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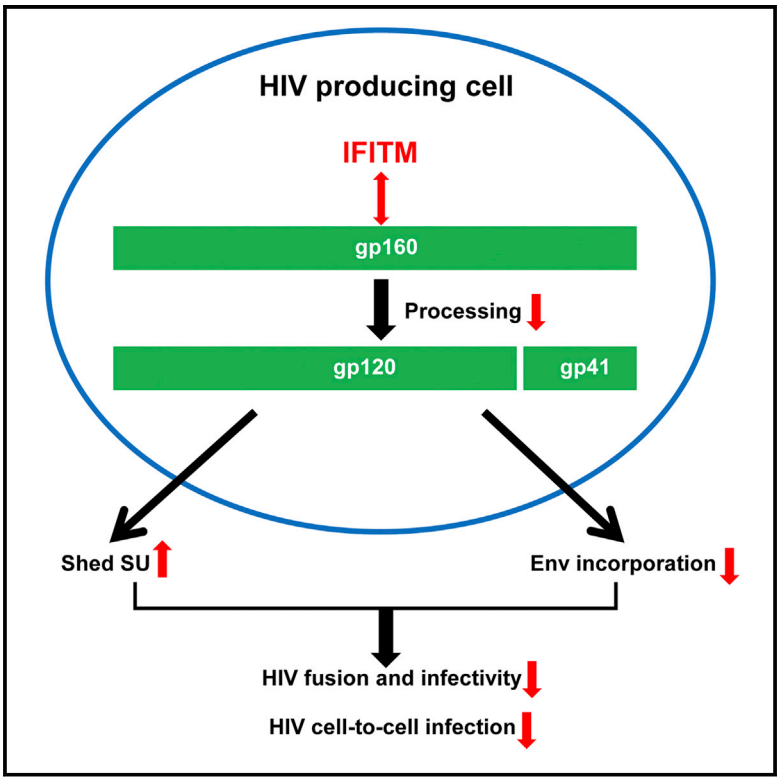
IFITM Proteins Restrict HIV-1 Infection by Antagonizing the Envelope Glycoprotein

Graphical Abstract



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In Brief

Yu et al. show that IFITM proteins, particularly IFITM2 and IFITM3, specifically antagonize the HIV-1 Env glycoprotein to inhibit infection. IFITM proteins interact with HIV-1 Env and impair its processing and incorporation into virions. The effects of IFITMs on HIV-1, HIV-2, and SIVs are virus-strain dependent.

Highlights

- IFITMs inhibit HIV-1 cell-to-cell infection and impair viral infectivity
- IFITMs specifically interact with HIV-1 Env and inhibit Env processing
- IFITM incorporation into HIV-1 virions does not correlate with inhibition
- IFITM inhibition of primate lentiviruses is virus-strain specific

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IFITM Proteins Restrict HIV-1 Infection by Antagonizing the Envelope Glycoprotein

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SUMMARY

The interferon-induced transmembrane (IFITM) proteins have been recently shown to restrict HIV-1 and other viruses. Here, we provide evidence that IFITM proteins, particularly IFITM2 and IFITM3, specifically antagonize the HIV-1 envelope glycoprotein (Env), thereby inhibiting viral infection. IFITM proteins interact with HIV-1 Env in viral producer cells, leading to impaired Env processing and virion incorporation. Notably, the level of IFITM incorporation into HIV-1 virions does not strictly correlate with the extent of inhibition. Prolonged passage of HIV-1 in IFITM-expressing T lymphocytes leads to emergence of Env mutants that overcome IFITM restriction. The ability of IFITMs to inhibit cell-to-cell infection can be extended to HIV-1 primary isolates, HIV-2 and SIVs; however, the extent of inhibition appears to be virus-strain dependent. Overall, our study uncovers a mechanism by which IFITM proteins specifically antagonize HIV-1 Env to restrict HIV-1 infection and provides insight into the specialized role of IFITMs in HIV infection.

INTRODUCTION

Interferons are cytokines that inhibit viral infection by inducing expression of hundreds of the interferon-stimulated genes (ISGs) (Sadler and Williams, 2008). Among these, APOBEC3G, TRIM5α, Tetherin or Bst2, SAMHD1, and MxB have been reported as potent anti-HIV restriction factors (Goujon et al., 2013; Hrecka et al., 2011; Kane et al., 2013; Laguette et al., 2011; Liu et al., 2013; Neil et al., 2008; Sadler and Williams, 2008; Sheehy et al., 2002; Stremlau et al., 2004; Van Damme et al., 2008). The interferon-induced transmembrane (IFITM) proteins are recently identified ISGs that have been shown to inhibit a number of viruses, including influenza A virus (IAV), West Nile virus, Dengue virus, Marburg virus (MARV), Ebola virus (EBOV), SARS coronavirus (SARS-CoV), vesicular stomatitis virus (VSV), and HIV type 1 (HIV-1) (Brass et al., 2009; Chutiwitoonchai et al., 2013; Huang et al., 2011; Jiang et al., 2010; Li et al., 2013; Lu et al., 2011; Schoggins et al., 2011; Weidner et al., 2010). However, the underlying mechanism by which IFITMs broadly inhibit viral infection is currently not well understood.

One recently recognized mechanism underlying the IFITM-mediated inhibition of viral infection is inhibition of viral entry, particularly the Env-mediated virus fusion with target cell membranes (Diamond and Farzan, 2013; Perreira et al., 2013; Smith et al., 2014). We reported that IFITMs inhibit cell-cell fusion mediated by all three classes of viral fusion proteins, acting at the level of hemifusion initiation (Li et al., 2013). Additional studies revealed that IFITM proteins decrease membrane fluidity, possibly by adopting intramembrane topology and changing curvature (Li et al., 2013; Lin et al., 2013; Yount et al., 2012). Recent results using single-viral-particle fusion assays suggest that IFITMs can also inhibit formation of fusion pores in endosomes (Desai et al., 2014). The potent inhibitory effect of IFITMs on viral fusion has been linked to their localization to both endolysosomal compartments and plasma membrane, which is determined by post-translational modification and sorting signals (Chesarino et al., 2014; Huang et al., 2011; Jia et al., 2012, 2014). Interestingly, IFITMs have been recently shown to enhance infection of some viruses, such as human coronavirus HCoV-OC43 (Zhao et al., 2014). IFITM3 is known to interact with vesicle-membrane-protein-associated protein A (VAPA) and disrupt intracellular cholesterol homeostasis (Amini-Bavil-Olyaee et al., 2013), but the exact role of cholesterol in IFITM-mediated inhibition of the viral membrane remains elusive.

We initially reported that inducible expression of IFITMs suppresses the replication of HIV-1_{BH10} strain in SupT1 cells (Lu et al., 2011). However, other groups did not observe a significant effect of IFITMs on HIV-1_{NL43} infection in HeLa-TZM cells (Brass et al., 2009). Subsequent studies showed that overexpression of IFITMs can indeed inhibit HIV-1 replication in MT-4 cells (Schoggins et al., 2011) and that IFITMs also modestly reduce expression of some HIV-1 genes, including Gag (Chutiwitoonchai

et al., 2013; Ding et al., 2014; Lu et al., 2011). Furthermore, we recently observed that HIV-1 mutations in *env* and *vpu* genes can promote escape from IFITM1 restriction, and that these mutations appear to correlate with enhanced cell-to-cell transmission capability of these escape mutants (Ding et al., 2014). Most recently, Compton, Tartour, and colleagues demonstrated that IFITMs are incorporated into HIV-1 virions, leading to impaired viral fusion, infectivity, and spread (Compton et al., 2014; Tartour et al., 2014). However, the exact mechanism underlying the IFITM impairment of HIV-1 infection remains unknown.

Herein, we show that IFITM proteins, especially IFITM2 and IFITM3, specifically interact with HIV-1 Env in viral producer cells and antagonize its ability to mediate viral cell-to-cell infection. In contrast to the study of Compton and Tatour et al. (Compton et al., 2014; Tartour et al., 2014), we find that IFITM incorporation into HIV-1 particles does not strictly correlate with the ability of IFITMs to inhibit cell-to-cell infection. We confirm our previous finding (Ding et al., 2014) that prolonged passage of HIV-1 results in the emergence of Env mutations that overcome the IFITM-mediated restriction of cell-to-cell infection, resulting in enhanced viral replication. Overall, our work supports a model by which IFITMs specifically disrupt the function of HIV-1 Env so as to restrict HIV-1. Results from this study clarify the molecular mechanisms of IFITM restriction and provide insight into the specialized role of IFITMs in HIV-1 replication, with possible consequences for HIV-1 transmission and AIDS pathogenesis.

RESULTS

Expression of IFITM1, 2, and 3 Differentially Inhibits HIV-1 Infection

Despite the strong inhibition imposed by IFITMs on many enveloped viruses, the effect of IFITMs on HIV-1 has been shown to be comparatively modest (Diamond and Farzan, 2013; Perreira et al., 2013). Notably, most previous studies are based on cell-free HIV-1 infection, in which IFITMs are expressed in target cells. Here, we exploit a highly sensitive intron-*Gaussia* luciferase (inGLuc) system developed by David Derse and colleagues (Mazurov et al., 2010) to assess the effect of IFITM expression in donor cells on HIV-1 single-round cell-to-cell transfer. In this system, the Gluc gene can only be expressed upon HIV-1 vector infection of target cells. We first established Jurkat cell lines stably expressing IFITM1, IFITM2, or IFITM3 using Jurkat/inGLuc (a kind gift of Walther Mothes) previously engineered to express inGLuc HIV-1 vector (Agosto et al., 2014), and we infected these cells with wild-type (WT) HIV-1_{NL43} bearing VSV-G so that the majority of donor cells would be HIV-1 infected. We then cocultured these cells with parental Jurkat cells and measured GLuc activity 24–48 hr after coculture.

Expression of IFITM proteins, especially IFITM2 and IFITM3, in donor cells significantly inhibited HIV-1_{NL43} transmission from Jurkat/inGLuc to parental Jurkat cells (up to 10-fold) (Figure 1A). In contrast, IFITM expression in target Jurkat cells showed very modest effects, i.e., ~5%–40% inhibition, on HIV-1 cell-to-cell transmission (Figure 1A). Similar trends were also observed when 293T cells served as donor cells and Jurkat as target cells, although the inhibitory effect of IFITM1 in donor 293T cells was less compared to that in donor Jurkat/inGLuc cells (Figure 1B), possibly due to the expression of endogenous IFITMs in Jurkat cells (Lu et al., 2011). We also measured the infectivity of HIV-1 virions produced from 293T cells in HeLa-TZM and observed that IFITM2 and IFITM3 significantly diminished the viral infectivity (Figure 1C). The relatively low inhibitory effect of IFITM1 on HIV-1 cell-to-cell infection was not due to different levels of IFITM expression in 293T or Jurkat cells, as determined by western blotting (Figure 1D). We treated the cocultured 293T-Jurkat cells with AMD3100 (a CXCR4 inhibitor), latrunculin B (LAT-B, an actin polymerization inhibitor), or VRC01 (a broadly neutralizing antibody against the CD4 binding site of Env), and we observed that each of these inhibitors markedly reduced cell-to-cell HIV-1 infection (Figure 1E), showing that HIV-1 transmission from 293T to Jurkat cells is CXCR4 coreceptor and HIV-1 Env dependent and requires a dynamic actin cytoskeleton.

To directly visualize the effect of IFITMs on HIV-1 cell-to-cell spread, we performed live-cell imaging by transfecting 293T cells with an infectious HIV-1 clone that contains an internally inserted GFP in Gag (HIV-1_{NL43}-iGFP) along with IFITM expression vectors. Once again, expression of IFITM2 and IFITM3, but not IFITM1, in 293T cells significantly inhibited HIV-1_{NL43}-iGFP spread and syncytium formation from 293T to Jurkat (Figures 1F and S1A). Similar results were also obtained in HIV-1 transmission and cell-cell fusion from 293T to HeLa-TZM cells (Figure S1B). Collectively, these data demonstrate that expression of IFITMs in donor cells strongly but differentially inhibits HIV-1 cell-to-cell infection and cell-cell fusion. These findings confirm the recent reports (Compton et al., 2014; Tartour et al., 2014) that potent IFITM restriction of HIV-1 infection requires expression in the producer cell.

Knockdown of Endogenous IFITM Expression in a $CD4^+$ T Cell Line Promotes HIV-1 Cell-to-Cell Infection

$CD4^+$ PM1 T cells express detectable levels of endogenous IFITM1, IFITM2, and IFITM3 (Lu et al., 2011). We thus tried to knock down the IFITM expression in PM1 cells by transducing them with lentiviral small hairpin RNAs (shRNAs) against IFITM1, 2, or 3 and examined the effect on HIV-1 cell-to-cell infection. The expression level of IFITMs in these PM1 cells was substantially reduced by the shRNA, either in the presence or absence of interferon treatment (IFNα2b, 500 U), although cross-knockdown of different human IFITMs was observed (Figure 2A). These cell lines were infected with HIV-1_{NL43}-iGFP bearing VSV-G for 12 hr, resulting in ~97% Gag-iGFP-positive PM1 cells, which served as donor cells (Figure S2A). After extensive washes, the PM1 cells were cocultured with target Jurkat cells, which were pre-labeled with CMTMR (red). The cell-to-cell HIV-1 transmission efficiency was determined by calculating the GFP^+ populations over time in CMTMR-labeled target Jurkat cells using flow cytometry.

As shown in Figure 2B, 4 hr after coculture, the HIV-1 Gag-iGFP transfer from PM1 to Jurkat cells was slightly increased for PM1 cells expressing shRNA targeting IFITMs compared to that of cells transduced with scrambled shRNA control. Notably, the enhancement became apparent at 8 hr in PM1 cells

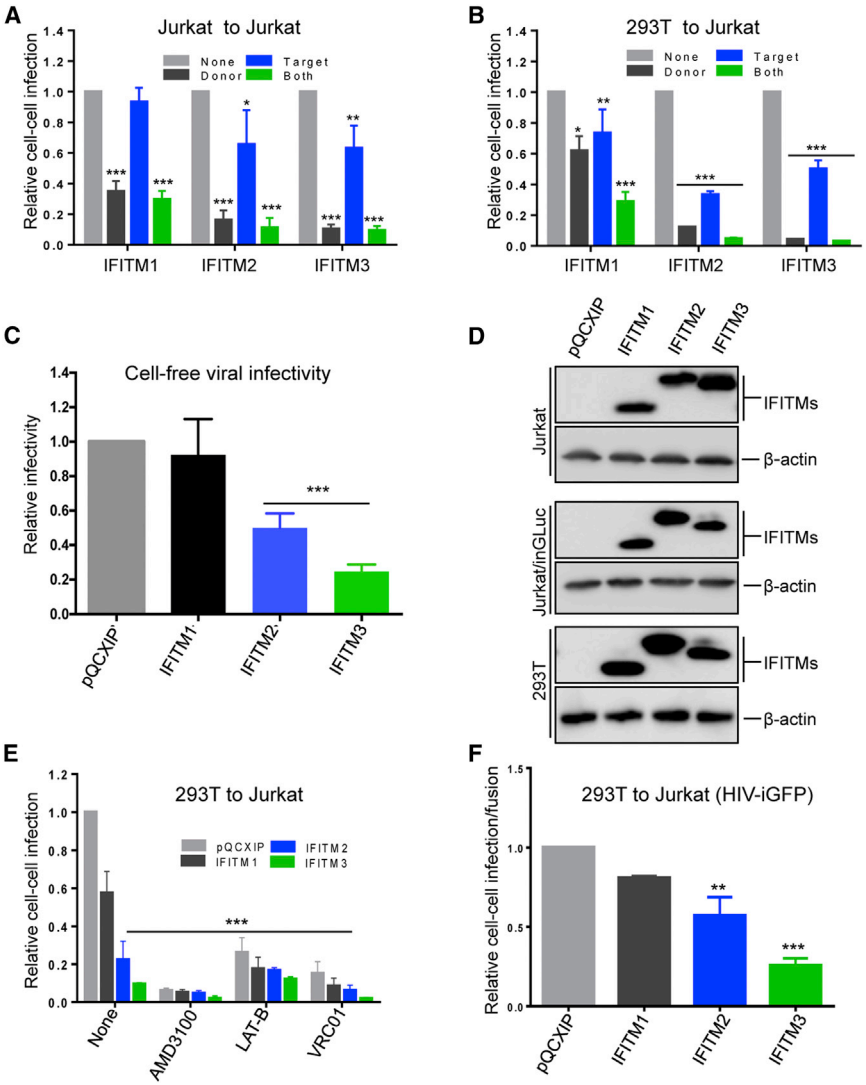

Figure 1. Expression of IFITMs Differentially Inhibits HIV-1 Cell-to-Cell Infection

(A) Donor Jurkat/inGLuc cells expressing or not expressing IFITMs were infected with HIV-1_{NL43} pseudotypes bearing VSV-G and cocultured with target Jurkat or Jurkat cells stably expressing IFITMs.

(B) HEK293T cells were transiently transfected with HIV-1 NL43/inGLuc plus Env in the presence or absence of IFITMs and were cocultured with Jurkat cells stably expressing or not expressing IFITMs.

(C) Effect of IFITMs on cell-free HIV-1 infection. HIV-1_{NL43} virions were produced from 293T cells expressing or not expressing IFITMs, and cell-free infectivity was determined by infecting HeLa-TZM cells.

(D) Expressions of IFITM proteins in Jurkat/inGLuc, Jurkat and 293T cells determined by western blotting. β-Actin serves as loading control.

(E) Effect of AMD3100 (4 μg/m), LAT-B (1 μg/ml), and VRC01 (5 μg/ml) on HIV-1 transfer from 293T to Jurkat cells. All reagents were added immediately upon coculturing and maintained throughout the entire assay.

(F) Quantification of HIV-1 transmission/fusion from 293T to Jurkat/tdTomato cells using live-cell imaging. HEK293T cells were transfected with HIV-1_{NL43} Gag-iGFP and cocultured with Jurkat/tdTomato cells for 20–24 hr. The numbers of GFP- and tdTomato-double-positive cells were scored and plotted relative to that of empty vector pQCXIP control.

For all figures applicable, the values are means and SDs of three to five independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. See also Figure S1.

IFITM Proteins Interact with HIV-1 Env in Viral Producer Cells and Impair Env Processing and Incorporation into Virions

One possible mechanism by which IFITM expression in donor cells could inhibit HIV-1 cell-to-cell infection and infectivity is through association with HIV-1 Env. As the first step to test this possibility, we performed western blotting to examine HIV-1 Env expression and processing in 293T cells transiently transfected with HIV-1_{NL43} in the presence or absence of IFITMs. We found that the ratio of gp120 or gp41 to gp160 in IFITM-expressing cells, especially that of IFITM2 and IFITM3, was considerably decreased compared to that observed in cells transfected with the pQCXIP vector control (Figures 3A and 3C). These results suggest that HIV-1 Env processing is impaired by IFITMs. We then carried out immunoprecipitation (IP) of the cell lysates (containing 1% NP-40 and 0.1% SDS) with anti-FLAG and observed that IFITM proteins, especially those expressing IFITM2 and IFITM3, readily pulled down HIV-1 Env, especially gp41 (Figure 3B, top and middle panels). IFITM1 was also found to interact with HIV-1 Env, but with much reduced efficiency (Figure 3B). Importantly, we did not detect HIV-1 Gag in immunoprecipitated cell lysates (data not shown), implying that the observed interaction is HIV-1 Env specific. The interaction between endogenous

expressing shRNA against IFITM2 (23.8%) and IFITM3 (32.8%) relative to the scrambled shRNA control (13.8%) (Figure 2B). Azidothymidine (AZT) treatment of cocultured cells at 8 hr did decrease HIV-1 transmission in IFITM-knockdown PM1 cells compared to untreated cells (Figure 2B), suggesting that knockdown of IFITMs can enhance productive HIV-1 infection following cell-to-cell infection (i.e., newly synthesized Gag-iGFP). The cell-free HIV-1_{NL43} infection of the mixed PM-1/Jurkat cell population (without spinoculation) was low (~1.6%), regardless of shRNA IFITM expression. We subsequently confirmed the effects of IFITMs knockdown, especially IFITM3, in primary peripheral blood mononuclear cells (PBMCs), which served as donor cells for HIV-1 cell-to-cell infection (Figures 2C–2E). We cannot exclude the possibility that the smaller effect of IFITM1 and 2 knockdown on viral transmission could be due to their relatively low knockdown efficiency in PBMCs. Nonetheless, these results demonstrate that knockdown of endogenous IFITM expression enhances HIV-1 transmission in $CD4^+$ T cells.

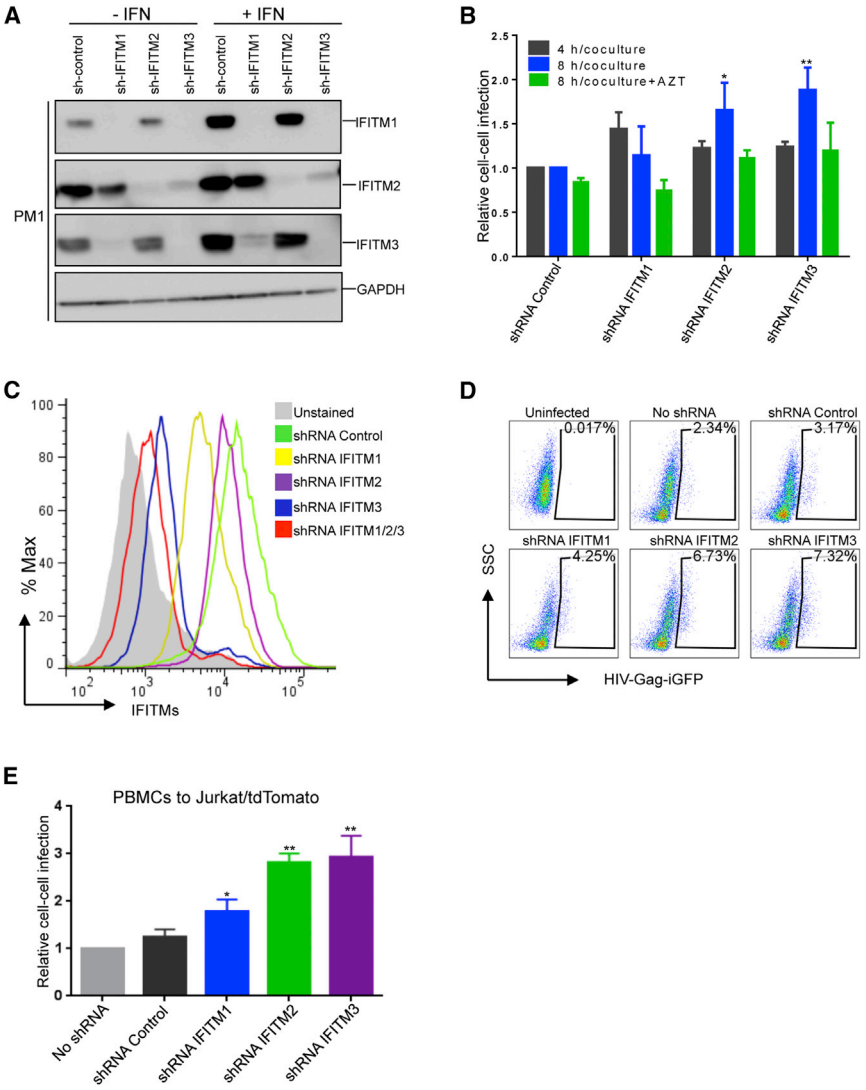

Figure 2. Knockdown of IFITM Expression in CD4⁺ PM1 Cells or PBMCs Promotes HIV-1 Transmission to Jurkat Cells

(A) Examination of IFITM protein expression in PM1 cells stably expressing shRNA IFITM1, 2, and 3. Cells were treated with or without 500 U IFNα2b for 18–24 hr, and lysates were subjected to western blotting using anti-IFITM1, anti-IFITM2, or anti-IFITM3 antibodies. Cross-knockdown and antibody cross-reactions among IFITM1, 2, and 3 are noticed. GAPDH serves as loading control due to weak signals for β-actin in PM-1 cells.

(B) Summary of flow cytometric analysis of HIV-1 Gag-GFP transfer from PM1 to Jurkat cells. PM-1 cells stably expressing shRNA IFITM1, 2, or 3 or scramble control were infected with NL43-Gag-iGFP; cells were cocutured for 4–8 hr with Jurkat cells that were prelabeled with CMTMR. Cells were gated for GFP (Green) and CMTMR (Red) by flow cytometry (see Figure S2). For 8 hr coculture, cells were also treated with AZT to ensure that productive HIV-1 replication had occurred. Data were from four independent experiments. All values shown are normalized against shRNA control either at 4 or 8 hr after coculture, *$p < 0.05$; **$p < 0.01$.

(C–E) Knockdown of IFITM expression in PBMCs enhances HIV-1 cell-to-cell infection. Activated PBMCs were treated with 500 U IFNα2b and infected with VSV-G lentiviral pseudotypes encoding shRNA IFITM1, 2, or 3 for 48 hr. The knockdown efficiency was examined by intracellular staining using pooled antibodies against IFITM1, 2, and 3 (1:1:1) by flow cytometry shown in (C). Note that shRNA against IFITM1 knocks down both IFITM1 and IFITM3, shRNA against IFITM2 only knocks down IFITM2, and shRNA against IFITM3 knocks down all three IFITMs. Next, the cells were infected with HIV-1_{NL43}-iGFP bearing VSV-G, which served as donor cells, and cocultured with target Jurkat cells stably expressing tdTomato (Jurkat/tdTomato). The cell-to-cell infection efficiency was determined by measuring GFP⁺ Jurkat cells 48 hr after coculture using flow cytometry (D) and summarized in (E). *$p < 0.05$. Results are from four independent experiments performed in duplicates.

See also Figure S2.

IFITM proteins and HIV-1 Env was also observed in PM1 cells (Figure 3D), although knockdown of individual IFITMs in PM1 cells only modestly increased HIV-1 Env processing (Figures S2B–S2D). Overall, these results demonstrated that IFITM proteins, especially IFITM2 and IFITM3, specifically interact with HIV-1 Env in viral producer cells, resulting in impaired Env processing.

IFITM incorporation into HIV-1 virions has been recently reported to be responsible for impaired viral infectivity (Compton et al., 2014; Tartour et al., 2014). Hence, we compared the incorporation efficiencies of IFITM1, IFITM2, and IFITM3 into HIV-1 virions and examined their possible correlations between incorporation efficiency and inhibitions of cell-to-cell infection. Surprisingly, we found that all three IFITMs were abundantly present in culture media, likely as exosomes, even in cells not expressing HIV-1 (Figure 3E, right three lanes). To determine if IFITMs are specifically incorporated into HIV-1 virions, we adopted an approach that we recently used to evaluate TIM-family protein incorporation (Li et al., 2014). We first immunoprecipitated purified HIV-1 virions using HIV-1 immunoglobulin (HIV-Ig, pooled HIV patient sera), and we then performed western blotting using anti-FLAG to detect IFITMs. We observed that all IFITMs were detected in the pulled down HIV-1 virions, consistent with reports of Compton and Tartout et al. (Compton et al., 2014; Tartour et al., 2014). However, IFITM1 was more abundantly incorporated into HIV-1 virions compared to IFITM2 and 3 (Figure 3F, top panel), which is not in accordance with their inhibition of cell-to-cell infection and diminished infectivity (Figure 1); this was despite the equivalent input of IFITMs (Figure 3G), as well as almost equivalent virion pull-down efficiency by HIV-Ig (Figure 3F, bottom panel). Notably, the signals of gp120 in the purified virions, either

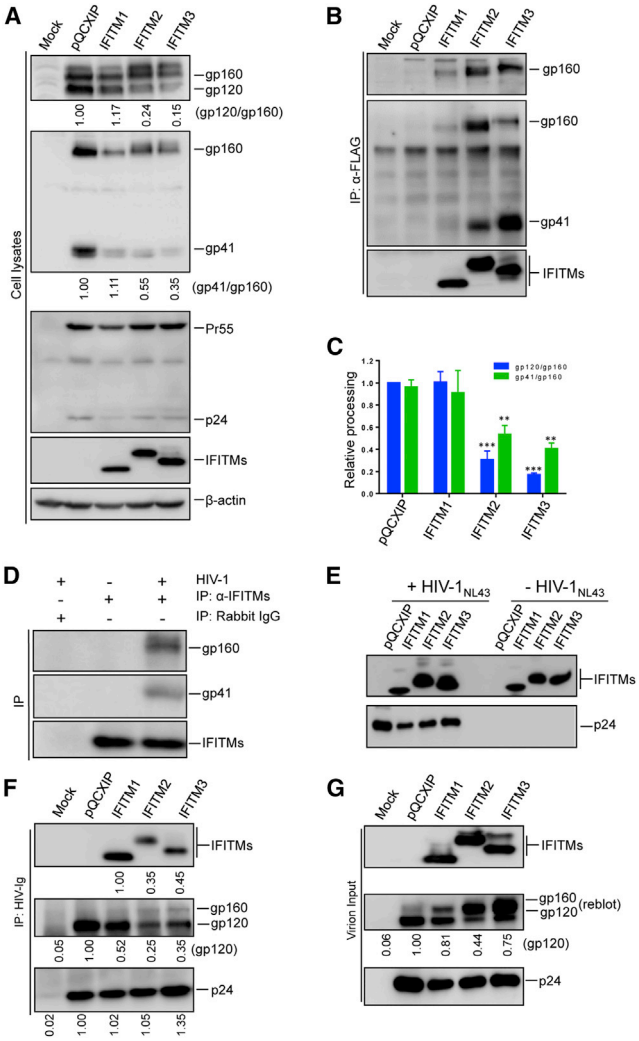

Figure 3. IFITM Proteins Interact with HIV-1 Env and Impair its Processing and Incorporation into Viral Particles

293T cells were transiently transfected with HIV-1_{NL43} in the presence or absence of IFITM plasmids. The cells were lysed with a membrane-disrupting RIPA buffer containing 0.1% SDS and 1% NP40, and lysates were immunoprecipitated with anti-FLAG antibody.

(A) The expression of HIV-1 Env, Gag, and IFITMs in transfected cells was examined by using anti-gp120, gp41, anti-p24, and anti-FLAG, respectively. β-Actin serves as a loading control. The ratios of gp120 versus gp160 and gp41 versus gp160 in the cell lysates were determined by quantifying the band intensities of gp120, gp41, and gp160 using *Quantity One* (Bio-Rad).

(B) Detection of the interaction between IFITMs and HIV-1 Env in viral producer cells. Western blotting was performed by using anti-gp120 (top panel) or anti-gp41 (middle panel) to detect HIV-1 Env.

(C) Quantification of HIV-1 Env processing. The results represent average ± SD of four independent experiments. $^{**}p < 0.01$; $^{***}p < 0.001$.

(D) Endogenous IFITMs interact with HIV-1 Env in PM1 cells. PM1 cells were infected with HIV-1_{NL43} or mock-infected, and cells were lysed by RIPA buffer 48 hr after infection. Lysates were subjected to immunoprecipitation using pooled antibodies against IFITM1, 2, or 3, or with a normal rabbit Ig that serves as negative control; western blots were performed using anti-gp41.

(E) Detection of IFITMs in supernatants of 293T cells not transfected with HIV-1 plasmid. Supernatants were concentrated by ultracentrifugation using 20% sucrose, and pellets were analyzed by western blotting using anti-FLAG or anti-p24.

(F and G) IFITMs are incorporated into HIV-1 virions with different efficiencies, which do not correlate with their phenotypes in inhibiting HIV-1 cell-to-cell infection. Viral supernatants were concentrated from cells used for Figures 3A and 3B by ultracentrifugation (20% sucrose), followed by immunoprecipitation (IP) using HIV-Ig. Western blotting was performed by using anti-FLAG (to detect IFITMs), anti-gp120, or anti-p24. The gp120 band intensities were quantified by *Quantity One*, with relative values to the vector control indicated. No IFITM protein can be pulled down by HIV-Ig from concentrated supernatant of cells not expressing IFITMs (data not shown). Results represent three independent experiments.

See also Figure S3.

before (referred to as "virion input") or after immunoprecipitation with HIV-Ig (referred to as "IP: HIV-Ig"), were substantially reduced by IFITM expression, especially IFITM2 and 3 (Figures 3F and 3G), consistent with the lower levels of gp120 in the cells. We also observed that gp160 in IFITM2 and IFITM3-containing HIV-1 virion input was significantly increased (Figure 3G, middle panel), which likely represents immature Env in microvesicles and/or exosomes, whose release might be promoted by IFITM2 and 3. Together, our data support the conclusion that IFITMs are abundantly present in the media of normally cultured cells, even without HIV-1, which is consistent with a recent study (Zhu et al., 2015), and that IFITM incorporation into HIV-1 virions, although detected, does not strictly correlate with the phenotype of IFITM inhibition of HIV-1 cell-to-cell infection.

IFITM3 Protein Promotes HIV-1 gp120 Shedding

Next, we explored the possibility that IFITM proteins, in particular IFITM3, may trigger gp120 (SU) shedding in viral producer cells, which could partly explain or contribute to the reduced levels of gp120/gp41expression in the cell and viral particles as well as impaired viral infectivity. Our previous work has already demonstrated that IFITM3 does not significantly inhibit HIV-1 production (Lu et al., 2011). We performed pulse-chase labeling experiments using 293T cells transiently transfected with HIV-1_{NL43}, in the presence or absence of IFITM3, and then measured gp120 shedding into culture media by immunoprecipitation using HIV-Ig. While the expression of gp160 decreased in a time-dependent manner, as would be expected, the level of gp160 in IFITM3-expressing cells suffered a more rapid decrease compared to cells expressing HIV-1 NL43 alone (Figures 4A and 4B). In contrast, HIV-1 Gag (Pr55) expression in both cell groups exhibited similar rates of decline, regardless of IFITM3 expression or not (Figures 4A and 4C). Notably, we consistently observed an enhanced SU shedding in IFITM3-expressing cells compared to cells expressing HIV-1 NL43 alone, especially at 2, 4, and 6 hr of the chase labeling time (Figures 4A and 4D). Overall, the pulse-chase labeling data indicate that IFITM expression in viral producer cells likely increases gp160 turnover and promotes gp120 shedding into culture media.

The IFITM C Terminus Contributes to Inhibition of HIV-1 Cell-to-Cell Transmission and Viral Membrane Fusion

Given strong similarities among the three human IFITM proteins, it is interesting that IFITM1 is much less potent than IFITM2 and

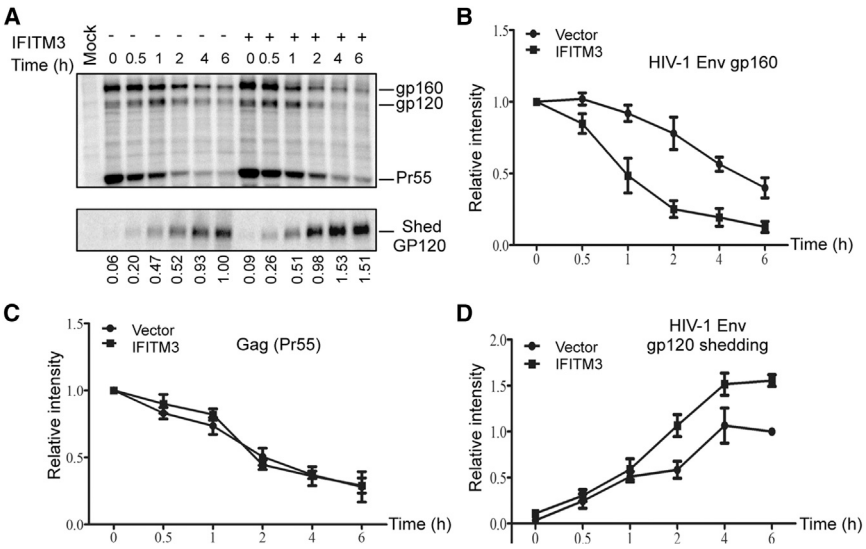

Figure 4. IFITM3 Protein Promotes HIV-1 gp120 Shedding and Increased gp160 Turnover

HEK293T cells were transfected with plasmids encoding HIV-1 NL4-3 provirus in the presence or absence of IFITM3. Cells were pulse-labeled with ^{35}S-Met/Cys at 37°C for 1 hr and chased in unlabeled medium for the times indicated. The cell lysates and supernatants were immunoprecipitated with HIV-Ig at 4°C overnight and separated by SDS-PAGE.

(A) Representative phosphorimages of cell lysates (top) and supernatants (bottom) from three independent experiments are shown.

(B–D) Quantifications of the phosphorimaging intensity of HIV-1 gp160 (B), Gag Pr55 (C), and shed gp120 (D). Note that in (B) and (C), we set the signals of HIV-1 gp160 or Gag Pr55 in the absence and presence of IFITM3 to 1.0, respectively. In (D), we set the signal of gp120 in the absence of IFITM3 at 6 as 1.0 for comparison.

IFITM3 at inhibiting HIV-1 viral infectivity and cell-to-cell transmission. We therefore examined several IFITM1/IFITM2 chimeras that we had previously generated; they differ primarily at the N and C termini (Li et al., 2015). Chimeric IFITM1112 and IFITM1222 contain the N terminus of IFITM1 and the C terminus of IFITM2, whereas chimeric IFITM2221 and IFITM2111 contain the N terminus of IFITM2 and the C terminus of IFITM1 (Li et al., 2015). We found that IFITM1112 and IFITM1222 strongly inhibited HIV-1 cell-to-cell infection, with efficiency similar to, or even higher than, that of IFITM2 (Figure 5A). In contrast, chimeras IFITM2221 and IFITM2111 showed modest inhibitions of HIV-1 cell-to-cell infection, almost identical to the phenotype of IFITM1 (Figure 5A). These data suggest that the C terminus of IFITM1 intrinsically suppresses inhibition of HIV-1 transmission from 293T to Jurkat cells. The negative impact of the C terminus of IFITM1 on inhibition of HIV-1 cell-to-cell transmission was further supported by the phenotypes of three IFITM1 truncation mutants (Li et al., 2015), i.e., Δ(108–125), Δ(112–125), and Δ(117–125), all of which exhibited enhanced inhibition of HIV-1 cell-to-cell transmission compared to WT IFITM1 (Figure 5A). The differential effects of IFITM chimeras as well as IFITM1 deletion mutants on HIV-1 cell-to-cell transmission seemed to correlate with their capability to associate with HIV-1 Env. For example, chimeric IFITM1112 and 1222 exhibited increased associations with HIV-1 Env relative to IFITM1 WT (Figure S3). The increased interaction was also observed for three IFITM1 C-terminal deletion mutants, e.g., Δ(117–125), despite their apparent downregulation of HIV-1 Env (Figure S3). Collectively, these results suggest that the C terminus of IFITM1 intrinsically suppresses association with HIV-1 Env, and this likely contributes to and explains, at least in part, its less potent ability to block cell-to-cell transmission.

We also examined several N-terminal point mutations or deletions of IFITM3, which had been previously shown to affect post-translational modification and endocytic trafficking of IFITM3 (Jia et al., 2012, 2014). However, we did not observe significant differences between these mutants and parental IFITM3 in inhibiting HIV-1 cell-to-cell transmission (Figure 5B), despite the fact that some of these constructs exhibited increased expression on the cell surface (Jia et al., 2012, 2014) (data not shown). The total protein expression of these IFITM1/2 chimeras and IFITM3 mutants had been previously shown to be comparable to their parental IFITM1, 2, or 3 (Jia et al., 2014; Li et al., 2015; Lu et al., 2011). Taken together, these results revealed that the C termini of IFITM proteins regulate their inhibitory effects on HIV-1 cell-to-cell transmission.

To directly measure the effect of IFITM expression on virion-cell fusion, we next performed HIV-1 BlaM-Vpr viral fusion assays in our cell-to-cell infection system. 293T cells were co-transfected with NL43 proviral DNA plus a plasmid expressing BlaM-Vpr, in the presence or absence of IFITMs. We cocultured the transfected 293T cells with target Jurkat cells stably expressing tdTomato (Jurkat/tdTomato); 2 hr after coculture, HIV-1 fusion within target Jurkat/tdTomato cells was measured by gating the tdTomato-positive population using flow cytometry. As shown in Figure 5C, expression of IFITMs, especially IFITM2 and IFITM3, in donor 293T cells substantially inhibited HIV-1 fusion (likely a mix of virus-cell and cell-cell fusion) in Jurkat/tdTomato cells, correlating with their effects on cell-to-cell infection (Figure 1B). Consistent with the negative influence of the C terminus of IFITM1 on inhibiting cell-to-cell infection shown in Figure 5A, chimeras IFITM1112 and IFITM1222, but not those of IFITM2221 and IFITM2111, effectively inhibited HIV-1 fusion in target cells to a similar extent as IFITM2 and IFITM3 (Figure 5C).

Given the above finding that HIV-1 Env is directly affected by IFITMs, we next carried out cell-cell fusion assays by transfecting 293T cells with plasmids encoding HIV-1 Env and Tat along with IFITMs; these cells were then cocultured with HeLa-TZM, and cell-cell fusion was quantified by measuring the firefly luciferase activity of cocultured cells. In particular, we compared the cell-cell fusion efficiency of HIV-1 Env when IFITM proteins are expressed at similar levels in effector 293T cells, the target HeLa-TZM cells (stably expressing IFITMs by retroviral

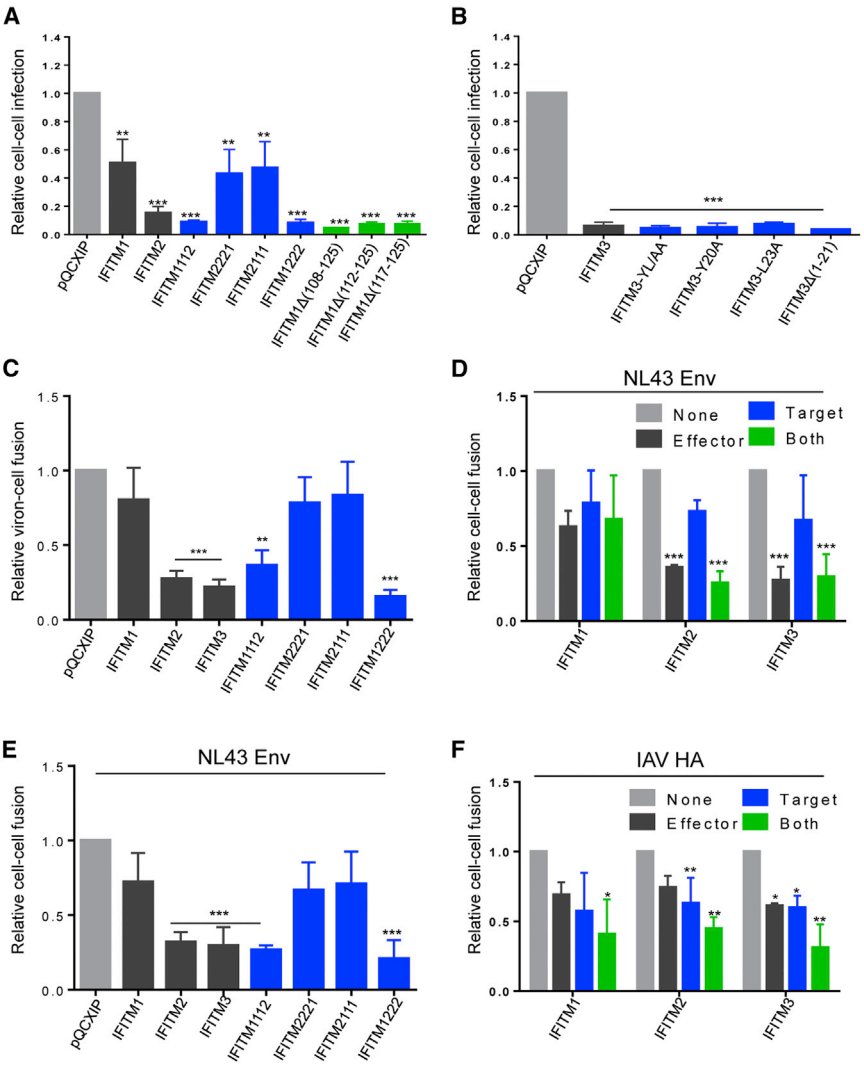

Figure 5. The IFITM C Terminus Regulates the Inhibition of HIV-1 Cell-to-Cell Infection and Membrane Fusion

(A and B) Effect of IFITM1/2 chimeras and IFITM3 N-terminal mutants on HIV-1 cell-to-cell infection. The experiments were performed using 293T as donor cells and Jurkat as target cells.

(C) Effect of parental IFITMs and IFITM1/2 chimeras on HIV-1 virion fusion during cell-to-cell infection. Note that the HIV-1 BlaM-Vpr-based fusion assay was implemented into cell-to-cell infection experiments (see details in Experimental Procedures).

(D and E) Effect of IFITMs and chimeras on HIV-1 Env-mediated cell-cell fusion. 293T cells were transfected with HIV-1_{NL43} along with LXSN that encodes HIV-1 Tat, in the presence or absence of IFITMs. The transfected 293T cells (effector cells) were cocultured with parental HeLa-TZM or HeLa-TZM stably expressing IFITMs (target cells) for 12–24 hr. Cells were lysed, proteins were quantified, and firefly activity was measured. All samples were done in duplicate. Results shown represent three to four independent experiments.

(F) Effect of IFITMs in effector cell, target cells, or both on IAV HA-mediated cell-cell fusion. Assays were carried out as described in (D) and (E), except that IAV HA was tested and cocultured cells were treated with pH 5.0 for 1 min following coculture. Results represent three to four independent experiments.

For all figures, relative numbers are presented. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.

transduction), and both. Consistent with previously published data (Compton et al., 2014), expression of IFITMs, especially IFITM2 and IFITM3, in effector cells, but to a much lesser extent in target cells, inhibited cell-cell fusion induced by HIV-1_{NL43} Env protein (Figure 5D). Extending these results, and in agreement with the cell-to-cell infection (Figure 5A) and virion-cell fusion data (Figure 5C), chimeric IFITM1112 and IFITM1222, but not IFITM2221 and IFITM2111, potently inhibited HIV-1_{NL43} Env-mediated cell-cell fusion (Figure 5E). In stark contrast to the strong inhibitory effect of IFITM expression in effector cells on HIV-1 Env, we found that cell-cell fusion mediated by influenza A virus (IAV) HA was equivalently inhibited by IFITM expression in effector cells and target cells (Figure 5F), similar to our previous results obtained with Jaagsiekte sheep retrovirus (JSRV) Env (Li et al., 2013). Altogether, these results strengthen the notion that IFITMs specifically antagonize the HIV-1 Env protein to inhibit viral cell-to-cell infection, and that this mechanism is distinct from the IFITM inhibition of IAV hemagglutinin (HA) and JSRV Env (Li et al., 2013).

HIV-1 Adapts to Overcome IFITM Restriction of Cell-to-Cell Infection by Acquiring Mutations in Env

We previously reported that mutations in HIV-1 Env were able to overcome IFITM-mediated restriction of HIV-1 replication (Ding et al., 2014). Next, we sought to extend these results to the IFITM constructs under analysis here. Given the apparently enhanced ability of IFITM1Δ(117–125) to inhibit HIV-1_{NL43} cell-to-cell infection compared to parental IFITM1, we performed long-term replication assays and observed that, indeed, IFITM1Δ(117–125) and IFITM1Δ(108–125) mutants profoundly inhibited HIV-1_{NL43} replication compared to WT IFITM1 (Figure 6A). We then carried out virus evolution experiments to interrogate if HIV-1 can escape from the inhibition of IFITM1Δ(117–125) following a series of prolonged passages. After 3 weeks of culturing HIV-1_{NL43} in IFITM1Δ(117–125)-expressing SupT1 cells, we observed pronounced cytopathic effects accompanied by increased viral RT activity in the culture supernatants, implying the possible emergence of HIV-1 escape mutants. We recovered the viral DNA from infected cells and identified two mutations: a stop codon at the 40th amino acid position in Vpu (Vpu40), which caused Vpu truncation, and an alanine-to-valine change at residue 539 in Env (EnvA539V). While the Vpu40 or EnvA539V alone marginally increased the replication of HIV-1_{NL4-3} in IFITM1Δ(117–125)-expressing SupT1 cells (Figure 6B),

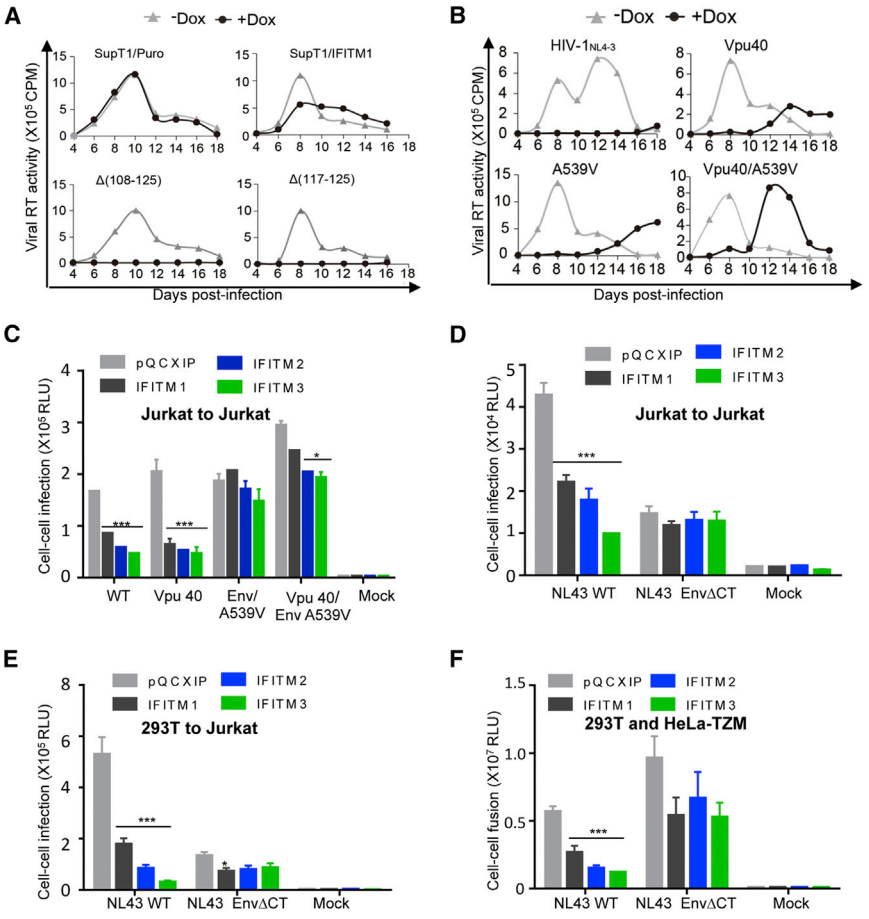

Figure 6. Mutations in the HIV-1 *env* Gene Provide Escape from IFITM-Mediated Inhibition of Viral Replication and Cell-to-Cell Infection

(A) Replication kinetics of HIV-1_{NL43} in SupT1 cells expressing IFITM1 WT or its two mutants, IFITM1Δ(117–125) and IFITM1Δ(108–125). The expression of IFITM1 and its mutants in the cells was induced by doxycycline (Dox) (500 ng/ml), viral replication was determined by measuring the levels of viral RT activity in culture supernatants.

(B) Replication kinetics of HIV-1_{NL43} WT and mutants Vpu40, EnvA539V, and Vpu40/EnvA539V in SupT1 cells expressing (with Dox) or not expressing IFITM1Δ(117–125) (without Dox).

(C) Effect of IFITMs on cell-to-cell infection of HIV-1 escape mutants. Experiments were performed as described in the legend of Figures 1A–1C, except HIV-1_{NL43} mutants Vpu40, EnvA539V, and Vpu40/EnvA539V bearing VSV-G were used for infection of Jurkat/inGLuc cells, which served as donors. Because of the difference between WT and HIV-1 mutants in infectivity in the absence of IFITMs, we plotted the absolute Gluc readouts (RLU) in this and other related figures.

(D) Effect of IFITMs on Jurkat-to-Jurkat transfer mediated by the Env cytoplasmic tail deletion mutant (NL43 EnvΔCT).

(E) Effect of IFITMs on 293T-to-Jurkat transfer of NL43 EnvΔCT. (F) Effect of IFITMs on cell-cell fusion (between 293T expressing Env and HeLa-TZM) mediated by the Env protein of NL43 EnvΔCT.

For all data shown in (A)–(F), results shown represent at least three independent experiments. *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$. See also Figure S4.

the double mutant Vpu40/EnvA539V allowed virus replication to peak at day 12 in Δ(117–125)-expressing SupT1 cells compared to the replication peak at day 8 in control SupT1 cells, which suggests a partial restoration of virus replication (Figure 6B). Consistent with this result, the Vpu40 mutant did not appear to exhibit any difference from WT HIV-1 in sensitivity of IFITM inhibition of cell-to-cell infection; however, EnvA539V, as well as the double mutant containing both Vpu40 and EnvA539V, substantially, though not completely, overcame the inhibition by IFITMs (Figure 6C). These results demonstrated that HIV-1 can acquire escape mutations that evade IFITM restriction, further supporting the notion that IFITMs antagonize HIV-1 Env to inhibit viral cell-to-cell infection, although these mutants may not be IFITM-specific (see below).

Truncation of the cytoplasmic tail of HIV-1 Env is known to increase cell-cell fusion, possibly by changing the conformation of the Env's extracellular domain (Freed and Martin, 1994, 1996; LaBranche et al., 1995). We thus examined the effect of IFITMs on truncated HIV-1_{NL43} Env, in which the last 144 amino acids of the C terminus was deleted (NL43 EnvCTdel-144) (Freed and Martin, 1996; Murakami and Freed, 2000). Interestingly, we found that IFITMs did not inhibit Jurkat-to-Jurkat and 293-to-Jurkat transfer of NL43 EnvCTdel-144 as potently as they did the WT, despite the reduced infectivity of this mutant relative to that of HIV-1_{NL43} WT (Figures 6D and 6E) (Murakami and Freed, 2000). We next analyzed cell-cell fusion by transfecting 293T cells with an Env-expression construct derived from NL43 EnvCTdel-144 and using HeLa-TZM as target cells, again with a similar trend of inhibition obtained (Figure 6F). Note that the cell-cell fusion activity of NL43 EnvCTdel-144 is relatively higher than the WT in the absence of IFITM (Figure 6F), consistent with numerous previously published reports. Altogether, these results showed that deletion of the cytoplasmic tail of HIV-1 Env can overcome the restriction of IFITMs on cell-to-cell infection and cell-cell fusion.

To determine whether IFITM proteins, especially IFITM3, have altered capability to inhibit the processing of A539V and CTdel-144 mutants, and to determine whether or not IFITM3 still interacts with them, we carried out coimmunoprecipitation experiments as described for the WT HIV-1_{NL43}. We found that the processing of Env A539V mutant was still inhibited by IFITM3, as evidenced by increased gp160 and decreased gp120 and gp41 in the cell lysates, and that A539V also interacted with IFITM3 as efficiently as did the WT Env (Figure S4A, top two panels). These suggest that A539V mutant likely arose by gaining replication fitness. Notably, we found that CTdel-144 Env had substantially lost its capability to associate with IFITM3 (Figure S4A, bottom panels). Furthermore, we observed

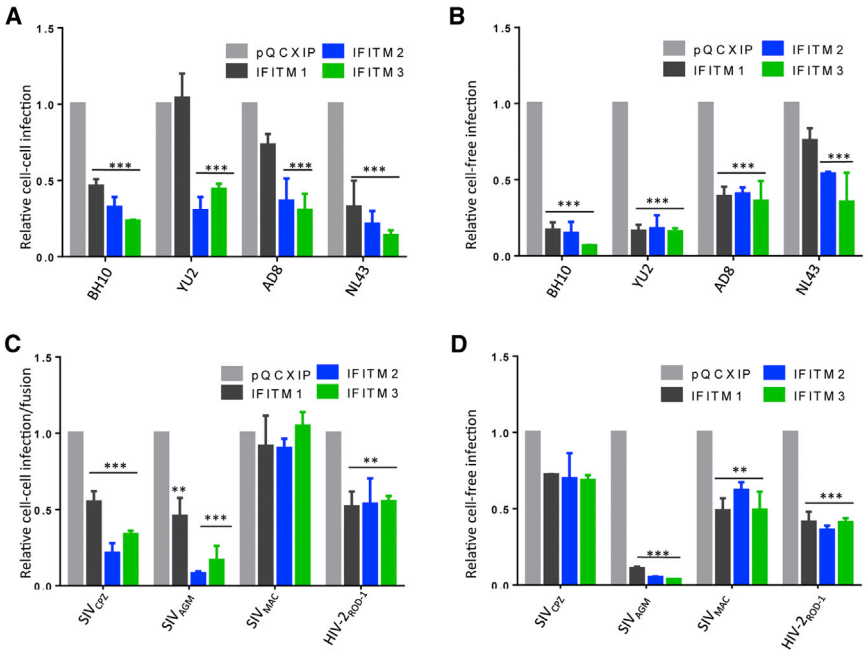

Figure 7. The Ability of IFITMs to Inhibit Cell-to-Cell Infection/Fusion by HIV-1 BH10, YU2, AD8, HIV-2, and SIVs Are IFITM-Species and Virus-Strain Dependent

293T cells were transfected HIV-1 proviral DNAs encoding BH10, YU2, NL43(AD8) (abbreviated AD8), NL43 along with a VSV-G-expressing plasmid (A) and (B), or the molecular clones of HIV-2 or SIVs (C) and (D). For HIV-1 (A) and (B), the produced viruses were used to infect Jurkat/inGLuc cells overnight, and after thorough washing with PBS, the infected Jurkat/inGLuc cells were cocultured with Jurkat cells (for BH10 and NL43) or $CD4^+$ PBMCs (for YU2 and AD8). For HIV-2 and SIVs (C and D), the transfected 293T cells were cocultured with HeLa-TZM. Cell-to-cell infection/fusion efficiency was determined by measuring GLuc (A) or firefly luciferase activity (D) 24–48 hr after coculture. Cell-free viral infection was performed in parallel, with exactly the same numbers of normal donor plus target cells as described in the text and Experimental Procedures (B and D). **p < 0.01; ***p < 0.001. Because different HIV and SIV isolates have different infectivities in the absence of IFITMs, we also plotted the data using absolute Gluc readouts shown in Figures S5A–S5D. For comparisons between cell-to-cell and cell-free viral infection, please refer to Figures S5E and S5F.

a comparable gp41/g160 ratio for CTdel-144 in cells expressing or not expressing IFITM3 (Figure S4A) as well as comparable gp120 levels in virions produced by the NL4-3/CTdel-144 mutant (Figure S4B). These results demonstrate that IFITM3 has a smaller effect on the CTdel-114 mutant as compared to the HIV-1_{NL43} WT. Altogether, these data suggest that the CT of HIV-1 Env is critical for Env association with IFITM3 and that the A539V Env escape mutant is more fit than WT and is thus able to non-specifically overcome IFITM restriction.

IFITMs Inhibit Cell-to-Cell Infection/Fusion of HIV-2 and SIVs in a Species-Specific Manner

We further determined the ability of IFITMs to inhibit cell-to-cell infection by other strains of HIV-1, including some primary isolates, as well as by some commonly used HIV-2 and SIV isolates. Similar to HIV-1_{NL43}, the transmission of HIV-1_{BH10} and AD8 from Jurkat/inGLuc to parental Jurkat cells or PBMCs was strongly inhibited by IFITMs, especially IFITM2 and IFITM3 (Figures 7A and S5A). Interestingly, the transmission of primary isolate YU2 from Jurkat/inGLuc cells to primary PBMCs was solely inhibited by IFITM2 and IFITM3, but not at all by IFITM1 (Figures 7A and S5A). Surprisingly, and distinct from NL43, the cell-free infection of BH10, YU2 and AD8 was almost equivalently inhibited by three IFITMs (Figures 7B and S5B). In all cases, cell-to-cell infection was more efficient than cell-free infection, although the fold differences were virus-strain dependent (Figure S5E).

Because, like HIV-1, HIV-2 and SIVs use either CCR5 or CXCR4 coreceptors for infection in addition to CD4, for simplicity, we decided to use HeLa-TZM, which are permissive to both HIV-2 and SIVs, as target cells. We thus transfected 293T cells with molecular clones of HIV-2_{Rod-1}, SIVagm, SIVmac, or SIVcpz, and cocultured the transfected 293T cells with HeLa-TZM; the cell-to-cell infection/fusion and cell-free viral infection of these viruses were measured in parallel as described above. The cell-to-cell infection/fusion mediated by SIVcpz and SIVagm were greatly inhibited by IFITMs, especially IFITM2 and 3; however, the cell-to-cell infection/fusion of SIVmac was unaffected, despite similar infection efficiencies in mock cells transfected with an empty pQCXIP vector (Figures 7C and S5C). Notably, the cell-free infection of SIVagm was profoundly inhibited by all three IFITMs (>20-fold) compared to SIVcpz, SIVmac, and HIV-2, which were modestly inhibited by the three human IFITMs (Figures 7D and S5D). Similar to HIV-1, the cell-to-cell infection/fusion of HIV-2 and SIVs are more efficient than cell-free infection (Figure S5F). However, we cannot rule out the possibility that the differences between cell-to-cell and cell-free infection for SIVcpz and HIV-2 were due to their extremely low cell-free infection in HeLa-TZM cells. Altogether, these results demonstrated that while cell-to-cell infection/fusion of CCR5-using HIV-1 YU2 and AD8, as well as HIV-2 and SIVs, are inhibited by human IFITMs, the extents of inhibition differ depending on the specific IFITMs and the viruses tested.

DISCUSSION

Cell-to-cell transfer by HIV-1 is a more efficient means of infection than cell-free infection and is likely the dominant route of HIV-1 infection in vivo (Dale et al., 2013; Zhong et al., 2013). Here, we provide evidence that IFITM proteins inhibit HIV-1 cell-to-cell infection by antagonizing Env, thus clarifying the mechanism by which IFITMs restrict HIV and possibly other viruses. During the preparation of our manuscript, a paper by

Compton et al. was published showing that IFITMs are incorporated into HIV-1 virions thus impairing the viral spread through cell contact; however, the exact underlying mechanism was not determined (Compton et al., 2014). While the incorporation of IFITMs into HIV-1 virions was confirmed in our work, we did not observe a strict correlation between levels of IFITM incorporation and inhibition of cell-to-cell infection. For example, despite its high levels in HIV-1 particles, IFITM1 in general displayed the lowest inhibitory activity among the three human IFITMs. Significantly, we discovered that IFITM proteins interact with HIV-1 Env in viral producer cells, impair Env processing, and promote SU shedding that altogether reduce virion fusion activity. While we find that IFITMs also inhibit the cell-to-cell infection/fusion of HIV-2 and SIVs, the extents of inhibition are both IFITM- and virus-species dependent. We were able to confirm and extend our previous finding (Ding et al., 2014) that HIV-1 can adapt to overcome the IFITM restriction by mutating its *env* gene during prolonged replication. Hence, results from our study offer insights into understanding the mechanisms of IFITM restriction of viral infection as well as HIV-host co-evolution.

How can the expression of IFITMs in donor cells, rather than in target cells, inhibit HIV-1 cell-to-cell infection? One obvious possibility is that IFITM proteins somehow modulate HIV-1 gene expression and/or their functions in viral producer cells, resulting in reduced viral output and/or defective virions. Alternatively, IFITMs may modulate the expression of HIV-1 receptor, coreceptors, and/or even cellular adhesion molecules, such as ICAM-1 or LFA-1, all of which are known to be important for formation of the virological synapse (VS) and therefore cell-to-cell infection. However, our flow cytometric analyses did not show significant changes in CD4, CXCR4, CD63, CD81, ICAM-1, or LFA-1 on the cell surface upon IFITM overexpression (Figure S6). Additionally, at the early stage of cell-to-cell infection (e.g., 4 hr), HIV-1 Gag-iGFP or iCherry can be transferred from donor to target cells with similar efficiency between mock and IFITM-expressing cells (Figures S7A and S7B), further arguing against an IFITM-mediated block in VS formation. By using an Amnis flow cytometric imaging system (Swartz et al., 2014), we also observed comparable stable associations between donor Jurkat expressing or not expressing IFITMs with target PBMCs (Figure S7C), again suggesting that the formation of the VS is not affected by IFITMs.

We provide evidence that IFITM proteins, especially IFITM2 and IFITM3, specifically interact with HIV-1 Env, resulting in impaired Env processing and incorporation into HIV-1 virions, which correlates with their inhibition of HIV-1 cell-to-cell infection. This mechanism of action is apparently distinct from that observed in the context of IAV, JSRV, and possibly other viruses in cell-free viral infections (Perreira et al., 2013). Previously, we and others have shown that IFITM proteins inhibit cell-cell fusion induced by a variety of viral fusion proteins on the plasma membrane and that IFITMs can also block the virus-cell fusion in endosomes (Desai et al., 2014; Li et al., 2013). We demonstrated that expression of IFITMs in effector cells is as potent as their expression in target cells in inhibiting Jaagsiekte sheep retrovirus (JSRV) Env-mediated cell-cell fusion (Li et al., 2013). In the current study, we extended this finding by showing that cell-cell fusion induced by IAV HA is also equivalently inhibited by IFITM expression in effector and target cells. These results are in striking contrast to what we have found for HIV-1, where IFITMs must be present in effector or donor cells in order to efficiently inhibit HIV-1 Env-mediated cell-cell fusion and cell-to-cell infection. In addition to the impaired processing of HIV-1 Env, we also find that IFITM3 promotes HIV-1 SU shedding into culture media. This observation, together with our data showing that IFITM proteins specifically interact with HIV-1 Env gp41, suggests that IFITM proteins, especially IFITM2 and 3, may alter the conformation of HIV-1 Env and likely result in premature triggering and impaired infectivity. The increased rate of gp160 turnover observed here also suggests that IFITM-bound gp160 may be targeted for degradation. Work is ongoing to determine the detailed interactions between IFITMs and HIV-1 Env as well as possible conformational changes in Env.

Similar to the escape of HIV-1_{BH10} from IFITM1 inhibition that we recently reported (Ding et al., 2014), we demonstrated in this work that HIV-1_{NL43} also mutates Vpu and Env to overcome the inhibition not only by IFITM1Δ(117–125) mutant, from which these mutants were selected, but also by the parental IFITM1, 2, and 3. Interestingly, both HIV-1_{BH10} and HIV-1_{NL43} acquired mutations in Vpu that caused the production of a truncated Vpu of 33–39 amino acids. However, the escape mutations in Env differ considerably between these two viruses. HIV-1_{BH10} has the G367E mutation that attenuates the binding of Env to receptor CD4 and therefore profoundly diminishes the infectivity of HIV-1_{BH10}. In the case of HIV-1_{NL43}, the A539 residue within heptad repeat 1 (HR1) of gp41 was changed to a valine. However, preliminary data show that the A539V Env mutant still interacts with IFITM proteins (Figure S4A), indicating that additional mechanisms are involved. While the escape mutants we discovered may not be specific for IFITMs, these data support an active role of Env in overcoming host restrictions of HIV-1 infection.

It is interesting that, despite strong sequence similarity between IFITM1 and IFITM2/3, IFITM1 shows a relatively weak ability to inhibit HIV-1 cell-to-cell infection as compared to IFITM2 and 3, especially from 293T to Jurkat cells. By examining the chimeras between IFITM1 and IFITM2, we discovered that the C terminus of IFITM1 inherently suppresses the inhibition of HIV-1 cell-to-cell infection while the N terminus does not play a major role. Further deletion mutants revealed that the C-terminal nine amino acids of IFITM1 are sufficient to confer this effect. While it remains to be determined how exactly these nine residues determine the differential effects of IFITMs on HIV-1 cell-to-cell infection, it is possible that deletion of the last nine amino acids may change the conformation or the topology of IFITM1, rendering it capable of interacting with HIV-1 Env as does IFITM3. It is also possible that the predominant localization of IFITM1 is different from that of IFITM2 and IFITM3 in the cell, and this may be determined, in part, by the C terminus of IFITM1, which could interact with other cellular factors involved in HIV-1 cell-to-cell transmission (Puryear et al., 2013; Roy et al., 2014; Wen et al., 2014). Future experiments will address these possibilities.

EXPERIMENTAL PROCEDURES

Plasmids, Cells, and Reagents

Details are described in the Supplemental Experimental Procedures.

Transfection, Virus Production, and Infection

Cells were transfected for viral production and infection as described in the Supplemental Experimental Procedures.

Cell-to-Cell Infection

Cell-to-cell infection was performed in several setups, including (A) HEK293T-to-Jurkat cells, (B) T cell-to-T cells, (C) PBMCs-to-T cells, and (D) T cell to PBMCs. For Jurkat cell to other T cell transmission, we use an HIV-1_{NL43}-intron *Gaussia*-based system, which was originally described by Derse and colleagues (Mazurov et al., 2010). This assay takes advantage that an intron, which was inserted into an HIV-1 vector in an opposite orientation to the Luc open reading frame, can be removed upon lentiviral vector transduction of target cells; the Gluc gene is not expressed in transfected or infected donor cells. Briefly, we infected Jurkat/inGLuc cells expressing or not expressing IFITMs, or PM1 cells stably expressing shRNA IFITMs or scramble RNA, with VSV-G pseudotyped HIV-1_{NL43}. The VSV-G pseudotyped HIV-1 was used because it can efficiently infect donor cells (almost 100%) and therefore enhance our capability of detecting cell-to-cell infection. Target cells include Jurkat, HeLa-TZM, and PBMCs; 24–48 hr after coculture or infection, GLuc activity was measured for cell-to-cell infection and cell-free HIV-1 infection. In all cases, no effect of IFITM expression on cell viability or proliferation capacity of the donor cells was found. Detailed information is provided in the Supplemental Experimental Procedures.

Live-Cell Imaging

The procedures are outlined in the Supplemental Experimental Procedures.

Immunoprecipitation and Western Blotting

Western blotting and IP was performed as previously described (Côté et al., 2012; Liu et al., 2003). In particular, we used a RIPA buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% SDS), which disrupts membrane-associated proteins, to lyse cells. The detailed steps are outlined in the Supplemental Experimental Procedures.

Pulse-Chase Labeling, Cell-to-Cell Virion Fusion, and Cell-Cell Fusion

Details are provided in the Supplemental Experimental Procedures.

Cell Surface or Intracellular Staining by Flow Cytometry

Cells were washed and incubated with different primary antibodies for 1 hr. After incubation with secondary antibodies, cells were analyzed by flow cytometry (see details in the Supplemental Experimental Procedures).

Viral Replication and Selection of HIV-1 Escape Mutants

The experiments were carried out described in the Supplemental Experimental Procedures.

Statistical Analysis

All statistical analyses were carried out in GraphPad Prism5, with Student's t tests or one-way ANOVA used unless otherwise noted. Typically, data from three to five independent experiments were used for the analysis.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures and seven figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.055.

AUTHOR CONTRIBUTIONS

J.Y. and M.L. contributed equally to this manuscript. J.Y. performed most experiments. M.L carried out key initial experiments leading to critical conclusions. J.W. performed live-cell imaging and quantified cell-to-cell infection. S.D. carried out selection of HV-1 escape mutants. T.H.S. and A.M.E. performed cell-to-cell infection using Amnis flow cytometry. Y.-M.Z. made essential IFITM constructs. J.Y., M.L., E.O.F., C.L., B.K.C., and S.-L.L. analyzed and interpreted data, assembled the figures, and wrote the manuscript.

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