

1 **TITLE**

2 Short 5' UTRs serve as a marker for viral mRNA translation inhibition by the IFIT2-IFIT3 antiviral
3 complex

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32 **ABSTRACT**

33 Recognition of “non-self” nucleic acids, including cytoplasmic dsDNA, dsRNA, or mRNAs
34 lacking proper 5' cap structures, is critical for the innate immune response to viruses. Here, we
35 demonstrate that short 5' untranslated regions (UTRs), a characteristic of many viral mRNAs,
36 can also serve as a molecular pattern for innate immune recognition via the interferon-induced
37 proteins IFIT2 and IFIT3. The IFIT2-IFIT3 heterodimer, formed through an intricate domain
38 swap structure resolved by cryo-EM, mediates viral mRNA 5' end recognition, translation
39 inhibition, and ultimately antiviral activity. Critically, 5' UTR lengths <50 nucleotides are
40 necessary and sufficient to sensitize an mRNA to translation inhibition by the IFIT2-IFIT3
41 complex. Accordingly, diverse viruses whose mRNAs contain short 5' UTRs, such as vesicular
42 stomatitis virus and parainfluenza virus 3, are sensitive to IFIT2-IFIT3-mediated antiviral activity.
43 Our work thus reveals a pattern of antiviral nucleic acid immune recognition that takes
44 advantage of the inherent constraints on viral genome size.

45

46 **KEYWORDS**

47 Innate antiviral immunity, host-virus interactions, interferon-stimulated genes, viral mRNA
48 translation, non-self RNA recognition, IFIT proteins

49

50 **MAIN**

51 Recognition of foreign RNA is a critical component of immune sensing during viral infection.
52 Interferon (IFN) serves as the first line of defense against viral infection, and its induction is
53 triggered by one of several mechanisms sensing “non-self” RNA or DNA in host cells. IFN
54 signaling induces expression of a number of proteins (e.g., OAS, MDA5, and PKR), which
55 themselves bind to double-stranded RNA (dsRNA) in the cytoplasm to amplify innate immune
56 signaling and activation, promote RNA degradation, or inhibit mRNA translation^{1, 2, 3, 4, 5}.
57 Analogously, dsDNA sensing in the cytoplasm by cGAS and other proteins is essential for
58 activation of IFN responses^{6, 7, 8}. Other RNAs can be recognized as foreign, including RNAs with
59 an uncapped 5' tri- or di-phosphate end by RIG-I^{1, 2, 3}, mRNAs with high CG-dinucleotide content
60 by zinc finger antiviral protein (ZAP/PARP13)⁹, and endosomal single-stranded RNA (ssRNA)
61 by TLR7 and TLR8¹⁰. Notably, some of these patterns are also found on “self” nucleic acids,
62 including dsDNA and CG-dinucleotides. Despite this, these nucleic acid sensing proteins
63 comprise a multifaceted barrier to infection by a wide range of viruses.

64
65 Among the many RNA-sensing innate immune proteins are the IFIT (IFN-induced proteins with
66 tetratricopeptide repeats) proteins, a family of genes that is highly upregulated during viral
67 infection. Vertebrates encode a varying repertoire of IFITs, with humans encoding five IFIT
68 genes (*IFIT1*, *IFIT1B*, *IFIT2*, *IFIT3*, *IFIT5*), and mice encoding six (*IFIT1*, *IFIT1B*, *IFIT1C*, *IFIT2*,
69 *IFIT3*, *IFIT3B*)^{11, 12, 13}. Notably, IFIT1 and IFIT1B proteins recognize “non-self” methylation
70 patterns on the 5' cap structures of viral mRNAs, leading to inhibition of their translation^{13, 14, 15,}
71 ^{16, 17, 18, 19, 20}. In contrast, the role of IFIT2 and IFIT3 in the antiviral response is less clear.

72 Several studies have implicated IFIT2 and IFIT3 in the restriction of viral infection *in vitro* and *in*
73 *vivo*^{11, 21, 22, 23, 24, 25}, and human IFIT3 interacts with and potentiates the effects of human IFIT1²⁶.
74 In other studies, IFIT2 has been shown to have a proviral effect on influenza virus replication²⁷
75 and activate apoptosis^{28, 29, 30}, whereas IFIT3 may abrogate IFIT2-induced apoptosis^{28, 31}. While
76 these studies indicate that IFIT2 and IFIT3 have important roles in the innate immune response,
77 the manner in which IFIT2 and IFIT3 exert direct antiviral effects has remained unclear.

78
79 In this study, we demonstrate that IFIT2 and IFIT3 are necessary and sufficient for antiviral
80 activity against vesicular stomatitis virus (VSV) and parainfluenza virus (PIV3). Using cryo-
81 electron microscopy (cryo-EM), virological experiments, and mRNA translation reporter assays,
82 we demonstrate that IFIT2 and IFIT3 form a stable heterodimeric complex that facilitates
83 recognition and translation inhibition of mRNA from several viruses. We determine that the

84 molecular pattern that leads to IFIT2-IFIT3-mediated inhibition is the presence of short (<50
85 nucleotide (nt)) 5' untranslated regions (5' UTRs), which is a feature of mRNAs from many viral
86 families. Our data elucidate a previously undescribed antiviral role for IFIT2 and IFIT3, and
87 reveal that 5' UTR length is a marker for non-self recognition of viral mRNAs during the innate
88 immune antiviral response.

89

90 RESULTS

91 IFIT2 and IFIT3 combine to exert potent antiviral effects *in vitro*

92 *IFIT2* and *IFIT3* are among the most highly upregulated IFN-stimulated genes (ISGs) following
93 IFN treatment or viral infection³². Studies with knockout mice have shown that IFIT2 and/or
94 IFIT3 are required for antiviral activity against several RNA viruses, including VSV^{11, 22, 23, 24, 25, 33}.
95 Based on these results, we first determined whether IFIT2 and IFIT3 contribute to the antiviral
96 effects of type I IFN against VSV in human cells. As a positive control, we also evaluated IFIT1,
97 which has been shown to have antiviral activity against VSV^{13, 14, 20}. Knockdown of either *Ifit1*,
98 *Ifit2*, or *Ifit3* using gene-specific siRNAs in the A549 human lung epithelial cell line partially
99 rescued viral replication following IFN- α treatment (**Fig. 1a, Extended Data Fig. 1**), suggesting
100 that all three IFITs contribute to – but are not individually the entire driver of – the antiviral effect
101 of type I IFN.

102

103 Human IFIT1, IFIT2, and IFIT3 can interact with and regulate one another^{14, 26, 34, 35, 36}, making it
104 difficult to determine from the above experiments whether IFIT2 and IFIT3 have antiviral activity
105 distinct from IFIT1. To establish whether IFIT2 and IFIT3, alone or in combination, are sufficient
106 for antiviral activity in the absence of an IFIT1 interaction, we took advantage of the observation
107 that mouse IFIT2 and IFIT3 do not interact with IFIT1^{26, 34}. Because HEK293 cells basally
108 express low levels of IFIT2 and IFIT3¹² ([https://www.proteinatlas.org/ENSG00000119922-
109 IFIT2/cell+line](https://www.proteinatlas.org/ENSG00000119922-IFIT2/cell+line); <https://www.proteinatlas.org/ENSG00000119917-IFIT3/cell+line>) and do not
110 mount functional inflammatory responses³⁷, we generated HEK293 cell lines that express
111 mouse IFIT2 and mouse IFIT3 alone or in combination under the inducible control of
112 doxycycline (DOX) (**Extended Data Fig. 1**). Upon DOX induction, we observed no decrease in
113 VSV titers when mouse IFIT2 or IFIT3 was expressed alone (**Fig. 1b**). However, when mouse
114 IFIT2 and IFIT3 were co-expressed in HEK293 cells, we observed a >1,000-fold decrease in
115 VSV titers (**Fig. 1b**). Notably, a significant difference in GFP expression from the viral genome
116 can be observed as early as 4 hours post-infection (**Fig. 1c,d**). Together with our knockdown
117 data, these results indicate that IFIT2 and IFIT3 are both necessary for an effective IFN-

118 mediated antiviral activity against VSV and sufficient to attenuate VSV replication when
119 expressed in the absence of IFN stimulation.

120

121 Several IFITs have been shown to bind RNA and repress mRNA translation^{13, 18, 19, 34}. We
122 therefore evaluated the effect of mouse IFIT2 and IFIT3 expression on VSV-encoded protein
123 levels during infection. At 0, 2, 4, 6, and 8 h post-infection, infected cells were harvested and
124 analyzed by western blotting. Expression of IFIT2 and IFIT3 together, but not alone, prevented
125 detectable accumulation of VSV-G and -N proteins compared to control cells (**Fig. 1e**). These
126 data support a model in which IFIT2 and IFIT3 cooperate during the antiviral response to disrupt
127 an early step of viral infection, resulting in lower viral protein expression.

128

129 **Structure of the mouse IFIT2-IFIT3 heterodimer reveals the basis for antiviral complex** 130 **assembly**

131 Although IFIT2 and IFIT3 were previously shown to form a stable heterodimer³⁴, the structural
132 basis of IFIT2-IFIT3 complex formation had not been characterized. To determine the basis for
133 its antiviral activity and inform structure-guided mutations to disrupt function, we recombinantly
134 expressed and purified a 1:1 complex of mouse IFIT2 and IFIT3 and determined its structure to
135 3.2 Å resolution by cryo-EM (**Supplementary Table 1, Extended Data Fig. 2**).

136

137 IFIT proteins are composed of tandem α -helical tetratricopeptide repeats (TPR) that can fold
138 into superhelical spiral structures^{12, 15, 32, 38}. In the case of monomeric IFIT1 and monomeric
139 IFIT5, the superhelical structure is broken into four domains termed subdomain (SD) I, SD II,
140 Pivot, and SD III¹⁵, where RNA is bound in a conserved cleft in SD II (α -helices 7-14) and the
141 non-TPR Pivot α -helices (α -helices 15-16)¹⁵. In our structure, mouse IFIT2 and IFIT3 also adopt
142 all- α -helical structures, with IFIT2 containing 22 α -helices and IFIT3 containing 19 α -helices
143 (**Fig. 2a,c, Extended Data Fig. 3**). However, in the IFIT2-IFIT3 heterodimer, the two proteins
144 are oriented parallel to one another and associate through a domain swap involving α -helices 7-
145 9 in SD II. The large buried surface area that results from this domain swap ($>4,000 \text{ \AA}^2$ per
146 protomer) provides a molecular explanation for the stability of the IFIT2-IFIT3 complex³⁴, and is
147 similar to the domain topology of an earlier crystal structure of an IFIT2 homodimer³⁸.
148 Importantly, in RNA-bound IFIT1 and IFIT5, α -helices 7-9 form a clamp around the bound 5' end
149 of the mRNA, with the rest of SD II and the Pivot helices forming a deep positively-charged cleft
150 that cradles the mRNA (**Fig. 2d**) whereas in IFIT2-IFIT3, α -helices 7-9 are domain-swapped,
151 such that the equivalent deep clefts in this complex include structural elements from both IFIT2

152 and IFIT3. As a consequence, SD II is shorter in IFIT2 and IFIT3 relative to IFIT1 and IFIT5, and
153 the α -helices C-terminal to SD II (α -helices 10-22 in IFIT2 and α -helices 10-19 in IFIT3) form a
154 single continuous superhelix that comprises SD III (**Fig. 2a-c, Extended Data Fig. 3**).

155

156 To investigate the importance of the domain-swapped SD II for IFIT2-IFIT3 function, we
157 performed evolutionary analyses on IFIT2 and IFIT3 across species. Consistent with the
158 importance of SD II for function, we observed strong signatures of purifying selection acting on
159 many residues in this region of the protein in both rodents (**Fig. 2e, Supplementary Table 2**)
160 and primates (**Extended Data Fig. 4, Supplementary Table 2**). These evolutionary analyses
161 also revealed strong signatures of positive selection acting on several residues in SD I and
162 especially SD III of both IFIT2 and IFIT3 in both rodents (**Fig. 2e, Supplementary Table 2**) and
163 primates (**Extended Data Fig. 4, Supplementary Table 2**). Such signatures of recurrent
164 positive selection are characteristic of host immunity proteins that are engaged in direct
165 evolutionary arms races with viral antagonists^{39, 40, 41, 42} and similarly strong signatures of
166 positive selection has recently been described for IFIT1²⁰. Our evolutionary analyses thus reveal
167 not only the strong conservation of SD II, but also suggest that IFIT2 and IFIT3 are engaged in
168 host-virus arms races as a result of their antiviral activities.

169

170 We next evaluated whether mutations at the heterodimer interface in SD II would alter antiviral
171 function. At the core of the SD II domain swap in the IFIT2-IFIT3 heterodimer is a pair of
172 reciprocal salt bridges (**Fig. 2f**). Wild-type IFIT2 and IFIT3 have glutamic acid residues at amino
173 acid positions 155 and 153, respectively, and lysine residues at positions 158 and 156,
174 respectively. These residues form a pair of E-K salt bridges that we hypothesized are important
175 for complex formation and antiviral activity. To test this idea, we generated single E-to-K
176 mutations in both IFIT2 (E155K) and IFIT3 (E153K). As expected, co-transfection of IFIT2 and
177 IFIT3 single mutant constructs showed less antiviral activity against VSV-GFP than WT IFIT2
178 and IFIT3, consistent with the predicted K-to-K charge clash at the core of the heterodimeric
179 interface (**Fig. 2g**). To corroborate this hypothesis, we restored the predicted salt bridges by
180 introducing a second set of K-to-E mutations. Indeed, when we co-transfected these double
181 mutant proteins (IFIT2 E155K/K158E and IFIT3 E153K/K156E), which are predicted to alleviate
182 the charge clash, we restored the antiviral activity of IFIT2-IFIT3 (**Fig. 2g**). These structural and
183 functional data describe the basis for IFIT2-IFIT3 complex formation and its importance for
184 antiviral activity.

185

186 **The IFIT2-IFIT3 antiviral complex binds VSV mRNAs near the start codons**

187 Several IFITs have been described as having RNA binding capabilities^{13, 18, 19, 34}. In the case of
188 IFIT1 and IFIT1B, specific recognition of viral mRNAs, by nature of their “non-self” methylation
189 patterns, is required for their antiviral activity^{13, 14, 18, 19, 20, 34}. Accordingly, we assessed whether
190 IFIT2 or IFIT3, alone or in combination, interacted with specific regions of VSV RNAs by
191 performing eCLIP (enhanced UV-crosslinking and immunoprecipitation) on cells expressing
192 mouse IFIT2 and/or IFIT3 and infected with VSV.

193

194 We first performed these experiments in the cells ectopically expressing mouse IFIT2 and IFIT3
195 alone or in combination and infected with VSV. When IFIT2 and IFIT3 were expressed together,
196 we observed strong immunoprecipitation of RNA sequences toward the 5' end of the positive
197 (protein-coding) strand of each VSV gene (**Fig. 3a, Extended Data Fig. 5a**). The profile of
198 enriched viral RNA was nearly identical for IFIT2 and IFIT3 when co-expressed, whereas we did
199 not observe such enrichment when IFIT2 or IFIT3 was expressed alone. These data further
200 support the model that the IFIT2-IFIT3 heterodimer acts as a single unit, with both IFITs in close
201 proximity to the viral mRNA. Moreover, we observed the strongest enrichment of sequences
202 cross-linking to IFIT2 and IFIT3 in regions that overlap the 5' UTR and start codons of the VSV
203 mRNA transcripts (**Fig. 3a,b**). These data suggest that the IFIT2-IFIT3 heterodimer associates
204 with VSV transcripts at sequences proximal to the start codons.

205

206 To test whether IFIT2 and IFIT3 associate with the 5' regions of viral mRNAs during an
207 endogenous IFN response, we performed eCLIP experiments in mouse embryonic fibroblasts
208 (MEFs) in which *Ifit2* or *Ifit3a* and *Ifit3b* had been knocked out (**Fig. 3c,d, Extended Data Fig.**
209 **5b**). Consistent with our ectopic expression data, we observed that in WT MEFs induced with
210 IFN, IFIT2 and IFIT3 cross-linked with RNA sequences near the start codons of VSV mRNAs. In
211 contrast, this association was lost when either *Ifit2* or *Ifit3a* and *Ifit3b* were knocked out.
212 Although there are differences between our data with ectopically-expressing cells and IFN-
213 induced cells, we observe a similar overall pattern in IFIT2 and IFIT3 association with the 5'
214 ends of VSV mRNAs and a necessity for both IFIT2 and IFIT3 together during the antiviral
215 response. These data further support that the IFIT2-IFIT3 complex associates with viral mRNAs
216 near their start codons during an innate immune response in infected cells.

217

218 **The IFIT2-IFIT3 complex inhibits translation of mRNAs with short viral 5' UTRs**

219 Both human IFIT1 and mouse IFIT1B inhibit viral mRNA translation by recognizing different
220 methylation patterns on the 5' cap of viral mRNA¹³. In contrast, our eCLIP data indicate that the
221 IFIT2-IFIT3 complex interacts with mRNA downstream of the cap, at regions near the start
222 codon (**Fig. 3b,d**). If a specific cap structure is not required for IFIT2-IFIT3 to recognize viral
223 mRNAs, then we reasoned that viral mRNAs transcribed by the host-encoded RNA polymerase
224 II may also be susceptible to IFIT2-IFIT3 mediated repression. To evaluate this idea, we first
225 cloned the 5' UTR and ORF (open reading frame) of VSV-N, -P, -M, -G, and -L with C-terminal
226 V5 tags into a plasmid that expresses genes from a CMV promoter. We co-transfected these
227 into HEK293T cells in the presence or absence of mouse IFIT2 and IFIT3-expressing plasmids,
228 and then analyzed viral protein expression by western blotting. Despite containing the
229 sequences bound by IFIT2-IFIT3 in our eCLIP experiments, we did not observe changes in viral
230 protein levels in the presence of IFIT2-IFIT3 expression (**Fig. 4a**).

231
232 We next considered features of VSV mRNAs near the start codon that could differentiate them
233 from most host mRNAs. One apparent difference is the vast discrepancy in 5' UTR length
234 between VSV mRNAs, which range from 10 nt to 41 nt, and human mRNAs, which have a
235 median 5' UTR length of 218 nt⁴³. Based on this observation, we hypothesized that 5' UTR
236 length might serve as a molecular pattern for viral mRNA recognition by IFIT2-IFIT3. We first
237 used 5' RACE (Rapid Amplification of cDNA Ends) to determine that the transcription start site
238 (TSS) of our plasmid was 105 nt upstream of where we cloned our viral sequences, resulting in
239 a much longer 5' UTR than natural VSV mRNAs (**Fig. 4a, Extended Data Fig. 6a**). Accordingly,
240 we removed this extraneous sequence between the TSS and our viral 5' UTR, leaving only 3 nt
241 upstream of the viral 5' UTR that provided a consistent TSS (**Fig. 4b, Extended Data Fig. 6b**).
242 With this construct, we now observed a striking reduction in VSV-N, -P, -G, and -L protein
243 expression when IFIT2-IFIT3 was expressed (VSV-M was excluded as it failed to express when
244 transfected, independent of IFIT2-IFIT3) (**Fig. 4b**). These data, which recapitulate the
245 decreased protein expression during viral infection when IFIT2-IFIT3 is expressed (**Fig. 1d**),
246 suggest that 5' UTR length, not 5' cap structure, determines the sensitivity of an mRNA to IFIT2-
247 IFIT3-mediated inhibition.

248
249 To facilitate more quantitative and higher throughput experiments, we established a
250 fluorescence-based reporter assay. Because VSV-P has the shortest of the VSV 5' UTRs (10
251 nt), we cloned eGFP as a C-terminal fusion to VSV-P in our “long” (105 nt plasmid derived UTR
252 followed by 10 nt VSV-P 5' UTR) and “short” (3 nt followed by 10 nt VSV-P 5' UTR alone)

253 plasmids (**Fig. 4c**). Consistent with our western blotting data (**Fig. 4a,b, Extended Data Fig.**
254 **6c**), GFP fluorescence signal was decreased only when it was expressed with a “short” 5' UTR
255 and IFIT2-IFIT3 was co-expressed (**Fig. 4d, Extended Data Fig. 6d**). We quantified a 10-fold
256 decrease in normalized GFP intensity with the short but not the long 5' UTR construct in cells in
257 which IFIT2-IFIT3 was co-expressed (**Fig. 4e, Extended Data Fig. 6d**).

258

259 Using this reporter assay, we determined whether any part of the VSV-P ORF contributes to the
260 sensitivity to IFIT2-IFIT3. We truncated the VSV-P ORF to the N-terminal 16 or 6 codons, or
261 removed the entire VSV-P coding sequence, leaving only the 10-nt VSV-P 5' UTR upstream of
262 the eGFP-V5 ORF (**Fig. 4f**). These three transcripts were all sensitive to IFIT2-IFIT3 mediated
263 inhibition (**Fig. 4g**), indicating that only the short viral 5' UTR is required to sensitize an ORF to
264 repression. Importantly, GFP became insensitive to IFIT2-IFIT3 expression when the 10-nt
265 VSV-P 5' UTR was lengthened to include the original 105-nt plasmid-derived UTR (**Extended**
266 **Data Fig. 6e**), and neither IFIT2 nor IFIT3 alone inhibited translation of the VSV-P 5' UTR alone
267 construct (**Extended Data Fig. 6f**). To confirm that the observed effects on protein expression
268 were due to mRNA translation inhibition rather than mRNA degradation, we demonstrated that
269 IFIT2-IFIT3 expression had no negative effect on levels of the either “short” or “long” mRNA
270 transcripts (**Extended Data Fig. 6g**). Additionally, though the 5' UTRs for all five VSV genes
271 share a conserved 5' end sequence (AACAG)⁴⁴, they differ in length and downstream
272 sequence. We therefore confirmed that the 5' UTRs from VSV-N, -M, -G, and -L are all sensitive
273 in our reporter system as well (**Extended Data Fig. 6h**), further demonstrating that multiple viral
274 5' UTRs are susceptible to IFIT2-IFIT3-mediated mRNA translation inhibition.

275

276 These data show that 5' UTRs below a certain length become sensitive to IFIT2-IFIT3 inhibition.
277 To identify the 5' UTR threshold length determinants of IFIT2-IFIT3-driven translation inhibition,
278 we progressively added length to the sensitive 10 nt VSV-P UTR, increasing it back to the
279 complete plasmid-derived 105 nt (**Fig. 4h**). Using this approach, we demonstrated that the
280 strongest IFIT2-IFIT3-mediated translation inhibition occurs when the total length of the 5' UTR
281 is less than 50 nt (**Fig. 4i**).

282

283 **IFIT2-IFIT3 has broad antiviral activity driven by the length of 5' UTRs**

284 The data above suggests that a 5' UTR that is <50 nt in length can serve as a molecular pattern
285 that sensitizes an mRNA to an IFIT2-IFIT3 complex. Based on this idea, we hypothesized that
286 mRNA from other viruses with short 5' UTRs would be sensitive to IFIT2-IFIT3 antiviral activity.

287 We first cloned the 5' UTRs from rabies virus (RABV) into our reporter construct, as RABV is in
288 the same *Rhabdoviridae* family as VSV. All RABV 5' UTRs are <50 nt, ranging from 12-30 nt,
289 and we found that all RABV transcripts are sensitive to IFIT2-IFIT3 mediated inhibition (**Fig. 5a**).
290 These data are consistent with prior work showing that RABV is more pathogenic in mice in
291 which either *Ifit2* or *Ifit3* is knocked out^{21, 23}.

292
293 To extend our studies, we tested another non-segmented negative-sense RNA virus with
294 several short 5' UTRs, human parainfluenza virus 3 (PIV3). PIV3 encodes two mRNAs with 5'
295 UTRs that fall under the 50 nt length threshold (gp5, 32 nt; gp8, 22 nt). When these were tested
296 in our reporter assay, we found that both transcripts were sensitive to IFIT2-IFIT3-mediated
297 inhibition (**Fig. 5b**). We therefore hypothesized that PIV3 would be sensitive to IFIT2-IFIT3
298 expression during infection. As with VSV (**Fig. 1b**), neither IFIT2 nor IFIT3 alone exerted an
299 antiviral effect on PIV3, but their co-expression significantly inhibited infection (**Fig. 5c**). Also, as
300 with VSV (**Fig. 1a**), knockdown of *Ifit2* and *Ifit3* expression in human A549 cells blunted the
301 antiviral effects of type I IFN on PIV3 replication (**Fig. 5d**). Together, our data on VSV, RABV,
302 and PIV3 indicate that IFIT2-IFIT3 co-expression confers inhibitory activity against diverse
303 viruses that encode mRNAs with 5' UTRs shorter than 50 nt in length.

304
305 Finally, we evaluated whether a virus with a long 5' UTR would be insensitive to the antiviral
306 effects of IFIT2 and IFIT3. We cloned the single 742-nt 5' UTR of coxsackievirus B3 (CVB3)
307 upstream of eGFP and tested it for sensitivity to IFIT2-IFIT3-mediated translation repression.
308 Indeed, the CVB3 5' UTR was insensitive to IFIT2-IFIT3 expression, with reporter levels actually
309 increasing (**Fig. 5e**), suggesting that IFIT2-IFIT3 may be proviral in some contexts as previously
310 shown by Tran et al.²⁷. Moreover, as predicted from our reporter system data, ectopic
311 expression of IFIT2 and IFIT3 did not inhibit CVB3 infection (**Fig. 5f**). These data suggest that
312 viruses with long 5' UTRs may evade recognition and translation inhibition by the IFIT2-IFIT3
313 complex.

314 315 **DISCUSSION**

316 Recognition of non-self viral mRNA is critical to controlling infection. Host cells must sense and
317 distinguish viral mRNAs to prevent their translation and subsequent viral replication and
318 formation of virions. Here, we show that the ISGs IFIT2 and IFIT3 form a heterodimeric complex
319 capable of exerting a potent antiviral effect against VSV (*Rhabdoviridae*) and PIV3

320 (*Paramyxoviridae*), and that this activity is driven by recognition and translation inhibition of viral
321 mRNAs containing short 5' UTRs.

322

323 RNA viruses have an average genome length of ~9 kilobases⁴⁵. With such limited genetic
324 space, many viruses – especially small non-segmented negative strand RNA viruses, encode
325 short 5' UTRs, presumably to maximize nucleotides available for protein coding sequences.
326 Though RNA viruses have high rates of mutation and can undergo rapid change to genome
327 sequences^{46, 47}, the length of viral 5' UTRs is likely constrained due to constraints on overall
328 genome size⁴⁶, enabling IFIT2 and IFIT3 to have evolved recognition of this feature as a
329 hallmark of RNA virus infection. Indeed, nearly every non-segmented negative-sense RNA virus
330 that infects humans contains at least one mRNA with a 5' UTR <50 nt, and our data with three
331 of these viruses (PIV3, VSV, and RABV) suggest IFIT2-IFIT3 may be a broad restriction
332 mechanism against them. In comparison, viruses in the henipavirus genus, including Nipah and
333 Hendra viruses, are unique among the *Paramyxoviridae* in not having 5' UTRs that are <50 nt
334 (ranging from 57 nt to 284 nt). This observation, in addition to our data indicating that the long 5'
335 UTR of CVB3 and CVB3 infection are insensitive to IFIT2-IFIT3 expression, indicate that
336 evolving longer viral 5' UTRs to mimic the length of host 5' UTRs is one potential viral escape
337 mechanism. Combined with studies showing that viruses can evolve RNA secondary structures,
338 host-mimicking RNA methylation machinery, and additional mechanisms to evade IFIT1^{16, 20},
339 these results suggest that IFITs may have had a profound effect on the evolution of the 5' ends
340 of viruses. Additionally, viruses likely have developed additional strategies to evade translation
341 inhibition by IFIT2-IFIT3. As has been shown with IFIT1²⁰, we find that primate and rodent IFIT2
342 and IFIT3 have evolved under strong recurrent positive selection, especially in SD III. These
343 evolutionary data mirror the strong signatures of positive selection described for other antiviral
344 ISGs that are targeted by viral antagonists^{16, 20}, and indicate that some viruses that otherwise
345 might be sensitive to IFIT2-IFIT3-mediated repression likely encode species-specific IFIT
346 antagonists.

347

348 The precise mechanism by which the IFIT2-IFIT3 complex selectively inhibits translation of
349 mRNAs with short 5' UTRs requires further study. Previous models based on ribosome
350 structures and functional data have described a “blind spot” of 40-50 nt at the very 5' end of
351 human mRNAs, where efficient translation initiation occurs at the first start codon downstream
352 of the blind spot⁴⁸. That viruses with short 5' UTR-containing mRNAs are able to efficiently
353 translate their proteins during infection suggests that there are exceptions to the blind spot

354 model. As such, viruses that express mRNAs with short 5' UTRs likely co-opt non-canonical
355 translational pathways. Indeed, VSV translation initiation is strongly dependent on the large
356 ribosomal subunit protein, RPL40 (eL40), whereas bulk cellular translation is not⁴⁹. Likewise,
357 some host mRNAs contain short 5' UTRs, several of which contain a specific sequence known
358 as a TISU (Translation Initiator of Short 5' UTR)^{50, 51}, which rely on the small ribosomal subunit
359 proteins RPS3 and RPS10⁵², and the initiation factor eIF1 for efficient translation^{52, 53}. It is likely
360 that other non-canonical translation mechanisms exist as well. The differential requirements for
361 efficient translation of host mRNAs with short 5' UTRs leads us to hypothesize that IFIT2 and
362 IFIT3 might selectively inhibit a specialized translation pathway, although further work will be
363 required to understand this mechanism completely.

364

365 In summary, our study identifies short mRNA 5' UTRs as a molecular pattern that at least some
366 mammalian hosts can use to selectively inhibit viral replication. This pattern is present on a wide
367 range of viral mRNAs and is sufficient to sensitize an mRNA to translation inhibition by the
368 IFIT2-IFIT3 antiviral complex. Thus, 5' UTR length is another point of conflict in the battle
369 between viruses and hosts for mRNA translation control.

370

371 **METHODS**

372 **Cell culture and transient transfection**

373 HEK293T cells were obtained from ATCC (catalog # CRL-3216) and grown in complete medium
374 containing DMEM (Gibco, Carlsbad, CA), 10% FBS, 100 U/mL penicillin, and 100 µg/mL
375 streptomycin (Gibco, Carlsbad, CA). For transient transfections, HEK293T cells were seeded
376 the day prior to transfection in a 24-well plate (Genesee, El Cajon, CA) with 500 µL of complete
377 media. Cells were transiently transfected with 500 ng of total DNA and 1.5 µL of TransIT-X2
378 (Mirus Bio, Madison, WI) following the manufacturer's protocol.

379

380 For generation of inducible cell lines, sequences for mCherry, IFIT2, IFIT3, or IFIT2 and IFIT3
381 separated by a P2A site were cloned into the Flp-In vector pcDNA5/FRT/TO. Flp-In T-REx
382 HEK293 cells (Invitrogen) maintained in 5 µg/mL of blasticidin were transfected at 70%
383 confluency with mCherry or IFIT constructs and the vector containing the Flp recombinase
384 pOG44 in a 1:10 molar ratio using TransIT-X2 (Mirus Bio). After one day, cells were transferred
385 to new dishes, and on the following day, hygromycin (100 µg/mL) was added to cells. Following
386 selection, cells were maintained in 5 µg/mL of blasticidin and 100 µg/mL of hygromycin. For
387 induction of mCherry or IFIT proteins, cells were treated with 500 ng/mL of doxycycline for 24 h.

388

389 MEFs from *Ifit2*^{-/-11} and Δ *Ifit3a/b* mice were prepared from day 13.5-14.5 embryos according to
390 published protocols⁵⁴. Isolated MEFs were maintained in DMEM supplemented with 10% heat-
391 inactivated, fetal bovine serum (FBS) (Cytiva), 100 U/mL penicillin-streptomycin (Invitrogen),
392 non-essential amino acids (Cellgro), and Glutamax (Gibco). Passage 0 (P0 MEFs were frozen
393 or split 1:4 when approximately 80% confluent (around 3 days). To generate transformed MEFs,
394 5×10^6 P1 primary MEFs were transfected with 10 μ g of a plasmid (SV2)⁵⁵ encoding the SV40 T
395 antigen under control of a CMV promoter, using FuGene reagent (Promega) (3:1 μ L FuGene to
396 μ g DNA ratio). Upon achieving confluence, MEF cultures were split 1:10. This process was
397 repeated for approximately ten passages, at which time the transformed MEFs were frozen or
398 used for experiments.

399

400 **IFIT siRNA knockdowns**

401 Specific siRNAs against *ifit1* (Integrated DNA Technologies; 5'-
402 UAGACGAACCCAAGGAGGCUCAAGCUU -3'), *ifit2* (Horizon Discovery, cat. # M-012582-01-
403 0050), and *ifit3* (Integrated DNA Technologies, TriFECTa RNAi Kit - hs.Ri.IFIT3.13.1) were
404 obtained from their respective manufacturers. A549 cells were seeded into 24-well plates.
405 Twenty-four hours after seeding, cells were transfected with 20 pmol of siRNA in Lipofectamine
406 2000 Transfection Reagent (Invitrogen) and allowed to incubate for 24-hours before being used
407 in subsequent infection experiments or being harvested for western blot to validate knockdown
408 efficiency.

409

410 **Viral stocks and infections**

411 VSV-GFP was a generous gift of Dr. John Rose⁵⁶ (Yale University) and was propagated in BHK
412 cells. For siRNA experiments, siRNA-treated A549 cells in 24-well plates were induced with 500
413 U/mL of IFN- α for 24 hours. Cells were then infected at a multiplicity of infection (MOI) of 0.01
414 for 16 h before harvesting, and virus was quantified by plaque assay. For ectopic
415 overexpression experiments, Flp-In T-REx HEK293 cells in 24-well plates were induced with
416 doxycycline for 24 h. Cells were then infected at an MOI of 0.01 for 16 h before harvesting, and
417 virus was quantified by plaque assay. For high MOI experiments, Flp-In 293 cells in 96-well
418 (imaging) or 24-well (western blotting) plates were infected at an MOI of 3.0, and cells were
419 harvested or plates were imaged at 0, 2-, 4-, 6-, and 8-hours post-infection. For evaluation of
420 structure guided mutations, 293T cells cultured in 24-well plates were transfected 6 h post-

421 seeding with IFIT constructs. Eighteen hours post-transfection, cells were infected at an MOI of
422 0.05 for 16 h before imaging.

423

424 PIV3-GFP was obtained from Dr. Benhur Lee (Icahn School of Medicine at Mount Sinai)⁵⁷. For
425 siRNA experiments, siRNA-treated A549 cells in 24-well plates were induced with 500 U/mL of
426 IFN- α for 24 h. Cells were then infected at an MOI of 0.01, and supernatant was harvested 40
427 hpi. Virus was quantified by TCID₅₀ analysis. For ectopic expression experiments, Flp-In T-REx
428 HEK293 cells in 24-well plates were induced with doxycycline for 24 h before being infected at
429 an MOI of 0.1 for 48 h. Supernatant was then harvested, and virus was quantified by TCID₅₀.

430

431 CVB3 stocks were generated by co-transfection of CVB3-Nancy infectious clone plasmids with
432 a plasmid expressing T7 RNA polymerase (generous gifts of Dr. Julie Pfeiffer, University of
433 Texas Southwestern Medical Center) as previously described⁵⁸. For ectopic overexpression
434 experiments, Flp-In T-REx HEK293 cells in 24-well plates were induced with doxycycline for 24
435 h before being infected at an MOI of 0.1. Supernatant was harvested 40 hpi and quantified by
436 plaque assay.

437

438 **Western blotting and antibodies**

439 Twenty-four hours post-transfection, cells were resuspended in supernatant and harvested,
440 followed by centrifugation for 5 minutes at 2500 RPM. Cell pellets were washed with 1X PBS
441 and lysed with 1X Bolt LDS Sample Buffer (Life Technologies, San Diego, CA) containing 5% β -
442 mercaptoethanol at 98°C for 7 min. The lysed samples were centrifuged at 15,000 RPM for 2
443 minutes, followed by loading into a 4-12% Bolt Bis-Tris Plus Mini Protein Gels (Life
444 Technologies, San Diego, CA) with 1X Bolt MOPS SDS Running Buffer (Life Technologies, San
445 Diego, CA). Following electrophoresis, gels were wet transferred onto nitrocellulose membranes
446 using a Mini Blot Module (Life Technologies, San Diego, CA). Membranes were blocked with
447 PBS-T containing 5% bovine serum albumin (BSA) (Spectrum, New Brunswick, NJ), followed by
448 incubation with primary antibodies (1:1000) for VSV-G [8G5F11] and VSV-N [10G4] (Kerafast),
449 V5 [D3H8Q] (Cell Signaling Technology), or GAPDH [14C10] (Cell Signaling Technology).
450 Membranes were rinsed three times in PBS-T and then incubated with the appropriate HRP-
451 conjugated secondary antibodies (1:10000; Bio-Rad). Membranes were rinsed again three
452 times in PBS-T and developed with SuperSignal West Pico PLUS Chemiluminescent Substrate
453 (Thermo Fisher Scientific, Carlsbad, CA). Blots were imaged on a Bio-Rad ChemiDoc MP using
454 the Image Lab Software suite (Bio-Rad).

455

456 **Plasmids, constructs, and molecular cloning**

457 The coding sequences of mouse IFIT2 (NCBI accession BC050835) and mouse IFIT3 (NCBI
458 accession BC089563) were cloned separately into the pcDNA5/FRT/TO backbone (Invitrogen,
459 Carlsbad, CA) with an N-terminal 3xFLAG tag or the pQCXIP backbone (Takara Bio, Mountain
460 View, CA) with an N-terminal HA tag, respectively, and both were cloned into the
461 pcDNA5/FRT/TO backbone (an N-terminal HA tag, followed by IFIT3, a p2a site, a 3xFLAG tag,
462 and IFIT2). IFIT2 and IFIT3 point mutants were generated using overlapping stitch PCR and
463 cloned into their respective backbones. The 5' UTR and coding sequence for each VSV gene
464 (GenBank accession number NC_038236.1) was cloned into the pQCXIP backbone with a C-
465 terminal V5 tag. Following 5' RACE, "short" 5' UTR constructs were generated by truncating the
466 pQCXIP 5' UTR sequence. The "long" and "short" backbone constructs expressing VSV-P-V5
467 were used to further clone the VSV-P fluorescence reporter plasmids by subcloning GFP in
468 between the VSV-P 5' UTR and V5 tag. The mCherry normalization construct was generated by
469 subcloning mCherry and a C-terminal 3xFLAG tag into the pcDNA5/FRT/TO backbone. All
470 additional reporter constructs (including VSV-P truncations, UTR length constructs, and viral 5'
471 UTRs [RABV (NCBI accession NC_001542)]; [PIV3 (NCBI accession NC_001796)]; [CVB3
472 (NCBI accession NC_038307)]) were cloned using primers and inserted into the "short" UTR
473 reporter backbone upstream of GFP-V5. All generated plasmids were sequenced across the
474 entire inserted region to verify that no mutations were introduced during the cloning process.
475 Plasmids and primers used in this study can be found in **Supplementary Table 3**. Gene
476 Fragments were ordered from Twist Bioscience (South San Francisco, CA). All newly created
477 plasmids will be made available upon request.

478

479 **Protein expression and purification**

480 For expression of the IFIT2-IFIT3 complex, we cloned codon-optimized genes encoding *M.*
481 *musculus Ifit2* and *Ifit3* into separate plasmid vectors for expression in *E. coli*, with *Ifit2* cloned
482 into UC Berkeley Macrolab vector 2-BT (Addgene #29666; ampicillin resistant) encoding a TEV
483 protease-cleavable His6-tag, and *Ifit3* cloned into UC Berkeley Macrolab vector 13S-A
484 (Addgene #48323; spectinomycin resistant) with no tag.

485

486 Plasmids were co-transformed into *E. coli* Rosetta pLysS cells (EMD Millipore) and grown
487 overnight at 37°C in LB plus carbenicillin and spectinomycin. Saturated overnight cultures were
488 used to inoculate six 1 L cultures of 2XYT media plus carbenicillin and spectinomycin, and

489 cultures were grown at 37°C with shaking at 180 RPM to an OD600 of 0.8. Protein expression
490 was induced by the addition of 0.25 mM IPTG, then cultures were shifted to 20°C and grown
491 another 16 h with shaking. Cells were harvested by centrifugation and resuspended in Nickel
492 Wash Buffer (20 mM Tris-HCl pH 7.5, 500 mM NaCl, 20 mM imidazole pH 8.0, 2 mM beta-
493 mercaptoethanol, and 10% glycerol).

494

495 For protein purification, resuspended cells were lysed by sonication (Branson Sonifier), then cell
496 debris was removed by centrifugation at 14,000 RPM in a JA-17 rotor in an Avanti J-E
497 centrifuge (Beckman Coulter) for 30 min. Clarified lysate was passed over a nickel column (5
498 mL HisTrap HP, Cytiva) in Nickel Wash Buffer, then bound protein was eluted with Nickel
499 Elution Buffer (20 mM Tris-HCl pH 7.5, 75 mM NaCl, 250 mM imidazole pH 8.0, 2 mM beta-
500 mercaptoethanol, and 10% glycerol). Eluted protein was concentrated and buffer-exchanged
501 into Nickel Elution Buffer containing 20 mM imidazole (Amicon Ultra, EMD Millipore), and the
502 His₆-tag on IFIT2 was cleaved by addition of 1:10 w/w ratio of purified TEV protease (S219V
503 mutant, purified in-house from expression vector pRK793; AddGene #8827)⁵⁹, followed by
504 incubation at 4°C for 48 h. The reaction mixture was passed over a nickel column to remove
505 cleaved His₆-tags, uncleaved IFIT2, and His₆-tagged TEV protease. The flow-through was
506 concentrated, then passed over a size exclusion column (Superdex 200 Increase, Cytiva) in
507 Size Exclusion Buffer (20 mM Tris-HCl pH 7.5, 150 mM NaCl, and 1 mM DTT) and fractions
508 containing both proteins were pooled and concentrated.

509

510 **Cryo-EM grid preparation**

511 Prior to use, UltrAuFoil 1.2/1.3 300 mesh grids were plasma cleaned for 12 sec using a Solarus
512 II plasma cleaner (Gatan). Purified IFIT2-IFIT3 at 3 mg/mL was applied to the grid in a 3 µL drop
513 within the environmental chamber adjusted to 4°C temperature and approximately 95% humidity
514 in a Vitrobot Mark IV (ThermoFisher Scientific). After a 4 sec incubation, the grids were blotted
515 with a blot force of 4 for 4 sec; the sample was then plunged frozen into liquid nitrogen-cooled
516 liquid ethane.

517

518 **Cryo-EM data acquisition and image processing**

519 All data was acquired at the UCSD Cryo-EM Facility on a Titan Krios G3 electron microscope
520 (Thermo Fisher Scientific) operating at 300 kV and equipped with a Gatan BioContinuum energy
521 filter. All images were collected at a nominal magnification of 165,000x in EF-TEM mode (with a
522 calibrated pixel size of 0.854 Å) on a Gatan K2 detector using a 20-eV slit width and a

523 cumulative electron exposure of ~ 65 electrons/ \AA^2 over 50 frames (**Supplementary Table 1**).
524 Data were collected automatically using EPU (Thermo Fisher Scientific) with aberration-free
525 image shift with a defocus range of -1 to -2.5 μm . Data collection was monitored live using
526 cryoSPARC Live (Structura Bio)⁶⁰ where movies were patch motion-corrected and patch CTF-
527 estimated on the fly. Micrographs with a CTF estimation worse than 7 \AA and/or a cumulative
528 motion of more than 150 pixels were discarded.

529
530 An initial 484,841 particle picks were obtained using crYOLO⁶¹ picker using a general model,
531 and these picks were imported into cryoSPARC⁶⁰. Particles were extracted with a box size of
532 384 pixels and Fourier-cropped to 96 pixels at (3.34 $\text{\AA}/\text{pixel}$). These particles were subjected to
533 three rounds of two-dimensional (2D) classification, where classes with proteinaceous features
534 were chosen to move forward. The selected particles were subjected to an *ab initio*
535 reconstruction to generate a starting model and carried forward to a non-uniform (NU)
536 refinement using C1 symmetry. These particles were then re-extracted at a box size of 384
537 pixels with a Fourier crop to 128 pixels (1.708 $\text{\AA}/\text{pixel}$) and a second NU refinement was
538 performed. The particle stack was then subjected two rounds of a two-class heterogeneous
539 refinement with one volume being the volume from the previous NU refinement, and the other
540 volume from EMD-4877 (20S proteasome)⁶², followed by NU refinement. In each round, the
541 particles that contributed to the best volume that resembled the IFIT2-IFIT3 dimer were
542 selected. A final 3-class heterogenous refinement was performed using two IFIT2-IFIT3
543 volumes and a 20S proteasome volume. The particles associated with the volume that showed
544 the best secondary structure features was selected, and NU refinement was performed,
545 resulting in a 3.51 \AA resolution map. These particles were then re-extracted at a box size of 384
546 pixels with no Fourier cropping (0.854 $\text{\AA}/\text{pixel}$). These particles were then NU refined, resulting in
547 a 3.57 \AA resolution map. Due to heterogeneity that was observed in the map, particles were
548 then exported from cryoSPARC into RELION⁶³, where they were extracted at a box size of 256
549 with a Fourier crop of 64 (3.34 $\text{\AA}/\text{pixel}$). These particles were subjected to a round of 2D
550 classification in which obvious junk classes were discarded. Selected particles were then re-
551 extracted at a box size of 384 (0.854 $\text{\AA}/\text{pixel}$) and subjected to 3D auto refinement, CTF
552 refinement, and a second 3D auto refinement⁶⁴. The particles were then Bayesian particle
553 polished⁶⁵ before another round of two 3D auto refinements and a CTF refinement. This final
554 particle stack was then imported back into cryoSPARC for a final NU refinement that resulted in
555 a 3.22 \AA resolution map (**Supplementary Table 1**).

556

557 To generate a starting model, we used ModelAngelo⁶⁶ and supplied our final 3.22Å map and
558 sequence files for IFIT2 and IFIT3. This resulting model was then iteratively real-space refined
559 using Phenix⁶⁷ and manually adjusted in COOT⁶⁸. After the final refinement, the model was
560 checked for accuracy in COOT (**Supplementary Table 1**).

561

562 **Evolutionary analyses**

563 For evolutionary analyses of primate and rodent IFIT2 and IFIT3, Uniprot reference protein
564 sequences for human IFIT2, human IFIT3, mouse IFIT2, and mouse IFIT3 were used as a
565 search query against NCBI's non-redundant (NR) database using tBLASTn⁶⁹. Searches were
566 restricted to simian primates and the Muroidea superfamily of rodents respectively. For each
567 species, the nt sequence with the highest bit score was downloaded and aligned to the human
568 or mouse ORF nt sequence using MAFFT⁷⁰ implemented in Geneious software (Dotmatics;
569 geneious.com). Poorly aligning sequences or regions were removed from subsequent analyses.
570 Accession numbers of final sequences used for analyses are in **Supplementary Table 2**. Using
571 these aligned sequences, FUBAR⁷¹ was performed on Datamonkey.org using 50 grid points and
572 a 0.5 concentration parameter of the Dirichlet prior to infer codons evolving under positive and
573 negative selection. Codons with a posterior probability of 0.9 or higher are in **Supplementary**
574 **Table 2**. PAML⁷² was used to infer gene-wide positive selection, as well as codon-based
575 estimates of positive selection. Aligned sequences were analyzed using the NS sites models
576 disallowing (M7) or allowing (M8) positive selection. The p-value reported is the result of a chi-
577 squared test on twice the difference of the log likelihood (lnL) values between the two models
578 using two degrees of freedom. Analyses were performed using two models of frequency (F61
579 and F3x4) and both sets of values are reported in **Supplementary Table 2**. For each codon
580 model, we confirmed convergence of lnL values by performing each analysis using two starting
581 omega (dN/dS) values (0.4 and 1.5). Positively selected codons with a posterior probability
582 greater than 0.90 using a Bayes Empirical Bayes (BEB) analysis and the F61 codon frequency
583 model are in **Supplementary Table 2**.

584

585 **eCLIP experimental methods**

586 Flp-In T-REx HEK293 cells in 10 cm culture dishes were induced with doxycycline for 24 h.
587 Cells were then infected at an MOI of 3.0, and dishes were crosslinked using a UV cross-linker
588 (254 nm; CL-1000 from UVP/Analytik Jena) at 400 mJ/cm². Downstream sample processing
589 and eCLIP were performed as previously described⁷³, using antibodies against FLAG (M2 /
590 F1804, Sigma) and HA (HA.11 / 901502, Biolegend) for 293T experiments, and IFIT2 (PA3-845,

591 ThermoFisher) and IFIT3 (ABF1048, Millipore) for MEF experiments. Most experiments were
592 performed in biological duplicate, excepting the uninfected 293T samples (which were single
593 replicates).

594

595 **eCLIP computational analysis**

596 Standard processing of eCLIP data was performed as previously described⁷³, with mapping
597 performed to a custom genome index that included both the VSV genome and either hg19 (for
598 293T experiments) or mm10 (for MEF experiments). Data (**Fig. 3, Extended Data Fig. 5**) are
599 plotted as normalized reads per million (RPM), where reads per million are normalized to
600 density of reads mapped to viral and human genomes.

601

602 **Animal Ethics statement and generation of *Ifit3a/b* DKO mice**

603 Experiments was carried out in accordance with the recommendations in the Guide for the Care
604 and Use of Laboratory Animals of the National Institutes of Health. The protocols were approved
605 by the Institutional Animal Care and Use Committee at the Washington University School of
606 Medicine (Assurance number A3381-01). Wild-type C57BL/6J were commercially obtained from
607 Jackson Laboratories (Strain #000664; Bar Harbor, ME). To generate *Ifit3* deficient mice, single
608 guide RNAs (sgRNAs) were designed to target exon two in *Ifit3a* and *Ifit3b*. Two sgRNAs were
609 chosen that target conserved sequences between *Ifit3a* and *Ifit3b*: sgRNA-4, 5'-
610 ATTTACCTGGAATTTATTCNGG-3', and sgRNA-30, 5'- AATGGCACTTCAGCTGTGGANGG
611 -3'. Two additional sgRNAs were chosen that target only *Ifit3a* due to polymorphisms: sg-RNA-
612 21, 5'- AATTCGTCGACTGGTCACCTNGG -3', and sgRNA-22, 5'-
613 ATTCGTCGACTGGTCACCTGNGG -3'. The sgRNAs were selected based on their low off-
614 target profile. Guide RNAs and Cas9 protein were complexed and electroporated concurrently
615 into C57BL/6J zygotes. Using this approach, we identified a mouse that had both *Ifit3a* and
616 *Ifit3b* targeted (22 nucleotide (nt) and 20 nt frameshift deletions, respectively), two mice in which
617 only *Ifit3a* was targeted (2 nt and 119 nt frameshift deletions), and two mice in which only *Ifit3b*
618 was targeted (1 nt and 2 nt frameshift insertions). After genotyping and two rounds of
619 backcrossing, five founder lines (*Ifit3a* del22/*Ifit3b* del20; *Ifit3a* del2; *Ifit3a* del119; *Ifit3b* ins1;
620 and *Ifit3b* ins2) were generated. The generation of gene-edited mice was accomplished with the
621 aid of the Genome Engineering and iPSC center and Department of Pathology Micro-Injection
622 Core (Washington University School of Medicine).

623

624 **5' RACE (Rapid Amplification of cDNA Ends)**

625 HEK293T cells were maintained as described above and subcultured into 6-well plates
626 (Genesee, El Cajon, CA) in 2 mL of complete media 24 h before transfection. Cells were
627 transiently transfected with 2500 ng of total DNA and 7.5 μ L of TransIT-X2 (Mirus Bio, Madison,
628 WI) following the manufacturer's protocol. 24 hours post-transfection, cells were harvested and
629 pelleted; cell pellets were washed with 1x PBS, pelleted again, and supernatant was aspirated.
630 RNA was extracted from pellets using the Takara Bio NucleoSpin RNA Plus kit following the
631 manufacturer's protocol. Downstream processing of RNA was performed using the Takara Bio
632 SMARTer RACE 5'/3' Kit according to the manufacturer's protocol.

633

634 **Fluorescent reporter assay**

635 Cells (HEK293T or inducible Flp-In lines) were maintained as described above and subcultured
636 into 24-well plates for transfection. Transfections were performed as described above with the
637 exception of the addition of 100 ng of an mCherry-expressing DNA plasmid, resulting in 600 ng
638 of total DNA transfected along with 1.8 μ L of TransIT-X2. 24 hours post-transfection, cells were
639 imaged using the BioTek Cytation 5 Cell Imaging Multimode Reader. Four images were taken at
640 fixed positions in each well using a 20X objective lens, with each condition in two replicates;
641 both GFP and RFP images were collected for each well position. Non-transfected wells were
642 imaged for use as background subtraction. Images were pre-processed in the Gen5 3.1
643 software package (BioTek) using the default rolling ball algorithm settings, and mean
644 fluorescence values for GFP and RFP were quantified by the Gen5 software.

645

646 **Image analyses**

647 Normalized GFP intensity for each image was calculated as follows: (experimental GFP signal –
648 background GFP signal) / (experimental RFP signal – background RFP signal). Background
649 GFP and RFP signals are the average of the quantified pre-processed values from 8 total
650 images taken of non-transfected wells. Once the normalized GFP intensity was calculated for
651 each image, we averaged the normalized GFP intensity of each set of four images (*i.e.* for each
652 well). Finally, we calculated the relative GFP intensity for each well by dividing the average
653 normalized GFP intensity from each well by the average of the two IFIT-untreated wells, thus
654 representing each data point as relative to 100%.

655

656 **qRT-PCR**

657 HEK293T cells were maintained as described above in 24-well plates. Cells were harvested in
658 1x PBS and pelleted at 500 x g for 1 minute. RNA was then extracted using the New England

659 BioLabs Monarch Total RNA Miniprep Kit according to the manufacturer's protocol. 100 ng of
660 RNA was then subjected to reverse transcription using the Applied Biosystems High-Capacity
661 cDNA Reverse Transcription Kit according to the manufacturer's protocol. The resulting cDNA
662 was then used to conduct quantitative PCR on the Applied Biosystems StepOnePlus machine
663 with gene-specific primers (GFP-F, 5'- CCGACCACTACCAGCAGAACAC -3'; GFP-R, 5'-
664 GGACCATGTGATCGCGCTTCTC -3'; 18S-F, 5'- TCGCTCGCTCCTCTCCTACTTG -3'; 18S-R,
665 5'- GCTGACCGGGTTGGTTTTGATCTG -3') and the Luna Universal qPCR Master Mix
666 according to the manufacturer's protocol.

667

668 **Statistical analyses**

669 Statistical analyses and data visualization were performed using GraphPad Prism 10 software.
670 Tests were performed as indicated in figure legends. All error bars represent SEM.

671

672 **Material availability statement**

673 All unique materials/reagents generated in this study are available through the lead contact
674 upon request.

675

676 **DATA AVAILABILITY**

677 All data reported in this paper will be shared by the lead contact upon request. The cryo-EM
678 structure has been deposited under PDB 9MK9 and EMDB code EMD-48323. Sequencing data
679 from eCLIP experiment have been deposited in GEO record GSE284636.

680

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692

693 **AUTHOR INFORMATION**

694 **Contributions**

695 Conceptualization, D.R.G., M.D.D.; Methodology, D.R.G., C.T., A.D., E.E., J.D.W., M.D.D.;
696 Investigation, D.R.G., C.T., B.T., S.K., A.D., E.E., J.D.W., P.F., J.P., K.H., B.A.Y., K.M.E., Q.L.,
697 S.R.H., J.B.C., E.L.V.N.; Formal Analysis, D.R.G., M.D.B., K.D.C., E.L.V.N., M.D.D.; Resources,
698 J.B.C., K.D.C., M.S.D.; Writing – Original Draft, D.R.G., B.C., K.D.C., M.D.D.; Writing – Review
699 & Editing, All authors except S.R.H., who has tragically passed away since making important
700 initial discoveries for this work; Visualization, D.R.G., B.C., M.D.B., K.D.C., E.L.V.N., M.D.D.;
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702 E.L.V.N., G.W.Y., K.D.C.

703

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705 Requests for further information and resources should be directed to and will be fulfilled by the
706 lead contact, Matthew D. Daugherty (mddaugherty@ucsd.edu).

707

708 **ETHICS DECLARATIONS**

709 **Competing interests**

710 M.S.D. is a consultant or advisor for Inbios, Moderna, IntegerBio, Merck, GlaxoSmithKline,
711 Bavarian Nordic, and Akagera Medicines. The Diamond laboratory has received unrelated
712 funding support in sponsored research agreements from Emergent BioSolutions, Bavarian
713 Nordic, Moderna, Vir Biotechnology, and IntegerBio. E.L.V.N. is co-founder, member of the
714 Board of Directors, on the SAB, equity holder, and paid consultant for Eclipse BioInnovations,
715 on the SAB of RNACConnect, and is inventor of intellectual property owned by University of
716 California San Diego. E.L.V.N.'s interests have been reviewed and approved by the Baylor
717 College of Medicine in accordance with its conflict-of-interest policies. G.W.Y. is a SAB member
718 of Jumpcode Genomics and a co-founder, member of the Board of Directors, on the SAB, equity
719 holder, and paid consultant for Eclipse BioInnovations. G.W.Y. is a visiting professor at the
720 National University of Singapore. G.W.Y.'s interest(s) have been reviewed and approved by the
721 University of California, San Diego in accordance with its conflict-of-interest policies. All other
722 authors declare no other competing financial interests.

723

724 **FIGURE LEGENDS**

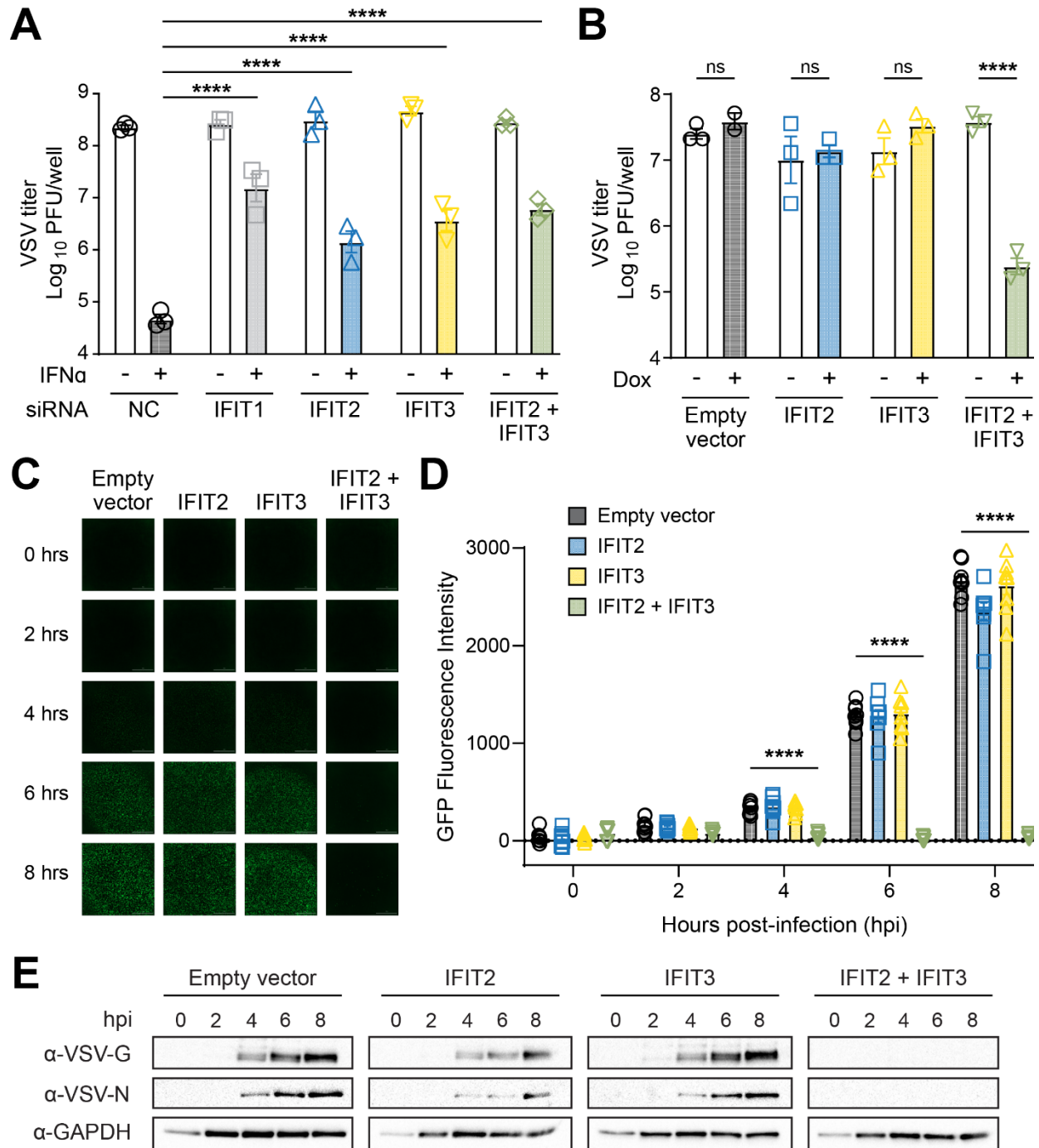


Fig. 1: IFIT2 and IFIT3 act together to inhibit viral replication. (A) Human A549 cells were treated with a non-targeting control siRNA (NC, black circles) as a negative control, or siRNA targeting IFIT1 (light grey squares), IFIT2 (blue triangles), IFIT3 (yellow triangles), or IFIT2 and IFIT3 in combination (green diamonds). In indicated conditions, cells were induced with

IFN- α (1000 U/mL). All cells were infected with VSV-GFP (0.01 MOI), and supernatant was collected at 16 hpi for titration. **(B)** Inducible Flp-In T-REx HEK293 cells expressing no IFIT (black circles), mouse IFIT2 (blue squares), mouse IFIT3 (yellow triangles), or mouse IFIT2 and IFIT3 together (green triangles) were mock treated or treated with doxycycline (500 ng/ μ L) for 24 h and then infected with VSV-GFP (0.01 MOI). Supernatant was harvested 24 hpi for titration. **(C-E)** Using the same cell lines as in as Figure 1B, each cell line was treated with doxycycline (500 ng/ μ L) for 24 h and infected with VSV-GFP (3.0 MOI). **(C)** Images were taken at 0, 2, 4, 6, and 8 hpi. **(D)** Images from experiments shown in panel C were processed and quantified for GFP fluorescence intensity. **(E)** Lysates from infected cells were harvested at 0, 2, 4, 6, and 8 hpi and analyzed by western blotting for expression of two viral proteins, VSV-G and VSV-N, and a loading control, GAPDH. All experiments were performed with three or more biological replicates with individual data points shown **(A, B, D)** or representative images shown **(C, E)**. Statistical analyses: Data are represented as mean \pm SEM. **(A, D)** Ordinary two-way ANOVA with Tukey's post-test and a single pooled variance. **** = $p < 0.0001$, ns = not significant; **(B)** ordinary two-way ANOVA with Šídák's post-test and a single pooled variance. **** = $p < 0.0001$.

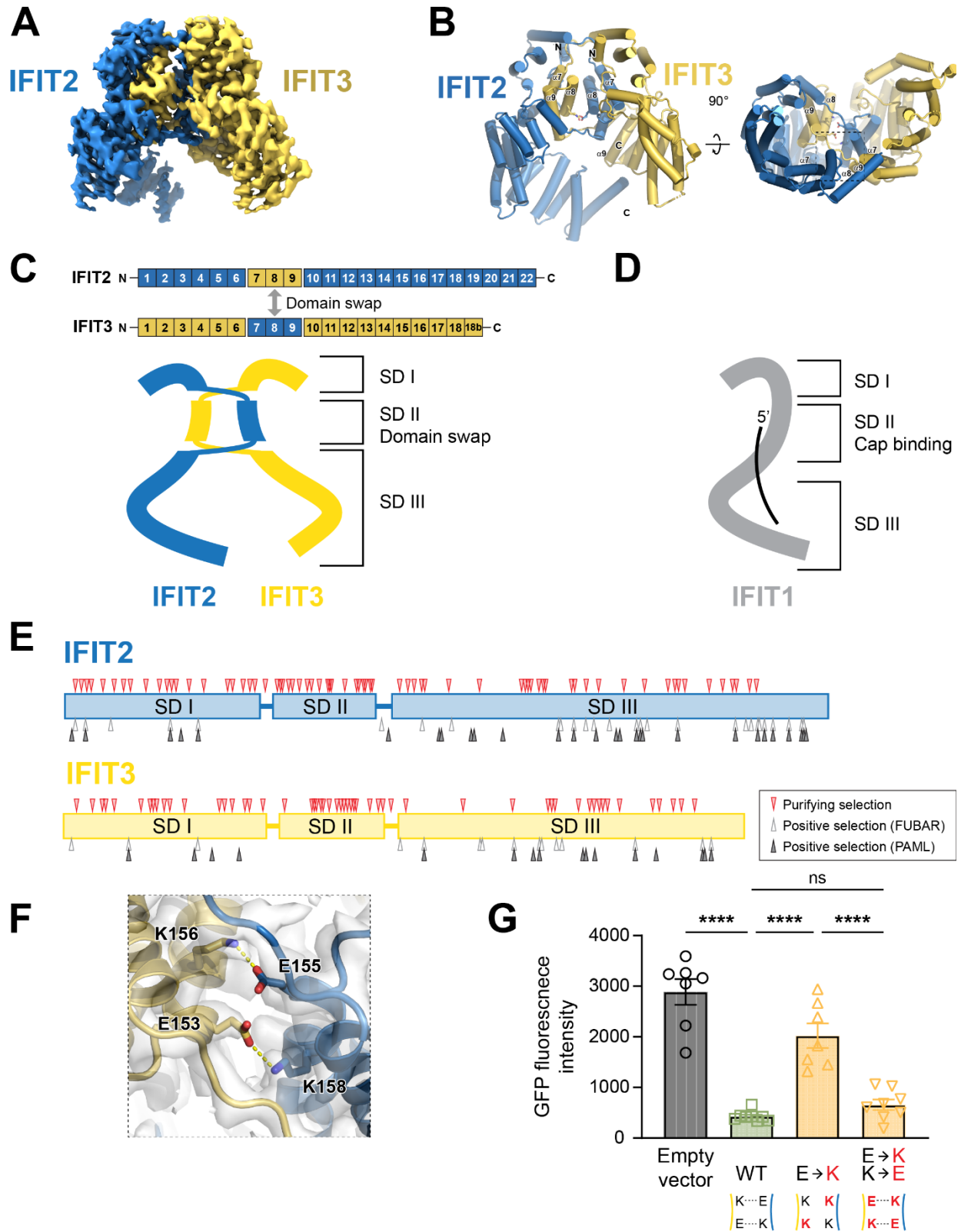


Fig. 2: Structure of the IFIT2-IFIT3 heterodimer reveals a conserved interaction surface required for antiviral activity. (A) Cryo-EM density map of the mouse IFIT2-IFIT3 heterodimer. (B) Structural model of the IFIT2-IFIT3 heterodimer. (C) Schematic of the IFIT2-IFIT3 heterodimer, with subdomains (SDs) marked. The SD II domain swap, which forms a continuous superhelix with SD I and SD III, is illustrated. (D) Cartoon of the RNA-bound IFIT1 based on PDB 6C6K, highlighting the importance of SD II for 5' cap binding. (E) Evolutionary analyses of IFIT2 and IFIT3 across >20 rodent species, revealing codons evolving under purifying selection (red triangles, from FUBAR analysis) and positive selection (grey and black triangles, from FUBAR and PAML analyses respectively). (F) Zoomed-in view of the central helices in SD II that form a reciprocal salt bridge between IFIT2 (blue) and IFIT3 (yellow). Residue numbers are indicated. (G) Importance of salt bridge residues for antiviral activity. HEK293T cells were transiently transfected with empty vector, wild-type (WT) IFIT2 and IFIT3, single E->K mutant IFIT2 (E155K) and IFIT3 (E153K), or double mutant E->K/K->E IFIT2 (E155K K158E) and IFIT3 (E153K K156E). Twenty-four hours post-transfection, cells were infected with VSV-GFP (0.05 MOI), and fluorescence images were taken and quantified at 16 hpi. Data (n>5) are represented as mean \pm SEM and were analyzed with an ordinary one-way ANOVA with Tukey's multiple comparisons test. **** = $p < 0.0001$, ns = not significant.

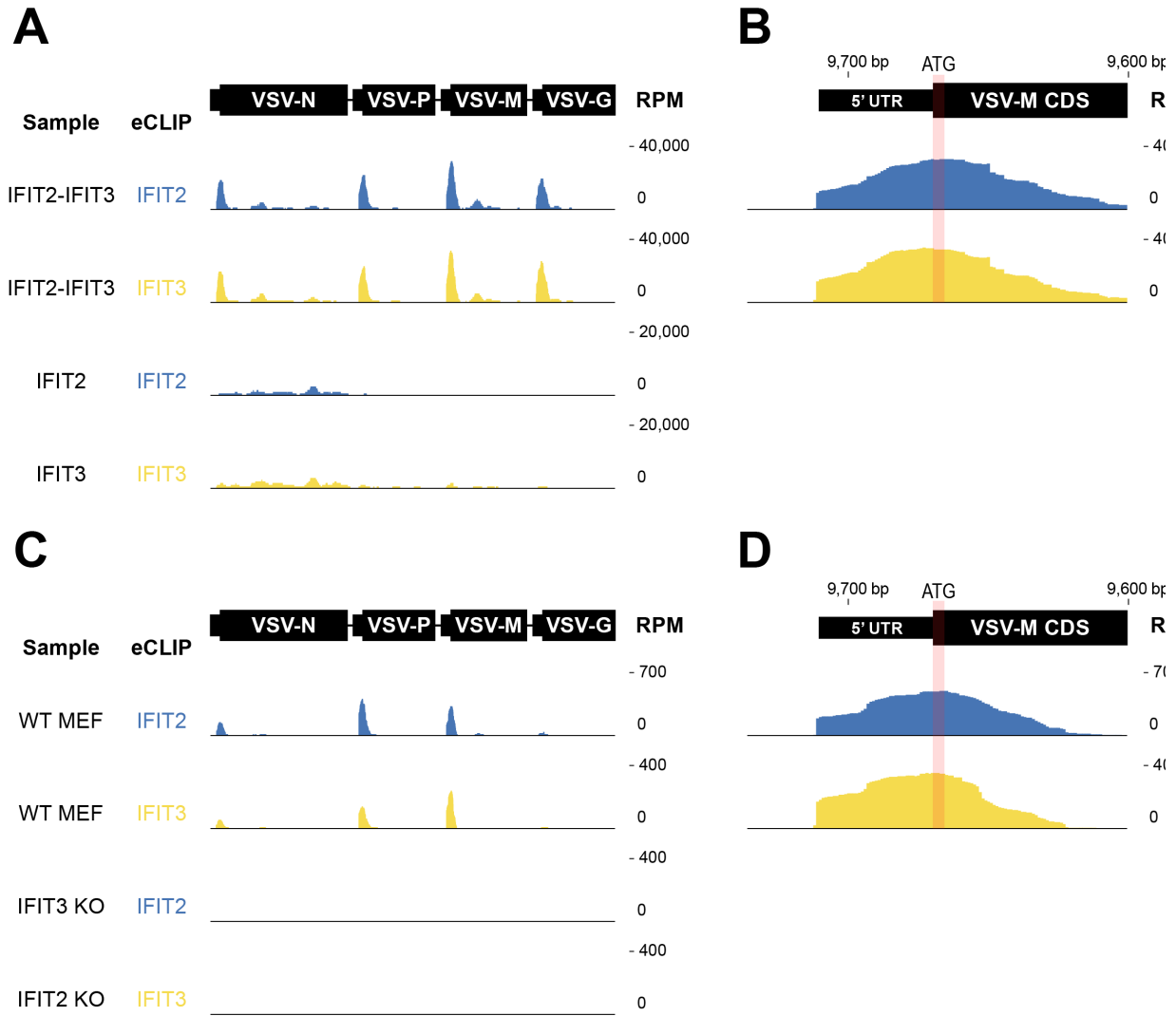


Fig. 3: The IFIT2-IFIT3 heterodimer interacts with the 5' end of VSV mRNAs near the start codons. Browser tracks of VSV reads uniquely mapped to the viral genome as captured by eCLIP of IFIT2 or IFIT3 in **(A-B)** IFIT-expressing Flp-In T-REx HEK293 lines and **(C-D)** wild-type and *Ifit2* or *Ifit3* knockout MEFs. eCLIP data are plotted as reads per million (RPM) mapped across the VSV genome normalized to the total RNA from that region of the viral genome. **(B, D)** Zoomed-in browser tracks for **(B)** 293 and **(D)** MEF lines highlighting peaks of IFIT2-IFIT3 binding proximal to the start codon of the VSV-M gene.

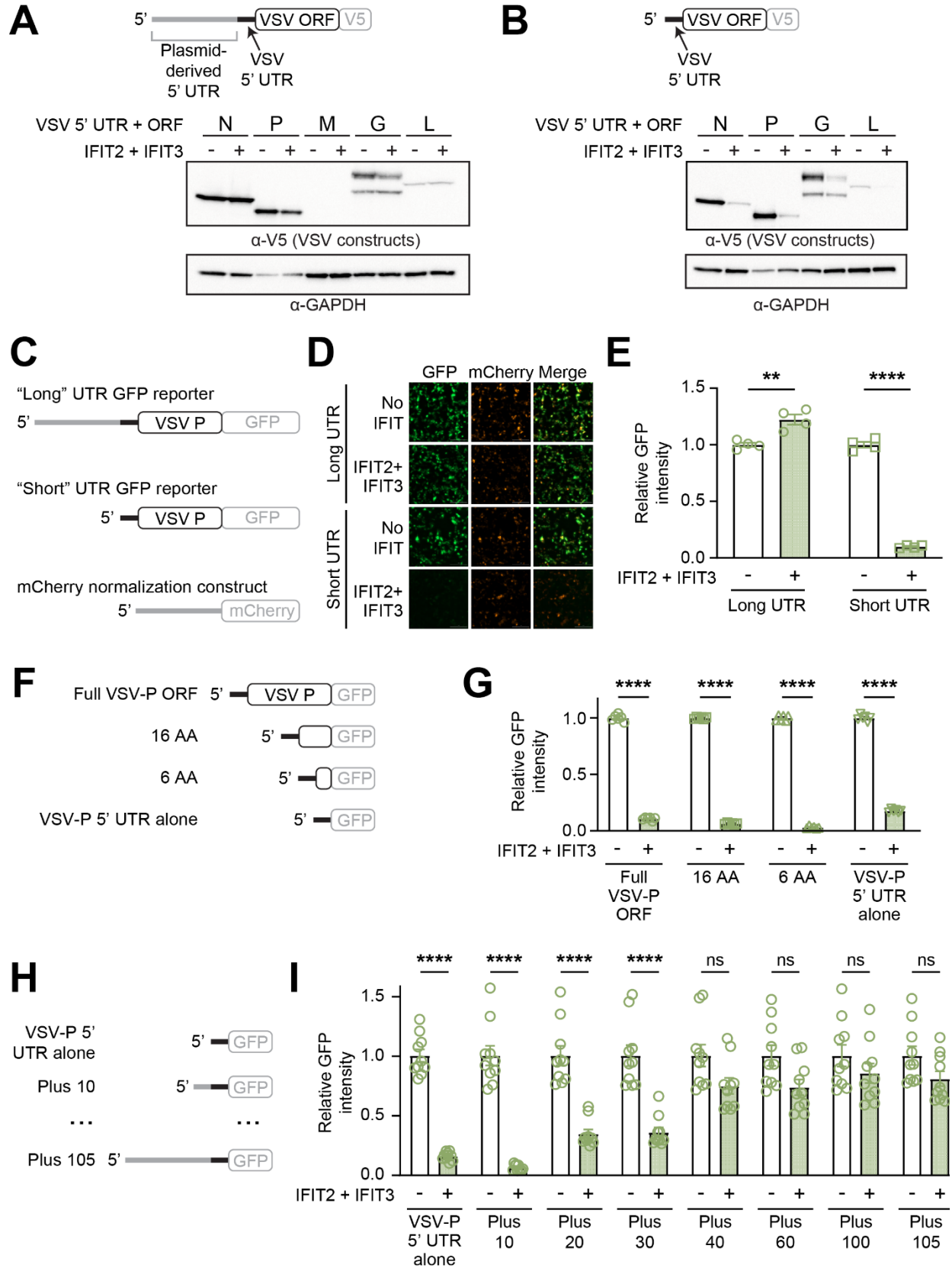


Fig. 4: IFIT2-IFIT3 recognizes short viral 5' UTRs. (A) The 5' UTR and open reading frame (ORF) of each VSV gene was cloned into a mammalian expression plasmid fused to a C-terminal V5 tag (schematic at top). Based on 5' RACE data (**Extended Data Fig. 6A**), we identified a 108 nt plasmid-derived extension (grey) appended to the 5' end of the cloned VSV 5' UTR (black). Each viral gene-expressing construct was co-transfected in the absence or presence of IFIT2-IFIT3 into HEK293T cells. Twenty-four hours post-transfection, cells were harvested and lysates were analyzed by western blotting. (B) Following plasmid engineering, only 3 nt of plasmid derived sequence remained at the 5' end of each VSV UTR and ORF (schematic at top; **Extended Data Fig. 6B**). Transfections and western blotting were performed as in panel A. (C) 5' UTR and ORF schematics for fluorescence reporter constructs shown in panels D and E. (D) HEK293T cells were transfected with the indicated GFP reporters in the absence or presence of co-transfected IFIT2-IFIT3. All wells were transfected with the control mCherry normalization construct. Images were taken 24 h post-transfection. (E) Experiments were performed as in panel D. For each well (n=4 biological replicates), four images were taken and the ratio of GFP:mCherry signal intensity (see **Extended Data Fig. 6D**) was calculated for each image. All values were normalized to the average of the condition in which IFIT2-IFIT3 was not transfected. (F) 5' UTR and ORF schematics for GFP reporter constructs shown in panel G. AA=amino acids. (G) Experiments with the indicated constructs were performed and quantified as in panel E. (H) 5' UTR and ORF schematics for GFP reporter constructs shown in panel I. Plasmid derived sequence from the “long” construct shown in panel C was added back by the indicated number of nt (e.g. Plus 10). (I) Experiments with the indicated constructs were performed and quantified as in panel E (n=10 biological replicates). Statistical analyses: Data are represented as mean \pm SEM. (E, G, I) Ordinary two-way ANOVA with Šídák's post-test and a single pooled variance. **** = $p < 0.0001$, ns = not significant.

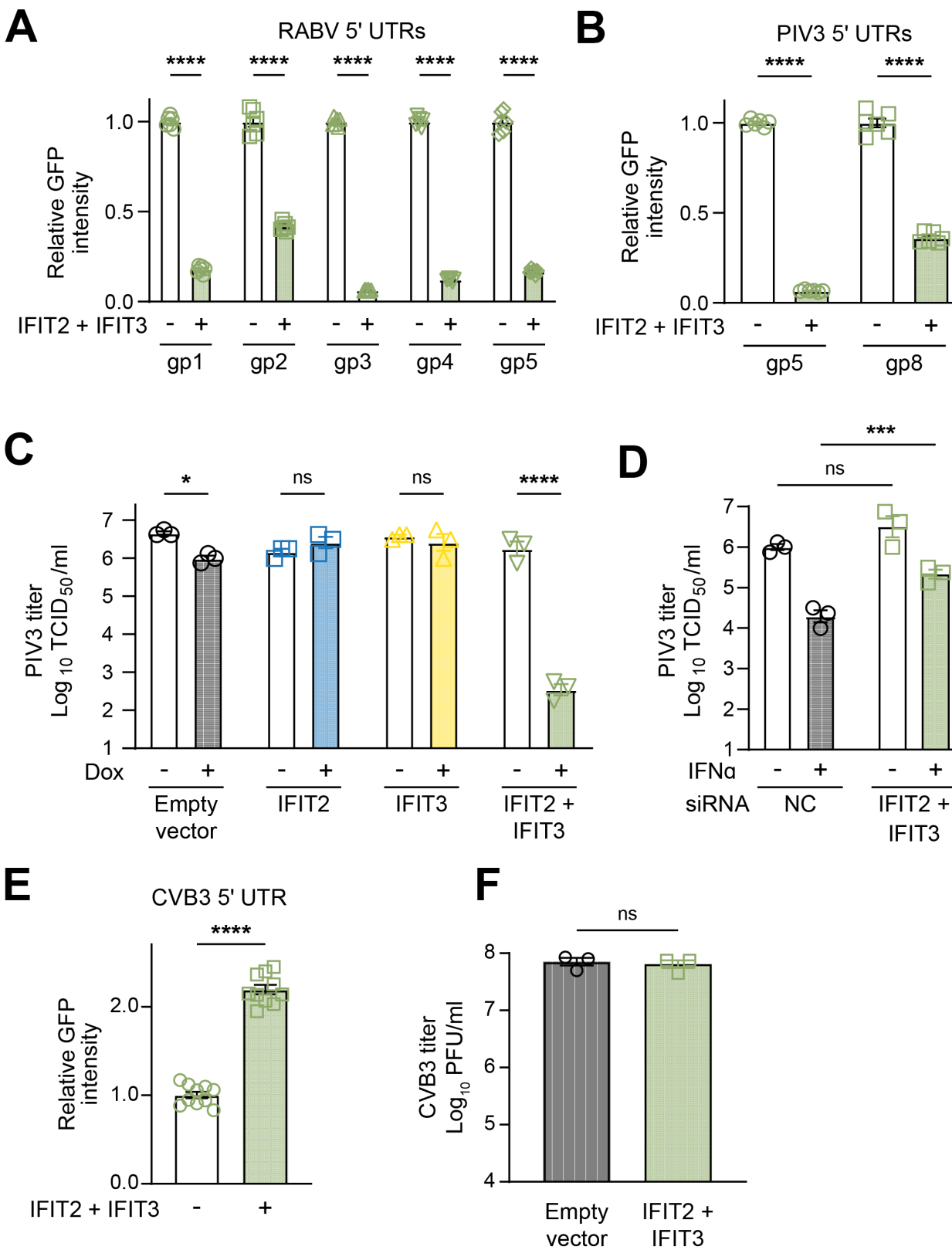
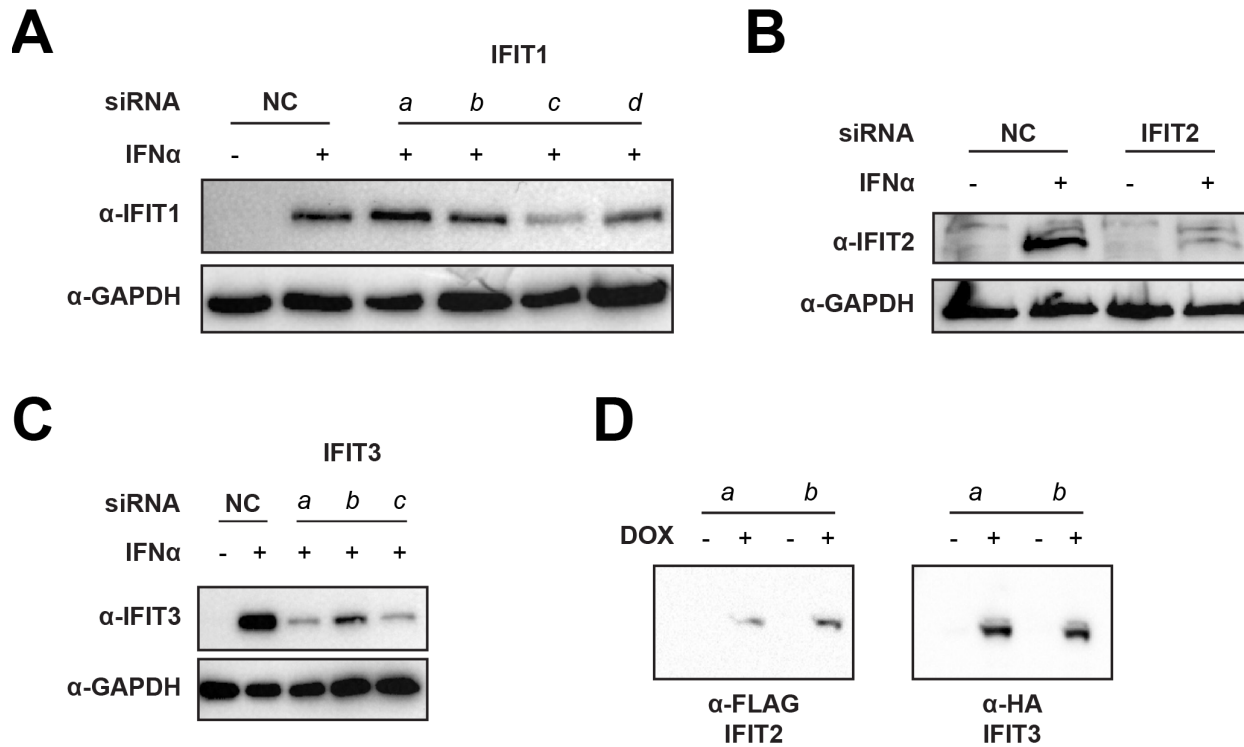


Fig. 5: IFIT2-IFIT3 has broad antiviral activity driven by length of 5' UTRs. (A-B)

HEK293T cells were transfected with eGFP expression plasmids containing the indicated viral 5' UTRs in the absence or presence of co-transfected IFIT2-IFIT3. Experiments were performed as in Figure 4E. **(C)** Inducible Flp-In T-REx HEK293 cells expressing no IFIT (black circles), IFIT2 (blue squares), IFIT3 (yellow triangles), or IFIT2 and IFIT3 together (green triangles) were mock-treated or treated with doxycycline (500 ng/ μ L) for 24 h and then infected with VSV-GFP (0.1 MOI). Supernatant was collected at 48 hpi and quantified by TCID₅₀. **(D)** A549 cells were treated with a non-targeting control siRNA (NC, black circles) as a negative control, or siRNA targeting *IFIT2* and *IFIT3* in combination (green diamonds). In indicated conditions, cells were induced with IFN- α (1000 U/mL). All cells were infected with PIV3-GFP (0.1 MOI), and supernatant was collected at 48 hpi for quantification by TCID₅₀. **(E)** HEK293T cells were transfected with an eGFP expression plasmid containing the 742 nt CVB3 5' UTR in the absence or presence of co-transfected IFIT2-IFIT3. **(F)** Inducible Flp-In T-REx HEK293 cells expressing no IFIT (black circles), IFIT2 (blue squares), IFIT3 (yellow triangles), or IFIT2 and IFIT3 together (green triangles) were mock-treated or treated with doxycycline (500 ng/ μ L) for 24 h and then infected with CVB3 (0.1 MOI). Supernatant was collected at 24 hpi and titered. **(A-F)** All experiments were performed with three or more biological replicates with individual data points shown. Statistical analyses: Data are represented as mean \pm SEM. **(A-D)** Ordinary two-way ANOVA with Šídák's multiple comparisons test and a single pooled variance. * = $p < 0.05$, *** = $p < 0.001$, **** = $p < 0.0001$, ns = not significant; **(E-F)** unpaired parametric t test. **** = $p < 0.0001$, ns = not significant.

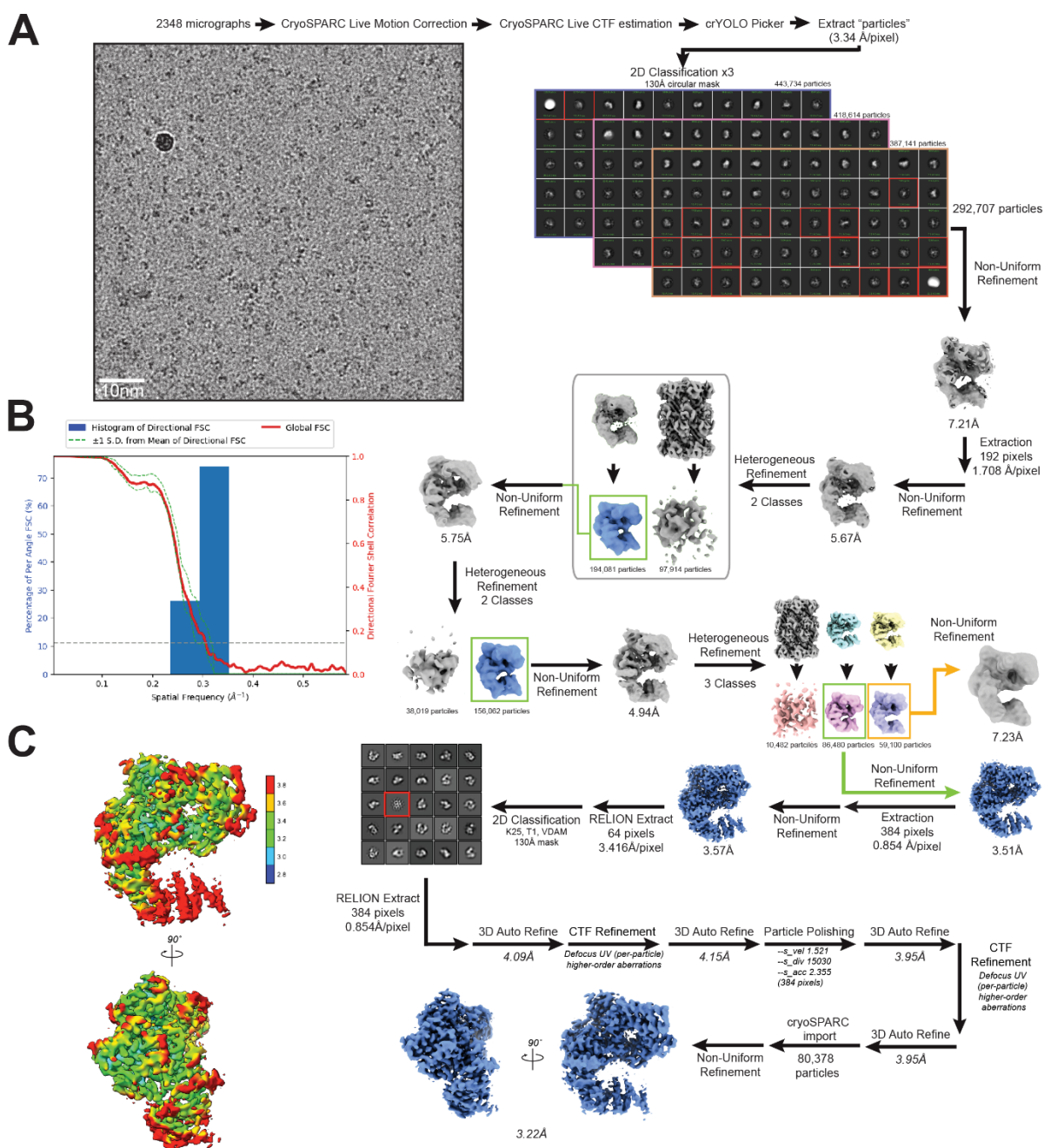
730 EXTENDED DATA

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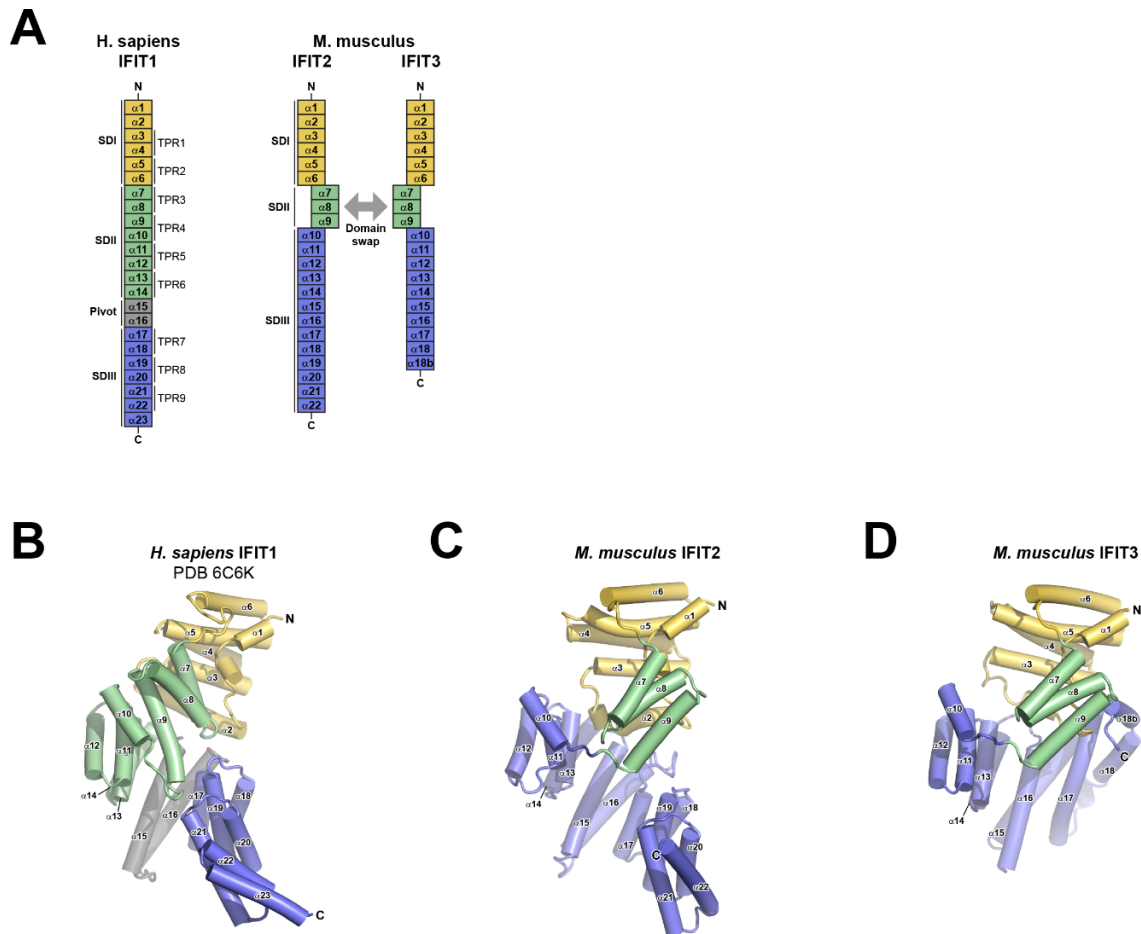
Extended Data Fig. 1: siRNA knockdown of IFITs in A549 cells and expression of IFIT2 and IFIT3 in Flp-In lines, related to Fig. 1. (A-C) A549 cells were treated with a non-targeting siRNA as a negative control or siRNA targeting human (A) *IFIT1*, (B) *IFIT2*, or (C) *IFIT3*. Twenty-four hours post-treatment, cells were harvested and subsequently processed and analyzed by western blotting. Representative images shown; 2 independent experiments. For downstream infection experiments, siRNA *IFIT1*-c and siRNA *IFIT3*-a were used. (D) We generated inducible Flp-In T-REx HEK293 cell lines co-expressing mouse *IFIT2* and *IFIT3*. Cells were induced with 500 ng/mL DOX for 24 hours before harvesting cell pellets for analysis by western blot. For all associated downstream experiments, Flp-In line *b* was used.

732



Extended Data Fig. 2: Cryo-EM data processing for IFIT2-IFIT3, related to Fig. 2. (A)

Workflow for structure determination. See **Methods** for a detailed explanation of data processing. For 2D classes, classes outlined in red were excluded from later steps. For 3D classes, classes outlined in green were selected for later steps. The final refined map showed a resolution of 3.22 Å (gold-standard FSC criterion of 0.143). **(B)** Directional Fourier Shell Correlation analysis for the final map, calculated by 3DFSC⁷⁴. **(C)** Two views of the final map, colored by local resolution (blue: 2.8 Å or better; red: 3.8 Å or worse).



Extended Data Fig. 3: Comparison of IFIT structures, related to Fig. 2. (A)

Representation of human IFIT1 and mouse IFIT2 and IFIT3 domains. (B-D) Representations of (B) human IFIT1, (C) mouse IFIT2, and (D) mouse IFIT3. (C-D) The white, semitransparent helices represent the domain-swapped helices during IFIT2-IFIT3 heterodimer formation.

Rodent IFIT2 Whole gene p-value for positive selection: **<0.0001**

	SD I	SD II	SD III
	1	136	186
			470
Negatively-selected codons (FUBAR)	30	16	31
Positively-selected codons (FUBAR)	5	0	23
Positively-selected codons (PAML)	5	0	23
Subdomain dN/dS	0.28	0.11	0.75
Subdomain p-value for positive selection	0.02	1.0	<0.0001

Rodent IFIT3 Whole gene p-value for positive selection: **<0.0001**

	SD I	SD II	SD III
	1	136	188
			434
Negatively-selected codons (FUBAR)	22	15	23
Positively-selected codons (FUBAR)	3	0	12
Positively-selected codons (PAML)	4	0	12
Subdomain dN/dS	0.37	0.18	0.69
Subdomain p-value for positive selection	0.009	1.0	<0.0001

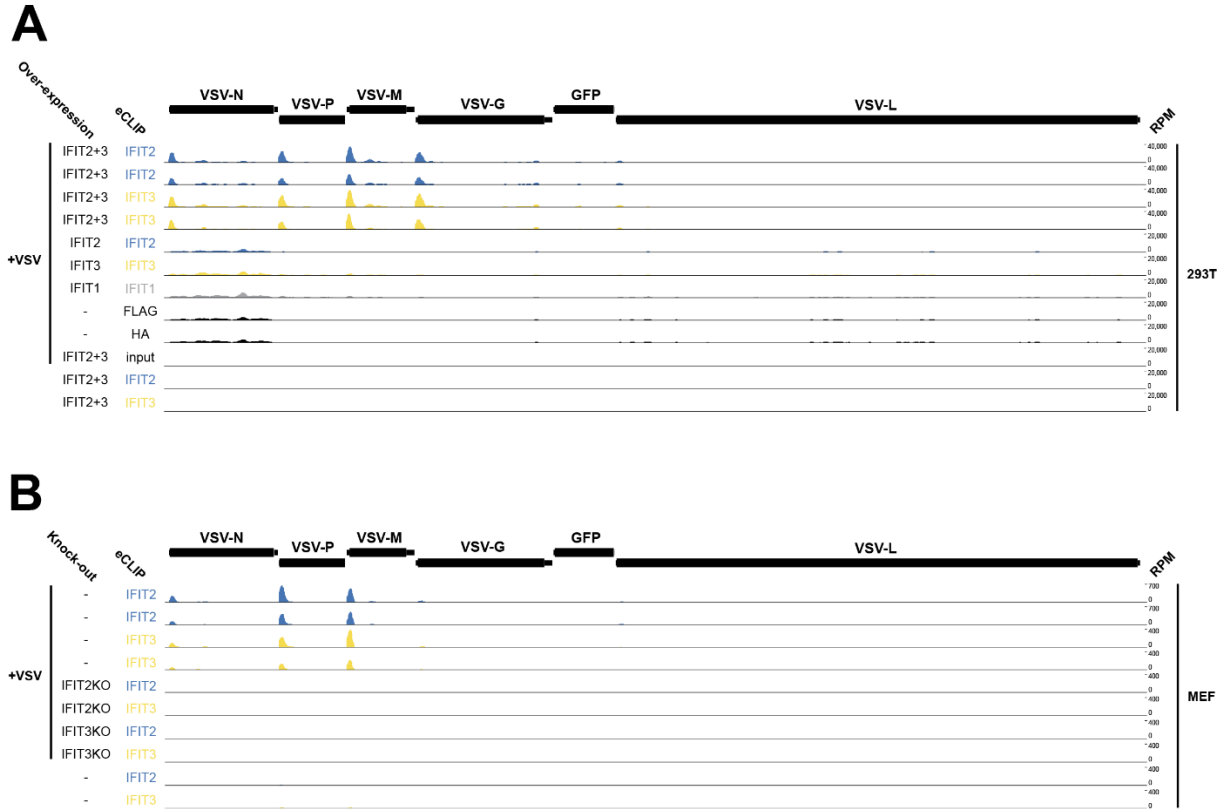
Primate IFIT2 Whole gene p-value for positive selection: **<0.0001**

	SD I	SD II	SD III
	1	136	186
			472
Negatively-selected codons (FUBAR)	15	6	28
Positively-selected codons (FUBAR)	7	0	12
Positively-selected codons (PAML)	5	0	8
Subdomain dN/dS	0.33	0.12	0.54
Subdomain p-value for positive selection	0.03	1.0	<0.0001

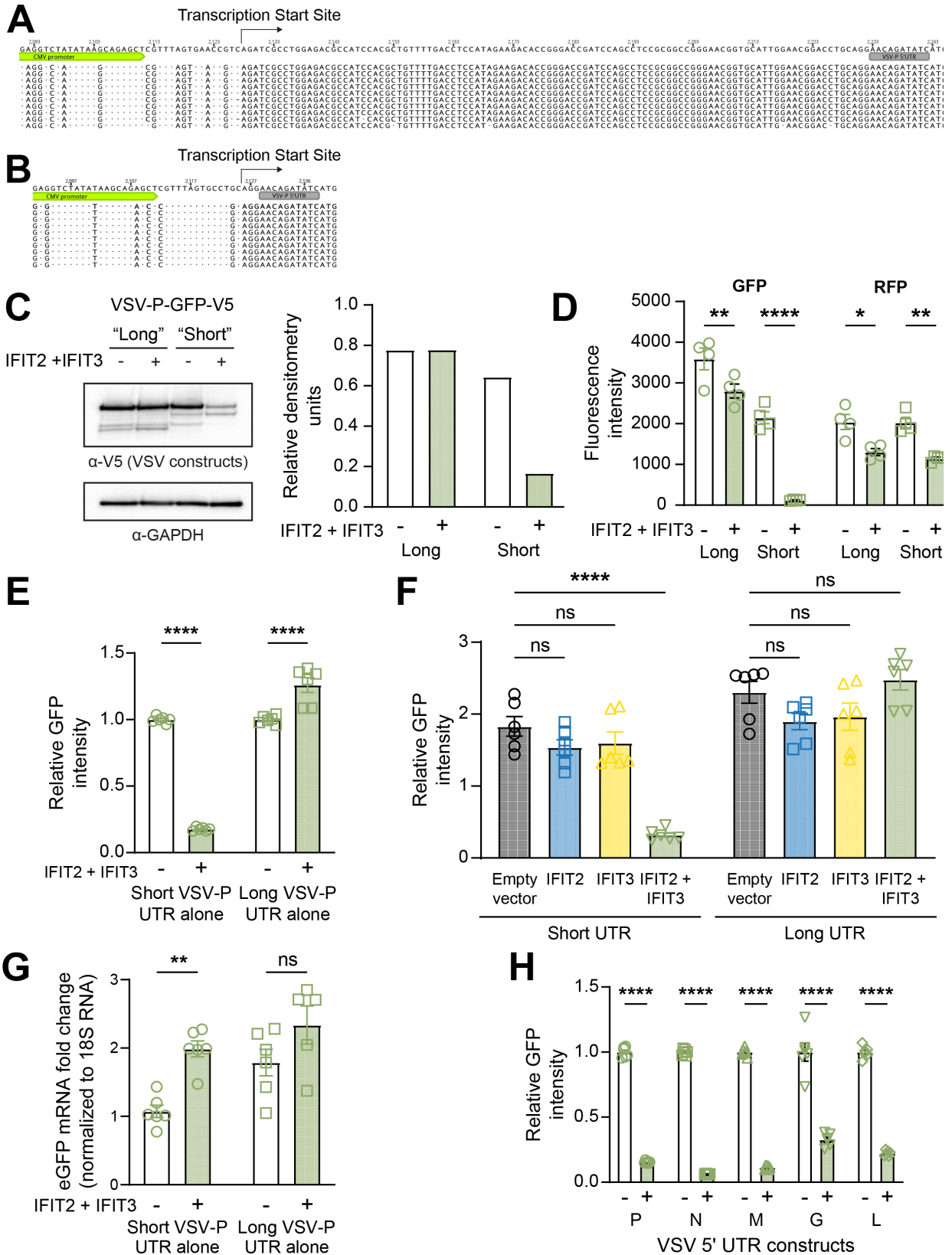
Primate IFIT3 Whole gene p-value for positive selection: **<0.0001**

	SD I	SD II	SD III
	1	136	188
			490
Negatively-selected codons (FUBAR)	20	5	25
Positively-selected codons (FUBAR)	2	0	7
Positively-selected codons (PAML)	2	0	7
Subdomain dN/dS	0.28	0.18	0.69
Subdomain p-value for positive selection	0.13	1.0	<0.0001

Extended Data Fig. 4: Evolutionary analysis, related to Fig. 2. Whole gene and subdomain evolutionary analyses on rodent and primate IFIT2 and IFIT3. Domain boundaries for each subdomain (SD) are indicated by amino acid numbers below each schematic. Statistical evidence for positive selection, determined by PAML software, is shown for each gene and each subdomain, with statistically significant p-values shown in bold red text. Overall dN/dS (omega) values for each subdomain are also shown. The number of specific codons within each subdomain that show statistical evidence for positive and negative selection, as determined by PAML and FUBAR software as indicated. Additional statistics and analysis parameters, complete lists of codons, and accession numbers and species of sequences analyzed are found in Table S2.



Extended Data Fig. 5: Complete set of eCLIP data, related to Fig. 3. Full VSV genome coverage for additional samples from the eCLIP experiment described in **Fig. 3**, including uninfected controls, for both **(A)** human IFIT-expressing Flp-In T-REx HEK293 lines and **(B)** mouse wild-type and *Ifit2* or *Ifit3* knockout MEFs. Values shown are normalized reads per million (RPM) out of all reads mapped to the VSV genome.



Extended Data Fig. 6: IFIT2-IFIT3 recognizes short viral 5' UTRs, related to Fig. 4. (A-B)

Plasmids expressing either a **(A)** long plasmid-derived 5' UTR or **(B)** no plasmid-derived 5' UTR immediately upstream of the VSV-P 5' UTR were transfected into 293T cells. Twenty-four hours post-transfection, cells were harvested, and RNA was isolated for 5' RACE experiments. Ten clones from each construct were sequenced, and the transcription start site is marked for each plasmid. **(C)** Experiment was carried out as described in Figure 4C, but instead of imaging, cells were harvested and lysates were analyzed by western blotting. Densitometry values were calculated using ImageJ. **(D)** Raw values from normalized data in panel C. **(E-F)** 293T cells were transfected with plasmids expressing the 10 nt VSV-P 5' UTR and eGFP with either a short or long plasmid-derived 5' UTR upstream of the viral sequence, co-transfected in the absence or presence of **(E)** IFIT2-IFIT3 or **(F)** IFIT2 or IFIT3 alone. Experiments were performed as described in Figure 4F-G. **(G)** Cells were transfected as in panel E, and 24 hours post-transfection, cells were harvested, RNA was extracted, and qRT-PCR was conducted. Data are pooled from two independent experiments, and data are normalized to the short VSV-P UTR alone, no IFIT2-IFIT3 condition. **(G)** Cells were transfected with plasmids expressing GFP and the 5' UTR of each VSV gene in the absence or presence of IFIT2-IFIT3. Experiments were performed as described in Figure 4F-G. Statistical analyses: Data are represented as mean \pm SEM. **(D, E, G, H)** Ordinary two-way ANOVA with Šídák's multiple comparisons test and a single pooled variance. * = $p < 0.05$, ** = $p < 0.01$, **** = $p < 0.0001$, ns = not significant; **(F)** ordinary two-way ANOVA with Dunnett's post-test and a single pooled variance. Comparisons are to the empty vector condition within each column. **** = $p < 0.0001$, ns = not significant.

735

736 **SUPPLEMENTARY INFORMATION**

737 **Supplementary Table 1. Cryo-electron microscopy data collection and structure**

738 **determination, related to Figure 2**

739 **Supplementary Table 2. Excel file containing evolutionary analyses-related information,**

740 **related to Figure 2 and S4.** Individual tables contain accession numbers and species for

741 sequences analyzed, complete lists of codons determined to be evolving under purifying and

742 positive selection, and parameters and statistics for whole-gene and subdomain analyses.

743 **Supplementary Table 3. Excel file containing information on plasmids and primers,**

744 **related to Experimental Procedures.** Spreadsheet containing all plasmids used in this study,

745 along with a brief description and primers and/or Gene Fragments used for cloning.

746

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748

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