IFITM-Family Proteins: The Cell's First Line of Antiviral Defense

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Abstract

Animal cells use a wide variety of mechanisms to slow or prevent replication of viruses. These mechanisms are usually mediated by antiviral proteins whose expression and activities can be constitutive but are frequently amplified by interferon induction. Among these interferon-stimulated proteins, members of the IFITM (interferon-induced transmembrane) family are unique because they prevent infection before a virus can traverse the lipid bilayer of the cell. At least three human IFITM proteins—IFITM1, IFITM2, and IFITM3—have antiviral activities. These activities limit infection in cultured cells by many viruses, including dengue virus, Ebola virus, influenza A virus, severe acute respiratory syndrome coronavirus, and West Nile virus. Murine Ifitm3 controls influenza A virus infection in vivo, and polymorphisms in human *IFITM3* correlate with the severity of both seasonal and highly pathogenic avian influenza virus. Here we review the discovery and characterization of the IFITM proteins, describe the spectrum of their antiviral activities, and discuss potential mechanisms underlying these effects.

INTRODUCTION

Interferon-Stimulated Genes and Viral Restriction Factors

IFITM: interferon-induced transmembrane

Our innate and adaptive immune responses use a number of mechanisms to detect infectious pathogens—for example, activation of cytosolic viral sensors such as RIG-I and MDA5 or detection of viral nucleic acids by members of the Toll-like receptor family. These mechanisms usually result in the expression of type I or type II interferons. Interferons signal through interferon receptors to recruit and activate JAKs (Janus kinases) and TYK (tyrosine-specific kinase), which in turn phosphorylate STAT (signal transducers and activators of transcription) proteins. Following phosphorylation, STAT proteins translocate to the nucleus, where they associate with interferon regulatory proteins to promote expression of a wide array of genes. More than 1,000 interferon-stimulated genes have been identified. These genes can be specifically induced by either or both types of interferons and vary widely in their antipathogen activities and their constitutive and interferon-induced expression levels (1–4).

The term restriction factor emerged from the study of intra- and interspecies variation in susceptibility to retroviral infection and has been applied to proteins whose primary or sole function is to suppress viral replication. The retrovirus field has also made a distinction between so-called intrinsic and innate immunity (3). Intrinsic factors are those with high constitutive levels of expression and activity, whereas classical innate immune factors, such as the RNA-activated PKR (protein kinase R), RNase L, and 2′,5′-oligo-adenylate synthases, usually require a pathogen recognition event and induction by interferon. However, expression of intrinsic factors such as the antiretroviral factors Trim5α and tetherin can be further increased by interferons, whereas a number of interferon-stimulated genes also have high levels of basal expression. Other investigators have categorized antiviral proteins expressed in target cells as cell-intrinsic factors (2) to distinguish their functions from the multiple innate immune activities of macrophages, neutrophils, and other immune cells that specialize in responding to infection.

The IFITM Family of Viral Restriction Factors

The IFITM (interferon-induced transmembrane) proteins comprise a family of interferon-induced antiviral cell-intrinsic restriction factors with high constitutive expression in many cells, including barrier epithelial cells. As their names imply, the expression of human IFITM1, IFITM2, and IFITM3 is also strongly upregulated by both type I and type II interferons (5, 6). Uniquely among known restriction factors, these proteins prevent viruses from traversing the lipid bilayer of the cell and accessing the cytoplasm (1). The IFITM proteins potently restrict a number of viral pathogens important to human health, such as dengue virus (DENV) and influenza A virus (IAV), but intriguingly have little or no effect on many other viruses—for example, the arenavirus that causes Lassa fever (LASV) (7). Much has been learned since the IFITM proteins were identified as anti-IAV restriction factors in 2009, but the exact molecular mechanisms mediating their antiviral activities are not yet fully understood. Here we discuss their identification, their structures and activities, and what is known or can be guessed about their mechanisms of restriction.

IFITM PROTEINS AS RESTRICTION FACTORS

Early Characterization

IFITM proteins were identified several decades ago and indeed were among the first interferon-stimulated genes to be discovered (5). They were originally named 9-27 (IFITM1), 1-8D

(IFITM2), and 1-8U (IFITM3) (or, in mice, fragilis2, fragilis3, and fragilis, respectively) (8). *IFITM1* was found to encode the Leu-13 antigen (later designated as CD225), indicating that at least some part of IFITM1 was exposed at the plasma membrane (9). IFITM1 is associated with components of the B cell receptor including CD19, CD21, and, most directly, CD81/TAPA-1 (10–12). Antibodies cross-linking IFITM1 promote homotypic adhesion of leukemic B and T cells (13, 14), inhibit the proliferation of B cell lines, and downregulate L-selectin (15). The significance of these observations remains unclear. Moreover, the topology of IFITM proteins suggests that they are unlikely to have natural ligands that could function directly in the same manner and, therefore, that these anti-IFITM1 antibodies likely function by cross-linking IFITM1-associated proteins.

In parallel with the study of IFITM1 in lymphocytes, several investigators explored the roles of IFITM proteins in germ cell homing and maturation. In the murine embryo, Ifitm3 (fragilis) is specifically expressed in primordial germ cells (PGCs) but not in adjacent somatic cells and can be used as a marker of germ cell competence in mouse embryos (16, 17). Ifitm3 confers the homing properties of PGCs to somatic cells. In contrast, Ifitm1 may mediate the transit of primordial germ cells from the mesoderm to the endoderm (18). However, the relevance of these observations was called into question when it was shown that mice homozygous for a deletion of the *Ifitm3* gene or of the entire *Ifitm* locus (*IfitmDel*$^{-/-}$ mice) have no apparent developmental defects or indeed any overt phenotype (19). These knockout mice have since been repurposed to study the antiviral activities of Ifitm3 and other murine Ifitm proteins in vivo.

Discovery of the Antiviral Activities of IFITM Proteins

An early clue that IFITM proteins function primarily to control viral infections was published in 1996 by Alber & Staeheli (20). These authors observed that overexpressed IFITM1 inhibits replication of vesicular stomatitis virus (VSV), albeit less potently than the interferon-induced protein MxA (20). These investigators also observed that mouse cells overexpressing human IFITM1 were more refractory than control cells to VSV infection. Much less pronounced effects were observed with IAV. Although these results differ from more recent studies that indicate more potent restriction of IAV relative to VSV (21), this study marked the first description of antiviral activity for an IFITM protein. Despite this report, a passing reference to activity against hepatitis C virus (HCV) by IFITM3 (22), and abundant evidence that IFITM proteins are potently induced by type I and II interferons, it took an additional 13 years to rediscover the antiviral activities of the IFITM proteins.

IFITM3 was first identified as a potential IAV restriction factor in 2009 by Brass et al. (7) and Shapira et al. (23), in two of five similar IAV-targeting RNA interference screens published within weeks of one another. Further work reported by Brass et al. (7) validated the initial screen by demonstrating that small interfering RNA (siRNA) targeting IFITM3 strongly promoted H1N1 (A/PR/8/34) replication in U2OS cells and that IFITM3-specific siRNA could, to a large extent, overcome suppression of viral replication mediated by interferon-γ. Overexpression of human IFITM1, IFITM2, or IFITM3 suppressed replication of H1N1 (A/PR/8/34) and H3N2 (A/Udorn/72) but not that of murine leukemia virus (MLV) in A549, U2OS, and MDCK cell lines as well as in chicken embryo fibroblasts. Murine embryonic fibroblasts (MEFs) from *IfitmDel*$^{-/-}$ mice were markedly more susceptible to IAV infection than were MEFs from their wild-type littermates, and type I and type II interferons had a less pronounced effect on IAV replication in *IfitmDel*$^{-/-}$ MEFs. Moreover, infection by retroviruses pseudotyped with various H1, H3, H5, and H7 hemagglutinin (HA) proteins, but not with the entry proteins of the Machupo virus (MACV) or MLV, was efficiently suppressed by IFITM1, IFITM2, and IFITM3, establishing that restriction targets an HA-mediated process, presumably viral entry. The same study also

showed that flaviviruses, including DENV and West Nile virus (WNV), are similarly susceptible to IFITM-mediated restriction. Collectively, these results established that IFITM proteins function in cell culture as potent restriction factors against two important families of enveloped viruses and that their activities may contribute significantly to the anti-IAV activities of type I and type II interferons.

Vertebrate Orthologs

The IFITM proteins belong to a larger family of dispanin proteins found in both eukaryotes and prokaryotes (24). IFITM proteins are defined by the presence of a central highly conserved region, named after CD225 (IFITM1). The IFITM proteins can be further divided into three clades based on sequence similarity and putative functions (25). Clade I includes all the human proteins shown to have antiviral activities, namely IFITM1, IFITM2, and IFITM3—the so called immunity-related IFITMs. IFITM2 and IFITM3 are highly homologous, whereas IFITM1 diverges modestly from these two proteins. Clade I also includes murine orthologs of these proteins as well as murine Ifitm6 and Ifitm7, the latter of which is encoded by an intronless retrogene derived from the *Ifitm1* gene. Most mammals appear to have orthologs of IFITM1, IFITM2, and IFITM3, as do chickens (25–28). In other species, such as rhesus macaques, it is difficult to assign the two *IFITM2/3*-like genes as orthologs of human *IFITM2* or *IFITM3*. The human *IFITM* locus is located on chromosome 11, whereas the mouse and chicken loci are on chromosomes 7 and 5, respectively (**Figure 1**). Murine *Ifitm7*, likely retrotranscribed from *Ifitm1*, is found on chromosome 16. With the exception of *Ifitm7*, all human and mouse genes encoding IFITM proteins include an intron that separates two coding exons. Most of these genes are also initiated by an additional noncoding exon.

The diversity and multiplicity of the immunity-related IFITMs suggest a good deal of positive selection, consistent with their role as viral restriction factors (25). In contrast, IFITM clades II and clade III are each composed of a single protein, IFITM5 and IFITM10, respectively. Although the *IFITM10* gene is near the human *IFITM* locus, and *IFITM5* is clearly part of that locus, neither gene is interferon inducible and neither has an antiviral function (25). Both genes reflect purifying rather than positive selection, and both have clear orthologs in most vertebrates. Although expression of the immunity-related IFITM proteins appears to be ubiquitous, especially following interferon stimulation, IFITM5 is primarily expressed in osteoblasts (29, 30). Human IFITM5 variants have been implicated in osteogenesis imperfecta type V, a brittle bone disease (31, 32). The expression patterns and functions of IFITM10 have not been characterized.

In Vivo Studies in Mice and Humans

Lange et al. (19) established two strains of knockout mice to explore the role of murine Ifitm proteins in primordial germ cell development. *IfitmDel*$^{-/-}$ mice bear a 120-kb deletion of the entire *Ifitm* locus on chromosome 7, resulting in deletion of the *Ifitm1*, *Ifitm2*, *Ifitm3*, *Ifitm5*, and *Ifitm6* genes (see **Figure 1**). *Ifitm10* and the *Ifitm7* retrogene on chromosome 16 remain intact. These mice are viable, do not display gross developmental defects, and reproduce similarly to their wild-type counterparts. Careful study of *Ifitm5*-knockout mice, however, revealed subtle changes in skeletal morphology during embryonic development (30), which makes it likely that *IfitmDel*$^{-/-}$ mice share this phenotype. Fortuitously, Lange et al. also established *Ifitm3*$^{-/-}$ mice by disrupting the first exon of *Ifitm3* via insertion of sequences encoding enhanced green-fluorescent protein. Again, these animals showed no obvious developmental defects.

Two groups characterized the susceptibility of *Ifitm3*$^{-/-}$ mice to IAV infection. Everitt et al. (33) challenged these mice and their wild-type littermates with a relatively nonpathogenic

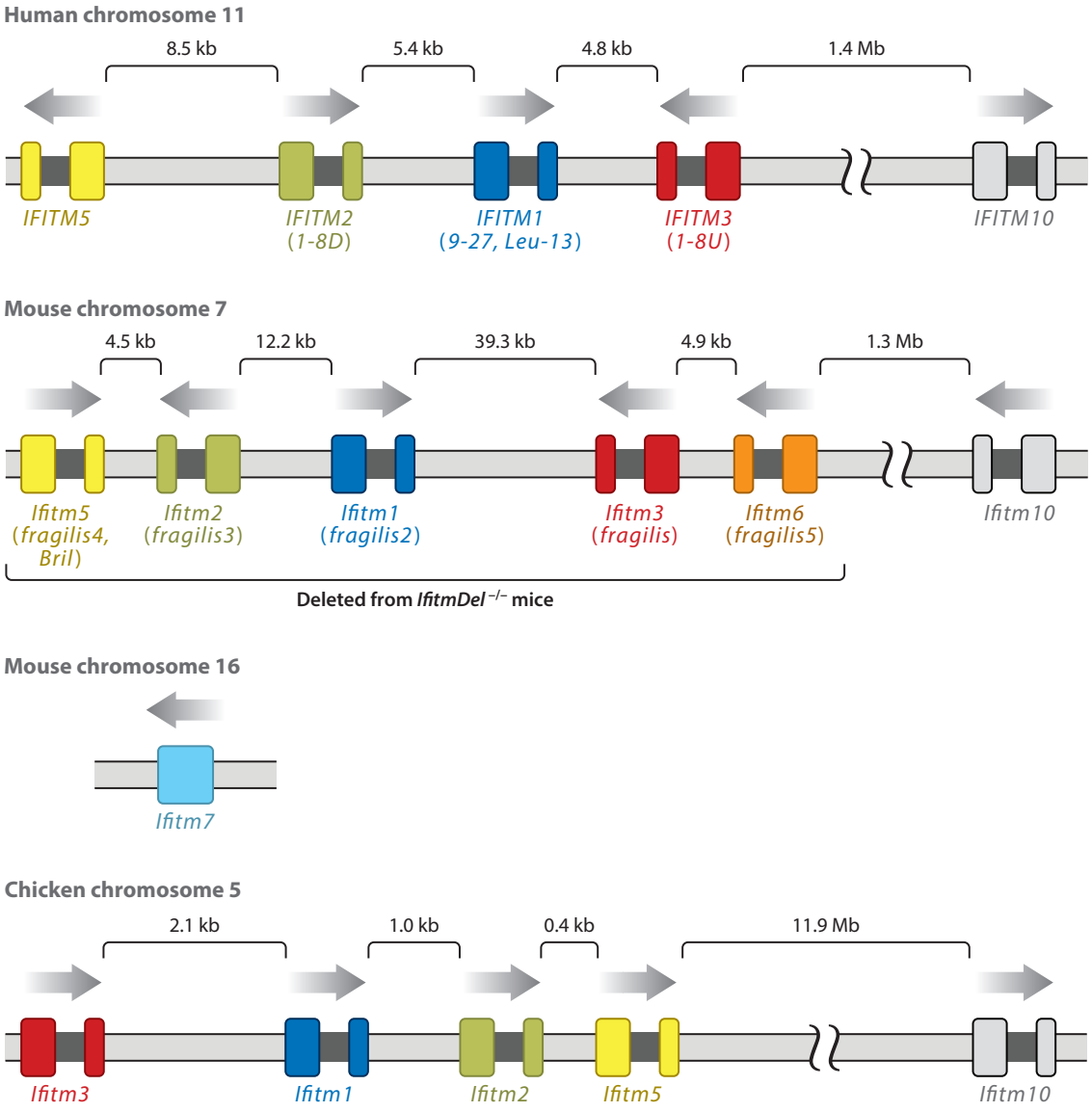

Figure 1

The immunity-related *IFITM* genes (*IFITM1*, *IFITM2*, and *IFITM3*), together with the osteoblast-restricted *IFITM5*, form a gene cluster in humans that is conserved in mice and chickens (25, 26). Alternative gene names are given in parentheses. Exons are depicted in color and introns in dark gray. Arrows indicate the direction of transcription. Mice possess two additional immunity-related *Ifitm* genes, *Ifitm6* and *Ifitm7* (19). *Ifitm6* lies within the chromosome 7 *Ifitm* cluster, but *Ifitm7* is a retrogene located on chromosome 16. *IFITM10*, a gene of unknown function, lies outside the *IFITM* cluster but remains on the same chromosome (25).

murine-adapted H3N2 IAV strain (A/X-31) as well as with a 2009 H1N1 pandemic strain (A/09 Eng/195). *Ifitm3*$^{-/-}$ mice consistently lost >25% of their body weight by day 6 and were euthanized. In contrast, their wild-type littermates lost <25% of their body weight and recovered. Bailey et al. (34) challenged both *Ifitm3*$^{-/-}$ and *IfitmDel*$^{-/-}$ with a pathogenic H1N1 strain (A/PR/8/34) and observed a more rapid loss of weight in these mice compared with their wild-type littermates.

Respiratory epithelium: the ciliated columnar epithelial cells that line the upper airways; one of the key targets of IAV in vivo

As observed by Everitt et al., all of the knockout mice exhibited weight loss of >20% by day 6 and were euthanized. Approximately 60% of wild-type mice also lost >20% of their body weight by day 7–8, whereas the remainder recovered. Intermediate responses were noted with heterozygotes of both lines of mice, demonstrating a gene-dosage effect for *Ifitm3*. No differences were observed between *IfitmDel*$^{-/-}$ and *Ifitm3*$^{-/-}$ mice or between *IfitmDel*$^{+/-}$ and *Ifitm3*$^{+/-}$ heterozygotes, indicating that the murine Ifitm3 plays an especially important role in controlling an IAV infection. Both groups noted that lesions were confined to the respiratory tract. Although Bailey et al. noted the presence of viral RNA in the spleens of *IfitmDel*$^{-/-}$ animals, they found no histologic evidence of infection at this site. Bailey et al. also demonstrated constitutive expression of Ifitm3 in the lungs of uninfected wild-type mice, particularly in respiratory epithelium, the visceral pleura, and endothelial cells. These tissues all form anatomic barriers between the host and the environment or between different anatomic compartments, suggesting that Ifitm3 prevents dissemination of local infection. In addition to respiratory epithelial cells, Ifitm3 is expressed in another key IAV target cell type—alveolar type II pneumocytes—following IAV infection (34). Collectively, these studies make a compelling case for a critical role of Ifitm3 in the in vivo control of acute influenza.

Everitt et al. (33) provided the first evidence for an in vivo role for IFITM3 in humans. Caucasians hospitalized for infection with 2009 pandemic H1N1 were noted to display a significant enrichment of a minor *IFITM3* allele, SNP 12252-C. Individuals with two such alleles (rs12252-C/C) were found in 5.7% of 53 patients hospitalized with H1N1 IAV in Great Britain, whereas only 0.3% of 1,000 Europeans were homozygous for rs12252-C. SNP 12252-C has been proposed to create a novel splice acceptor site resulting in an IFITM3 variant, truncated in its first 21 amino acids, that is much less efficient at restricting IAV. Although this shorter splice variant was not identified in lymphoblastoid cell lines homozygous for rs12252-C, lower overall IFITM3 expression was observed in these cells. The rs12252-C allele is rare in Caucasians, but it is much more prevalent among Han Chinese. A study of this population showed that 69% of Chinese patients with severe H1N1/09 infection were homozygotes (35). In contrast, rs12252-C/C homozygotes constituted only 25% of individuals with a milder infection. Similarly, in a study of individuals infected with pathogenic avian-origin H7N9, rs12252-C/C homozygotes sought medical attention sooner, developed acute respiratory distress more quickly, and had higher mortality than heterozygotes or rs12252-T/T patients (36). In general, the sample size of these studies is small—for example, Everitt et al. relied on only three rs12252-C/C homozygotes—and at least one study has failed to confirm these results (33, 37). Collectively, however, there appears to be a trend in which at least one polymorphism in the *IFITM3* gene increases susceptibility to IAV.

IFITM3 expression also can influence the quality of adaptive immune responses to IAV. For example, a population of lung-resident memory CD8$^+$ T cells (T_{RM}) in mice strongly expresses Ifitm3 following an IAV infection (38). Furthermore, Ifitm3 upregulation by these T_{RM} cells is not the result of nonspecific stimulation by interferons during infection but is instead contingent on antigen recognition. This point was demonstrated by introducing ovalbumin (OVA)-specific CD8$^+$ T cells into mice that were subsequently infected with either wild-type IAV or an OVA-expressing strain. OVA-specific CD8$^+$ T cells persistently upregulated Ifitm3 following OVA-IAV challenge but not following challenge with wild-type virus. Because Ifitm3 rendered these cells resistant to viral infection, they were not lost following a second challenge with IAV and contributed to more rapid clearance of this virus. A similar population of Ifitm3-high CD8$^+$ memory T cells was also observed in the brains of mice responding to VSV, suggesting that Ifitm3 generally protects the CD8$^+$ T_{RM} cells from viral infection (39). These findings suggest that IFITM protein expression can shape the adaptive immune response by selectively protecting subsets of immune effector cells.

THE SPECTRUM OF IFITM-RESTRICTED VIRUSES

A number of viruses and retroviruses pseudotyped with various entry proteins are restricted by one or more IFITM proteins. In some cases, the relative susceptibility or resistance of a virus to IFITM-mediated restriction can vary depending on cell type, virus titers, the IFITM protein in question, its expression level, and indirect consequences of IFITM overexpression. More robust investigations include a positive control (typically IAV or a retrovirus pseudotyped with IAV HA) and a negative control (typically MLV or a retrovirus pseudotyped with LASV, MACV, or MLV entry proteins) and use siRNA depletion of endogenous IFITM proteins (or *IfitmDel*$^{-/-}$ or *Ifitm3*$^{-/-}$ MEFs) in parallel with overexpression studies. Interpretation of experimental results can be further confounded by real or artifactual perturbation of downstream steps in the virus life cycle and potential interference with host factors important in the viral entry process. Ideally, reports of IFITM restriction should include restriction of a pseudotyped retrovirus in addition to live virus studies to differentiate between entry restriction and inhibition of other phases of the viral life cycle.

Influenza A Viruses

Restriction of IAV by IFITM proteins is well established both in cultured cells and in vivo. Cell culture studies suggest that IFITM3 has a more pronounced effect on IAV entry than IFITM1 or IFITM2, but with one exception, all human and mouse immunity-related IFITM proteins can restrict IAV. The single exception is murine Ifitm6, which fails to inhibit IAV entry when overexpressed in human cells and does so inefficiently in murine cells under conditions in which Ebola virus (EBOV) pseudoviruses are efficiently restricted (21). In mouse studies, *Ifitm3* plays the predominant role in controlling IAV, with the other Ifitm proteins contributing modestly or not at all (34). Also of note, every IAV assayed, including H1N1, H3N2, H5N1, H7N1, and H7N9 isolates, appears to be restricted by IFITM proteins, and there are no reports of IAV escape from IFITM restriction.

Flaviviruses

IFITM-mediated restriction of flaviviruses, including DENV1 and DENV2, Japanese encephalitis virus (JEV), and WNV, has also been demonstrated by several laboratories (7, 21, 40, 41). Flavivirus replication is amplified by loss of endogenous IFITM proteins, and IFITM-protein overexpression markedly suppresses viral replication. IFITM-mediated restriction is presumed to target the flavivirus entry process. However, there is a caveat with this interpretation. Because the structural proteins of flaviviruses cannot pseudotype retroviruses or VSV, all studies to date have been performed with infectious virions or virus-like particles (VLPs). Because readouts from both infectious virions and VLPs require the early activity of these nonstructural proteins, it is possible that IFITM proteins restrict steps following viral entry. However, IFITM expression does not appear to affect the efficiency of electroporated DENV replicons (40), and given that the site of fusion of flaviviruses is similar to that of IAV, it is possible that these viruses are restricted through the same mechanism. Although not investigated with the same precision as IAV, the pattern of restriction also appears similar to that of IAV, with IFITM3 restricting these viruses and VLPs more efficiently than IFITM1 and IFITM2.

Filoviruses and the Severe Acute Respiratory Syndrome Coronavirus

The IFITM proteins also efficiently restrict the filoviruses EBOV and Marburg virus (MARV) as well as severe acute respiratory syndrome coronavirus (SARS-CoV), as demonstrated by

overexpression and short hairpin RNA (shRNA)-depletion studies using both infectious viruses and pseudoviruses (21). Although these viruses are from different families, they share a dependency on the enzymatic activities of lysosomal cathepsins to activate their fusion proteins (42, 43). Thus, they are thought to fuse later in the endocytic pathway than IAV or flaviviruses. IFITM expression does not detectably change the level of cathepsin L activity in cell lysates or the surface expression of the SARS-CoV receptor ACE2 (21, 44). SARS-CoV and filoviruses also share a pattern of restriction that is different from that of IAV. For example, SARS-CoV and filoviruses appear to be more sensitive to IFITM1 than is IAV, and unlike IAV, they can be efficiently restricted by murine Ifitm6 expressed in human cells (21). These studies provide the first suggestion that different IFITM proteins might specialize in targeting different viruses.

Rhabdoviruses

Although VSV, a rhabdovirus, was the first virus reported to be restricted by an IFITM protein (20), the effect of IFITM proteins on VSV-G pseudotyped retroviruses is relatively modest compared with that on EBOV or IAV pseudoviruses (7). Studies of viable VSV also suggest a modest level of restriction (45). It has been proposed that VSV fuses with the intraluminal vesicles of the late endosome/multivesicular body, which then backfuse with the limiting membrane of the multivesicular body. Although the efficiency of this process is not well understood, it may facilitate partial escape from IFITM restriction. This speculation presumes that restriction does not extend to the intraluminal vesicles and that IFITM proteins inhibit intraluminal vesicle backfusion less efficiently than viral fusion. Overexpressed human and chicken IFITM proteins also inhibit infection by retroviruses pseudotyped with the entry proteins of rabies virus and Lagos bat virus, a virus from the same genus (26).

Bunyaviruses

Rift Valley fever virus (RVFV) is efficiently restricted by overexpressed IFITM2 and IFITM3 but not, interestingly, by IFITM1 (46). Similarly, siRNA targeting of IFITM2 and IFITM3 enhanced RVFV replication. Although studies with pseudoviruses have not been reported, postentry replication kinetics of RVFV are not affected by IFITM protein expression. Other bunyaviruses including Andes virus, Hantaan virus, and La Crosse virus are restricted by IFITM2 and IFITM3, but unlike RVFV, these viruses are also restricted by IFITM1. However, with the exception of La Crosse virus, the magnitude of restriction is less than that observed for RVFV. Interestingly, Crimean Congo hemorrhagic fever virus (CCHFV), which is in the same virus family, is unaffected by IFITM restriction.

HIV-1

Both groups contributing to the initial discovery of IFITM proteins as IAV restriction factors attempted independently to determine whether these proteins restrict HIV-1 infection. Both concluded that IFITM1, IFITM2, and IFITM3 overexpression does not significantly impede HIV-1 entry in TZMbl and GHOST-CCR5 reporter-cell lines (7). A large-scale screen of overexpressed interferon-stimulated genes in Huh cells, however, identified IFITM2 and IFITM3 as inhibitors of HIV-1 replication (4). A parallel shRNA screen using 293T cells also identified IFITM2 and IFITM3 as limiting HIV-1 replication. Subsequent overexpression experiments using tetracycline-inducible IFITM constructs demonstrated that IFITM1, IFITM2, and IFITM3 each suppress HIV-1 replication and showed that IFITM2 and IFITM3, but not IFITM1, inhibit HIV-1 entry (47). Although direct comparisons were not performed, the magnitude of

the entry-inhibition effect of IFITM2 and IFITM3 appears to be lower than that observed with IAV or flaviviruses, with approximately 50% of infection inhibited, although larger effects were observed with HIV-1 replicating in SupT1 cells over a 2-week period. IFITM protein expression has no effect on cell-surface CD4 expression, but coreceptor levels have not been measured. IFITM1, which suppresses HIV-1 replication but not entry, inhibits an early step in the HIV-1 life cycle between integration of viral DNA and expression of the Gag protein.

A subsequent investigation revealed that IFITM protein expression diminishes the expression of several HIV-1 proteins expressed either from a proviral construct or individually (48). Unexpectedly, this effect is specific to proteins expressed from constructs containing transactivation-response or Rev-response elements, suggesting that IFITM proteins may modulate translation from RNA transcripts with particular secondary structures. Mutation of the palmitoylated cysteine residues of IFITM3, which causes IFITM3 mislocalization and attenuates its entry-restriction activity (49), does not alter its capacity to suppress HIV-1 protein synthesis. Collectively, these studies suggest a moderate but reproducible restriction of HIV-1 replication by the IFITM proteins. It is likely that the mechanisms of HIV-1 restriction are mostly distinct from those of IAV restriction and result from a combination of entry-specific and entry-independent effects.

Hepatitis C Virus

It was reported as early as 2003 that IFITM3 could slow replication of HCV RNA (22). Brass et al. (7), however, did not observe an effect of IFITM3 on HCV replication. A 2011 study showed that IFITM1 blocks replication but does not interfere with the entry of VSV pseudotyped with HCV structural proteins (50). A third group was unable to reproduce the IFITM1 effect on RNA replication but did observe a marked effect on HCV entry into hepatocytes at least in part by relocalizing the HCV receptor CD81 into tight junctions, thereby disrupting subsequent coreceptor interactions (51). Consistent with this observation, a physical association between CD81 and IFITM1 had been established before the antiviral activities of IFITM1 were appreciated (10). Thus, the effect of IFITM1 on HCV replication appears to be cell-type dependent and, by interfering directly with HCV receptors and coreceptors, operates through a mechanism distinct from that of IAV restriction.

Respiratory Syncytial Virus

Everitt et al. (57) noted that *Ifitm3*$^{-/-}$ mice are more susceptible to RSV infection than their wild-type littermates. The scale of this effect is less dramatic than in parallel studies with nonpathogenic IAV, although given the different pathologies produced by IAV and RSV, such comparisons are imperfect. Like HIV-1, RSV fuses at the plasma membrane. Therefore, RSV, and perhaps HIV-1 and HCV, may be an exception to the general rule that viruses fusing late in the endocytic pathway are most susceptible to IFITM-mediated restriction. However, RSV restriction has not been examined in cultured cells, and it is possible that IFITM proteins protect mice from RSV challenge via means other than inhibition of viral entry. An alternative explanation for this protection is that, in vivo, Ifitm3 appears to be concentrated on the apical plasma membranes of ciliated respiratory epithelial cells, the principal site of RSV entry (34, 52).

Reoviruses

IFITM3 restricts two strains of reovirus (53), which is the only nonenveloped virus known to date to be restricted by an IFITM protein. Reoviruses enter into the cytoplasm of host cells by

perforating late endosomal membranes (54). Like filoviruses (43) and SARS-CoV (42), reoviruses require acid-dependent cathepsins to remove outer-capsid proteins to allow delivery of the viral core into the cytoplasm (55). Protease-independent infectious subvirion particles of reovirus bypass IFITM3 restriction in a manner similar to SARS-CoV, which can bypass IFITM protein–mediated restriction when its entry protein is exposed to exogenous proteases (21, 53, 56). It is not known whether IFITM1 or IFITM2 restricts reovirus replication more or less efficiently than IFITM3. Nonetheless, these observations provide evidence that IFITM proteins target a process common to enveloped and nonenveloped viruses.

Intramembrane domain: a region of a protein that enters and exits the lipid bilayer through the same leaflet without spanning the membrane

CD225 domain: the conserved region of IFITM proteins that includes the cytosolic loop and a portion of the intramembrane domain

Pathogens Not Restricted by IFITM Proteins

A host of viruses and pseudoviruses are not restricted by IFITM proteins. These include murine leukemia virus; every arenavirus tested thus far, including LASV, lymphocytic choriomeningitis virus, and MACV; every alphavirus assayed thus far, including chikungunya virus, Sindbis virus, and Venezuelan encephalitis virus; and CCHFV (1, 7, 21, 46). In general, these enveloped viruses do not fuse in late endosomes or lysosomes. In addition, *Ifitm3*$^{-/-}$ mice are no more susceptible than wild-type mice to a range of bacterial and protozoan pathogens including *Citrobacter rodentium*, *Mycobacterium tuberculosis*, *Salmonella typhimurium*, and *Plasmodium berghei* (57).

IFITM PROTEIN STRUCTURE AND POSTTRANSLATIONAL MODIFICATIONS

Domains of the IFITM Proteins

The IFITM proteins have been divided into five domains reflecting their hydrophobicity and conservation (**Figure 2**) (7). These include a variable, hydrophobic N-terminal domain (NTD; human IFITM3 residues 1–57); a conserved hydrophobic intramembrane domain (IMD; residues 58–80; previously denoted IM1 or TM1); a conserved intracellular loop (CIL; residues 81–104); a variable, hydrophobic transmembrane domain (TMD; residues 105–126; previously denoted IM2 or TM2); and a short, highly variable C-terminal domain (CTD; residues 127–133). The CD225 domain (residues 58–108), present in many prokaryotic and eukaryotic proteins, encompasses the IMD and CIL domains.

IFITM Protein Topology

The topology of the IFITM proteins in the membrane has been a source of considerable debate. Although the CIL is intracellular (58), evidence for the orientations of the N and C termini is conflicting. Early literature proposed a type III, two-pass transmembrane topology with both N and C termini oriented toward the endoplasmic reticulum (ER) lumen or extracellular space (**Figure 3*a***). For example, early investigators noted that antibodies against the IFITM1 N terminus caused aggregation of leukemia cell lines, suggesting exposure of extracellular IFITM1 epitopes (14). Furthermore, labeled IFITM1 could be recovered by immunoprecipitation after enzymatic cell-surface radiolabeling (10). Moreover, several groups observed that IFITM proteins epitope tagged at either their N or C terminus could be recognized at the plasma membrane by flow cytometry (7, 45, 59). However, two studies provided persuasive evidence supporting localization of the IFITM3 NTD in the cytosol. Yount et al. (49) demonstrated ubiquitylation of Lys24 of the NTD of IFITM3, implying access of this residue to cytosolic ubiquitin ligases, and noted a lack of *N*-linked glycosylation of the NHT motif of the IFITM3 N terminus. Jia et al. (60)

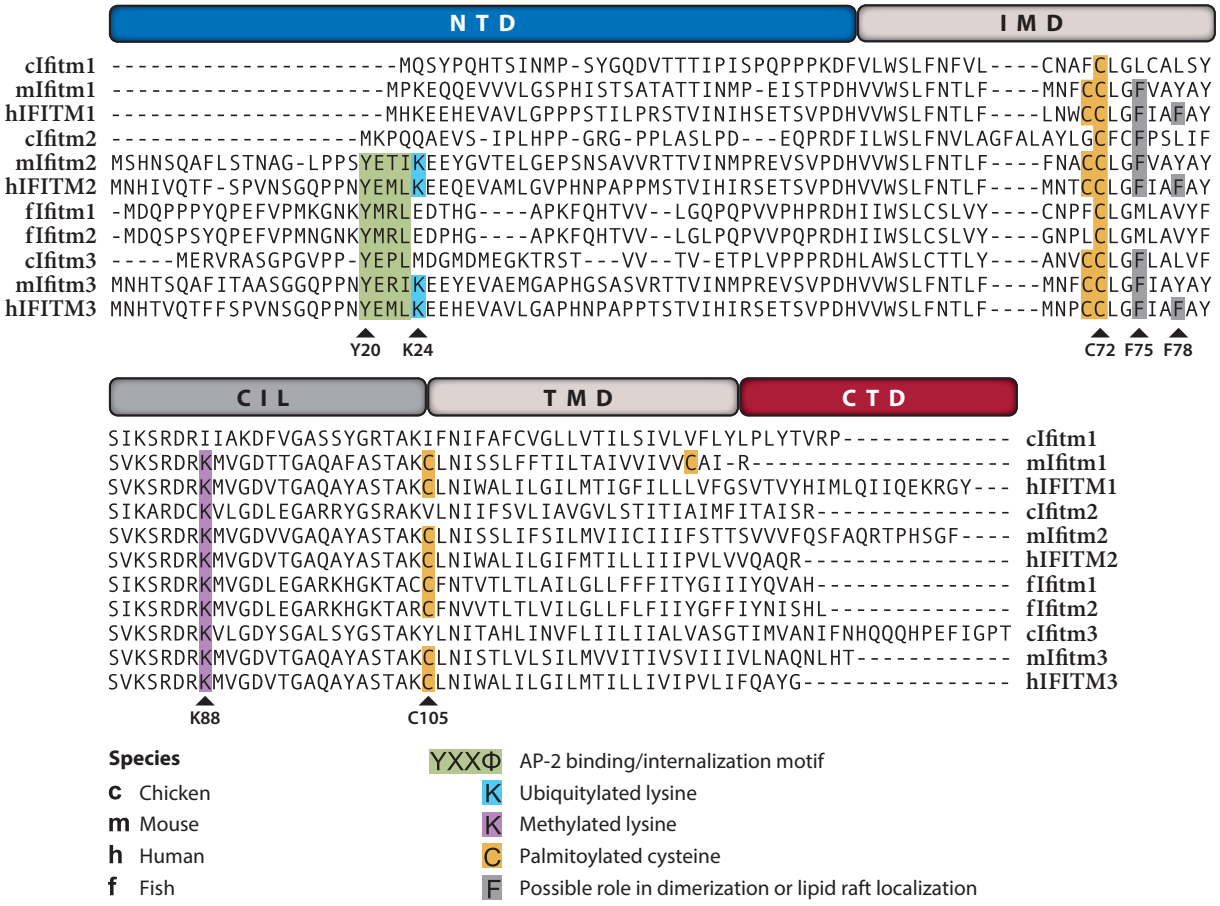

Figure 2

The topological domains of the IFITM proteins are shown above an alignment of immunity-related IFITM proteins of humans (h), mice (m), chickens (c), and fish (rainbow trout) (f). Domains consist of the N-terminal domain (NTD), intramembrane domain (IMD), conserved intracellular loop (CIL), transmembrane domain (TMD), and C-terminal domain (CTD). The junction of the TMD and CTD is poorly defined. Residues are numbered according to human IFITM3, and where applicable, highlighting of important amino acids has been extended to include orthologous residues in other species. The NTD is poorly conserved, but the NTDs of human IFITM2 and IFITM3 and their orthologs all contain YXXΦ motifs (*green*) that promote their internalization into endolysosomes (69). Phosphorylation of Tyr20 by Fyn kinase inhibits this internalization (60). Ubiquitylation of Lys24 in human IFITM3 (*blue*) promotes its degradation (49). Palmitoylated cysteines (*orange*) are required for proper subcellular localization and restriction of viral entry (49). Two phenylalanines (*gray*) in the IMD have been proposed to promote dimerization (41). Methylation of Lys88 (*purple*) negatively regulates restriction activity (64).

observed phosphorylation of IFITM3 Tyr20 and identified the kinase Fyn as the phosphorylating kinase, again consistent with the accessibility of the N terminus to cytosolic enzymes. Yount et al. also provided data that the IFITM3 C terminus is localized to the cytosol. These data included the capacity of a prenylation reporter to bind an artificial C-terminal CLVL motif (49), but the prenylation motif was positioned only in a construct that also included an artificial N-terminal myristoylation motif and mutation of the palmitoylated cysteine residues. Subsequently, Hach et al. (61) detected palmitoylation of a C-terminal cysteine of murine Ifitm1, which supported a cytosolic orientation of the C terminus. On the basis of these results, Yount et al. proposed a

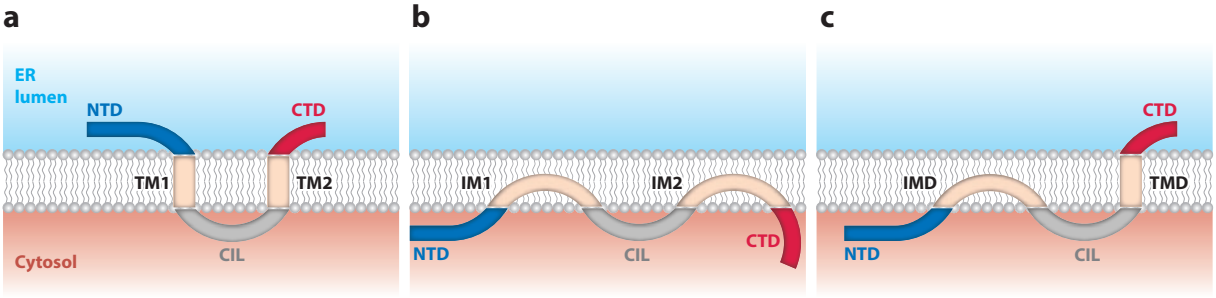

Figure 3

Three models of IFITM protein transmembrane topology. (*a*) The predicted type III transmembrane topology with ER-luminal N and C termini. In support of this model, several flow cytometry studies have localized IFITM1 or IFITM3 N termini to the cellular exterior (7, 45, 59, 62). (*b*) An intramembrane topology was proposed based on the findings of posttranslational modifications. Ubiquitylation and phosphorylation of NTD amino acids support cytosolic localization of the N terminus (49, 60). Palmitoylation of a C-terminal cysteine in the short TM2/IM2 region of mouse Ifitm1 is consistent with a cytosolic orientation of the C terminus (61). (*c*) More recent work has shown that murine Ifitm3 can adopt at least two different topologies. The predominant topology, however, has an intracellular N terminus and extracellular C terminus (62). Abbreviations: CIL, conserved intracellular loop; CTD, C-terminal domain; ER, endoplasmic reticulum; IMD, intramembrane domain (previously denoted as IM1 or TM1); NTD, N-terminal domain; TMD, transmembrane domain (previously denoted as IM2 or TM2).

topology in which both the NTD and CTD faced the cytosol, as did the CIL, and both IM1 and IM2 insert into but do not traverse the lipid bilayer (**Figure 3*b***).

A recent reexamination of the topology of murine Ifitm3 confirmed through several means the cytosolic orientation of the NTD but demonstrated that the CTD localizes to the ER lumen (**Figure 3*c***) (62). Evidence for the latter included retention in the ER of an Ifitm3 variant with a C-terminal KDEL ER retention motif. In addition, markedly greater antibody recognition of C-terminal epitope tags relative to that of N-terminal tags was seen in nonpermeabilized cells. Following permeabilization, however, N- and C-terminal tags were recognized comparably. Finally, the IM2/TM2 region traverses the membrane when expressed in isolation. Collectively, these results suggest that IM2/TM2 is indeed a transmembrane domain and that Ifitm3 exists primarily as a type II transmembrane protein (62). Thus, IM1/TM1 is an intramembrane domain (i.e., the IMD), and IM2/TM2 is a transmembrane domain (i.e., the TMD). However, a minority fraction of expressed Ifitm3 is oriented in an alternative topology, with the NTD facing outward. It is unclear whether this topology is a complete inversion of the protein, or whether in this orientation both the NTD and the CTD face the ER lumen. The frequency of this minority topology is also not clear, and notably, it has been observed in only one of two cell lines tested. Nonetheless, the observation of two topologies helps to reconcile the older literature with clear evidence for a cytosolic orientation of the NTD.

Type II transmembrane protein: a protein with a cytosolic N terminus, an extracellular or ER-luminal C terminus, and a single membrane-spanning domain

Lipid rafts: cholesterol-rich microdomains of the cell membrane resistant to lysis by nonionic detergents

Posttranslational Modifications

IFITM proteins have two highly conserved and palmitoylated cysteines within the IMD (IFITM3 Cys71 and Cys72) and one more poorly conserved palmitoylated cysteine at the junction between the CIL and TMD (IFITM3 residue 105) (**Figure 2**). Cysteine palmitoylation is necessary for the restriction activity of IFITM3, with palmitoylation of Cys72 playing an especially important role (49, 63). It is likely that these palmitoyl groups contribute to the stability of IFITM proteins, their appropriate subcellular localization, and their association with lipid rafts. The IFITM

proteins also have four lysines that can be ubiquitylated: one in the NTD (IFITM3 Lys24) and three in the CIL (Lys83, Lys88, and Lys104). Lys48 ubiquitin linkages are observed at all four positions but are most efficient at Lys24. Lys63-linked polyubiquitin is also observed at Lys24. In general, ubiquitin appears to interfere with the antiviral activity of IFITM3, and IFITM3 with ubiquitin is more stable and better localized to the endolysosomal sites of IAV fusion (49). It is thought that ubiquitin primarily functions to regulate IFITM3 expression, and indeed the extent of ubiquitylation observed may reflect its overexpression in HEK239T cells. It is noteworthy that all four conserved and potentially ubiquitylated IFITM3 lysines can be altered to alanine without significant loss of restriction activity. This observation suggests that their conservation is due to their role in tightly regulating IFITM protein levels and that IFITM protein overexpression may be deleterious in a physiologic context. Indeed, in some cell lines, overexpression of IFITM proteins can lead to enlarged vacuoles, consistent with dysregulation of vesicle trafficking. In addition, Lys88 is methylated by the lysine methyltransferase SET7, which inhibits IFITM3 activity for reasons that are unclear (64).

IFITM3 is phosphorylated at Tyr20 in the NTD (60) and, less efficiently, at Tyr99 in the CIL. Tyr20 is highly conserved in IFITM2 and IFITM3 but absent from IFITM1 and its orthologs (**Figure 2**). Phosphorylation of this residue regulates subcellular localization and restriction activity of IFITM3. Mutation of Tyr99 to alanine interferes with IFITM restriction of IAV but not DENV. This Y99A mutation, however, does not grossly alter IFITM3 localization, and DENV and IAV are thought to fuse in similar late endosomal vesicles (41).

Subcellular Localization of IFITM Proteins

Early reports provided conflicting evidence about the subcellular localization of IFITM proteins. They were first detected at the cell surface by flow cytometry or through radiolabeling and immunoprecipitation (10). An early set of confocal and immuno–electron microscopy experiments demonstrated colocalization of IFITM3 with MHC class I in the cytoplasmic vesicles of D10 and ME15 melanoma cell lines (65). A subsequent fluorescence microscopy study of LA7 rat mammary cells identified Ifitm3 at the plasma membrane, and further investigation showed an association with Lyn and Fyn kinases in lipid rafts (66). Subcellular distribution of these proteins varies depending on the cell or tissue type and expression level. In most monolayer cultures, IFITM proteins are concentrated in cytoplasmic vesicles that stain with late endosomal and lysosomal markers such as CD63 and LAMP2 (21, 67). Expression of high levels exogenous IFITM proteins appears to overwhelm the cellular machinery responsible for their internalization, resulting in plasma membrane–localized protein (**Figure 4*a***). IFITM protein overexpression also results in enlargement of endolysosomal organelles, a phenomenon best observed with IFITM1 (**Figure 4*b***) (21, 67). Confocal imaging of lung tissue from IAV-infected mice confirms the endosomal localization of Ifitm3 in vivo in most cell types, including type II pneumocytes, mesothelial cells, fibroblasts, and various leukocytes (**Figure 4*c***) (34, 38). Interestingly, the ciliated columnar cells of the upper airways are an exception to this rule; in these cells, Ifitm3 is localized almost entirely to the apical plasma membrane and cilia (**Figure 4*d***) (34). Although IFITM1, IFITM2, and IFITM3 all exhibit partial colocalization with endolysosomal markers such as CD63 (21, 67), IFITM1 appears to reside in subcellular compartments distinct from those of IFITM2 and IFITM3, with little or no overlap observed by microscopy (46, 47). Furthermore, the enlarged cytoplasmic vesicles that accompany overexpression of IFITM1 differ from those of IFITM3 as revealed by electron microscopy. IFITM1-associated vesicles are typically larger, and apparently empty (**Figure 4*e***). In contrast, IFITM3-associated endolysosomes

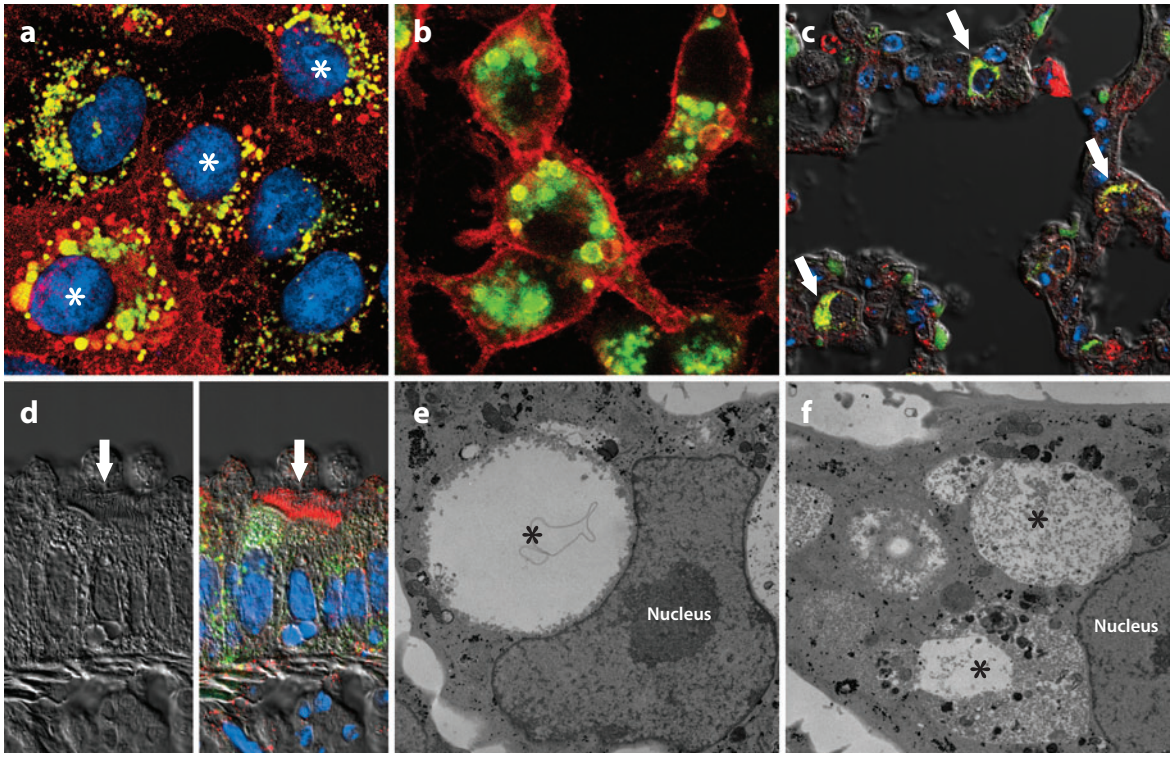

Figure 4

IFITM protein localization depends on the particular IFITM protein, cell type, and expression level. (*a*) A549 cells were transduced with an N-terminally Myc-tagged IFITM3 construct. Myc-IFITM3 (*red*) colocalizes with endogenous CD63 (*green*) in cytoplasmic puncta. Not all IFITM3-positive vesicles are CD63 positive, and not all CD63-positive vesicles are IFITM3 positive. In cells expressing high levels of the protein (*asterisks*), IFITM3 is visible at the plasma membrane. (*b*) A549 cells were transduced with Myc-tagged IFITM1 and stained for Myc (*red*) and endogenous LAMP2 (*green*). IFITM1 expression caused marked enlargement of the LAMP2-positive vesicles. (*c*) Laser scanning confocal microscopy of influenza A virus (IAV)-infected mouse lung parenchyma revealed that alveolar type II pneumocytes (*arrows*) express Ifitm3 (*red*) on lysosome-related organelles [lamellar bodies marked by DC-LAMP (*green*)]. Punctate cytoplasmic distribution of Ifitm3 also is seen in adjacent type I pneumocytes. (*d*) Ifitm3 is localized to the cilia and apical plasma membrane of ciliated columnar epithelial cells of the upper airways. The left panel shows a phase-contrast image of two ciliated cells (*arrows*) from a bronchus of an IAV-infected mouse. In the right panel, staining for Ifitm3 (*red*) and lysosomal antigen MAC-3 (*green*) has been superimposed onto the image. (*e*) Electron micrograph (5,000× magnification) of a Vero E6 cell overexpressing human IFITM1. The enlarged vesicles (*asterisk*) are mostly empty. (*f*) Electron micrograph (5,000× magnification) of a Vero E6 cell overexpressing human IFITM3. The enlarged vesicles (*asterisks*) are packed with round membranous structures consistent with intraluminal vesicles.

contain intraluminal vesicles and other debris, similar to multivesicular bodies (**Figure 4*f***) (68).

YXXΦ motif: a four amino acid motif that marks proteins as cargo for clathrin-mediated intracellular transport pathways

IFITM2 and IFITM3 contain a highly conserved YXXΦ motif in their NTD (**Figure 2**) (60). This motif binds the clathrin adaptor protein AP-2 and promotes internalization (69). The comparatively short NTD of IFITM1 lacks this YXXΦ motif. Therefore, IFITM1 likely uses an alternative pathway for its internalization. In fact, IFITM1 shows partial colocalization with caveolin at the plasma membrane and coimmunoprecipitates with this protein (70). Thus, whereas IFITM2 and IFITM3 are internalized via clathrin-mediated endocytosis, IFITM1 may instead follow the caveolin pathway. The phosphorylation of Tyr20 of the IFITM3 YXXΦ motif by Fyn

kinase inhibits internalization of IFITM3 (60). Cell-type variability in Fyn kinase activity may therefore explain conflicting reports about the plasma membrane or endosomal localization of IFITM3.

Artificial modifications of the N terminus (epitope tagging) can also influence subcellular localization and restriction activity. For example, our attempt to engineer an N-terminal fluorescent fusion of IFITM3 resulted in a protein that exhibited exaggerated perinuclear localization. This construct retained full restriction activity against MARV but exhibited markedly attenuated restriction activity against IAV (C.C. Bailey, G. Zhong, I. Huang & M. Farzan, unpublished observation). C-terminal epitope tags do not appear to influence IFITM protein–mediated restriction activity. Because they are cleaved or degraded in acidic subcellular compartments, however, these tags have limited utility for colocalization or expression analysis (62).

MODELS AND MECHANISMS

Since the rediscovery of IFITM proteins as viral restriction factors, insights into possible mechanisms of action have begun to emerge. Perhaps the clearest advance in this direction was the observation that IFITM proteins inhibit not only replication of infectious viruses (e.g., IAV) but also cell entry of retroviral particles pseudotyped with the entry proteins of these same viruses (7). Importantly, retroviruses pseudotyped with the entry proteins of other viruses (e.g., MLV) are not inhibited by IFITM proteins (7, 21). Therefore, IFITM restriction must target a process mediated by the entry proteins of restricted viruses.

What, then, are the relevant differences between the entry processes of IFITM-restricted viruses (DENV, IAV, MARV, reovirus, RVFV, and VSV) and IFITM-insensitive viruses (LASV, MACV, and MLV)? One potential explanation is receptor downregulation; however, although not all receptors of restricted viruses have been identified, surface expression of the receptors for IAV (sialic acid) and SARS-CoV (ACE2) is not affected by IFITM protein expression (7, 21). Thus, receptor modulation alone cannot explain the entire spectrum of restriction. Another possible mechanism for restriction is that restricted viruses are not internalized into IFITM protein–expressing cells. Microscopy studies of IAV and reovirus internalization, however, have not identified any gross defects of virion trafficking into acidified endosomes (21, 53, 67).

A more compelling explanation is that IFITM proteins block viral entry at specific sites of fusion. Favoring this explanation, most IFITM-restricted viruses (e.g., IAV, MARV) are thought to fuse in a pH- or cathepsin-dependent manner, whereas IFITM-insensitive viruses (e.g., MLV) fuse at the plasma membrane. Exceptions to this rule include HIV-1, which uses a pH-insensitive entry pathway but is partially restricted by IFITM3 (47), and the IFITM-insensitive arenaviruses (e.g., LASV, MACV), which use pH-dependent entry processes (21, 71). However, despite dependence on factors like cathepsins or low pH, different viruses are internalized via different endocytic pathways. For example, DENV uses a clathrin-mediated endocytosis pathway (72), but LASV can apparently enter cells using clathrin- and caveolin-independent mechanisms (73, 74).

The site-of-fusion model is intriguing but raises additional questions. For example, does restriction require localization of IFITM proteins to the sites of viral fusion? Evidence suggests that it might. Jia et al. (60) noted that mutation of the NTD YXXΦ motif of IFITM3 inhibits its internalization into endolysosomal compartments and impairs its restriction activity against IAV. Feeley et al. (67) observed that IAV genomes accumulate in IFITM3-positive vesicles in infected cells, implying that these compartments are a dead end for viral endocytosis. Also in support of a location-dependent mechanism is the observation that the IFITM-restricted viruses SARS-CoV and reovirus, which use entry processes that require acid-dependent proteases, become IFITM insensitive if the protease dependence is circumvented experimentally (21, 53). Similarly, IFITM1

and IFITM3, which localize to different subcellular compartments (46, 47), also possess distinct patterns of viral entry restriction. IFITM1 is a potent inhibitor of filovirus entry but exhibits less efficient restriction of IAV. IFITM3 shows the opposite pattern, restricting IAV more potently than MARV or EBOV (21). A simple explanation for these differences in restriction activity is the different localization of these IFITM proteins.

Evidence against a localization-dependent mechanism of restriction includes the absence to date of any evidence that redirecting IFITM proteins to the plasma membrane increases restriction of viruses that fuse there. For example, in their study of IFITM3 NTD variants that mislocalized to the plasma membrane, Jia et al. (60) noted no gain of activity against HIV-1. MACV poses another such paradox. MACV presumably follows the route of its cellular receptor, transferrin receptor 1, as it is internalized via clathrin-mediated endocytosis. Transferrin, however, appears in the IFITM2- and IFITM3-positive endosomes of HEK293 cells within 10 min of its addition to culture (47), yet neither IFITM2 nor IFITM3 restricts MACV in HEK293 cells (21). Similarly, LASV fusion is thought to occur in multivesicular bodies—compartments enriched in IFITM3—but LASV entry is not restricted by IFITM proteins (74).

Considering these observations in aggregate it appears that (*a*) IFITM proteins probably act at the site of fusion; (*b*) they are, for whatever reason, less effective at the plasma membrane; and (*c*) there may be occasional exceptions to rules *a* and *b*. Accepting these premises, how exactly do IFITM proteins inhibit viral entry? We envision two possibilities. First, IFITM proteins may alter properties of the endolysosomal lumina, rendering these compartments inhospitable to viral fusion. This explanation is attractive because it would explain the relative failure of IFITM proteins to inhibit viral entry at the plasma membrane. Evidence in favor of this model comes from the study of C-terminally epitope-tagged murine Ifitm3. C-terminal Ifitm3 epitope tags are cleaved upon exposure to the lysosomal lumen. Imaging of cells expressing doubly N- and C-terminally tagged Ifitm3 revealed that in most cytoplasmic vesicles, C-terminal epitope tags are cleaved from Ifitm3, but these tags are retained in the large, perinuclear vesicles that accompany IFITM protein overexpression (62). This finding suggests a loss of proteolytic activity in these compartments. However, the physiologic relevance of these enlarged, perinuclear compartments is not clear, which questions the significance of this observation.

In further support for this model, IFITM proteins inhibit syncytium formation induced by cell-expressed viral entry proteins (59). Although there may be fundamental differences between the mechanisms of virus-cell fusion and cell-cell fusion, these observations suggest that IFITM proteins can be functional at the plasma membrane. Other investigators have shown, using pH-sensitive dye studies and ex vivo enzymatic activity assays, that endosomal acidification and cathepsin activity are not inhibited by IFITM protein expression (21, 67). These results should be interpreted with caution, however, as total acidic compartment volume and bulk cathepsin activity are not necessarily indicative of the microenvironment in the lumina of IFITM protein–positive vesicles. Ideally, these properties would be measured by microscopy of living cells, but difficulties in engineering physiologically relevant GFP-IFITM fusion proteins preclude this experimental approach.

A second explanation for IFITM protein activity, with mounting evidence in its favor, is that IFITM proteins alter physical properties of cellular membranes to inhibit the formation or expansion of fusion pores. Using fluorescence lifetime imaging microscopy (FLIM), Li et al. (59) found that overexpression of IFITM proteins causes loss of fluidity of host cell membranes. Lin et al. (75) obtained similar results using fluorescence recovery after photobleaching (FRAP) microscopy to show that IFITM1 inhibits lateral diffusion of plasma membrane proteins. One possible mechanism for these observations was provided by Amini-Bavil-Olyaee et al. (68), who found that IFITM3 overexpression resulted in the accumulation of cholesterol in the membranes of

CD63- and LBPA-positive cellular compartments, which are presumably late endosomes. This group also identified a physical interaction between IFITM3 and VAPA (vesicle-associated membrane protein–associated protein A), which was mapped to the IFITM3 TMD. VAPA is an ER-resident protein that modulates cholesterol homeostasis by interaction with oxysterol-binding proteins. The model is unusual in that the IFITM3-VAPA interaction was mapped to the poorly conserved TMD of IFITM3 and that the ER-resident VAPA and endosomal IFITM3 would not be expected to interact in intact cells. However, the IFITM3-mediated cholesterol enrichment in endolysosomal membranes is robust and has been independently confirmed (75). Reports currently differ regarding the importance of the amino acid sequence of the TMD. For example, replacement of the IFITM3 TMD with the transmembrane domains of type I (CD4) or type II (transferrin receptor) transmembrane proteins resulted in a near-complete loss of IAV restriction activity (68). However, alanine-scanning mutagenesis of the IFITM3 TMD failed to identify any mutants with diminished restriction activity against IAV, even when blocks of six consecutive residues were replaced by alanines (41). Overexpression of VAPA alone does appear to abrogate IFITM3-mediated viral entry restriction, but again, reports differ about the magnitude of the effect (68, 75).

Lin et al. (75) found that treatment of cells with the antifungal compound amphotericin B counteracts IFITM2/3-mediated restriction of IAV but has little or no effect on IFITM1-mediated viral entry restriction. The same phenomenon was observed following treatment with the antifungal agent nystatin. Amphotericin B and nystatin are polyene antifungals that share a putative mechanism: They bind membrane sterols (with a preference for the fungus-specific molecule ergosterol) and form ion leak channels. Lin et al. performed a thorough analysis of this phenomenon to identify the mechanism by which polyenes prevent IFITM3-mediated IAV restriction. Using fluorescent dyes, they noted that polyene antifungals abolish Na^+ gradients across endolysosomal membranes. However, when Na^+ gradients were disrupted by non-pore-forming ionophores, no effect on IFITM3 activity was detected. These findings suggest that Na^+ gradients do not contribute to IFITM restriction, but they do not formally exclude a role for other ion gradients. Lin et al. also investigated whether polyenes interfere with IFITM protein activity by disrupting cholesterol. They treated cells with a variety of cholesterol-modulating drugs including methyl β-cyclodextran, statins, and cholesterol itself. Ultimately, neither these treatments nor overexpression of VAPA mitigated the effect of amphotericin B on IFITM3 function. Thus, the mechanism underlying the effects of the polyenes remains unclear. It is possible that amphotericin B restores membrane fluidity, perhaps by disrupting a higher-order complex of IFITM proteins. Evidence for such a model could be obtained by FLIM studies of IFITM-expressing, amphotericin-treated cells, but results of such experiments have not been reported.

Other investigators have proposed that IFITM proteins may act alone. John et al. (41) demonstrated that HA-tagged IFITM3 coimmunoprecipitates with untagged IFITM3 molecules and that this interaction is lost through the combined mutation of IMD residues Phe75 and Phe78 to alanines. Furthermore, this mutant shows no IAV restriction activity. The authors proposed that, like other proteins with intramembrane domains, IFITM3 induces membrane curvature. This model suggests that IFITM3 dimers deform endosomal membranes and make fusion pore formation energetically unfavorable. This possibility is intriguing, especially considering that IFITM proteins from distantly related species (e.g., chickens) retain much of their activity in human cells, but there are caveats to the interpretation of these studies. For example, IFITM3 also coimmunoprecipitates with tagged IFITM1, yet in living cells these two proteins show little colocalization (46); this suggests that the observed interactions may occur following cell lysis. Another caveat arises from the use of 0.5% CHAPSO detergent in these coimmunoprecipitation studies. IFITM3 is localized to detergent-resistant microdomains (lipid rafts) and may coimmunoprecipitate nonspecifically with other lipid raft proteins (e.g., other IFITM3 molecules) following lysis with a

detergent as gentle as CHAPSO. Therefore, an alternative interpretation of these results is that Phe75 and Phe78 are required for lipid raft incorporation.

CONCLUDING REMARKS

Over the past several years, IFITM proteins have been recognized as key components of cell-intrinsic defense against several pathogenic viruses. In particular, IFITM3 and its orthologs play a direct and important role in controlling the severity of influenza in mice and likely in humans. These proteins also account for a surprisingly large fraction of the capacity of interferon-treated cells to suppress IAV replication. IFITM proteins are expressed constitutively by cells and tissues where their protective effects would be especially important, for example, barrier epithelial cells in the lung or lung-resident $CD8^+$ memory T cells. Based on cell culture data, IFITM3 is likely to make a similar contribution to reducing the severity of diseases caused by flaviviruses, including DENV and WNV, which appear to be internalized through pathways similar to those used by IAV. Similarly, IFITM1 or IFITM3 might contribute to the control of EBOV, reovirus, SARS-CoV, and various bunyaviruses through similar mechanisms. Multiple or alternative IFITM-mediated mechanisms might also assist in the control of HCV, HIV-1, RSV, and perhaps others. Of equal interest are those viruses that remain unaffected by expression of IFITM proteins. It remains unclear how these viruses evade restriction and why restricted viruses do not readily escape restriction through the same means.

Important questions about mechanisms of IFITM-mediated restriction are unanswered. There is a good deal of circumstantial evidence that IFITM proteins alter the site of fusion, but it remains unclear exactly how this occurs; whether these changes occur primarily in the lumina, membrane, or cytoplasm; and what cofactor and target proteins are involved. Beyond the puzzle of defining mechanisms, a final challenge is to translate these emerging insights into strategies to prevent and treat viral diseases. There are several means by which this might be accomplished. First, once we understand the mechanism of restriction, we might be able screen for compounds that mimic this activity. Second, we might find an interferon-independent means of inducing expression of IFITM proteins at the transcriptional level—also, ideally, with a small molecule. Third, extending from the observation that cytokines other than type I and II interferons, namely those that use the signaling receptor gp130, also stimulate IFITM expression (34), it might be possible to specify with more precision which cell types, or perhaps which IFITM proteins, can be expressed. This approach could bypass the undesirable or unpleasant effects of interferon administration. Another approach targets key animal intermediates in zoonosis. Transgenic chickens, ducks, or pigs overexpressing Ifitm3 in IAV target cells are likely to be viable and more resistant to influenza. Finally, as we begin to better understand how human polymorphisms in the *IFITM* locus contribute to varying responses to viral infections, we can respond more effectively with efforts to protect against or treat these infections.

SUMMARY POINTS

1. IFITM proteins are a family of type II transmembrane proteins that are present in vertebrates and share a highly conserved cytosolic loop.
2. These proteins restrict the cellular entry processes of a number of viruses (EBOV, DENV, IAV, RVFV, reovirus, and SARS-CoV) and inhibit the replication of other viruses (HCV and HIV-1) at a postentry step.
3. Viral entry restriction occurs following virion endocytosis but prior to membrane fusion.

4. IFITM protein expression is potently induced by interferons and acute-phase cytokines, but the expression of these proteins is constitutive in many cell types in vivo.
5. IFITM proteins exhibit distinct signatures of viral entry restriction activity; IFITM1, for example, is a potent inhibitor of EBOV entry but is less effective against IAV, whereas IFITM3 is highly effective against IAV but less so against EBOV.
6. In mouse models of influenza, deletion of *Ifitm3* increases the severity of disease; of five members of the murine *Ifitm* cluster, *Ifitm3* alone contributes to influenza resistance in vivo.
7. IFITM2, IFITM3, and, to a lesser extent, IFITM1 concentrate in endolysosomal organelles, but IFITM1 localizes to subcellular compartments distinct from those of IFITM2 and IFITM3.
8. IFITM protein overexpression causes loss of membrane fluidity and accumulation of cholesterol in endolysosomal membranes, suggesting that IFITM proteins may inhibit viral entry by altering the physical properties of host cell membranes.

FUTURE ISSUES

1. The high degree of conservation of the intracellular loop suggests the possibility of unidentified, shared cofactors for the IFITM proteins. Identification of such cofactors would provide insight into the mechanisms of viral entry restriction. Does such a cofactor exist, or is the loop conserved because some intrinsic structural motif is necessary for restriction activity?
2. A thorough analysis of virion trafficking in IFITM-expressing cells has yet to be completed despite the availability of pseudotyped retroviruses for studies of IFITM-mediated viral entry restriction. IAV virions, for example, appear to traffic into IFITM3-positive compartments and accumulate there. Do IFITM1-restricted filoviral particles likewise become arrested within IFITM1-positive compartments? Do IFITM-insensitive arenaviruses such as MACV traffic to IFITM-positive endosomes and fuse there despite the presence of IFITM proteins, or do they escape by fusing before encountering IFITM-positive compartments?
3. Although cell culture data suggest that IFITM proteins restrict a wide variety of pathogens, in vivo studies have focused on the effects of murine *Ifitm3* deletion. Are the other *Ifitm* proteins involved in the control of different pathogens in vivo?
4. Why don't IFITM-restricted viruses alter their entry processes to be more like viruses that avoid restriction? Are the entry processes of restricted viruses advantageous in other ways? What differences determine sensitivity or resistance to restriction?
5. Different groups have shown that IFITM protein overexpression results in the accumulation of cholesterol in endolysosomal compartments, but the physiologic relevance of this observation has not been established. Does interferon treatment also result in endolysosomal cholesterol accumulation? Do cells from wild-type and *IfitmDel*$^{-/-}$ mice fundamentally differ in their cholesterol content and distribution following interferon treatment? More generally, what is the mechanism of IFITM-mediated restriction?

6. Expression of IFITM proteins is constitutive in some cells and regulated in others by cytokines other than interferon. Can IFITM protein expression be induced by small molecules while avoiding the undesirable, pleiotropic effects of interferon? Can insight into the mechanism of restriction suggest other therapeutic approaches to viral infection?

DISCLOSURE STATEMENT

The authors are not aware of any affiliations, memberships, funding, or financial holdings that might be perceived as affecting the objectivity of this review.

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