



**HAL**  
open science

## Functional Heterogeneity of Mammalian IFITM Proteins against HIV-1

Federico Marziali, Mathilde Delpuch, Anuj Kumar, Romain Appourchaux,  
Jérémy Dufloo, Kevin Tartour, Lucie Etienne, Andrea Cimorelli

► **To cite this version:**

Federico Marziali, Mathilde Delpuch, Anuj Kumar, Romain Appourchaux, Jérémy Dufloo, et al..  
Functional Heterogeneity of Mammalian IFITM Proteins against HIV-1. *Journal of Virology*, 2021,  
95 (18), 10.1128/JVI.00439-21 . hal-03370239

**HAL Id: hal-03370239**

**<https://hal.science/hal-03370239>**

Submitted on 13 Oct 2021

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

# 1 Functional heterogeneity of mammalian IFITM proteins against

## 2 HIV-1

3  
4  
5 Federico Marziali, Mathilde Delpeuch, Anuj Kumar, Romain Appourchaux, Jérémy Dufloo<sup>1</sup>,  
6 Kevin Tartour<sup>2</sup>, Lucie Etienne, Andrea Cimorelli §

7  
8 Centre International de Recherche en Infectiologie (CIRI)  
9 Univ Lyon,  
10 Inserm, U1111,  
11 Université Claude Bernard Lyon 1,  
12 CNRS, UMR5308,  
13 ENS de Lyon,  
14 46 Allée d'Italie-69007 Lyon-France  
15  
16

17 **Running title:** IFITM functional heterogeneity against HIV

18  
19 1. Present address: Institut Pasteur, Paris, France

20 2. Present address: IGFL, Lyon, France

21  
22 § **Correspondent footnote:** correspondence should be addressed to Andrea Cimorelli, CIRI,  
23 46 Allée d'Italie, 69364 Lyon, France. E-mail: [acimarel@ens-lyon.fr](mailto:acimarel@ens-lyon.fr)

24  
25 **Keywords:** IFITM, Interferon, HIV, virus, innate immunity, restriction factor

26

27 **ABSTRACT**

28 IFITMs are a family of interferon-inducible proteins that inhibit a broad range of viruses by  
29 interfering with viral to cellular membrane fusion. The antiviral activity of IFITMs is highly  
30 regulated by several post-translational modifications and by a number of protein domains  
31 that modulate steady-state protein levels, trafficking and antiviral effectiveness.

32 Taking advantage of the natural diversity existing among IFITMs of different animal species,  
33 we have compared twenty-one IFITMs for their ability to inhibit HIV-1 at two steps: during  
34 virus entry into cells (target cell protection) and during the production of novel virion  
35 particles (negative imprinting of virion particles' infectivity).

36 We found a high functional heterogeneity among IFITM homologs with respect to both  
37 antiviral modalities, with IFITM members that exhibit enhanced viral inhibition, while others  
38 have no ability to block HIV-1. These differences could not be ascribed to known regulatory  
39 domains and could only be partially explained through differential protein stability, implying  
40 the existence of additional mechanisms. Through the use of chimeras between active and  
41 inactive IFITMs, we demonstrate that the cross-talk between distinct domains of IFITMs is  
42 an important contributor of their antiviral potency. Finally, we identified murine IFITMs as  
43 natural variants competent for target cell protection, but not for negative imprinting of virion  
44 particles' infectivity, suggesting that the two properties may, at least in principle, be  
45 uncoupled.

46 Overall, our results shed new light on the complex relationship between IFITMs and viral  
47 infection and point to the cross-talk between IFITM domains as a novel layer of regulation  
48 of their activity.

49

## 50 **AUTHOR SUMMARY**

51 IFITMs are broad viral inhibitors capable of interfering with both early and late phases of the  
52 replicative cycle of many different viruses. By comparing twenty-one IFITM proteins issued  
53 from different animal species for their ability to inhibit HIV-1, we have identified several  
54 that exhibit either enhanced or impaired antiviral behavior. This functional diversity is not  
55 driven by differences in known domains and can only be partly explained through differential  
56 protein stability. Chimeras between active and inactive IFITMs point to the cross-talk  
57 between individual IFITM domains as important for optimal antiviral activity. Finally, we  
58 show that murine IFITMs are not capable of decreasing the infectivity of newly-produced  
59 HIV-1 virion particles, although they retain target cell protection abilities, suggesting that  
60 these properties may be in principle disconnected.

61 Overall, our results shed new light on the complex layers of regulation of IFITM proteins  
62 and enrich our current understanding on these broad antiviral factors.

63

64

65

66

**67 INTRODUCTION**

68 The interferon-induced transmembrane proteins (IFITMs) are a family of membrane-bound  
69 proteins that play an important role in innate immune responses, due to an exquisite ability  
70 to inhibit a large spectrum of viruses (1).

71 Members of this family present a highly similar structural organization characterized by an  
72 intramembrane domain (IMD, previously referred to as TM1), an intracellular loop (CIL), a  
73 transmembrane domain (TMD, previously referred to as TM2) and N and C-termini of  
74 variable length and regulatory functions (2, 3, 4).

75 By virtue of this organization, IFITMs are localized in endo-lysosomal vesicles, plasma  
76 membrane and Golgi, and display a heterogeneous distribution influenced by both membrane  
77 dynamics and specific protein domains. As such, while the intracellular distribution of human  
78 IFITM1 is skewed towards the plasma membrane, the presence of endocytic signals at the  
79 longer N-terminus of human IFITM2/3 confer them an higher endosomal/lysosomal  
80 localization (5–8) (9, 10)(11).

81 IFITMs present at least two peculiar features that distinguish them from many antiviral  
82 factors. First, they can inhibit a broad spectrum of viruses. Second, by acting on viral-to-  
83 cellular membrane fusion, they can interfere with two distinct phases of the replicative cycle  
84 common to most viruses: (i) during the step of virus entry in target cells (property defined as  
85 target cell protection and historically the first associated to IFITM inhibition (2, and reviewed  
86 in 4), and (ii) by triggering the production of novel virion particles of decreased infectivity  
87 (property that we refer to as the negative imprinting of virion particles' infectivity, 12–15).

88 In target cells, very recent studies have visually shown how IFITMs lead to the sequestration

89 of incoming virion particles in endosomes and enhance their trafficking to lysosomes for  
90 degradation (17–19).

91 Instead, in infected cells undergoing active virion production, IFITMs lead the production of  
92 novel virion particles that incorporate IFITMs and exhibit decreased infectivity (13–15, 20).

93 Although whether the physical incorporation of IFITMs in virion particles is required for this  
94 effect remains to be formally demonstrated, the presence of IFITMs during the virion

95 assembly process leads to virions with a reduced propensity to undergo membrane fusion,  
96 similarly to what has been described during infection of target cells (14, 15, 19, 21–23), as

97 well as in more recent functions associated to the biology of IFITMs (24–26). In the case of  
98 target cell protection, the membrane fusion defect has been proposed to be due to the direct

99 rigidification of membranes in which IFITMs are inserted and oligomerize (21, 23, 27).

100 Whether the same mechanism applies to membrane fusion inhibition in IFITM-containing  
101 virion particles remains unclear.

102 IFITMs are regulated by numerous post-translational modifications (palmitoylation,  
103 methylation, phosphorylation and ubiquitination) that control their intracellular levels, their

104 pattern of distribution in cells and ultimately their antiviral activities (5, 6, 10). In this respect,

105 human IFITM2 and IFITM3 exhibit long N-termini containing two juxtaposed regulatory

106 domains: a PPxY domain that serves as a docking site for the E3-ubiquitin ligase neural  
107 precursor cell expressed developmentally down-regulated protein 4 (NEDD4) and a Yxx $\phi$

108 domain (where  $\phi$  stands for bulky amino acid residues) that directs Adaptor protein 2 (AP2)

109 complex-mediated endocytosis (5, 6, 10) and these activities can be modulated by the kinase

110 Fyn through phosphorylation of the tyrosine residue common to both domains (5, 28). In

111 contrast, relatively little is known about regulatory domains in human IFITM1 that lacks the

112 above-mentioned domains due to a shorter N-terminus and possesses instead a longer C-  
113 terminal tail. A single study reported the existence of a non-canonical dibasic sequence  
114 (KRxx) at the IFITM1 C-terminus that triggers AP3-mediated endocytosis and lysosomal  
115 targeting (8). This domain appears however unique to human IFITM1.

116 While several studies have examined the antiviral properties of individual animal IFITM  
117 molecules (albeit essentially during target cell protection) (29–35)(11), none has so far  
118 compared the antiviral properties of a large number of animal IFITMs in a single  
119 homogeneous setup, comparison that may reveal differences in terms of species specificity  
120 and that may point to IFITM domains involved in protein stability, intracellular distribution,  
121 antiviral potency and so forth.

122 Using a homogeneous setting, we have compared here 21 different animal IFITM proteins  
123 for their antiviral activities against HIV-1 in human cells in terms of target cell protection  
124 and negative imprinting of virion particles. Our findings highlight a remarkable heterogeneity  
125 in the action of these proteins against HIV-1, with homologs that have either completely lost  
126 their ability to inhibit the virus, while others on the contrary can do it more efficiently. The  
127 antiviral activities of the different IFITMs cannot simply be ascribed to the presence or  
128 absence of known regulatory domains in the N terminus of IFITMs that clearly distinguish  
129 IFITM2/3 molecules from IFITM1s and it can only be partially explained through protein  
130 stability, suggesting the existence of additional layers of regulation. The phenotype of  
131 chimeras obtained between antiviral and non-antiviral IFITMs highlights the importance of  
132 a cross-talk between individual domains for optimal antiviral activity. Finally, while past  
133 results from our lab failed to dissociate the two antiviral properties ascribed to IFITMs (20),  
134 we herein identify murine IFITMs as molecules intrinsically deficient in their ability to

135 mediate the negative imprinting of HIV-1 virions, while maintaining their effect during target  
136 cell protection. Thus, these results indicate for the first time that these two properties may be,  
137 at least in principle, uncoupled.

138

## 139 RESULTS

140 **Description of the mammalian IFITMs used in this study.** To benefit from the natural  
141 variability existing among IFITMs, twenty different IFITM proteins derived from a wide  
142 range of mammalian species were selected, based on cells availability and successful cloning  
143 and compared to an identically HA-tagged human IFITM3 (H3), the antiviral functions of  
144 which have been well characterized with respect to HIV-1 (Fig. 1A) (14–16, 36, 37). For  
145 clarity's purposes, the *IFITM* genome reference nomenclature was maintained, despite the  
146 caveat that this may be misleading, because some are from poorly annotated genomes and  
147 *IFITM* gene family annotations do not follow its evolutionary history. Two referenced  
148 *IFITM1-like* genes were also included in our analysis (*Canis Lupus*, D (dog), D1La and  
149 D1Lb), along with murine (*Mus musculus*, M) *IFITM6* and *IFITM7* (Fig. 1A for a schematic  
150 representation of the genomic organization of *IFITMs* used here). At a general level, the  
151 percentage identity across the mammal IFITMs analyzed here was high with a minimum of  
152 50% for the most divergent members (M6 and M7) (Fig. 1B, for alignment purposes human  
153 IFITM1 and IFITM2 were also included). A major difference between human IFITM1 and  
154 IFITM2/3 is the presence in the former of a shorter N-terminus devoid of regulatory  
155 sequences that play important roles in IFITM trafficking and stability and of a longer C  
156 terminus that contributes to its antiviral properties, at least in human IFITM1.

157 A simple survey of the lengths of the N and C termini among the mammalian IFITMs tested  
158 here indicates that while the N-terminus appears homogeneous in IFITM2/3-like and  
159 IFITM1-like members (53 to 55 and 31-32 amino acids, respectively), the C-terminus varies  
160 to a greater extent from 1 amino acid in M1 to 18 in M2 (Fig. 1C). In addition, a number of  
161 IFITM molecules present combinations of either long N and C termini (B3, P2 and P3, M2,  
162 D3, C3 and G3) or short N and C ones (M1 and R1).

163 All IFITM2/3-like members exhibited the adjacent PPxY and YxxΦ domains important for  
164 NEDD4 and AP2 recruitment respectively (6, 28), with the sole exception of the rabbit  
165 IFITM3 protein (R3) that presented only the former. Lastly, while the Nter regions of  
166 mammalian IFITMs present enough homology to allow their good alignment, the Cter  
167 regions exhibit very little sequence homology between the selected species (Fig. 1C).

168

169 Next, the intracellular distribution of the different IFITMs was examined by confocal  
170 microscopy upon ectopic transfection in HEK293T cells (Fig. 2). Several IFITMs exhibited  
171 a punctuate intracellular staining similar to H3 (B3, P2, P3, M2, M3, M6, G3, D3), in some  
172 cases marked by accrued perinuclear accumulation (C3, R3 and to a lower measure M7)  
173 compatible with their accumulation in the Golgi. Instead, higher plasma membrane  
174 distribution was observed for the remaining members (B1, B2, P1, M1, D1 and D1La, D1Lb,  
175 C1 and R1). Overall, the different IFITM proteins display a heterogeneous distribution  
176 skewed towards either the plasma membrane or internal membranes, as described for their  
177 human counterparts (4, 20). The only exception was B2 that, despite its nomenclature,  
178 displays both an IFITM1-like plasma membrane distribution and sequence features.

179

180 **Mammalian IFITMs display a heterogeneous behavior with respect to their ability to**  
181 **protect target cells from infection and to negatively imprint HIV-1 virion particles**  
182 **infectivity.** To date, animal IFITM antiviral activities have been mostly evaluated in  
183 homologous host-species-virus settings; i.e. in cells of the same species and against viruses  
184 specific for that species (29, 30, 38–41), making a direct comparison of their antiviral potency  
185 difficult. Here, we aimed at comparing the antiviral behavior of the different IFITMs in a  
186 single cellular setting and to this end, both target cell protection and negative imprinting of  
187 virion's infectivity abilities of the different IFITMs were assessed against HIV-1. Target cell  
188 protection was evaluated by challenging CD4/CXCR4- HEK293T cells expressing the  
189 different IFITMs with a NL4-3-Envelope HIV-1 GFP-coding virus, prior to flow cytometry  
190 analysis two days post-infection (Fig. 3A). Negative imprinting of virion particles was  
191 determined by producing HIV-1 viruses in cells expressing the different IFITMs and by using  
192 purified and normalized amounts of virion particles to challenge target cells (Fig. 3A).  
193 Transfections were carried out with DNA levels that allowed a comparable IFITM expression  
194 with IFN-stimulated primary cells, at least as appreciated for human IFITMs, as precedently  
195 described in (14, 20). The same DNA levels were then used for the remaining IFITM  
196 orthologs. Upon ectopic expression, the steady-state levels of the different IFITMs varied  
197 considerably following WB analyses with few members either barely detectable or detectable  
198 only upon overexposure (as M6, M7 and D3, Fig. 3B and see below Fig. 4 for a quantification  
199 by intracellular flow cytometry). In the case of HIV-1 virions produced in the presence of  
200 IFITMs, the levels of virion-associated IFITM proteins mirrored their intracellular levels of  
201 expression, in agreement with our previous mutagenesis study conducted on human IFITM3  
202 (14, 20). As we and others already documented (14–16, 20), IFITM expression exerted a

203 minor but detectable effect on the extent of virion released (Fig. 3B), so that subsequent  
204 experiments made use of exo-RT normalized virions.

205 Under the conditions used here, H3 expression in target cells induced a two-fold protection  
206 of cells from HIV-1 infection (Fig. 3C, white bars for target cell protection results). Several  
207 mammalian IFITMs exhibited antiviral activities comparable to the ones of H3 (in particular  
208 B3, P1, M2, M3, G3 and R3), while others displayed enhanced inhibitory capacities (ten-fold  
209 reduction for B1, B2, D1La and R1; four to eight-fold reduction for P2, P3, M1 and D1Lb).  
210 Interestingly, several IFITMs were found to display no discernable antiviral effect during  
211 target cell protection (C1, C3, D1, D3, M6 and M7). While this could be foreseen for the  
212 most divergent IFITM members (specifically M6 and M7 that no data has so far linked to  
213 IFN responses), the lack of antiviral activities in the remaining IFITM members was  
214 unexpected.

215 Next, the different IFITMs were tested for their ability to alter the infectivity of normalized  
216 HIV-1 virions (Fig 3C, blue bars). As expected, H3 drove the production of virions exhibiting  
217 a three-fold decrease in infectivity over a single round of infection assay. In addition, several  
218 IFITMs behaved as H3 (B2, B3, P1, P2, P3, D1La, R1 and R3). In stark contrast, a more  
219 drastic phenotype (six to ten-fold reduction in infectivity) was observed for B1, G3 and  
220 D1Lb, while no antiviral activity was detected for C1, C3, D1, D3, M1, M2, M3, M6 and  
221 M7. Lastly, the expression of M2, M6 and D3 exerted a positive effect on the infectivity of  
222 virion particles.

223 At a general level, a strong positive correlation was observed between the ability of individual  
224 IFITMs to interfere with the infectivities measured in target cell protection and negative  
225 imprinting of virions (Fig 3D;  $R=0,77$ ,  $p\text{-value}<0.0001$ ), suggesting a strong interdependence

226 between these two activities, as previously suggested (20). The only exceptions were noted  
227 in murine IFITMs M1, M2 and M3 proficient in target cell protection, but lacking detectable  
228 effects on the infectivity of newly-formed HIV-1 virions. Overall, the results obtained here  
229 highlight the heterogeneous behavior of animal IFITMs with respect to their antiviral  
230 properties, as assessed in a unique cellular and viral setting.

231

232 **The intracellular levels of the different mammalian IFITMs is an important, but not**  
233 **unique parameter of their antiviral properties.**

234 The antiviral potency of IFITM proteins is influenced by a number of factors and among  
235 them those governing the protein's steady-state levels (6, 11, 16, 20). To determine whether  
236 protein stability (at least at steady state), could relate to the antiviral behavior of the IFITMs  
237 examined here, we used intracellular staining and flow cytometry analysis (Fig. 4A for  
238 representative panels and 4B for cumulative data). When thus analyzed and compared to  
239 human IFITM3, the intracellular levels of accumulation of IFITMs varied from 2-fold higher  
240 to 4-fold lower. Variations spanned animal clade division in that IFITMs within one species  
241 could present increased or decreased steady state levels. The intracellular levels of IFITM  
242 expression (as assessed by the mean fluorescent intensity, MFI) was inversely correlated with  
243 the infectivities measured in both target cell and negative imprinting, indicating that IFITMs  
244 present at higher levels were generally more antiviral (Fig. 4C,  $r=-0.7601$ ;  $p=0.0001$  and  $r=-$   
245  $0.54$ ;  $p=0.01$ , respectively). This correlation was however lower for the negative imprinting  
246 of virion particles defect, indicating that this property is less reliant on the levels of IFITMs  
247 in cells, than target cell protection. Overall, this analysis indicates that protein stability at  
248 steady state is an important parameter of the antiviral properties of IFITMs in agreement with  
249 data present in the literature.

250

251 To assess the contribution of protein levels to the lack of antiviral activity during target cell  
252 protection, property more influenced by the intracellular levels of IFITMs, increasing doses  
253 of D1 and C1 were used in a comparison with other IFITMs exhibiting antiviral activity  
254 (D1Lb and R1, respectively). To this end, target cells transfected with different amounts of  
255 IFITM-coding DNAs were challenged with WT HIV-1, prior to flow cytometry analysis two  
256 days later (Fig. 5A). Protein levels were quantified by densitometry following WB, as this  
257 yielded similar results than intracellular staining and flow cytometry analyses, under the  
258 conditions used here.

259 Increasing doses of D1 did not significantly alter virion infectivity and when D1 was  
260 expressed at similar levels than D1Lb the different antiviral behavior was still significant  
261 (Fig. 5A). A similar behavior was observed upon the expression of increasing doses of C1,  
262 although in this case we were unable to achieve a notable increase in C1 protein levels. To  
263 circumvent this issue, we used a reciprocal setup in which a fixed dose of C1 was compared  
264 to decreasing levels of R1 (Fig. 5B). In this case, despite the fact that the antiviral activity of  
265 R1 was gradually lost upon dilution, R1 was still able to inhibit viral infection when  
266 expressed at similar levels than C1. Altogether, these results indicate that protein stability is  
267 important, but is not the sole contributor of the observed differences in the antiviral behavior  
268 of IFITMs.

269

270 **Elements described as crucial for IFITM antiviral activities are largely conserved**  
271 **among mammalian IFITMs.** To investigate potential amino acid differences or  
272 evolutionary paths between the mammalian IFITMs with and without antiviral activities, we

273 performed comparative genetic and phylogenetic analyses. Benefiting from the natural  
274 variations existing among mammalian IFITMs, we grouped members according to their non-  
275 antiviral (C1, D1, C3 and D3, only members devoid of both antiviral activities were grouped  
276 here) and antiviral (the remaining ones) behavior, omitting M6 and M7 from the analysis due  
277 to their higher divergence (Fig. 6A and 6B). No single common amino acid change was able  
278 to discriminate IFITMs according to their antiviral behavior. Indeed, most IFITM members  
279 displayed three conserved cysteines (C71, 72, 105; amino acid positions relative to H3)  
280 important for palmitoylation and membrane association (7, 42), with the exception of C71  
281 replaced by phenylalanine in antiviral D1La and D1Lb IFITMs. Most IFITMs also displayed  
282 four conserved phenylalanines (F63, F67, F75, F78) reported as relevant for antiviral  
283 functions (43, 44). Residues F75 and F78 were previously proposed to mediate IFITM3  
284 oligomerization, but this was not the case when examined in living cells (45) (46). Therefore,  
285 these four phenylalanines are important functional determinants for unknown reasons. The  
286 only exceptions found in our analysis were the F75 and F78 residues that were replaced by  
287 isoleucine and tyrosine in C1 and M1, M2 and M3, respectively. Moreover, IFITMs possess  
288 several lysines that can be ubiquitinated, one specific to the long Nter of IFITM2/3-like  
289 proteins and three others shared by all IFITMs (K83, K88 and K104) (6, 47). Of these, K83  
290 and K88, but not K104 were strictly conserved among mammalian IFITMs, despite the fact  
291 that ubiquitination of K104 together with K83 is required for a recently-identified scaffolding  
292 property of IFITM3 involved in B cell signaling (26). Remarkably, in none of the positions  
293 mentioned above and described in the literature to modulate the antiviral properties of  
294 IFITMs a strict correlation existed between antiviral and non-antiviral IFITMs. Furthermore,  
295 residues described as forming an amphipathic helix in the IMD of human IFITMs  
296 (VWSLFNTL/I/VF) (48), as well as a very recently identified GxxxG motif with crucial

297 relevance in membrane rigidification (27), were also conserved among all mammalian  
298 IFITMs tested. Finally, the “non-antiviral” IFITMs did not group together on a phylogenetic  
299 analysis (Fig. 6C), suggesting that they do not share a common ancestor and that the absence  
300 of antiviral activity is not due to an ancient single event.

301 Therefore, the main elements previously described as crucial for the antiviral activity of  
302 IFITMs are largely conserved among mammalian IFITMs, indicating that additional ones are  
303 likely to contribute to the fine regulation of their overall antiviral behavior.

304

305 **Chimeras between active and inactive IFITM homologues highlight the importance of**  
306 **the cross-talk between individual domains to finely tune antiviral behavior.** To identify  
307 the domain(s) at the basis of the distinct behavior of the different IFITMs, we generated  
308 chimeric IFITM proteins in which domains of the antiviral R1 were inserted into the non-  
309 antiviral C1 either individually, or in combination (Fig. 7A). Contrarily to other individual  
310 domains, the Cter of R1 (C1-C<sub>R1</sub>) promoted a substantial increase in protein accumulation at  
311 steady state and its combination with the TMD domain (C1-(TMD+C)<sub>R1</sub>) further increased  
312 it (Fig. 7B and 7C). The different chimeras did not exhibit gross changes in their intracellular  
313 distribution after confocal microscopy analysis, with the exception of the C1-TMD<sub>R1</sub> chimera  
314 that appeared to have lost the plasma membrane distribution typical of IFITM1-like proteins  
315 (Fig. 7D). Interestingly, when cells expressing the different chimeras were challenged with  
316 HIV-1 in target cell protection, a single chimera was able to exhibit significant antiviral  
317 activity (C1-(TMD+C)<sub>R1</sub>, Fig. 7E), indicating the importance of the cross-talk between the  
318 TMD and the Cter domains for the antiviral activity.

319 A similar approach was used to engineer chimeras between D1 and D1Lb, respectively  
320 inactive and active against HIV-1 (Fig. 8A). Also in this case, the Cter of D1Lb (D1-C<sub>b</sub>) was  
321 able to substantially rescue the low protein expression levels of D1. However, in this case it  
322 was the combination of the Cter with the Nter domains (D1-(N+C)<sub>b</sub>) that yielded the highest  
323 increase in protein levels (Fig. 8B and 8C). No gross modifications of the intracellular  
324 distributions of the different chimeras were observed with the exception of the D1-C<sub>b</sub> and the  
325 D1-(TMD+C)<sub>b</sub> chimeras that exhibited higher perinuclear accumulation than WT (Fig. 8D).  
326 When the chimeras were tested for their ability to mediate target cell protection against HIV-  
327 1, we again found that the cross-talk between the TMD and the Cter was important for the  
328 antiviral activity (Fig. 8E). Interestingly, in this case the combination between the Nter and  
329 the IMD domains was also important to restore full antiviral activities. These results indicate  
330 that cross-talks between individual domains are important for optimal antiviral activity with  
331 the combination between the TMD and the Cter being important for both chimeric  
332 configurations examined, while the one between Nter with IMD and Cter with TMD being  
333 important only in the case of the D1/D1Lb chimera.

334 Finally, the antiviral chimeras were also tested for their ability to mediate the negative  
335 imprinting of virion particles infectivity. For this, HIV-1 virions produced in cells ectopically  
336 expressing the IFITMs were used to challenge target cells (Fig. 9A for a schematic  
337 presentation of the chimeras and the experimental setup). All chimeric forms of IFITMs  
338 decreased HIV-1 virions' infectivity, with C1-(TMD+C)<sub>R1</sub> exerting an antiviral effect largely  
339 comparable to the parental R1 homologue and the D1-(N+IMD)<sub>b</sub> and D1-(TMD+C)<sub>b</sub>  
340 chimeras still antiviral despite the fact that the latter appeared less potent than D1Lb (Fig.

341 9B). Hence, the domain crosstalks showed above as relevant for target cell protection activity  
342 plays an important role also during negative imprinting of virions infectivity.

343

344 **Murine IFITM3 is a natural IFITM variant devoid of negative imprinting of HIV-1**  
345 **virion particles ability.** An important result highlighted from our study is the fact that  
346 despite being proficient in target cell protein, murine IFITM 1, 2 and 3 seem not to affect the  
347 infectivity of newly-produced HIV-1 virion particles. To determine whether this could be  
348 dependent on insufficient levels of expression, HIV-1 virions were produced in the presence  
349 of increasing doses of M3, prior to virion normalization and challenge of target cells. Even  
350 under conditions of increased doses of M3 (Fig. 10A), a consistent lack of antiviral effects  
351 was noted suggesting a true deficiency in negative imprinting of HIV-1 virion's infectivity,  
352 at least under the experimental conditions used here.

353 As mentioned above, we noticed that F78 (numbering with respect to human IFITM3, see  
354 Fig 6) is commonly substituted by tyrosine in murine IFITMs (M1, M2 and M3) that lack  
355 negative imprinting of virion particles abilities. As such, we substituted this residue back to  
356 phenylalanine (Y78F) in the context of M3 and tested its ability to impair the infectivity of  
357 newly-produced virion particles, using the same experimental setup. However, no change in  
358 infectivity could be measured when virion particles were produced in the presence of  
359 increasing levels of the M3 Y78F mutant (Fig. 10B), indicating that this residue does not  
360 play an important role in this setting.

361 Overall, these results indicate that murine IFITM3 is a natural variant impaired in their ability  
362 to negatively imprint the infectivity of newly-produced virion particles, at least in the case of  
363 HIV-1 and also further highlight that protein stability is not the sole contributor of the  
364 observed differences in the antiviral behavior of IFITMs.

365

366

367 **DISCUSSION**

368 In this study, we have taken advantage of the natural variability existing between a large  
369 panel of mammalian IFITMs to compare their potency against HIV-1 in a single experimental  
370 system through expression in human cells. In doing so, we have uncovered IFITM homologs  
371 that exhibit either enhanced, or completely absent, ability to affect both target cell protection  
372 and negative imprinting of HIV-1 virions infectivity. Interestingly despite few exceptions,  
373 the domains described in previous studies as important for either post-translational  
374 modifications, multimerization or overall antiviral properties are conserved across all the  
375 IFITMs tested here independently of their antiviral behavior, pointing to the existence of  
376 additional determinants for the antiviral activities of IFITMs. In this respect, the results  
377 obtained with our chimeras indicate that one such determinant may be the cross-talk between  
378 individual IFITM domains.

379 A general distinction between human IFITM1 and IFITM2/3 proteins is the reciprocal  
380 presence of long C and N termini, respectively. Long Nter are indeed well maintained in  
381 mammalian IFITM2/3 that also exhibit a strict conservation of the NEDD4 and AP2  
382 recruitment domains with one sole exception (R3). However, the Cter appears more  
383 heterogeneous, and long C-termini are in some cases present in combination with long Nter  
384 in IFITM2/3 molecules (B3, P1, P2, M3, D3, C3 and G3). Whether this latter endows such  
385 molecules with additional regulatory features with respect to those described for their human  
386 counterparts remains unknown. Relatively few studies have examined the functions of the  
387 Cter of IFITM proteins and have been essentially focused on human IFITM1 in which a

388 regulatory dibasic KRxx domain has been shown to regulate association to AP3 (8).  
389 However, this domain is absent in all the remaining IFITMs, so that how and whether the  
390 Cter regulates more commonly the behavior and properties of IFITM1-like proteins remain  
391 unclear. In this respect, it is of interest that M1 and R1 present very short N and C-termini  
392 which may indicate them as *core* minimal IFITM homologs devoid of some of the multiple  
393 regulatory possibilities described for other IFITMs.

394 It is evident from our comparative analysis that no common specific residue or described  
395 domain can discriminate active from inactive IFITMs. A recent report highlighted the  
396 importance of the polymorphism of residues (P/W/T/F/Y) in the rates of palmitoylation on  
397 adjacent cysteines (C71/C72) and in the restriction capacity of bat IFITMs (33) and another  
398 determined the functional relevance of a polymorphism within the AP2 binding site of  
399 African green monkeys IFITM3 alleles (I22) (49). While variations at these residues do not  
400 explain the antiviral behavior of the IFITM tested here, these findings raise the possibility  
401 that additional polymorphism may finely tune IFITM functions, possibly in a species-specific  
402 context.

403 Previous work from a number of laboratories including ours has highlighted the importance  
404 of the intracellular levels of accumulation for the antiviral effects of human IFITM3 (14–16,  
405 20, 36). However, the results obtained with two non-antiviral IFITM proteins (C1 and D1),  
406 as well as with chimeras clearly indicate that protein levels are not the sole determinant of  
407 antiviral behavior, pointing to the existence of additional layers of regulation.

408 With the caveat that we have not examined the dynamic distribution of the different IFITMs  
409 and that for a given mutation it is difficult to completely separate effects on protein levels  
410 from those on antiviral behavior, the results we have obtained with two sets of chimeras  
411 indicate that the cross-talk between two distinct domains (in particular the TMD and the Cter)

412 is an important modulator of antiviral functions. While the Cter and TMD domains in R1 are  
413 very different from those in C1, they exhibit only four and five amino acid changes between  
414 D1 and D1Lb, respectively. Yet, the transfer of the Cter and TMD domains is sufficient to  
415 restore antiviral functions. The fact that the combination of Nter and Cter can also exert  
416 similar effects in the context of the D1/D1Lb but not of the C1/R1 chimera suggests that such  
417 cross-talks are likely to be different according to the specific IFITM examined, probably as  
418 a result of species-specific adaptations.

419

420 At present the reasons for the absence of antiviral activities by some IFITM molecules remain  
421 unclear and, in terms of evolution, it is entirely possible that this has resulted from a loss of  
422 antiviral activity that has been compensated by other IFITM members of a given species not  
423 tested here. However, an alternative, and not mutually exclusive, possibility we favor is that  
424 certain IFITMs may be subjected to host species-specific regulation and/or exhibit optimal  
425 activity against viruses naturally encountered in the same species. In this respect, the feline  
426 C1 and C3 IFITMs examined here are poorly expressed in human cells and inactive against  
427 HIV-1, but they may exhibit better stability and antiviral properties in a feline cell-virus  
428 context.

429 To support this contention, it is interesting to note that murine IFITMs (M1, M2 and M3)  
430 were found in this study to inhibit HIV-1 during target cell protection, but not to alter the  
431 infectivity of newly-formed HIV-1 virion particles. While on one hand this finding is  
432 important because it suggests for the first time that these two properties may be dissociated,  
433 on the other it raises the possibility that murine IFITM3 may have been optimized for activity  
434 against viruses of its own species. The latter would be in agreement with a recent study  
435 reporting that murine IFITM3 is capable of interfering with the production of infectious

436 virion particles of the murine leukemia virus (MLV) (50). Thus, these results highlight the  
437 possibility that species-specific adaptations may have shaped the optimal antiviral activity of  
438 a given IFITM against viruses of the same species.

439 In conclusion, our study highlights a broad functional heterogeneity among mammals  
440 IFITMs against HIV-1 and indicates that the cross-talk between IFITM domains finely tunes  
441 the antiviral properties of the different IFITMs, adding a novel layer of complexity to the  
442 regulation of the activities of these broad antiviral factors.

443

444

## 445 MATERIAL AND METHODS

446 **Cell culture, plasmids and antibodies.** HEK293T epithelial cell line ATCC (CRL-3216)  
447 and HeLa P4/P5 expressing the CD4 receptor along with the CXCR4 and CCR5 co-receptors  
448 were cultured in complete DMEM supplemented with 10% FCS. IFITMs were amplified by  
449 PCR from cDNAs obtained from relevant animal cell lines (cell lysates were kind gifts of the  
450 CelluloNet facility of the SFR-Biosciences in Lyon Gerland or of Frederick Arnaud at the  
451 IVPC, Lyon, France): MBDK, *Bos Taurus*; PK(15), *Sus scrofa*; RK15, *Oryctolagus*  
452 *cuniculus*; TIGMEC, *Capra hircus*; CRFK, *Felis catus*. Murine and canine IFITMs were  
453 obtained by gene synthesis (Genewiz). All IFITMs were cloned as BamH1/NotI fragments  
454 into a pcDNA-HA vector. The species abbreviations used in this manuscript are: H= *Homo*  
455 *sapiens*; M= *Mus musculus*; G= *Capra hircus*; D= *Canis lupus familiaris*; C= *Felis catus*; P=  
456 *Sus scrofa*; B= *Bos Taurus*; R= *Oryctolagus cuniculus*. Retrieved sequences were strictly  
457 identical to the following gene ID numbers: H3, 10410; M1, 68713; M2, 80876; M3, 66141;  
458 M6, 213002; M7, 74482; G3, 102180655; D1, 483397; D1La, 475935; D1Lb, 483396; D3,

459 606890; C1, 101086846; C3, 101100838; P1, 100127358; P2, 100620056; P3, 100518544;  
460 B1, 353510; B2, 615833; B3, 777594; R1, 103347593; R3, 100353245. D1/D1Lb and C1/R1  
461 chimeras were obtained by gene synthesis (Genewiz). CD4, CCR5 and CXCR4-coding  
462 plasmids were obtained from the AIDS Reagent and Reference Program of the NIH (cat.  
463 Numbers: 158, 3325, 3326, respectively, respectively). The anti-HA (H3663) and anti- $\alpha$ -  
464 Tubulin (T8203) antibodies were purchased from SIGMA. The anti-HIV-1 Gag and anti-  
465 HIV-1 Env antibodies were similarly obtained from the AIDS Reagent and Reference  
466 Program of the NIH (clone 183-H5C and cat. number 288, respectively).

467

468 **Virus production, purification, normalization and infections.** Standard single-round of  
469 infection vectors were produced by transient calcium phosphate transfection with DNAs  
470 coding for: a mini viral genome bearing GFP (pRRL-CMV-GFP); the Gag and Gag-Pol  
471 structural proteins (8.2); the NL4-3 Env and the HIV-1 Rev protein (Rev) (4:4:1 and 0.5  $\mu$ g  
472 respectively for a 10cm plate), as described in (13, 14).

473 The target cell protection properties of the different IFITMs were assessed by using the  
474 above-mentioned virus at a multiplicity of infection (MOI) comprised between 0.1 and 0.2  
475 on HEK293T cells ectopically transfected thirty-six hours earlier with DNAs coding CD4,  
476 CXCR4 and the different IFITMs (0.2: 0.2 and 1  $\mu$ g, respectively).

477 To assess the negative imprinting of virion infectivity abilities of IFITMs, HIV-1-derived  
478 lentiviral particles were produced in the presence of IFITMs by calcium phosphate DNA co-  
479 transfection of HEK293T cells using Gag-Pro-Pol, viral GFP genome, Env, Rev and IFITMs  
480 or pcDNA control plasmid (using 4: 4: 0.5: 0.5: 3  $\mu$ g each for a 10-cm dish plate), as  
481 previously described (13, 14, 20). We have already shown that in the case of human IFITMs,

482 these levels were similar to those observed for the endogenous form in primary dendritic cells  
483 stimulated with type I interferon.

484 In this case, forty-eight hours after transfection, cell-free supernatants were syringe-filtered  
485 (0.45  $\mu\text{m}$ ) and virion particles were purified by ultracentrifugation through a 25% sucrose  
486 cushion (w/v) for two hours at 110.000g. Virion pellets were then resuspended in PBS for  
487 further analyses and normalized by exogenous-reverse transcriptase (exo-RT), as described.  
488 Infections were carried out on HeLaP4/P5 cells with normalized amounts of virions for 2-4  
489 hours and the extent of infection was assessed three days later by flow cytometry.

490

491 **Immunofluorescence and confocal microscopy.** HEK293T cells growing on glass  
492 coverslips coated with poly-L-Lysine 0.01% were transfected with plasmids coding animal  
493 IFITMs with Lipofectamine 3000, according to the manufacturer's instructions (Invitrogen,  
494 LifeTechnologies). Twenty-four hours later, cells were fixed with PFA (3.7%),  
495 permeabilized with Triton 0.1% in PBS and incubated first with the primary anti-HA  
496 antibody overnight (at a 1/100 dilution), then with a secondary antibody conjugated to  
497 Alexa488 (Molecular Probes, LifeTechnologies; #A21202; 1/1000 dilution). Coverslips  
498 were stained with a DAPI-containing solution (1/10000 dilution in PBS) and mounted using  
499 the anti-bleaching solution Fluormount G (Southern Biotech). Fluorescent confocal images  
500 were collected using a Zeiss LSM 880 AiryScan confocal microscope and pictures were  
501 analyzed with the ImageJ software.

502

503 **Intracellular staining and flow cytometry analysis.** HEK293T cells expressing the  
504 different IFITMs were permeabilized with the Cytotfix/Cytoperm plus kit (cat: n°554715,

505 BD), according to the manufacturer's instructions, before incubation with a phycoerythrin-  
506 conjugated anti-HA antibody (Miltenyi Biotec, #130-092-257) and flow cytometry analysis.  
507 The mean fluorescent intensity of HA-positive cells was then determined using the FlowJo  
508 software.

509

510 **Sequence analyses and phylogenetics.** Sequence alignment of the various IFITMs were  
511 performed using amino acid sequences with PRANK -F with a gap rate of 0.005 and minor  
512 adjustments (51). The N-/C-termini of the selected mammalian IFITMs are highly variable  
513 (low homology) and are the result of complex evolutionary histories. They aligned very  
514 differently depending on the alignment method and gap parameters. All in all, only the central  
515 portion of IFITMs is enough homologous and can be correctly aligned. Visualization in  
516 Figure 6 was produced using Geneious (Biomatters, Inc.), with color-coding according to  
517 polarity, and logo plots. Phylogenetics in Figure 6 were performed using PhyML with JTT +  
518 G + I as a model and aLRT for node support (52, 53). The sequence alignment in fasta format  
519 and the phylogenetic tree in nwk format are available at  
520 <https://doi.org/10.6084/m9.figshare.13365893> and  
521 <https://doi.org/10.6084/m9.figshare.13365893>, respectively.

522

523 **Statistical analysis.** One-Way Anova, one-tail Student t tests or Pearson's correlation  
524 coefficient were used, as indicated in the legend to figures.

525

526 **Data Availability.** All relevant data are within the paper.

527

528 **ACKNOWLEDGMENTS**

529 We wish to acknowledge the AIDS Reagents and Reference Program of the NIH for  
530 antibodies and plasmids cited in this manuscript and Frederick Arnaud for sharing material.

531 We acknowledge the contribution of the flow cytometry (ANIRA-Cytométrie), microscopy  
532 (LYMIC-PLATIM) and genetic analysis (ANIRA-AGC CRB Cellulonet) platforms of SFR  
533 BioSciences Gerland Lyon Sud (UMS3444/US8).

534

535 **Funding**

536 The work described here in the laboratory of AC was supported by grants from the ANRS  
537 and Sidaction (2017-1 and 2019-1). AK and FM are post-doctoral fellows of the ANRS. AC  
538 and LE are supported by the CNRS. LE is supported by grants from amfAR (Mathilde Krim  
539 Phase II Fellowship no. 109140-58-RKHF), the ANR LABEX ECOFECT (ANR-11-LABX-  
540 0048 of the Université de Lyon, within the program Investissements d’Avenir [ANR-11-  
541 IDEX-0007] operated by the French National Research Agency), the Fondation pour la  
542 Recherche Médicale (FRM Projet Innovant no. ING20160435028), the FINOVI (“recently  
543 settled scientist” grant), the ANRS (no. ECTZ19143 and ECTZ118944), and a JORISS  
544 incubating grant. The funders had no role in study design, data collection and analysis,  
545 decision to publish, or preparation of the manuscript.

## 546 REFERENCES

- 547 1. Friedman RL, Manly SP, McMahon M, Kerr IM, Stark GR. 1984. Transcriptional and  
548 posttranscriptional regulation of interferon-induced gene expression in human cells. *Cell*  
549 38:745–755.
- 550 2. Bailey CC, Zhong G, Huang I-C, Farzan M. 2014. IFITM-Family Proteins: The Cell’s First Line  
551 of Antiviral Defense. *Annu Rev Virol* 1:261–283.
- 552 3. Liao Y, Goraya MU, Yuan X, Zhang B, Chiu S-H, Chen J-L. 2019. Functional Involvement of  
553 Interferon-Inducible Transmembrane Proteins in Antiviral Immunity. *Front Microbiol* 10:1097.
- 554 4. Brass AL, Huang I-C, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van  
555 der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ. 2009. The IFITM proteins  
556 mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell*  
557 139:1243–1254.
- 558 5. Chesarino NM, McMichael TM, Hach JC, Yount JS. 2014. Phosphorylation of the antiviral  
559 protein interferon-inducible transmembrane protein 3 (IFITM3) dually regulates its endocytosis  
560 and ubiquitination. *J Biol Chem* 289:11986–11992.
- 561 6. Chesarino NM, McMichael TM, Yount JS. 2015. E3 Ubiquitin Ligase NEDD4 Promotes  
562 Influenza Virus Infection by Decreasing Levels of the Antiviral Protein IFITM3. *PLoS Pathog*  
563 11.
- 564 7. Yount JS, Moltedo B, Yang Y-Y, Charron G, Moran TM, López CB, Hang HC. 2010.  
565 Palmitoylome profiling reveals S-palmitoylation-dependent antiviral activity of IFITM3. *Nat*  
566 *Chem Biol* 6:610–614.
- 567 8. Li K, Jia R, Li M, Zheng Y-M, Miao C, Yao Y, Ji H-L, Geng Y, Qiao W, Albritton LM, Liang  
568 C, Liu S-L. 2015. A Sorting Signal Suppresses IFITM1 Restriction of Viral Entry. *J Biol Chem*  
569 290:4248–4259.
- 570 9. Smith SE, Busse DC, Binter S, Weston S, Soria CD, Laksono BM, Clare S, Nieuwkoop SV,  
571 Hoogen BGV den, Clement M, Marsden M, Humphreys IR, Marsh M, Swart RL de, Wash RS,  
572 Tregoning JS, Kellam P. 2019. Interferon-Induced Transmembrane Protein 1 Restricts  
573 Replication of Viruses That Enter Cells via the Plasma Membrane. *Journal of Virology* 93.
- 574 10. Jia R, Xu F, Qian J, Yao Y, Miao C, Zheng Y, Liu S, Guo F, Geng Y, Qiao W, Liang C. 2014.  
575 Identification of an endocytic signal essential for the antiviral action of IFITM3. *Cell Microbiol*  
576 16:1080–1093.
- 577 11. Compton AA, Roy N, Porrot F, Billet A, Casartelli N, Yount JS, Liang C, Schwartz O. 2016.  
578 Natural mutations in IFITM3 modulate post-translational regulation and toggle antiviral  
579 specificity. *EMBO Rep* 17:1657–1671.
- 580 12. Shi G, Schwartz O, Compton AA. 2017. More than meets the I: the diverse antiviral and cellular  
581 functions of interferon-induced transmembrane proteins. 1. *Retrovirology* 14:53.
- 582 13. Tartour K, Nguyen X-N, Appourchaux R, Assil S, Barateau V, Bloyet L-M, Burlaud Gaillard J,  
583 Confort M-P, Escudero-Perez B, Gruffat H, Hong SS, Moroso M, Reynard O, Reynard S,

- 584 Decembre E, Ftaich N, Rossi A, Wu N, Arnaud F, Baize S, Dreux M, Gerlier D, Paranhos-  
585 Baccala G, Volchkov V, Roingard P, Cimarelli A. 2017. Interference with the production of  
586 infectious viral particles and bimodal inhibition of replication are broadly conserved antiviral  
587 properties of IFITMs. *PLoS Pathog* 13.
- 588 14. Tartour K, Appourchaux R, Gaillard J, Nguyen X-N, Durand S, Turpin J, Beaumont E, Roch E,  
589 Berger G, Mahieux R, Brand D, Roingard P, Cimarelli A. 2014. IFITM proteins are  
590 incorporated onto HIV-1 virion particles and negatively imprint their infectivity. *Retrovirology*  
591 11:103.
- 592 15. Compton AA, Bruel T, Porrot F, Mallet A, Sachse M, Euvrard M, Liang C, Casartelli N,  
593 Schwartz O. 2014. IFITM proteins incorporated into HIV-1 virions impair viral fusion and  
594 spread. *Cell Host Microbe* 16:736–747.
- 595 16. Lu J, Pan Q, Rong L, He W, Liu S-L, Liang C. 2011. The IFITM proteins inhibit HIV-1 infection.  
596 *J Virol* 85:2126–2137.
- 597 17. Kummer S, Avinoam O, Kräusslich H-G. 2019. IFITM3 Clusters on Virus Containing  
598 Endosomes and Lysosomes Early in the Influenza A Infection of Human Airway Epithelial Cells.  
599 *Viruses* 11.
- 600 18. Spence JS, He R, Hoffmann H-H, Das T, Thinon E, Rice CM, Peng T, Chandran K, Hang HC.  
601 2019. IFITM3 directly engages and shuttles incoming virus particles to lysosomes. *Nat Chem*  
602 *Biol* 15:259–268.
- 603 19. Suddala KC, Lee CC, Meraner P, Marin M, Markosyan RM, Desai TM, Cohen FS, Brass AL,  
604 Melikyan GB. 2019. Interferon-induced transmembrane protein 3 blocks fusion of sensitive but  
605 not resistant viruses by partitioning into virus-carrying endosomes. *PLoS Pathog* 15:e1007532.
- 606 20. Appourchaux R, Delpuch M, Zhong L, Burlaud-Gaillard J, Tartour K, Savidis G, Brass A,  
607 Etienne L, Roingard P, Cimarelli A. 2019. Functional Mapping of Regions Involved in the  
608 Negative Imprinting of Virion Particle Infectivity and in Target Cell Protection by Interferon-  
609 Induced Transmembrane Protein 3 against HIV-1. *Journal of Virology* 93.
- 610 21. Li K, Markosyan RM, Zheng Y-M, Golfetto O, Bungart B, Li M, Ding S, He Y, Liang C, Lee  
611 JC, Gratton E, Cohen FS, Liu S-L. 2013. IFITM Proteins Restrict Viral Membrane Hemifusion.  
612 *PLoS Pathog* 9.
- 613 22. Lin T-Y, Chin CR, Everitt AR, Clare S, Perreira JM, Savidis G, Aker AM, John SP, Sarlah D,  
614 Carreira EM, Elledge SJ, Kellam P, Brass AL. 2013. Amphotericin B increases influenza A virus  
615 infection by preventing IFITM3-mediated restriction. *Cell Rep* 5:895–908.
- 616 23. Desai TM, Marin M, Chin CR, Savidis G, Brass AL, Melikyan GB. 2014. IFITM3 restricts  
617 influenza A virus entry by blocking the formation of fusion pores following virus-endosome  
618 hemifusion. *PLoS Pathog* 10:e1004048.
- 619 24. Zani A, Zhang L, McMichael TM, Kenney AD, Chemudupati M, Kwiek JJ, Liu S-L, Yount JS.  
620 2019. Interferon-induced transmembrane proteins inhibit cell fusion mediated by trophoblast  
621 syncytins. *J Biol Chem* 294:19844–19851.
- 622 25. Buchrieser J, Degrelle SA, Couderc T, Nevers Q, Disson O, Manet C, Donahue DA, Porrot F,  
623 Hillion K-H, Perthame E, Arroyo MV, Souquere S, Ruigrok K, Dupressoir A, Heidmann T,

- 624 Montagutelli X, Fournier T, Lecuit M, Schwartz O. 2019. IFITM proteins inhibit placental  
625 syncytiotrophoblast formation and promote fetal demise. *Science* 365:176–180.
- 626 26. Lee J, Robinson ME, Ma N, Artadji D, Ahmed MA, Xiao G, Sadras T, Deb G, Winchester J,  
627 Cosgun KN, Geng H, Chan LN, Kume K, Miettinen TP, Zhang Y, Nix MA, Klemm L, Chen  
628 CW, Chen J, Khairnar V, Wiita AP, Thomas-Tikhonenko A, Farzan M, Jung JU, Weinstock  
629 DM, Manalis SR, Diamond MS, Vaidehi N, Müschen M. 2020. IFITM3 functions as a PIP3  
630 scaffold to amplify PI3K signalling in B cells. *Nature* 1–7.
- 631 27. Rahman K, Coomer CA, Majdoul S, Ding SY, Padilla-Parra S, Compton AA. 2020. Homology-  
632 guided identification of a conserved motif linking the antiviral functions of IFITM3 to its  
633 oligomeric state. *Elife* 9.
- 634 28. Jia R, Pan Q, Ding S, Rong L, Liu S-L, Geng Y, Qiao W, Liang C. 2012. The N-Terminal Region  
635 of IFITM3 Modulates Its Antiviral Activity by Regulating IFITM3 Cellular Localization. *J Virol*  
636 86:13697–13707.
- 637 29. Smith SE, Gibson MS, Wash RS, Ferrara F, Wright E, Temperton N, Kellam P, Fife M. 2013.  
638 Chicken Interferon-Inducible Transmembrane Protein 3 Restricts Influenza Viruses and  
639 Lyssaviruses In Vitro. *J Virol* 87:12957–12966.
- 640 30. Wang J, Wang C-F, Ming S-L, Li G-L, Zeng L, Wang M-D, Su B-Q, Wang Q, Yang G-Y, Chu  
641 B-B. 2020. Porcine IFITM1 is a host restriction factor that inhibits pseudorabies virus infection.  
642 *Int J Biol Macromol* 151:1181–1193.
- 643 31. Xing H, Ye L, Fan J, Fu T, Li C, Zhang S, Ren L, Bai J. 2020. IFITMs of African Green Monkey  
644 Can Inhibit Replication of SFTSV but Not MNV In Vitro. *Viral Immunol*  
645 <https://doi.org/10.1089/vim.2020.0132>.
- 646 32. Chen S, Wang L, Chen J, Zhang L, Wang S, Goraya MU, Chi X, Na Y, Shao W, Yang Z, Zeng  
647 X, Chen S, Chen J-L. 2017. Avian Interferon-Inducible Transmembrane Protein Family  
648 Effectively Restricts Avian Tembusu Virus Infection. *Front Microbiol* 8:672.
- 649 33. Benfield CT, MacKenzie F, Ritzefeld M, Mazzon M, Weston S, Tate EW, Teo BH, Smith SE,  
650 Kellam P, Holmes EC, Marsh M. 2019. Bat IFITM3 restriction depends on S-palmitoylation and  
651 a polymorphic site within the CD225 domain. 1. *Life Sci Alliance* 3.
- 652 34. Benfield CTO, Smith SE, Wright E, Wash RS, Ferrara F, Temperton NJ, Kellam P. 2015. Bat  
653 and pig IFN-induced transmembrane protein 3 restrict cell entry by influenza virus and  
654 lyssaviruses. *J Gen Virol* 96:991–1005.
- 655 35. Bailey CC, Huang I-C, Kam C, Farzan M. 2012. Ifitm3 limits the severity of acute influenza in  
656 mice. *PLoS Pathog* 8:e1002909.
- 657 36. Foster TL, Wilson H, Iyer SS, Coss K, Doores K, Smith S, Kellam P, Finzi A, Borrow P, Hahn  
658 BH, Neil SJD. 2016. Resistance of Transmitted Founder HIV-1 to IFITM-Mediated Restriction.  
659 *Cell Host Microbe* 20:429–442.
- 660 37. Ding S, Pan Q, Liu S-L, Liang C. 2014. HIV-1 mutates to evade IFITM1 restriction. *Virology*  
661 454–455:11–24.
- 662 38. Blyth GAD, Chan WF, Webster RG, Magor KE. 2016. Duck Interferon-Inducible

- 663 Transmembrane Protein 3 Mediates Restriction of Influenza Viruses. *J Virol* 90:103–116.
- 664 39. Xie J, Bi Y, Xu S, Han Y, Idris A, Zhang H, Li X, Bai J, Zhang Y, Feng R. 2020. Host antiviral  
665 protein IFITM2 restricts pseudorabies virus replication. *Virus Research* 287:198105.
- 666 40. Xu Z, Li X, Xue J, Shen L, Zheng W, Yin S, Xu J. 2020. S-palmitoylation of swine interferon-  
667 inducible transmembrane protein is essential for its anti-JEV activity. *Virology* 548:82–92.
- 668 41. Zhang Y, Huang Y, Wang L, Huang L, Zheng J, Huang X, Qin Q. 2020. Grouper interferon-  
669 induced transmembrane protein 3 (IFITM3) inhibits the infectivity of iridovirus and nodavirus  
670 by restricting viral entry. *Fish Shellfish Immunol* 104:172–181.
- 671 42. McMichael TM, Zhang L, Chemudupati M, Hach JC, Kenney AD, Hang HC, Yount JS. 2017.  
672 The palmitoyltransferase ZDHHC20 enhances interferon-induced transmembrane protein 3  
673 (IFITM3) palmitoylation and antiviral activity. *J Biol Chem* 292:21517–21526.
- 674 43. John SP, Chin CR, Perreira JM, Feeley EM, Aker AM, Savidis G, Smith SE, Elia AEH, Everitt  
675 AR, Vora M, Pertel T, Elledge SJ, Kellam P, Brass AL. 2013. The CD225 Domain of IFITM3  
676 Is Required for both IFITM Protein Association and Inhibition of Influenza A Virus and Dengue  
677 Virus Replication. *Journal of Virology* 87:7837–7852.
- 678 44. Zhao X, Sehgal M, Hou Z, Cheng J, Shu S, Wu S, Guo F, Marchand SJL, Lin H, Chang J, Guo  
679 J-T. 2018. Identification of Residues Controlling Restriction versus Enhancing Activities of  
680 IFITM Proteins on Entry of Human Coronaviruses. *Journal of Virology* 92.
- 681 45. Rahman K, Coomer CA, Majdoul S, Ding SY, Padilla-Parra S, Compton AA. 2020. Homology-  
682 guided identification of a conserved motif linking the antiviral functions of IFITM3 to its  
683 oligomeric state. *Elife* 9.
- 684 46. Winkler M, Wrensch F, Bosch P, Knoth M, Schindler M, Gärtner S, Pöhlmann S. 2019. Analysis  
685 of IFITM-IFITM Interactions by a Flow Cytometry-Based FRET Assay. *Int J Mol Sci* 20.
- 686 47. Yount JS, Karssemeijer RA, Hang HC. 2012. S-Palmitoylation and Ubiquitination Differentially  
687 Regulate Interferon-induced Transmembrane Protein 3 (IFITM3)-mediated Resistance to  
688 Influenza Virus. *J Biol Chem* 287:19631–19641.
- 689 48. Chesarino NM, Compton AA, McMichael TM, Kenney AD, Zhang L, Soewarna V, Davis M,  
690 Schwartz O, Yount JS. 2017. IFITM3 requires an amphipathic helix for antiviral activity. *EMBO*  
691 *Rep* 18:1740–1751.
- 692 49. Qian J, Le Duff Y, Wang Y, Pan Q, Ding S, Zheng Y-M, Liu S-L, Liang C. 2015. Primate  
693 lentiviruses are differentially inhibited by interferon-induced transmembrane proteins. *Virology*  
694 474:10–18.
- 695 50. Ahi YS, Yimer D, Shi G, Majdoul S, Rahman K, Rein A, Compton AA. 2020. IFITM3 Reduces  
696 Retroviral Envelope Abundance and Function and Is Counteracted by glycoGag. *mBio* 11.
- 697 51. Löytynoja A, Goldman N. 2008. Phylogeny-aware gap placement prevents errors in sequence  
698 alignment and evolutionary analysis. *Science* 320:1632–1635.
- 699 52. Guindon S, Dufayard J-F, Lefort V, Anisimova M, Hordijk W, Gascuel O. 2010. New algorithms  
700 and methods to estimate maximum-likelihood phylogenies: assessing the performance of  
701 PhyML 3.0. *Syst Biol* 59:307–321.

- 702 53. Anisimova M, Gascuel O. 2006. Approximate likelihood-ratio test for branches: A fast, accurate,  
703 and powerful alternative. *Syst Biol* 55:539–552.

704

705 **FIGURE LEGENDS**

706 **Figure 1. Presentation of the animal IFITMs used here.** A) Schematic genomic  
707 organization of *IFITMs*. Black boxes indicate IFITMs that have been functionally analyzed  
708 here. Given that the anti-HIV-1 activities of human IFITM1, 2 and 3 have been well  
709 characterized, only human IFITM3 was used here as functional standard for the remaining  
710 IFITMs. B) Identity matrix of the different IFITMs. C) Comparison of the N and C termini  
711 of animal IFITMs. Left, Species cladogram. Right, Comparison of N and C terminal  
712 sequences of selected IFITMs. Nter sequences were aligned as described in Methods, while  
713 the Cter sequences have little sequence homology and are shown here as sequence list.

714

715 **Figure 2. Confocal microscopy analysis of animal IFITMs.** HEK293T cells ectopically  
716 transfected with DNAs coding the indicated IFITMs were fixed and analyzed by  
717 immunofluorescence and confocal microscopy analyses. The pictures are representative of  
718 the major distribution pattern observed by examining >50 positive cells per construct.

719

720 **Figure 3. Mammalian IFITMs exhibit a broad functional heterogeneity in their ability**  
721 **to inhibit HIV-1 during either target cell protection or negative imprinting of virion**  
722 **particles infectivity.** A) Schematic representation of the experimental system used here. To  
723 assess the activity of IFITMs in mediating the protection of target cells from infection,  
724 HEK293T cells were transiently transfected with plasmids coding CD4, CXCR4 and the  
725 indicated IFITMs. Cells were challenged 36 hours later with a GFP coding NL4-3-bearing  
726 HIV-1 vector for a single round of infection assay. The extent of infection was assessed by  
727 flow cytometry. To determine the ability of IFITMs to affect the infectivity of newly-

728 produced virion particles, HEK293T cells were instead transfected with DNAs coding the  
729 HIV-1 virus in addition to IFITMs. The amount of virion particles released in the supernatant  
730 was determined by exo-RT assay after ultracentrifugation through a 25% sucrose cushion.  
731 Virion infectivity was assessed after exo-RT normalization on HeLaP4 cells that stably  
732 express both CD4 receptor and CXCR4 co-receptor. The extent of infection was again  
733 assessed by flow cytometry. B) WB panels display representative results obtained when  
734 IFITMs were expressed in target cells (cell lysates, upper panels) or in virion-producing cells  
735 (purified virion particles, lower panels). C) Infectivities measured during target cell  
736 protection and negative imprinting of virion particles (avg, SEM  $n \geq 4$ ; one-way Anova tests  
737 between the indicated conditions: only statistically significant p values are color-coded). D)  
738 Correlation between the two antiviral properties for each IFITM molecule: \*,  $p < 0.0001$   
739 following a Pearson's correlation coefficient analysis.

740

741 **Figure 4. The steady state levels of the different IFITM proteins indicate a correlative,**  
742 **not absolute, trend between antiviral phenotype and levels of expression of IFITMs.**

743 Intracellular staining was used to measure the intracellular levels of IFITMs by flow  
744 cytometry in function of their MFI. A and B) Representative histograms and cumulative  
745 variations in the MFIs, in this case normalized to human IFITM3 (grey in the panels of A  
746 present staining of negative controls). Graphs present avg and SEM obtained from 3  
747 independent experiments. C) Correlations between the antiviral effects of IFITMs and their  
748 intracellular accumulation levels. \*:  $p = 0.0001$  (left) and  $p = 0.01$  (right), following a Pearson's  
749 correlation coefficient analysis between the indicated conditions.

750

751 **Figure 5. The phenotypic differences observed among selected IFITM molecules are**  
752 **also governed by their intrinsic behavior and not only by protein stability.** Increasing  
753 doses of the non-functional D1 and C1 IFITMs were compared to a fixed dose (starting from  
754 equimolar to a 4-fold excess) of antiviral D1Lb and R1, respectively in target cell protection.  
755 IFITM expressing cells were either analyzed by WB or challenged prior to flow cytometry  
756 analysis three days later. IFITM levels were in this case measured by densitometry following  
757 WB, as this yielded comparable results than intracellular staining and FACS. B) as in A, but  
758 using decreasing doses of the antiviral R1 molecule, over a fixed dose of the non-functional  
759 C1. Graphs present avg and SEM of at least 4 independent experiments and WB panels  
760 present typical results obtained. \*:  $p < 0.05$ , following a one-tail Student t test between the  
761 indicated conditions.

762

763 **Figure 6. Anti-viral and non-antiviral IFITMs do not present obvious distinguishing**  
764 **marks.** A and B) Amino acid alignment of the tested IFITMs (A) and corresponding logo  
765 plots (B) for the “Antiviral” and “Non-antiviral” (D3, C3, C1, and D1; highlighted in grey)  
766 IFITMs, as described in this study. Grey underlines strictly non-antiviral IFITMs.  
767 Visualization was performed with Geneious (Biomatters, Inc.), with color-coding according  
768 to polarity. In panel A, H3 is set as the reference sequence; dots correspond to residues similar  
769 to the reference sequence, while hyphens correspond to gaps. Numbering is according to H3  
770 (human IFITM3) in panel A, and to the consensus sequence of the alignment in panel B. C)  
771 Phylogenetic tree of the IFITMs tested here. Node supports are aLRT. The scale bar  
772 represents number of amino acid substitutions per site.

773

774 **Figure 7. A chimera approach between C1 and R1 IFITM proteins identifies the**  
775 **importance of the cross-talk between the TMD and the Cter domains for optimal**  
776 **antiviral activity.** A) Schematic representation of the chimeras used. B and C) Western blot  
777 analysis and densitometric quantification following ectopic DNA transfection in HEK293T  
778 cells. D) Representative images of the intracellular distribution of the different chimeras  
779 obtained by confocal microscopy. E) Chimeras were tested in target cell protection by  
780 challenging HEK293T cells expressing the indicated chimeras thirty-six hours post DNA  
781 transfection with GFP-coding NL4-3-bearing HIV-1. The extent of infection was measured  
782 two days later by flow cytometry. The graphs present avg and SEM of at least 4 independent  
783 experiments and WB panels present typical results obtained. \*:  $p < 0.05$ , following a One-way  
784 Anova test between the indicated conditions.

785

786 **Figure 8. D1/D1Lb chimeras further stresses the importance of the cross-talks between**  
787 **individual IFITM domains for optimal antiviral activity.** As in the legend to figure 7. A)  
788 Schematic representation of the chimeras used. B and C) Western blot analysis and  
789 densitometric quantification following ectopic DNA transfection in HEK293T cells. D)  
790 Representative images of the intracellular distribution of the different chimeras obtained by  
791 confocal microscopy. E) Chimeras were tested in target cell protection by challenging  
792 HEK293T cells expressing the indicated chimeras thirty-six hours post DNA transfection  
793 with GFP-coding NL4-3-bearing HIV-1. The extent of infection was measured two days later  
794 by flow cytometry. The graphs present avg and SEM of at least 4 independent experiments  
795 and WB panels present typical results obtained. \*:  $p < 0.05$ , following a One-way Anova test  
796 between the indicated conditions.

797

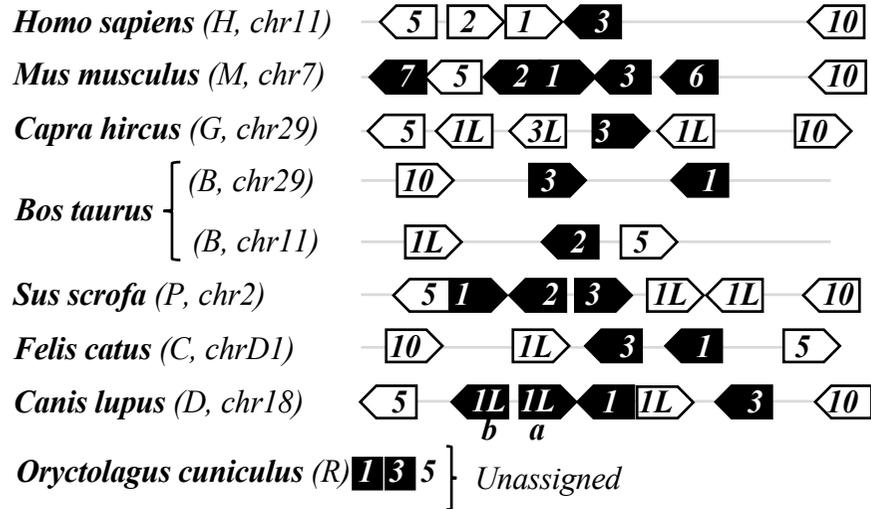
798 **Figure 9. Chimeras between C1/R1 and D1/D1Lb that are active for target cell**  
799 **protection conserve their ability to negatively imprint HIV-1 virions infectivity.** A)  
800 Experimental scheme and chimeras tested here. B) Virions were produced in the presence of  
801 the indicated chimeras, normalized and used to challenge target cells. Infectivity was  
802 determined forty-eight hours later by flow cytometry. The graph presents avg and SEM of 3  
803 independent experiments. \*:  $p < 0.05$ , following a One-way Anova test between the indicated  
804 conditions.

805

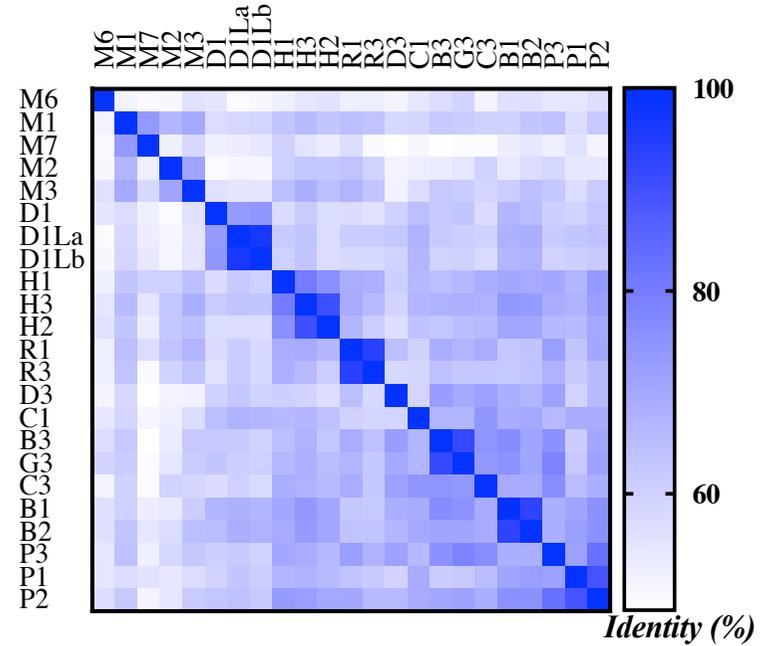
806 **Figure 10. Murine IFITM3 intrinsically lacks the ability to negatively modulate the**  
807 **infectivity of newly-produced HIV-1 virion particles.** A) Increasing doses of human and  
808 murine IFITM3s (H3 and M3, respectively) were used to produce HIV-1 particles and to  
809 determine whether M3 would hinge on HIV-1 virions infectivity. The panels and the graph  
810 present typical results obtained (n=3, avg and SEM). \*:  $p < 0.05$ , following a One-Way Anova  
811 test between the indicated conditions. B) The Y78F substitution was introduced in M3 and  
812 the mutant was tested for its ability to negatively imprint newly-produced HIV-1 virions. The  
813 panels and the graph present typical results obtained (n=3, avg and SEM). No statistically  
814 significant difference was observed between control and mutant.

**A**

INTERFERON-INDUCED TRANSMEMBRANE PROTEINS



**B**

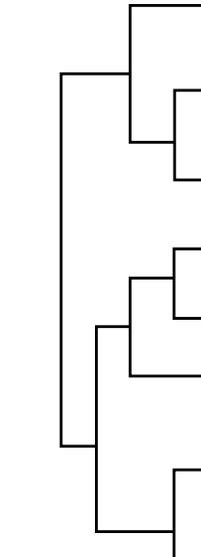


**C**

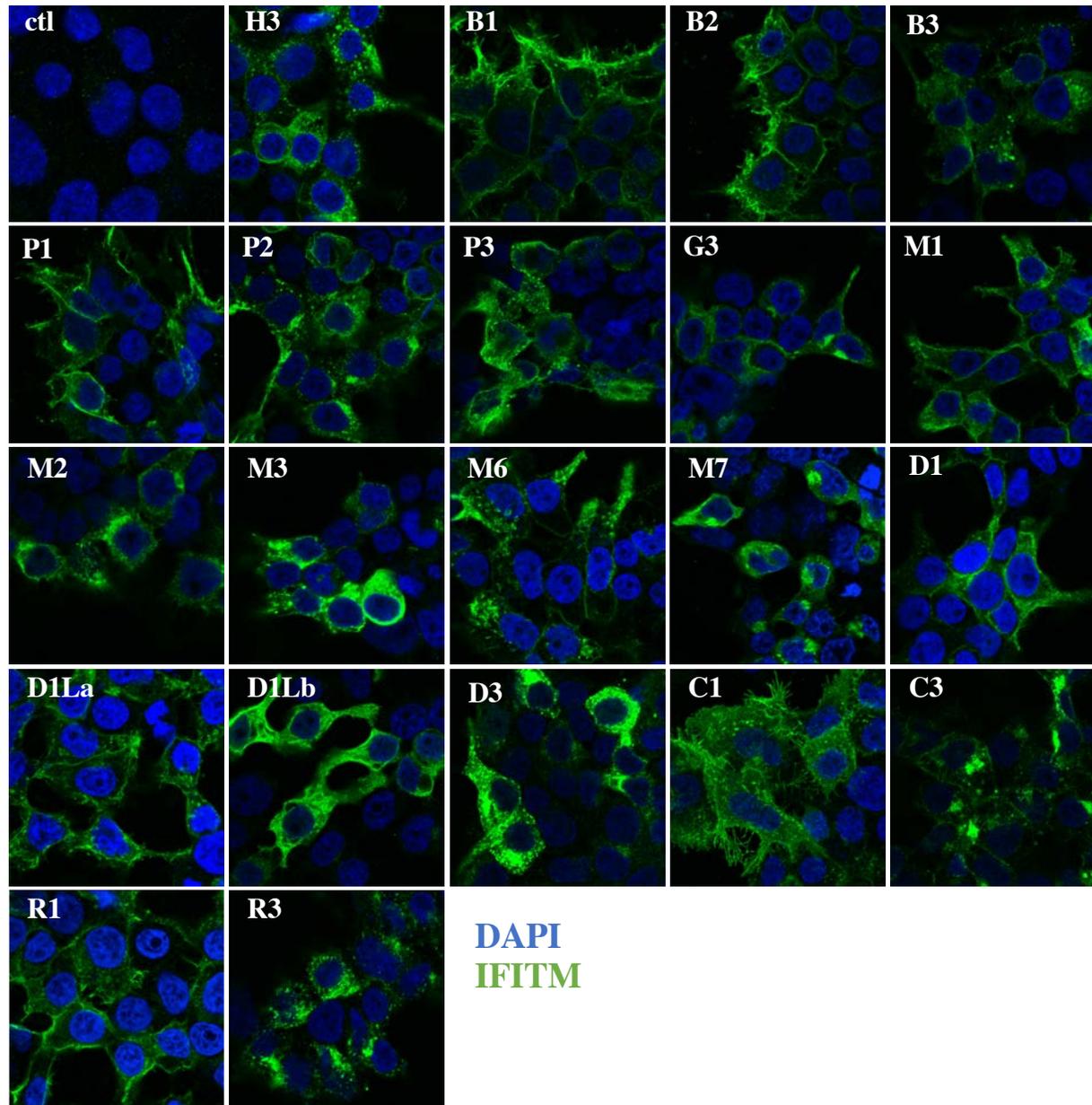
Domains

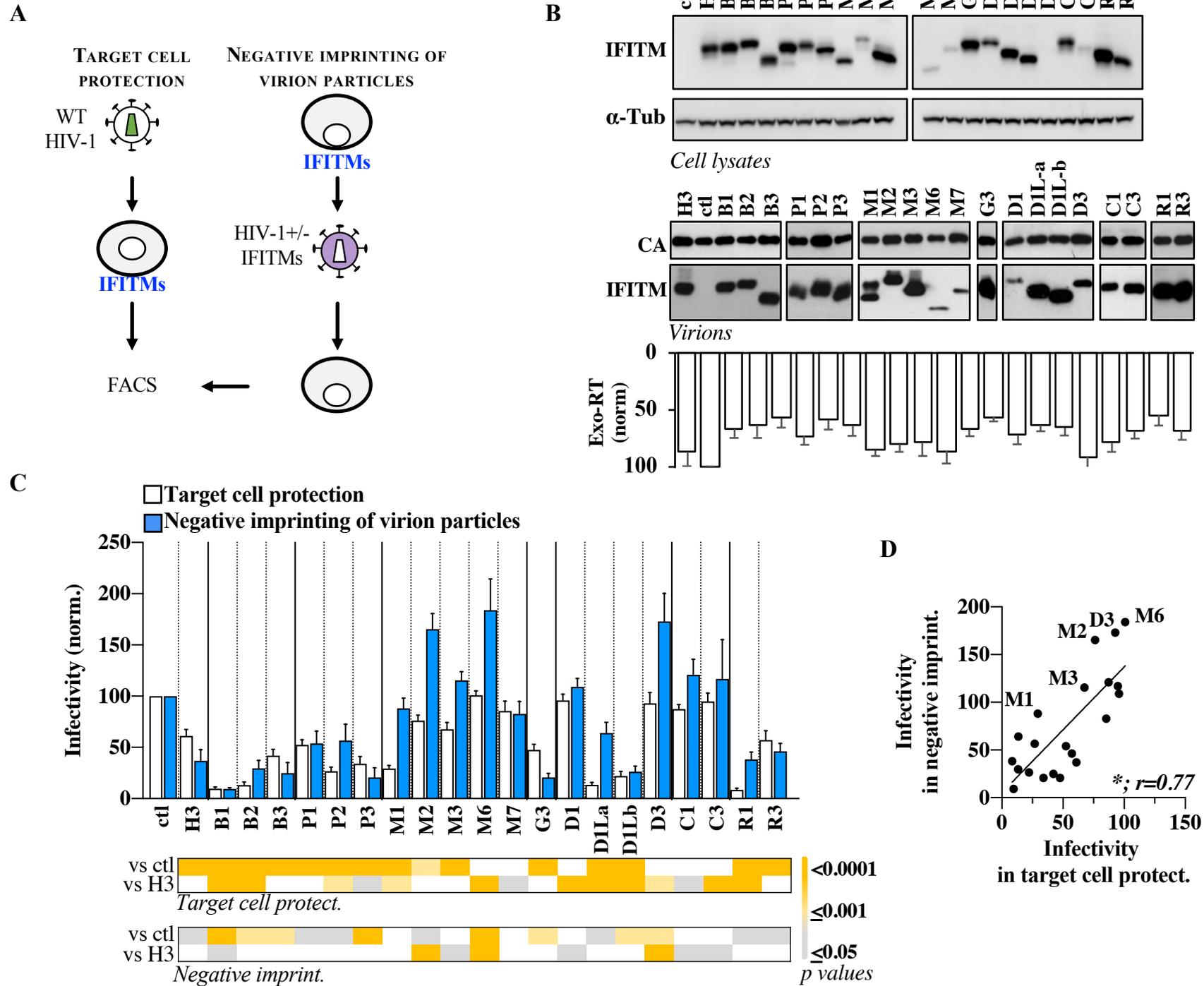
PPxY (*Nedd4*)

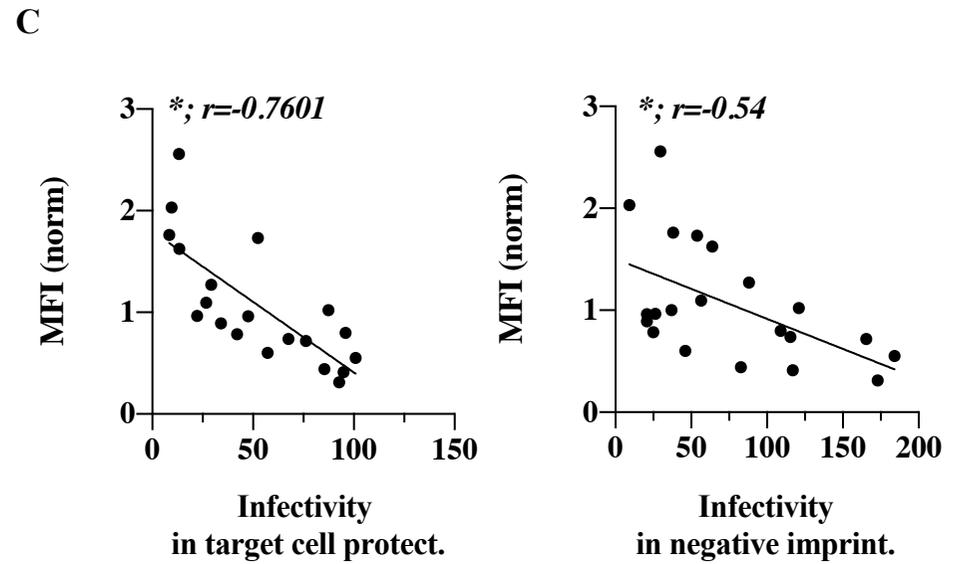
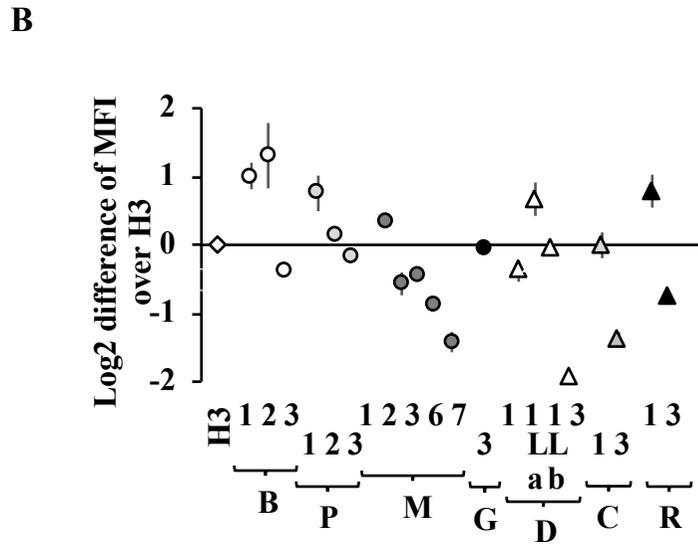
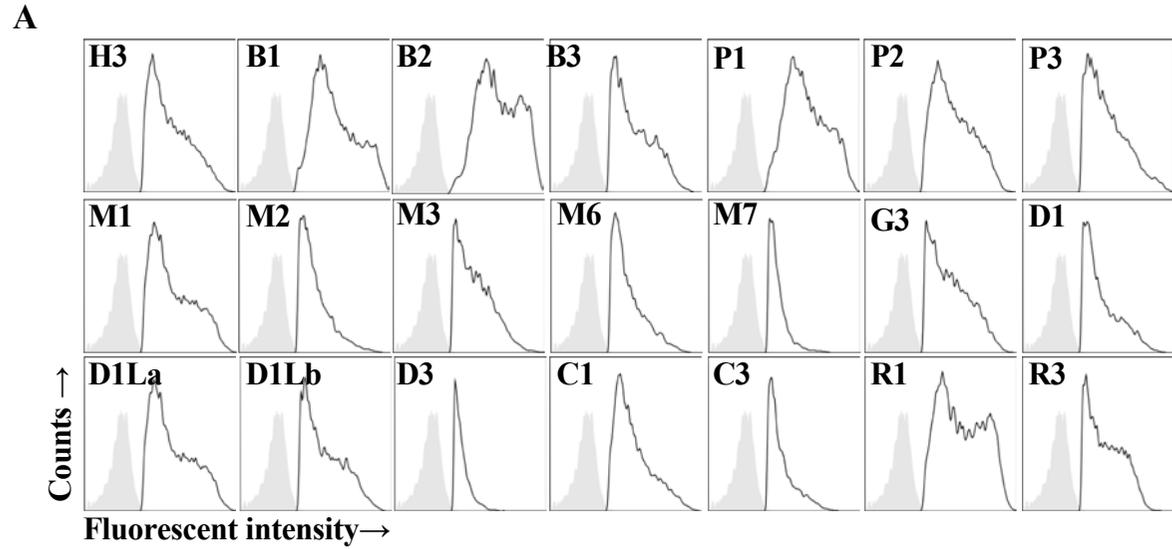
Yxxφ (*AP2*)



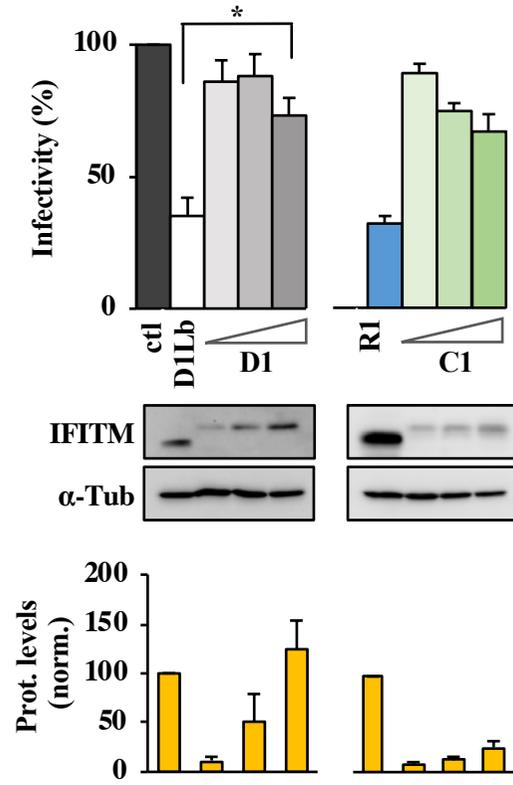
|      | ← N-Term. →   | ← C-Term. →           |
|------|---|-----------------------|
| H3   | MNHTVQTFVSPVNSGQPPNYEMLKEEHEVAVLG-APHNAPPTSTVIHIRSETSVDPH   | IFQAYG                |
| H2   | MNHIVQT-FSPVNSGQPPNYEMLKEEQEVAMLG-VPHNPAPMSTVIHIRSETSVDPH   | VVQAQR                |
| H1   | -----MHKEEHEVAVLG-APPSTILPRSTVINIHSETSVDPH                  | GSVTVYHIMLQIIQEKRGY   |
| M3   | MNHTSQAFITAAASGGQPPNYERIKEEYEVAEMG-APHGASVVRTTVINMPREVSVPDH | IVLNAQNLHT            |
| M2   | MSHNSQAFVST-NAGLPPSYETIKEEYGVTELG-EPNSAVVRTTVINMPREVSVPDH   | TSVVVFQSFQRTPHSGF     |
| M1   | -----MPKEQQEVVVLG-SPHISTSATATTINMP-EISTPDH                  | R                     |
| R3   | MSNVSKTFFSTASTSRTPNYEELREEHQIIVLG-APH-SAAVASTVINVHSEVSVPDH  | SSAIFHAISP            |
| R1   | -----MSKEEHQVEMLG-APR-SAAVASTVINVHSEVSVPDH                  | SSAIFHAISP            |
| B3   | MNRTSQPLFTGAHGAVPPAYEVLKEEHEVAVLG-APQSAPLTTVINIRSDTAVPDH    | GSLMIVQAVSELMQNYGGH   |
| B2   | -----MLKEEHEVAVLG-APQSAPVTTVINIPRENSVPDH                    | VYMAAYETALRISRHHGH    |
| B1   | -----MI-EEHEVAVLG-APQSAPLTTVINIRSDTAVPDH                    | VYMAAYETALRISRHHGH    |
| G3   | MNCTSQPLFTGAHGAVPPGYEVLKEEHEVAVLG-APQSAPVTTVINIRSDTAVPDH    | GSLMIVQAILLELIQNYGGH  |
| P3   | MNCASQPFVFTGAHGG-PPTYEMLKEEHEVAVLG-APQTSAPVATTVINIRSETSVDPH | GSLVIFQAVLQLIKDYRGY   |
| P2   | MNCASQPFVFTGAHGG-PPTYEMLKEEHEVAVLG-APQTSAPVATTVINIRSETSVDPH | VYITAYQMLERAKSNRGY    |
| P1   | MI-KSQ-----H-----EM--D---G-LG-APQTSAPVATTVITIPRETSVPDH      | VYITAYHMLERAKSNRGY    |
| D3   | MSRAPRLLPGARAAGPPTYEMLKEEHEVVVLGGAPQSAAPATTVINIRGDTVVPDH    | GSLVIFETVSEMVKHYGGS   |
| D1   | -----MDQDQYKVPETG-APQSMVPVTTVINIRSDTVVPDH                   | AFGAAYWALLQVMQERSRYH  |
| D1La | -----M-QN--KVDVRG-APLSTAPATTVINVPVETVVPDH                   | AYAAAYGALLQAMQESGRYH  |
| D1Lb | -----M-QN--KVDVRG-ALLSTAPATTVINVPEETVVPDH                   | AYAAAYGALLQVMQESGRYH  |
| C3   | MNRNAQPFVLPVHTGVPPTYEMLKEEHEVAVLG-GPQSPAPMATTVINIQTETSVPDH  | GSLVVFQAI SEMMKGYGGY  |
| C1   | -----MLKD-NKVDILE-GPQSSAPMATTVINIQTETSVPDH                  | AYLTAYSII LRVMQNSRGHH |



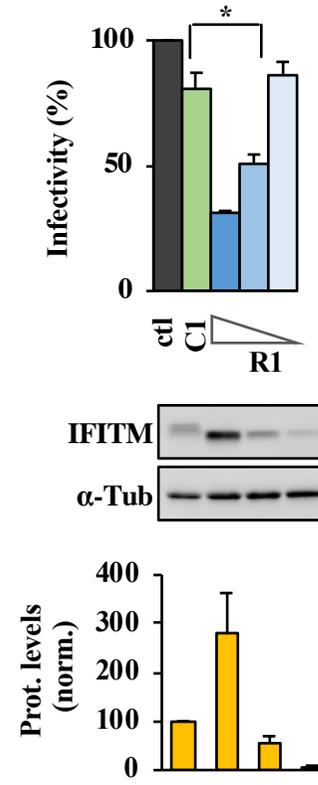




**A**

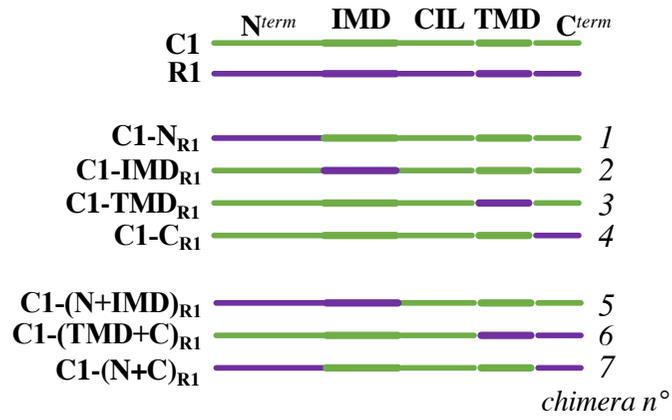


**B**

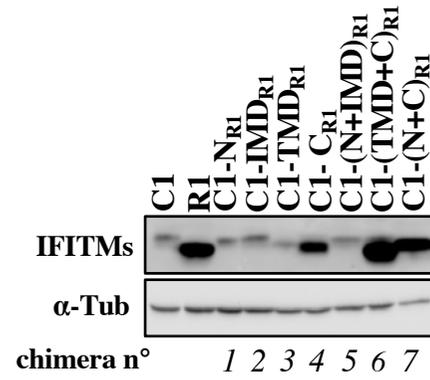




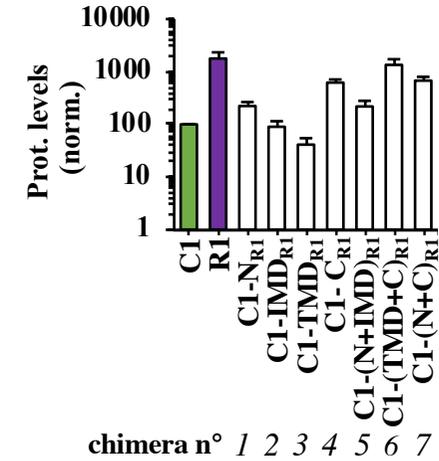
**A**



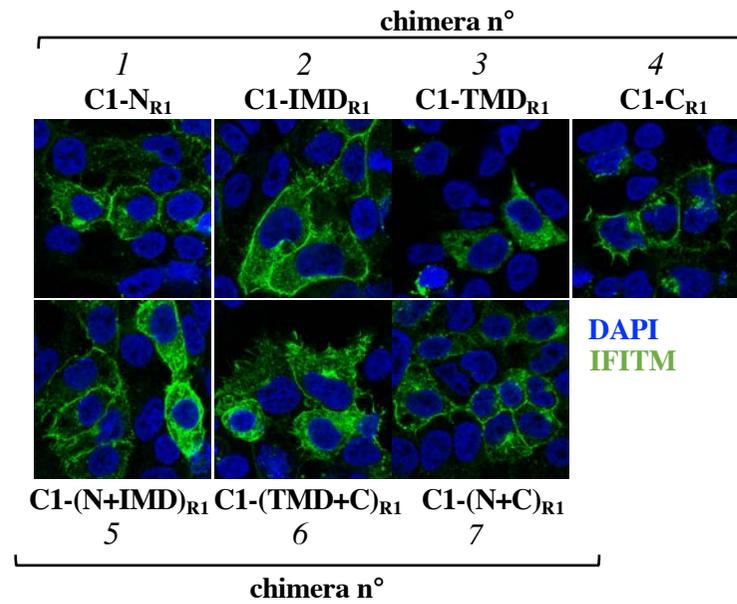
**B**



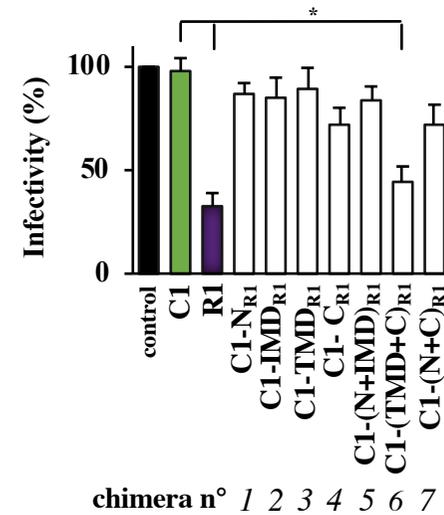
**C**



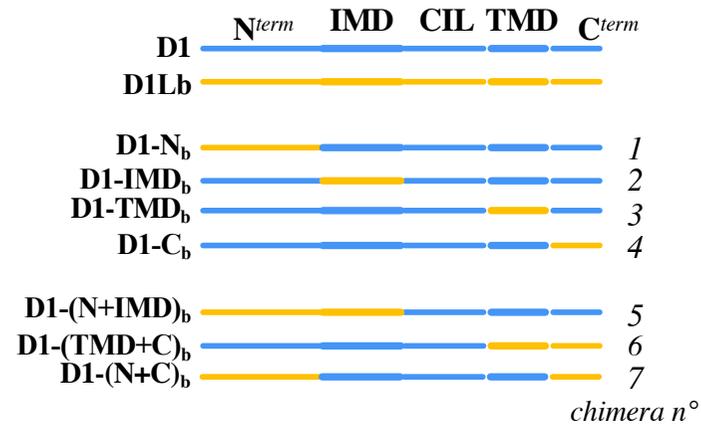
**D**



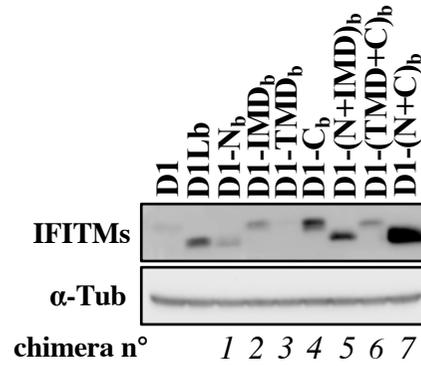
**E**



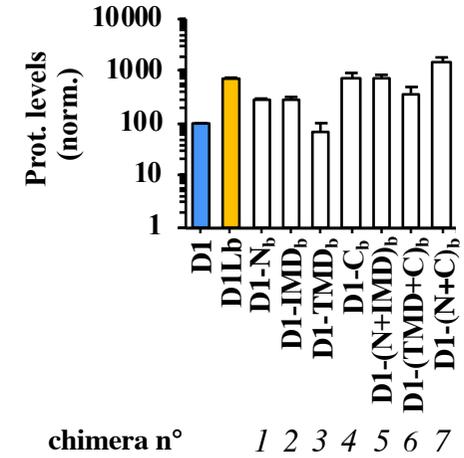
**A**



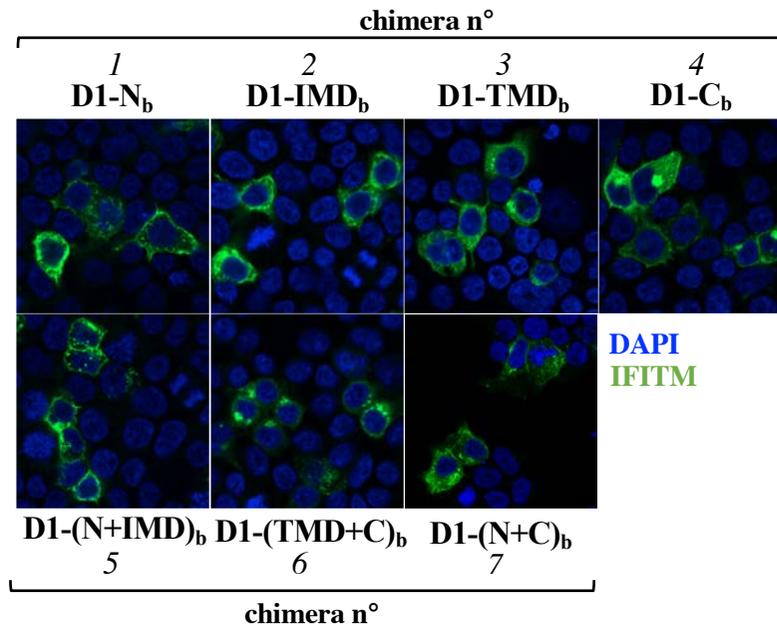
**B**



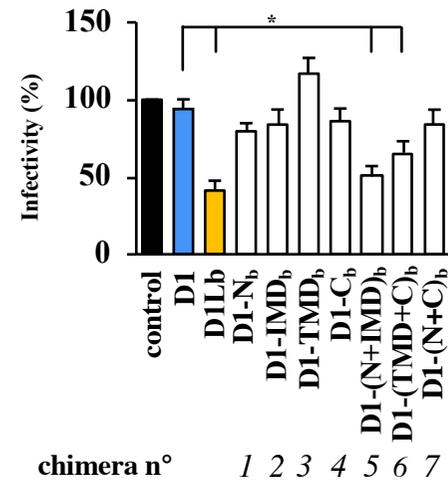
**C**



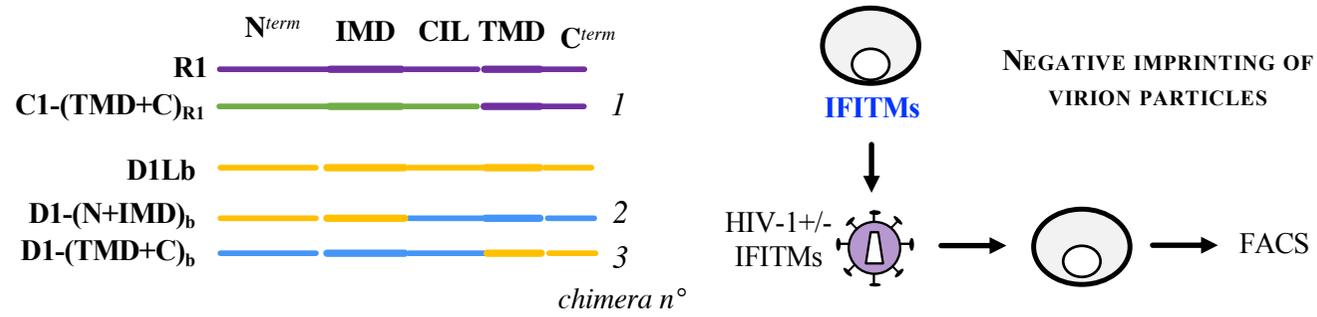
**D**



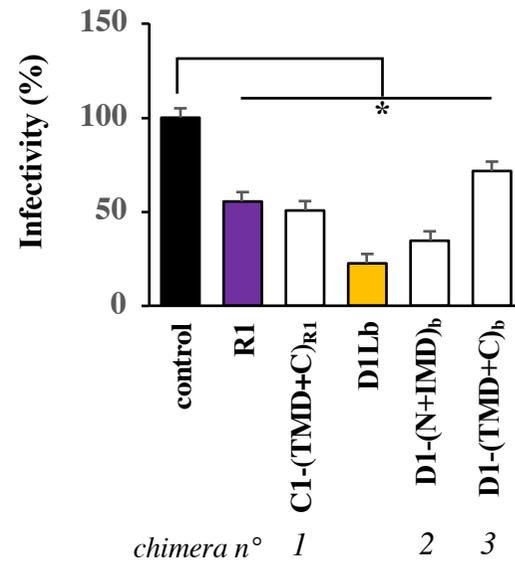
**E**



A



B



NEGATIVE IMPRINTING OF VIRION PARTICLES

