow.

1105

1106 (C) 293T cells were infected for 24 h with TBEV or LIV at an MOI of 0.5 and 0.05 respectively. Cell
1107 lysates were immunoprecipitated using antibodies specific for NS5 of tick-borne flaviviruses.
1108 Western blot analysis was performed on whole cells lysates (Input) and NS5-immunoprecipitates (IP
1109 NS5) using the indicated antibodies. The presented western blot is representative of three independent
1110 biological replicates.

1111

1112

1113 **Figure 4. The RdRp domain, but not the MTase domain, of TBEV and LIV NS5 binds TYK2**
1114 **and antagonizes the JAK/STAT pathway.**

1115

1116 (A) The flavivirus NS5 contains two domains: an N-terminal methyltransferase (MTase) domain (30
1117 kDa) and an RNA-dependent RNA polymerase (RdRp) domain (90 kDa). The full-length proteins and
1118 the domains were fused to an N-terminal FLAG tag.

1119

1120 (B) 293T cells were mock-transfected (NT), transfected with plasmids expressing either FLAG-tagged
1121 full length (FL) NS5 or individual domains (MTase or RdRp), together with V5-tagged TYK2
1122 plasmid. Cells transfected with empty vectors (EV) or YFV NS5 plasmids were used as negative and
1123 positive controls, respectively. Cells were lysed 24 h post-transfection. Western blot analyses were
1124 performed on whole-cell lysates with the indicated antibodies (Input). Immunoprecipitation assays
1125 were performed on the same samples with anti-FLAG magnetic beads (IP FLAG). Lysates were
1126 revealed using FLAG and V5 antibodies. Results are representative of three independent biological
1127 replicates.

1128

1129 (C) The interactions between NS5 (full-length or RdRp domains) and the C-terminal domain of TYK2
1130 were assessed by Yeast Gap Repair assays. Protein expression and interaction enable yeast growth on
1131 a medium deprived of leucine, tryptophan and histidine and supplemented with 5 mM 3-aminotriazole
1132 (3-AT). Yeast transformed with circular pPC86 were used as positive recombination control (see
1133 Experimental procedures section) and yeasts transformed with linear pPC86 alone provided a negative
1134 control of interaction. Yeast expressing NS5 of Yellow Fever virus (FL YFV) was used as a negative
1135 interaction control. Results are representative of three independent biological replicates.

1136

1137 (D) 293T cells were co-transfected with Firefly luciferase reporter plasmid p-ISRE-luc, TK Renilla
1138 luciferase control plasmid pRluc-TK and plasmids expressing TBEV or LIV full length (FL) NS5 or
1139 individual domains (MTase or RdRp). Empty vectors (EV) and plasmids expressing YFV NS5 protein
1140 were used as negative and positive controls, respectively. Cells were stimulated 7 h post-transfection
1141 with IFN γ at 200 IU/ml and assayed for luciferase activity 24 h later. The data were analyzed by first

1142 normalizing the Firefly luciferase activity to the Renilla luciferase (Rluc) activity and then to EV
1143 samples, which were set at 100%. The data are means +/- SD of three independent biological
1144 replicates. One-way ANOVA tests with Dunnett's correction were performed, **: $p < 0.01$, ***:
1145 $p < 0.001$, ****: $p < 0.0001$.

1146

1147 (E) 293T cells were mock-transfected (NT), transfected with empty plasmids (EV) or plasmids
1148 expressing full-length versions or individual domains of NS5 from TBEV or LIV for 24 h. Cells
1149 expressing YFV NS5 were used in parallel. Cells were left untreated (NT) or stimulated with IFN γ 2
1150 (400 IU/ml) 30 min before lysis. Whole-cell lysates were analysed by Western blotting with antibodies
1151 against the indicated proteins. Data are representative of three independent experiments.

1152

1153

1154 **Figure 5. A variable region of tick-borne RdRp is involved in TYK2 binding and IFN**
1155 **antagonism.**

1156

1157 (A) 293T cells were mock-transfected (NT) or transfected with plasmids expressing FLAG-tagged
1158 versions of NS5 from flaviviruses transmitted by ticks (TBEV, LIV and LGTV), *Culex* mosquitoes
1159 (WNV, USUV or JEV) or *Aedes* mosquitoes (ZIKV, DENV or YEV), together with empty plasmids
1160 (EV) or plasmids expressing TYK2-V5. Cells were lysed 24 h post-transfection. Western blot analysis
1161 was performed on whole-cell lysates with the indicated antibodies (Input). Immunoprecipitation
1162 assays were performed on the same samples using anti-FLAG magnetic beads (IP FLAG). Lysates
1163 were revealed using FLAG and V5 antibodies. Results are representative of three independent
1164 biological replicates.

1165

1166 (B) Structure-based alignment of tick- and mosquito-borne flavivirus NS5, from amino acids 616 to
1167 658. Red boxed residues in the variable region are the residues predicted to interact with TYK2 after
1168 molecular docking simulations.

1169

1170 (C) Superimposed structures of the NS5 RdRp domain of YFV (PDB:6QSN; blue) and TBEV (PDB:
1171 7D6N; green). NS5 YFV presents an insertion (DES) located within the variable region that extends
1172 the alpha helix with negatively charged surface-exposed residues (dotted red circle).

1173

1174 (D) Protein sequence of TBEV and YFV NS5 (from amino acids 616 to 658), as well as of a TBEV-
1175 VR_{YFV} construct where TBEV residues from the variable region (VR) within the inter B-C domain
1176 have been replaced by the corresponding YFV aa.

1177

1178 (E) 293T cells were co-transfected with Firefly luciferase reporter plasmid p-ISRE-luc, TK Renilla
1179 luciferase control plasmid pRluc-TK and full-length TBEV NS5 (wild-type or TBEV-VR_{YFV} mutant)
1180 or empty vectors (EV) as negative controls. Cells were stimulated 7 h post-transfection with IFN γ 2 at
1181 200 IU/ml and assayed for luciferase activity at 24 h post-transfection. The data were analyzed by first
1182 normalizing the Firefly luciferase activity to the Renilla luciferase (Rluc) activity and then to EV
1183 samples, which were set at 100%. Data are mean \pm SD of three independent biological replicates.
1184 One-way ANOVA tests with Dunnett's correction were performed, **: p<0.01, ***: p<0.001, ****: p
1185 < 0.0001.

1186
1187 (F) 293T cells were mock-transfected (NT), transfected with plasmids encoding FLAG-tagged
1188 versions of NS5 TBEV (full-length or RdRp domain), either wild-type or TBEV VR_{YFV} mutant,
1189 together with empty plasmid (EV) or plasmid encoding TYK2-V5. Cells were lysed 24 h post-
1190 transfection. Western blot analysis was performed on whole-cell lysates with the indicated antibodies
1191 (Input). Immunoprecipitation assays were performed on the same samples with anti-FLAG magnetic
1192 beads (IP FLAG). Lysates were revealed using FLAG and V5 antibodies. Results are representative of
1193 three independent biological replicates.

1194
1195 (G) Huh7 cells were mocked-electoporated or electoporated with *in-vitro* synthesized RNAs derived
1196 from wild-type (rTBEV) or mutated TBEV replicons (rTBEV-VR_{YFV} or rTBEV-GAA). Three days
1197 later the relative amounts of cell-associated viral RNA were measured by RT-qPCR analysis and were
1198 expressed as genome equivalents (GE) per μ g of total cellular RNAs. Data are mean \pm SD of three
1199 independent biological replicates. T-tests were performed, ns: non-significant, **: p<0.05

1200
1201 (H) Huh7 cells were mocked-electoporated or electoporated with *in-vitro* synthesized RNA derived
1202 from wild-type or mutated TBEV replicons (VR_{YFV} or GAA). Three days later, they were stimulated
1203 or not with IFN γ 2 (2000 IU/ml) for 30 min. Whole-cell lysates were analysed by Western blotting
1204 with antibodies against the indicated proteins. Data are representative of three independent biological
1205 replicates.

1206

1207

1208 **Figure 6. TBEV and LIV NS5 proteins interact with the tyrosine kinase domain of TYK2 and**
1209 **affect its catalytic activity.**

1210

1211 (A) 293T cells were co-transfected or not (NT) with plasmids expressing FLAG-tagged versions of
1212 NS5 TBEV or LIV together with V5-tagged TYK2 from different mammalian species or control
1213 empty vectors. Cells were lysed 24 h post-transfection. Western blot analysis was performed on cell
1214 lysates with the indicated antibodies (Input). Immunoprecipitation assays were performed on the same

1215 samples using anti-FLAG magnetic beads (IP FLAG). Immunoprecipitates were revealed with anti-
1216 FLAG and anti-V5 antibodies. Results are representative of three independent biological replicates.

1217 (B) Interactions between NS5 from TBEV and LIV with the C-terminal domain of human, bovine,
1218 sheep and goat TYK2 were assessed by yeast Gap Repair assays. Protein expression and interaction
1219 enable yeast growth on a medium devoid of leucine, tryptophan and histidine and supplemented with
1220 5mM 3-aminotriazole (3-AT). Yeast transformed with circular pPC86 were used as positive
1221 recombination control (see Experimental procedures section) and yeast transformed with linear pPC86
1222 alone provided a negative control of interaction. Results are representative of three independent
1223 biological replicates.

1224 (C) TYK2 is composed of four domains: an N-terminal FERM domain, a SH2 domain, a kinase-like
1225 (KL) domain and a C-terminal tyrosine kinase (TK) domain. Mutants of TYK2 were generated as
1226 indicated. All are carrying a C-terminal V5 tag.

1227

1228 (D) 293T cells were mock-transfected (NT), transfected with empty plasmids (EV) or plasmids
1229 expressing full-length versions of TBEV or LIV NS5, together with different versions of V5-tagged
1230 TYK2 plasmids. Cells were lysed 24 h post-transfection. Western blot analysis was performed on
1231 whole-cell lysates with the indicated antibodies (Input). Immunoprecipitation assays were performed
1232 on the same samples using anti-FLAG magnetic beads (IP FLAG). Lysates were revealed using FLAG
1233 and V5 antibodies. Results are representative of three independent biological replicates.

1234

1235 (E) Structural cartoon of the interaction between the TBEV RdRp domain (green) and the kinase
1236 domain of TYK2 (gray), as predicted by molecular docking. Data presented in figure 5 suggest that
1237 TBEV NS5 RdRp interacts with the TYK2 via a variable region of its B-C loop (yellow).
1238 Intermolecular hydrogen bonds are represented as dotted lines in the enlarged box. The residue K930,
1239 which is involved in ATP binding and kinase activity, is colored in magenta.

1240

1241 (F) 293T cells were mock-transfected (NT), transfected with empty plasmids (EV) or plasmids
1242 expressing NS5 from TBEV, LIV or YFV. Twenty-four hours later, cells were stimulated with IFN α 2
1243 (400 IU/ml) for 15 min. Whole-cell lysates were analysed by Western blotting with antibodies against
1244 the indicated proteins. Data are representative of three independent biological replicates. For
1245 densitometric analysis of band intensities see Fig. EV6A.

1246

1247 (G) TYK2 kinase activity was assessed *in vitro* using the kinase domain (aa871-1187) of TYK2, a
1248 peptide derived from IRS-1 as substrate and ATP. Kinase reactions were carried with or without
1249 recombinant NS5 proteins as indicated. The level of ATP, which decreases during the kinase reaction,
1250 was inversely correlated to the luciferase activity. The activity of TYK2 in the absence of viral
1251 proteins was set at 100%. The data are means +/- SD of at least six independent biological replicates.

1252 One-way ANOVA tests with Dunnett's correction were performed, **: $p < 0.01$, ***: $p < 0.001$, ****: p
1253 < 0.0001 .

1254

1255 (H) *In vitro* kinase assays were performed as in (G) by replacing IRS-1 peptides by recombinant
1256 STAT1. The samples were analyzed by Western blot with the indicated antibodies. Data are
1257 representative of three independent biological replicates. For densitometric analysis of band intensities
1258 see Fig. EV6B.

1259

1260

1261 **EV figure legends.**

1262

1263 **Figure EV1. Viral protein expression in 293T cells.** 293T cells were mock-transfected (NT),
1264 transfected with empty plasmids (EV) or with plasmids encoding FLAG-tagged TBEV or LIV viral
1265 proteins. A plasmid coding a FLAG-tagged version of YFV NS5 protein was included in the analysis.
1266 Cells were harvested 24 hours post-transfection and protein expression was assessed by Western
1267 blotting with anti-FLAG and anti-actin antibodies. Data are representative of three independent
1268 experiments.

1269

1270 **Figure EV2. YFV NS5 protein reduces ISRE activation in 293T cells.** 293T cells were co-
1271 transfected with Firefly luciferase reporter plasmid p-ISRE-luc, TK Renilla luciferase control plasmid
1272 pRluc-TK and increasing amounts (ranging from 0.1 ng to 70 ng) of plasmids encoding the NS5 of
1273 YFV. Cells were stimulated 7 h post-transfection with IFN β 2 at 200 IU/ml and assayed for luciferase
1274 activity at 24 hours post-transfection. Cells transfected with empty vector (EV) were used as negative
1275 controls. The data were analyzed by first normalizing the Firefly luciferase activity to the Renilla
1276 luciferase (Rluc) activity and then to EV samples, which were set at 100%. Data are mean \pm SD of
1277 three independent biological replicates. One-way ANOVA tests with Dunnett's correction were
1278 performed, **: $p < 0.01$, ***: $p < 0.001$, ****: $p < 0.0001$). Western blot analyses were performed with
1279 anti-FLAG and anti-actin antibodies on the same samples. Non-transfected (NT) cells added in the
1280 Western blotting analysis for comparison. Data are representative of at least three independent
1281 experiments.

1282

1283 **Figure EV3. TBEV and LIV NS5 proteins localize both in the cytoplasm and the nucleus of**
1284 **transfected Huh7 cells.** Huh7 cells were mock-transfected (mock) or transfected with plasmids
1285 expressing FLAG-tagged NS5 from TBEV or LIV. Twenty-four hours later, cells were fixed and
1286 stained with antibodies recognizing the FLAG tag (green), and NucBlue® (blue). Images are
1287 representative of three independent biological replicates. Scale bars, 40 μ m.

1288

1289 **Figure EV4. TBEV and LIV NS5 proteins localize both in the cytoplasm and the nucleus of**
1290 **infected Huh7 cells.** Huh7 cells were left uninfected (NI) or were infected with TBEV or
1291 LIV at an MOI 0.02 for 48 h. They were stimulated or not with IFN γ 2 at 2000 IU/ml for 30 min
1292 before fixation. Cells were stained with antibodies recognizing the viral E protein (red), NS5 protein
1293 (green) and NucBlue® (blue). Images are representative of two independent biological replicates.
1294 Scale bars, 40 μ m.

1295

1296 **Figure EV5. Evolutionary history of NS5.** Using the neighbor-joining method on NS5 protein
1297 sequences. Numbers correspond to bootstrap values inferred from 10,000 replicates. Evolutionary
1298 distances were computed using Kimura-Protein model.

1299

1300 **Figure EV6. Alignment of human JAKs Tyrosine Kinases and TYK2 orthologs, in NS5**
1301 **interacting region.** Residues boxed in red correspond to NS5 interaction residues identified by
1302 molecular docking analysis. Residue K930, which is involved in ATP binding and kinase activity, is
1303 colored in magenta. Residues Y1054 and Y1055, which are phosphorylated during activation, are
1304 boxed in green. Numbering is relative to human TYK2.

1305

1306 **Figure EV7 (related to figures 6F, 6G and 6H).**

1307 (A) Densitometric analysis of Western blots from three independent biological replicates showing the
1308 relative abundances of pTYK2 to total TYK2. Data are expressed as a percentage of the values of cells
1309 transfected with an empty plasmid (EV). They are means \pm SD. One-way ANOVA tests with
1310 Dunnett's correction were performed (ns: non-significant, **: $p < 0.01$, ***: $p < 0.001$).

1311 (B) Viral protein expression *in vitro*. Coomassie Gel of purified NS5 (full-length protein or individual
1312 domain).

1313 (C) Densitometric analysis of Western blots from three independent biological replicates showing the
1314 relative abundances of pSTAT1 to total STAT1. Data are expressed as a percentage of the samples
1315 containing no NS5 (mock), and are means \pm SD. One-way ANOVA tests with Dunnett's correction
1316 were performed (ns: non-significant, **: $p < 0.01$, ***: $p < 0.001$).

1317

1318 **Table EV1. Mass Spectrometry analysis of NS5 interacting partners in 293T cells.** The FLAG-
1319 tagged versions of TBEV and LIV NS5 proteins were expressed and affinity purified from human
1320 293T cells. Cells transfected with empty vectors (EV) were used as controls. NS5 interacting partners
1321 were analyzed by mass spectrometry (MS). Three analyses were performed: one was based on peptide
1322 intensities (NS5-TBEV-vs-EV and NS5-LIV-vs-EV) and the other 2 were conducted MiST ('Mist-
1323 hits') and SAINTexpress ('SaintExpress-hits').

Figure 1

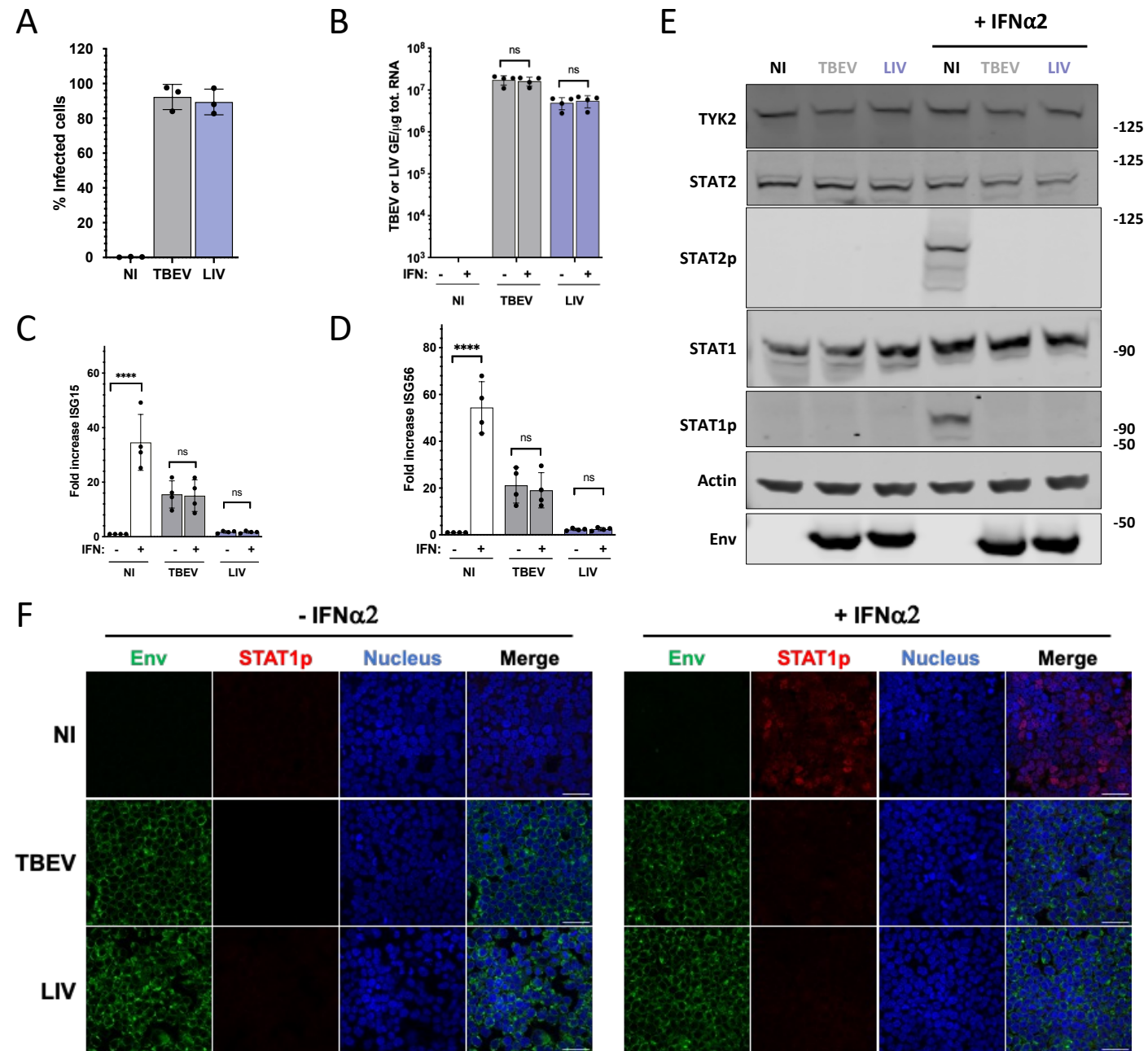


Figure 2

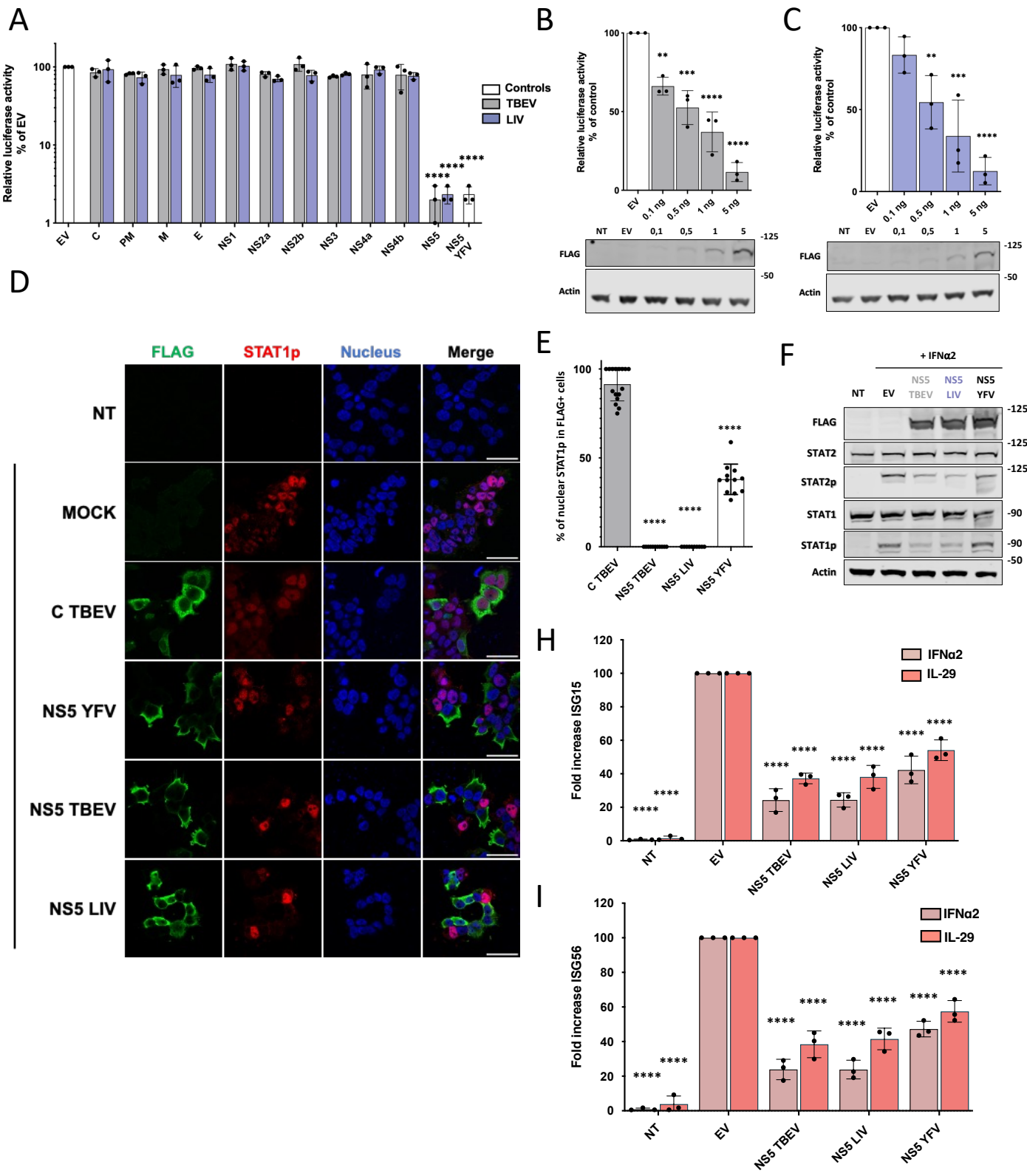


Figure 3

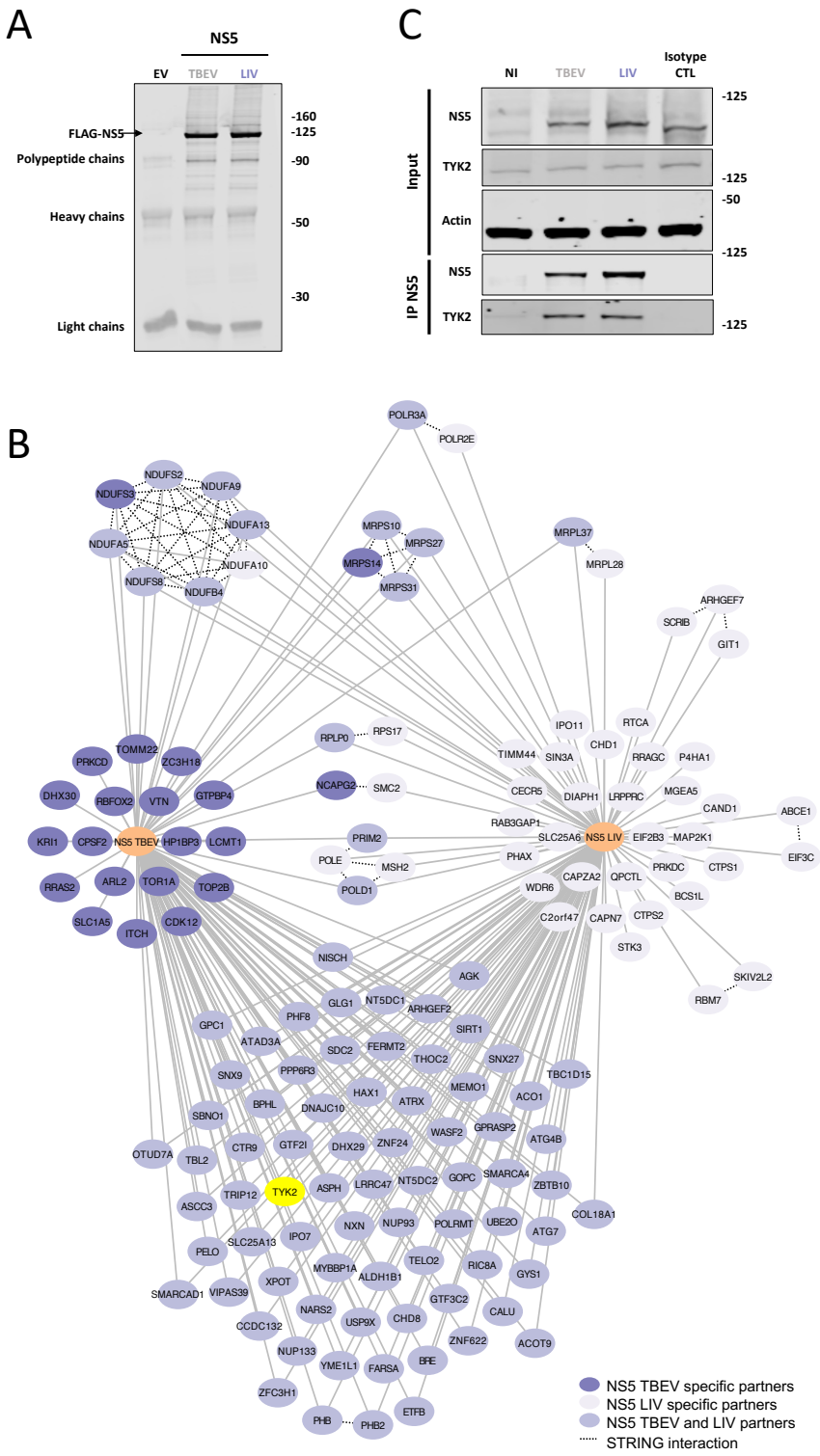
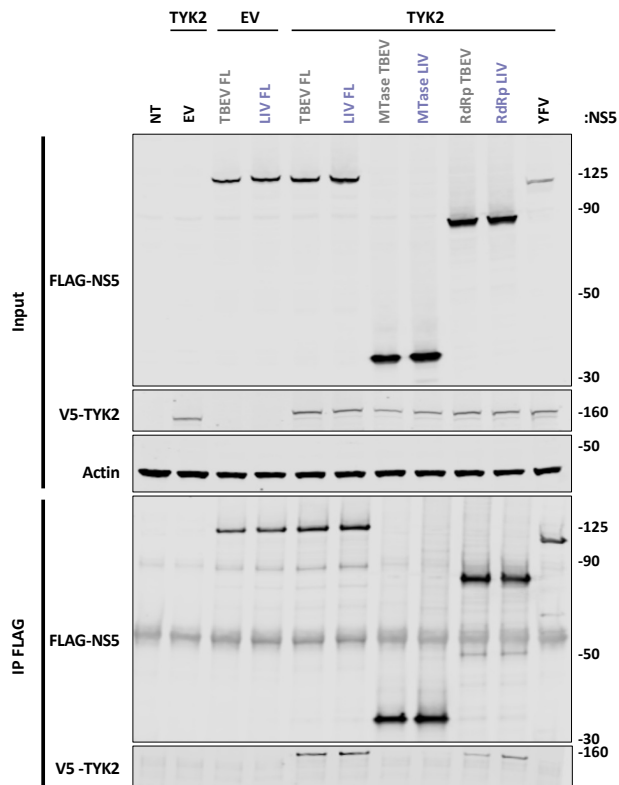


Figure 4

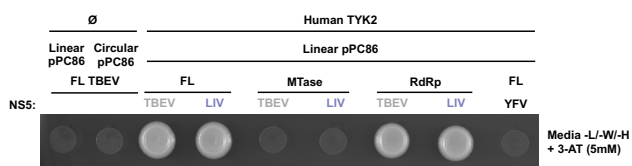
A



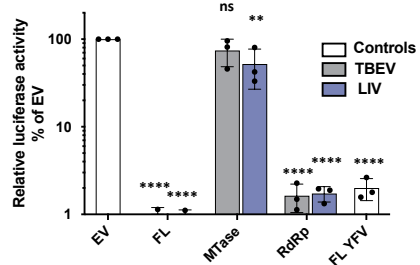
B



C



D



E

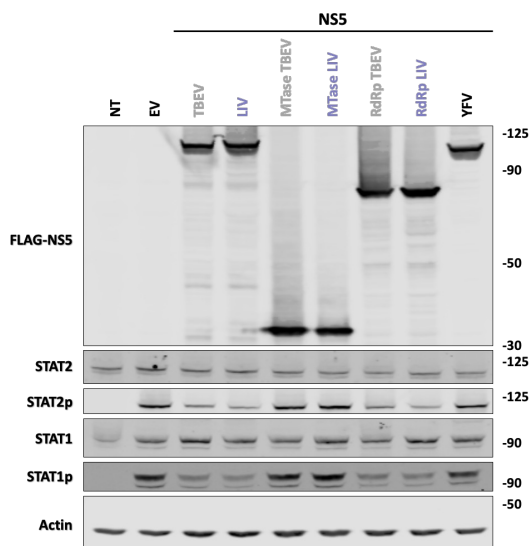


Figure 5

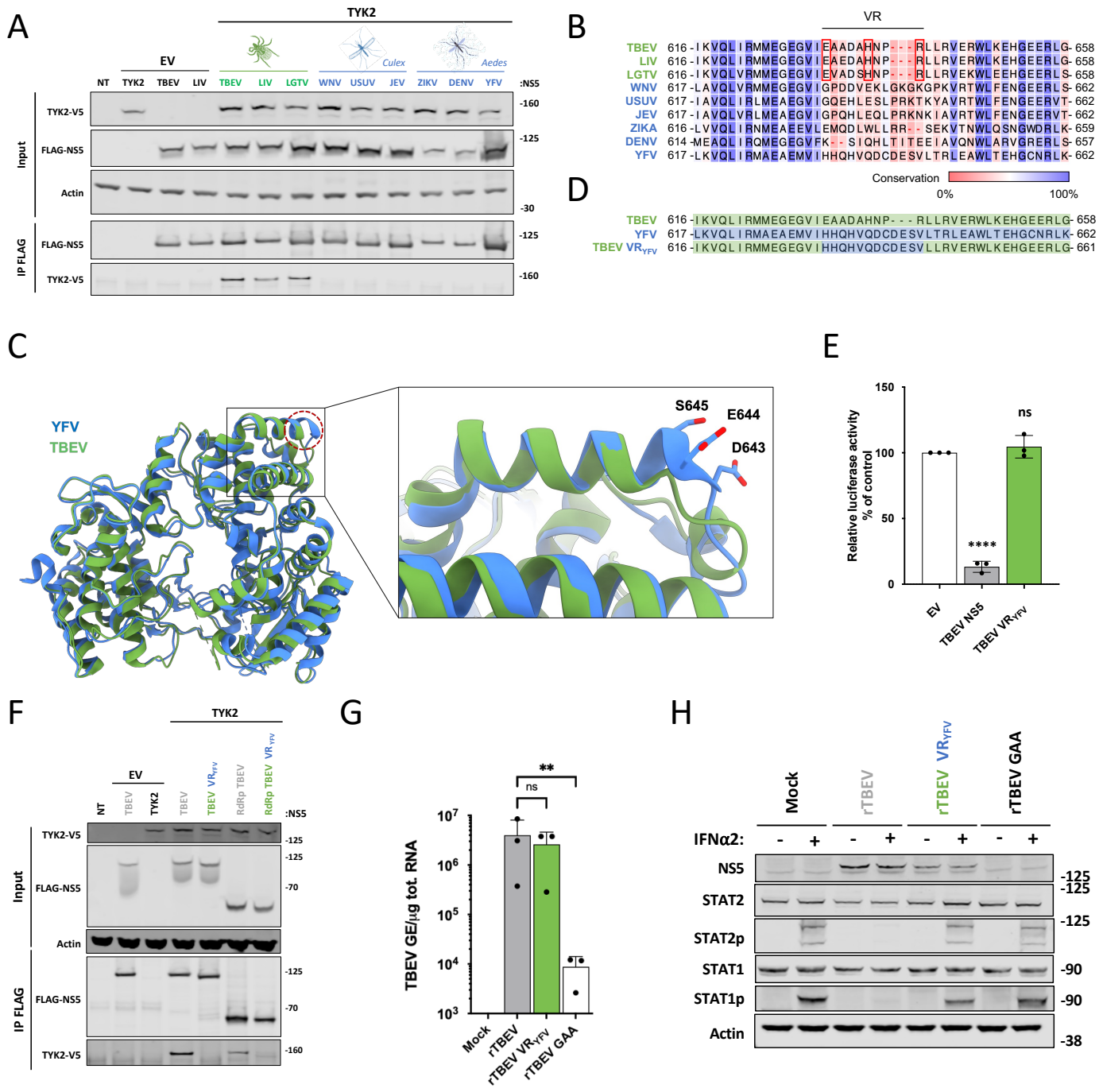


Figure 6

