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► **To cite this version:**

Giulio Superti-Furga, Andreas Pichlmair, Caroline Lassnig, Christoph L Baumann, Tilmann Bürckstümmer, et al.. IFIT1 is an antiviral protein that recognises 5'-triphosphate RNA. *Nature Immunology*, 2011, 10.1038/ni.2048 . hal-00648046

**HAL Id: hal-00648046**

**<https://hal.science/hal-00648046>**

Submitted on 5 Dec 2011

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# **IFIT1 is an antiviral protein that recognises 5'- triphosphate RNA**

Andreas Pichlmair<sup>1</sup>, Caroline Lassnig<sup>2,3</sup>, Carol-Ann Eberle<sup>1</sup>, Maria W Górna<sup>1</sup>, Christoph L  
Baumann<sup>1</sup>, Thomas R Burkard<sup>1</sup>, Tilmann Bürckstümmer<sup>1</sup>, Adrijana Stefanovic<sup>1</sup>, Sigurd Krieger<sup>5</sup>,  
Keiryn L Bennett<sup>1</sup>, Thomas Rüllicke<sup>3,4</sup>, Friedemann Weber<sup>6</sup>, Jacques Colinge<sup>1</sup>, Mathias Müller<sup>2,3</sup>  
and Giulio Superti-Furga<sup>1\*</sup>

<sup>1</sup> CeMM- Research Center for Molecular Medicine of the Austrian Academy of Sciences,  
Vienna, Austria; <sup>2</sup> Institute of Animal Breeding and Genetics, <sup>3</sup> Biomodels Austria and <sup>4</sup> Institute  
of Laboratory Animal Science, Vetmeduni Vienna, Austria; <sup>5</sup> Department of Clinical Pathology,  
Medical University of Vienna, Vienna, Austria; <sup>6</sup> Institute for Virology, Philipps University  
Marburg, Germany

**\* Corresponding author:**

Giulio Superti-Furga  
CeMM - Research Center for Molecular Medicine of the Austrian Academy of Sciences  
Lazarettgasse 14, AKH BT25.3  
1090 Vienna, Austria  
Email: [gsuperti@cemm.oeaw.ac.at](mailto:gsuperti@cemm.oeaw.ac.at)  
Telephone: +43 664 4042300

22

23 **Abstract**

24 Antiviral innate immunity relies on recognition of microbial structures. One such structure is viral RNA  
25 that carries a triphosphate group on its 5'terminus (PPP-RNA). In an affinity proteomics approach with  
26 PPP-RNA as bait we identified interferon induced protein with tetratricopeptide repeats 1 (IFIT1) to  
27 mediate binding of a larger protein complex containing other IFIT family members. IFIT1 bound PPP-  
28 RNA with nanomolar affinity and required R187 in a highly charged C-terminal groove of the protein. In  
29 the absence of IFIT1 growth and pathogenicity of PPP-RNA viruses were severely increased. In contrast,  
30 IFITs were dispensable for clearance of pathogens not generating PPP-RNA. Based on this specificity and  
31 the high abundance of IFITs after infection we propose that the IFIT complex antagonises viruses by  
32 sequestering specific viral nucleic acids.

33

34 Pattern recognition receptors (PRRs) sense molecular signatures associated with microbes<sup>1</sup>. Viral nucleic  
35 acid delivered and generated during the viral life cycle can activate PRRs to initiate the innate antiviral  
36 defence<sup>2</sup>. Recently, triphosphorylated RNA (PPP-RNA), which is constituent of genomic, antigenomic  
37 and certain transcript RNAs associated with some viruses like influenza and vesicular stomatitis virus,  
38 was identified as one such component that can be recognised by the innate immune system<sup>3-5</sup>. Binding of  
39 PPP-RNA to the PRR Retinoic acid inducible gene-I (RIG-I) mediates activation of a signalling cascade  
40 that culminates in the expression of type-I interferon (IFN- $\alpha/\beta$ ) and other cytokines<sup>5,6</sup>. Most likely  
41 through evolutionary pressure exerted by the innate immune system, some viruses evolved sophisticated  
42 mechanisms to avoid presentation of PPP-RNA<sup>7,8</sup>. These viruses are often sensed through atypical  
43 nucleic acids components such as long double-stranded RNA (dsRNA), which activates Melanoma  
44 differentiated associated gene-5 (Mda5) to initiate expression of IFN- $\alpha/\beta$ <sup>5,9</sup>. Beside their interferon-  
45 inducing capabilities, viral RNAs are known to trigger additional cellular functions that are unrelated to  
46 transcriptional control of cytokine expression<sup>2</sup>. Thus, the cellular machinery not only discriminates  
47 between host and invading molecules but often selectively targets the same structures as part of an  
48 antiviral program execution. Several interferon-stimulated proteins only reveal their antiviral potential  
49 after binding to dsRNA<sup>10</sup>. However, some viruses like influenza and Rift valley fever virus appear to  
50 generate only limited amounts of long dsRNA<sup>3,11</sup>, yet they are antagonised by IFN- $\alpha/\beta$  consistent with  
51 the notion that alternative viral nucleic acid structures like PPP-RNA may be key to inhibiting their  
52 replication. Moreover, there are many early and strongly IFN- $\alpha/\beta$ -induced proteins that have unclear  
53 molecular function and could in principle participate in the machinery involved in engagement of viral  
54 nucleic acid some of which have been revealed through viral or host genetics<sup>12-14</sup>. In particular, little is  
55 known about the cellular repertoire of proteins that have the potential to bind the type of PPP-RNA that is  
56 generated during viral infection. Here we used an unbiased proteomic-centred survey to identify cellular  
57 proteins that engage microbial structures<sup>15</sup> and report the identification and functional characterisation of

58 a class of proteins binding to PPP-RNA.

59

## 60 **Results**

### 61 **IFIT1 and IFIT-5 are PPP-RNA binding molecules**

62 We used agarose beads coupled to PPP-RNA (mimicking viral RNA) or the same RNA not containing a  
63 triphosphate group (OH-RNA), which is known not to activate the innate immune system<sup>3</sup>, to affinity  
64 purify potentially interacting proteins from HEK293 cells that were or were not pre-treated with  
65 recombinant Interferon  $\beta$  (IFN- $\beta$ ). Pulled-down proteins were identified by mass spectrometry  
66 (**Supplementary Fig. 1a**). The proteins predominantly precipitated with PPP-RNA from interferon  
67 treated cells were interferon stimulated proteins with tetratricopeptide repeats (IFIT) 1-5 (**Supplementary**  
68 **Fig. 1b**). A double-logarithmic plot of the spectral counts as well as the exponential modified protein  
69 abundance index (emPAI)<sup>16</sup> confirmed the specific IFN- $\beta$  dependent enrichment of IFIT1, IFIT2 and  
70 IFIT3 (**Fig. 1a, Supplementary Fig. 1c**). IFITs were expressed at low levels at steady-state but highly  
71 induced by type-I interferon (IFN- $\alpha/\beta$ ) and virus infection<sup>17</sup>. 16 h after treatment with 1000 U/ml hIFN-  
72  $\beta$ , HeLa cells contained 216 pg/ $\mu$ g IFIT1 roughly corresponding to 2.4 million copies per cell (**Fig. 1b**),  
73 while in 293T cells the IFIT1 levels were 126 pg/ $\mu$ g, corresponding to some 1.4 million copies  
74 (**Supplementary Fig. 2**), placing IFIT1 amongst the most abundant cellular proteins<sup>18</sup>. The IFIT protein  
75 family contains four known human (IFIT1, IFIT2, IFIT3, IFIT5) and three mouse members (Ifit1, Ifit-2,  
76 Ifit-3) (**Supplementary Fig. 3**). IFITs consist mainly of tetratricopeptide repeats (TPRs) but no annotated  
77 nucleic acid binding domain<sup>17</sup>. We tested the binding of IFITs to RNA by using PPP-RNA-coated beads  
78 to precipitate human IFITs from IFIT overexpressing 293T cells or using recombinant protein expressed  
79 in bacteria. Overexpressed and recombinant IFIT1 and IFIT5, but little IFIT2 and no IFIT3, associated  
80 with PPP-RNA beads (**Fig. 1c, d**). The two members of the family that best bound to PPP-RNA, IFIT1  
81 and IFIT5, share the highest sequence homology within the IFIT family (**Supplementary Fig. 3**). We

82 hypothesised that IFIT2 and IFIT 3 associate with PPP-RNA indirectly and be part of a molecular  
83 complex that only assembles after IFN- $\alpha/\beta$  induction.

#### 84 **IFITs form an interferon-dependent multiprotein complex**

85 To study the putative cellular complex assembling around the IFIT family members we performed affinity  
86 purification-mass spectrometry (AP-MS) analysis using IFIT1, IFIT 2 and IFIT 3 as baits. We expressed  
87 IFIT1, IFIT 2 and IFIT 3 in doxycycline-inducible HEK-FlpIN cells in the presence or absence of IFN-  
88  $\alpha/\beta$ . Doxycycline treatment of HEK-FlpIN cells elicited expression of IFIT1 protein that was comparable  
89 to the endogenous levels measured in cells treated with 50 to 500 U/ml of hIFN- $\beta$  (**Supplementary Fig.**  
90 **4a**). Moreover, using a green fluorescent protein (GFP)-expressing isogenic cell line it was possible to  
91 ascertain that expression in this system is highly homogenous among the cell population (**Supplementary**  
92 **Fig. 4b**). Protein complexes were purified by tandem affinity purification and analysed by Liquid  
93 Chromatography-Mass Spectrometry (LC-MSMS)<sup>19,20</sup>. IFIT proteins interacted with a limited number of  
94 cellular proteins in unstimulated cells (**Supplementary Fig. 5**; IntAct database<sup>21</sup> identifier IM-15277).  
95 However, IFN- $\alpha/\beta$  treatment drastically changed the interaction profile in terms of number of identified  
96 proteins and peptide count per protein. In purifications from IFN- $\alpha/\beta$  stimulated cells, IFIT2 and IFIT3  
97 co-purified with IFIT1 with high enough sequence coverage to suggest a stoichiometric interaction among  
98 the three proteins (**Table 1**). IFIT5 did not co-precipitate with any other IFIT protein. IFITs do not require  
99 IFN- $\alpha/\beta$ -induced factors to bind to each other since tagged versions of IFIT proteins co-precipitated after  
100 overexpression of single proteins (**Supplementary Fig. 6a**). Similarly, recombinant purified IFIT1 and  
101 IFIT2, IFIT1 and IFIT3 but not IFIT1 and IFIT5 associated in gel filtration experiments, suggesting a  
102 direct interaction at a roughly 1:1 ratio (**Fig. 2a, Supplementary Fig. 6b**), consistent with the results  
103 obtained by mass spectrometry on cellular complexes. Compiling the individual interaction profiles into a  
104 network analysis revealed several interesting features. First, IFN- $\alpha/\beta$  induced a dramatic change in the  
105 number of nodes (**Fig. 2b**), reflecting the fact that the bait proteins are naturally expressed at high levels

106 only after IFN- $\alpha/\beta$  induction when they find partners. Also the topology of the network is affected by  
107 IFN- $\alpha/\beta$  stimulation with the dramatic increase of proteins interacting with all three baits from 1 node to  
108 14 nodes (**Fig. 2b**, red dots). At the same time the high degree of connectivity after IFN- $\alpha/\beta$  validated the  
109 quality of the analysis, as contaminants would interact also in non-induced cells. Importantly, the network  
110 also suggested that a few inducible components, in this case mainly the IFIT members, may exert their  
111 function by recruiting cellular proteins to assemble IFN-triggered cellular machines (**Fig. 2c**).  
112 Interestingly, IFIT1B, a poorly characterised member of the IFIT family interacts with both IFIT1 and  
113 IFIT3 making it a possible component of the larger complex or of a subcomplex worth investigating in  
114 the future (**Fig. 2c**). Among the group of proteins interacting with more than one IFIT member are hnRNP  
115 components, known to bind RNA and regulate transport and translation, small nuclear Ribonucleoprotein  
116 particle (SNRP) components, RNA binding proteins involved in RNA processing, as well as polyA-  
117 binding proteins. While we cannot exclude that these proteins co-precipitate through binding an RNA  
118 species that simultaneously binds to IFITs, this is unlikely as it would have to be via an IFN- $\alpha/\beta$ -  
119 inducible RNA. Overall the protein complex suggests a role of IFIT family members in RNA biology. In  
120 future, it may be worth investigating the contribution of several members of the IFIT interactome in the  
121 antiviral program. Here we initially focus on IFIT1 being the component mediating association of the  
122 IFIT complex to PPP-RNA.

### 123 **Molecular basis for IFIT1 interaction to PPP-RNA**

124 Interferon-stimulated proteins partially re-distribute upon engagement of the respective viral ligands<sup>22</sup>.  
125 We examined the subcellular localisation of murine Ifit1 in IFN- $\beta$  stimulated NIH3T3 cells after  
126 transfection of biotinylated PPP-RNA or OH-RNA. Ifit1 is equally distributed in IFN- $\beta$  treated cells and  
127 re-localises to discrete intracellular foci after stimulation with PPP-RNA in roughly half of all cells  
128 examined (**Fig. 3a**). In contrast, only a small fraction of cells showed relocalisation of Ifit1 after  
129 transfection of OH-RNA.



130 To further assess the association of PPP-RNA with IFIT1 we investigated the requirement for  
131 triphosphates in RNA precipitations comparing cells expressing c-Myc-tagged IFIT1 to cells expressing  
132 GFP-RIG-I as positive control. In both cases PPP-RNA was considerably more efficient than its OH  
133 counterpart in purifying the two proteins (**Fig. 3b**). Similarly, PPP-RNA efficiently and specifically  
134 purified endogenous IFIT1 from both interferon treated HEK293 cells and mouse embryonic fibroblasts  
135 (MEFs) (**Fig. 3c**), suggesting that the PPP-RNA binding property of IFIT1 is common to different cells  
136 and species. To further assess the PPP-RNA binding properties of IFIT1 we took advantage of  
137 *Escherichia coli* purified proteins in gel mobility assays. IFIT1 but not IFIT3 caused mobility retardation  
138 of a PPP-RNA and not a OH-RNA probe (**Fig. 3d**). Antibodies directed against the recombinant IFIT1  
139 caused an increased retardation in mobility confirming that IFIT1 is a major component of the retarded  
140 complex. IFIT1 contains no recognised RNA binding domain and to identify a potential interaction  
141 mechanism we relied on homology modelling with the closest homologue in the PDB database, O-linked  
142  $\beta$ -N-acetylglucosamine transferase (PDB code 1w3b; **Fig. 3e, Supplementary Fig. 7**)<sup>23</sup>. The model  
143 shows a superhelical structure of the several tetratricopeptide repeats with an extended groove winding  
144 along the longitudinal axis of the protein (**Fig. 3e, Supplementary Fig. 7**). Large patches of positively  
145 charged surfaces (blue) can be seen both in the central part of the groove and in C-terminal part of the  
146 protein. We identified individual residues different between IFIT1 and IFIT3, mutated these residues into  
147 the IFIT3 identity and tested for PPP-RNA binding. Only R187H showed a significant loss of association  
148 (**Fig. 3f, Supplementary Fig. 8a**). In these experiments tagged IFIT3 was co-expressed, allowing the  
149 demonstration that without a functional PPP-RNA binding moiety, as in the case of the IFIT1(R187H),  
150 IFIT3 will not co-purify with PPP-RNA (**Fig. 3f**). Importantly, IFIT1(R187H) maintained its ability to  
151 associate with IFIT3 as shown by co-immunoprecipitation experiments and gel filtration (**Supplementary**  
152 **Fig. 8b, c**) indicating that the R187H mutation is not associated with a major folding problem of the  
153 protein. To quantify the binding capabilities of wild-type (wt) IFIT1 compared to the mutant we used  
154 PPP-RNA- and OH-RNA-coated ELISA plates and found that only the intact IFIT1 displayed a  
155 significant affinity for PPP-RNA and none of the other combinations (**Fig. 3g**). To obtain binding

156 affinities we then used surface plasmon resonance and measured an estimated binding constant of  
157 recombinant IFIT1 for PPP-RNA of 242 nM and a 10-20 fold lower affinity of IFIT1 for OH-RNA (3.14  
158 mM) or IFIT1(R187H) for PPP-RNA (4.36 mM) and OH-RNA (2.64 mM) (**Fig. 3h**). Altogether these  
159 experiments demonstrate that IFIT1 has the ability to bind directly and specifically to PPP-RNA.  
160 Moreover, the data strongly suggest that TPR motifs, such as the ones present in the IFIT1 protein, have  
161 the ability to convey specific interactions with nucleic acids, further expanding their well characterised  
162 protein-protein interaction property <sup>24</sup>.

### 163 **Sequestration of PPP-RNA by IFIT proteins**

164 Previous studies suggested that IFIT1 suppresses *in vitro* translation through binding eIF3e <sup>17,25</sup>. While  
165 we were able to confirm an overall negative effect of IFITs in PPP-RNA programmed translation assays  
166 using rabbit reticulocyte lysates <sup>17,25</sup>, in our experiments it strongly correlated with the RNA-binding  
167 properties of the different IFITs. Since the commonly used templates generated by *in vitro* transcription  
168 are not capped and contain a triphosphate group at the 5' end our findings suggest a simple mechanism  
169 involving PPP-RNA sequestration for the observed inhibitory effects. Accordingly, IFIT1 and IFIT5, the  
170 only two family members capable of binding PPP-RNA directly, most efficiently interfered with the assay  
171 (**Fig. 4a**). If sequestration was indeed involved it should be antagonised by excess template. To directly  
172 test this hypothesis we increased the amount of template RNA and assayed the ensuing translation  
173 efficiency. The inhibitory effect of IFIT1 was inversely proportional to the amount of template RNA used  
174 in these assays (**Fig. 4b**) and depended on the presence of triphosphates on the 5' end (**Fig. 4c**). To finally  
175 prove that it is PPP-RNA binding that lies at the center of the inhibitory effect we used the IFIT1 mutated  
176 in R187 to find that IFIT1(R187H) was indeed less effective (**Fig. 4d**). To further exclude any possible  
177 interference with the translational machinery based on protein-protein interaction properties we choose  
178 the translational assay obtained from wheat germ extract. IFIT1 had an inhibitory effect comparable to the  
179 one observed with rodent-derived extracts (**Fig. 4e**). As evolutionary distance between plants and animals  
180 dates more than 1.5 billion years <sup>26</sup> and plants do not appear to encode IFIT orthologs <sup>27</sup> it makes specific,

181 mechanistically meaningful effects on the translational machinery through a protein-protein interaction  
182 extremely unlikely. Altogether, this set of data is compatible with the ability of IFIT1 to sequester PPP-  
183 RNA and offers a simple mechanism for the negative effects in translational assays.

184 To test whether IFIT1 has the ability to engage viral RNA also in infected cells, we precipitated tagged  
185 IFIT1 or tagged GFP as control from cells infected with vesicular stomatitis virus (VSV) or influenza A  
186 virus (FluAV) and tested the association of viral RNA. IFIT1 but not GFP precipitated viral RNA (**Fig.**  
187 **4f, g**) suggesting that IFIT1 can also bind and potentially sequester viral RNA in cells.

### 188 **Antiviral effects of IFIT family members**

189 As IFIT1 participates in a protein complex containing stoichiometric amounts of IFIT2 and IFIT3, to test  
190 antiviral activity we addressed all three family members and also where appropriate the IFIT1 ortholog  
191 IFIT5. Consistent with the requirement for the formation of a protein complex, overexpression of  
192 individual family members did not impair virus growth (**Supplementary Fig. 9** and data not shown).  
193 siRNA knockdown of IFIT members in HeLa cells effectively and specifically caused reduction of  
194 transcript levels and expression of the cognate protein (**Fig. 5a, b, Supplementary Fig. 10 a-d**) but did  
195 not influence induction of IFN- $\beta$  mRNA (**Supplementary Fig. 10e**). Loss of IFIT family members led to  
196 an increase in growth of VSV, VSV-M2 (mutated in the Matrix protein, M51R leading to IFN- $\beta$   
197 induction) and Rift valley fever virus (RVFV Clone13) to different degrees, with IFIT1 and IFIT2 being  
198 most efficient (**Fig. 5c-e, Supplementary Fig. 11a-e**). In contrast, growth of encephalomyocarditis virus  
199 (EMCV) was not significantly affected by the siRNA treatments (**Fig. 5f, Supplementary Fig. 11f**),  
200 consistent with the notion that EMCV does not generate PPP-RNA during its replication cycle<sup>28</sup>. Similar  
201 to other PPP-RNA generating viruses, also the replication of FluAV, as measured by activation of a  
202 polymerase-I promoter read-out, increased in the absence of IFIT1, IFIT2 and IFIT3, suggesting that the  
203 entire IFIT1 complex is involved in antiviral activities against influenza (**Fig. 5g**). Collectively our data  
204 suggest that members of the IFIT family contribute to the antiviral response against several PPP-RNA

205 producing viruses. The contribution of the different family members may differ depending on the nature  
206 of the microbial agent. As the affinity of IFIT1 to PPP-RNA constitutes a central feature of the IFIT1  
207 complex, we directly tested its importance for antiviral activity. For this we expressed siRNA-resistant  
208 versions of wt IFIT1 and the PPP-RNA binding mutant IFIT1(R187H) (**Fig. 5h**), respectively, in cells  
209 that were treated with siRNA against IFIT1. We used as read-out the FluAV polymerase-I dependent  
210 transcriptional assay to observe that the PPP-RNA binding impaired IFIT1(R187H) mutant was  
211 considerably less able to constrain viral replication as compared to wt IFIT1 (**Fig. 5i**). Taken together  
212 these data clearly show that the requirements for an efficient antiviral activity include the presence of all  
213 three family members, IFIT1, IFIT2 and IFIT3, and the PPP-RNA binding capability of IFIT1.

#### 214 **Ifit1 displays antiviral activity *in vivo***

215 Mice should be a particularly suitable model system to study IFIT activity since mouse *Ifit1* is the only  
216 family member binding PPP-RNA and knockdown cell lines using shRNA against *Ifit1* were impaired in  
217 their ability to contain virus growth in the presence of IFN- $\beta$  (**Supplementary Fig. 11 g-j**). We generated  
218 mice with a deletion in the *Ifit1* gene (**Fig. 6a**). *Ifit1* deficiency was confirmed by quantitative PCR (**Fig.**  
219 **6b**) and immunoblotting of lysates from IFN- $\beta$  stimulated MEFs (**Fig. 6c**). The absence of mouse *Ifit1*  
220 was not due to defective signalling downstream of the type-I interferon receptor since the interferon  
221 responsive protein DAI<sup>29</sup> was induced upon IFN- $\beta$  treatment (**Fig. 6c**). Under specific pathogen-free  
222 (SPF) conditions, mice lacking *Ifit1* showed no phenotypic abnormalities and were undistinguishable  
223 from wt C57BL/6 mice.

224 IFITs have been proposed to regulate cytokine expression<sup>30,31</sup>. However, *Ifit1* deficiency did not change  
225 phosphorylation of the transcription factor IRF3 in response to transfection of innate immune stimuli  
226 (**Supplementary Fig. 12a**). Transfecting PPP-RNA, viral RNA derived from VSV particles (vRNA),  
227 poly-I:C, interferon stimulatory DNA (ISD) or poly-dA:dT, or activation of the TLR pathway through  
228 CpG and LPS did not yield significantly different amounts of type-I IFN- and IL-6 protein in MEFs, *ex*

229 *in vivo* bone-marrow, bone marrow-derived macrophages and bone marrow-derived dendritic cells (**Fig. 6d**,  
230 **Supplementary Fig. 12b-g**). Neither was the induction of IFN- $\alpha/\beta$  and IL-6 protein by viral infection  
231 affected by *Ifit1* deficiency (data not shown). We therefore concluded that Ifit1 is dispensable for  
232 induction of antiviral cytokines. In contrast, Ifit1-deficient cells allowed consistent higher VSV  
233 accumulation compared to wt counterparts at three different time points tested (**Fig. 6e**). EMCV infected  
234 MEFs showed equal viral loads irrespective of the genetic status of the *Ifit1* gene (**Fig. 6f**).

235 To establish an antiviral function of Ifit1 *in vivo* we infected *Ifit1* knockout mice with VSV. At all doses  
236 tested, Ifit1 deficient mice showed significantly reduced survival as compared to control mice (**Fig. 6g**,  
237 **Supplementary Fig. 13a** and data not shown), suggesting that control of VSV infection required Ifit1  
238 also *in vivo*. In contrast, absence of Ifit1 did not affect viability of mice infected with EMCV (**Fig. 6h**,  
239 **Supplementary Fig. 13b**). Similar to EMCV, Ifit1 seemed to be dispensable for the clearance of *Listeria*  
240 *monocytogenes*, a bacterium known to predominantly engage DNA-sensing pathways<sup>32, 33</sup> (**Fig. 6i**,  
241 **Supplementary Fig. 13c**). We concluded that *in vivo*, Ifit1 manifests a critical activity against VSV and  
242 presumably other PPP-RNA-expressing viruses but not against the other pathogens tested here.

243 Overall we conclude that the IFIT proteins contribute to an executing branch of the PPP-RNA innate  
244 immunity molecular network. While RIG-I represents the PPP-RNA sensing module that signals towards  
245 type-I interferon production, interferon causes a feed-back mechanism that ensures the arming of cells  
246 with PPP-RNA-binding antiviral proteins, such as IFIT1, IFIT5 and the protein complexes that they form.  
247 (**Supplementary Fig. 14**).

248

249

## 250 **Discussion**

251 IFIT1 demands the regular attention of immunologists, since it is encoded by one of the most abundantly  
252 IFN- $\alpha/\beta$  induced mRNAs. So far most evidence has been gathered for it being a general inhibitor of  
253 protein translation<sup>17</sup>. Recently, however, elegant studies using viruses defective in their ability to  
254 methylate mRNA CAP structures at the 2'O-position and *Ifit1* and *Ifit2* deficient mice identified an  
255 intriguing correlation between specific 5' nucleic acid conformations and Ifit function<sup>14</sup> for which the  
256 present study offers a mechanistic rationale. While IFIT1 is shown here to bind PPP-RNA, IFIT2 and  
257 IFIT3 also have a virus-containing function and all three proteins form a complex that contains yet other  
258 family members as well as other RNA-binding proteins. This raises the possibility that the IFIT complex  
259 represents multiple RNA-binding valencies able to recognise and counteract a yet to be determined  
260 spectrum of microbes. The IFIT versatility may well reside in the modular use of TPRs, shown here to  
261 have nucleic acid binding capability, in analogy to the role of leucine-rich repeats that confer binding  
262 plasticity to another family of PRR, namely the Toll-like receptors. Unlike these, IFITs are strongly  
263 induced during infection and reach expression levels beyond a million copies per cell. This abundance,  
264 rather than with the signalling roles of receptors, may be more compatible with an executing function. We  
265 therefore suggest a general model whereby IFIT proteins exert their antiviral activity by physically  
266 engaging microbial elements. In particular the present work focuses on the 5' conformation of RNAs such  
267 as it is present on the genomic, antigenomic and some transcripts of certain virus species. While members  
268 of the RIG-I helicases represent the PPP-RNA binding components of the sensing and interferon  
269 induction branch of the innate immunity molecular network, we here propose that IFIT family members  
270 represent the PPP-RNA binding component of an executing antiviral branch of the network. The final fate  
271 of the PPP-RNA physically sequestered by the IFIT complex remains to be elucidated. Sequestration of  
272 viral components has been described before in the case of orthomyxovirus resistance (Mx) proteins  
273 known to physically inhibit assembly of viral particles though binding viral proteins<sup>34</sup>. Some viruses

274 generate large amounts of small triphosphorylated leader-RNAs which could potentially antagonise IFIT  
275 activity<sup>35</sup>. We suggest that similarly to the diverse set of proteins sensing the variety of PAMPs and  
276 triggering the anti-pathogen response, also the abundant proteins executing the response itself need to  
277 maintain specificity for defined pathogen structures to limit interference with vital host processes.

278

279 **Database accession numbers**

280 Mass spectrometry data presented in Figure 2 was deposited in the IntAct database <sup>21</sup>, identifier: IM-  
281 15277.



282

283 **Table 1**

|              | <b>Bait protein</b> |              |               |              |               |              |
|--------------|---------------------|--------------|---------------|--------------|---------------|--------------|
|              | <b>IFIT1</b>        |              | <b>IFIT2</b>  |              | <b>IFIT3</b>  |              |
|              | <b>No IFN</b>       | <b>+ IFN</b> | <b>No IFN</b> | <b>+ IFN</b> | <b>No IFN</b> | <b>+ IFN</b> |
| <b>IFIT1</b> | 19                  | 34           | 5             | 29           | 14            | 32           |
| <b>IFIT2</b> | 0                   | 17           | 24            | 25           | 0             | 19           |
| <b>IFIT3</b> | 0                   | 29           | 5             | 25           | 32            | 28           |
| <b>IFIT5</b> | 0                   | 0            | 0             | 0            | 0             | 0            |

284

285 HEK-FlpIN cells were stimulated with 1  $\mu\text{g/ml}$  doxycycline for 24 h to induce expression of IFIT1. Cells  
286 were left untreated or treated overnight with approximately 1000 U/ml IFN- $\alpha/\beta$  that was generated by  
287 transfecting HEK293 cells with poly-I:C. Protein complexes isolated by tandem affinity purification were  
288 analysed by LC-MSMS. The table shows number of identified IFIT peptides in precipitations of IFITs  
289 (Bait proteins) in presence or absence of IFN- $\alpha/\beta$ , as indicated.

290

## 291 **Figure Legends**

292 **Figure 1: Identification of an IFN- $\alpha/\beta$ -induced IFIT containing complex as a PPP-RNA binding**  
293 **entity**

294 **(a)** HEK293 cells were left untreated or treated with 1000 U/ml IFN- $\beta$  overnight. Cells were lysed and  
295 incubated with PPP-RNA or OH-RNA coupled to streptavidin beads. After precipitation, bead-associated  
296 proteins were eluted, separated by 1D SDS PAGE electrophoresis and whole lanes analysed by Liquid  
297 Chromatography-Mass Spectrometry (LC-MSMS). Identified proteins are represented as dots with  
298 detection strength (log of spectral count) in OH-RNA pull downs (x-axis) and PPP-RNA pull downs (y-  
299 axis), both in IFN- $\beta$  stimulated conditions. Red dots represent proteins with no detection in the absence of  
300 IFN- $\beta$  in both OH-RNA and PPP-RNA pull downs. IFIT proteins are by far the strongest hits. IFIT5 is  
301 gray due to detection in the pull down done in the absence of IFN- $\beta$  priming. Data from four experiments  
302 is shown. **(b)**  $10^6$  HeLa cells were treated with the indicated amount of recombinant IFN- $\beta$  for 16h and  
303 the lysates, alongside a recombinant IFIT1 standard, were analysed by immunoblotting for IFIT1 and  
304 tubulin. The signal was quantified using infrared imaging. The cellular copy number of IFIT1 in per HeLa  
305 cells treated with 1000 U/ml IFN- $\beta$  was determined to be  $2,4 * 10^6$ . One of two experiments done in  
306 duplicate is shown. **(c, d)** Lysates from 293T cells transfected with plasmids for c-Myc-tagged IFITs **(c)**  
307 and *E. coli* expressing His-GST-tagged IFITs **(d)** were used for affinity precipitation with PPP-RNA and  
308 associated proteins analysed by immunoblotting.

309

310 **Figure 2: Formation of a complex containing IFIT proteins**

311 **(a)** Recombinant IFIT proteins and their binary complexes were analyzed by size-exclusion  
312 chromatography. Shown are overlaid elution profiles from Superdex 200 10/300 GL column (the void  
313 volume is ~8.3 ml), and the indicated peak fractions were analyzed by SDS-PAGE followed by  
314 coomassie staining. His-tagged IFIT1 binds His-GST-tagged IFIT2. **(b, c)** Network analysis of the IFIT  
315 protein complex based on data described in Table 1. **(b)** The IFIT proteins (large balls) in absence of IFN-  
316  $\alpha/\beta$  stimulation (left) are interacting with fewer proteins (small balls) whereas upon IFN- $\alpha/\beta$  stimulation  
317 IFITs recruit many new partners. Interactions between IFIT-1, -2, and -3 are also stronger. Proteins  
318 identified by all IFITs are shown in red. **(c)** Protein interaction network for the IFN- $\alpha/\beta$  stimulated  
319 condition and annotated protein functions using Gene Ontology (GO) molecular functions and manual  
320 curation. Obvious non-specific proteins or contaminants were removed (keratin, albumin from MS BSA  
321 quality control runs, and MCC12 and PCCAB which bind to the Strep-tactin affinity resin in high  
322 abundance). Many of the shared IFIT partners have the ability to bind to RNA (red) and some are  
323 involved in mRNA translation (green). IFIT bait proteins are shown in blue.

324

### 325 **Figure 3: Triphosphate-dependent RNA-binding of IFIT1 requires an Arginine at position 187**

326 **(a)** Ifit1 redistribution (white arrows) in IFN- $\beta$  - treated NIH 3T3 cells transfected with biotinylated PPP-  
327 RNA and OH-RNA for 3 h. Shown is the average % relocalisation of Ifit1 (+/- standard deviation) in 100  
328 randomly selected cells in two independent experiments. \* =  $p < 0,05$ . **(b, c)** PPP-RNA or OH-RNA  
329 beads were used for affinity purification from lysates of 293T cells expressing c-Myc-IFIT1 or GFP-RIG-  
330 I **(b)** or IFN- $\beta$  treated HEK293 cells and MEFs **(c)**. Precipitates were analysed by immunoblotting. **(d)**  
331 Mobility shift assay of PPP-RNA and OH-RNA by recombinant His- GST-IFIT1 and -IFIT3. Where  
332 indicated an antibody against GST was added. Numbers on the right indicate free probe (1), shifted probe  
333 (2) and supershifted probe (3). **(e)** Surface charge of an IFIT1 structure model based on O-linked  $\beta$ -N-  
334 acetylglucosamine transferase (PDB code 1w3b). Surface colour represents electrostatic potential, red is

335 negative, blue is positive charge, N is N-terminus, C is C-terminus. Proteins with targeted point mutations  
336 of the indicated residues were used for further functional characterisation. **(f)** c-Myc-tagged IFIT1  
337 mutants and HA-IFIT3 were co-expressed in 293T cells and 24 h later used for affinity purification using  
338 PPP-RNA as bait. **(g)** PPP-RNA or OH-RNA were bound to ELISA plates and incubated with the  
339 indicated amounts (ng) of recombinant IFIT1 or IFIT1(R187H). RNA-associated proteins were detected  
340 using secondary reagents. Shown is substrate conversion at OD 450, error bars show standard deviation of  
341 triplicate measurements. One representative experiment of three is shown. **(h)** The affinity of IFIT1 and  
342 IFIT1(R187H) to PPP-RNA and OH-RNA was measured by surface plasmon resonance using  
343 biotinylated RNA as immobilised ligand and increasing amounts of recombinant protein. Shown are the  
344 response units of the indicated combinations of binding partners with standard deviation from duplicate  
345 measurements.

346

#### 347 **Figure 4: IFIT1 sequesters PPP-RNA *in vitro***

348 **(a-d)** Rabbit reticulate lysate (RRL) or **(e)** wheat germ extract (WGE) was supplemented with RNA  
349 template expressing firefly-luciferase and recombinant IFITs or no protein was added. **(a, d, e)** 0.2 µg of  
350 *in vitro* transcribed PPP-RNA template (that is commonly used in such assays) was incubated with the  
351 indicated amounts of recombinant IFITs or no protein. **(b)** As in **(a)** but 0.2 µg and 0.05 µg template RNA  
352 were used. **(c)** RNA that was not (PPP-luc) or was treated with calf intestinal phosphatase (OH-luc) was  
353 supplemented together with 35 µM IFIT1, as indicated. **(d)** Translation of PPP-luc mRNA template in the  
354 presence of 35 µM IFIT3, IFIT1 or IFIT1(R187H). **(a-e)** The graph shows luciferase activity after an 1 h  
355 incubation period at 37 °C. Error bars show standard deviation of at least two experiments done in  
356 triplicate measurements. \* = p<0,05, n.s. = non significant. **(f-g)** HEK-FlpIN IFIT1 or HEK-FlpIN GFP  
357 cells were stimulated with doxycycline for 24 h and infected with VSV-GFP and FluAV (both MOI: 5)  
358 for 9 h. Cells were then lysed and proteins precipitated using Strep-tactin beads. RNA before (Input) and

359 after precipitation (SII-IP) was analysed by qRT-PCR for VSV **(f)** or FluAV sequences **(g)**. The graph  
360 shows arbitrary units +/- standard deviation of duplicate measurements of one representative experiment  
361 of three **(f)** or two **(g)**.

362

### 363 **Figure 5: Influence of IFIT RNA interference on virus growth**

364 **(a)**  $10^5$  HeLa cells were transfected with 0.5  $\mu\text{g}$  of the indicated IFIT expression vector and 5 nM siRNA  
365 directed against the indicated IFIT family member. Expression of c-Myc-tagged proteins was evaluated  
366 by immunoblot 48 h later. **(b-f)** HeLa cells were transfected with 5 nM siRNA for 48 h. **(b)** Cells were  
367 stimulated with 0.25  $\mu\text{g}$  PPP-RNA for 16 h and expression of IFIT1 or IFIT3 was tested by  
368 immunoblotting. **(c-f)** siRNA treated HeLa cells were infected at a multiplicity of infection (MOI) of 0.01  
369 with VSV **(c)**, VSV-M2 (with a M51R mutation in the matrix protein)<sup>36</sup> **(d)**, RVFV (Clone13) **(e)** or  
370 EMCV **(f)** and virus accumulation was tested by TCID50 at 48 h **(c, d, f)** and 72 h **(e)** after infection.  
371 Graphs in **(c-f)** show the average of three independent experiments, error bars indicate standard deviation.  
372 **(g)** HeLa cells were co-transfected with Pol-I ff-luc (0.1  $\mu\text{g}$ ), pRL-TK (0.05  $\mu\text{g}$ ) reporter plasmids and the  
373 indicated siRNAs. 48 h later cells were left uninfected or infected with FluAV at a MOI of 1 and reporter  
374 activity analysed after over-night incubation. The graph shows the ratio between firefly- and renilla  
375 luciferase +/- standard deviation of one representative experiment of two done in duplicate measurements.  
376 **(h)** HeLa cells were co-transfected with siRNA against IFIT1 or control siRNA together with plasmids  
377 coding for c-Myc-tagged versions of parental or silencing-resistant IFIT1. Immunoblots 48 h after  
378 transfection are shown. **(i)** as in **(g)** but plasmids coding for silencing-resistant IFIT1 were co-transfected  
379 as indicated. The graph shows the ratio between firefly- and renilla luciferase +/- standard deviation of  
380 one representative experiment of three done in duplicate measurements.

381

### 382 **Figure 6: Ifit1 is necessary to contain virus growth and *in vivo* pathogenicity**

383 **(a)** Targeting strategy for mouse *Ifit1*. **(b, c)** Loss of *Ifit1* in *Ifit1*<sup>+/+</sup> MEFs (+/+) and *Ifit1*<sup>-/-</sup> MEFs (-/-) was  
384 validated by PCR **(b)** and by immunoblotting in MEFs that were stimulated with IFN- $\beta$  for 16 h **(c)**. **(d)**  
385 MEFs ( $2 \times 10^5$  cells/ml) were left unstimulated or transfected with PPP-RNA (0.4  $\mu$ g/ml and 0.08  $\mu$ g/ml),  
386 viral RNA isolated from VSV particles (vRNA) (0.2  $\mu$ g/ml), poly-I:C (1  $\mu$ g/ml) or poly-dA:dT (1  $\mu$ g/ml)  
387 and accumulation of IFN- $\alpha/\beta$  was tested using a cell line stably containing an ISRE-luc reporter. **(e, f)**  
388 MEFs of the indicated genotype were infected with VSV **(e)** or EMCV **(f)** at a MOI of 0.01 and virus  
389 accumulation in the cell supernatant was measured by TCID50 after 48 h. Graphs show average virus  
390 titers from two independent experiments. Error bars show standard deviation. \* =  $p < 0.05$  tested by two  
391 way Anova for two independent experiments done in hexaplicate measurements. **(g-i)** Survival of *Ifit1*  
392 deficient (*Ifit1*<sup>-/-</sup>) (red lines) and C57BL/6 mice (*Ifit1*<sup>+/+</sup>) (black lines). **(g)** Male animals (n = 14) were  
393 anesthetized with ketamine-xylazine and infected intranasally with  $10^5$  pfu of VSV and monitored twice  
394 daily for survival over a two week period. Wt mice survived significantly longer than *Ifit1* deficient  
395 animals (Mantel-Cox Test  $p < 0.01$ ). **(h)** Sex-matched *Ifit1*<sup>-/-</sup> and *Ifit1*<sup>+/+</sup> mice (n = 17) were infected  
396 intraperitoneally with 500 pfu of EMCV and monitored for survival. **(i)** Female *Ifit1*<sup>-/-</sup> and *Ifit1*<sup>+/+</sup> mice (n  
397 = 9) were infected intraperitoneally with  $10^6$  CFU *L. monocytogenes*. d.p.i.: days post infection.

398

## 399 **Acknowledgements**

400 We want to thank the NIH Knock-out Mouse Project (KOMP) for providing ES cells with a targeted Ifit1  
401 allele. We want to thank Lill Andersen for expansion of ES cells, Kumaran Kandasamy for bioinformatics  
402 support, Michael Bergmann for providing FluAV matrix protein antibody. The work in the authors'  
403 laboratories was funded by the Austrian Academy of Sciences, the i-FIVE ERC grant to GSF, an EMBO  
404 long-term fellowship to AP (ATLF 463-2008), a Marie-Curie and an EMBO fellowship to CB, DFG grant  
405 We 2616/5-2 and SFB 593/B13 to FW. TR and MM are funded by the Austrian Federal Ministry for  
406 Science and Research GEN-AU programme Austromouse; MM is funded by the Austrian Science Fund  
407 FWF SFB F28.

408

## 409 **Methods**

### 410 **Reagents, proteins and viruses**

411 IFN- $\alpha$  and IFN- $\beta$  were from PBL Interferonsource. IFN- $\alpha/\beta$  was generated by transfecting HEK293 cells  
412 with poly-I:C. Expression constructs were generated by PCR amplification and cloned into pCS2-6myc-  
413 GW, pCDNA-HA-GW, pTO-SII-HA-GW<sup>20</sup> or pETG30A-GW and pETG10A-GW. Point mutations were  
414 introduced by site directed mutagenesis. Pol-I ff-luc was from Georg Kochs<sup>37</sup>. p7SK-as and pGFP-RIG-I  
415 were described earlier<sup>3</sup>. pRL-TK was from Promega. *In vitro* translation was done with Rabbit  
416 reticulocyte lysate or Wheat germ extract (Promega) using the provided luciferase mRNA or SP6-  
417 polymerase transcribed luciferase mRNA as template. Strep-tacin beads were from IBA, HA-agarose  
418 from Sigma, Protein G sepharose was from GE Healthcare and Streptavidin beads from Pierce.  
419 Antibodies for  $\alpha$ -tubulin and  $\beta$ -actin were from Alexis. Phospho-IRF3 was from Cell Signalling. IRDye -  
420 conjugated anti-myc antibody, anti-mouse and anti-rabbit secondary reagents were from Rockland.  
421 Streptavidin Alexa-800, Streptavidin Alexa-488 and goat anti-mouse Alexa-548 were from Molecular  
422 probes. Polyclonal antibodies against rb- $\alpha$ -DAI, rb- $\alpha$ -IFIT1, ms- $\alpha$ -Ifit1 and rb- $\alpha$ -IFIT3 were generated  
423 by immunisation of animals with full-length recombinant protein. RT-PCR reagents were from Qiagen.  
424 Biotinylated PPP-RNA (7SK-as) was described earlier<sup>3</sup>. PPP-RNA was dephosphorylated using Calf  
425 intestinal phosphatase (New England Biolabs). LPS (E.coli K12), CpG (CpG-DNA-ODN1826), poly-  
426 (I:C) and poly-(dA:dT) were from Invitrogen. ISD<sup>33</sup> was synthesised at Microsynth. vRNA was isolated  
427 using Trizol (Invitrogen). For stimulation TLR agonists were added other stimuli were transfected with  
428 Lipofectamine 2000 (Invitrogen) or Polyfect (Qiagen). Total IFN- $\alpha/\beta$  was measured as described<sup>38</sup>. IL-6  
429 was measured by ELISA (BD).

430 Recombinant IFITs were expressed in *E. Coli* and purified on a HisTrap HP column (GE Healthcare).

431 EMCV, FluAV (A/PR/8/34), VSV (strain: Indiana)<sup>38</sup>, VSV-GFP<sup>39</sup>, VSV-M2 (M51R, originally named



432 AV1)<sup>36</sup>, RVFV (Clone 13)<sup>40</sup> and *Listeria monocytogenes* (EGD)<sup>41</sup> were described earlier. Viruses were  
433 titrated on Vero cells using the TCID50 method of Reed and Muench.

#### 434 **Cells, mice and *in vivo* experiments**

435 293T, NIH3T3 and HEK293 cells were described earlier<sup>3</sup>. IRF3 deficient MEFs were a gift of Thomas  
436 Decker. Doxycycline regulatable HEK-FlpIN cells were from Invitrogen. MEFs were generated from  
437 embryos of mated *Ifit1*<sup>+/-</sup> mice. BM macrophages (BMMs) were cultured in the presence of M-CSF  
438 (Preprotech), BM dendritic cells (BM-DC) in presence of GM-CSF (Preprotech). Fibroblasts were kept in  
439 DMEM (PAA) and primary cells cultured in RPMI (PAA) supplemented with 10 % fetal calf serum  
440 (Invitrogen) and antibiotics (100 U/ml penicillin, 100 µg/ml streptomycin). For inducible transgene  
441 expression HEK-FlpIN cells were treated with 1 µg/ml doxycycline for 24-48 h. For siRNA knockdown,  
442 5nmol siRNA was mixed with HiPerfect (Qiagen) and added to 10<sup>5</sup> HeLa cells. 48 h later cells were used  
443 for experiments. Sequences of shRNA vectors and siRNA knockdown oligos are available on request.  
444 *Ifit1* knockout mice were generated using ES cells clones (VGB6; C57BL/6NTac background) with a  
445 targeted *Ifit1* locus. ES cells were provided by the NIH-knockout mouse project (KOMP, NIH). C57BL/6  
446 wild-type control mice were purchased from Charles River. All mice were kept under specific pathogen  
447 free conditions according to FELASA recommendations. For EMCV infections age (9-11 weeks) and sex-  
448 matched mice were infected intraperitoneally, for *Listeria monocytogenes* (EGD) age-matched (8-11  
449 weeks) females were infected intraperitoneally. For VSV challenge, age-matched (8-11 weeks) male mice  
450 were anesthetised with ketamine-xylazine and inoculated intranasally with VSV. All animal experiments  
451 were approved by the institutional ethics committee and the Austrian laws (GZ 68.205/0057-II/10b/2010).

#### 452 **RT-PCR, Immunofluorescence, gel shift assays, protein quantification**

453 RNA was isolated using RNeasy kit (Qiagen) and reverse transcribed using oligo-dT primers and the  
454 RevertAID RT-PCR kit (Fermentas). NIH3T3 cells were grown overnight on coverslips and stimulated as  
455 described in figure legends. Cells were stained with murine anti-*Ifit1* antibodies, followed by anti-mouse

456 Alexa-548, Alexa-488-Streptavidin and DAPI. Images were acquired with a Leica AF6000 deconvolution  
457 microscope. For gel shift assays 200 ng biotinylated 7SK-as RNA<sup>3</sup> supplemented with Alexa-800-  
458 Streptavidin was incubated with 12,5 µg recombinant His-GST-IFIT1 or His-GST-IFIT3 protein solved in  
459 PBS supplemented with RNAsin (Promega) (1:20), DTT (final volume 400 mM) and 100 mM NaCl.  
460 Where shown, GST antibody (1 µg) was added. Samples were run on a 1 % Agarose gel and RNA was  
461 visualised using a LI-COR Odyssey system. To estimate the protein copy number of IFIT1 in cells,  
462 recombinant IFIT1 was used as calibration standard and compared to lysates of IFN-β stimulated HeLa  
463 and 293T cells. The signal intensity on western blots was quantified using a LI-COR Odyssey system.

#### 464 **Affinity purifications and measurements, mass spectrometry and homology modelling**

465 For RNA precipitation 5 µg PPP-RNA or OH-RNA (both 7SK-as) were added to streptavidin resin, and  
466 incubated with 6 mg of HEK293 cell lysate for 60 minutes. Beads were washed three times in TAP-buffer  
467 (50 mM Tris pH 7.5, 100 mM NaCl, 5 % (v/v) glycerol, 0.2 % (v/v) Nonidet-P40, 1.5 mM MgCl<sub>2</sub> and  
468 protease inhibitor cocktail (Complete, Roche)), proteins eluted by boiling in SDS sample buffer and  
469 analysed by one-dimensional SDS-PAGE. Entire gel lanes were analysed by mass spectrometry using a  
470 hybrid LTQ-Orbitrap XL (ThermoFisher Scientific) or a quadrupole time-of-flight mass spectrometer  
471 (QTOF Premier; Waters) coupled to an 1100/1200 series HPLC (Agilent Technologies) with an analytical  
472 column packed with C18 material. Data generated by LC-MSMS was searched against  
473 UniProtKB/SwissProt version 57.12<sup>42</sup> integrating Mascot<sup>43</sup> and Phenyx<sup>44</sup> search engines. A false  
474 discovery rate of less than 1 % on the protein groups was estimated. HEK-FlpIN cells and isolation of  
475 protein complexes for LC-MSMS analysis is described elsewhere<sup>20</sup>. 293T cells were transfected with  
476 respective expression plasmids for 48 h and lysates used for immunoprecipitation using HA-agarose or  
477 RNA-coated beads. For surface plasmon resonance measurements biotinylated 7SK-as RNA was loaded  
478 on a streptavidin coated SA sensor chip (GE Healthcare) and probed with recombinant wild-type or  
479 IFIT1(R187H) diluted in running buffer (0.01 M HEPES, pH 7.4, 0.25 M NaCl, 0.005 % surfactant P20).  
480 Sensorgrams were fitted to a single site binding model (1:1 Langmuir binding), using the numerical

481 integration functions of the BIAevaluation 3.1 software package. To determine the dissociation constant  
482 (KD) the equilibrium-state binding values were plotted as a function of the applied protein concentrations  
483 and fitted to first-order kinetics assuming a monovalent RNA-protein interaction. Comparative modelling  
484 was done using the I-TASSER server (<http://zhanglab.cmb.med.umich.edu/I-TASSER/>)<sup>45</sup> to obtain a  
485 model for full-length IFIT1. The model was based on the structure of O-linked  $\beta$ -N-acetylglucosamine  
486 transferase (PDB code 1w3b), with 17 % sequence identity. Surface charge potential was calculated by  
487 APBS as implemented in PyMOL (DeLano Scientific).

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Figure 1

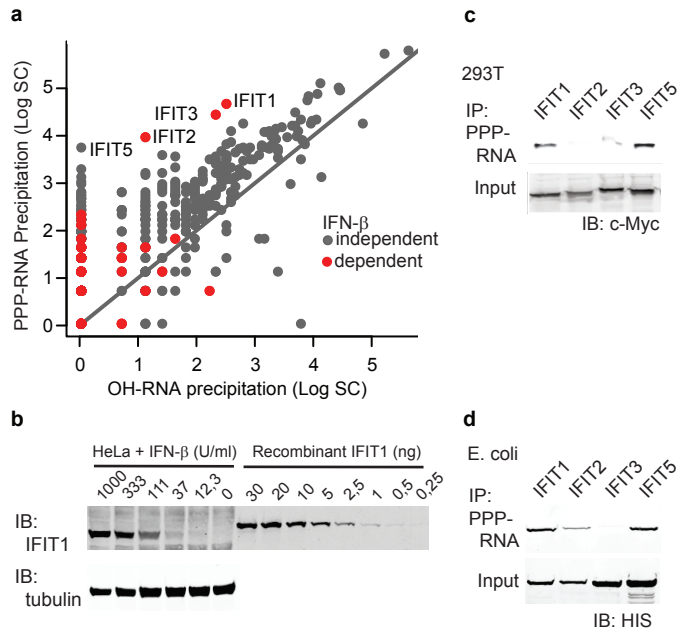


Figure 2

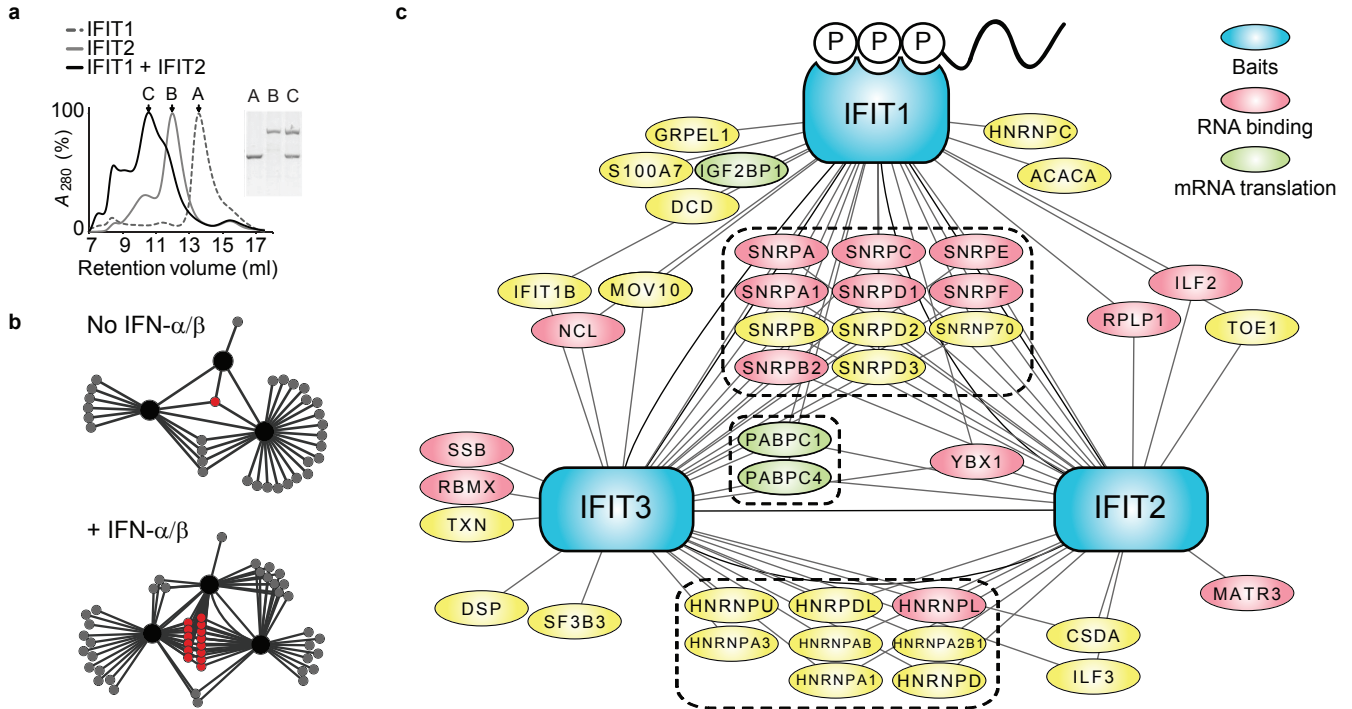




Figure 3

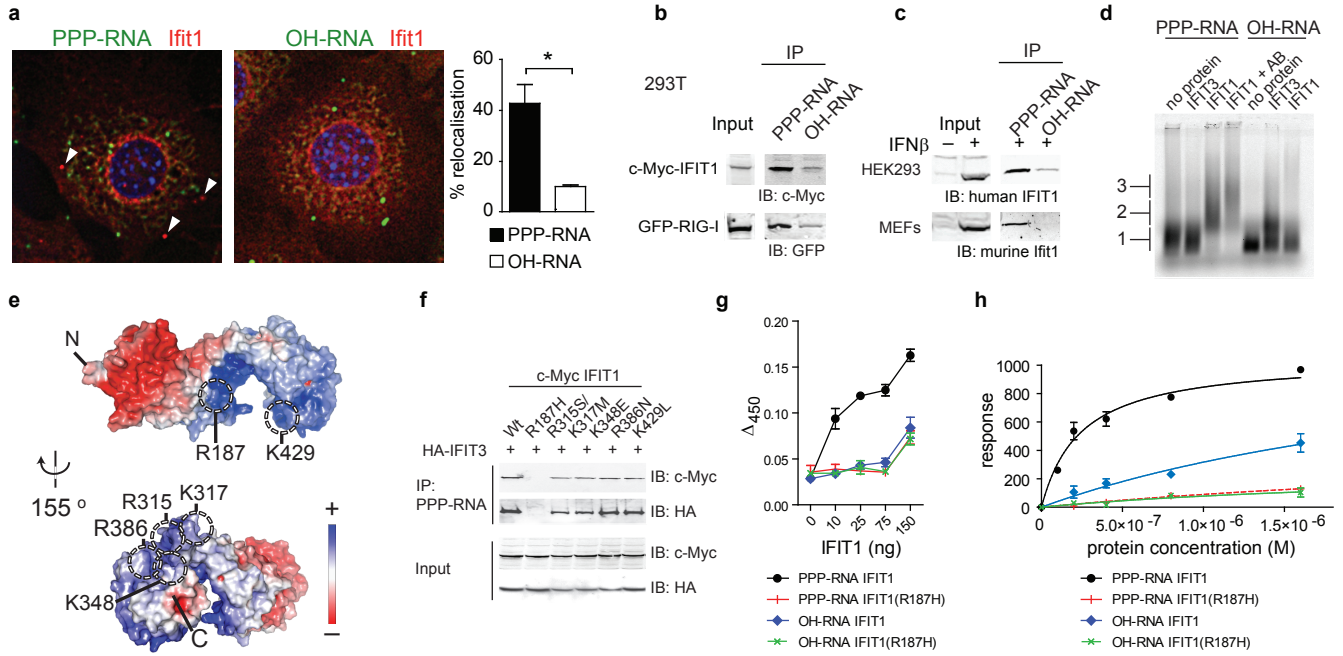


Figure 4

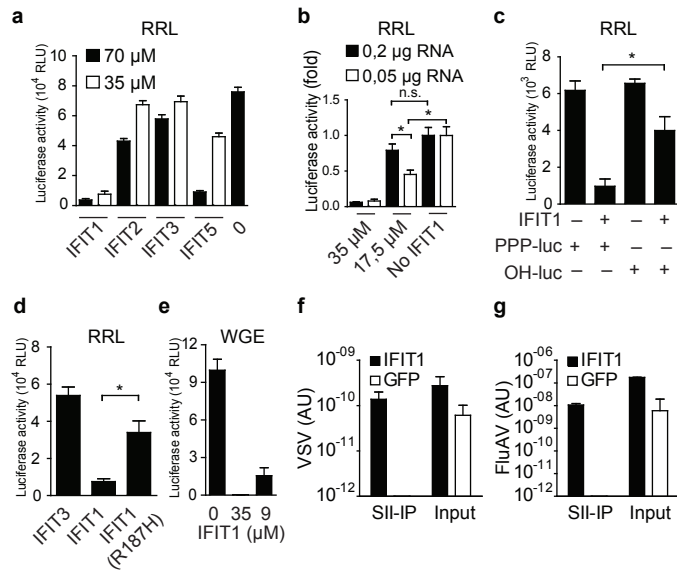


Figure 5

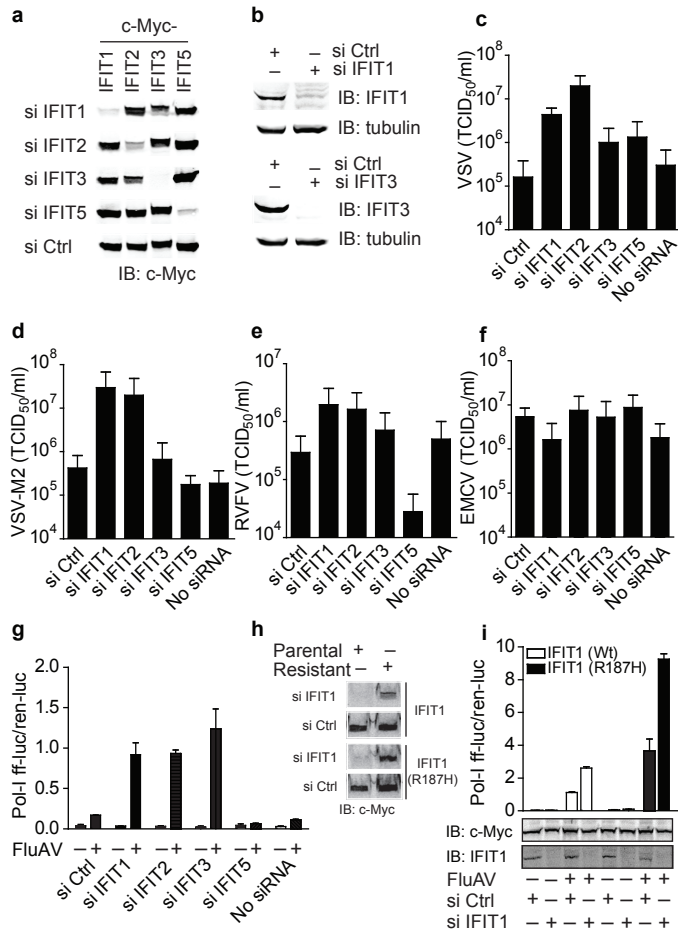


Figure 6

