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Identification of residues controlling restriction versus enhancing activities of IFITM proteins on the entry of human coronaviruses

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Running Title: IFITMs modulate human coronavirus entry

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ABSTRACT

Interferon-induced transmembrane proteins (IFITM) are restriction factors that inhibit the infectious entry of many enveloped RNA viruses. However, we demonstrated previously that human IFITM2 and IFITM3 are essential host factors facilitating the entry of human coronavirus (HCoV)-OC43. In a continuing effort to decipher the molecular mechanism underlying IFITM differential modulation of HCoV entry, we investigated the role of structural motifs important for IFITM protein post-translational modifications, intracellular trafficking and oligomerization in modulating the entry of five HCoVs. We found that three distinct mutations in IFITM1 or IFITM3 converted the host restriction factors to enhance the entry driven by the spike proteins of severe acute respiratory syndrome coronavirus (SARS-CoV) and/or Middle East respiratory syndrome coronavirus (MERS-CoV). First, substitution of IFITM3 tyrosine 20 with either alanine or aspartic acid to mimic the unphosphorylated or phosphorylated IFITM3 reduced its activity to inhibit the entry of HCoV-NL63 and 229E, but enhanced the entry of SARS-CoV and MERS-CoV. Second, substitution of IFITM3 tyrosine 99 with either alanine or aspartic acid reduced its activity to inhibit the entry of HCoV-NL63 and SARS-CoV, but promoted the entry of MERS-CoV. Third, deletion of carboxyl-terminal 12 amino acid residues from IFITM1 enhanced the entry of MERS-CoV and HCoV-OC43. These findings suggest that these residues and structural motifs of IFITM proteins are key determinants for modulating the entry of HCoVs, most possibly through interaction with viral and/or host cellular components at the site of viral entry to modulate the fusion of viral envelope and cellular membranes.

Importance

The differential effects of IFITM proteins on the entry of HCoVs that utilize divergent entry pathways and membrane fusion mechanisms even when using the same receptor make the HCoVs a valuable system for comparative investigation of the molecular mechanisms underlying IFITM restriction or promotion of virus entry into host cells. Identification of three distinct mutations that converted IFITM1 or IFITM3 from inhibitors to enhancers of MERS-CoV or SARS-CoV spike protein mediated entry reveals key structural motifs or residues determining the biological activities of IFITM proteins. These findings have thus paved a way for further identification of viral and host factors that interact with those structural motifs of IFITM proteins to differentially modulate the infectious entry of HCoVs.

INTRODUCTION

The interferon (IFN)-mediated innate immune response is the first line of defense against virus infections in vertebrates (1, 2). IFNs execute antiviral activity by binding to their cognate receptors on the cell surface to activate a signaling cascade leading to induction of hundreds of IFN-stimulated genes (ISGs) (3, 4). Among those ISGs, IFN-induced transmembrane (IFITM) proteins, including IFITM1, IFITM2 and IFITM3, are widely expressed and can be induced by all three types of IFNs in many cell types. The IFITMs localize at the cell plasma membrane and endocytic vesicles and restrict the entry of enveloped RNA viruses from nine viral families (5), including some medically important human pathogens, such as influenza A virus (IAV), dengue virus (DENV), West Nile virus, Zika virus, chikungunya virus, Ebola virus (EBOV), Rift Valley fever virus, human immunodeficiency virus (HIV) and hepatitis C virus (3, 6-15).

Coronaviruses (CoVs) are a large family of enveloped, positive-stranded RNA viruses with a broad host range and primarily cause respiratory or enteric diseases, but some of them cause hepatitis, neurological disorders or cardiomyopathy (16, 17). Human coronaviruses (HCoV) 229E, OC43, NL63, and HKU1 circulate globally and cause mild upper respiratory tract infections (18), but are occasionally associated with more severe lower respiratory tract diseases in the elderly and immunocompromised patients (19). On the contrary, the recently emerging HCoVs, such as severe acute respiratory syndrome coronavirus (SARS-CoV) and Middle East respiratory syndrome coronavirus (MERS-CoV), cause severe diseases among infected individuals (20, 21). Thus far, several groups have reported that IFITMs inhibited entry of HCoV-229E, HCoV-NL63, SARS-CoV and MERS-CoV into their host cells with varying efficiency (8, 22, 23).

Concerning the molecular mechanism underlying IFITM restriction of virus entry, the currently favored hypothesis postulates that existence of IFITM proteins in the endocytic membranes either alters the membrane curvature or fluidity to make the endosomal membrane rigid and harder to fuse with viral envelopes (24, 25) (26-28). However, several recent findings challenge this hypothesis. First, human IFITM2 and IFITM3 efficiently enhance the infectious entry of HCoV-OC43 *via* a post receptor binding/endocytosis mechanism (29). Second, mutation of the SVKS motif in the CD225 domain required for IFITM1 to inhibit HIV-1 entry and IFITM3 to restrict IAV and dengue virus infection (24, 30) enhances Lloviu virus (LLOV) glycoprotein-mediated entry (Fig. 11) (31). Third, human IFITM proteins are required for the formation of human cytomegalovirus virion assembly complex (VAC) and infectious virion secretion (32). The VAC is a perinuclear membrane structure where the vesicles with endosomal markers occupy the central area and the vesicles with the Golgi markers are wrapped around it to form a circle (33). It is possible that IFITMs modulate endosomal trafficking/fusion during the VAC formation. All those findings strongly suggest that the IFITM-induced membrane curvature and/or fluidity alterations may not always make the endocytic membranes too “rigid” to fuse, but at least under certain condition, may facilitate membrane fusion.

In order to further understand the molecular mechanism underlying the differential modulation of IFITM proteins on HCoV entry, we set out to identify structural motifs important for IFITM protein post-translational modifications, intracellular trafficking and oligomerization in modulating the entry of five HCoVs. We found that although both SARS-CoV and NL63 use angiotensin-converting enzyme 2 (ACE2) as their entry receptor, IFITMs differentially modulate the entry of these two viruses. We also found that three distinct mutations in IFITM1 or IFITM3 converted the host restriction factors to enhancers of SARS-CoV and/or MERS-CoV entry.

These findings imply that restriction or promotion of a virus entry by an IFITM may rely on its fine-tuned interaction with viral and host cellular factors *via* those structural motifs at the site of viral envelope and cellular membrane fusion. Moreover, post-translation modification of those structural motifs by host cellular factors may alter IFITM interaction with the components of viral entry machinery and consequentially change its potency and/or nature of modulating the entry of a virus.

RESULTS

Host cellular factors other than viral receptors have a strong impact on antiviral activity of IFITM proteins. Viral envelope proteins and cellular receptors are the major players of virus entry into their host cells. It is conceivable that IFITM interaction with viral envelope and/or cellular receptors may play an important role in restriction of virus entry. Angiotensin-converting enzyme 2 (ACE2), as the common receptor for both SARS-CoV and NL63, provides a unique opportunity to investigate the role of viral receptor in IFITM modulation of HCoV entry (34). Accordingly, we examined the effects of three human IFITMs proteins on the entry of four HCoVs, with lassa fever virus (LASV) and influenza A virus (IAV) as negative and positive controls, in HEK293 and hepatoma cell line Huh7.5 that express detectable and undetectable basal levels of IFITMs, respectively (29) (Fig. 1). As anticipated, expression of any of the three IFITMs in either HEK293 or Huh7.5 cells did not inhibit the infection of lentiviral particles pseudotyped with the envelope proteins of lassa fever virus (LASVpp) (Figs. 1B and 1C). However, all the three IFITMs significantly inhibited the infection of lentiviral particles pseudotyped with IAV hemagglutinin 1 (H1) and neuraminidase 1 (N1) (IAVpp), Spike protein

(S) of HCoV-229E (229Epp), HCoV-NL63 (NL63pp), SARS-CoV (SARSpp) and MERS-CoV (MERSpp) in both HEK293 (Fig.1B) and Huh7.5 cells (Fig. 1C). However, comparing the extent of IFITM inhibition between the two cell lines (Figs. 1B and C), IFITM2 and IFITM3 inhibition of IAVpp infection was 4- and 20-fold more potent, respectively, in HEK293 cells than that in Huh7.5 cells. On the contrary, IFITM2 and IFITM3 more efficiently inhibited entry of all four HCoVpp, particularly for MERSpp and 229Epp, in Huh7.5 cells. The steady state levels of IFITM1 were lower than that of IFITM2 and IFITM3 in both HEK293 and Huh7.5 cells, which may, at least in part, explain its lower activity to inhibit the infection of all the tested pseudoviruses, except for 229Epp. Interestingly, IFITM1 more potently inhibited 229Epp infection than IFITM2/3 in HEK293 cells, but was less effective in Huh7.5 cells. While the viral envelope proteins are obviously the primary determinants of the potency of IFITMs to restrict virus entry, the more potent inhibition by all three IFITMs on the infection of NL63pp than that of SARSpp suggests that host cellular factors other than viral receptors have a strong impact on antiviral activity of IFITMs.

Substitution of Y20 in IFITM3 enhances SARS-CoV and MERS-CoV entry. We next aimed at identifying IFITM structural motifs that control the modulation of HCoV entry. IFITM proteins contain a conserved CD225 domain flanked by sequence-divergent N- and C-terminal variable regions (5). The N-terminal 21 amino acid residues unique to IFITM2 or 3 has been demonstrated to be important for IFITM3 to inhibit IAV infection in cultured cells and *in vivo* in humans (35-38). It has been shown recently that the N-terminal region of IFITM3 contains a 20-YEML-23 tetrapeptide that is consistent with the canonical YxxΦ endocytic sorting signal (x can be any amino acid, Φ denotes Val, Leu, or Ile) (39-41). Furthermore, the Y20 can be

149 phosphorylated by tyrosine kinase Fyn, which regulates the IFITM3 trafficking and metabolism
150 (42). We thus investigated how the phosphorylation of IFITM3 at Y20 may regulate its function
151 of modulating HCoV entry. The results showed that compared to wild-type IFITM3, substitution
152 of Y20 with alanine (A) and aspartic acid (D) or glutamic acid (E) to mimic the non-
153 phosphorylated (Y20A) or phosphorylated (Y20E or Y20D) IFITM3, respectively, did not alter
154 the steady-state levels of expression (Figs. 2A and F) and activity to enhance the entry of HCoV-
155 OC43 in both HEK293 and Huh7.5 cells (Figs. 2B and G). However, the mutant IFITM3
156 proteins showed significantly reduced activity to inhibit NL63pp and 229Epp infection (Figs. 2C,
157 D, H and I). On the contrary, mutant IFITM3 proteins enhanced the infection of SARSpp and
158 MERSpp in both cell lines (Figs. 2C, E, H and J). Consistent with previous reports (39, 40),
159 wild-type IFITM3 was accumulated in the perinuclear region and primarily co-localized with
160 Rab9, a later endosome marker (43) (Fig. 3A). In contrast, IFITM3 proteins bearing Y20A or
161 Y20D mutation were primarily accumulated in the regions closed to the plasma membrane (Fig.
162 3B and 3C). These results indicate that Y20 is critical for endocytic sorting, which is regulated
163 by tyrosine phosphorylation.

164 In order to investigate whether the enhanced infection of SARSpp and MERSpp by the
165 mutant IFITM3 proteins is due to the induction of membrane fusion on the plasma membrane,
166 we examined the effect of endosomal pH on HCoVpp infection of Huh7.5 cells expressing wild-
167 type or Y20A IFITM3 (Fig. 4). As shown in Figs. 4A, among the five tested HCoVpp, MERSpp
168 and NL63pp infection were less sensitive to NH_4Cl treatment that elevates endosomal pH,
169 suggesting that the membrane fusion for those two viruses may occur in early endosomal
170 compartments. Interestingly, IFITM3 Y20A-enhanced SARSpp and MERSpp infection were
171 efficiently inhibited by NH_4Cl treatment in a concentration-dependent manner (Figs. 4B and C).

The results thus suggest that although Y20 mutant IFITM3 proteins are primarily accumulated in the plasma membrane regions, the enhanced infection of SARSpp and MERSpp still occurs in low pH endosomal compartments.

Substitution of Y99 in IFITM3 enhances MERS-CoV entry. In addition to Y20, Y99 had been shown to be phosphorylated in cells by mass spectrometry analysis and play a role in restricting the infectious entry of IAV, but not dengue virus (24). Therefore, we performed phosphomimetic analysis on this amino acid residue. As shown in Fig. 5A, the Y99A or Y99D mutant IFITM3 was expressed to a level similar to that of wild-type IFITM3. Compared with wild-type IFITM3, Y99A or Y99D mutants had a slightly increased activity to enhance OC43pp infection, but significantly reduced activity to inhibit the infection of SARSpp, NL63pp, IAVpp and VSVpp (Figs. 5B to E). Intriguingly, both Y99A and Y99D IFITM3 enhanced MERSpp infection by approximately 10-fold (Fig. 5F). The results imply that Y99 plays a critical role in IFITM3 modulation of the entry of different HCoVs.

Oligomerization of IFITM3 is essential for its suppression of the entry of HCoVs, except for NL63. In addition to phosphorylation, the function of IFITM proteins is regulated by cysteine palmitoylation (44, 45), ubiquitination (46, 47) as well as homo- and hetero-oligomerization (24, 29). To investigate the role of these post-translational modifications in IFITM3 inhibition of HCoV entry, HEK293 cell lines inducibly expressing wild-type or mutant IFITM3 proteins bearing mutations that preclude the cysteine palmitoylation or ubiquitination were established. Specifically, the conserved cysteine residues C71 and C72 or one additional cysteine C105, that are critical for IFITM3 palmitoylation, were substituted with alanine to yield

two mutants, IFITM3/2CA and IFITM3/3CA, respectively. As shown in Figs. 6A and 6B, the mutations had minimal impacts on protein expression, but completely abolished activity to restrict the infection of all the five pseudoviruses sensitive to IFITM3. However, IFITM3/4KA, that was generated by substitution of K24, K83, K88 and K104 with alanines, accumulated in a significantly reduced level in cells and failed to inhibit the infection of all the pseudoviruses examined. Moreover, IFITM3 containing F75A and F78A mutations (IFITM3/2FA), that disrupt its oligomerization (29), completely lost ability to inhibit the infection of SARSpp, 229Epp, MERSpp, and IAVpp, but could still significantly inhibit the infection of NL63pp, despite reduced activity (Fig. 6B). The results thus suggest that unlike other viruses (24, 29), suppression of NL63 spike protein triggered membrane fusion does not absolutely require the oligomerization of IFITM3.

C-terminal domain of IFITM1 differentially regulates the entry of HCoVs. We showed previously that sequential truncation of the C-terminal 18 amino acid residues from IFITM1 did not apparently affect its activity to inhibit the infection of IAVpp, but converted the antiviral protein to an increasingly potent enhancer of OC43pp infection (29). In our efforts toward further dissecting the role of C-terminal domain (CTD) in IFITM1 modulation of HCoV entry, we found that deletion of C-terminal 3, 6 or 9 amino acids did not apparently affect the activity of IFITM1 to inhibit the infection of SARSpp, but further deletion of C-terminal 12, 15 or 18 amino acids significantly compromised or abolished the ability of IFITM1 to inhibit the infection of SARSpp (Fig. 7B). In contrast, deletion of C-terminal 3, 6 and 9 amino acids enhanced the activity of IFITM1 to inhibit the infection of NL63pp by 5, 10 and 3 fold, respectively. However, further truncation of the C-terminal 12, 15 or 18 amino acids abolished the enhanced inhibitory

effect on NL63pp infection (Fig. 7B). Interestingly, sequential truncation of the CTD gradually attenuated and ultimately abolished the activity of IFITM1 to inhibit 229Epp infection (Fig. 7C). On the contrary, sequential truncation of C-terminal 12 amino acids gradually increased its activity to enhance MERSpp infection, but further deletion of C-terminal 15 or 18 amino acids reduced its activity to enhance MERSpp infection (Fig. 7D).

To rule out the potential interference of endogenous IFITM proteins on mutant IFITM1 in HEK 293 cells (29), we further confirmed the observation in Huh7.5 cells that express undetectable endogenous IFITM proteins. Four Huh7.5 cell lines were established by transduction with empty retroviral vector or retroviral vector expressing wild-type IFITM1, IFITM1/TC6 or IFITM1/TC18 protein. Consistent with the observations made in HEK293 cells, deletion of C-terminal 18, but not 6, amino acids significantly compromised IFITM1 to suppress SARSpp infection and deletion of C-terminal 6, but not 18, amino acids significantly enhanced activity to inhibit NL63pp infection (Fig. 8B). In agreement with the results obtained with NL63pp infection, we also observed that deletion of C-terminal 6, but not 18, amino acids significantly increased the ability of IFITM1 to inhibit the infection of HCoV-NL63, as judged by significant reduction in infected cell percentage and intracellular viral RNA (Figs. 8C and D).

Taken together, the results suggest that two partially overlapped functional motifs exist in the CTD of IFITM1. While its C-terminal 9 to 12 amino acid residues contain a motif that down-regulates the antiviral activity against HCoV-NL63 and suppresses the enhancing activity to MERS-CoV infection, the motif located in the N-terminal 9 amino acid residues of the CTD is important for IFITM1 to suppress SARS-CoV and HCoV-229E entry.

To investigate the molecular mechanisms underlying the differential modulation of HCoV entry by the CTD, we examined the subcellular localization of wild-type and mutant

IFITM1 proteins. As shown in Fig.9, similar to wild-type IFITM1, IFITM1/TC6 and IFITM1/TC18 are primarily accumulated in regions close to or at the plasma membrane. However, a modestly increased intracellular localization of both IFITM1/TC6 and IFITM1/TC18 is evident. Specifically, IFITM1/TC6 tends to more frequently co-localize with Rab5 and Rab9, whereas IFITM1/TC18 is more frequently co-localized with EEA1, an early endosomal marker (48), and Rab9, a later endosomal marker. Moreover, as shown in Fig. 10, compared to wild-type IFITM1, IFITM1/TC6, but not IFITM1/C18, demonstrated reduced mono- and di-ubiquitination. As anticipated, substitution of four residues of lysine in IFITM3 (IFITM3/4KA) completely abolished ubiquitination. The results thus indicate that the CTD of IFITM1 contains structural motif(s) that regulates its subcellular trafficking and ubiquitination, which may consequentially affect its activity to modulate the entry of HCoVs.

DISCUSSION

In spite of the relatively broad spectrum of antiviral activities, IFITM proteins do not restrict the infection of MLV, Sendai virus and several members of *Arenaviridae* family as well as all the DNA viruses tested thus far (6, 8, 49, 50). The molecular determinants that control the viral specificity and potency of IFITMs remain to be fully understood. Because viral envelope proteins and cellular receptors are the two major players of viral membrane fusion, it is plausible to consider that modulating the interaction between viral envelope proteins and cellular receptors might be the key mechanism of IFITM restriction of virus entry. Indeed, it was reported recently that IFITM sensitivity of HIV-1 strains is determined by the co-receptor usage of viral envelope glycoproteins (51, 52). However, the difference in the potency and requirement of IFITM

oligomerization in inhibition of SARSpp and NL63pp infection (Figs. 1 and 6) and particularly, the results that IFITM3/Y20 phosphomimetic mutations only enhanced the infection of SARSpp, but not NL63pp (Fig. 2) strongly suggest that IFITM interaction with viral and host cellular factors, other than viral receptors, such as ACE2, play a critical role in IFITM modulation of virus entry. Furthermore, our findings that IFITM2 and IFITM3 promoted HCoV-OC43 infection, and three distinct mutations converted IFITM1 or IFITM3 from inhibitors to enhancers of SARS-CoV and/or MERS-CoV spike protein-mediated entry challenge the "rigid" membrane hypothesis and suggest that IFITM proteins may also promote the membrane fusion, under selected conditions, to facilitate virus entry.

Based on those new findings, we hypothesize that depending on the fine-tuned interaction with the entry machinery of a given virus, which consists of viral envelope components as well as viral receptors and other host entry factors at the site of membrane fusion, IFITM proteins can either promote or arrest the fusion between viral envelope and endosomal membranes (29). It is possible that the three structural motifs identified herein mediate interactions with key host factors to determine either to arrest or to enhance the membrane fusion. Along this line, recent studies revealed that zinc metallopeptidase STE24 forms complexes with IFITM proteins and is required for IFITMs to inhibit the entry of many different viruses (53). In addition, the sensitivity of IAVs to IFITM3 appears to depend on the pH value at which the viral HA undergoes a conformational transition and mediates membrane fusion (54). More interestingly, IFITM expression promotes the uptake of avian sarcoma leukosis virus (ASLV) and the acidification of endosomal compartments, resulting in an accelerated membrane fusion when driven by the glycosylphosphatidylinositol-anchored, but not by the transmembrane isoform of the ASLV receptor (55). These recent findings clearly highlight the regulation of multiple viral and host

287 cellular components on IFITM modulation of the fusion between viral envelope and endosomal
288 membranes during viral infections.

289 Our new hypothesis predicts that in order to modulate virus entry, IFITM proteins ought
290 to be at the site of viral membrane fusion (26-28). Indeed, previous studies demonstrated in a
291 variety of virus infection systems that localization at the subcellular compartment where a virus
292 enters into the cytoplasm is important for the IFITM protein to inhibit its infectious entry (5). For
293 instance, disruption of the canonical endocytic signal in IFITM3 by Y20A/E/D mutations
294 resulted in its plasma membrane accumulation (Fig. 11). As a consequence, the mutant IFITM3
295 demonstrated a reduced antiviral activity against IAV that enter cells by fusion with lysosomal
296 membrane (39-41), but enhanced the activity to restrict the infection of parainfluenza virus 3, a
297 virus that enters cells by fusion with the plasma membrane (56). However, the subcellular
298 localization of IFITM proteins is not always strictly correlated with antiviral activity. For
299 example, while IFITM3 Y99A mutation does not apparently alter the subcellular localization, the
300 mutation significantly compromises the antiviral activity of IFITM3 against IAV, but not dengue
301 virus (24). In this study, we further demonstrated that although Y20A mutant IFITM3 was
302 predominantly accumulated in the plasma membrane regions, its enhanced infection of SARSpp,
303 MERSpp and OC43pp was still low pH dependent (Fig. 4) (29), suggesting that the enhanced
304 entry of those viruses still occurs in low pH intracellular endosomal compartments. The apparent
305 contradiction between the subcellular localization of the mutant IFITM3 proteins and site of
306 membrane fusion implies that a small fraction of the mutant IFITM3 might still traffic to the sites
307 where the viral spike protein-induced membrane fusion occurs. In addition, phosphomimetic
308 analyses suggest that Y20 or Y99 tyrosine phosphorylation regulates the metabolism, trafficking
309 and biological function of IFITM3.

In addition to tyrosine phosphorylation, IFITM3 can also be post-translationally modified at more than 8 different amino acid residues with at least three different types of modifications, including palmitoylation, ubiquitination and methylation (42, 44-47, 57). Our mutagenesis studies showed that both palmitoylation and ubiquitination are absolutely required for IFITM3 to modulate the entry of all the tested HCoVs (Fig. 6). Moreover, oligomerization of IFITM proteins has been demonstrated to be essential for their restriction of IAV and dengue virus infection as well as enhancement of HCoV-OC43 infection (24, 29). Interestingly, hetero-oligomerization between IFITM1 and IFITM3, that inhibits and enhances HCoV-OC43 infection, respectively, antagonize each other's functions (29). In this study, we further revealed that IFITM3 bearing F75A and F78A mutations that disrupt its oligomerization completely lost its ability to inhibit the infection of SASRpp, 229Epp and MERSpp, but still partially inhibited NL63pp infection (Figs. 6 and 11). While the results reinforce the notion that oligomerization is important for IFITMs to modulate the entry of many viruses, NL63 appears to be an exception.

It was reported previously that the CTD of IFITM1, illustrated in Fig. 11, plays an important role in modulating the entry of HCoV-OC43 (29), HIV1 (58), jaagsiekte sheep retrovirus and 10A1 amphotropic murine leukemia virus (59). In this study, we showed that the CTD of IFITM1 plays distinct roles in modulating the entry of different HCoVs. It appears that the CTD contains two overlapping functional motifs. While the C-terminal 9 and 12 amino acid residues negatively regulate IFITM restriction of HCoV-NL63 entry and enhancement of MERS-COV infection, the motif located in the N-terminal 9 amino acid residues of the CTD is important for IFITM1 to suppress SARS-CoV and HCoV-229E entry. In search for the underlined mechanism, a study identified a dibasic ^{122}KRxx^{125} motif at the C-terminus of IFITM1 regulates IFITM1 intracellular trafficking with reduced localization in LAMP1-positive

lysosomes, but increased levels in CD63-positive multivesicular bodies (59). IFITM1 binds to cellular adaptor protein complex 3 (AP-3), an association that is lost when the dibasic motif is altered (59). However, we found that partial or complete deletion of the CTD does not dramatically alter its subcellular distribution (Fig. 9). Instead, deletion of 6, but not 18 amino acid residues from the C-terminus reduced IFITM1 ubiquitination (Fig. 10). The results collectively indicate that the CTD is a key regulator of IFITM function. However, its effects on the entry of different viruses imply a versatile function of the CTD. Further investigation into the structure, post-translational modification and membrane topology of the CTD as well as identification of the cellular and/or viral proteins interacting with the CTD will shed light on the mechanism by which the CTD regulates the function of IFITM1.

In summary, we demonstrated in this study that in addition to viral envelope proteins and cellular receptors, IFITM protein oligomerization, post-translational modification and intracellular trafficking, which can be regulated by host cellular pathobiological cues, play critical roles in determining the extent and nature of IFITMs to modulate the entry of HCoVs. More importantly, identification of the three structural motifs that reverse the function of IFITM1 and IFITM3 on virus entry paves the way for uncovering viral and host factors that interact with those structural motifs to differentially modulate the infectious entry of HCoVs and other viruses (53).

MATERIALS AND METHODS

Cell lines, viruses, and antibodies. LLC-MK2 cells were cultured in minimal essential medium (MEM), which was prepared by mixing Hanks MEM (Invitrogen, Cat. No. 11575-032) and Earle's MEM (Invitrogen, Cat. No. 11095-080) in 2:1 ratio, and supplemented with 10% heat-

357 inactivated fetal bovine serum (FBS) (Invitrogen). Huh7.5 cells were cultured with Dulbecco's
358 modified Eagle's medium (DMEM) [Corning] supplemented with 10% FBS, 1x nonessential
359 amino acids (Invitrogen) and 2 mM L-glutamine (Invitrogen). Human colon cancer cell line
360 HCT-8 was grown in RPMI-1640 medium (ATCC, Cat. No. 30-2001) supplemented with 10%
361 FBS. GP2-293 and Lenti-X 293T cell Lines were purchased from Clontech and cultured in
362 DMEM supplemented with 10% FBS and 1 mM Sodium pyruvate (Invitrogen). FLP-IN T Rex
363 293 cells were purchased from Invitrogen and maintained in DMEM supplemented with 10%
364 FBS, 10 μg/ml blasticidin (Invitrogen) and 100 μg/ml Zeocin (Invivogen) (60). HCoV-NL63 was
365 purchased from ATCC through BEI Resource (Cat. No. NR470) and amplified in LLC-MK2
366 cells. Virus titers were determined by a plaque assay (29). Monoclonal antibody against FLAG
367 tag (anti-FLAG M2), rabbit anti-FLAG polyclonal antibody and β-actin antibody were purchased
368 from Sigma (Cat. No. F1804, F7425 and A2228, respectively). A mouse monoclonal antibody
369 against HCoV-NL63 nucleocapsid (N) protein was purchased from Ingenansa, Spain (Cat. No.
370 M.30.HCo.I2D4).

371

372 **Plasmids.** pcDNA5/FRT-derived plasmids expressing chloramphenicol acetyl transferase (CAT),
373 N-terminally FLAG-tagged human IFITM1, IFITM2 and IFITM3 as well as C-terminally
374 truncated IFITM1 mutants were reported previously (7, 60, 61) (29). Plasmids expressing N-
375 terminally FLAG-tagged mutant IFITM3 proteins with point mutations were constructed by
376 overlap extension PCR (60). All resulting plasmids were sequenced to verify the desired
377 mutation(s). N-terminally FLAG-tagged human IFITM1, IFITM2 and IFITM3 and their mutants
378 were also cloned into pQCXIP vector (Clontech) between the NotI and BamHI sites (29).

379 Plasmids expressing HCoV-OC43 spike (S) and HE proteins, VSV G protein, H1N1 IAV
380 (A/WSN/33) hemagglutinin (HA) and neuraminidase (NA), Ebola virus (EBOV) GP protein,
381 LASV GP protein, murine leukemia virus (MLV) envelope protein, HCoV-NL63 and SARS-
382 CoV spike protein were described previously (62, 63). MERS-CoV spike gene (GenBank
383 accession number AFS88936) was synthesized by GeneScript, cloned into pCAGGS vector, and
384 confirmed by DNA sequence analyses. Plasmid pNL4-3.Luc.R^-E^- was obtained through the NIH
385 AIDS Research and Reference Reagent Program (64, 65). Angiotensin I converting enzyme 2
386 (ACE2), aminopeptidase N (APN), and dipeptidyl peptidase-4 (DPP4) cDNA clones were
387 obtained from Origene, and cloned into a pcDNA3 vector (Invitrogen) to yield plasmid pcDNA3
388 /ACE2, pcDNA3 /APN and pcDNA3/DDP4, respectively (66).
389
390 **Package of pseudotyped retroviral particles.** The various viral envelope protein pseudotyped
391 lentiviruses bearing luciferase reporter gene as well as VSV G protein pseudotyped retroviruses
392 expressing wild-type and mutant IFITM mutant proteins were packaged as reported previously
393 (66, 67). Each pseudotype was titrated by infection of cells with a serial dilution of pseudotype
394 preparations. The modulation of IFITM on the transduction of a given pseudotype was
395 determined with a titrated amount of pseudotypes that yield luicferase signal between 10,000 to
396 1,000,000 light units per well of 96-well plates. For a given pseudotype, the input of pseudoviral
397 particles is consistent across all the experiments.
398
399
400 **Establishment of cell lines stably expressing wild-type and mutant IFITM proteins.** Huh7.5
401 cells in each well of 6-well plates were incubated with 2 ml of Opti-MEM medium containing

402 pseudotyped retroviruses and centrifuged at 20 °C for 30 minutes at 4,000×g. Forty-eight h post
403 transduction, cells were cultured with media containing 2 µg/ml of puromycin for two weeks.
404 The antibiotic resistant cells were pooled and expanded into cell lines stably expressing human
405 wild-type or mutant IFITM proteins (66). FLP-IN T Rex 293-derived cell lines expressing
406 mutant IFITM proteins in a tetracycline (tet) inducible manner were established as previously
407 described (7, 60).
408
409 **Western blot assay.** Cell monolayers were washed once with Phosphate Buffered Saline (PBS)
410 and lysed with 1× Laemmli buffer. An aliquot of cell lysate was separated on NuPAGE® Novex
411 4-12% Bis-Tris Gel (Invitrogen) and electrophoretically transferred onto a nitrocellulose
412 membrane (Invitrogen). The membranes were blocked with PBS containing 5% nonfat dry milk
413 and probed with the desired antibody. The bound antibodies were visualized with IRDye
414 secondary antibodies and imaging with LI-COR Odyssey system (LI-COR Biotechnology).
415
416 **Real-time RT-PCR.** Total cellular RNA was extracted by using TRIzol reagent (Invitrogen) and
417 reverse transcribed by using SuperScript III (Invitrogen). Quantitative PCR (qPCR) reaction was
418 performed as previously described on LightCycler 480II (Roche) with modified forward primer
419 [5`-AAA CCT CGT TGG AAG CGT GTTC-3`] and reverse primer [5`- CTG TGG AAA ACC
420 TTT GGC ATC-3`] under the following conditions: denature at 95 °C for 10 min and 45 cycles
421 of amplification (15 s at 95 °C and 1 min at 60 °C). The PCR reaction amplifies a fragment of
422 HCoV-NL63 N gene (GenBank Gene ID: 2943504).
423

Luciferase assay. T REX 293-derived IFITM-expressing cell lines were seeded into 96-well plates with black wall and clear bottom and transfected with plasmids encoding ACE2, APN, or DPP4 to express viral receptors. For Huh7.5-derived IFITM-expressing cell lines, cells were seeded into black wall 96-well plates. Cells were infected at 24 h post transfection or seeding with desired pseudotyped lentiviral particles for 2 h, and then replenished with fresh media. Two days post infection, the media were removed and cells were lysed with 20 µl/well of cell lysis buffer (Promega) for 15 min, followed by adding 50 µl/well of luciferase substrate (Promega). The firefly luciferase activities were measured by luminometry in a TopCounter (Perkin Elmer) (66).

Immunofluorescence To visualize HCoV-NL63 infected cells, the infected cultures were fixed with 4% paraformaldehyde for 20 min. After permeabilization with 0.1% Triton X-100, cells were stained with a monoclonal antibody recognizing HCoV-NL63 N protein (Ingenansa, Spain, Cat. No. M.30.HCo.I2D4). Bound antibodies were visualized by using Alexa Fluor 488-labeled goat anti-mouse IgG (Abcam, Cat. No. ab150113). Cell nuclei were counterstained with 4′,6-Diamidine-2′-phenylindole dihydrochloride (DAPI).

For determination of mutant IFITM1 or IFITM3 subcellular localization, T REX 293-derived cell lines inducibly expressing mutant IFITM proteins were fixed and permeabilized as described above. The cells were then stained with anti-FLAG monoclonal antibody together with a rabbit derived polyclonal antibody against EEA1 (Cell Signaling, Cat. No. 2411), Rab5 (Cell Signaling, Cat. No. 2143) or Rab9 (Cell Signaling, Cat. No. D52G8), respectively. The bound antibodies were visualized using Alexa Fluor 594-labeled goat anti-mouse IgG (red) and Alex Fluor 488-labeled goat anti-rabbit IgG (green). Cell nuclei were counterstained with DAPI.

Images were sequentially acquired on a FV1000 confocal microscope (Olympus) with a PlanApoN 60×/1.42 N objective (Olympus). Pinhole size was adjusted to 1 airy unit. Optimal diffraction-limited spatial resolution was obtained using a pixel size of 82 nm /pixel. DAPI was excited at 405 nm and its fluorescence emission collected between 430 nm and 470 nm. Alexa Fluor 488 was excited at 488nm and its fluorescence emission collected between 505 and 525 nm. Alexa Fluor 594 was excited at 543nm and its fluorescence emission collected between 560 and 660 nm. Negative controls were performed to make sure that there were no significant spectral bleed-through artifacts between channels.

$TCID_{50}$. Confluent LLC-MK2 cells cultured in 96-well plates were infected with 200 µl of OPTI-MEM containing a serial 10-fold dilution of viral stock for 2 h at 33 °C. Cells were then cultured at 33 °C with MEM with 2.5% FBS for 6 days. The cells of each well with cytopathic effect (CPE) were visualized and counted under microscope. The $TCID_{50}$ of virus stock was measured and converted to pfu/ml (68).

Immunoprecipitation To detect ubiquitination of IFITM protein and its mutants, IP-western blot was performed as reported previously (29, 44). Briefly, 293T cells were transfected with plasmids expressing FLAG-tagged IFITM1, IFITM3 and their mutants, respectively. Cells were lysed at 48 h post transfection with lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 and protease inhibitors (Roche) and immunoprecipitated with a monoclonal antibody against FLAG epitope followed by incubation with Protein A/G-Agarose beads (PIERCE) and washing with TBS buffer. The immunocomplexes were resolved in a NuPAGE® Novex 4-12% Bis-Tris Gel in MES buffer

(Invitrogen) and transferred on to a PVDF membrane. The membrane was probed with rabbit polyclonal antibody against Flag epitope (Sigma, Cat. No. F7425) or anti-ubiquitin rabbit polyclonal antibody (Proteintech, Cat. No. 10201-2-AP). The bound antibodies were visualized with IRDye secondary antibodies and imaged with LI-COR Odyssey system (LI-COR Biotechnology).

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REFERENCES

1. **Cullen BR, Cherry S, tenOever BR.** 2013. Is RNA interference a physiologically relevant innate antiviral immune response in mammals? Cell Host Microbe **14:**374-378.
2. **Chang J, Block TM, Guo JT.** 2012. The innate immune response to hepatitis B virus infection: implications for pathogenesis and therapy. Antiviral Res **96:**405-413.
3. **Diamond MS, Farzan M.** 2013. The broad-spectrum antiviral functions of IFIT and IFITM proteins. Nat Rev Immunol **13:**46-57.
4. **Bailey CC, Zhong G, Huang IC, Farzan M.** 2014. IFITM-Family Proteins: The Cell's First Line of Antiviral Defense. Annual review of virology **1:**261-283.
5. **Perreira JM, Chin CR, Feeley EM, Brass AL.** 2013. IFITMs restrict the replication of multiple pathogenic viruses. J Mol Biol **425:**4937-4955.
6. **Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ.** 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. Cell **139:**1243-1254.
7. **Jiang D, Weidner JM, Qing M, Pan XB, Guo H, Xu C, Zhang X, Birk A, Chang J, Shi PY, Block TM, Guo JT.** 2010. Identification of five interferon-induced cellular proteins that inhibit west nile virus and dengue virus infections. J Virol **84:**8332-8341.
8. **Huang IC, Bailey CC, Weyer JL, Radoshitzky SR, Becker MM, Chiang JJ, Brass AL, Ahmed AA, Chi X, Dong L, Longobardi LE, Boltz D, Kuhn JH, Elledge SJ, Bavari S, Denison MR, Choe H, Farzan M.** 2011. Distinct patterns of IFITM-mediated restriction of filoviruses, SARS coronavirus, and influenza A virus. PLoS Pathog **7:**e1001258.
9. **Mudhasani R, Tran JP, Retterer C, Radoshitzky SR, Kota KP, Altamura LA, Smith JM, Packard BZ, Kuhn JH, Costantino J, Garrison AR, Schmaljohn CS, Huang IC, Farzan M, Bavari S.** 2013. IFITM-2 and IFITM-3 but not IFITM-1 restrict Rift Valley fever virus. J Virol **87:**8451-8464.
10. **Anafu AA, Bowen CH, Chin CR, Brass AL, Holm GH.** 2013. Interferon-inducible transmembrane protein 3 (IFITM3) restricts reovirus cell entry. J Biol Chem **288:**17261-17271.
11. **Wilkins C, Woodward J, Lau DT, Barnes A, Joyce M, McFarlane N, McKeating JA, Tyrrell DL, Gale M, Jr.** 2013. IFITM1 is a tight junction protein that inhibits hepatitis C virus entry. Hepatology **57:**461-469.
12. **Yu J, Li M, Wilkins J, Ding S, Swartz TH, Esposito AM, Zheng YM, Freed EO, Liang C, Chen BK, Liu SL.** 2015. IFITM Proteins Restrict HIV-1 Infection by Antagonizing the Envelope Glycoprotein. Cell reports **13:**145-156.
13. **Savidis G, Perreira JM, Portmann JM, Meraner P, Guo Z, Green S, Brass AL.** 2016. The IFITMs Inhibit Zika Virus Replication. Cell reports **15:**2323-2330.
14. **Gorman MJ, Poddar S, Farzan M, Diamond MS.** 2016. The Interferon-Stimulated Gene Ifitm3 Restricts West Nile Virus Infection and Pathogenesis. J Virol **90:**8212-8225.
15. **Poddar S, Hyde JL, Gorman MJ, Farzan M, Diamond MS.** 2016. The Interferon-Stimulated Gene IFITM3 Restricts Infection and Pathogenesis of Arthritogenic and Encephalitic Alphaviruses. J Virol **90:**8780-8794.

16. **Weiss SR, Navas-Martin S.** 2005. Coronavirus pathogenesis and the emerging pathogen severe acute respiratory syndrome coronavirus. Microbiol Mol Biol Rev **69:**635-664.
17. **Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA.** 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. N Engl J Med **367:**1814-1820.
18. **Gaunt ER, Hardie A, Claas EC, Simmonds P, Templeton KE.** 2010. Epidemiology and clinical presentations of the four human coronaviruses 229E, HKU1, NL63, and OC43 detected over 3 years using a novel multiplex real-time PCR method. Journal of clinical microbiology **48:**2940-2947.
19. **Dijkman R, Jebbink MF, Gaunt E, Rossen JW, Templeton KE, Kuijpers TW, van der Hoek L.** 2012. The dominance of human coronavirus OC43 and NL63 infections in infants. J Clin Virol **53:**135-139.
20. **Hilgenfeld R, Peiris M.** From SARS to MERS: 10 years of research on highly pathogenic human coronaviruses. Antiviral Res **100:**286-295.
21. **Graham RL, Donaldson EF, Baric RS.** A decade after SARS: strategies for controlling emerging coronaviruses. Nat Rev Microbiol **11:**836-848.
22. **Wrensch F, Winkler M, Pohlmann S.** IFITM proteins inhibit entry driven by the MERS-coronavirus spike protein: evidence for cholesterol-independent mechanisms. Viruses **6:**3683-3698.
23. **Bertram S, Dijkman R, Habjan M, Heurich A, Gierer S, Glowacka I, Welsch K, Winkler M, Schneider H, Hofmann-Winkler H, Thiel V, Pohlmann S.** 2013. TMPRSS2 activates the human coronavirus 229E for cathepsin-independent host cell entry and is expressed in viral target cells in the respiratory epithelium. J Virol **87:**6150-6160.
24. **John SP, Chin CR, Perreira J, Feeley EM, Aker A, Savidis G, Smith SE, Elia AE, Everitt AR, Vora M, Pertel T, Elledge SJ, Kellam P, Brass AL.** 2013. The CD225 Domain of IFITM3 is Required for both IFITM Protein Association and Inhibition of Influenza A Virus and Dengue Virus Replication. J Virol.
25. **Amini-Bavil-Olyaee S, Choi YJ, Lee JH, Shi M, Huang IC, Farzan M, Jung JU.** 2013. The antiviral effector IFITM3 disrupts intracellular cholesterol homeostasis to block viral entry. Cell Host Microbe **13:**452-464.
26. **Li K, Markosyan RM, Zheng YM, Golfetto O, Bungart B, Li M, Ding S, He Y, Liang C, Lee JC, Gratton E, Cohen FS, Liu SL.** 2013. IFITM proteins restrict viral membrane hemifusion. PLoS Pathog **9:**e1003124.
27. **Desai TM, Marin M, Chin CR, Savidis G, Brass AL, Melikyan GB.** 2014. IFITM3 restricts influenza A virus entry by blocking the formation of fusion pores following virus-endosome hemifusion. PLoS Pathog **10:**e1004048.
28. **Lin TY, Chin CR, Everitt AR, Clare S, Perreira JM, Savidis G, Aker AM, John SP, Sarlah D, Carreira EM, Elledge SJ, Kellam P, Brass AL.** 2013. Amphotericin B increases influenza A virus infection by preventing IFITM3-mediated restriction. Cell reports **5:**895-908.
29. **Zhao X, Guo F, Liu F, Cuconati A, Chang J, Block TM, Guo JT.** 2014. Interferon induction of IFITM proteins promotes infection by human coronavirus OC43. Proc Natl Acad Sci U S A **111:**6756-6761.
30. **Lu J, Pan Q, Rong L, He W, Liu SL, Liang C.** 2011. The IFITM proteins inhibit HIV-1 infection. J Virol **85:**2126-2137.

31. **Wrensch F, Karsten CB, Gnirss K, Hoffmann M, Lu K, Takada A, Winkler M, Simmons G, Pohlmann S.** 2015. Interferon-Induced Transmembrane Protein-Mediated Inhibition of Host Cell Entry of Ebolaviruses. J Infect Dis **212 Suppl 2:**S210-218.
32. **Xie M, Xuan B, Shan J, Pan D, Sun Y, Shan Z, Zhang J, Yu D, Li B, Qian Z.** 2015. Human cytomegalovirus exploits interferon-induced transmembrane proteins to facilitate morphogenesis of the virion assembly compartment. J Virol **89:**3049-3061.
33. **Tandon R, Mocarski ES.** 2012. Viral and host control of cytomegalovirus maturation. Trends Microbiol **20:**392-401.
34. **Li W, Moore MJ, Vasilieva N, Sui J, Wong SK, Berne MA, Somasundaran M, Sullivan JL, Luzuriaga K, Greenough TC, Choe H, Farzan M.** 2003. Angiotensin-converting enzyme 2 is a functional receptor for the SARS coronavirus. Nature **426:**450-454.
35. **Everitt AR, Clare S, Pertel T, John SP, Wash RS, Smith SE, Chin CR, Feeley EM, Sims JS, Adams DJ, Wise HM, Kane L, Goulding D, Digard P, Anttila V, Baillie JK, Walsh TS, Hume DA, Palotie A, Xue Y, Colonna V, Tyler-Smith C, Dunning J, Gordon SB, Gen II, Investigators M, Smyth RL, Openshaw PJ, Dougan G, Brass AL, Kellam P.** 2012. IFITM3 restricts the morbidity and mortality associated with influenza. Nature **484:**519-523.
36. **Bailey CC, Huang IC, Kam C, Farzan M.** 2012. Ifitm3 limits the severity of acute influenza in mice. PLoS Pathog **8:**e1002909.
37. **Zhang YH, Zhao Y, Li N, Peng YC, Giannoulatou E, Jin RH, Yan HP, Wu H, Liu JH, Liu N, Wang DY, Shu YL, Ho LP, Kellam P, McMichael A, Dong T.** 2013. Interferon-induced transmembrane protein-3 genetic variant rs12252-C is associated with severe influenza in Chinese individuals. Nature communications **4:**1418.
38. **Wang Z, Zhang A, Wan Y, Liu X, Qiu C, Xi X, Ren Y, Wang J, Dong Y, Bao M, Li L, Zhou M, Yuan S, Sun J, Zhu Z, Chen L, Li Q, Zhang Z, Zhang X, Lu S, Doherty PC, Kedzierska K, Xu J.** 2014. Early hypercytokinemia is associated with interferon-induced transmembrane protein-3 dysfunction and predictive of fatal H7N9 infection. Proc Natl Acad Sci U S A **111:**769-774.
39. **Jia R, Pan Q, Ding S, Rong L, Liu SL, Geng Y, Qiao W, Liang C.** 2012. The N-terminal region of IFITM3 modulates its antiviral activity by regulating IFITM3 cellular localization. J Virol **86:**13697-13707.
40. **Jia R, Xu F, Qian J, Yao Y, Miao C, Zheng YM, Liu SL, Guo F, Geng Y, Qiao W, Liang C.** 2014. Identification of an endocytic signal essential for the antiviral action of IFITM3. Cell Microbiol **16:**1080-1093.
41. **Compton AA, Roy N, Porrot F, Billet A, Casartelli N, Yount JS, Liang C, Schwartz O.** 2016. Natural mutations in IFITM3 modulate post-translational regulation and toggle antiviral specificity. EMBO Rep **17:**1657-1671.
42. **Chesarino NM, McMichael TM, Hach JC, Yount JS.** 2014. Phosphorylation of the antiviral protein interferon-inducible transmembrane protein 3 (IFITM3) dually regulates its endocytosis and ubiquitination. J Biol Chem **289:**11986-11992.
43. **Ganley IG, Carroll K, Bittova L, Pfeffer S.** 2004. Rab9 GTPase regulates late endosome size and requires effector interaction for its stability. Mol Biol Cell **15:**5420-5430.

44. **Yount JS, Karssemeijer RA, Hang HC.** 2012. S-palmitoylation and ubiquitination differentially regulate interferon-induced transmembrane protein 3 (IFITM3)-mediated resistance to influenza virus. J Biol Chem **287:**19631-19641.
45. **Yount JS, Moltedo B, Yang YY, Charron G, Moran TM, Lopez CB, Hang HC.** 2010. Palmitoylome profiling reveals S-palmitoylation-dependent antiviral activity of IFITM3. Nat Chem Biol **6:**610-614.
46. **Chesarino NM, McMichael TM, Yount JS.** 2015. E3 Ubiquitin Ligase NEDD4 Promotes Influenza Virus Infection by Decreasing Levels of the Antiviral Protein IFITM3. PLoS Pathog **11:**e1005095.
47. **Chesarino NM, McMichael TM, Yount JS.** 2014. Regulation of the trafficking and antiviral activity of IFITM3 by post-translational modifications. Future Microbiol **9:**1151-1163.
48. **Lawe DC, Sitouah N, Hayes S, Chawla A, Virbasius JV, Tuft R, Fogarty K, Lifshitz L, Lambright D, Corvera S.** 2003. Essential role of Ca2+/calmodulin in Early Endosome Antigen-1 localization. Mol Biol Cell **14:**2935-2945.
49. **Hach JC, McMichael T, Chesarino NM, Yount JS.** 2013. Palmitoylation on conserved and nonconserved cysteines of murine IFITM1 regulates its stability and anti-influenza A virus activity. J Virol **87:**9923-9927.
50. **Warren CJ, Griffin LM, Little AS, Huang IC, Farzan M, Pyeon D.** 2014. The antiviral restriction factors IFITM1, 2 and 3 do not inhibit infection of human papillomavirus, cytomegalovirus and adenovirus. PLoS One **9:**e96579.
51. **Foster TL, Wilson H, Iyer SS, Coss K, Doores K, Smith S, Kellam P, Finzi A, Borrow P, Hahn BH, Neil SJ.** 2016. Resistance of Transmitted Founder HIV-1 to IFITM-Mediated Restriction. Cell Host Microbe **20:**429-442.
52. **Wu WL, Grotefend CR, Tsai MT, Wang YL, Radic V, Eoh H, Huang IC.** 2017. Delta20 IFITM2 differentially restricts X4 and R5 HIV-1. Proc Natl Acad Sci U S A **114:**7112-7117.
53. **Fu B, Wang L, Li S, Dorf ME.** 2017. ZMPSTE24 defends against influenza and other pathogenic viruses. J Exp Med **214:**919-929.
54. **Gerlach T, Hensen L, Matrosovich T, Bergmann J, Winkler M, Peteranderl C, Klenk HD, Weber F, Herold S, Pohlmann S, Matrosovich M.** 2017. pH Optimum of Hemagglutinin-Mediated Membrane Fusion Determines Sensitivity of Influenza A Viruses to the Interferon-Induced Antiviral State and IFITMs. J Virol **91**.
55. **Desai TM, Marin M, Mason C, Melikyan GB.** 2017. pH regulation in early endosomes and interferon-inducible transmembrane proteins control avian retrovirus fusion. J Biol Chem **292:**7817-7827.
56. **Rabbani MA, Ribaudo M, Guo JT, Barik S.** 2016. Identification of IFN-stimulated gene (ISG) proteins that inhibit human parainfluenza virus type 3. J Virol.
57. **Yount JS, Zhang MM, Hang HC.** 2013. Emerging roles for protein S-palmitoylation in immunity from chemical proteomics. Current opinion in chemical biology **17:**27-33.
58. **Jia R, Ding S, Pan Q, Liu SL, Qiao W, Liang C.** 2015. The C-terminal sequence of IFITM1 regulates its anti-HIV-1 activity. PLoS One **10:**e0118794.
59. **Li K, Jia R, Li M, Zheng YM, Miao C, Yao Y, Ji HL, Geng Y, Qiao W, Albritton LM, Liang C, Liu SL.** 2015. A sorting signal suppresses IFITM1 restriction of viral entry. J Biol Chem **290:**4248-4259.

60. **Jiang D, Guo H, Xu C, Chang J, Gu B, Wang L, Block TM, Guo JT.** 2008. Identification of three interferon-inducible cellular enzymes that inhibit the replication of hepatitis C virus. J Virol **82:**1665-1678.

61. **Weidner JM, Jiang D, Pan XB, Chang J, Block TM, Guo JT.** 2010. Interferon-induced cell membrane proteins, IFITM3 and tetherin, inhibit vesicular stomatitis virus infection via distinct mechanisms. J Virol **84:**12646-12657.

62. **Chang J, Warren TK, Zhao X, Gill T, Guo F, Wang L, Comunale MA, Du Y, Alonzi DS, Yu W, Ye H, Liu F, Guo JT, Mehta A, Cuconati A, Butters TD, Bavari S, Xu X, Block TM.** 2013. Small molecule inhibitors of ER alpha-glucosidases are active against multiple hemorrhagic fever viruses. Antiviral Res.

63. **Lin HX, Feng Y, Tu X, Zhao X, Hsieh CH, Griffin L, Junop M, Zhang C.** 2011. Characterization of the spike protein of human coronavirus NL63 in receptor binding and pseudotype virus entry. Virus Res **160:**283-293.

64. **He J, Choe S, Walker R, Di Marzio P, Morgan DO, Landau NR.** 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J Virol **69:**6705-6711.

65. **Connor RI, Chen BK, Choe S, Landau NR.** 1995. Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes. Virology **206:**935-944.

66. **Zhao X, Guo F, Comunale MA, Mehta A, Sehgal M, Jain P, Cuconati A, Lin H, Block TM, Chang J, Guo JT.** 2015. Inhibition of endoplasmic reticulum-resident glucosidases impairs severe acute respiratory syndrome coronavirus and human coronavirus NL63 spike protein-mediated entry by altering the glycan processing of angiotensin I-converting enzyme 2. Antimicrob Agents Chemother **59:**206-216.

67. **Chang J, Warren TK, Zhao X, Gill T, Guo F, Wang L, Comunale MA, Du Y, Alonzi DS, Yu W, Ye H, Liu F, Guo JT, Mehta A, Cuconati A, Butters TD, Bavari S, Xu X, Block TM.** 2013. Small molecule inhibitors of ER alpha-glucosidases are active against multiple hemorrhagic fever viruses. Antiviral Res **98:**432-440.

68. **Milewska A, Zarebski M, Nowak P, Stozek K, Potempa J, Pyrc K.** 2014. Human coronavirus NL63 utilizes heparan sulfate proteoglycans for attachment to target cells. J Virol **88:**13221-13230.

691 **FIGURE LEGENDS**

692

693 **Fig. 1 IFITM proteins inhibit the infection of pseudoviruses in FLIP-IN T REX 293 and**
694 **Huh7.5 cells. (A)** The expression of FLAG-tagged IFITMs in FLIP-IN T Rex 293 cells cultured
695 in the presence of 1μg/ml of tetracycline (tet) for 24 h and in Huh7.5-derived stable cell lines
696 was detected by a Western blot assay using a monoclonal antibody against Flag tag. β-actin
697 served as a loading control. **(B)** FLIP-IN T Rex 293 cells expressing chloramphenicol
698 acetyltransferase (CAT) or the indicated IFITM protein were cultured in the presence or absence
699 of tet for 24 h and then infected with 229Epp, NL63pp, SARSpp, MERSpp, IAVpp, or LASVpp.
700 Luciferase activities were determined at 48 h post infection (hpi). Relative infection efficiency is
701 the ratio of luciferase activity in cells cultured in the presence of tet over that in cells cultured in
702 the absence of tet. Error bars indicate standard deviations (n = 6). IFITMs do not significantly
703 affect the infection of LASVpp, but significantly (p <0.001) inhibit the infection of all other
704 tested pseudotyped viruses. **(C)** Huh7.5 cells expressing N-terminally FLAG-tagged human
705 IFITM proteins or transduced with empty vector (pQCXIP) were infected with indicated
706 pseudoviruses. Luciferase activities were determined at 48 hpi. Relative infection is the ratio of
707 luciferase activity of cells expressing the indicated IFITM protein over that of cells transduced
708 with empty vector. Error bars indicate standard deviations (n = 6). All three IFITM proteins
709 significantly (p<0.001) inhibited the infection of all the pseudoviruses, except for LASVpp.

710

711 **Fig. 2 Y20 of IFITM3 plays a critical role in modulating the entry of 229E, MERS-CoV**
712 **SARS-CoV and NL63 in both 293 and Huh7.5 cells. (A)** FLIP-IN T Rex cells expressing CAT

or the indicated wild-type or mutant IFITM proteins were cultured in the presence of 1μg/ml of tet for 24 h. Expression of FLAG-tagged IFITM mutants was detected by Western blot assay using anti-Flag monoclonal antibody. β-actin served as a loading control. **(B to E)** The above FLP-IN T Rex-derived cell lines were cultured in the presence or absence of tet for 24 h and then infected with HCoV-OC43pp (B), SARSpp or NL63pp (C), 229Epp (D) MERSpp and LASVpp (E). Luciferase activities were determined at 48 hpi. Relative infection efficiency is the ratio of luciferase activity in cells cultured in the presence of tet over that in cells cultured in the absence of tet. Error bars indicate standard deviations (n = 6). **(F)** Huh7.5 cells were stably transduced with empty retroviral vector (pQCXIP) or vectors expressing wild-type or mutant IFITM3 proteins. Expression of FLAG-tagged IFITM proteins was detected by Western blot assay using anti-Flag monoclonal antibody. β-actin served as a loading control. **(G to K)** The above Huh7.5-derived cell lines were infected with OC43pp **(G)**, SARSpp and NL63pp (H), 229Epp (I), MERSpp (J) or LASVpp (K). Luciferase activities were determined at 48 hpi. Relative infection is the ratio of luciferase activity of cells expressing the indicated IFITM protein over that of cells transduced with empty vector. Error bars indicate standard deviations (n = 6).

Fig. 3 Y20 mutation alters IFITM3 subcellular localization. FLP-IN T Rex cells expressing the indicated wild-type and mutant IFITM3 proteins were cultured in the presence of tet for 24 h to induce the IFITM expression. The localization of FLAG-tagged IFITM3 **(A)**, IFITM3/Y20A **(B)** and IFITM3/Y20D **(C)** was detected by immunofluorescent staining with an anti-Flag monoclonal antibody (red). EEA1, Rab5 or Rab9 were visualized by immunofluorescent staining with respective antibodies (green). Cell nuclei were stained with DAPI (Blue).

Fig. 4 IFITM3/Y20A-enhanced SARSpp and MERSpp infection is low pH dependent. (A) Huh7.5 cells were infected with OC43pp, MERSpp, SARSpp, NL63pp, or 229Epp in the absence (mock) or presence of indicated concentrations of NH_4Cl. Luciferase activities were determined at 48 hpi. Relative infection is the ratio of luciferase acyivity in cells treated with NH_4Cl over that in the mock-treated cells. Error bars indicate standard deviations (n = 6). **(B and C)** Huh7.5 cells stably expressing indicated wild-type or Y20A mutant IFITM3 proteins or transduced with empty vector were infected with MERSpp (B) or SARSpp (C) in the absence (mock) or presence of indicated concentrations of NH_4Cl. Luciferase activities were determined at 48 hpi. Relative infection is the ratio of luciferase acyivity in cells treated with NH_4Cl over that in the mock-treated cells. Error bars indicate standard deviations (n = 6).

Fig. 5 Y99 of IFITM3 plays a critical role in modulating the entry of HCoVs (A) FLIP-IN T Rex 293 cells expressing CAT, IFITM3, or indicated mutant IFITM3 proteins were cultured in the presence of 1μg/ml of tet for 24 h. Expression of FLAG-tagged IFITM proteins was detected by Western blot assay using anti-Flag monoclonal antibody. β-actin served as a loading control. **(B** to **F)** FLP-IN T Rex 293-derived cells were cultured in the presence of tet for 24 h to induce the IFITM mutant expression. Cells were then infected with HCoV-OC43pp (B), SARSpp or NL63pp (C), 229Epp (D), VSVpp or IAVpp (E), and MERSpp(F), respectively. Luciferase activities were determined at 48 hpi. Relative infection efficiency represents luciferase activity of cells cultured with tet normalized to that of cells cultured in the absence of tet. Error bars indicate standard deviations (n = 6).

Fig. 6 Palmiltoylation, ubiquitination and oligomerization are important for IFITM3 restriction of HCoV entry. (A) FLIP-IN T Rex 293 cells expressing CAT or the indicated wild-type and mutant IFITM3 proteins were cultured in the presence of tet for 24 h. Expression of the IFITM proteins was detected by Western blot assay using anti-Flag monoclonal antibody. β-actin served as a loading control. **(B)** The above FLP-IN T Rex-derived cells were cultured in the presence or absence of tet for 24 h and then infected with SARSpp, NL63pp, 229Epp, MERSpp, IAVpp, or MLVpp. Luciferase activities were determined at 48 hpi. Relative infection efficiency is the ratio of luciferase activity in cells cultured in the presence of tet over that in cells cultured in the absence of tet. Error bars indicate standard deviations (n = 6).

Fig. 7 Role of the IFITM1 C-terminal motifs in modulating the entry of HCoVs. (A) FLIP-IN T Rex cells expressing CAT, wild-type or mutant IFITM1 proteins were cultured in the presence of of tet for 24 h. Expression of FLAG-tagged IFITM1 proteins was detected by Western blot assay using anti-Flag monoclonal antibody. β-actin served as a loading control. **(B to D)** The above cell lines were left untreated or treated with 1μg/ml of tet for 24 h to induce the IFITM expression. Cells were then infected with SARSpp, NL63pp, or MLVpp (B), 229Epp (C), or MERSpp (D). Luciferase activities were determined at 48 hpi. Relative infection efficiency is the ratio of luciferase activity in cells cultured in the presence of tetracycline over that in cells cultured in the absence of tetracycline. Error bars indicate standard deviations (n = 6). ** compared to wild-type IFITM1, $p<0.001$.

Fig. 8 Role of the IFITM1 C-terminal motifs in modulating the infection of HCoV-NL63 in Huh7.5 cells. (**A**) Huh7.5 cells were stably transdused with retroviral vectors expressing wild-

type IFITM1, IFITM1/TC6, IFITM1/TC18 or empty vector (pQCXIP). Expression of FLAG-tagged IFITM1 proteins was detected by Western blot assay using anti-Flag monoclonal antibody. β-actin served as a loading control. **(B)** The above cell lines were infected with SARSpp, NL63pp or MLVpp. Luciferase activities were determined at 48 hpi. Relative infection is the ratio of luciferase activity of cells expressing the indicated IFITM protein over that of cells transduced with empty vector. Error bars indicate standard deviations (n = 6). **(C)** The above cell lines were infected with HCoV-NL63 virus at a M.O.I. of 5. Infected cells were visualized immunofluorescent staining using NL63 NP monoclonal antibody (green). Cell nuclei were stained with DAPI (blue). Percentages of cells infected by NL63 were expressed as average ± standard deviation. **(D)** The amounts of intracellular HCoV-NL63 RNA at 24 hpi were quantified by a qRT-PCR assay and expressed as the ratio of viral RNA in IFITM-expressing cells over that in the cells transduced with empty vector. Error bars indicate standard deviations (n = 4).

Figure 9. Subcellular localization of wild-type and C-terminally truncated IFITM1 proteins. FLP-IN T Rex 293 cells expressing the indicated wild-type and mutant IFITM1 proteins were treated with 1μg/ml of tetracycline for 24 h to induce the IFITM expression. The localization of FLAG-tagged IFITM1 (**A**), IFITM1/TC6 (**B**) and IFITM1/TC18 (**C**) was detected by immunofluorescent staining with an anti-Flag monoclonal antibody (red). EEA1, Rab5 or Rab9 were visualized by immunofluorescent staining with respective antibodies (green). Cell nuclei were stained with DAPI (Blue).

Figure 10. C-terminal motif of IFITM1 is required for its ubiquitination. 293T cells were transfected with a plasmid expressing the indicated FLAG-tagged wild-type and mutant IFITM1 or IFITM3 proteins. Immunoprecipitation was performed by using an anti-FLAG monoclonal antibody. The precipitated proteins were resolved by SDS-PAGE and blotted onto a membrane. IFITM proteins and their ubiquitinated species were visualized by probing with an anti-FLAG rabbit polyclonal antibody **(A)** or anti-ubiquitin rabbit polyclonal antibody **(B)**. The mono-ubiquitinated and di-ubiquitinated form of IFITM were indicated by a single- and double-asterisk, respectively. IFITM proteins without ubiquitination with the molecular weight smaller than 15kDa serve as loading controls.

Figure 11. Illustration of IFITM protein structural domains and motifs important for subcellular trafficking, oligomerization and post-translational modification. An alignment of human IFITM1, IFITM2 and IFITM3 protein sequence by using Vector NTI 8.0 software is shown. Five structural domains including N-terminal domain (NTD), intramembrane domain (IMD), intracellular loop (CIL), transmembrane domain (TMD), and C-terminal domain (CTD) are indicated. IMD and CIL domains comprise the canonical CD225 domain (shown in red). ^{20}YXXΦ^{23} motif required for IFITM3 endocytosis and ^{122}KRXX^{125} motif that serves as a sorting signal for IFITM1 are underlined with purple lines. The putative phosphorylation, palmitoylation and ubiquitination sites are indicated. Two phenylalanine residues in the IMD that promote IFITM oligomerization are indicated with red up-triangles.