

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 Yxx Φ 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

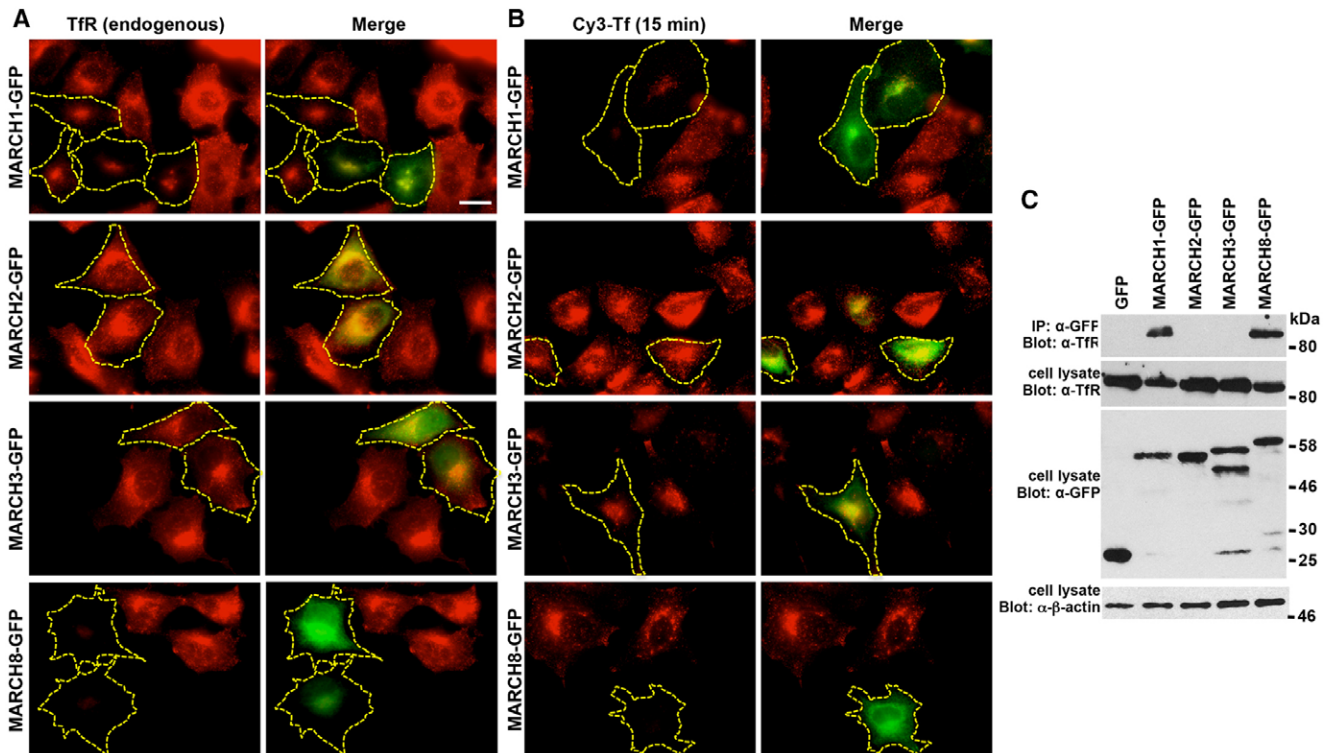


Fig. 1. MARCH1 and 8, but not 2 and 3, specifically downregulate TfR. HeLa cells transfected with plasmids encoding MARCH-GFP chimeric proteins for 24 hours were processed for either IF analysis with an anti-TfR antibody (A) or examined for Cy3-Tf uptake for 15 minutes (B). The cells expressing MARCH-GFP (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 μ m. (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.

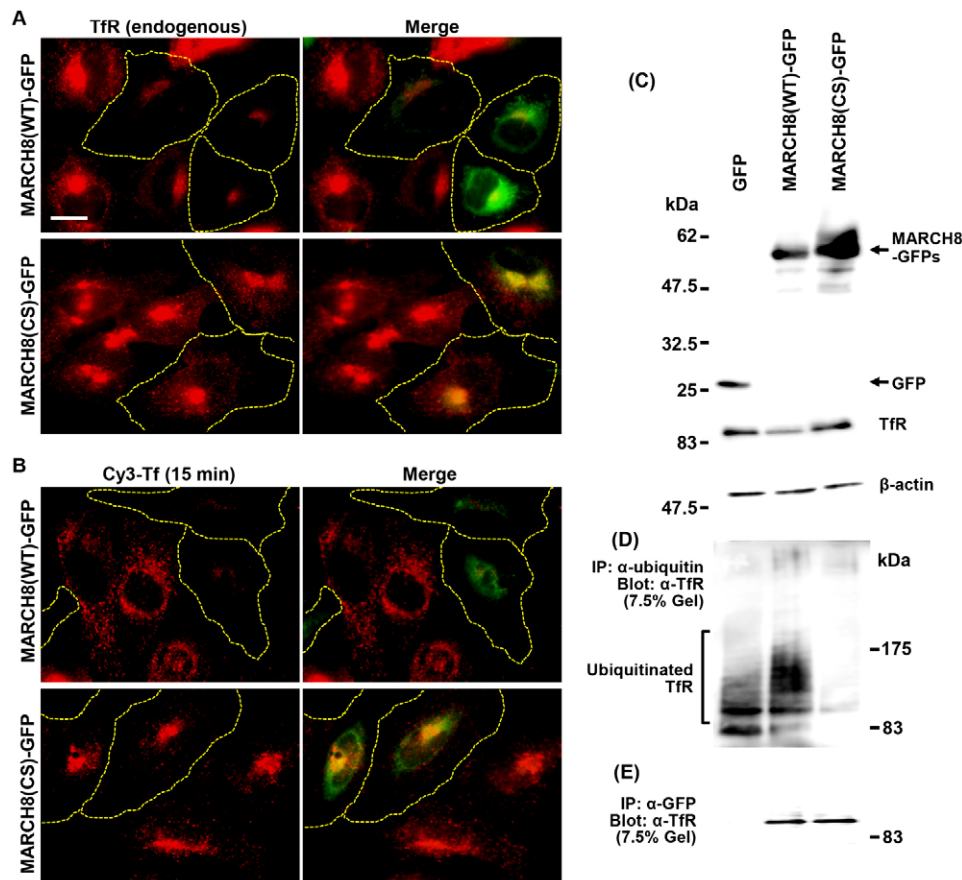


Fig. 2. WT MARCH8, but not the CS mutant, specifically ubiquitinates and downregulates TfR. HeLa cells transfected with plasmids encoding MARCH8-GFP (WT or CS mutant) for 24 hours were processed for either IF analysis with an anti-TfR antibody (A) or examined for Cy3-Tf uptake for 15 minutes (B). The 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)

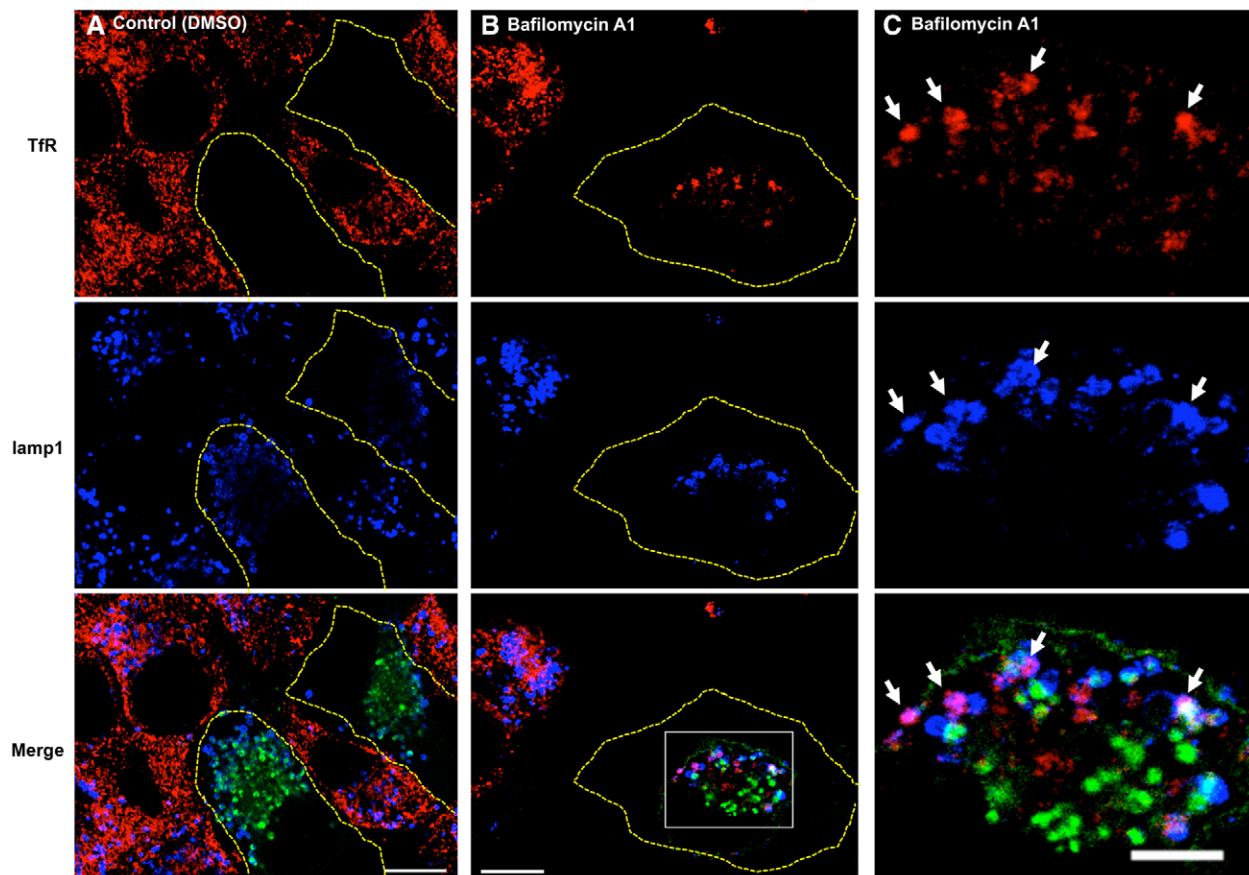


Fig. 3. MARCH8 induces the lysosomal degradation of TfR. HeLa cells expressing WT MARCH8-GFP for 24 hours were further cultured for 12 hours in the presence of either DMSO (control; A) or bafilomycin A1, an inhibitor of the vacuolar-type proton pump (0.5 μ M; B,C) and processed for IF analysis with anti-TfR (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 μ m (A,B); 5 μ m (C).

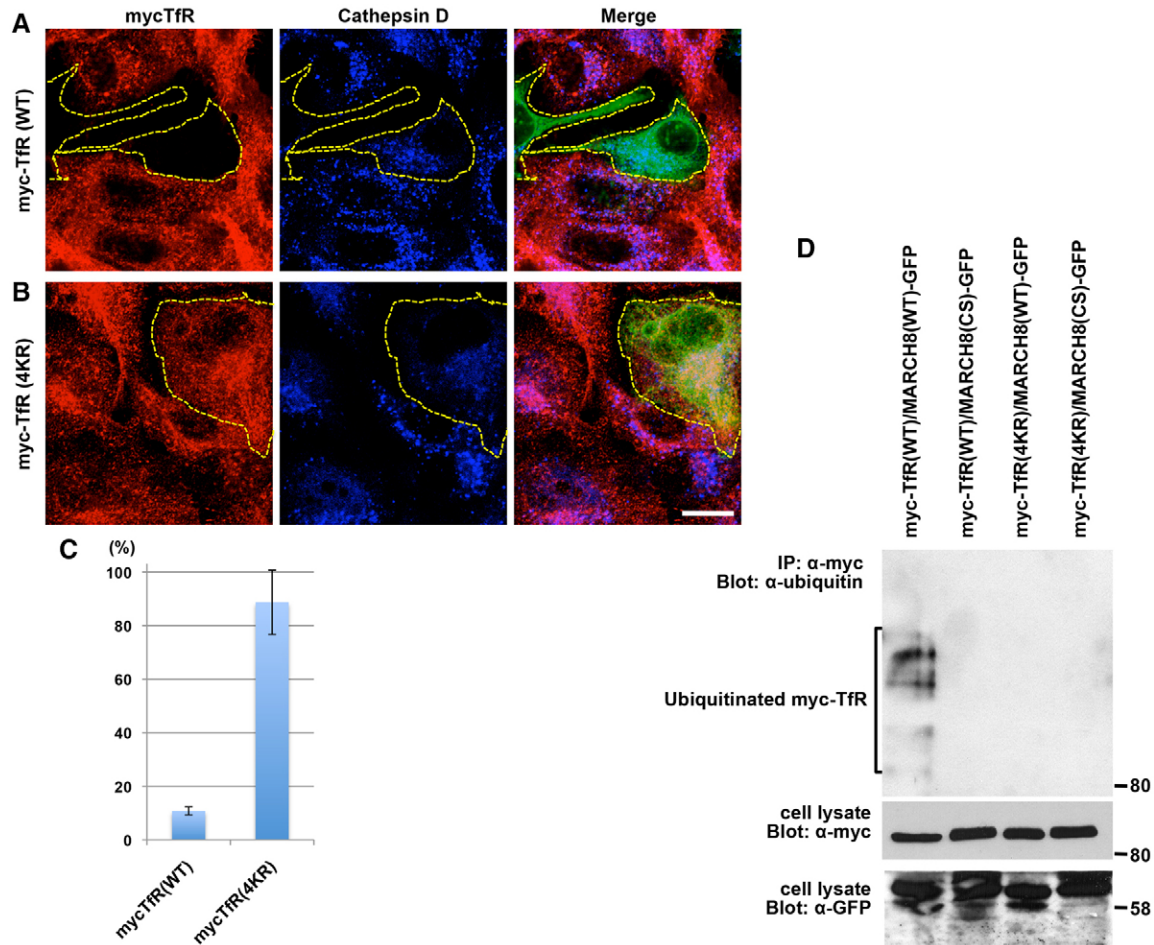


Fig. 4. Lysine residues in the cytoplasmic region of TfR are involved in the MARCH8-induced downregulation of TfR. (A,B) HeLa cells stably expressing myc-TfR (WT in A or 4KR mutant in B) were transfected with WT MARCH8-GFP and processed for IF analysis with anti-myc (red) and anti-cathepsin D (blue) antibodies. The images were obtained by confocal laser microscopy. The cells expressing WT MARCH8-GFP (green in Merge) are indicated by the yellow dotted lines. Scale bar: 20 μ m. (C) The percentage of cells expressing myc-TfR (WT or 4KR mutant) in cells transfected with MARCH8-GFP. The number of cells examined was 394 for WT and 356 for 4KR. (D) The ubiquitination of myc-tagged TfRs (WT or 4KR mutant) in cells expressing MARCH8-GFP (WT or CS mutant) was examined. COS7 cells transfected with plasmids encoding myc-tagged TfRs (WT or 4KR mutant) and MARCH8-GFP (WT or CS mutant) for 24 hours were processed for IP with an anti-myc antibody followed by immunoblot analysis with a ubiquitin antibody. To confirm the expression of TfR and MARCH8 proteins, cell lysates were subjected to immunoblot analysis with anti-myc (middle panel) and anti-GFP (lower panel) antibodies, respectively.

between TfR and bone marrow stromal antigen 2 (BST-2), which is another type-II TM protein (see upper scheme in Fig. 5A) to determine the domains of TfR involved in its downregulation by MARCH8. FACS analysis showed that WT MARCH8 selectively reduced the cell surface levels of the BST-2/TfR chimeric proteins containing the TM domain of TfR (TfRct/tm-BST2 and TfRtm-BST2), but did not affect proteins lacking the TM domain (BST2 and TfRct-BST2). The MARCH8 CS mutant showed no effects on any of the chimeric proteins (Fig. 5A). These results were confirmed by IF analysis (Fig. 5B), which showed that WT MARCH8 efficiently reduced the cell surface TfRct/tm-BST2 and TfRtm-BST2, whereas BST2 and TfRct-BST2 were not affected. Consistent with these results, a ubiquitination assay showed that the MARCH8-induced ubiquitination of TfR only requires the TM domain of TfR (Fig. 5C). Interestingly, while the sequences of the CT domains of TfR and BST2 are not similar, both TfRct/tm-BST2 and TfRtm-BST2 were effectively ubiquitinated when co-transfected

with WT MARCH8-GFP (Fig. 5C). In the case of TfRtm-BST2, it is likely that a lysine residue located in the CT domain of BST2 is ubiquitinated by MARCH8. Taken together, these results suggest that MARCH8 specifically recognizes the TM domain of TfR and induces its ubiquitination and downregulation.

The TM and CT domains of TfR interact with MARCH8

Based on these results, we assumed that MARCH8 binds to TfR through TM-TM interactions. We therefore performed co-IP analysis using MARCH8-GFP and GFP (as negative control) together with the myc-tagged BST-2/TfR chimeric proteins (Fig. 6). The expression of all proteins in the transfected cells was confirmed by immunoblotting (middle and lower panels in Fig. 6). As expected, the BST-2/TfR chimeric proteins containing the TM domain of TfR (TfRct/tm-BST2-myc and TfRtm-BST2-myc) were specifically co-immunoprecipitated with MARCH8-GFP. Unexpectedly, TfRcyt-BST2-myc, which harbors only the CT domain of TfR and is defective for

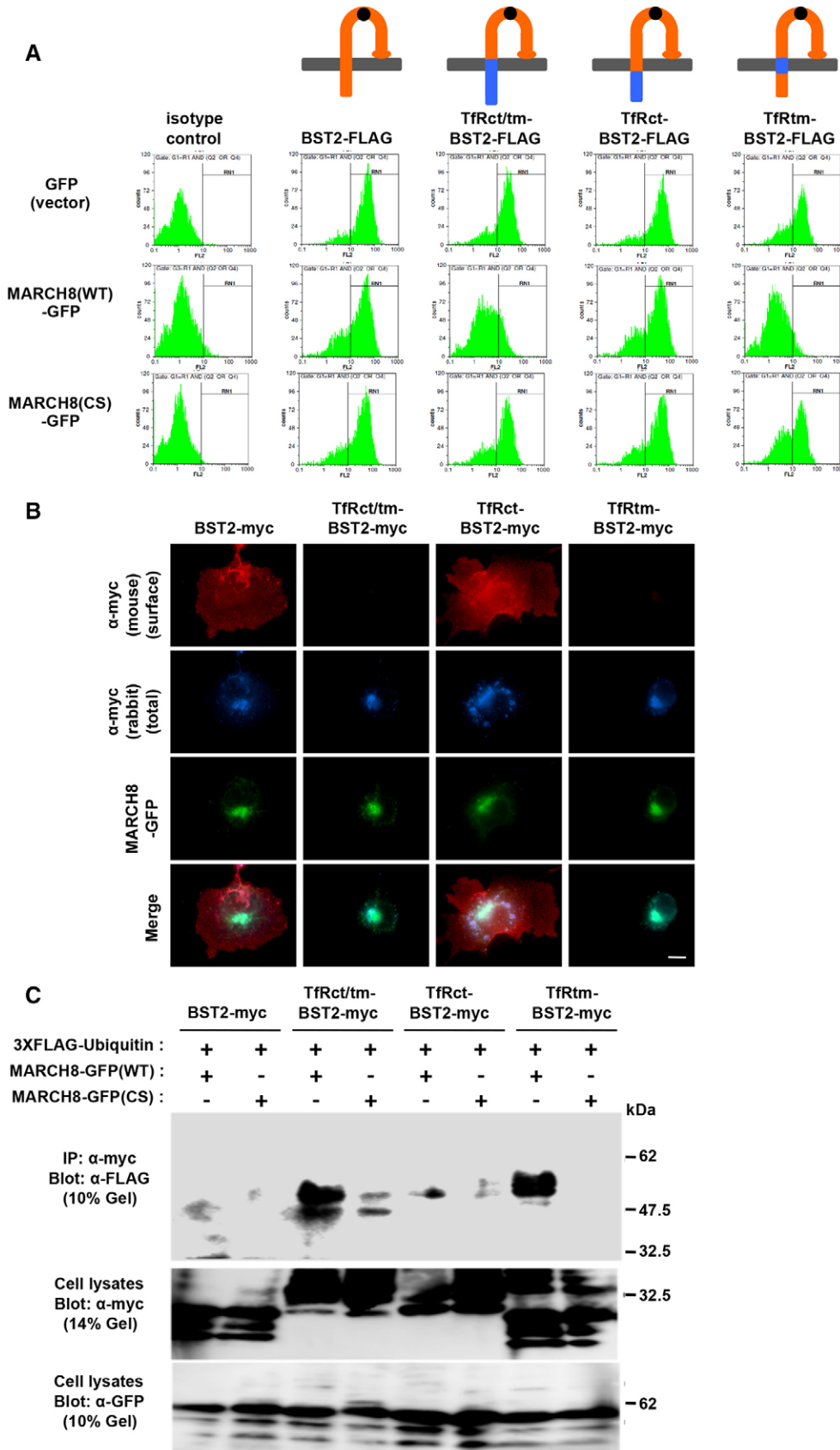


Fig. 5. The TM domain of TfR is specifically recognized by MARCH8.

(A) The predicted topologies of the BST-2/TfR chimeric proteins are shown above the histograms. The CT, TM and extracellular domains of BST-2 are shown in orange, and the CT and TM domains of TfR are shown in blue. The inserted FLAG tag is shown as a black circle. The PM is depicted in gray. The cell surface expression of the chimeric BST-2 proteins is shown. 293T cells transfected with plasmids encoding GFP (upper panels) or MARCH8-GFP (WT or CS mutant, middle and lower panels, respectively) together with FLAG-tagged BST-2 WT, TfRct/tm, TfRct, or TfRtm were stained for cell surface BST-2 chimera expression using an anti-FLAG monoclonal antibody and analyzed by two-color flow cytometry. (B) COS7 cells transfected with plasmids encoding WT MARCH8-GFP, together with myc-tagged BST-2 WT, TfRct/tm, TfRct, or TfRtm were stained for cell surface BST-2/TfR chimera expression using an anti-myc mouse monoclonal antibody before fixation. After washing, the cells were fixed and processed for IF with an anti-myc rabbit polyclonal antibody that mainly detected the intracellular localization of the BST-2/TfR chimeric proteins. Scale bar: 20 μ m. (C) MARCH8 specifically ubiquitinates the chimeric proteins containing the TM domain of TfR. COS7 cells transfected with plasmids encoding 3 \times FLAG-ubiquitin and MARCH8-GFP (WT or CS mutant), together with myc-tagged BST-2 WT, TfRct/tm, TfRct, or TfRtm were subjected to IP with an anti-myc antibody. The ubiquitination of BST-2/TfR chimeric proteins by MARCH8-GFP was examined by immunoblotting with an anti-FLAG antibody. To confirm the expression of the transfected plasmids, aliquots of cell lysates were subjected to immunoblotting with anti-myc (middle panel) and anti-GFP (lower panel) antibodies, respectively.

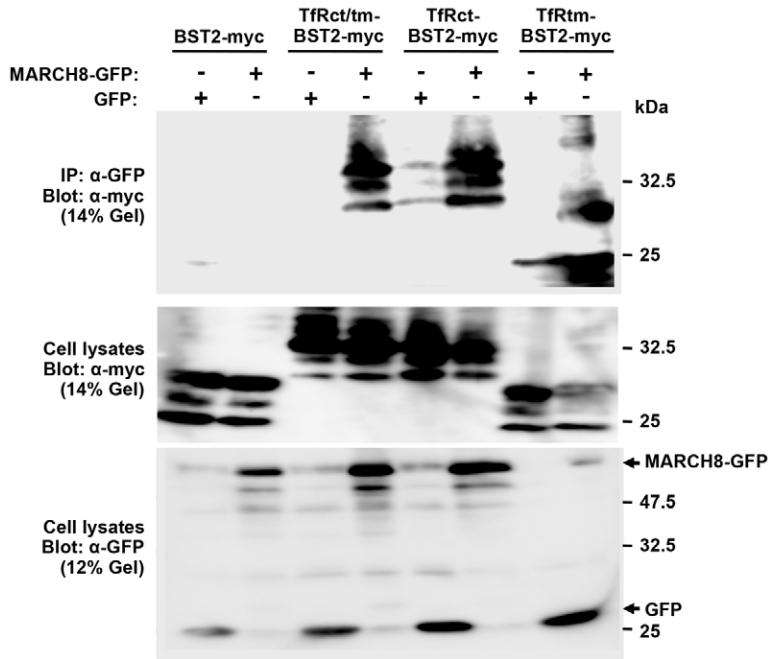


Fig. 6. The TM and CT domains of Tfr interact with MARCH8. MARCH8 interacts with the TM and CT domain of Tfr. COS7 cells transfected with plasmids encoding either GFP (as control) or WT MARCH8-GFP together with myc-tagged BST-2 WT, Tfrct/tm, Tfrct, or Tfrtm were subjected to IP with an anti-GFP antibody. The association of the BST-2/Tfr chimeric proteins with MARCH8-GFP was revealed by immunoblotting with an anti-myc antibody (upper panel). To confirm the expression of the transfected plasmids, aliquots of cell lysates were subjected to immunoblotting with anti-myc (middle panel) and anti-GFP (lower panel) antibodies, respectively.

A CLUSTAL 2.1 multiple sequence alignment

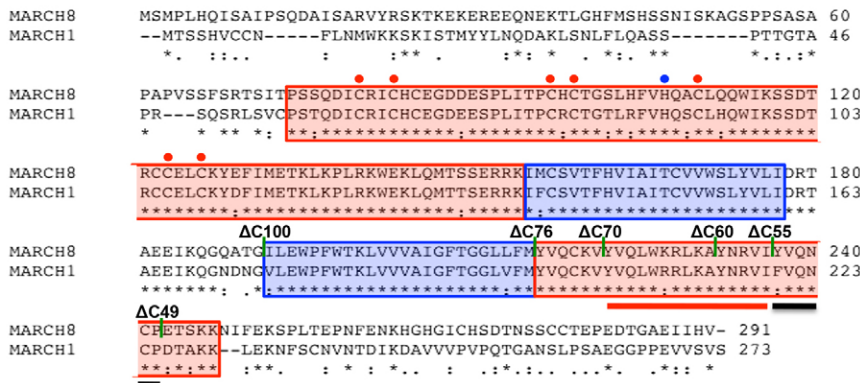
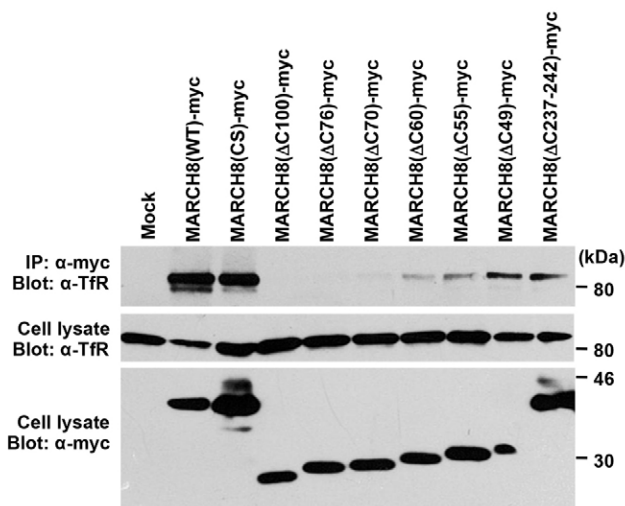


Fig. 7. The C-terminal membrane-proximal region of MARCH8 interacts with Tfr.

(A) Sequence alignment of MARCH8 and MARCH1 was performed using CLUSTAL 2.0.11. The blue boxes indicate the TM domains of MARCH8 and MARCH1. The red boxes indicate the highly homologous regions located in both N- and C-terminal CT domains of MARCH8 and MARCH1. Seven cysteine residues and one histidine residue potentially required for the formation of the RING-CH structure are shown by red and blue dots, respectively. The green lines indicate the C-terminal positions of the MARCH8 deletion mutants (Δ C100, Δ C76, Δ C70, Δ C60, Δ C55, and Δ C49). The solid red line indicates the region that is responsible for MARCH8 association with Tfr. The solid black lines indicate the minimum region that is required for MARCH8 downregulation of Tfr. (B) COS7 cells transfected with plasmids encoding WT, CS, and C-terminal deletion mutants of MARCH8-myc chimeras were subjected to co-IP with an anti-myc antibody. The association of endogenous Tfr with MARCH8-myc was examined by immunoblotting with an anti-Tfr antibody (upper panel). The expression of endogenous Tfr and MARCH8-myc chimeras was confirmed by immunoblotting with antibodies against Tfr and myc, respectively (middle and lower panels).

B



MARCH8-induced ubiquitination and downregulation (Fig. 5A–C), was also co-immunoprecipitated with MARCH8–GFP (upper panel in Fig. 6). These results suggest that the interaction between MARCH8 and TfR involves the TM and CT domains of TfR. However, the interaction of MARCH8 with the CT domain of TfR is not sufficient for its ubiquitination and downregulation, whereas interaction with the TM domain of TfR is sufficient for its activity (Fig. 5A–C). This would imply that the subsequent actions (ubiquitination and lysosomal degradation of TfR) cannot be explained by the mere interaction between these two proteins, as discussed below.

The membrane-proximal region of the C-terminal CT domain of MARCH8 interacts with TfR

As shown above (Fig. 1), MARCH8 and MARCH1 showed similar activity in the downregulation of TfR. The deduced amino acid sequence of MARCH8 shares 60.5% identity and 79.4% similarity with that of MARCH1 (Fig. 7A). In particular, the region containing the RING-CH domain at the N-terminal CT domain (Fig. 7A, first red box) and the region in the C-terminal CT domain (Fig. 7A, second red box) are highly conserved between MARCH8 and MARCH1. In addition to the RING-CH domain, the C-terminal CT domain of mouse MARCH1 was suggested to be functionally required for the downregulation of CD86 and MHC-II (Jabbour et al., 2009). Therefore, we next

examined the role of this domain of human MARCH8 in the downregulation of TfR. We generated plasmids expressing serial C-terminal deletion mutants of myc-tagged MARCH8 and performed co-IP analysis to determine whether endogenous TfR would be co-immunoprecipitated with myc-tagged mutants (Fig. 7B). The expression of endogenous TfR and MARCH8-myc mutant proteins in the transfected cells was confirmed by immunoblotting (Fig. 7B, middle and lower panels, respectively). As observed for GFP-tagged MARCH8 (Fig. 2E), WT and the CS mutant of MARCH8-myc effectively associated with endogenous TfR (Fig. 7B, upper panel). The $\Delta C100$, $\Delta C76$, and $\Delta C70$ mutants showed no or little association with TfR, whereas the $\Delta C60$, $\Delta C55$, and $\Delta C49$ mutants showed weak but visible association with TfR. These results suggest that the membrane-proximal region of the C-terminal CT domain of MARCH8 (amino acids 222–231, shown by a solid red line in Fig. 7A) is responsible for the association with TfR.

The highly conserved six-amino-acid region in the C-terminal CT domain of MARCH8 promotes the downregulation of TfR

Next, we examined the ability of deletion mutants of MARCH8 to downregulate TfR and their subcellular localization. WT, but not the CS mutant of MARCH8-myc, effectively downregulated TfR (Fig. 8, top and second panels in the left columns). Both the

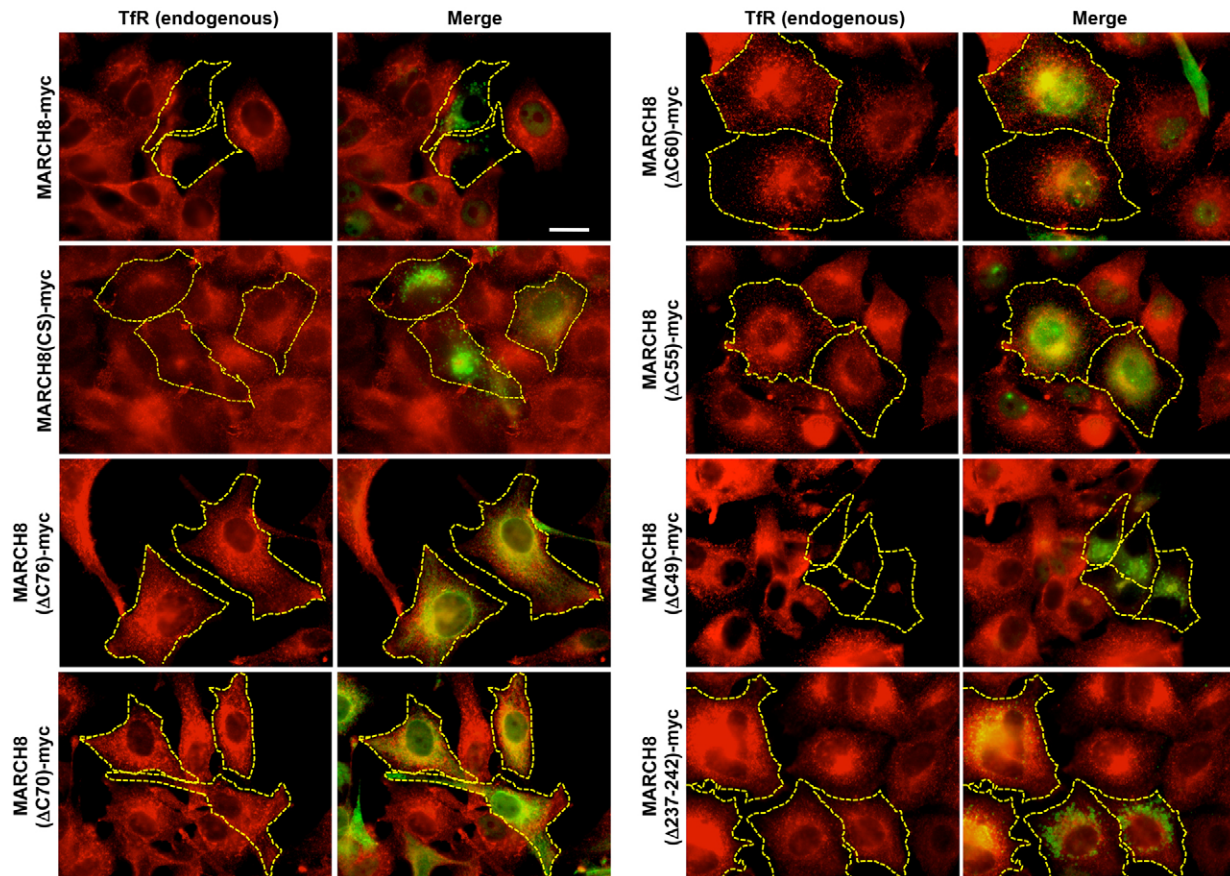


Fig. 8. A highly conserved six-amino-acid region in the C-terminal CT domain of MARCH8 is responsible for its ability to downregulate TfR. HeLa cells transfected with plasmids encoding the C-terminal deletion mutants of MARCH8–myc chimeras were processed for IF analysis with anti-myc and anti-TfR antibodies. The cells expressing MARCH8–myc (green in Merge) are indicated by the yellow dotted lines. The localization and expression of endogenous TfR are shown in red. Scale bar: 20 μ m.

WT and the CS mutant of MARCH8-myc localized to vesicular compartments, which most likely corresponded to lysosomes, the final destination of MARCH8 (green signals in merged images). As expected, deletion mutants (Δ C100, Δ C76, and Δ C70) that showed no or little association with TfR were unable to downregulate TfR (Fig. 8, third and bottom panels, left columns; Δ C100; data not shown). Importantly, these mutants exclusively localized to the ER and did not reach the post-Golgi compartments where MARCH8 interacts with TfR. Unexpectedly, the Δ C60 and Δ C55 mutants, which showed weak interaction with TfR, lost the ability to downregulate TfR (Fig. 8, right top and second panels). These mutants localized to peri-nuclear regions that most likely correspond to the TGN, which would account for their association with TfR (see Fig. 7B). By contrast, the Δ C49 mutant protein downregulated TfR (Fig. 8, third panel, right columns) and localized to the vesicular compartments as efficiently as the WT. Together, these results suggest that the membrane-proximal region of the C-terminal CT domain of MARCH8 is responsible for its exit from the ER. Importantly, the association of Δ C60 and Δ C55 mutants with TfR was insufficient to downregulate TfR, because these mutants lack the six-amino-acid region present in the Δ C49 mutant. Finally, we examined an internal deletion mutant (Δ 237–242) of MARCH8 that lacks only the six amino acid region. Although this mutant associated with TfR (Fig. 7B) and localized correctly to the vesicular compartments, as did the Δ C49 mutant, it failed to downregulate TfR (Fig. 8, bottom panel, right columns). In agreement with this, the internal deletion mutant (Δ 237–242) of MARCH8 also failed to ubiquitinate TfR (supplementary material Fig. S1). These results suggest that the ability of MARCH8 to downregulate TfR requires the sequence between Tyr237 and Pro242 (YVQNCP) located in the C-terminal CT domain of MARCH8 (solid black line in Fig. 7A). The amino acid sequence of this region is completely conserved among various species (identical in human, chimpanzee, mouse, rat, cow, and dog proteins). It is also noteworthy that the distance from the membrane (21 amino acids) and the amino acid sequence of this region of MARCH8 are also conserved in human MARCH1, in which only a tyrosine residue is replaced with a phenylalanine.

MARCH8 knockdown stabilizes TfR in HepG2 cells

To verify the physiological significance of MARCH8 in TfR downregulation, we examined the effect of knockdown of MARCH8 on the expression level of TfR in HepG2 human hepatocellular carcinoma cells. Immunoblot analysis showed that endogenous MARCH8 expression was suppressed by an siRNA directed against MARCH8 (Fig. 9, top panel). Importantly, the expression level of TfR was markedly increased in the cells treated with siRNA directed against MARCH8 (Fig. 9, second panel). It should be noted that the increased expression of another MARCH8 substrate CD98 (Eyster et al., 2011) was confirmed by knockdown of MARCH8 (Fig. 9, third panel). These results reveal that endogenous MARCH8 is involved in TfR protein turnover as well as that of CD98.

Discussion

TfR is constitutively internalized from the PM and selectively recycled back from the early and/or recycling endosomes to the PM (Ciechanover et al., 1983; Dautry-Varsat et al., 1983; Hopkins and Trowbridge, 1983; Schneider et al., 1984). Because

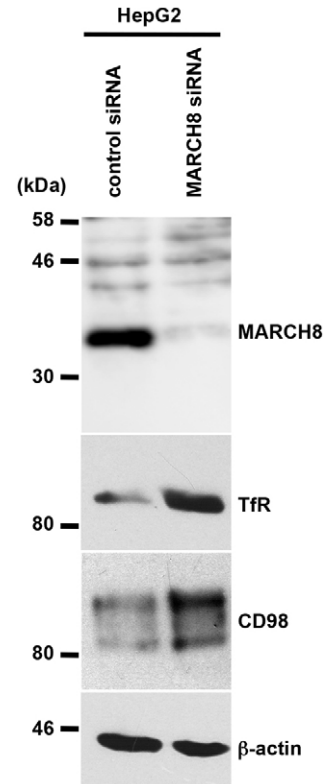


Fig. 9. Effect of the siRNA-mediated knockdown of endogenous MARCH8 on the expression levels of MARCH8 substrates, TfR and CD98, in HepG2 cells. HepG2 cells transfected with either control siRNA or siRNA directed against MARCH8 were cultured for 72 hours. Equivalent amounts of protein extracted from the cells were subjected to SDS-PAGE. The expression of endogenous MARCH8, TfR, CD98 and β -actin was confirmed by immunoblotting with specific antibodies. A representative of three independent experiments is shown.

of its relatively long half-life, it is likely that only a small proportion of TfR is segregated from the early and/or recycling endosomes and directed to the late endosomes/MVBs for its physiological turnover. However, the mechanism by which TfR is targeted for degradation is largely unknown. In this study, we first demonstrated that MARCH8 is a ubiquitin ligase specific for TfR. Our results showed that MARCH8 induces the ubiquitination and lysosomal degradation of TfR. Our results showing the accumulation of TfR in the late endosomes/lysosomes in cells expressing MARCH8 (Fig. 3B) suggest the following model: MARCH8 recognizes and ubiquitinates TfR at the PM or in early and/or recycling endosomes. The ubiquitinated TfR is selectively removed from the recycling pathway by the ESCRT system at the early and/or recycling endosomes, targeted to the inner vesicles of the MVBs, and finally degraded in lysosomes.

Partially consistent with this model, we previously reported the accumulation of ubiquitinated TfR in cells expressing a dominant-negative form of SKD1/Vps4B (E235Q), which abrogates the proper function of the ESCRT system resulting in the accumulation of ubiquitinated cargo proteins in aberrant endosomes, the so-called class E vps compartments (Tachiyama et al., 2011). Intriguingly, in the same study, our proteomics analysis showed that different types of PM proteins including

CD44 and CD98 were among the ubiquitinated proteins accumulating in Class E Vps compartments (Tachiyama et al., 2011). Eyster et al. showed that MARCH8 induces the ubiquitination of CD98 and alters the trafficking of CD44 and CD98 to the late endosomes (Eyster et al., 2011). They also showed that TSG101, an ESCRT-I subunit, is required for MARCH8-induced alteration of trafficking to the late endosomes. These results suggest that an ESCRT-dependent MVB pathway is involved in the downregulation of PM proteins targeted by MARCH8.

We also demonstrated that the TM domain of TfR is responsible for MARCH8-induced TfR ubiquitination and downregulation (Fig. 5A–C). MARCH1 also recognizes the TM domains of its substrates (Goto et al., 2003; Tze et al., 2011), suggesting that TM domain-dependent recognition may be common to the MARCH family of ubiquitin ligases. Indeed, Tze et al. reported that the MARCH1-induced downregulation of CD86 and MHC-II is affected by the presence of the TM domain of CD83 (Tze et al., 2011) and proposed that CD83 is an endogenous inhibitor of the MARCH1-induced downregulation of CD86 and MHC-II. In our study, however, the co-transfection of CD83-HA with either MARCH1 or MARCH8 did not impair the downregulation of TfR (supplementary material Fig. S2), suggesting the existence of different regulators that control the activity of MARCH proteins depending on their substrate.

One of the major findings of this study was the identification of a six-amino-acid region in the C-terminal CT domain of MARCH8, which is responsible for the downregulation of TfR. Although this region is conserved between MARCH1 and MARCH8 (Fig. 7A), no known motifs were found in this F(Y)VQNCP sequence. Consistent with our findings, Jabbour et al. reported that the ability of mouse MARCH1 to downregulate CD86 and MHC-II was lost in a CT domain deletion mutant (Jabbour et al., 2009) that completely lacks this six-amino-acid region. These observations suggest that the six-amino-acid region in the C-terminal CT domain of MARCH1 and MARCH8 plays a pivotal role in the downregulation of their substrate membrane proteins. The precise role of this six-amino-acid region is not clear. At least, this region was found to be dispensable for the targeting of MARCH8 to the post-Golgi compartments, because the internal deletion mutant lacking this region properly localized to lysosomes (Fig. 8).

Based on these results, we propose the following model in which MARCH8 recognizes and ubiquitinates TfR (supplementary material Fig. S3). We postulate the existence of an unknown co-factor that interacts with MARCH8 through the six-amino-acid region [supplementary material Fig. S3, (1)]. In the absence of such a co-factor, MARCH8 might not be able to ubiquitinate (supplementary material Fig. S1) and downregulate TfR (Fig. 8). The TM domain of TfR may be recognized and interacted with the TM domain of the unknown co-factor for the ubiquitination of the CT domain of the former protein by MARCH8 [supplementary material Fig. S3, (2)]. The membrane-proximal region of the CT domain of MARCH8 may be responsible for both its association with the CT domain of TfR [Fig. 6; supplementary material Fig. S3, (3)] and its exit from the ER (Fig. 8).

It has recently been reported that MARCH8 associates with BAP31, a membrane protein localized to the ER and possibly involved in vesicle formation and/or cargo selection at the ER exit sites (Bartee et al., 2010). These authors proposed that

BAP31 is required for the proper trafficking of MARCH8 to the PM. BAP31 could therefore be a candidate co-factor in the interaction between TfR and MARCH8. However, siRNA-mediated knockdown of BAP31 did not impair the MARCH8-induced downregulation of TfR (supplementary material Fig. S4), indicating that BAP31 is not the co-factor in this process.

MARCH8 targets multiple substrates; however, the physiological significance of its ubiquitin ligase activity has remained unknown. In this study, we found that endogenous MARCH8 is involved in the turnover of TfR in the hepatocyte cell line HepG2 (Fig. 9). Since the liver plays a crucial role in iron metabolism, the identification of a previously unknown mechanism for MARCH8-induced downregulation of TfR should increase understanding of iron homeostasis in the human body.

Materials and Methods

Reagents

The protease inhibitor cocktail was obtained from Nacalai Tesque (Kyoto, Japan). *N*-ethylmaleimide and bafilomycin A1 were purchased from Sigma Aldrich (St. Louis, MO). Protein A-coupled Sepharose 4B was purchased from GE Healthcare UK Ltd (Buckinghamshire, UK).

Antibodies

The antibodies against c-myc and FLAG (mouse monoclonal and rabbit polyclonal, respectively) and the mouse monoclonal antibody to β -actin were obtained from SIGMA Aldrich. Rabbit antibody to GFP was obtained from Molecular Probes (Eugene, OR). The mouse monoclonal antibodies to ubiquitin (FK2) and human TfR were obtained from Nippon Bio-Test Laboratories, Inc. (Tokyo, Japan) and Zymed Laboratories, Inc. (San Francisco, CA), respectively. The rabbit polyclonal antibody to CD98 was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The antibody to human lamp1 (mouse monoclonal) was a gift from Dr K. Furuta (National Cancer Center Research Institute, Tokyo, Japan). The generation of an antibody against human TfR was described previously (Tachiyama et al., 2011). Polyclonal antibody to human MARCH8 was generated by immunizing a rabbit with a GST-tagged fusion protein (GST–MARCH8:Met1–Ser158) as an immunogen. The secondary goat anti-rabbit or -mouse antibodies conjugated with Cy3 or Cy5 were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA). Alexa-Fluor-488-labeled goat anti-mouse and -rabbit secondary antibodies were purchased from Molecular Probes.

Cell culture

HeLa, COS7, and HepG2 cells were obtained from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum.

Plasmid construction and selection of cells stably expressing myc–TfR

The cDNAs for human MARCH1 (accession number BC148531), MARCH2 (accession number BC111388), MARCH3 (accession number BC047569), and MARCH8 (accession number BC025394) were obtained from Open Biosystems (Huntsville, AL). They were amplified by PCR and subcloned into GFP-N1 (Clontech, Palo Alto, CA) or pcDNA3.1/Myc-His(-) B (Invitrogen, Carlsbad, CA). The following sets of primers were used: MARCH1 (5'-atagaattcatgaccacagccactgtttg-3', 5'-ataggatccatgagactgatacaactcagggg-3'); MARCH2 (5'-atagaattcatgacaggggtgactgtc-3', 5'-ataggatccatcactgtgtctcctgccacc-3'); MARCH3 (5'-atagaattcatgacaaccagccgctgcagtcacc-3', 5'-ataggatcccaacaactgtctctttgagttctctt-3'); and MARCH8 (5'-atagaattcatgagcagcactgcacag-3', 5'-ataggatccacagcgtgaatgatttctgctcc-3'). The mutants of MARCH8 were constructed with the following sets of primers: CS mutant (5'-agccacagcacaggaagcctcactcgtgaccagccagc-3', 5'-gctgctggtgacaggaagtgaggctctgtgctgct-3', 5'-gacatacagcagcagcactgtg-3', 5'-cacagtgctgactcctgctgactc-3', 5'-cgctgcagcagcactcagcaagtatg-3', 5'-catactgtgagctcgtgcagc-3'), Δ C100 (5'-ataggatccatcctgtgctcgcctgctgtgact-3'), Δ C76 (5'-ataggatccacataaaaagaagctcctcggtgaag-3'), Δ C70 (5'-ataggatccacactttacactgaacatacataaaaag-3'), Δ C60 (5'-ataggatccagccttgagtcttccacaaattg-3'), Δ C55 (5'-ataggatccagcactctattatagcccttgag-3'), Δ C49 (5'-ataggatccatggacagtttgaacatagatac-3'), and Δ C237–242 (5'-cctataatagagtgcgaacaagcaaaaag-3', 5'-cttttctgtttcgcactctattatagg-3'). The plasmids encoding the BST-2/TfR chimeric proteins were described previously (Iwabu et al., 2009). The plasmids encoding 3 \times FLAG-ubiquitin and N-terminally myc-tagged human TfR (WT and 4KR), and HeLa cells stably expressing myc-tagged human TfR (WT and 4KR) were described previously (Tachiyama et al., 2011). Plasmid transfection was performed by lipofection with FUGEN6 (Roche

Molecular Biochemicals, Indianapolis, IN) following the manufacturer's instructions.

Immunofluorescence microscopy

Cells were cultured on 13 mm diameter glass coverslips, fixed with 4% paraformaldehyde for 30 minutes, permeabilized with 0.05% saponin, and immunostained as described previously with the primary antibodies at the following dilutions: anti-TfR (rabbit, 1:200; mouse, 1:300), anti-lamp1 (mouse, 1:300), and anti-myc (rabbit, 1:200; mouse, 1:300). The secondary goat anti-mouse and -rabbit antibodies that were conjugated with Alexa Fluor 488, Cy3, or Cy5 were used at 5 µg/ml. All IF images were observed on a Leica DMRB microscope (Wetzlar, Germany) equipped with a 63× 1.32 NA oil immersion lens (PL APO), acquired through a cooled CCD camera, MicroMAX (Princeton Instruments, Trenton, NJ), and digitally processed using IPLab Software (Scanalytics, Fairfax, VA). Confocal IF images were obtained via a Leica TCS SP8 system (Fig. 3) and Zeiss 510 meta microscopy (Fig. 4), respectively. All images were assembled using Adobe Photoshop (Adobe Systems, Mountain View, CA) and labeled using PowerPoint2008 (Microsoft Corporation, Tokyo, Japan).

Cy3-Tf uptake

Transfected cells were incubated in DMEM containing 20 µg/ml of Cy3-labeled Tf (Cy3-Tf; Molecular Probes) for 15 minutes at 37°C. The cells were washed three times with phosphate-buffered saline (PBS) before fixation and processed for IF microscopy with the appropriate antibodies.

Immunoprecipitation

The cells were lysed in TBS-T buffer [50 mM Tris-HCl buffer (pH 7.5), 0.15 M NaCl, 1% Triton-X100, and 0.5% deoxycholic acid] containing protease inhibitor cocktail and 10 mM *N*-ethylmaleimide. After centrifugation (21,500 g for 15 minutes), the supernatant was used as total cell lysate for either immunoblotting or immunoprecipitation. Protein A-coupled Sepharose 4B was pre-incubated for 2 hours at 4°C with the appropriate antibodies. The total cell lysate was incubated with the antibody-coupled Sepharose for 20 hours at 4°C, and then washed three times with TBS-T buffer. Immunoprecipitated proteins were eluted with SDS sample buffer and subjected to SDS-PAGE.

Flow cytometry

Flow cytometry analysis was described previously (Iwabu et al., 2009). Briefly, 293T cells were co-transfected with the extracellular FLAG-tagged series of BST-2/TfR chimera expression plasmids together with GFP or MARCH8-GFP. After 48 hours, the transfected cells were incubated with an anti-FLAG M2 mouse monoclonal antibody (Sigma) or an isotype control antibody (Immunotech, Marseille, France) followed by staining with a goat anti-mouse IgG conjugated to R-phycoerythrin (Molecular Probes) for 30 minutes on ice. The cells were then washed extensively with PBS plus 0.1% BSA, fixed with 4% formaldehyde in PBS, and analyzed by FACS on a CyFlow flow cytometer (Partec, Görlitz, Germany). The data were analyzed by using FlowJo software (Tree Star, Inc., Ashland, OR).

siRNA transfection

siRNA for MARCH8 (ON-TARGETplus Human MARCH8 (220972)) was obtained from Dharmacon (Waltham, MA). Transfection was done by lipofection with Oligofectamine (Invitrogen).

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Author contributions

H.F. conceived the study, designed and performed the experiments, analyzed the data and wrote the paper. Y.I. performed the experiments. K.T. performed the experiments, analyzed the data and wrote the paper. Y.T. edited the paper. All authors read and approved the final manuscript.

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