Natural mutations in IFITM3 modulate post-translational regulation and toggle antiviral specificity

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Abstract

The interferon-induced transmembrane (IFITM) proteins protect host cells from diverse virus infections. IFITM proteins also incorporate into HIV-1 virions and inhibit virus fusion and cell-to-cell spread, with IFITM3 showing the greatest potency. Here, we report that amino-terminal mutants of IFITM3 preventing ubiquitination and endocytosis are more abundantly incorporated into virions and exhibit enhanced inhibition of HIV-1 fusion. An analysis of primate genomes revealed that IFITM3 is the most ancient antiviral family member of the IFITM locus and has undergone a repeated duplication in independent host lineages. Some IFITM3 genes in nonhuman primates, including those that arose following gene duplication, carry amino-terminal mutations that modify protein localization and function. This suggests that “runaway” IFITM3 variants could be selected for altered antiviral activity. Furthermore, we show that adaptations in IFITM3 result in a trade-off in antiviral specificity, as variants exhibiting enhanced activity against HIV-1 poorly restrict influenza A virus. Overall, we provide the first experimental evidence that diversification of IFITM3 genes may boost the antiviral coverage of host cells and provide selective functional advantages.

Keywords evolution; HIV; IFITM; innate immunity; virus

Subject Categories Microbiology, Virology & Host Pathogen Interaction; Immunology; Evolution

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Introduction

Viruses depend on numerous cellular factors and pathways to complete the viral life cycle and spread to new target cells. Known as virus cofactors, these are host molecules that are commandeered by the virus in order to promote efficient infection [1]. To prevent virus takeover, cells express a battery of antiviral effectors that repress viral replication at multiple stages. These proteins make up the cell-intrinsic innate immune response and are also known as host restriction factors. The establishment of a hostile intracellular environment sets the stage for ongoing host–virus coevolution, a phenomenon that is particularly well characterized between primates and lentiviruses [2]. As their name implies, host restriction factors influence the host tropism of virus infections in vitro and in vivo because they are rapidly evolving and thus divergent between species.

The immune-related IFITM proteins (IFITM1, IFITM2, and IFITM3 in humans) may represent the earliest acting restriction factors yet identified. The IFITM family also includes the IFITM5 and IFITM10 genes with no known immune function [3]. IFITM genes are present among a wide range of vertebrate animal species, and they may have originated in early unicellular eukaryotes via horizontal gene transmission from a bacterium [4]. Since then, expansions within genomes have given rise to unique IFITM gene repertoires that vary at the level of sequence and copy number. To date, all immune-related IFITM proteins identified in animals (metazoans) display antiviral function [5–7]. Even IFITM-like genes in Mycobacteria inhibit virus infection when expressed in human cells [8].

As residents of membranes at the interior and exterior of the cell, they block the entry step of diverse viruses [9,10] by inhibiting virus–cell fusion. The mechanisms behind this protection involve altering the biophysical properties [11–13] or cholesterol content [14] of the cellular membranes in which they are found. These proteins inhibit many enveloped viruses, including influenza A virus (IAV), West Nile virus, dengue virus, severe acute respiratory syndrome coronavirus, hepatitis C virus, and Ebola virus [15], as well as lentiviruses including HIV-1 and SIV [16–18]. While the majority of studies have relied on in vitro infection systems, it is well established that IFITM3 restricts virus infection in vivo.
IFITM3 is detectable at the surface of transfected 293T cells and in lysosomal membranes [24], we observed that a portion of IFITM proteins are restriction factors of increasingly broad scope. Furthermore, very little is known about natural, genome-encoded antiviral gene family that improves the function against one virus can inhibit its activity against other viruses. As a testament to their functional importance, periodic diversification of residues within the two regulatory motifs of IFITM3 is observed during primate evolution, resulting in variants with different potency and specificity. Finally, our multifaceted analysis has allowed us to retrace the origins of IFITM in primates and provides an explanation for the recurrent gene duplication observed in this antiviral gene family.

Results

Mutation in IFITM3 alters subcellular localization and anti-HIV activity

IFITM3 contains two hydrophobic domains conserved in other IFITM family members, but is divergent in its amino- and carboxy-termini. The amino-terminal sequence motifs recognized by NEDD4 and the AP-2 complex are adjacent and overlap at the tyrosine at residue 20, found just upstream of an internal methionine (which serves as the translation start site in IFITM1 and the Δ1–21 construct of IFITM3) [25] (Fig 1A).

293T cells were selected as a suitable system to study the impact of post-translational modifications on IFITM3 function because they express very low levels of constitutive IFITM3. Transfection of FLAG-tagged IFITM3 into 293T cells results in expression levels comparable to, but less uniform than, those induced by type-I interferon (Fig EV1A) and localization to disperse intracellular vesicles and the plasma membrane (Fig 1B). As previously described [16], the subcellular localization of the Δ1–21 variant is mostly restricted to the plasma membrane (Fig 1B). A mutant deficient in cysteine residues (referred to as Dpalm because it prevents palmitoylation of IFITM3 [27]) results in a predominantly perinuclear localization with additional diffuse staining throughout the cytoplasm. Western blot and flow cytometric analysis reveal quantitative differences in protein expression. Levels of the Δ1–21 variant are elevated relative to full-length protein, while Dpalm is expressed at lower levels (Fig 1C and D).

To address how mutation in IFITM3 affects its activity against HIV-1, virus was produced in 293T cells via co-transfection with the HIV-1 molecular clone pNL4-3, pVpr-Blam (which allows the detection of virion access into the cytoplasm), and plasmids expressing IFITM3 mutants (as performed in [21]). Consistent with its enhanced localization to the cell surface, which also serves as the site for virus assembly and budding, the Δ1–21 variant exhibits increased levels of virion incorporation relative to full-length IFITM3 (Fig 1E). Importantly, viruses produced in the presence of the Δ1–21 variant are further reduced in virion infectivity when incubated with fresh SupT1 T-cell targets, as evidenced by reduced virus–cell fusion at 4 h (Fig 1F) and reduced productive viral infection at 48 h (Fig 1G). Virus produced in the presence of MxA was used as an additional negative control. Confocal immunofluorescence microscopy confirms that surface-associated IFITM3 can be localized to sites of HIV-1 budding tagged by the presence of mature proteins.
Figure 1.

A. IFITM3 and IFITM3 Δ1-21 protein domains.

B. Immunofluorescence images of DAPI (blue), FLAG (green), Empty, IFITM3, Δ1-21, Δpalm.

C. Western blot analysis of Flag (IFITM3) and actin.

D. Western blot analysis of Flag (IFITM3) and p24 (CA) in virus lysates.

E. Western blot analysis of Flag (IFITM3) and actin.

F. Virus fusion assay showing IFITM3 and Δ1-21 inhibition.

G. Virus infectivity assay showing IFITM3 and Δ1-21 inhibition.

H. Immunofluorescence images of DAPI (blue), FLAG (green), Empty, IFITM3, Δ17-18, Δ17-20.

I. Western blot analysis of Flag (IFITM3) and actin.

J. Western blot analysis of Flag (IFITM3) and p24 (CA) in virus lysates.

K. Virus fusion assay showing IFITM3 and Δ17-18, Δ17-20 inhibition.

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Gag protein (Fig EV1B). Results with an IFITM3 mutant encoding a single residue deletion at position 20 (AY20) reproduced those of Δ1–21, strongly suggesting that the tyrosine residue and the two overlapping regulatory motifs to which it belongs are crucial to the enhancement of anti-HIV activity observed (Fig EV1C–E). Thus, the extent of inhibition of HIV-1 infectivity is associated with cell surface localization and the increased detection of IFITM3 in virions. We previously demonstrated that IFITM1, which naturally lacks the amino-terminus present in IFITM3, only modestly restricts HIV-1 infectivity [21]. Thus, the absence of an amino-terminus per se is not sufficient to enhance anti-HIV-1 activity. Rather, IFITM3 contains other intrinsic determinants that enable a potent HIV-1 restriction that can be amplified by the removal of its amino-terminus.

To assess the involvement of distinct regulatory activities in the regulation of IFITM3 activity, we took advantage of additional IFITM3 mutants in which the NEDD4-binding site alone (Δ17–18) or both the NEDD4- and the AP-2-binding sites (Δ17–20) are disrupted. Immunofluorescence microscopy confirmed previously published results [16] showing that Δ17–20 is enriched at the cell surface (Fig 1H). Similar to Δ1–21, the Δ17–18 mutant exhibits slightly elevated protein levels relative to full-length IFITM3. Importantly, the Δ17–20 variant does not share this feature, and it is even expressed at lower levels relative to full-length IFITM3 (Figs 1I and EV1F). Both the Δ17–18 and Δ17–20 variants display an increased propensity for virion incorporation, with the mutant in which endocytosis is disrupted (Δ17–20) showing the greatest phenotype (Fig 1J). Consistently, the Δ17–20 variant demonstrates the most profound inhibition of HIV-1 particle fusogenicity (Fig 1K) and infectivity (Fig EV1G). Therefore, the enhancement of IFITM3 antiviral activity is associated with its subcellular localization and is not solely dependent on protein levels. When we employed a L23Q mutant to inhibit protein endocytosis [26] without disturbing the NEDD4-binding motif, we found that this single amino acid change also results in more pronounced restriction of HIV-1 infectivity relative to wild-type IFITM3 (Fig EV1H and I). These results collectively suggest that motifs interacting with endogenous NEDD4 and AP-2 act in an additive fashion to negatively regulate the anti-HIV activity of IFITM3.

The E3 ubiquitin ligase NEDD4 regulates the activity of IFITM3 against HIV-1

We directly tested the regulatory effect of NEDD4 in virus-producing cells using a system in which NEDD4 is co-expressed by transfection with IFITM3 or the Δ1–21 variant in 293T cells. The co-expression of NEDD4 resulted in lower levels of IFITM3 in cell lysates, an effect that was not observed for the catalytically inactive NEDD4 mutant C867A (Fig EV2A). These results confirm that NEDD4-mediated ubiquitination accelerates the turnover of IFITM3 [31]. Moreover, NEDD4 co-expression diminished the levels of IFITM3 incorporated into HIV-1 virions (Fig EV2A). In contrast to its typical expression pattern in the cytoplasm and on the cell surface, confocal immunofluorescence microscopy revealed that IFITM3 becomes mostly intracellular in cells co-expressing NEDD4, but not in those with inactive NEDD4 C867A (Fig EV2B and C). NEDD4 induced the accumulation of IFITM3 in large vesicular compartments that partially colocalize with LAMP1 (Fig EV2D). In contrast, protein levels of the Δ1–21 variant in cell lysates or in virions were unaffected by NEDD4, and the localization remained predominantly cell surface oriented (Fig EV3D). Collectively, these findings suggest that NEDD4 regulates IFITM3 levels in cells producing HIV-1 and thus may regulate its antiviral effect. They also highlight how variants of IFITM3 missing key elements of the amino-terminus may confer escape from NEDD4 and lead to enhanced antiviral function in the cell.

The amino-terminus of IFITM3 regulates activity against HIV-1 in T cells

To study the regulation of IFITM3 protein in the natural target cells of HIV-1, we took advantage of T-lymphocyte cell lines (SupT1)
engineered to allow for Dox-inducible expression of IFITM3 protein variants [17,21]. Following Dox treatment of these cells, expression levels are uniform and full-length IFITM3 localizes mostly to the cell interior, while the Δ1–21 variant is predominantly found at the surface (Fig 2A), mirroring the results in 293T cells. Importantly, we observed the induction of higher levels of full-length IFITM3...
compared to the Δ1–21 variant in its respective cell line (Fig 2B). Using this T cell-based system, we tested the relative antiviral potential of IFITM3 in both donor and target cells. We produced virus in the presence or absence of IFITM3 variants and subsequently infected target T cells in which IFITM3 variants were induced or not. As previously reported [21], IFITM3 restricts HIV-1 infection within donor and target cells in an additive fashion (Fig 2C). However, the Δ1–21 variant exhibits superior antiviral activity at both stages, such that infection is almost completely blocked when it is present in both donor and target cells (Fig 2C). These data demonstrate that removal of the amino-terminus enhances both antiviral functions fulfilled by IFITM3 during HIV-1 infection. Despite the relatively lower level of protein induction in this system, and in agreement with its localization at the cell surface, the Δ1–21 variant is enriched in virions (Fig 2D). Importantly, we did not detect IFITM3 in supernatants from noninfected cells, strongly suggesting that the majority of signal detected originates from within virus particles [21] (Fig 2D). Nonetheless, because IFITM proteins can also be found in exocytotic vesicles [36], we performed density gradient purification of virions produced in the presence of full-length IFITM3 and the Δ1–21 variant. As previously reported [21], full-length IFITM3 co-sediments with HIV-1 Gag protein and is nearly absent from fractions containing lower-density microvesicles, and the pattern was similar for Δ1–21 (Fig EV3). A recent publication reported that IFITM3 antagonizes HIV-1 envelope protein, altering both the maturation of gp120 and inhibiting its incorporation into virions produced in 293T cells [23]. However, and consistent with our previous publication [21], the induction of IFITM3 or the Δ1–21 variant did not affect gp120 levels in virions produced in T-cell lines (Fig 2D). To explore further the effect of IFITM3 on HIV-1 Env in virus-producing cells, we co-infected 293T cells with HIV-1 pNL4-3 and titrating amounts of plasmid encoding IFITM3, the Δ1–21 variant, or MxA. We observed that gp120 levels were slightly reduced only at the highest level of IFITM3 overexpression (Fig 2E). The Δ1–21 variant reduced gp120 levels somewhat further in a manner consistent with its elevated expression, while MxA overexpression had no effect. Interestingly, we also found that reduction in gp120 was accompanied by a concomitant decrease in HIV-1 p24 levels, possibly suggestive of a global effect on virus translation (Fig 2E). Therefore, while IFITM3-mediated restriction of HIV-1 infectivity may involve Env antagonism, this effect is cell type dependent and expression level dependent. We propose that the mechanistic basis for IFITM3 antiviral function against HIV-1 lies primarily with its cell surface localization, which is associated with its incorporation into virions budding from the same compartment.

**Recurrent duplication and divergence of primate IFITM3 over evolutionary time**

Given our finding that synthetic mutants in the amino-terminus of IFITM3 exhibit enhanced anti-HIV-1 activity, we chose to examine the functional consequences of standing variation in *IFITM3* genes from nonhuman primates. Dozens of species harbor simian immunodeficiency virus (SIV) strains in the wild, infections that have likely persisted for millions of years in coevolution with their hosts [2]. Experimental approaches combining *in silico* genomic analysis and *in vitro* or *in vivo* infection assays have revealed that primate immunity genes, such as APOBEC3G, TRIM5, tetherin, and SAMHD1, are undergoing adaptive evolution in ways that impact lentiviral infections [37]. Our results prompted us to ask whether the antiviral function and specificity of IFITM3 may also be dynamic and subject to natural selection on evolutionary timescales. Some previous reports focusing on the evolution of the *IFITM* gene family in vertebrates have shown that, compared to the five *IFITM* genes present on human chromosome 11, the number of *IFITM* genes per species may vary as a result of gene duplication events [4,38]. To build a comprehensive understanding of *IFITM* evolution in primates, we used comparative genomics to identify *IFITM* genes from full reference genomes. Aided by the Genomicus toolkit [39] and the Ensembl gene database (www.ensembl.org), we characterized the *IFITM* locus in simian and prosimian primate species. Simians consist of haplorhine primates, including hominoids (humans, chimpanzee, gorilla, orangutan, and gibbon), Old World Monkeys [rhesus macaque, olive baboon, and vervet monkey (African Green Monkey)], and New World Monkeys (common marmoset), while prosimians consist of strepsirrhine primates (mouse lemur and bushbaby) as well as a Tarsiiforme relict species (tarsier). This analysis yielded several novel insights into *IFITM* evolution. First, the “canonical” *IFITM* gene locus found in humans is atypical with regard to gene number and gene composition. For example, the *IFITM* locus of the prosimian bushbaby species is syntenic with that of humans, but it does not contain *IFITM1* or *IFITM2*. Instead, it contains two adjacent copies of *IFITM3* resulting from gene duplication (a third copy of *IFITM3*, also the product of gene duplication, is located on a separate chromosome) (Fig 3A). In fact, while the last common primate ancestor (LCPA) is predicted to contain only three *IFITM* genes, primate evolution is punctuated by *IFITM* gene gain and gene loss events (Fig 3B). Many of the modern primate genomes analyzed show evidence of *IFITM3* gene duplication, while the tarsier contains only a single *IFITM* gene, and it is *IFITM3* (Fig 3B). The number of *IFITM3* genes listed for each species is a minimal estimate, as we only considered *IFITM3* sequences predicted to be protein coding by Ensembl (sequences containing an intact open reading frame and lacking premature stop codons). These data suggest that *IFITM3* is the oldest of the *IFITM* family members with known antiviral activities. Furthermore, the genomes of hominoids (apes) revealed that *IFITM2* is a relatively recent genetic innovation specific to humans, chimpanzee, and gorilla (Fig 3B). Overall, the evolutionary trajectory of *IFITM* in primates suggests a recurrent need for functional diversification.

We used BLAST analysis to confirm the identity of sequences in Ensembl and also to supplement our *IFITM3* sequence list with additional species (such as an additional strepsirrhine species (the sifaka) and an Old World Monkey of the *Colobinae* subfamily, the colobus monkey). From these combined analyses, we compiled an alignment of *IFITM3* coding sequences and performed phylogenetic reconstructions (Fig 4A and B, and Appendix Fig S1). Notably, our analysis uncovered recurrent mutation in the amino-terminus of primate *IFITM3*, including many examples found in the motifs controlling ubiquitination and endocytosis (selected examples shown in Fig 4A). *IFITM3* sequences from prosimians demonstrate that these motifs were likely intact in the common ancestor of all primate species (Fig 4A). However, each one of the strepsirrhine species contains two *IFITM3* genes in their respective *IFITM* locus.
and one copy of each pair contains mutations that disrupt motifs for ubiquitination and/or endocytosis. This finding suggests that a tandem duo of IFITM3 genes with potentially divergent functional properties has evolved independently on multiple occasions.

Meanwhile, similar observations were made in simian species. It was previously recognized that the marmoset monkey genome contains dozens of IFITM3 sequences in its genome [38]. Interestingly, the array of 25 IFITM3 variants present in this species carry

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**Figure 3. Retracing the origins of IFITM in primates.**

A. A comparison of the IFITM locus on chromosome 11 of the human reference genome (GRCh38.p5) and the corresponding locus in the bushbaby reference genome (OtoGar3) visualized using Genomicus v84.01. Synteny is observed between human IFITM and bushbaby IFITM, as evidenced by the conserved flanking genes B4GALT4 and ATHL1. The bushbaby locus lacks IFITM2 and IFITM1 genes. In addition, it contains two adjacent IFITM3 genes, as well as a third IFITM3 gene located in a different genomic context, indicative of gene duplication events.

B. A gene gain/loss tree summarizing the phylogenetic history of the IFITM gene family in primates, using annotated reference genomes in Ensembl. The number at a branch tip refers to the number of IFITM genes present in a given extant species, while the number at each node refers to the number present in an ancestral species. Differences in gene number at tips and nodes represent significant gene gain events (expansions) or gene loss events (contractions) based on a P-value calculation of < 0.01 determined by CAFE (Computational Analysis of gene Family Evolution) [55]. The makeup of the IFITM repertoire for each species, as determined by comparative genomics and the IFITM gene family tree in Ensembl, is detailed to the right. When multiple copies of IFITM genes were identified, the total number is listed inside the box. The gene numbers estimated for the mouse lemur and bushbaby in Ensembl were adjusted following phylogenetic analysis of IFITM homologs in Genomicus as well as BLAST searches of the NCBI GenBank database. Blue and red arrows indicate the emergence of IFITM1 and IFITM2 genes, respectively.
### A

| Species          | IFITM3 | Loc. |
|------------------|--------|------|
| Human            | PPNYEMLK | 11*  |
| Human Δ1-21      | --     | n/a  |
| Chimp            | PPNYEMLK | 11*  |
| Gorilla          | PPNYEMLK | 11*  |
| Gorilla          | PLNYEMLK | 2a   |
| Gorilla          | PPNYEMLK | 11*  |
| Orangutan        | LPNYEMLK | 8    |
| Orangutan        | PPNYEMLK | 11*  |
| Rhesus           | PPNYEMLK | 14*  |
| Rhesus           | PPSYEMLK | 14*  |
| Vervet-AGM       | PPNYEMLK | 19   |
| Vervet-AGM       | --     | 14   |
| Colobus          | PPNYE-   | unk  |
| Marmoset         | PPNYEMLK | many |
| Marmoset         | PLNYEMLK | 4,8  |
| Marmoset         | PLNYEMPK | 1    |
| Marmoset         | APSYEMPK | 5    |
| Marmoset         | PP-YEMLK | 5    |
| Marmoset         | PH-----K | 11   |
| Marmoset         | --     | 22   |
| Tarsier          | PPKYDMLK | unk  |
| Mouse Lemur      | PPNYEVLK | unk* |
| Mouse Lemur      | HSNSEMLK | unk* |
| Sifaka           | PPNYEVLK | unk* |
| Sifaka           | PNSYEGLK | unk* |
| Bushbaby         | PPNEYTLK | unk* |
| Bushbaby         | PPNEYTRK | unk* |

A partial protein alignment of select IFITM3 genes from various primate species for which a reference genome is available and accessible in Genomicus. Sequences from the mouse lemur and colobus monkey were identified by BLAST analysis of reference genomes in NCBI GenBank. Gene location is indicated, when available, by chromosome number, and an asterisk indicates that the given gene sequence falls into a canonical IFITM locus with flanking B4GALT4 and A4GALT genes. The suborder and parvorder (from left to right) indicate taxonomical relationships between species. Residues bolded in red highlight nonsynonymous substitutions that disrupt canonical motifs for the recruitment of NEDD4 and AP-2. A complete protein alignment of all IFITM sequences analyzed in this study is available in Appendix.

### B

A phylogenetic gene tree (cladogram) indicates the relationship among IFITM3 sequences analyzed in this study. The final consensus tree was made by merging results from neighbor-joining and maximum-likelihood phylogenetic reconstructions using both nucleotide and amino acid sequences in Ensembl (see Fig EV4 for robustness measurement by Bayesian inference). Branch lengths are normalized and arbitrary. The size of triangles at the tree tips roughly corresponds to the number of sequences contained therein. Note that the IFITM2 gene is the most recent member of the IFITM locus, appearing only in humans, chimpanzee, and gorilla genomes.

### C

A graphical representation of naturally occurring truncated forms of IFITM3 identified in the vervet and marmoset reference genomes, as compared to the putative Δ1–21 variant in humans.

**Figure 4. Recurrent duplication and divergence of primate IFITM3 over evolutionary time.**

A. A partial protein alignment of select IFITM3 genes from various primate species for which a reference genome is available and accessible in Genomicus. Sequences from the mouse lemur and colobus monkey were identified by BLAST analysis of reference genomes in NCBI GenBank. Gene location is indicated, when available, by chromosome number, and an asterisk indicates that the given gene sequence falls into a canonical IFITM locus with flanking B4GALT4 and A4GALT genes. The suborder and parvorder (from left to right) indicate taxonomical relationships between species. Residues bolded in red highlight nonsynonymous substitutions that disrupt canonical motifs for the recruitment of NEDD4 and AP-2. A complete protein alignment of all IFITM sequences analyzed in this study is available in Appendix.

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C. A graphical representation of naturally occurring truncated forms of IFITM3 identified in the vervet and marmoset reference genomes, as compared to the putative Δ1–21 variant in humans.
many iterations of mutations or deletions that disrupt the motifs necessary for interaction with NEDD4 and/or AP-2 (Fig 4A and Appendix Fig S1). These cases of IFITM3 duplication and divergence were not isolated to far-removed primate species, since we also found similar patterns in the catarrhine parvorder to which humans belong. We identified a single IFITM3 sequence in the colobus monkey that contains a deletion (Δ22–24) eliminating the motif necessary for AP-2-mediated endocytosis as well as a neighboring lysine residue (K24) targeted for ubiquitination [27] (Fig 4A). Furthermore, codons 17 and 18 of IFITM3, corresponding to the critical proline residues necessary for its interaction with and ubiquitination by NEDD4 [31], have diversified in IFITM3 genes in the vervet, gorilla, and orangutan. This latter species contains an IFITM3 that is syntenic with human IFITM3 on chromosome 11 and contains a P17L mutation. Phylogenetic analysis of primate IFITM3 showed branching patterns that reproduce major taxonomic groups, with a few exceptions (Figs 4B and EV4A). While IFITM3 from prosimians clearly represents the basal clade, some IFITM3 sequences found in gorilla, vervet, and orangutan genomes form two outlier branches (in gray). These sequences appear to predate other IFITM3 sequences found in the same species, suggesting that they most likely represent duplication events that took place in simian ancestors. Importantly, the maintenance of these sequences in multiple species until present day may indicate that they retain functional importance (Figs 4B and EV4A).

Since we found that many naturally occurring mutations identified in primate IFITM3 effectively mimic the synthetic mutants tested in this study, we predicted that they might similarly confer escape from the cellular processes of ubiquitination and/or endocytosis. A further observation that is especially pertinent to the data shown in this study is that certain IFITM3 sequences identified in the vervet and marmoset genome closely resemble the human Δ1–21 construct, with each lacking the amino-terminus and initiating translation at an internal methionine (Fig 4C). In addition, we observed a few IFITM3 sequences in which the initial methionine is absent or mutated (Appendix Fig S1), which could also give rise to truncated IFITM3 proteins similar to the Δ1–21 construct.

Naturally occurring mutations in IFITM3 disrupt regulatory motifs and toggle antiviral activity

We next characterized the functional consequences of select nonhuman IFITM3 mutations by introducing them into the human IFITM3 background. Transfection into 293T cells and confocal immunofluorescence microscopy showed that Δ22–24 resulted in a shift to the cell surface (Fig 5A). Predominant plasma membrane staining was also observed for marmoset-specific IFITM3 mutations, reiterating an effect on protein endocytosis through ablation of the AP-2-recruiting motif (Fig 5A). As expected, primate mutations in the NEDD4-binding motif (P17L found in orangutan and P18H found in vervet) did not grossly affect the subcellular localization of IFITM3. To assess the extent to which each variant could be ubiquitinated in cells, we immunoprecipitated IFITM3 from cells co-expressing NEDD4 or catalytically inactive NEDD4 C867A. In agreement with a previous report [31], ubiquitinated forms of human full-length IFITM3 can be detected following the overexpression of NEDD4, while the Δ1–21 variant (which lacks the NEDD4-binding motif) was less ubiquitinated (Fig 5B). The P17L and P18H mutations also led to lower levels of ubiquitination, especially pools modified with two or three ubiquitin moieties (as indicated by ** and ***, respectively). The IFITM3-specific antibody used here recognized all but the Δ22–24 variant. We confirmed the presence of ubiquitin using an antibody capable of recognizing mono- and polyubiquitinated proteins (Fig 5B, colored image). The direct labeling of ubiquitin showed that the Δ22–24 variant is modified by NEDD4 to an intermediate extent, since it contains an intact NEDD4-binding site yet lacks one nearby lysine residue. Ubiquitination was also detected in the presence of catalytically inactive NEDD4 C867A, albeit at lower levels (Fig 5B). These results reveal that the natural P17L, P18H, and Δ22–24 mutations prevent normal protein ubiquitination not only by ectopic NEDD4, but also by endogenous E3 ligases in 293T cells. Examination of whole-cell lysate fractions confirmed the expression of all IFITM3 variants (Fig 5C). In accordance with the degree of ubiquitination observed in the immunoprecipitated fractions, certain NEDD4-sensitive variants of IFITM3 were detected at lower levels (relative to the inactive NEDD4 C867A control). Notably, these naturally occurring mutations that disrupt subcellular localization (Fig 5A) and/or diminish NEDD4-mediated ubiquitination (Fig 5B) also exhibit enhanced restriction of HIV-1 virion infectivity relative to full-length human IFITM3 (Fig 5D).

We previously showed that IFITM3 performs two antiviral roles in the setting of HIV-1 infection: (i) inhibition of virus particle infectivity within infected cells (donor cell effect) and (ii) protection of uninfected cells from incoming cell-free virus particles (target cell effect) [21]. To measure the functional impact of IFITM3 variation in both of its antiviral roles, we tested the ability of primate IFITM3 variants to inhibit HIV-1 entry when expressed in uninfected target cells. To this end, we achieved the stable expression of IFITM3 variants in 293T cells transduced with CD4 and CXCR4 (293T 4 × 4 cells) [40] and tested their relative resistance to infection by HIV-1. Importantly, we achieved similar levels of protein expression across all conditions (Fig EV5A), and the differential subcellular localization displayed between IFITM3 variants was maintained (Fig EV5B). When these cells were challenged with HIV-1, synthetic and natural IFITM3 variants containing amino-terminus mutations conferred elevated resistance to incoming HIV-1 relative to human IFITM3 (Fig 5E). The greatest degree of virus restriction was achieved by mutants with the enhanced surface localization (ΔY20, Δ1–21, and Δ22–24).

Since synthetic mutations in IFITM3 that affect regulation by NEDD4 and the AP-2 complex have previously been shown to alter sensitivity to RNA viruses such as IAV and VSV, we also examined the extent of IAV entry restriction in the same 293T 4 × 4 cell lines. In line with previous findings [9], we confirmed that IFITM3 is a more potent inhibitor of IAV compared to IFITM1 and IFITM2 (Fig EV5C). While human IFITM3 restricts IAV infection by nearly 50-fold, ΔY20 and the Δ1–21 variant are markedly less active (Fig 5F), consistent with previous studies [20]. Furthermore, while IFITM3 carrying the P17L and P18H mutations retained the potent restriction of IAV, the Δ22–24 deletion specific to colobus monkey IFITM3 reduces anti-IAV activity by approximately 20-fold (Fig 5G). We also tested the antiviral activity of a full-length “native” colobus IFITM3 protein, which encodes all amino acid substitutions that differ from human IFITM3 (Appendix Fig S1), and the results were similar (Fig 5G). These findings suggest that the functionally informative
Figure 5. Natural mutations in IFITM3 disrupt regulatory motifs and toggle antiviral activity.

A Confocal fluorescence microscopy of 293T cells transfected with 0.5 μg of pQCXIP-FLAG-IFITM3 mutated at the indicated residues and immunostained with anti-FLAG M2 antibody. Scale bars, 5 μm. The P17L, P18H, Δ22-24, P28L/L23P, and P18H/Δ19-23 mutations were introduced into the human IFITM3 background.

B SDS–PAGE of FLAG-immunoprecipitated fractions from 293T cells following transfection of 0.5 μg of pQCXIP-FLAG-IFITM3 variants and 0.5 μg of pCI-HA-NEDD4 or pCI-HA-NEDD4 C867A. Immunoblotting was performed with anti-FLAG M2, anti-IFITM3 (Abcam, EPR5242), and an anti-ubiquitin antibody (Enzo Life Sciences, recognizing K29-, K48-, and K63-linked mono- and polyubiquitinylated proteins). “L” indicates the presence of light chain antibody derived from the FLAG antibody-coupled beads used for immunoprecipitation. *, **, and *** indicate mono-, di-, and triubiquitinated forms of IFITM3.

C SDS–PAGE of whole-cell lysate input fractions from 293T cells used in (B). Immunoblotting was performed with anti-FLAG M2, anti-NEDD4, and anti-actin.

D 293T cells were transfected with 1.0 μg of pNL4-3 and 0.5 μg of pQCXIP-FLAG-IFITM3 variants. Virus-containing supernatants were harvested and 10 ng p24 equivalents were used to reinfect fresh SupT1 T cells. Productive infection was scored by immunostaining with the KC57 antibody and flow cytometry at 48 h postinfection. Mean ± SD of three experiments is shown.

E About 2.0 × 10⁵ cells were seeded and challenged with 10 ng of HIV-1 NL4-3 in the presence of 2 μg/ml DEAE-dextran. Productive infection was scored by immunostaining with KC57 antibody and flow cytometry at 48 h postinfection. The “Colobus native” sequence represents the full IFITM3 sequence identified in the colobus monkey.

F As in (E), except that cells were challenged with influenza A virus (H1N1 PR/8/34, Charles River Laboratories), at a dose that resulted in ~50% infection of control (Empty) cells (equivalent to 10⁴ TCID₅₀/0.2 ml). Infection was scored by immunostaining with an anti-IAV NP antibody and flow cytometry at 18 h postinfection. Results are presented in a logarithmic scale. Mean ± SD of 3–5 experiments is shown.

Data information: Unpaired t-test, *P < 0.05, **P < 0.005.
mutations that govern antiviral potency and specificity are primarily those found among the NEDD4- and AP2-binding motifs.

These results demonstrate that select IFITM3 sequences derived from nonhuman primates exhibit elevated activity against HIV-1 when expressed in either donor or target cells, suggesting that naturally occurring mutation has led to IFITM3 variants with enhanced anti-lentiviral potential. However, we show that mutations arising in IFITM3 have opposing effects against HIV-1 and IAV, suggesting a protective trade-off for host antiviral immunity.

Discussion

Despite the conserved antiviral activities displayed by immune-related IFITM proteins, regulatory mechanisms are in place to control their abundance and localization in the cell, allowing for a fine-tuning of the antiviral response. This study identifies the determinants that regulate the potency and specificity with which IFITM3 proteins inhibit HIV-1 infection. We show that post-translational modifications targeting the amino-terminus of IFITM3 impact antiviral activity, and furthermore, the sequence motifs found therein may be subject to natural selection over evolutionary time. Together, these data provide a functional rationale for the recurrent duplication of IFITM genes observed in vertebrate genomes. The duplication and subsequent divergence of IFITM genes may allow them to be conferred with novel cellular or antiviral functions, a process known as neo-functionalization. Following gene duplication, diversification of IFITM3 coding sequence via alternative splicing (in the case of the putative Δ1–21 form in humans) or by germ line-encoded codon substitution mutations (in the case of nonhuman primates) may allow the cell to expand its antiviral arsenal and to protect different compartments from viral invaders.

Specifically, our findings suggest that synthetic and naturally occurring mutation of single amino acid residues allows IFITM3 to escape regulation by the E3 ubiquitin ligase NEDD4 and the endocytosis adaptor complex AP-2. However, our description of natural mutations in primate IFITM3 sequences offers only a first glimpse at the potential variation that exists. It was previously reported that the IFITM3 locus is duplicated or polymorphic in the rhesus macaque and the African Green Monkey [18,41]. Due to a lack of complete genome sequences, we did not assess IFITM3 variation in additional members of the primate family Cercopithecinae, which comprises most of the monkey species that naturally harbor SIV (and other virus) infections in the wild. Previous reports have demonstrated that these species carry adaptive mutations in the restriction factor genes APOBEC3G [42,43], SAMHD1 [44], and TRIM5α [45,46], which impact lentivirus infection in vitro and in vivo. Thus, cloning of further IFITM3 genes in diverse species is warranted and may reveal other instances of functional diversification. Future experiments focused on the NEDD4–IFITM3 interaction need to be performed in cells from different tissue types and from different species. For example, while we show that certain primate genomes contain IFITM3 genes in which the NEDD4-binding site (PPxY) is altered, experiments were performed in human cells (and thus with human NEDD4). It is unknown whether NEDD4 from different species targets the same structural motif as human NEDD4. The prospect that NEDD4 E3 ligases in different species exhibit different substrate specificities is improbable, however, because the NEDD4 homolog in yeast, Rsp5, also targets substrates bearing the same PPxY signature [47]. A departure from this expectation would be exciting, however, since species-specific NEDD4 activities may be indicative of coevolution between NEDD4 and its cellular substrates in a novel form of genetic conflict [37]. Similar questions regarding the cell- or species-specific function of the endocytic adaptor complex, AP-2, must also be addressed.

Previous publications examining the antiviral activity of IFITM3 mutants demonstrated that the hypervariable amino-terminus governs its subcellular localization and, as a result, antiviral activity against IAV. As such, variants of IFITM3 carrying Δ1–21 or ΔY20 were deemed “defective” or “nonfunctional” [20]. On the contrary, we report here that these and other mutations to the amino-terminus of IFITM3 retain and even enhance antiviral function during HIV infection. Furthermore, the use of these hyperactive mutants allowed us to assess the involvement of two distinct, but not mutually exclusive, mechanisms underlying this restriction activity: (i) IFITM3 incorporation into viral membranes and (ii) inhibition of HIV-1 Env maturation and virion incorporation. While our results in 293T cells show that IFITM3 overexpression can lead to slightly decreased gp120 levels in virus-producing cells, as proposed by Yu et al [23], we do not observe this phenomenon when IFITM3 is induced in T cells. Previously published studies illustrating an effect of IFITM proteins on the synthesis and maturation of many lentiviral proteins [48], including Gag [17], and even other cellular proteins [49], raise the possibility that IFITM proteins, when overexpressed, promote wholesale changes that may affect protein trafficking in general. Furthermore, an HIV-1 Env-specific effect on restriction activity cannot explain the finding that VSV-G pseudotyping of virions produced in the presence of IFITM3 proteins does not rescue their infectivity [22]. Overall, while changes to Env may be partially responsible for restriction in 293T cells, our results suggest that the situation may be different in lymphocytes.

The roles played by human IFITM3 in susceptibility to infection have been actively researched, yet many questions remain. Numerous studies have drawn an association between the human SNP rs12252-C in IFITM3 and a severe outcome to IAV infection, yet the protein variant (Δ1–21) predicted to derive from this SNP has not been directly detected in human cells. Paradoxically, a study has recently reported an association between rs12252-C and rapid progression to AIDS in HIV-infected individuals in China [50]. Since we show here that the Δ1–21 variant exhibits an elevated anti-HIV-1 activity in both of its antiviral roles when ectopically expressed in human cells, efforts are needed to establish a direct functional link between rs12252-C and the truncated protein variant. Importantly, other polymorphisms exist in human IFITM3, such as one in the promoter that is associated with susceptibility to tuberculosis [51], further experimental advances will be necessary to directly assess the relationship between genotype, IFITM3 protein, and disease susceptibility. There is evidence for recent positive selection in human IFITM3 [20], so a scenario whereby “hitchhiking” (linkage disequilibrium) occurs between neighboring SNPs must also be considered.

While mutations to the NEDD4-binding motif (PPxY) did not interfere with anti-IAV function, our description of single amino acid changes and deletions disrupting the endocytosis motif (YxxL)
demonstrates that some naturally occurring mutations carry a cost in the form of a protective trade-off. The recurrent appearance of similar mutations in different primate lineages may reflect the presence of a common selective pressure in the past and may even reveal the nature of the selective pressure itself. Our results suggest that certain nonhuman primate IFITM3 may exhibit greater activity against HIV-1, suggesting that evolution may have fine-tuned their function against lentiviruses or other viruses entering and exiting from the cellular plasma membrane. Future studies examining the activities of primate IFITM3 proteins against their autologous SIV strains will be important for confirming their anti-lentiviral potential and may reveal viral countermeasures that allow antagonism or evasion of IFITM3 proteins. Regardless, this enhancement of function against lentiviruses comes with a loss of activity against other viruses entering at endosomes, like IAV and VSV. Considering that IFITM3 is critical to the antiviral response to IAV in mice and humans [19,20], the appearance of mutations in nonhuman IFITM3 that suppress this activity is surprising. Thus, our observation that many primate genomes contain multiple IFITM3 genes may reflect an evolutionary mechanism to provide balanced antiviral protection to the cell. Since some species covered in this study, such as the orangutan, the gorilla, and the common marmoset, have been studied with regard to susceptibility to human virus infections [52,53] it will be of great interest to address how IFITM3 genotype and gene copy number impact in vivo virus replication and transmission.

Materials and Methods

Cells, viruses, and reagents

HEK293T cells (293T, ATCC) and derivatives were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. Primary CD4+ T cells and HeLa cells were treated with type-I interferon as previously described [21]. 293T 4 × 4 cells were generated by lentiviral transduction with constructs encoding CD4 and CXCR4, as previously described [40]. pQCXIP plasmids encoding IFITM3 variants with amino-terminal FLAG (FLAG-IFITM3) were previously described [16,17], and additional mutants were generated by site-directed mutagenesis. 293T cells were transfected with Metafectene (Biontex) or Lipofectamine 2000 (Invitrogen) reagent. Tet-ON SupT1 cells were treated with doxycycline (500 ng/ml) overnight to induce FLAG-IFITM3 variants as previously described [21]. Stocks of HIV-1 were produced via CaPO4 transfection of 293T cells with pNL4-3. HIV-1 incorporating Vpr-B-lactamase (Vpr-Blam) fusion protein was produced via co-transfection of pNL4-3, pVpr-Blam, and pQCXIP encoding IFITM3 variants or an empty control or pCMV-MxA, at a respective plasmid ratio of 3:1:5:1. HIV-1 produced from Tet-ON SupT1 cell lines was produced by first infecting the cells with VSV-G-pseudotyped NL4-3 in the presence of DEAE-dextran (2 μg/ml) followed by overnight doxycycline treatment to induce IFITM3 protein expression.

Western blot

Cells were lysed in 1% Triton-X/PBS in the presence of protease inhibitor cocktail (cOmplete, Roche) and clarified by centrifugation. Virions were purified from supernatants by overlaying on a 6% OptiPrep cushion and pelleted by ultracentrifugation (50,000 × g, 4°C) for 1 h. An aliquot was removed for p24 Gag ELISA, and normalized amounts were loaded on a Bis-Tris 12% acrylamide SDS–PAGE gel. The following antibodies were used: mouse anti-FLAG-M2 (Sigma), mouse anti-IFITM3 (Proteintech, 66081-1-lg), rabbit anti-IFITM3 (Abcam, EPR5242), mouse anti-Gag p24 (183-H12-SC, NIH #1513), sheep anti-Env gp120 (NIH #288), mouse anti-HA (Covance, HA.11 clone 16B12), rabbit anti-actin (Santa Cruz Biotechnology), mouse anti-tubulin (Santa Cruz Biotechnology) and mouse anti-ubiquitin antibody (FK2, Enzo Life Sciences, recognizing K29-, K48-, and K63-linked mono- and poly-ubiquitinated proteins). DyLight-coupled secondary antibodies (Thermo Fisher) were used for protein detection on a Li-Cor Odyssey imaging system.

Flow cytometry

Cells were fixed in 4% PFA and permeabilized with 0.5% saponin. The same antibodies used for Western blot were also used for flow cytometry. In addition, the mouse anti-Gag KC57 (Beckman Coulter) and mouse anti-influenza A group (NP) (Argene) antibodies were used to score HIV-1 and IAV infections, respectively. FLAG M2-PE (Abcam) was used to measure the expression of FLAG-IFITM3 from pQCXIP. Acquisition was performed on a FACS Canto II (Becton Dickinson).

Confocal immunofluorescence microscopy

Transfected 293T cells were cultured on 12-mm glass coverslips, fixed in 4% PFA, and permeabilized with 0.5% saponin. The same antibodies used for Western blot were also used for confocal microscopy. In addition, the mouse anti-p17 Gag (ARP342, AIDS Reagents from Programme EVA Centre) and the mouse anti-LAMP1 (clone HA43, Santa Cruz Biotechnology) antibodies were used. Analysis was carried out on a Zeiss LSM700 using a 63× objective. Images were analyzed using FIJI software and assembled with the Magic Montage plugin.

Vpr-Blam virus fusion assay

A total of 25 ng p24 equivalent of Vpr-Blam-containing virions was incubated with 1 × 10^5 SupT1 cells. After 2 h, the cells were washed and incubated with the CCF2-AM substrate (CCF2-AM loading kit, Invitrogen) for an additional 2 h. Following fixation in 4% PFA, cleavage of CCF2-AM was measured by flow cytometry.

IFITM3 co-immunoprecipitation and ubiquitination assays

About 5 × 10^5 293T cells were transfected with 0.5 μg pCI-HA-NEDD4 or pCI-HA-NEDD4 C867A and 0.2 μg pQCXIP encoding FLAG-tagged IFITM3 variants. The cells were harvested at 24 h post-transfection and lysed for 30 min on ice in a lysis buffer consisting of 50 mM Tris, 300 mM NaCl, 1 mM EDTA, 1% NP-40, H2O, and a protease inhibitor cocktail. Cell lysates were incubated with prewashed FLAG-M2 agarose beads (Sigma) for 2 h at 4°C. The beads were washed and mixed directly with SDS–PAGE loading buffer (2× Laemmli plus reducing agent). Samples were then denatured by heating at 95°C for 5 min and stored at −20°C until ready for SDS–PAGE loading.
Purification of HIV-1 particles by velocity gradients

About 7 × 10^6 Tet-ON SupT1 cells (encoding inducible FLAG-IFITM3 or FLAG-IFITM3Δ1–21) were infected with HIV-1 NL4-3 to achieve approximately 50% Gag^+ cells. IFITM3 variants were induced by doxycycline addition (500 ng/ml). After two additional days, supernatants from HIV-1-infected, IFITM3^+ cells were filtered on a 0.22-µm filter. Clarified supernatants were ultracentrifuged at 70,000 × g for 1 h. Pellets containing both cellular microvesicles and viral particles were then suspended in PBS and subjected to second ultracentrifugation at 200,000 × g for 1.5 h through a discontinuous 6–18% OptiPrep (iodixanol) gradient, stratified by increments of 1.2%. Ten to eleven fractions were collected and further ultracentrifuged at 435,000 × g for 30 min to concentrate the samples. Pellets were resuspended in 30 µl of 1% Triton X-lysis buffer supplemented with protease inhibitors and directly loaded on a 4–12% acrylamide Bis–Tris SDS–PAGE gel.

Primate IFITM3 sequence retrieval, molecular analysis, and cloning

IFITM sequences were assembled from reference genome sequences for the following species: mouse lemur (Microcebus murinus; micMur1), bushbaby (Otolemur garnettii; OtoGar3), tarsier (Tarsius syrichta; tarSyr1), common marmoset (Callithrix jaccus; C_jacchus3.2.1), rhesus macaque (Macaca mulatta; MMUL 1.0), olive baboon (Papio anubis; PapAnu2.0), vervet (African Green Monkey-sabaeus) (Chlorocebus sabaeus; ChiSab1.1), orangutan (Pongo abelii; PpyG2), gorilla (Gorilla gorilla gorilla; gorGor3.1), chimpanzee (Pan troglodytes; CHIMP2.1.4), humans (Homo sapiens; GRCH38.p5), and gibbon (Nomascus leucogenys; Nleu1.0) using Genomicus v84.01 and Ensembl (www.ensembl.org), with additional sequences provided by BLASTN or BLASTP query in NCBI GenBank. Nucleotide and protein sequence alignments were performed using T-Coffee or ClustalW, with dNucDist and dProteDist as input, and additional sequences provided by BLASTN or BLASTP query in NCBI GenBank. Nucleotide and protein sequence alignments were performed using T-Coffee or ClustalW, with dNucDist and dProteDist as input.
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