3.2. The cell surface expression, total expression level, and intracellular localization of IFITMs are unaffected by HIV-1 proteins

Tetherin and APOBEC3G are well-characterized HIV-1 restriction factors, but it is also known that HIV-1 proteins counteract their activities. Vpu and Vif induce the downregulation of the cell surface expression of tetherin [14—19] and the degradation of APOBEC3G [21—27], respectively. Indeed, tetherin inhibited the release of the Vpu (−) viruses more strongly than it inhibited the release of the wild-type viruses (Fig. 1A). On the other hand, IFITM3 exhibited comparable inhibitory activity to these two viruses (Fig. 1A). Therefore, we next examined whether HIV-1 proteins affect the localization or expression of IFITM family proteins. The Flag-tagged IFITM1, 2, or 3 expression plasmid was co-transfected with the proviral plasmid (JRFL or NL43), but we did not detect any obvious changes in the cell surface expression of IFITMs (Fig. 2A). Although IFITM3 has been found to localize not only to the plasma membrane but also to the perinuclear region [7], we did not detect any obvious changes in the intracellular distribution of IFITMs after their co-transfection with the proviral JRFL plasmid (Fig. 2B). We also tested the effect of the over-expression of individual HIV-1 proteins. The Flag-tagged IFITM1, 2, or 3 expression plasmid was co-transfected with the codon-optimized version of the Vpu (Vphu) or Vif (HVif) plasmid, which bypassed the complicated viral-specific expression machinery and allowed efficient expression [31], and the cell surface expression of Flag-IFITMs was analyzed by flow cytometry using anti-Flag antibody. Nef was also added to the analysis, as it down-regulates the expression of multiple cell surface proteins including CD4 [36]. However, none of the HIV-1 proteins down-regulated the cell surface expression of IFITMs (data not shown). The expression levels of Vpu and Nef under our experimental conditions were sufficient to down-regulate tetherin and CD4, respectively (Fig. 2C). The expression level of Vif under our experimental conditions was sufficient to induce the degradation of APOBEC3G (Western blotting, data not shown). These results indicate that IFITMs are resistant to the down-regulation of cell surface expression or the degradation by HIV-1 proteins. These findings are important because they imply that any HIV-1 proteins, unlike those of tetherin and APOBEC3G, do not counteract the anti-HIV-1 activities of IFITMs.

3.3. IFITMs reduce the expression of several HIV-1 proteins when they were expressed by viral-specific machinery

We next attempted to understand how IFITMs suppress the production of HIV-1 viruses. There was no obvious difference in the inhibitory effect on viral production among IFITMs (Fig. 3A). All the IFITMs reduced the production of both the

Fig. 3. The effects of IFITMs on viral production and the expression of Gag, Nef, and Vif. (A) The 293 cells were co-transfected with the proviral NL43 plasmid (0.4 μg) and the indicated amount (0.4, 0.8, or 1.2 μg) of the IFITM expression plasmids. The cells were cultured for 2 days, and the concentration of p24 Gag in the culture supernatants was determined by ELISA. The results are expressed as a percentage of the value for the sample on the far left. Data are shown as the mean ± SD of triplicate assays. (B) The 293 cells were transfected with the proviral NL43 or JRFL plasmid (0.4 μg), or co-transfected with the empty vector (Empty, 1.2 μg) or IFITM expression plasmid (1.2 μg), as indicated. The cells were cultured for 2 days, and the concentration of p24 Gag in the culture supernatants was determined by ELISA and analyzed as described in panel A. Alternatively, the cells were lysed and analyzed for the expression of Gag, Nef, Vif, or Flag-tagged IFITMs by Western blotting (lower blots). The actin blot is a loading control. (A and B) Data shown are representative of two independent experiments with similar results. *p < 0.05.

NL43 and JRFL strain viruses (Fig. 3B, bar graph), and this effect was strongly associated with reduced Gag expression (Fig. 3B, Gag blot). Importantly, we found that IFITMs also reduced the expression of Nef and Vif (Nef and Vif blots). However, more important finding was that IFITM proteins did not induce any reduction in the expression of these viral proteins when they were co-transfected with the codon-optimized Gag expression plasmid (Fig. 4A, synGag–GFP blot), the Nef expression plasmid (Fig. 4A, Nef–GFP blot), or the codon-optimized Vif expression plasmid (Fig. 4B, HVif blot). It is well characterized that Gag and Vif RNAs contain a double-stranded region (Rev response element; RRE), and the incompletely spliced RNAs encoding these viral proteins

require the binding of HIV-1 Rev protein to the RRE sequences for their nuclear export and subsequent expression [37,38]. Codon-optimization bypasses this complicated mechanism and allows these proteins to be expressed in a Rev-independent manner [31,37,38]. Thus, our results raised the possibility that IFITMs selectively interfere with the Rev/RRE-mediated expression of Gag and Vif. Indeed, IFITMs displayed an inhibitory effect on Gag expression in an artificial Rev-dependent expression system (Fig. 4C). In this system, the codon-unoptimized and RRE sequence-containing Gag–Pol and Gag expression plasmids (Gag–Pol–RRE and Gag-RRE) were expressed at normal levels only in the presence of the Rev plasmid (Fig. 5B, first 2 lanes). As a result, we
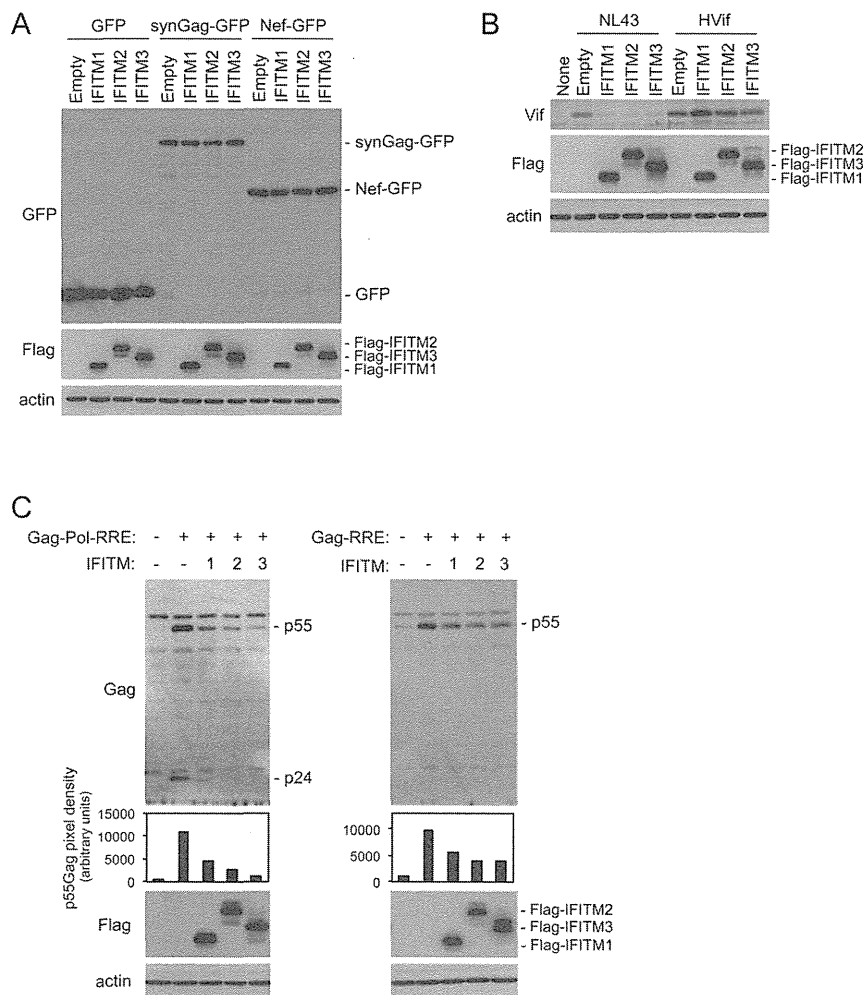

Fig. 4. The effects of IFITMs on the expression of the codon-optimized Gag, Nef and Vif, and the Rev/RRE-mediated expression of Gag. (A) The 293 cells were transfected with the GFP expression plasmid (0.1 μg), the codon-optimized Gag–GFP fusion expression plasmid (synGag–GFP, 0.2 μg), or the Nef–GFP expression plasmid, or co-transfected with the indicated IFITM expression plasmid (1.2 μg). (B) The 293 cells were transfected with the empty vector (None), the proviral NL43 plasmid (0.4 μg), or the codon-optimized Vif expression plasmid (HVif, 0.4 μg), or co-transfected with the indicated IFITM expression plasmid (1.2 μg). (A and B) The transfected cells were cultured for 2 days, lysed, and analyzed for the expression of GFP or GFP fusion proteins, Vif, and the Flag-tagged IFITM proteins by Western blotting. (C) The 293 cells were transfected with the Gag–Pol–RRE (0.3 μg) or Gag-RRE (0.3 μg) expression plasmid in combination with the Rev (0.1 μg) and/or IFITM (1.2 μg) expression plasmids, as indicated. The cells were cultured for 2 days, lysed, and analyzed for the expression of Gag and the Flag-tagged IFITM proteins by Western blotting. The profile created by quantifying the band pixel densities of p55Gag is also shown. (A–C) The actin blot is a loading control. Data shown are representative of two independent experiments with similar results.

found that IFITM1, 2, and 3 significantly reduced Rev/RRE-mediated Gag expression (lanes 3–5), as they did in the proviral plasmid-mediated expression system (see Fig. 3B).

However, the finding that IFITMs interfere with Rev/RRE-mediated expression does not explain the fact that they also reduced the proviral plasmid-mediated expression of Nef (see Fig. 3B) because Nef RNA does not contain RRE, and therefore its expression is independent of the function of Rev [37,38]. Thus, we next examined whether another viral double-stranded RNA, i.e., the *trans*-activation response (TAR) element, was involved in the inhibitory activity of IFITMs. All of the HIV-1 RNAs including that encoding Nef contain the TAR element at their 5' end, which binds and activates the double-stranded RNA-dependent protein kinase (PKR) [39]. Once activated, PKR phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α), which reduces the efficiency and rate of the translation initiation of proteins including viral proteins [39]. On the other hand, the HIV-1 Tat protein counteracts PKR activation via various complicated mechanisms, and the combination of the inhibitory pathways that prevent PKR activation determines the level of viral expression [39]. In this study, we found that the small molecule PKR inhibitor C16 restored the expression of p55Gag, Vif, and Nef, when it was added at effective but non-cytotoxic concentrations (0.2–0.8 μM) [40,41] to the cells that had been co-transfected with the proviral NL43 plasmid and

IFITM3 (Fig. 5A). Meanwhile, C16 did not restore Gag expression in the Tat/TAR-independent but Rev/RRE-dependent expression system (Fig. 5B), which was consistent with the fact that TAR, but not RRE, induces strong PKR activation [39]. Therefore, our results (Figs. 3–5) suggested that IFITMs reduced the expression of Gag, and possibly Vif, by interfering with both Rev/RRE- and Tat/TAR-mediated expression, and reduced the expression of Nef by interfering with Tat/TAR-mediated expression. Again, the inhibitory effect was not non-specific because IFITMs did not affect the levels of these viral proteins when they were expressed via the system that bypassed the viral-specific machinery (see Fig. 4A and B).

3.4. The S-palmitoylation of IFITMs is not required for their anti-HIV-1 activity

A previous study demonstrated that IFITM3 was post-translationally modified by S-palmitoylation, which is crucial for its activity against influenza virus infection [7]. Therefore, we finally examined whether the S-palmitoylation of IFITMs is important for their anti-HIV-1 activity. To this end, we prepared three different mutants of Flag-tagged IFITM3 and IFITM2, in which the S-palmitoylated cysteine residues were mutated singly or in combination to alanine (Fig. 6A). Consistent with the finding that the first two
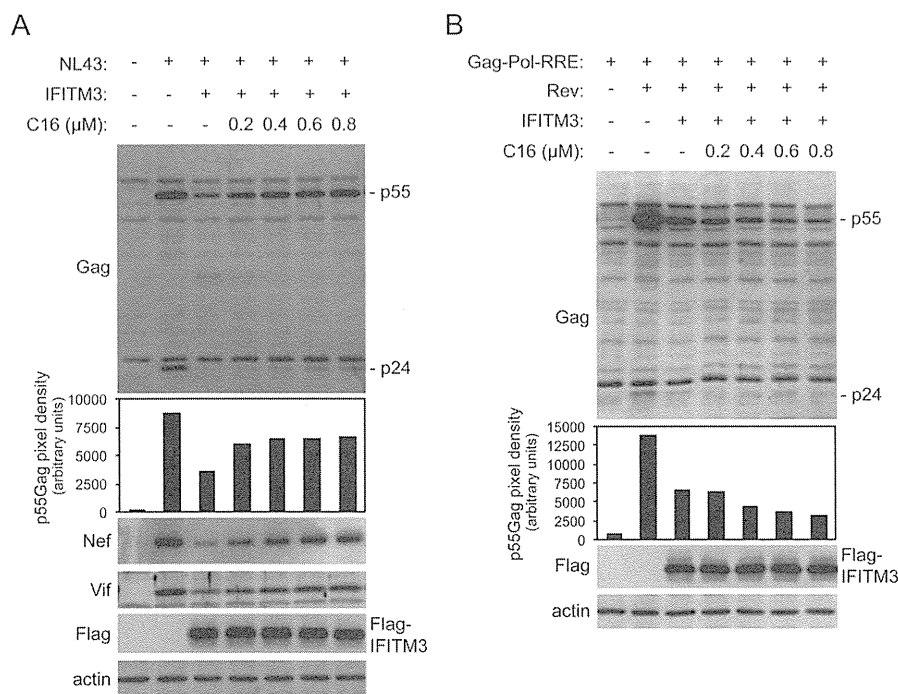

Fig. 5. The effect of the PKR inhibitor C16 on the suppression of Gag, Nef, and Vif expression by IFITM. (A) The 293 cells were transfected with the proviral NL43 plasmid (0.6 μg) and IFITM3 expression plasmid (1.0 μg), as indicated. (B) The 293 cells were transfected with the expression plasmids for Gag–Pol–RRE (0.3 μg), Rev (0.1 μg) and IFITM3 (1.0 μg), in the indicated combinations. (A and B) After 6 h transfection, the PKR inhibitor C16 was added to the culture at the indicated concentration (0.2–0.8 μM), and the cells were cultured for an additional 42 h. The cells were lysed and analyzed for the expression of Gag, Nef, Vif, and the Flag-tagged IFITM3 proteins. The actin blot is a loading control. The profile created by quantifying the band pixel densities of p55Gag is also shown. Data shown are representative of two independent experiments with similar results.
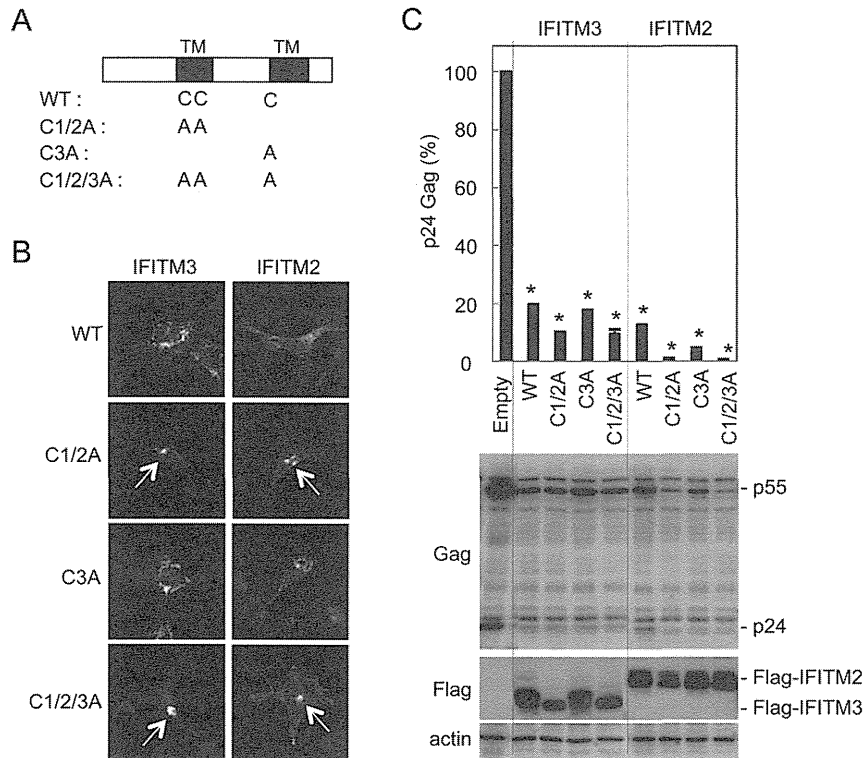
Fig. 6. The effects of S-palmitoylation-deficient IFITM mutants on viral production and Gag expression. (A) The IFITM mutants used are shown schematically. C, cysteine; A, alanine; TM, transmembrane. (B) The 293 cells were transfected with the expression plasmids (1.2 μg) for the wild-type (WT) or three mutants (C1/2A, C3A, and C1/2/3A) of IFITM3 or IFITM2. After 2 days transfection, the cells were fixed and co-stained with DAPI (blue) and anti-Flag antibody to detect Flag-tagged IFITM proteins (green). The C1/2A and C1/2/3A mutants displayed a distinct intracellular distribution (arrows) compared with the WT and C3A mutant. (C) The 293 cells were transfected with the proviral NL43 plasmid (1.0 μg), or co-transfected with the indicated IFITM expression plasmid (0.6 μg). The cells were cultured for 2 days, and the concentration of p24 Gag in the culture supernatants was determined by ELISA (bar graph). The results are expressed as percentages of the value for the sample on the far left. Data are shown as the mean ± SD of triplicate assays. Alternatively, the cells were lysed, and analyzed for the expression of Gag and the Flag-tagged IFITMs by Western blotting (lower blots). The actin blot is a loading control. (B and C) Data shown are representative of two independent experiments with similar results. *$p < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

cysteines were more heavily S-palmitoylated than the third cysteine [7], the mutants in which the first two cysteines were substituted (C1/2A and C1/2/3A) displayed more marked changes in their intracellular localization (Fig. 6B, arrows). However, unlike in the case of the anti-influenza virus activity of IFITM3, it was found that the S-palmitoylation was not necessary for the anti-HIV-1 activity of IFITMs because all of the mutants of IFITM3 and IFITM2 reduced the concentrations of p24 Gag in the supernatants (Fig. 6C, bar graph) and the expression levels of p55 and p24 Gag proteins in the cells (Gag blot). These results clearly indicated that IFITMs restrict HIV-1 and influenza viruses at distinct steps.

3.5. Conclusion

Both knockdown and enforced expression experiments demonstrated that IFITMs restrict HIV-1 replication [12,13]. In this study, we extended the findings of Lu et al. [12] and revealed that the enforced expression of IFITMs interfered with the production of HIV-1 proteins such as Gag, Vif, and Nef only when viral double-stranded RNAs (RRE and/or TAR) mediated their expression. These findings suggested that IFITM bind directly to viral double-stranded RNA. Indeed, a previous report raised the possibility that IFITM1 is an RRE-binding protein [P. Constantoulakis et al., Inhibition of Rev-mediated HIV-1 expression by an RNA binding protein encoded by the interferon-inducible 9—27 gene, Science 259 (1993) 1314—1318.]. However, as the report was retracted [M. Campbell et al., Science 264 (1994) 492.], careful studies will be necessary in order to clarify the exact mechanisms by which IFITMs interfere with the viral protein expression mediated by the double-stranded viral RNAs such as RRE and TAR. Studies will be also necessary to explain why there was no obvious difference in the anti-HIV-1 activity among three IFITM proteins in our transfection assay, in contrast to the study by Lu et al. [12]. Despite these unanswered questions, the present study demonstrated that IFITMs possess different characteristics from other anti-HIV-1 proteins such as tetherin and APOBEC3G and supported the idea that IFITMs restrict HIV-1 and influenza viruses at distinct steps.

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Membrane-associated RING-CH (MARCH) 8 mediates the ubiquitination and lysosomal degradation of the transferrin receptor

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Summary

The transferrin receptor (TfR) mediates the uptake of transferrin (Tf)-bound iron from the plasma into the cells of peripheral tissues. The TfR continuously recycles between the plasma membrane and early/recycling endosomes. TfR expression is tightly controlled by the intracellular iron concentration through the regulation of TfR mRNA stability. However, much less is known about the mechanism by which TfR is degraded in cells. Previously, we reported a correlation between TfR ubiquitination and its iron-induced lysosomal degradation. The identification and characterization of a specific ubiquitin ligase for TfR is important in understanding the mechanism of iron homeostasis. Here, we show that membrane-associated RING-CH (MARCH) 8 ubiquitinates TfR and promotes its lysosomal degradation. Similar to other RING-type ubiquitin ligases, the RING-CH domain of MARCH8, which is located in the N-terminal cytoplasmic domain, is essential for the ubiquitination and downregulation of TfR. MARCH8 specifically recognizes the transmembrane domain of TfR and mediates ubiquitination of its cytoplasmic domain. In addition, the six-amino-acid sequence located in the C-terminal domain of MARCH8, which is highly conserved among different species, is required for the downregulation of TfR. Finally, and most importantly, TfR expression was markedly increased by siRNA-mediated knockdown of endogenous MARCH8. These findings demonstrate that the endogenous level of MARCH8 regulates TfR protein turnover through the downregulation and ubiquitination of TfR.

Key words: Endocytosis, Iron metabolism, Lysosome, Multivesicular body, Transferrin receptor, Ubiquitin ligase

Introduction

Plasma membrane (PM) proteins have different half-lives and follow distinct trafficking routes (Hare, 1990). Although most PM proteins are degraded in lysosomes, there are different mechanisms that regulate their degradation in the cell. The endocytosis and subsequent degradation of certain PM proteins that lack lysosomal targeting signals are regulated by ubiquitination (Hicke, 1997; Mukhopadhyay and Riezman, 2007; Staub and Rotin, 2006; Strous and Govers, 1999). After endocytosis, ubiquitinated PM proteins are delivered to the late endosome/multivesicular body (MVB) pathway (Katzmann et al., 2002; Piper and Katzmann, 2007; Piper and Luzio, 2007). Ubiquitin molecules attached to the cytoplasmic (CT) domain of PM proteins are recognized by the endosomal sorting complex required for transport (ESCRT) machinery. The ESCRT machinery promotes the sequestration of ubiquitinated cargo in the inner vesicles of MVBs, which eventually fuse with lysosomes leading to the degradation of the ubiquitinated proteins. Accumulating evidence has indicated that ubiquitin ligases mediate the ubiquitination and degradation of PM proteins including receptors, transporters, and ion channels (Piper and Lehner, 2011). However, several associations

between PM proteins and their specific ubiquitin ligases remain unidentified.

Similar to most ubiquitin ligases, which target a variety of substrates for degradation, members of the membrane-associated RING-CH (MARCH) family target various membrane proteins (Bartee et al., 2004; Lehner et al., 2005; Nathan and Lehner, 2009; Ohmura-Hoshino et al., 2006). To date, 11 members of the MARCH family of proteins have been identified and characterized, all of which contain a RING-CH motif at their N-terminal CT domain that potentially interacts with an E2 enzyme, similar to the RING domains of RING-type ubiquitin ligases. They generally have several (two, four, or 12) transmembrane (TM) domains; however, MARCH7 and MARCH10 have no predicted TM domains. MARCH1 promotes the ubiquitination and degradation of MHC-II and CD86 (Baravalle et al., 2011; Corcoran et al., 2011; De Gassart et al., 2008; Ishido et al., 2009; Lapaque et al., 2009; Tze et al., 2011; Young et al., 2008). MARCH1-induced ubiquitination and degradation of PM proteins may be involved in the immune response by playing a role in dendritic cell maturation. MARCH10 and MARCH11 are highly expressed in the testis and are predicted to play a pivotal role in spermiogenesis and the

organization of spermatid flagella (Iyengar et al., 2011; Yogo et al., 2011; Morokuma, et al., 2007). Several other MARCH family members are widely expressed in various tissues, but their physiological roles are largely unknown.

The transferrin receptor (TfR) is a type-II membrane protein that mediates the uptake of transferrin (Tf)-bound iron from the plasma into cells in peripheral tissues (Hamilton et al., 1979; Omary and Trowbridge, 1981). The extracellular domain of TfR binds to Tf at the PM and its cytoplasmic domain contains a canonical signal for endocytosis, the YxxΦ motif (Collawn et al., 1990; Schneider et al., 1984). The Tf/TfR complex is internalized at the PM through clathrin-coated pits and transported to early endosomes. Acidification at the early endosomes promotes a conformational change in the Tf/TfR complex that causes the release of iron from Tf. The iron-free Tf/TfR complex is then recycled back to the PM through sorting and recycling endosomes (Ciechanover et al., 1983; Dautry-Varsat et al., 1983; Hopkins and Trowbridge, 1983; Schneider et al., 1984). TfR expression is regulated by the action of iron-regulatory proteins (IRP1 and IRP2) that modulate its mRNA stability (Hentze et al., 1987; Owen and Kühn, 1987). However, the mechanism of TfR degradation is poorly understood. Recently, we described the iron-induced ubiquitination and lysosomal degradation of TfR (Tachiyama et al., 2011). It is important to identify and characterize ubiquitin ligases responsible for this iron-induced ubiquitination and degradation of TfR. Certain

MARCH family members were shown to facilitate the degradation of TfR by fluorescence-activated cell sorting (FACS) analysis (Bartee et al., 2004).

In this study, we show that MARCH8 ubiquitinates TfR and promotes its lysosomal degradation. Furthermore, MARCH8 recognizes the TM domain of TfR and ubiquitinates its CT domain. Importantly, in addition to the RING-CH domain, a highly conserved six-amino-acid region in the C-terminal CT domain of MARCH8 is required for the downregulation of TfR. Our results suggest that an as-yet-unidentified co-factor may associate with the highly conserved six-amino-acid region of MARCH8 and the TM domain of TfR.

Results
MARCH1 and MARCH8 downregulate TfR
Phylogenetic analysis revealed that six members of the MARCH family containing two TM domains (MARCH1, 2, 3, 4, 8, and 9) were subdivided into three closely related pairs, namely MARCH1 and 8, MARCH2 and 3, and MARCH4 and 9 (Bartee et al., 2004; Wang et al., 2008). A previous study using FACS analysis showed that MARCH1 and MARCH8 downregulated the cell surface expression of TfR while MARCH2 or 3 had less or no effect, respectively, on TfR downregulation (Bartee et al., 2004). Based on these findings, GFP-fused MARCH1, 2, 3, and 8 were expressed in HeLa cells and their effect on TfR localization (Fig. 1A) and Cy3–Tf uptake
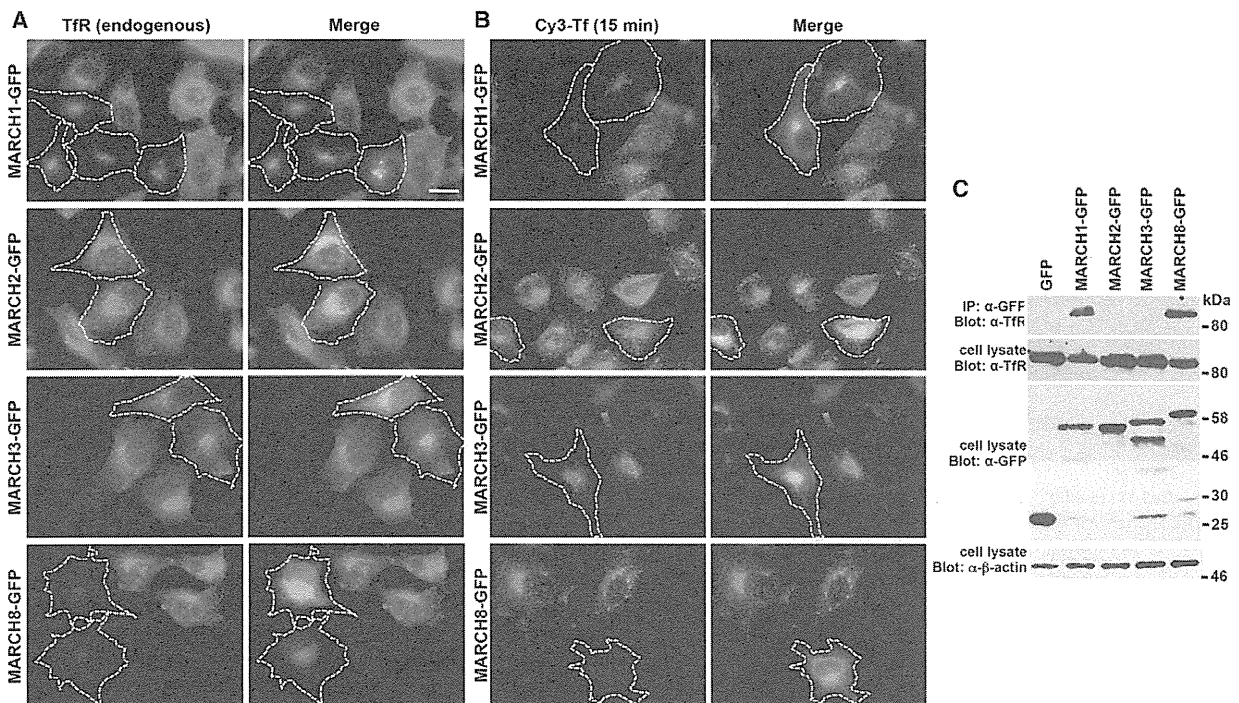

Fig. 1. **MARCH1 and 8, but not 2 and 3, specifically downregulate TfR.** HeLa cells transfected with plasmids encoding MARCH–GFP chimeric proteins for 24 hours were processed for either IF analysis with an anti-TfR antibody (**A**) or examined for Cy3–Tf uptake for 15 minutes (**B**). The cells expressing MARCH–GFP (green in Merge) are indicated by the yellow dotted lines. The localization and expression of endogenous TfR (A) or the uptake of Cy3–Tf (B) are shown in red. Scale bar: 20 μm. (**C**) COS7 cells transfected with plasmids encoding GFP or MARCH–GFP chimeras (MARCH1, 2, 3 and 8) for 24 hours were processed for co-IP analysis. The association of endogenous TfR with MARCH–GFP chimeras was examined by co-IP with an anti-GFP antibody followed by immunoblotting with an antibody to TfR (top panel). To confirm the expression levels of endogenous TfR, GFP-fused MARCH proteins, and β-actin (as an internal control), equivalent amounts of protein extracts were subjected to immunoblot analysis with antibodies directed against these proteins.

(Fig. 1B) were examined. Immunofluorescence (IF) analysis showed that TfR in non-transfected cells was distributed throughout the peripheral region (early endosomes and PM) and also densely localized at the perinuclear recycling endosomes. Overexpression of MARCH1 and 8, but not MARCH2 and 3, resulted in the disappearance of TfR from the peripheral regions and recycling endosomes (Fig. 1A). Consistent with these results, overexpression of MARCH1 and MARCH8, but not MARCH2 and 3, remarkably reduced the endocytosis of Cy3–Tf (Fig. 1B). These results are also consistent with the findings by Bartee et al. (Bartee et al., 2004). The ability of MARCH proteins to bind to endogenous TfR was also examined by immunoprecipitation (IP) using lysates from COS7 cells that overexpress MARCH–GFP proteins (MARCH1, 2, 3 and 8; Fig. 1C). In agreement with the data shown in Fig. 1A,B, GFP-fused MARCH1 and 8, but not MARCH2 and 3, bound specifically to TfR (top panel in Fig. 1C) and reduced intracellular TfR expression. These results suggest that both MARCH1 and 8 proteins specifically target TfR as a substrate. The expression of MARCH1 is restricted to immune system tissues, such as lymphoid tissues and antigen-presenting cells, whereas MARCH8 is widely expressed (Bartee et al., 2004); therefore, we hereafter focused on and analyzed the effect of MARCH8 on TfR.

MARCH8 RING-CH domain is required for ubiquitination and downregulation of TfR, but not for their interaction

We next examined the role of the MARCH8 RING-CH domain (located at the N-terminal CT domain; see Fig. 7A), which may mediate interaction with a specific E2 enzyme (Deshaies and Joazeiro, 2009). We generated a plasmid expressing an inactive mutant form of MARCH8–GFP in which all cysteine residues (Cys80, 83, 97, 99, 110, 123, and 126; shown by red dots in Fig. 7A) in the RING-CH domain were substituted with serine residues (designated as CS mutant). In HeLa cells, the MARCH8–GFP CS mutant did not downregulate endogenous TfR (Fig. 2A); therefore, cells expressing the CS mutant retained the ability to take up Cy3–Tf (Fig. 2B). We next examined the expression levels of endogenous TfR using COS7 cells transfected with either wild-type (WT) or the CS mutant MARCH8–GFP expression plasmid (Fig. 2C). WT, but not the CS mutant of MARCH8–GFP, remarkably reduced the levels of TfR expression (middle panel in Fig. 2C). Ubiquitination of TfR was examined by IP and western blotting analysis (Fig. 2D). In cells expressing WT MARCH8–GFP, TfR ubiquitination increased considerably. In cells expressing the CS mutant, however, the levels of ubiquitinated TfR were lower than in the control cells, suggesting that the CS mutant may act as a dominant-negative protein by competitively inhibiting TfR ubiquitination mediated by endogenous MARCH proteins. Importantly, both WT and the CS mutant MARCH8–GFP proteins efficiently associated with TfR (Fig. 2E). Taken together, these results suggest that the RING-CH domain of MARCH8 is required for its ubiquitination activity, most likely for its association with an E2 enzyme, but not with TfR.
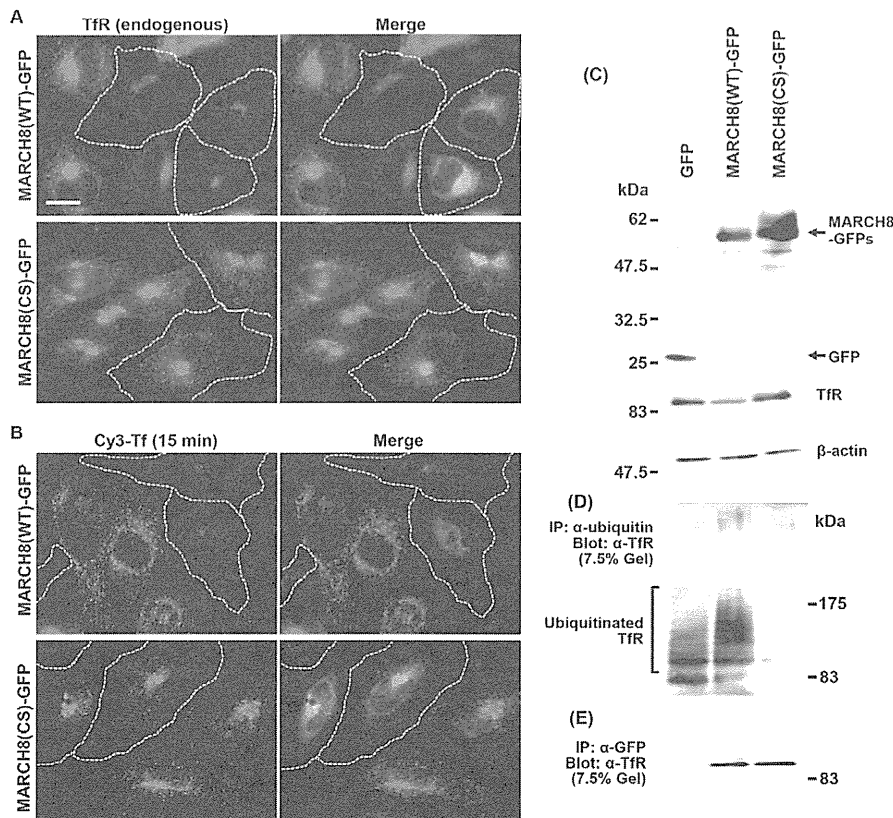

Fig. 2. **WT MARCH8, but not the CS mutant, specifically ubiquitinates and downregulates TfR.** HeLa cells transfected with plasmids encoding MARCH8–GFP (WT or CS mutant) for 24 hours were processed for either IF analysis with an anti-TfR antibody (A) or examined for Cy3–Tf uptake for 15 minutes (B). The cells expressing MARCH8–GFP (green in Merge) are indicated by the yellow dotted lines. The localization and expression of endogenous TfR (A) or the uptake of Cy3–Tf (B) are shown in red. Scale bar: 20 μm. (C) COS7 cells transfected with plasmids encoding GFP or MARCH8–GFP (WT or CS mutant) for 24 hours were processed for immunoblotting analysis with antibodies to GFP, TfR, and β-actin. (D) The ubiquitination of endogenous TfR in the cells expressing GFP or MARCH8–GFPs (WT or CS mutant) was examined by IP of ubiquitinated proteins with an anti-ubiquitin antibody followed by immunoblotting with an antibody to TfR. (E) The association of endogenous TfR with MARCH8 was examined by IP of MARCH8–GFP (WT or CS mutant) with an anti-GFP antibody followed by immunoblotting with an antibody to TfR.

MARCH8 induces lysosomal degradation of TfR

We next examined whether MARCH8-induced TfR degradation occurs in lysosomes. HeLa cells transfected with the MARCH8–GFP expression plasmid were cultured in the presence of either DMSO or bafilomycin A1, a specific inhibitor of vacuolar-type proton pump, that increases the luminal pH of late endosomes and lysosomes and inhibits acid-dependent lysosomal degradation. Cells were then analyzed by IF using antibodies against TfR and lamp1, a marker for late endosomes and lysosomes. In the DMSO-treated control cells expressing WT MARCH8–GFP, TfR was no longer detected (Fig. 3A); however, bafilomycin A1 restored the TfR signals (Fig. 3B,C), which were partly co-localized with those of MARCH8–GFP and lamp1 (arrows in Fig. 3C). These results suggest that MARCH8-induced downregulation of TfR is mediated by lysosomal degradation.

Lysine residues in the CT domain of TfR are involved in its degradation induced by MARCH8

The ubiquitination of TM proteins generally occurs at lysine residues located in the CT domain. We previously showed that a TfR mutant protein with no lysine residues (4KR mutant) was not ubiquitinated (Tachiyama et al., 2011). To determine whether the 4KR mutant of TfR is resistant to MARCH8-induced

degradation, HeLa cells stably expressing myc-tagged TfRs (WT or the 4KR mutant) were transfected with the MARCH8–GFP expression plasmid. While MARCH8 efficiently depleted WT myc–TfR (Fig. 4A), it did not affect the expression of the myc-tagged 4KR mutant (Fig. 4B). Importantly, more than 80% of cells expressing the myc-tagged TfR 4KR mutant were resistant to MARCH8 (Fig. 4C). Ubiquitination of the myc-tagged TfRs was next examined by IP and western blotting (Fig. 4D). The ubiquitination of myc-tagged TfR was readily detected in cells expressing both WT myc–TfR and WT MARCH8–GFP, but not in cells expressing either the myc-tagged TfR 4KR mutant or the MARCH8–GFP CS mutant. These results suggest that MARCH8 specifically ubiquitinates lysine residues in the CT domain of TfR, resulting in its lysosomal degradation.

The TM domain of TfR is required for MARCH8-induced ubiquitination and downregulation

TfR has a type-II topology that consists of an N-terminal CT domain and a TM domain. Because MARCH8 harbors only a small extracellular region, we predicted that it might recognize and interact with either the CT or TM domain of TfR. We used previously generated chimeric constructs (Iwabu et al., 2009)
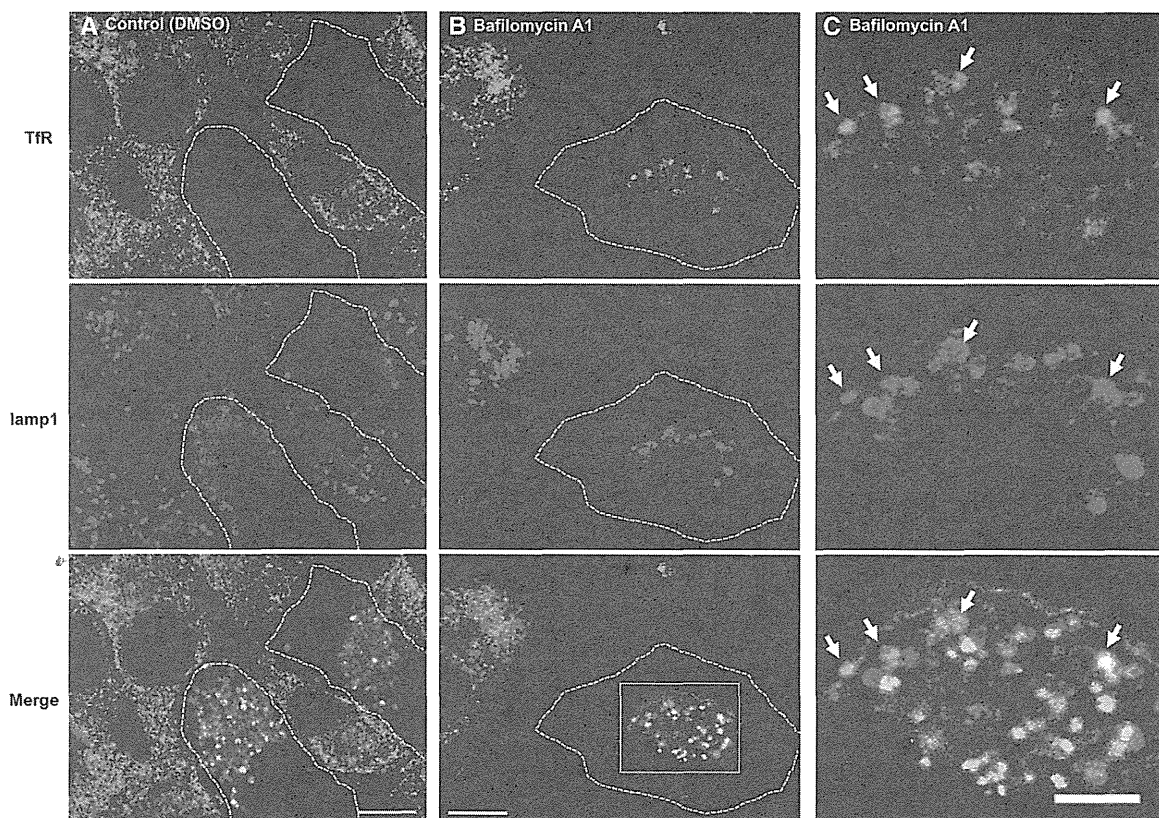

Fig. 3. **MARCH8 induces the lysosomal degradation of TfR.** HeLa cells expressing WT MARCH8–GFP for 24 hours were further cultured for 12 hours in the presence of either DMSO (control; **A**) or bafilomycin A1, an inhibitor of the vacuolar-type proton pump (0.5 μM; **B,C**) and processed for IF analysis with anti-TfR (red) and anti-lamp1 (blue) antibodies. The images were obtained by confocal laser microscopy. The cells expressing MARCH8–GFPs (green in Merge) are indicated by the yellow dotted lines. The colocalization of endogenous TfR with lamp1 and MARCH8–GFP in cells treated with bafilomycin A1 is indicated by the arrows in C, which are enlarged images of the boxed region in B. Scale bar: 20 μm A,B); 5 μm (C).