IFITM proteins inhibit HIV-1 protein synthesis

Wing-Yiu Jason Lee[1], Chen Liang[2] and Richard D Sloan[1,3*]

[1]Blizard Institute, Barts and The London School of Medicine and Dentistry, Queen Mary University of London, London E1 2AT, United Kingdom. [2]McGill University AIDS Centre, Lady Davis Institute, Montreal, Quebec H3T 1E2, Canada. [3]Division of Infection and Pathway Medicine, School of Biomedical Sciences, University of Edinburgh, Edinburgh EH16 4SB, United Kingdom.

*corresponding author: richard.sloan@ed.ac.uk

Abstract

Interferon induced transmembrane proteins (IFITMs) inhibit the cellular entry of a broad range of viruses, but it has been suspected that for HIV-1 IFITMs may also inhibit a post-integration replicative step. We show that IFITM expression reduces HIV-1 viral protein synthesis by preferentially excluding viral mRNA transcripts from translation and thereby restricts viral production. Codon-optimization of proviral DNA rescues viral translation, implying that IFITM-mediated restriction requires recognition of viral RNA elements. In addition, we find that expression of the viral accessory protein Nef can help overcome the IFITM-mediated inhibition of protein synthesis. Our studies identify a novel role for IFITMs in inhibiting HIV replication at the level of translation, but show that the effects can be overcome by the lentiviral protein Nef.

23 **Importance**

24 Cells can protect themselves from infection by producing antiviral proteins that directly
25 disrupt viral replication processes. The IFITM protein family has been identified to inhibit
26 a wide range of viruses in this way, including HIV. Inhibition of HIV infection in cells by
27 IFITM proteins is understood to occur due to a block in entry of viruses into cells, but
28 also due to a reduction in the infectivity of virus particles produced in cells that express
29 IFITMs. However, a third and additional block to HIV infection by IFITMs has been
30 suspected. We show here that IFITM proteins specifically block viral protein production
31 even after HIV enters target cells. Yet we also show that the virus can partially
32 overcome this block by producing a countermeasure protein Nef. Therefore our data
33 show that IFITM proteins are multifunctional antiviral proteins that can inhibit HIV
34 through three distinct mechanisms – blocking viral entry, reducing virus infectivity, and
35 inhibiting virus protein production.

36

37

38 **Introduction**

39 Viruses must replicate in cells; the consequence of such dependence is susceptibility.
40 Cells have thus evolved a series of antiviral factors that directly subvert viral replication.
41 Recent work has revealed the striking scale of antiviral mechanisms employed by cells -
42 almost every step of viral replication is apparently targeted by host encoded antiviral
43 factors. For HIV the best defined examples are APOBEC3G/3F and SamHD1 that
44 inhibit viral reverse transcription, TRIM5α that targets viral capsid uncoating, and
45 tetherin that prevents viral egress (reviewed in (1, 2)). However, genomic and proteomic

46 screens can aid identification of further antiviral factors, and so there is an expanding
47 list being described [3,4]. Such studies identified the MxB/Mx2 protein that targets HIV-1
48 replication around nuclear entry (3, 4), and the SERINC3 and SERINC5 proteins that
49 target HIV-1 entry (5, 6).

50 The interferon induced transmembrane proteins (IFITMs) have been similarly identified
51 in multiple independent screens as antiviral factors (7-10). Humans have three antiviral
52 IFITMs - IFITM1, IFITM2 and IFITM3 (reviewed in (11)). IFITM expression inhibits viral
53 entry into cells (7, 8), a phenotype that is enhanced by incorporation of IFITM proteins
54 into viral particles (12-14). Inhibition of viral entry is thought to occur via IFITM-mediated
55 changes in the physical characteristics of the host cell membrane thereby inhibiting
56 virus-cell membrane fusion (15), though other inhibitory mechanisms affecting entry
57 have been proposed (16). By employing this generic inhibitory mechanism, IFITMs can
58 then inhibit the entry of a broad range of viruses including influenza A virus, hepatitis C
59 virus, Ebola virus, SARs coronavirus, Dengue virus, Zika virus, and HIV-1 (7, 8, 17-19).

60 Yet since the discovery of their antiviral effect upon HIV-1, it was immediately apparent
61 that a viral replication step downstream of host genome integration might also be
62 inhibited by IFITM proteins, as IFITM1 knockdown increased HIV-1 titres from infected
63 $CD4^+$ T-cells (8). In the same study, IFITM1 inhibited the replication of HIV-1 in T-cell
64 culture despite the evidence that IFITM1 had no effect on viral replication steps from cell
65 entry to host genome integration for the viral strain used. Other studies have also noted
66 IFITM1-3 expression reduces viral particle production from cells (10, 12-14, 20-24).
67 When this occurs, IFITM expression is typically associated with reductions in HIV-1 Gag
68 levels, implying a block in viral protein synthesis.

69 Despite the frequency of these observations, there has been little to no attempt to
70 explain them. Why viral production should be affected by IFITM expression is unclear.
71 We therefore sought to investigate the potential block in protein synthesis induced by
72 IFITMs and now show that IFITM expression leads to an inhibition of lentiviral
73 production, which for HIV-1 occurs due to specific exclusion of viral mRNA from
74 polysomes. Yet we also find that expression of the lentiviral accessory protein Nef can
75 help relieve the inhibition in protein synthesis, allowing HIV-1 to better replicate in the
76 presence of IFITM proteins. Thus IFITM proteins are able to inhibit HIV-1 replication
77 through three distinct processes - inhibition of viral entry, reduction of viral particle
78 infectivity, and inhibition of viral protein synthesis.
79

80 **Results**

81 **IFITMs inhibit HIV-1 production**

82 We first wanted to confirm if IFITMs affect the production of HIV from virus producing
83 cells as noted in previous reports (8, 10, 12-14, 20-24). We used a co-transfection
84 scheme in which we co-transfected HEK293T cells with plasmids containing HIV-1
85 proviral DNAs together with expression vectors encoding human IFITM1-3, allowing the
86 effects on virus production to be studied independently of effects of IFITM proteins on
87 viral entry. The quantity of virus produced was then analyzed by supernatant p24 ELISA
88 and reverse transcriptase activity assays. Increasing levels of IFITM expression
89 decreased HIV-1 NL4-3 output by in a concentration-dependent manner consistent with
90 loss of intracellular p24 levels (Figure 1A, 1B), with a similar decrease seen in
91 supernatant when both p24 concentration (Figure 1C) and reverse transcriptase activity

92 (Figure 1D) were measured. Notably, the expression of IFITM1 and IFITM2 reduced the
93 HIV-1 output while the effect was weaker for IFITM3. We then studied the effect of
94 IFITMs on other viral proteins, and found IFITM1 and IFITM2 expression led to a
95 reduction in the levels of all HIV-1 NL4-3 proteins assayed in transfected cells (Figure
96 1E). Similar to HIV-1 NL4-3, viral production from the HIV-1 plasmid proviral DNAs 89.6
97 and Indie-C1 was also reduced by IFITM expression (Figure 1C-D).
98 In addition to HIV-1, the expression of IFITMs also reduced the quantity of virus
99 produced from cells transfected with plasmid proviral DNAs for HIV-2 and SIVs (Figure
100 1F-G). Of note, HIV-2_{ROD} and SIV_{AGM} appeared to be less susceptible to this IFITM-
101 mediated effect compared to the HIV-1 viruses studied. We also found similar inhibitory
102 effects upon HIV-1 with transfected African Green Monkey IFITM1 (Figure 1H).
103 We were concerned however that the loss of viral production we saw might be due to
104 generic inhibition of protein production during IFITM expression, but detection of GFP
105 from a transfected reporter plasmid was minimally, or totally unaffected by IFITM
106 expression (Figure 1I). We also measured the infectivity of the virus produced from
107 IFITM-expressing cells, in TZM-bl cells and showed that virus produced have lower
108 infectivity (Figure 1J), in agreement with previous results (12-14).
109 We next wanted to test if IFITMs inhibit viral production in infected cells rather than
110 transfected cells. We infected SupT1 cells expressing doxycycline inducible IFITM1-3
111 with wild type NL4-3 virus (8). To avoid inhibition of virus entry playing a role in our
112 experiments, after spinoculation, unbound virus was washed off and cells were
113 resuspended in new medium containing doxycycline and the CXCR4 antagonist
114 AMD3100 to induce IFITM expression only after virus entry and to limit viral replication

115 to a single round respectively. The quantity of wild type HIV-1 produced by IFITM-
116 expressing T-cells was inhibited across a range of viral inputs and was paired with
117 losses in viral protein levels (Figure 2), indicating that IFITMs inhibit viral production
118 independent of their effects on viral entry.

119 We next wished to investigate this effect using knockdown of endogenous IFITM levels,
120 and to assess the relative contribution of IFITMs towards inhibition of viral production
121 that is observed with type I interferon (IFN) treatment. We therefore analyzed viral
122 output from HIV-1 NL4-3 plasmid proviral DNA transfected and IFN-β treated TZM-bl
123 cells that had been transduced with shRNAs targeting different IFITMs (25). Entry of
124 virus produced was inhibited through the use of the CXCR4 antagonist AMD3100, such
125 that the level of viral output in our analysis is not confounded with entry inhibition of
126 IFITM expression. The efficiency of knockdown in IFITM expression was confirmed by
127 immunoblotting using monoclonal antibodies specific for individual IFITMs (Figure 3A).
128 As anticipated, IFN-β treatment reduced viral production in HIV-1 transfected TZM-bl
129 cells (Figure 3B). Despite some weak knockdown cross-reactivity, such that shRNA
130 targeting IFITM1 caused weak knockdown of IFITM2 (Figure 3A), and that it was not
131 possible to knockdown IFITM3 in isolation, knockdown of both IFITM1 and IFITM2
132 clearly rescued the level of virus produced in IFN-β treated cells (Figure 3C). Further,
133 knockdown of all three IFITMs gave a higher rescue, indicating that the inhibitory effects
134 of IFITM1 and IFITM2 on viral production are perhaps non-redundant in the context of
135 HIV-1 replication. These effects were paired with rescue of viral proteins levels during
136 IFITM knockdown (Figure 3D and 3E). These data then show that endogenous levels of

137 IFITMs can inhibit viral particle output and suggest that IFITMs are a significant
138 component of the antiviral response induced by type I interferon.
139 We next sought to confirm if the knockdown of physiologically relevant levels of IFITMs
140 in HIV-1 infected primary cells could rescue viral protein production. Primary human
141 $CD4^+$ T cells were infected with HIV-1 following transduction with lentiviral particles for
142 the expression of shRNAs targeting different IFITMs. Since IFN exposure upregulates
143 other antiviral factors from interferon-stimulated genes (ISGs), which would mask the
144 antiviral phenotype of IFITMs in our analysis, $CD4^+$ T cells were stimulated via the T cell
145 receptor (TCR) instead, to upregulate IFITMs (12) thereby mitigating effect of other
146 ISGs. The efficiency of knockdown in IFITM expression following TCR activation was
147 confirmed by immunoblotting using monoclonal antibodies specific for individual IFITMs
148 (Figure 4D and J). Transduction with shRNAs gave a specific ~30% downregulation of
149 IFITM1, and a specific ~90% downregulation of IFITM2, while one shRNA led to a ~50%
150 downregulation of all three IFITMs (Figure 4D, 4E, 4J and 4L). To mitigate the effects of
151 IFITMs affecting viral entry on our studies of viral production, we took advantage of
152 recent observations that different IFITM proteins vary in their inhibition of viral entry
153 depending on co-receptor usage (14, 20, 26). IFITM1 inhibits entry of CCR5-tropic
154 viruses and IFITM2/3 predominantly inhibits CXCR4-tropic viruses. We therefore
155 studied viral production effects driven by IFITM1 with HIV-1 NL4-3 (a CXCR4-tropic
156 virus that would be less inhibited by IFITM1 upon entry) or with dual-tropic HIV-1 89.6 in
157 cells pre-treated with the CCR5 antagonist Maraviroc that would direct viral entry via
158 CXCR4. Viral production effects driven by IFITM2 and IFITM3 were similarly studied
159 with HIV-1 89.6, but cells pre-treated with the CXCR4 antagonist AMD3100 which would

160 direct viral entry via CCR5 and thus mitigate entry inhibition by IFITM2 and IFITM3. We
161 used intracellular p24 staining of infected cells in single cycle infections to measure
162 uptake of virus after infection with trypsin treatment to remove cell surface bound virus
163 (27-29). Although cellular uptake of viral p24 is not an exact measure of virus entry due
164 to the uptake of virus into potentially non-productive endocytic pathways, p24 uptake is
165 still found to be inhibited by IFITM expression (12, 13, 22, 23), while endocytic uptake of
166 virus occurs at a lower rate in primary $CD4^+$ T-cells (30). We confirmed that viral uptake
167 was minimally impeded with these viruses during IFITM knockdown (Figure 4A, B, G,
168 H), consistent with prior observations (26). Therefore reductions in virus production
169 were considered to arise predominantly from effects other than inhibition of viral entry.
170 We were thus able to determine that weak knockdown of IFITM1 yielded only a very
171 weak recovery of NL4-3 viral production (25%) and protein expression (Figure 4C, D,
172 F). An effect was more readily detectable during knockdown of IFITM2, or IFITM1-
173 IFITM3, as this led to a clear rescue of viral particle output (7-fold and >10-fold
174 respectively) that was coupled with rescue in viral protein expression (Figure 4I, J, M).
175 This then confirms that physiologically relevant levels of IFITM proteins can clearly
176 inhibit HIV-1 production in primary cells independent of their effects upon entry and
177 infectivity, consistent with our observations in cell lines.
178

179 **IFITMs restrict HIV-1 protein synthesis.**

180 As we found that IFITM expression was associated with losses in viral protein
181 production and viral output, we next asked if IFITMs play a role in inhibiting the
182 transcription or stability of viral mRNA by analyzing the level of unspliced, singly-spliced,

183 and multiply-spliced transcripts detected by RT-qPCR from total RNA of whole cell
184 lysates. IFITM1 and IFITM2 weakly reduced the levels of singly-spliced and multiply-
185 spliced transcripts, while IFITM3 had no measureable effect (Figure 5A). The total level
186 of unspliced transcripts remained unaffected in the presence of IFITM proteins (Figure
187 5A) but nonetheless there were reductions of intracellular level of Gag (a product of
188 unspliced transcripts) in our prior analyses (Figures 1-4). This prompted us to
189 investigate if IFITM proteins affect translation of viral transcripts.

190 To achieve this we performed a polysome analysis for HIV-1 transcripts during IFITM
191 expression. We first analyzed the RNA content in cell lysates fractionated on a sucrose
192 gradient by ultracentrifugation. The total RNA profiles (Figure 5B) and the levels of
193 GAPDH RNA recovered from selected fractions (Figure 5C) of virus producing cells
194 expressing IFITMs are comparable to vector-transfected control, suggesting that IFITM
195 expression did not alter global translation in the cell.

196 Next we asked if the expression of IFITMs specifically affects translation of viral
197 transcripts. Reductions in the levels of viral transcript polysome fractions (in contrast to
198 whole cell lysates) would indicate translational inhibition. As such, we analyzed the level
199 of viral transcripts in selected fractions covering the whole RNA profile with RT-qPCR.
200 We used the level of ribosomal protein L7 as a guide for selection of fractions in our
201 analysis (Figure 5D) as it is a component of 60S ribosomes, which are enriched in 80S
202 monosomes (such as fraction 10 Figure 5D) as well as polysomes (such as fractions
203 14-18, Figure 5D). IFITM1 and IFITM2 significantly reduced the level of unspliced and
204 singly-spliced viral transcripts in polysomes (Figure 5E-G) and increased the level of
205 these transcripts in the ribosome-free fraction (fraction 6), suggesting that expression of

206 IFITM1 and IFITM2 inhibited translation of these viral transcripts. IFITM1 and IFITM2
207 also appeared to somewhat reduce the level of multiply-spliced transcripts (Figure 5G),
208 though the effect did not reach statistical significance, but was nonetheless coupled with
209 a clear accumulation of ribosome-free multiply-spliced transcripts in low concentration
210 sucrose fraction (fraction 6) relative to vector control, similar to other viral transcript
211 classes. These data were consistent with our analysis of viral production (Figure 1)
212 wherein IFITM1 and IFITM2 expression lead to a more potent block in viral production
213 than for IFITM3.
214 A recent study linked the production of abortive HIV-1 late mRNA transcripts to the
215 induction of type I interferon in dendritic cells (31), raising the possibility that a similar
216 induction of other antiviral responses could underpin our results rather than specific
217 IFITM-mediated effects. Notably we did not find strong induction of luciferase activity
218 driven by interferon-stimulated response element (ISRE) due to IFITM expression in
219 transfected HEK293T cells (Figure 5H). Thus IFITM proteins inhibit translation of HIV-1
220 transcripts by leading to their specific exclusion from polysomes, resulting in reduced
221 levels viral proteins and reduced viral output.
222

223 **HIV-1 RNA is a determinant of IFITM-mediated restriction of protein synthesis**

224 To better understand how IFITM proteins help suppressing viral translation, we explored
225 the possibility that they may be involved in a process that is able to distinguish viral
226 RNA from cellular RNA by recognizing viral RNA features. To address this, we
227 measured the level of unspliced viral RNA transcripts in the polysome fraction of
228 HEK293T cells transfected with a codon-optimized vector for HIV-1 NL4-3 Gag only (32)

229 with IFITM-expression vectors. Codon-optimization changes the codon bias of the
230 construct towards humans and therefore also alters the secondary structures of viral
231 RNA. Changing codon bias thus renders HIV-1 unspliced and singly-spliced transcript
232 expression independent of the HIV Rev responsive element (RRE), a *cis* acting RNA
233 structure necessary for transcript nuclear export (33). Codon optimization of *gag*
234 rescued the level of unspliced HIV-1 transcripts in polysomes during IFITM expression,
235 nor was there an accumulation of transcripts in low sucrose fractions as was seen with
236 wild type NL4-3 (fraction 7 in Figure 6A compared to fractions 6 in Figure 5E, F & G). As
237 a result, the level of extracellular p24 production from cells expressing IFITM1 and
238 IFITM2 was not only rescued but enhanced, and the production of the *gag* products p55
239 and p24 was also restored (Figure 6B, C and F), whereas contrasting data for full length
240 pNL4-3 DNA show clear losses of viral production and protein expression in similar
241 experiments (Figure 6D, E and F). These data suggest that IFITMs may recognize viral
242 RNA to suppress viral translation.
243 We then sought to identify the specific RNA features that render HIV-1 susceptible to an
244 IFITM-driven translational block. As our findings of transfected HEK293T cells showed
245 that unspliced and singly-spliced viral transcripts are seemingly more readily excluded
246 from polysomes by IFITM proteins than multiply-spliced transcripts in those cells (Figure
247 5), we reasoned that RRE, which is present in only unspliced and singly-spliced
248 transcripts may be somehow recognized due to IFITM protein expression leading to
249 translational inhibition. To address this, we exploited constructs in which singly-spliced
250 and unspliced transcripts had been rendered RRE and Rev independent via the
251 introduction of the Mason Pfizer Monkey Virus (MPMV) constitutive transport element

252 (CTE) (34). A modification which allows all transcripts to be exported from the nucleus
253 by the cellular TAP/NXT1 pathway, rather than RRE bearing Rev-dependent transcripts
254 exporting via the CRM1 pathway (35). We found that CTE dependent Rev independent
255 virus was still inhibited by IFITM proteins (Figure 6G), demonstrating at least that
256 nuclear export pathway does not affect the virus production inhibition seen with IFITM
257 proteins. Further introduction of the RRE into CTE bearing constructs did also not
258 augment the restriction.
259 Overall, these data suggests that the restriction of translation by IFITMs occurs at the
260 RNA level, though it is not clear that the RRE plays a significant role in susceptibility,
261 despite that RRE bearing transcripts are more affected in translation. Therefore some
262 other distinguishing feature of viral transcripts or translation determines their
263 susceptibility to IFITM proteins.
264

265 **HIV Nef can help overcome IFITM-mediated restriction of protein synthesis**

266 Under the selective pressure of cellular restriction factors, retroviruses have evolved to
267 counteract surveillance and restriction by host antiviral factors (reviewed in (1)). We
268 therefore sought to investigate if HIV is able to counteract the restriction of viral
269 production by IFITM proteins. We reasoned that HIV-1 Nef may influence this process.
270 IFITMs are membrane proteins and lentiviral Nef regulates the trafficking of many
271 membrane proteins such as CD4, MHC-I and tetherin to aid immune evasion and viral
272 replication (36-38). Further, the expression of a dominant negative form of HIV-1 Nef
273 (39), or *nef* deletion has previously been linked to deficits in viral production (40).

274 We therefore measured the level of virus production in HEK293T cells transfected with
275 expression vectors of IFITMs with wild type or *nef*-deleted HIV-1 NL4-3 proviral DNA.
276 The level of *nef*-deleted virus produced was inhibited 18-fold by IFITM1, 21-fold by
277 IFITM2 and 5-fold for IFITM3, suggesting a 4-fold enhancement of virus production due
278 to HIV-1 Nef during IFITM1 and IFITM2 expression (Figure 7A). We confirmed a
279 reduction in HIV-1 Gag levels in cells producing *nef*-deleted virus while IFITMs are
280 expressed (Figure 7B), indicating that Nef may rescue the translational suppression
281 driven by IFITMs. As these data were generated through transfection, they were
282 independent of any influence of Nef upon viral entry (5, 6). It is also of note that
283 exogenous IFITM levels were not reduced by HIV-1 Nef, perhaps indicating that
284 antagonism of IFITM proteins may not occur via degradation (Figure 7B).
285 We next investigated if Nef presented an advantage to viral growth in infected T-cells.
286 We infected SupT1 cells with either wild type or *nef*-deleted NL4-3 virus by post-entry
287 induction of IFITM expression via doxycycline (Figure 7C). As *nef*-deleted virus has an
288 inherent growth defect, we normalized the quantity of wild type and *nef*-deleted virus
289 produced from IFITM-induced cells to vector control cells. We chose to induce IFITMs
290 after viral entry and limit infections to a single round through the use of the (CXCR4
291 antagonist AMD3100) to mitigate the influence of IFITMs upon entry. In this regard, we
292 found that *nef*-deleted virus was no more susceptible to IFITM-mediated restriction of
293 entry than wild type virus (Figure 7D). Compared to wild type virus, production of *nef*-
294 deleted virus in infected SupT1 cells was reduced significantly when IFITM1 expression
295 was induced (~2-fold), while a trend of similar magnitude was noted for IFITM2.

296 However there was no rescue of virus infection by Nef in IFITM3 expressing cells
297 (Figure 7C).
298 To further confirm the role of IFITM1 in suppressing virus production in T cells, we
299 monitored the production of either wild type or *nef*-deleted HIV-1 NL4-3 virus in multiple
300 cycle infections in a C8166 T-cell line transduced to constitutively over-express IFITM1.
301 As IFITM1 does not typically affect the entry of CXCR4-tropic virus (Figure 4A, B and (8,
302 26)), any difference in replication measured between wild type and *nef*-deleted virus
303 could only arise from IFITM1-mediated effects on viral production, not cell entry.
304 Therefore this experiment was not pursued for IFITM2 and IFITM3, as their constitutive
305 expression would inhibit virus entry and complicate interpretation. We found that in the
306 presence of IFITM1, the growth of *nef*-deleted virus was significantly reduced relative to
307 wild type virus, with wild type virus showing 2-fold inhibition at 5 days post-infection,
308 while *nef*-deleted virus was inhibited 5-fold by IFITM1 (Figure 7E). Interestingly, the
309 benefit of Nef expression in the context of IFITM1 was lost in the later stages of viral
310 culture, when cell-to-cell infection typically predominates (41). As *nef*-deleted virus was
311 no more susceptible to IFITM-mediated restriction of entry than wild type virus (Figure
312 7D), these findings are likely due to the influence of Nef to partially rescue virus
313 production as seen in 293T cell transfections and SupT1 infections.
314 We then investigated if Nef from other lentiviruses was able to overcome IFITM-
315 mediated inhibition of protein synthesis using an HIV-1 NL4-3 plasmid proviral DNA into
316 which lentiviral *nef* alleles had been substituted (42). The level of virus production for
317 HIV-1 NL4-3 bearing other *nef* alleles during IFITM expression was generally
318 comparable to levels for wild type NL4-3 *nef* (Figure 7F), indicating that ability of

319 lentiviral Nef proteins to overcome IFITM mediated restriction of virus production is
320 common.
321 To further confirm the role of Nef in antagonizing IFITMs, we titrated a plasmid bearing
322 NA7 *nef* into cells transfected with IFITM expression plasmids and *nef*-deleted NL4-3
323 proviral HIV-1 DNA. Overexpression of Nef in cells transfected with 0.5μg Nef-encoding
324 plasmid resulted in a general lower level of viral proteins compared with cells
325 expressing lower levels of Nef. Despite this caveat, the level of viral production was
326 restored as the amount of Nef-encoding plasmid increased (0.005μg and 0.05μg in
327 Figure 7G). This was coupled with a dose dependent increase in viral protein production
328 (Figure 7G, H and I). These data show that Nef can help overcome IFITM-mediated
329 inhibition of HIV-1 production in a concentration-dependent manner. Taken together,
330 our data demonstrate that HIV-1 Nef is able to help overcome the inhibition of viral
331 protein production exerted by IFITM proteins and therefore enhance infection.
332

333 **Discussion**

334 We report here that IFITM proteins restrict HIV-1 by excluding viral mRNA from
335 polysomes thereby specifically inhibiting protein synthesis. Since the discovery of the
336 antiviral effect of IFITM proteins upon HIV-1 entry and infectivity, there have been data
337 suggesting that there may be a distinct IFITM function directed towards HIV-1
338 production (8, 10, 12, 13, 20-24). Despite the relative frequency of these passing
339 observations on viral production, the underlying processes leading to loss of viral
340 production during IFITM expression have not been explored. Nor was it known if HIV-1

341 can antagonize to this process as is seen for some antiviral restriction factors (reviewed
342 in (1)).
343 We have now advanced the understanding of this phenomenon by demonstrating the
344 breadth of the IFITM-mediated restriction of virus production, identifying the viral
345 replication process that is subverted, and determining a means by which HIV-1 may
346 partially overcome this effect. In addition, we find that restriction of viral production by
347 IFITM proteins is clear contributor towards the inhibition of HIV-1 production seen in
348 cells exposed to type I interferon, and have also shown that inhibition occurs in primary
349 $CD4^+$ T-cells at physiologically relevant levels of IFITM expression. From a mechanistic
350 perspective we have shown that HIV-1 RNA is the target of this inhibition, though the
351 viral determinant remains elusive. We therefore propose that inhibition of viral protein
352 synthesis is a genuine antiviral function exerted by IFITM proteins that is distinct from
353 previously described effects upon entry and infectivity.
354 Initial suggestions of a late-stage IFITM-mediated restriction have typically surrounded
355 two common laboratory HIV-1 strains, NL4-3 and BH10 (8, 10, 12, 23, 24). However, for
356 the putative anti-HIV restriction factor viperin/RSAD2, analysis demonstrated that the
357 phenotype could not be replicated beyond the laboratory strain NL4-3 (43). Therefore
358 our demonstration that IFITM proteins restrict production of multiple HIV-1, HIV-2 and
359 SIVs implies the phenotype we observe is likely not an artefact. This is supported by our
360 finding that African Green Monkey IFITM1 can exert a similar antiviral effect upon HIV-1
361 production as occurs with human IFITM1. Further work to fully define losses in viral
362 protein production for HIV-2 and SIVs will be of interest, particularly for $HIV\text{-}2_{ROD}$ and
363 SIV_{AGM} that showed some resistance in restriction relative to other viruses tested. In

364 contrast, HIV-1 89.6 showed remarkable sensitivity to the restriction, and the molecular
365 basis of this is not yet known. It will also be of interest to explore if the restriction to virus
366 production is exerted by additional non-human IFITM alleles.
367 The general mechanism of restriction we outline is specific exclusion of HIV-1 mRNA
368 from polysomes during IFITM expression. In HEK293T cells the effect principally occurs
369 with IFITM1 and IFITM2, but less so for IFITM3 was generally consistent throughout our
370 analysis. Yet inducible expression of IFITM3 in SupT1 cells showed an equivalent
371 antiviral effect to IFITM1 and IFITM2, though as this was also linked to viral protein loss
372 and independent of IFITM-driven effects of viral entry, we consider that the same
373 translational blocks measured in polysome analysis of 293T cells drive the result in
374 SupT1 cells. Indeed, we considered it very likely cell type specific differences would
375 occur, as IFITM localization and thus antiviral activity are governed by both
376 ubiquitination and palmitoylation (22, 44). Reasonably the degree to which IFITM-driven
377 viral entry inhibition or viral translation inhibition occurs in particular cells is then
378 influenced by the activity of these pathways, which are known to commonly vary,
379 especially in cancer cells. Equally, other cell specific factors influencing viral
380 transcription (such as NFkB translocation) might also account for variation in antiviral
381 activity between cell lines, as higher virus transcriptional activity may overcome
382 inhibition. Thus future work to determine the parameters regulating the activity of the
383 antiviral process we describe will be of value. However, it is important to note that we
384 were able to clearly confirm the inhibition of viral production in primary human $CD4^+$ T-
385 cells, demonstrating that inhibition of viral production by IFITMs occurs in HIV-1 target
386 cells at physiologically relevant levels of IFITM proteins.

387 Why IFITM1 and IFITM2 should act similarly in many instances, when IFITM2 and
388 IFITM3 have closer amino acid sequence identity and display greater overlap in cellular
389 localization is not yet clear (8, 17, 45, 46). IFITM-mediated antiviral effects upon viral
390 entry are principally determined by cellular localization, and we anticipate the inhibition
391 of protein synthesis to be similar (44, 45, 47). This is pertinent for differences we find
392 between IFITM2 and IFITM3. Despite their high sequence similarity, they typically show
393 distinct cellular localization.

394 The exclusion of viral transcripts from polysomes mainly seemed to affect unspliced and
395 singly-spliced transcripts. Yet despite a lack of significance, there was consistent but
396 weaker loss of multiply spliced transcripts from polysome fractions, coupled with
397 multiply-spliced transcript enrichment in low sucrose fractions. This is perhaps indicative
398 of some degree of multiply-spliced transcript translational blockage that explains the
399 loss of Nef protein seen throughout our experiments.

400 We saw no change in total unspliced mRNA levels in IFITM expressing cells yet there
401 was modest depletion of singly-spliced and multiply-spliced mRNA during IFITM1 and
402 IFITM2 expression. Though this overall depletion of total viral mRNA levels may partially
403 account for some loss of Vpu and Nef production, polysome analysis additionally
404 demonstrated clear exclusion of these transcript classes from translation. Why these
405 overall mRNA levels should be reduced is of interest. One possibility is a transcriptional
406 inhibition or pre-translational degradation, but degradation of mRNAs that have been
407 inhibited in translation is thought to be common (48). For example, the antiviral protein
408 ZAP, which degrades retroviral mRNA has also been shown to stall translation prior to

409 mRNA degradation, illustrating the fluid link between translational stalling and mRNA
410 stability (49, 50).
411 Susceptibility to inhibition occurred at the level of viral mRNA and so we sought to
412 identify the viral RNA susceptibility determinant. Our data showing *gag* codon
413 optimization relieves the late-stage IFITM inhibition of HIV-1 supports similar findings
414 (24). Singly-spliced and unspliced transcripts that bear the RRE were typically most
415 affected by IFITMs proteins in our analyses. Yet investigation of proviral constructs in
416 which the RRE was substituted for the CTE element were inconclusive, making it
417 difficult to reconcile a role for the RRE in determining inhibition, despite the appealing
418 observation that RRE bearing transcripts are typically most affected. Notably, RRE
419 bearing transcripts also have a non-human codon bias and surrounding splice sites that
420 may prove to influence restriction (51-54). As such further analysis to identify the HIV-1
421 RNA determinant of inhibition will then be of much value, though currently our further
422 attempts to identify the viral RNA determinant have been unsuccessful. Notably, a
423 recent study demonstrated that viral RNA CG dinucleotide frequency drives ZAP
424 mediated restriction, rather than a specific RNA structural determinant, thus the
425 determinant for IFITM-mediated restriction of protein synthesis may be similarly obscure
426 (55). Therefore further analysis of the differences between codon optimized vectors and
427 wild type vectors will then be revealing, as these were respectively enhanced and
428 inhibited during IFITM expression, and differ in a number of ways including codon bias,
429 splice sites, secondary structure and promoter. However, we were able to exclude the
430 broader possibilities of general translational arrest or further ISG induction due to IFITM
431 expression. Other aspects of the mechanism remain elusive also. IFITMs have no RNA

432 binding domain and so would require either an RNA-binding partner to directly achieve
433 inhibition, or would need to be involved in a pathway that could influence translation in
434 this manner.

435 If IFITMs are cofactors in direct inhibition of viral RNA translation, it is not yet clear how
436 a membrane protein can affect both soluble protein translation and membrane protein
437 translation, this may ultimately depend on the absolute strictness of translational site
438 partitioning, a concept which is under renewed debate (56). One unexplored possibility
439 is that the capacity of IFITM proteins to disrupt membrane fluidity or other membrane
440 physical characteristics to inhibition viral-cell membrane fusion (15, 57) on the cell
441 surface and in endosomal compartments similarly affects rough endoplasmic reticulum
442 membranes, leading to a disruption of membrane bound translational complexes.
443 Though how such a process could be specific to virus translation is unclear.

444 We demonstrated that the lentiviral protein Nef can help overcome the late-stage
445 inhibition of viral production exerted by IFITM proteins. Though notably this rescue is
446 not complete. Our data may help explain accounts of a Nef driven enhancement of HIV-
447 1 production (39, 40). The ability to overcome IFITM-mediated inhibition of virus
448 production was apparent with all HIV-1, HIV-2 and SIV *nef* alleles tested and it remains
449 to be seen if examples of enhanced or diminished Nef activity against IFITM will be
450 found (36).

451 Though the underlying mechanism of ability of Nef to counter IFITMs is unclear, we saw
452 no degradation of IFITMs in the context of viral protein expression. One possible
453 explanation may be re-trafficking, wherein Nef acts as an adaptor protein to direct
454 IFITMs away from their site of action on translation. This fits well with the sensitivity of

455 IFITM-mediated antiviral function to changes in IFITM localization (44, 45, 47), but also
456 fits with the ability of Nef to redirect a multitude of membrane proteins from their site of
457 action (37, 38, 58, 59). However, a second possibility is that due to the influence of Nef
458 on NFkB activity, viral transcription may be stimulated to a level that can saturate the
459 late-stage viral inhibition exerted by IFITM proteins (60).

460 We also found that, in multiple cycle infections, the benefit of Nef expression was only
461 seen early in culture but was latterly lost. Cell-free infection is prevalent in the early
462 stages of culture, but infection shifts towards a cell-to-cell mode in later stages (41). It
463 has been proposed that this represents a means of escape from immune factors in HIV-
464 1; as the high efficiency, high multiplicity of infection associated with cell-to-cell
465 transmission can overcome restriction (61, 62). For example, with the antiviral restriction
466 factor tetherin, antiviral effect is either severely weakened or absent during cell-to-cell
467 transmission of HIV-1 (29, 63, 64). The same effect may occur for IFITMs, especially as
468 we saw weaker inhibition at higher viral input in single cycle infections.

469 Thus we conclude that IFITMs are antiviral factors that can target HIV-1 translation. For
470 retroviruses this seems to be a recurring weak point in replication strategy that the
471 innate immune system has frequently evolved to target. For example, PKR senses viral
472 double stranded RNA and halts translation (65), while Schlaffen 11 inhibits viral protein
473 production by disrupting tRNA synthesis (66), and finally the protein ZAP which induces
474 viral mRNA degradation also stalls viral translation (50).

475 The model we put forward wherein IFITM expression specifically disrupts viral mRNA
476 translation may be applicable to other targets. Conceptually, other self and non-self
477 RNAs may be affected also. It is then of interest that a recent report found that LINE-1

478 transposon mobility could be inhibited by IFITM1 expression (67), which for a
479 retrotransposon cannot of course arise from viral entry effects. Future characterization
480 will therefore identify the viral RNA determinant of susceptibility, and the breadth of
481 antiviral and RNA regulatory response driven by IFITM proteins.
482

483 **Materials and Methods**

484 **Plasmids**

485 Human pQCXIP IFITM1-3 plasmids bearing an N-terminal FLAG were previously
486 described (57). The viral construct pBR4-3-eGFP-Nef, encoding for wild type HIV-1
487 NL4-3, pBR4-3-IeGFPΔNef, HIV-1 NA7, HIV-1 NL4-3 plasmids carrying *nef* alleles of
488 HIV-2 Ren or CBL and SIV AGM or MAC strains - all described in (42) - are kind gifts of
489 Professor Frank Kirchhoff (University of Ulm). HIV-2 MCR and MCN plasmids (68) are
490 kind gifts of Professor Áine McKnight (Queen Mary University of London). $pSIV_{agm}$Tan-
491 1, $pSIV_{cpz}$Tan1.910 and $pSIV_{mac}$1A11 are from NIH AIDS Reagent Program. Codon-
492 optimized HIV-1 NL4-3 is encoded by pCNC-SYNGP (32) (Oxford BioMedica). Plasmids
493 pCTEΔEnvΔRevΔRRE and pCTEΔEnvΔRev (34) are kind gifts of Professor Paul
494 Bieniasz (Rockefeller University).
495

496 **Antibodies, cells and antiviral compounds**

497 The following antibodies were used to detect IFITMs: human IFITM1 (clone 5B5E2,
498 Proteintech), IFITM2 (clone 3D5F7, Proteintech) and human IFITM3 (clone EPR5242,
499 Novus Biologicals). Anti-Flag (clone M2, Sigma) was used to detect FLAG-tagged
500 IFITMs. Anti-HIV1 Nef (clone 3F2, ThermoFisher), rabbit polyclonal anti-Vpu (Abcam)

501 and rabbit polyclonal anti-HIV1 Gag (Abcam) were used to detect viral proteins. As
502 loading control, β-actin (clone AC74, Sigma) and GAPDH (Abcam) antibodies were
503 used. Secondary antibodies were horseradish peroxidase conjugated goat anti-
504 rabbit/mouse IgG (Santa Cruz Biotech.). TZM-bl cells expressing shRNAs targeting
505 IFITMs were previously described (25), as were SupT1 cells inducible for IFITM
506 expression (8). Human Embryonic Kidney (HEK) epithelial 293T cells (HEK293T),
507 C8166 cells, TZM-bl cells, and SupT1 cells were maintained in standard conditions, and
508 were all originally procured from the NIH AIDS Reagents Program. Primary $CD4^+$ T cells
509 were isolated from leukocyte cones (NHS Blood Transfusion service, St. George's
510 Hospital, London) using the human $CD4^+$ T cell isolation kit according to manufacturer
511 instruction (Miltenyi Biotec). $CD4^+$ T cells were activated at $1x10^6$/ml with T cell activator
512 CD3/CD28 Dynabeads (ThermoFisher), at a bead-to-cell-ratio of 1:1, in complete
513 medium (RPMI 1640 medium supplemented with 10% heat-inactivated fetal bovine
514 serum, 2mM L-glutamine, 100U/ml streptomycin, 100U/ml penicillin, all from
515 ThermoFisher) and 30U/ml IL-2 (Peprotech) for 48 hours. AMD3100 and Maraviroc
516 were obtained via the NIBSC AIDS Reagent Program.
517

518 **Transfections**

519 HEK293T cells were plated at $2x10^4/cm^2$ in 48-well plates (for measurements of virus
520 production), 6-well plates (for measurements of whole cell viral transcripts by qPCR),
521 10cm dishes (for virus production, polysome analysis and immunoprecipitation, all from
522 Nunc), 48 hours before transfection. Plasmid DNA (total quantity of 1μg/well in 48-well
523 plates, 3μg/well in 6-well plates and 5μg/well in 10cm dishes) was diluted in OptiMEM

524 (ThermoFisher) at 10% volume of total cell culture medium. Equal quantity of different
525 plasmid DNA was added in co-transfection. Linear polyethylenimine (L-PEI) was added
526 at a w/w ratio of 5:1 into the diluted DNA. The transfection mixture was incubated at
527 room temperature for 10 minutes before added to cells.
528 TZM-bl cells were plated at $6x10^4/cm^2$ in 48-well plates 24 hours before transfection. In
529 a 48-well format, 1μg/well plasmid DNA and 6μl Lipofectamine 2000 (ThermoFisher)
530 were diluted in OptiMEM, incubated for 10 minutes at room temperature and then added
531 to cells. Medium was replaced with medium containing 5μM AMD3100 and 100IU/ml
532 IFN-β (Peprotech) 4 hours post-transfection.
533

534 **Infection**

535 HIV-1 virus was produced from transfected HEK293T cells. SupT1 cells were
536 spinoculated with the indicated concentrations of inoculum for 2 hours at 37 C at 1000x
537 *g* in 96-well U-bottom plates (Nunc) followed by incubation at 37 C for 1 hour. Infected
538 cells were then washed 3 times with phosphate-buffered saline (PBS, Sigma) and re-
539 suspended in medium with 1μg/ml doxycycline (Sigma) and 5μM AMD3100. C8166
540 cells were infected with the same protocol without AMD3100. Supernatant was
541 harvested at the indicated time-points after centrifugation at 500x *g* for 5 minutes at
542 room temperature.
543

544 **Transduction and infection of primary human $CD4^+$ T cells**

545 In the presence of CD3/CD28 T-cell activator beads, $5x10^5$/ml activated $CD4^+$ T cells
546 were transduced with IFITM-targeting or scrambled shRNA lentivirus with p24 at

547 100ng/ml for 2 hours at 1000 x*g*, 37°C and then for 1 hour incubation. Infected cells
548 were washed three times with PBS, re-suspended in complete medium with 30U/ml IL-2
549 and then incubated at 37°C for 48 hours. Prior to HIV infection, T cell activator beads
550 were removed from transduced $CD4^+$ T cells. Transduced $CD4^+$T cells were then
551 incubated with medium alone, 5μM Maraviroc or 5μM AMD3100 for 2 hours at 37°C.
552 Untreated cells and drug-treated cells were then infected with 100ng/ml p24 of NL4-3 or
553 89.6 virus, respectively, at 37°C, 1000 x*g* for 2 hours and then incubated for 1 hour.
554 Infected cells were then washed three times with PBS and re-suspended in complete
555 medium containing 30U/ml IL-2, 5μM Maraviroc and 5μM AMD3100. Aliquots of infected
556 cells were treated with 0.25% Trypsin/EDTA (ThermoFisher) for 15 minutes at 37°C,
557 washed three times with PBS, fixed in 2% paraformaldehyde, permeabilized in 0.2%
558 Triton X-100/PBS and stained with RD-1 conjugated monoclonal mouse anti-HIV-1-p24
559 (clone KC57, Beckman Coulter) for flow cytometry analysis of internalized virus. Level
560 of viral output in supernatant was measured by p24 ELISA 72 hours post-infection.
561

562 **p24 ELISA**

563 ELISA plates (Nunc) were pre-coated with 5μg/ml sheep anti-HIV-1 p24 antibody (Aalto
564 Bio Reagents) at 4 C overnight. Supernatant of transfected HEK293T, TZM-bl cells or
565 infected cells was treated with 1% Empigen BB (Sigma) for 30 minutes at 56°C, then
566 plated at 1:10 dilution in Tris-buffered saline (TBS) on anti-p24-coated plates and
567 incubated for 3 hours at room temperature. Alkaline phosphatase-conjugated mouse
568 anti-HIV-1 p24 monoclonal antibody (Aalto Bio Reagents) diluted in 20% sheep serum,
569 0.05% v/v Tween-20, TBS (all from Sigma) was then added and incubated for 1 hour at

570 room temperature. Plates were washed 4 times with 0.01% v/v Tween-20 in PBS and
571 twice with ELISA Light washing buffer (ThermoFisher). CSPD substrate with Sapphire II
572 enhancer (ThermoFisher) was added and incubated for 30 minutes at room temperature
573 before chemiluminiscence was read by a plate reader.
574

575 **Reverse transcriptase (RT) activity and luciferase activity assays**

576 Colorimetric reverse transcriptase activity assay kit (Roche) was used to determine
577 reverse transcriptase activity of HIV-1, HIV-2 and SIV in supernatants of transfected
578 HEK293T cells. Manufacturer instructions was followed except that supernatant was
579 first treated directly with lysis buffer (50mM Tris pH7.8, 80mM potassium chloride,
580 2.5mM DTT, 750μM EDTA and 0.5% Triton X-100, all from Sigma) for 30 minutes at
581 room temperature and then incubated in streptavidin-coated microplates for 15 hours at
582 37 C.
583 Luciferase activity of transfected HEK293T cells was analyzed with Bright-Glo luciferase
584 activity kit following manufacturer instructions (Promega).
585

586 **Polysome profiling**

587 Polysome analysis was performed with both manual fractionation and RNA analysis
588 (69). Transfected HEK293T cells were incubated with 100μg/ml cycloheximide (Sigma)
589 for 15 minutes for 37°C and then washed with ice-cold PBS with 100μg/ml
590 cycloheximide. Cells were then lysed in polysome buffer, 10mM Tris pH8, 140mM NaCl,
591 1.5mM $MgCl_2$, 0.5% v/v NP40, 100μg/ml cycloheximide, protease inhibitor cocktail (all
592 from Sigma) and 800U/ml RNase OUT (ThermoFisher), for 10 minutes on ice. Cell

593 lysate was centrifuged at 10, 000x *g* for 1 minute at 4°C and supernatant was then
594 adjusted to 200µg/ml cycloheximide and 700µg/ml heparin (Sigma). Following
595 centrifugation at 12, 000x *g* for 10 minutes at 4°C, an aliquot of supernatant was taken
596 as input and the rest was layered onto a 10% to 50% sucrose gradient prepared using
597 polysomal buffer. Gradients were then ultracentrifuged at 300,000x *g* for 16 hours at
598 4°C (Sorvall). After centrifugation, 20 550µl fractions were collected from the top of the
599 gradients for immunoblotting and quantitative PCR analyses. Level of RNA in fractions
600 was measured by absorbance at 254 nm (Nanodrop) and RNA was precipitated with 5x
601 volume of absolute ethanol (Sigma) overnight at −20°C.
602

603 **Quantitative PCR**

604 Total RNA of transfected HEK293T cells and precipitated RNA from polysome profiling
605 fractions were purified with RNeasy mini kit and on-column DNA digestion with RNase-
606 free DNase kit (both from Qiagen) following manufacturer instructions. 3ng of total RNA
607 per sample was analysed with Superscript III Platinum One-Step qRT-PCR kit with ROX
608 (for unspliced and multiply spliced viral transcripts, ThermoFisher) or QuantiTect SYBR
609 Green PCR kit (for singly-spliced viral transcript, Qiagen) and ABI 7500 Real Time PCR
610 system (Applied Biosystems). Cycling conditions were 50°C for 15 minutes, 95°C for 8
611 minutes, then cycling of 95°C for 15 s and 60°C for 30 s. Reactions carried out in the
612 absence of reverse transcriptase (Platinum *Taq* only) confirmed the absence of DNA
613 contamination. The samples were quantified against cloned standards.
614 Primers used to amplify unspliced (Forward, 5'-CCGTCTGTTGTGTGACTCTGG-3',
615 reverse, 5'-GAGTCCTGCGTCGAGAGATCT-3'), multiply-spliced (Forward, 5'-

616 CAGACTCATCAAGCTTCTCTATCAA-3', reverse, 5'-CTATTCCTTCGGGCCTGTC-3')
617 and singly-spliced (Forward, 5'-TAATCGGCCGAACAGGGACTTGAAAGCGAAAG-3',
618 reverse, 5'-CCCATCTCCACAAGTGCTGATACTTC-3') viral transcripts are described in
619 (70), (71), and (72), respectively. Oligonucleotide probes are labelled with 5'-FAM and
620 3'-TAMRA, (unspliced, 5'-TCTAGCAGTGGCGCCCGAACAGG-3' and multiply-spliced,
621 5'-AACCCACCTCCCAATCCCGAGG-3', all from ThermoFisher). Cellular GAPDH
622 (glyceraldehyde-3-phosphate dehydrogenase) mRNA was additionally amplified as a
623 loading control with primers (Forward, 5'-AGGTCGGAGTCAACGG ATTTGG-3',
624 reverse, 5'-GATGGCAACAATATCCACTTTACCA-3') and probe (5'-
625 TCTTATTGGGCGCCTGGTCAC-3', as described in (71).
626

627 **Immunoblotting**

628 Cells were washed once with PBS and then lyzed in radioimmunoprecipitation buffer
629 (RIPA, containing 20mM Tris pH7.5, 150mM NaCl, 1mM EDTA, 1mM EGTA, 1% NP40,
630 1% sodium deoxycholate, 250μM sodium pyrophosphate, 1mM β-glycerophosphate,
631 1mM sodium vanadate and protease inhibitor cocktail, all from Sigma) for 30 minutes at
632 4°C. Lysate was then centrifuged for 10, 000x *g* for 10 minutes at 4°C. Protein
633 concentration of supernatant was determined by bicinchoninic acid (BCA) assay
634 (ThermoFisher). 10μg of protein per sample was analyzed by immunoblotting,
635 developed with ECL Prime reagents (GE Healthcare Life Sciences) and captured on
636 CL-XPosure films (ThermoFisher) or ChemiDoc MP system (Bio-Rad). Intensity of
637 immunobands was analysed by ImageJ (on X-ray films) or ImageLab (Bio-Rad).
638

639 **Statistical analysis**

640 Levels of viral output in p24 ELISA and RT assay were normalized to vector-transfected
641 or scrambled shRNA-transduced control and expressed as the level of virus production
642 unless otherwise indicated. Statistical analysis was performed with Graphpad Prism 5.
643 Data shows mean + standard error of mean from a minimum of 3 independent
644 experiments.

645

646

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653

654

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875

876 **Figure Legends**

877

878 **Figure 1. IFITMs inhibit HIV viral output and viral protein production in transfected**
879 **293T cells.**

880 HEK293T cells were transfected with a titration of expression vectors for FLAG tagged

881 IFITMs. **(A)** Levels of virus production were measured by p24 ELISA 48h post-
882 transfection, and **(B)** cellular viral proteins and IFITMs were analyzed by
883 immunoblotting.
884 HEK293T cells were transfected with a fixed amount of FLAG-tagged IFITM expression
885 vectors and HIV-1 NL4-3 plasmid proviral DNA. Levels of virus production from
886 indicated HIV-1 plasmid proviral DNAs were measured by **(C)** p24 ELISA or **(D)** reverse
887 transcriptase activity (RT) 48h post-transfection. **(E)** Levels of cellular viral proteins and
888 IFITMs were analyzed by immunoblotting 48h post-transfection. Levels of virus
889 production from HEK293T cells transfected with expression vectors of IFITMs and
890 plasmid proviral DNA for **(F)** HIV-2 strains or **(G)** SIVs (AGM –African Green Monkey,
891 CPZ – Chimpanzee, MAC – Macaque). Viral production was measured by RT activity
892 assay and normalized. **(H)** Levels of virus production of HEK293T cells transfected with
893 expression vectors of African Green Monkey (AGM) IFITM1 and plasmid proviral DNA
894 of HIV-1 were measured by p24 ELISA and normalized. **(I)** Level of GFP expression in
895 HEK293T cells co-transfected with a CMV-driven GFP vector and IFITM-expression
896 vectors or empty vectors measured by flow cytometry 48 hours post-transfection. **(J)**
897 Infectivity of viral supernatants from HEK29T cells transfected with human IFITM1-3
898 expression plasmids with equal concentrations of p24 was measured in TZM-bl cells 48
899 hours post-infection and normalized to vector control. . Data show mean + S.E.M. from
900 3 independent experiments. All differences were assessed with Student's t-test and *
901 denotes $p<0.05$.
902
903 **Figure 2. Inducible expression of IFITMs after viral entry inhibits HIV viral output**
904 **and viral protein production in infected SupT1 cells. (A)** SupT1 cells were infected
905 with the indicated dilutions of wild type HIV-1 NL4-3 inoculum and then treated with
906 doxycycline to induce IFITMs post-entry, and AMD3100 to limit infection to a single
907 cycle. At 72h post-infection levels of virus production were measured by p24 ELISA and
908 differences assessed by Two-way ANOVA with Bonferroni's multiple comparison test.
909 **(B)** Cellular viral proteins and IFITMs (from cells infected with 100ng/ml virus) were
910 analyzed by immunoblotting and **(C)** densitometry. Data show mean + S.E.M from 3
911 independent experiments. Differences were assessed with Student's t-test. * indicates

912 p<0.05.

913

914 **Figure 3. Knockdown of IFITMs rescues HIV-1 output and viral protein production**
915 **in TZM-bl cells.** TZM-bl cells transduced with shRNAs against the indicated IFITMs or
916 scrambled control (sc) were treated with 100IU/ml IFNβ for 72 hours. **(A)** Levels of
917 IFITM expression were analyzed by immunoblotting. **(B)** Scrambled control TZM-bl cells
918 were transfected with HIV-1 NL4-3 proviral DNA and treated with 100IU/ml IFNβ. Level
919 of virus production was measured by p24 ELISA 72h post-transfection. TZM-bl cells
920 transduced with shRNAs against the indicated IFITMs or scrambled control were
921 transfected with HIV-1 NL4-3 proviral DNA and then treated with AMD3100 and IFNβ 4h
922 post-transfection. Level of virus output and viral proteins was measured by **(C)** p24
923 ELISA and **(D)** immunoblotting, respectively, at 72 hours post-transfection. **(E)**
924 Immunoblotting was further analyzed by densitometry.

925

926 **Figure 4. Knockdown of IFITMs rescues HIV-1 output and viral protein production**
927 **in primary CD4$^+$ T-cells.** Activated human CD4+ T cells were transduced with lentivirus
928 expressing indicated shRNAs against IFITMs or scrambled sequence (sc) for 48 hours.
929 Cells were then infected with **(panels A to F)** HIV-1 NL4-3 or **(panels G to M)** 89.6. For
930 experiments with 89.6 infection, cells were pre-treated with AMD3100 for 2 hours prior
931 to infection and throughout. Immediately after infection, cells were treated with trypsin
932 and washed before intracellular staining of p24 and flow cytometry to measure virus
933 uptake. Intracellular levels of p24 and median fluorescence intensity are shown in **(A**
934 **and G)** representative histograms and **(B and H)** summary bar charts, respectively. **(C**
935 **and I)** Level of virus production was measured by p24 ELISA 72 hours post-infection
936 and normalized. **(D and J)** Level of IFITM expression and viral proteins was analyzed
937 by immunoblotting and then **(E, F, K, L and M)** densitometry from one representative
938 experiment of two independent experiments with two different blood donors. All data
939 show mean + SEM. Differences were assessed by Student's t-test, and * denotes
940 p<0.05.

941

942 **Figure 5. IFITM proteins inhibit HIV-1 protein synthesis. (A)** Level of viral transcripts

943 in HEK293T cells transfected with HIV-1 NL4-3 and IFITM DNA was measured by
944 qPCR 48 hours post-transfection and the data normalized. **(B)** Level of RNA in
945 polysome analyses of HEK293T cells transfected with plasmid proviral HIV-1 NL4-3
946 DNA and expression vectors for IFITMs (or vector alone) was measured by absorbance
947 at 254nm. **(C)** Levels of GAPDH RNA was analyzed by qPCR and normalized to vector
948 control in the indicated fractions. **(D)** Level of RPL7 in the indicated fractions was
949 analyzed by immunoblotting. Levels of **(E)** unspliced, **(F)** singly-spliced and **(G)** multiply-
950 spliced viral transcripts in the indicated sucrose gradient fractions in **(B)** was analyzed
951 by qPCR then normalized with the levels of GAPDH RNA and input RNA. **(H)** Level of
952 luciferase activity driven by ISRE in HEK293T cells transfected with proviral DNA of
953 HIV-1 NL4-3 and plasmids for IFITMs or mitochondrial antiviral signalling protein
954 (MAVS) was measured 48 hours post-transfection and normalized with vector control.
955 Data shows mean + SEM from 3 independent experiments and differences were
956 assessed by Student's t-test, * denotes $p<0.05$ compared to vector control.
957

958 **Figure 6. HIV-1 RNA is a determinant of IFITM-mediated inhibition of protein**
959 **synthesis. (A)** Normalized levels of unspliced viral transcripts (measured by qPCR) in
960 the polysome fractions of HEK293T cells transfected with codon-optimized HIV-1 NL4-3
961 Gag DNA and IFITM expression vectors at 48h post-transfection. **(B)** Levels of
962 extracellular p24 in HEK293T cells transfected with codon-optimized HIV-1 Gag DNA
963 measured by p24 ELISA 48h post-transfection and normalized. **(C)** Cellular levels of
964 HIV-1 Gag (p55 and p24) were analyzed by immunoblotting.**(D)** Levels of virus
965 production in HEK293T cells transfected with wild-type HIV-1 NL4-3 proviral DNA and
966 IFITM-expression plasmids or empty vector was measured by p24 ELISA 48 hours
967 post-transfection and normalized. Cellular levels of HIV-1 Gag (p55 and p24) in cells
968 transfected were analyzed by **(E)** immunoblotting. **(F)** Normalized levels of p55 and p24
969 in (C) and (E) were further analyzed by desitometry. Data show mean + SEM from 3
970 independent experiments, differences were assessed by Student's t-test, * denotes
971 $p<0.05$ compared to vector control.
972

973 **Figure 7. HIV Nef can help overcome IFITM-mediated inhibition of protein**

974 **synthesis. (A)** Level of virus production in HEK293T cells transfected with expression
975 vectors for IFITMs and HIV-1 NL4-3 proviral DNA with a deletion (ΔNef) was measured
976 by p24 ELISA 48 hours post-transfection and the data normalized, fold change of virus
977 production compared to vector control is indicated. Differences were assessed with
978 Student's t-test. **(B)** Intracellular level of p55/p24 and IFITMs was measured by
979 immunoblotting.
980 **(C)** SupT1 cells inducible for IFITM expression were infected with the indicated dilutions
981 of wild type or Nef-deleted (ΔNef) HIV-1 NL4-3 inoculum and then treated with
982 doxycycline to induce IFITMs post-entry and AMD3100 to limit infections to a single
983 round. Differences were assessed with Two-way ANOVA. Level of virus production was
984 measured by p24 ELISA 72 hours post-infection and the data normalized.
985 **(D)** HIV-1 Nef does not overcome IFITM-mediated inhibition of early viral replication
986 steps. C8166 CD4+ T-cells were transduced to constitutively overexpress IFITM1-3.
987 Cells were then infected with the wild type pBR4-3-eGFP-Nef virus or pBR4-3-eGFP-
988 ΔNef virus. Cells were measured for GFP expression 48h post-infection by FACS to
989 determine infection rate. Data show fold-change in restriction of GFP infection rates
990 during IFITM expression relative to empty vector control cells.
991 **(E)** C8166 cells constitutively expressing either vector control or IFITM1 were infected
992 with either wild type or Nef-deleted (ΔNef) HIV-1 NL4-3. Levels of virus production were
993 measured by p24 ELISA at the indicated time-points post-infection and were normalized
994 to the levels of virus produced from vector controls. Differences were assessed with
995 Two-way ANOVA.
996 **(F)** HEK293T cells were transfected with expression vectors for IFITMs and HIV-1 NL4-
997 3 proviral DNA with either wildtype NL4-3 *nef* or the indicated lentiviral *nef* alleles. Virus
998 production was measured by p24 ELISA 48 hours post-transfection and the data
999 normalized.
1000 **(G)** HEK293T cells were transfected with ΔNef HIV-1 NL4-3 proviral DNA, expression
1001 vectors for IFITMs and an increasing proportion of HIV-1 Nef-encoding vector versus
1002 empty vector in a fixed total quantity. Level of virus production was measured by p24
1003 ELISA 48 hours post-transfection, while levels of viral proteins and IFITM-FLAG
1004 expression was analyzed by **(H)** immunoblotting and **(I)** densitometry. Data show mean

1005 + SEM of 3 independent experiments and differences were assessed by Student's t-
1006 test, * denotes $p<0.05$.
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Figure 1

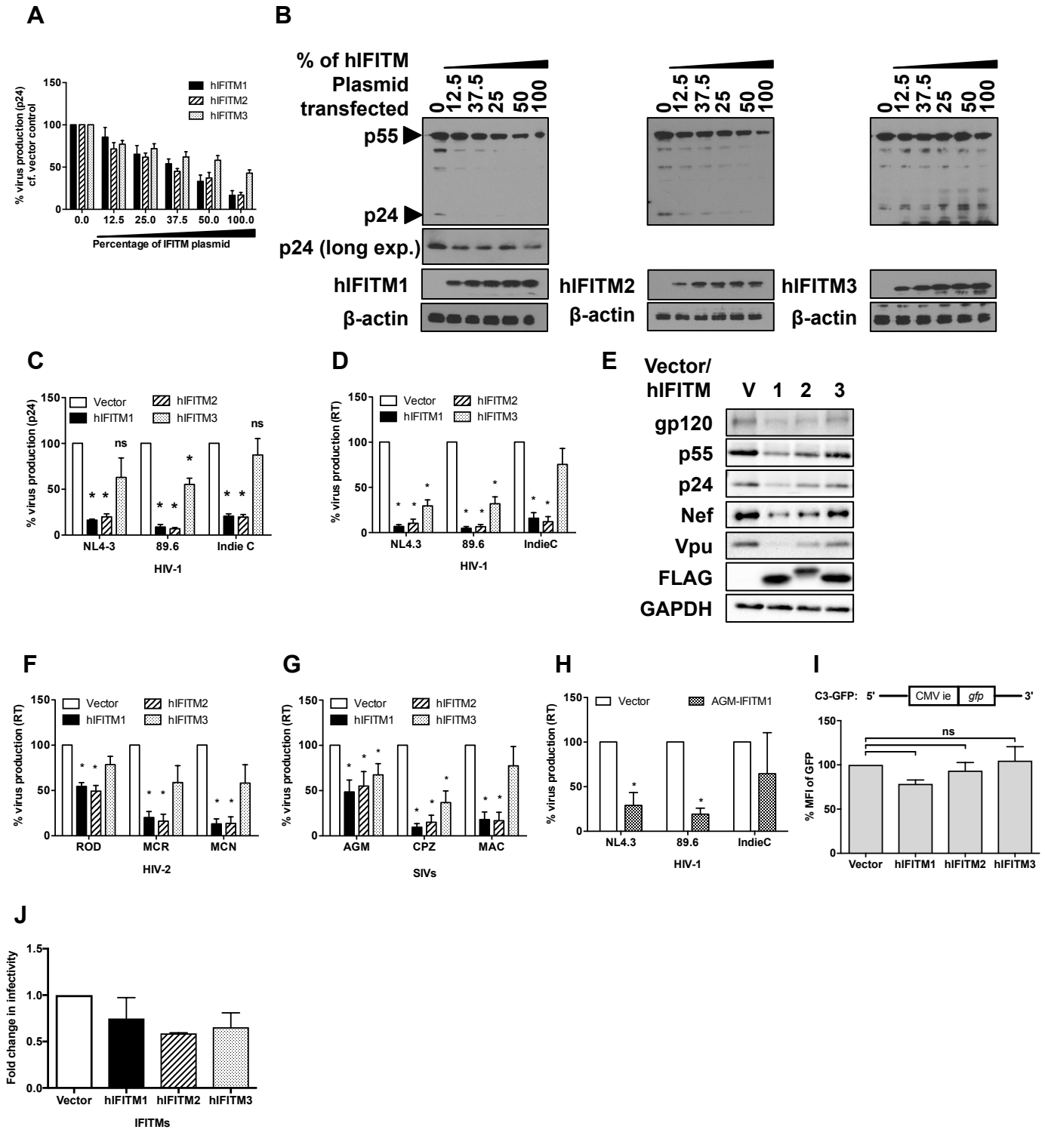

A
% virus production (p24) cf. vector control
hIFITM1
hIFITM2
hIFITM3
0.0
12.5
25.0
37.5
50.0
100.0
Percentage of IFITM plasmid
B
% of hIFITM Plasmid transfected
0
12.5
37.5
25
50
100
p55
p24
p24 (long exp.)
hIFITM1
hIFITM2
hIFITM3
β-actin
C
% virus production (p24)
Vector
hIFITM1
hIFITM2
hIFITM3
ns
NL4-3
89.6
Indie C
HIV-1
D
% virus production (RT)
NL4.3
89.6
IndieC
HIV-1
E
Vector/ hIFITM
V
1
2
3
gp120
p55
p24
Nef
Vpu
FLAG
GAPDH
F
% virus production (RT)
ROD
MCR
MCN
HIV-2
G
AGM
CPZ
MAC
SIVs
H
AGM-IFITM1
I
C3-GFP:
5'
CMV ie
gfp
3'
% MFI of GFP
J
Fold change in infectivity
IFITMs

Figure 2

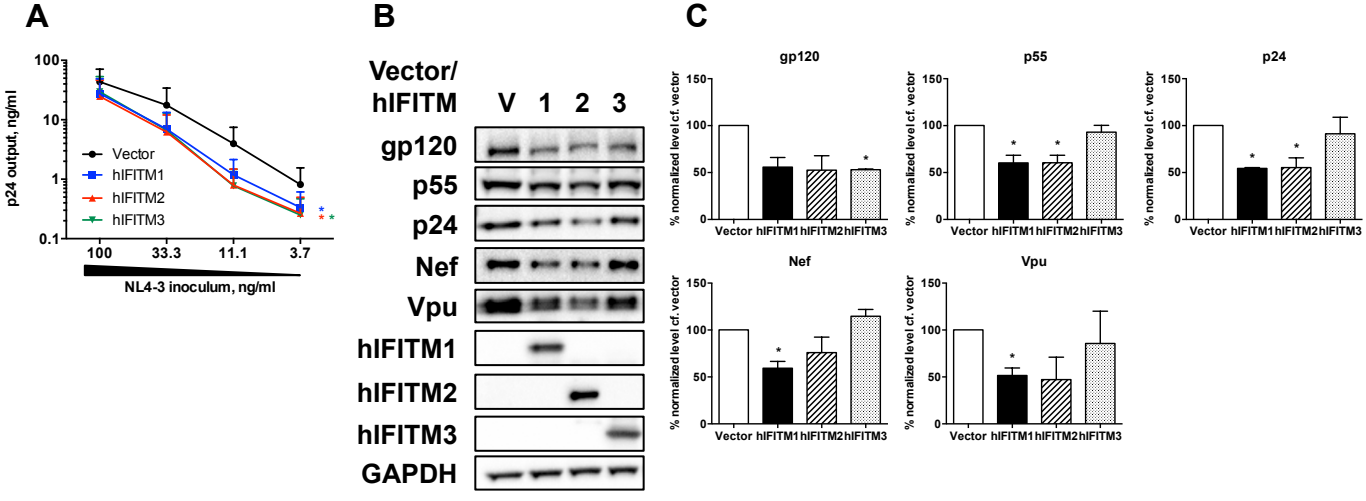

A
p24 output, ng/ml
100
10
1
0.1
Vector
hIFITM1
hIFITM2
hIFITM3
100
33.3
11.1
3.7
NL4-3 inoculum, ng/ml
B
Vector/
hIFITM
V 1 2 3
gp120
p55
p24
Nef
Vpu
hIFITM1
hIFITM2
hIFITM3
GAPDH
C
gp120
p55
p24
Nef
Vpu
% normalized level cf. vector
150
100
50
0
Vector hIFITM1 hIFITM2 hIFITM3

Figure 3

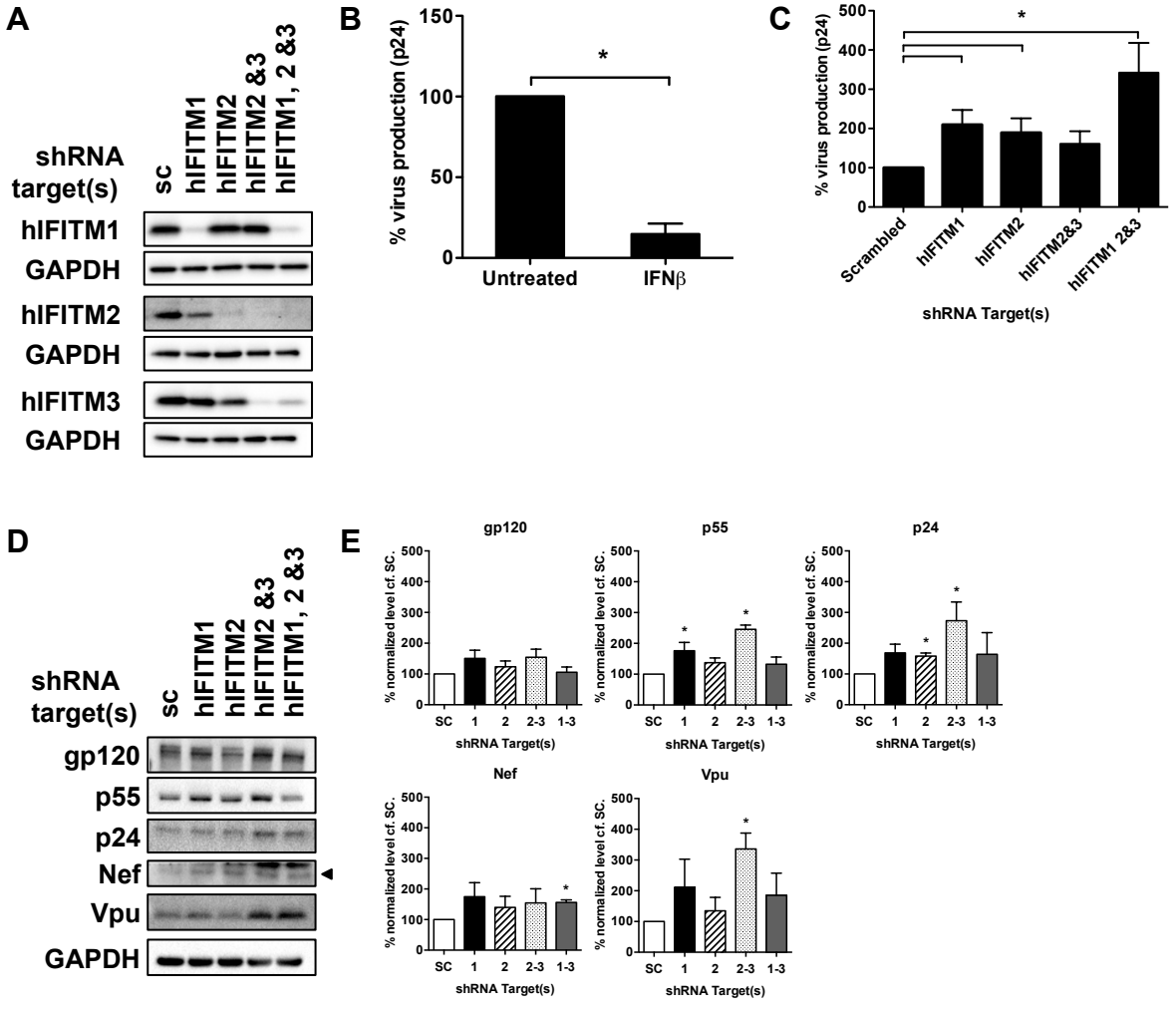

A
shRNA target(s)
sc
hIFITM1
hIFITM2
hIFITM2 &3
hIFITM1, 2 &3
hIFITM1
GAPDH
hIFITM2
GAPDH
hIFITM3
GAPDH
B
% virus production (p24)
150
100
50
0
*
Untreated
IFNβ
C
% virus production (p24)
500
400
300
200
100
0
*
Scrambled
hIFITM1
hIFITM2
hIFITM2&3
hIFITM1 2&3
shRNA Target(s)
D
shRNA target(s)
sc
hIFITM1
hIFITM2
hIFITM2 &3
hIFITM1, 2 &3
gp120
p55
p24
Nef
Vpu
GAPDH
E
gp120
p55
p24
Nef
Vpu
% normalized level cf. SC.
SC
1
2
2-3
1-3
shRNA Target(s)

Figure 4

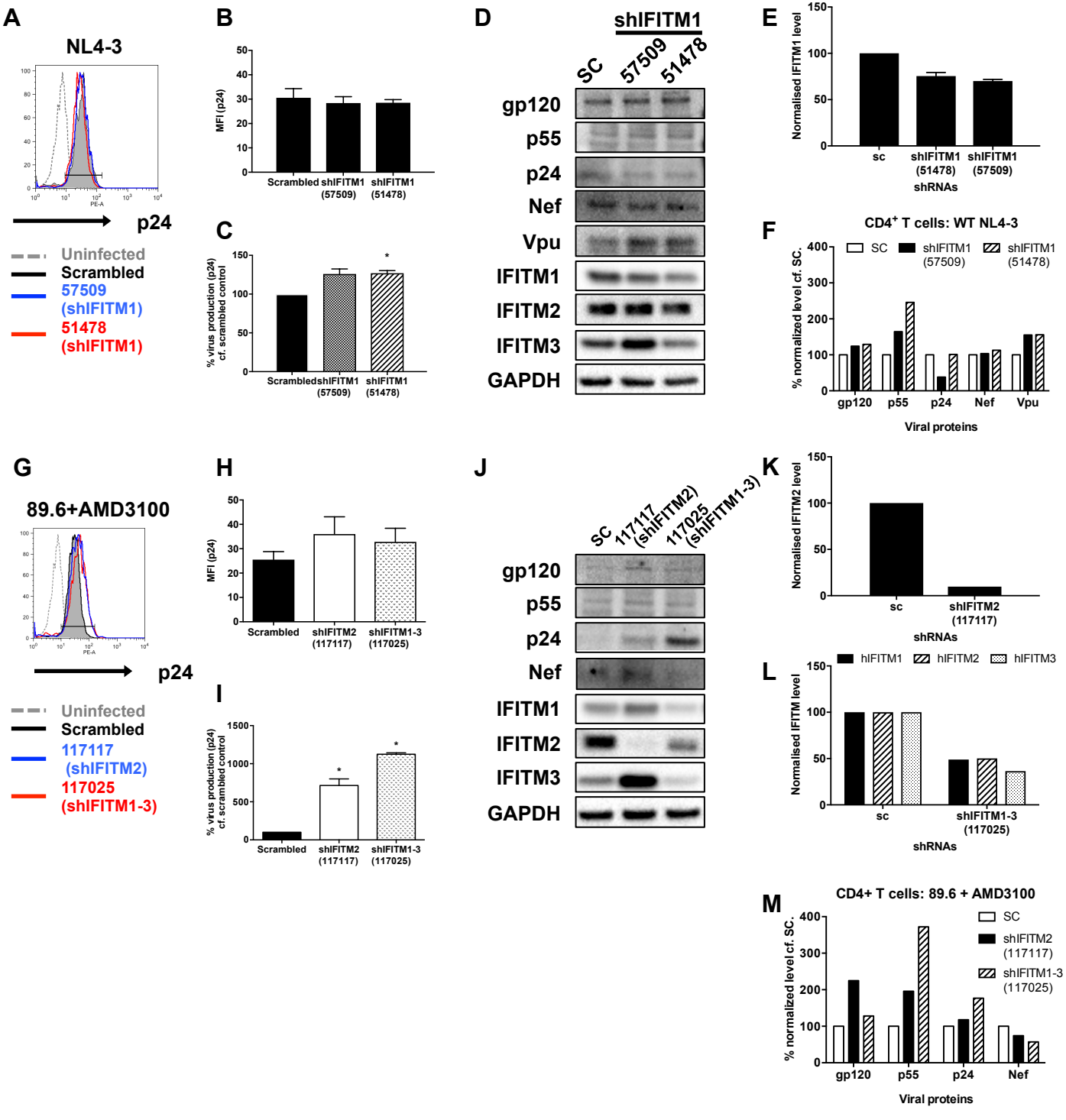

A
NL4-3
p24
Uninfected
Scrambled
57509 (shIFITM1)
51478 (shIFITM1)
B
MFI (p24)
Scrambled
shIFITM1 (57509)
shIFITM1 (51478)
C
% virus production (p24) cf. scrambled control
D
shIFITM1
SC
57509
51478
gp120
p55
p24
Nef
Vpu
IFITM1
IFITM2
IFITM3
GAPDH
E
Normalised IFITM1 level
sc
shIFITM1 (51478)
shIFITM1 (57509)
shRNAs
F
CD4+ T cells: WT NL4-3
SC
shIFITM1 (57509)
shIFITM1 (51478)
% normalized level cf. SC.
gp120
p55
p24
Nef
Vpu
Viral proteins
G
89.6+AMD3100
p24
Uninfected
Scrambled
117117 (shIFITM2)
117025 (shIFITM1-3)
H
MFI (p24)
Scrambled
shIFITM2 (117117)
shIFITM1-3 (117025)
I
% virus production (p24) cf. scrambled control
J
SC
117117 (shIFITM2)
117025 (shIFITM1-3)
gp120
p55
p24
Nef
IFITM1
IFITM2
IFITM3
GAPDH
K
Normalised IFITM2 level
sc
shIFITM2 (117117)
shRNAs
L
hIFITM1
hIFITM2
hIFITM3
Normalised IFITM level
sc
shIFITM1-3 (117025)
shRNAs
M
CD4+ T cells: 89.6 + AMD3100
SC
shIFITM2 (117117)
shIFITM1-3 (117025)
% normalized level cf. SC.
gp120
p55
p24
Nef
Viral proteins

Figure 5

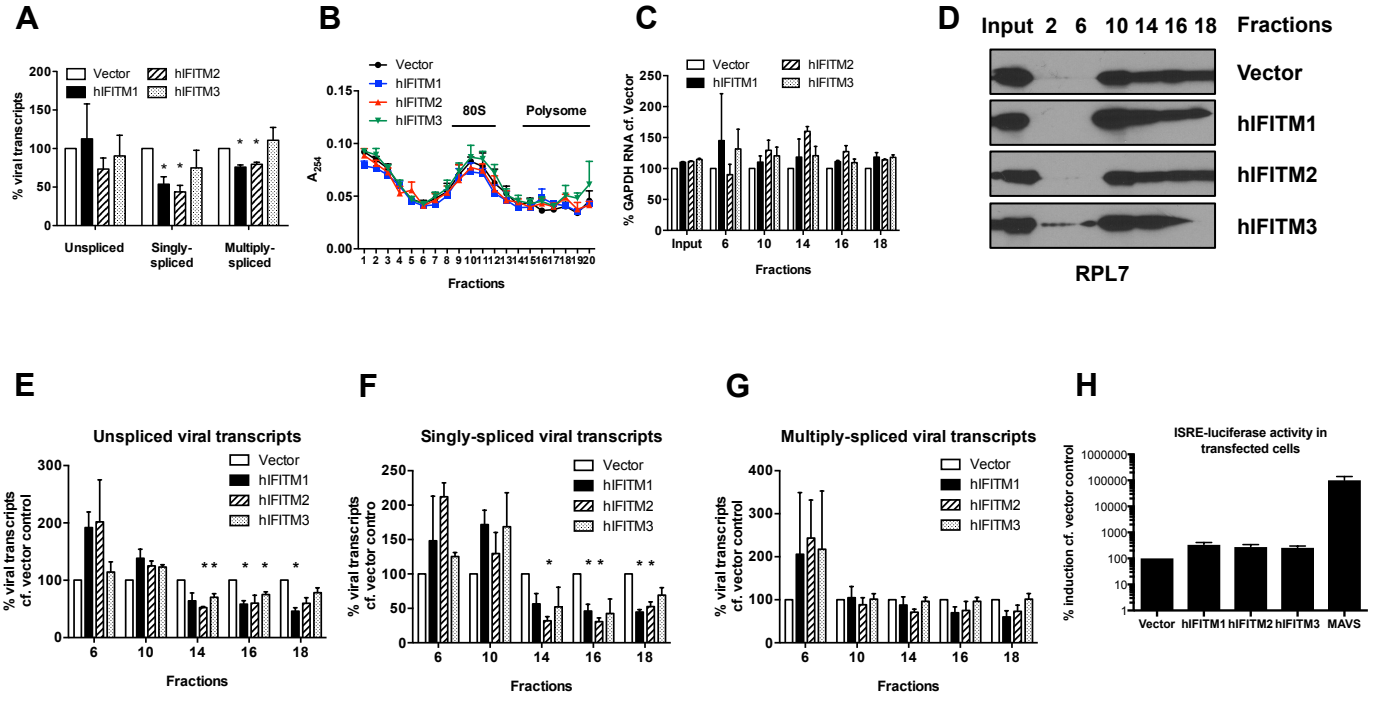

A
Vector
hIFITM1
hIFITM2
hIFITM3
% viral transcripts
Unspliced
Singly-spliced
Multiply-spliced
B
Vector
hIFITM1
hIFITM2
hIFITM3
80S
Polysome
A254
Fractions
C
Vector
hIFITM1
hIFITM2
hIFITM3
% GAPDH RNA cf. Vector
Input
Fractions
D
Input 2 6 10 14 16 18 Fractions
Vector
hIFITM1
hIFITM2
hIFITM3
RPL7
E
Unspliced viral transcripts
Vector
hIFITM1
hIFITM2
hIFITM3
% viral transcripts cf. vector control
Fractions
F
Singly-spliced viral transcripts
Vector
hIFITM1
hIFITM2
hIFITM3
% viral transcripts cf. vector contro
Fractions
G
Multiply-spliced viral transcripts
Vector
hIFITM1
hIFITM2
hIFITM3
% viral transcripts cf. vector control
Fractions
H
ISRE-luciferase activity in transfected cells
% induction cf. vector control
Vector
hIFITM1
hIFITM2
hIFITM3
MAVS

Figure 6

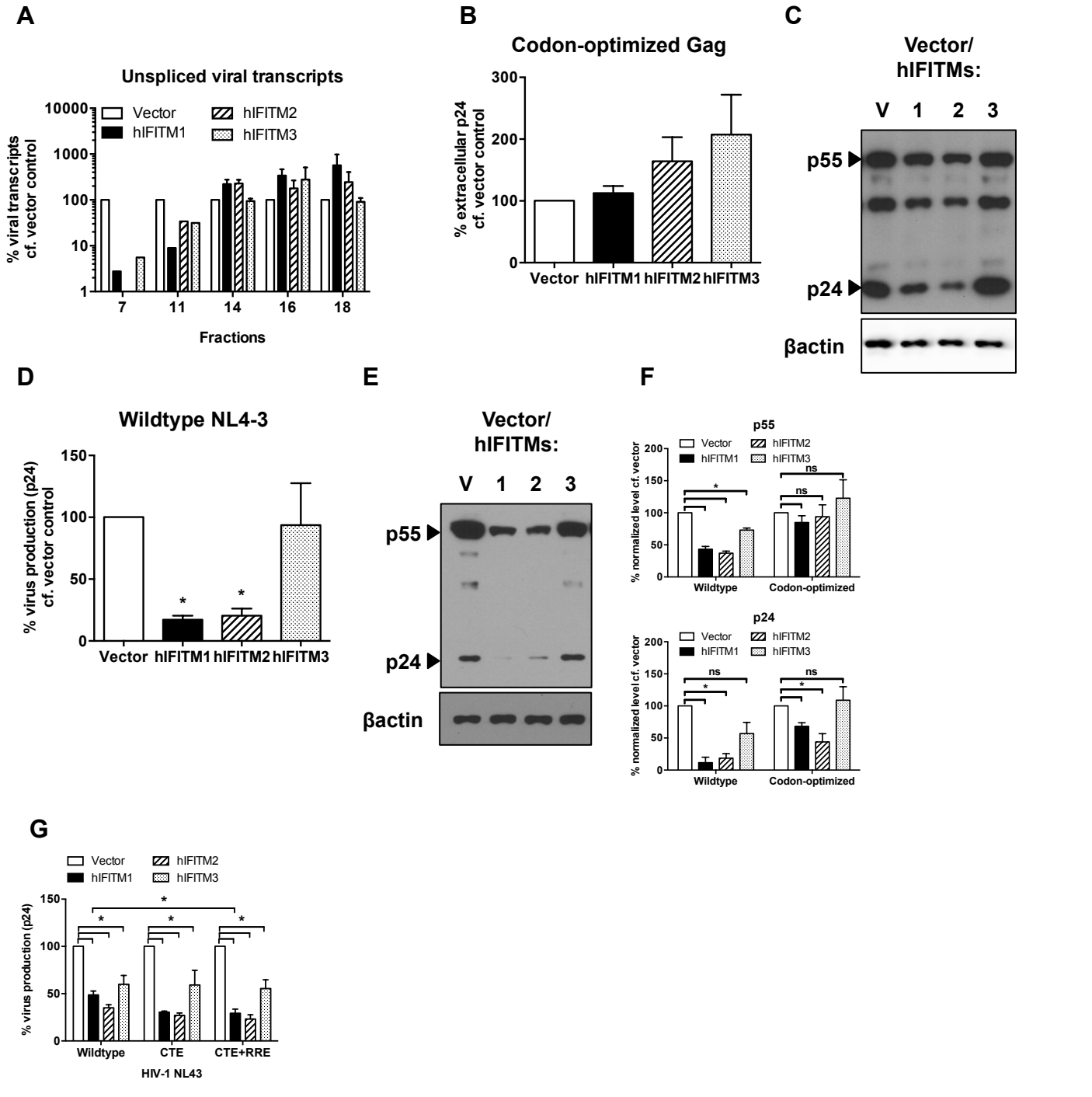
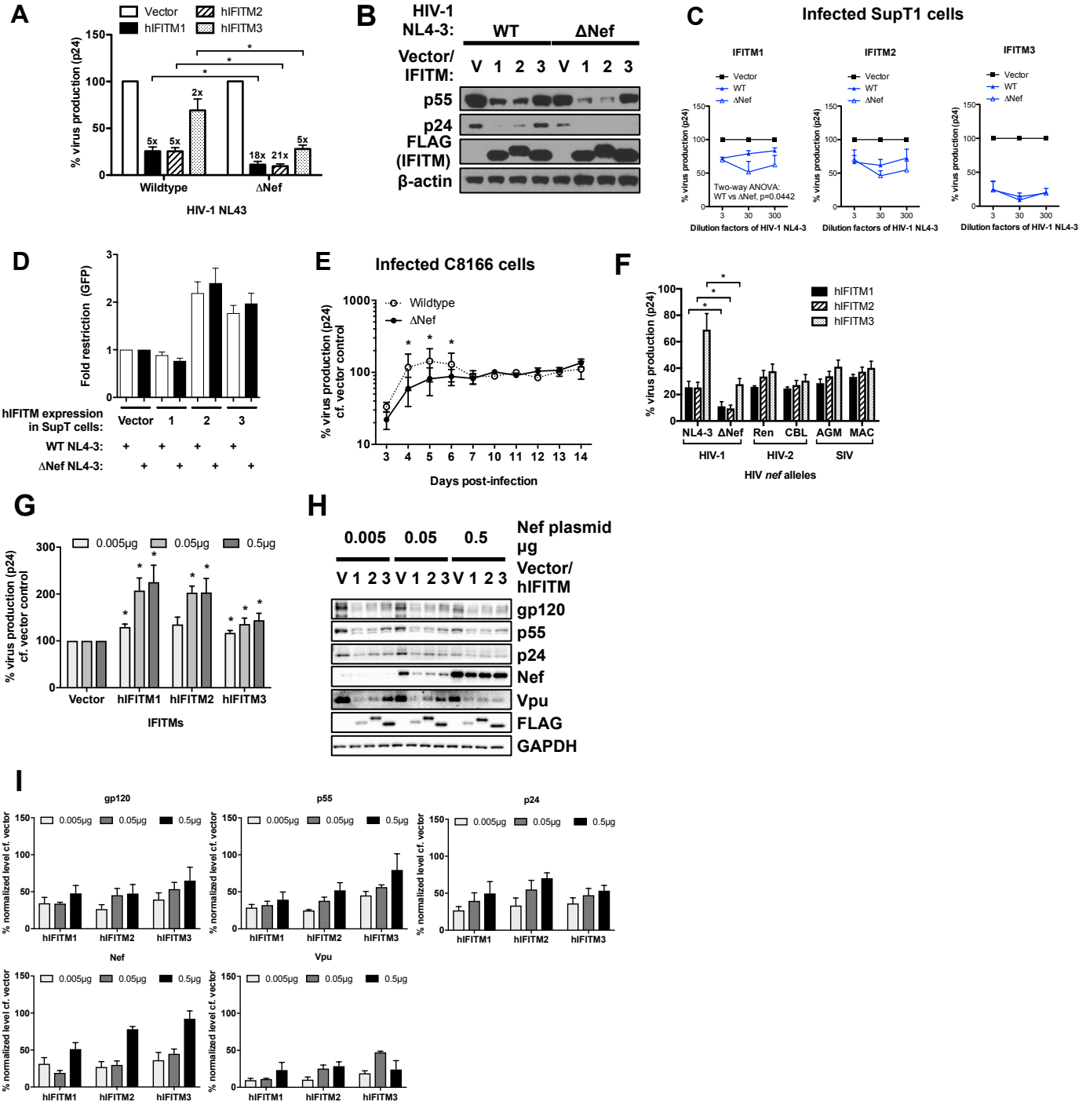

A
Unspliced viral transcripts
Vector
hIFITM1
hIFITM2
hIFITM3
% viral transcripts cf. vector control
10000
1000
100
10
1
7
11
14
16
18
Fractions
B
Codon-optimized Gag
% extracellular p24 cf. vector control
300
200
100
0
Vector
hIFITM1
hIFITM2
hIFITM3
C
Vector/ hIFITMs:
V 1 2 3
p55
p24
βactin
D
Wildtype NL4-3
% virus production (p24) cf. vector control
150
100
50
0
*
*
Vector
hIFITM1
hIFITM2
hIFITM3
E
Vector/ hIFITMs:
V 1 2 3
p55
p24
βactin
F
p55
Vector
hIFITM1
hIFITM2
hIFITM3
% normalized level cf. vector
200
150
100
50
0
*
ns
ns
Wildtype
Codon-optimized
p24
Vector
hIFITM1
hIFITM2
hIFITM3
% normalized level cf. vector
200
150
100
50
0
ns
*
ns
*
Wildtype
Codon-optimized
G
Vector
hIFITM1
hIFITM2
hIFITM3
% virus production (p24)
150
100
50
0
*
*
*
*
Wildtype
CTE
CTE+RRE
HIV-1 NL43

Figure 7


A
Vector
hIFITM1
hIFITM2
hIFITM3
% virus production (p24)
Wildtype
ΔNef
HIV-1 NL43
B
HIV-1 NL4-3:
WT
ΔNef
Vector/ IFITM:
V 1 2 3 V 1 2 3
p55
p24
FLAG (IFITM)
β-actin
C
Infected SupT1 cells
IFITM1
IFITM2
IFITM3
Vector
WT
ΔNef
Two-way ANOVA: WT vs ΔNef, p=0.0442
Dilution factors of HIV-1 NL4-3
D
Fold restriction (GFP)
hIFITM expression in SupT cells:
Vector 1 2 3
WT NL4-3:
ΔNef NL4-3:
E
Infected C8166 cells
Wildtype
ΔNef
% virus production (p24) cf. vector control
Days post-infection
F
hIFITM1
hIFITM2
hIFITM3
% virus production (p24)
NL4-3 ΔNef Ren CBL AGM MAC
HIV-1
HIV-2
SIV
HIV nef alleles
G
0.005μg
0.05μg
0.5μg
% virus production (p24) cf. vector control
Vector hIFITM1 hIFITM2 hIFITM3
IFITMs
H
0.005 0.05 0.5
Nef plasmid μg
V 1 2 3 V 1 2 3 V 1 2 3
Vector/ hIFITM
gp120
p55
p24
Nef
Vpu
FLAG
GAPDH
I
gp120
p55
p24
Nef
Vpu
% normalized level cf. vector
hIFITM1 hIFITM2 hIFITM3