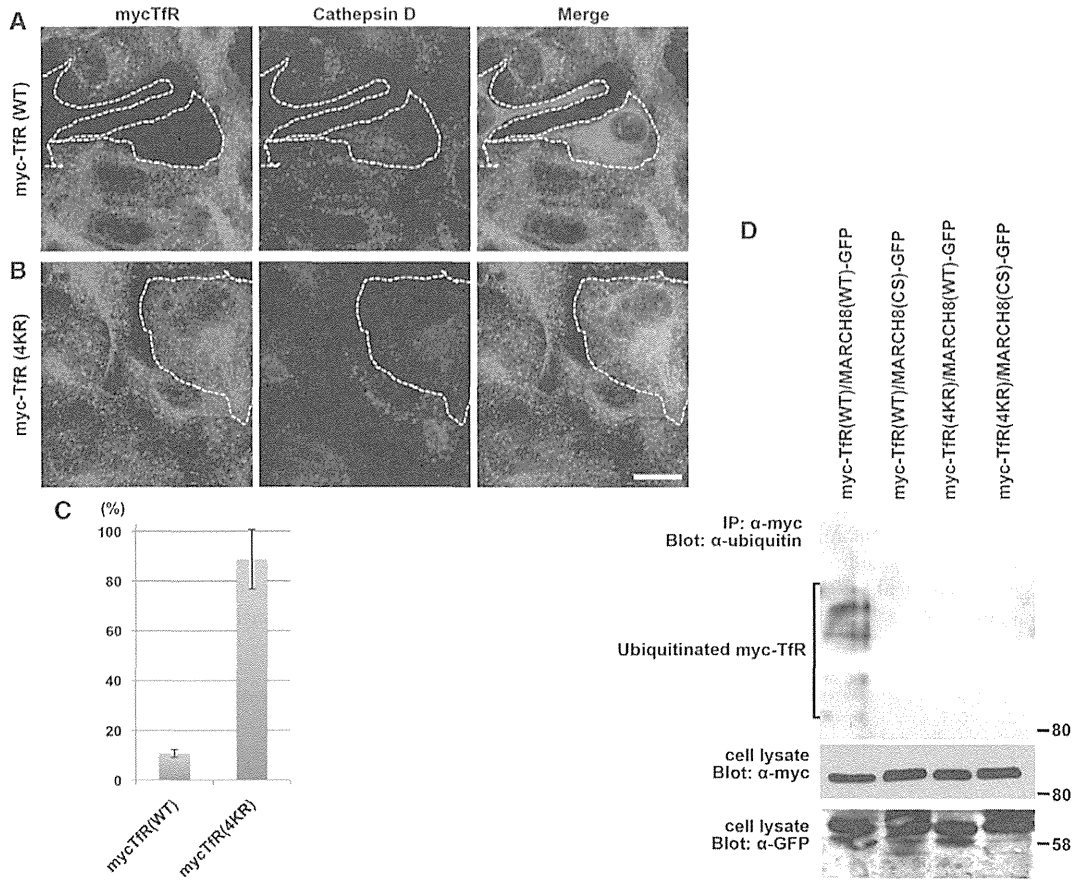

Fig. 4. Lysine residues in the cytoplasmic region of TfR are involved in the MARCH8-induced downregulation of TfR. (**A**,**B**) HeLa cells stably expressing myc–TfR (WT in A or 4KR mutant in B) were transfected with WT MARCH8–GFP and processed for IF analysis with anti-myc (red) and anti-cathepsin D (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 μm. (**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

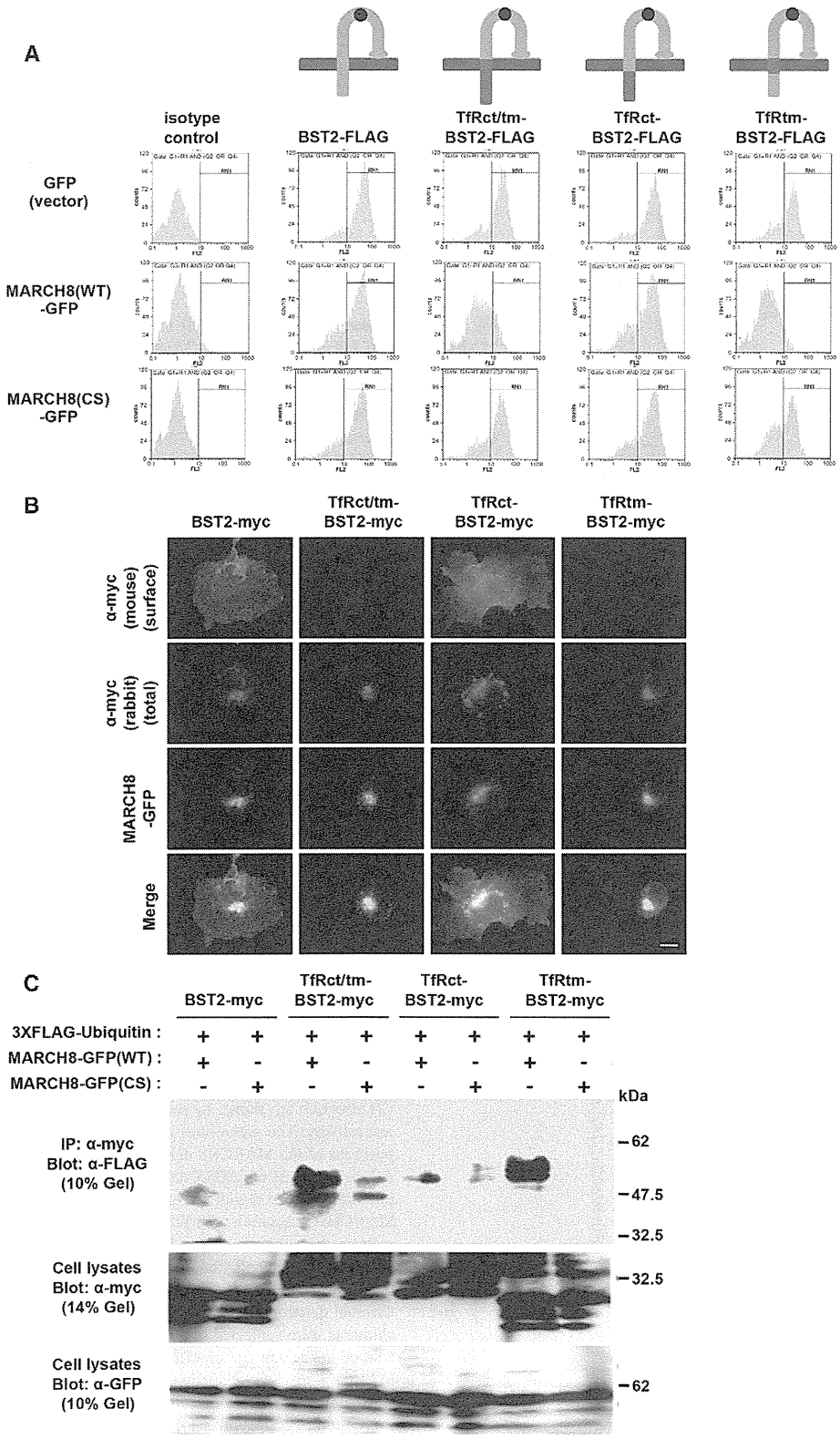

Fig. 5. The TM domain of TfR is specifically recognized by MARCH8. (**A**) The predicted topologies of the BST-2/TfR chimeric proteins are shown above the histograms. The CT, TM and extracellular domains of BST-2 are shown in orange, and the CT and TM domains of TfR are shown in blue. The inserted FLAG 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 μm. (**C**) MARCH8 specifically ubiquitinates the chimeric proteins containing the TM domain of TfR. COS7 cells transfected with plasmids encoding 3× 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.

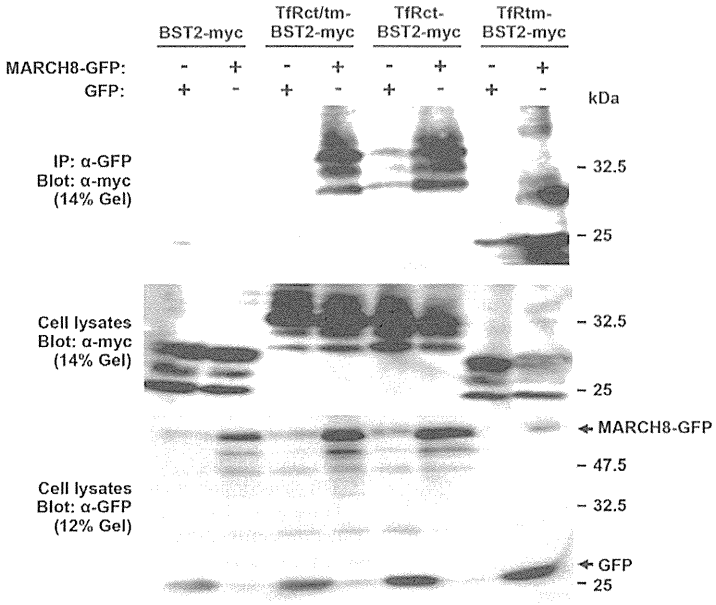

Fig. 6. The TM and CT domains of TfR interact with MARCH8. MARCH8 interacts with the TM and CT domain of TfR. COS7 cells transfected with plasmids encoding either GFP (as control) or WT MARCH8–GFP together with myc-tagged BST-2 WT, TfRct/tm, TfRct, or TfRtm were subjected to IP with an anti-GFP antibody. 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.

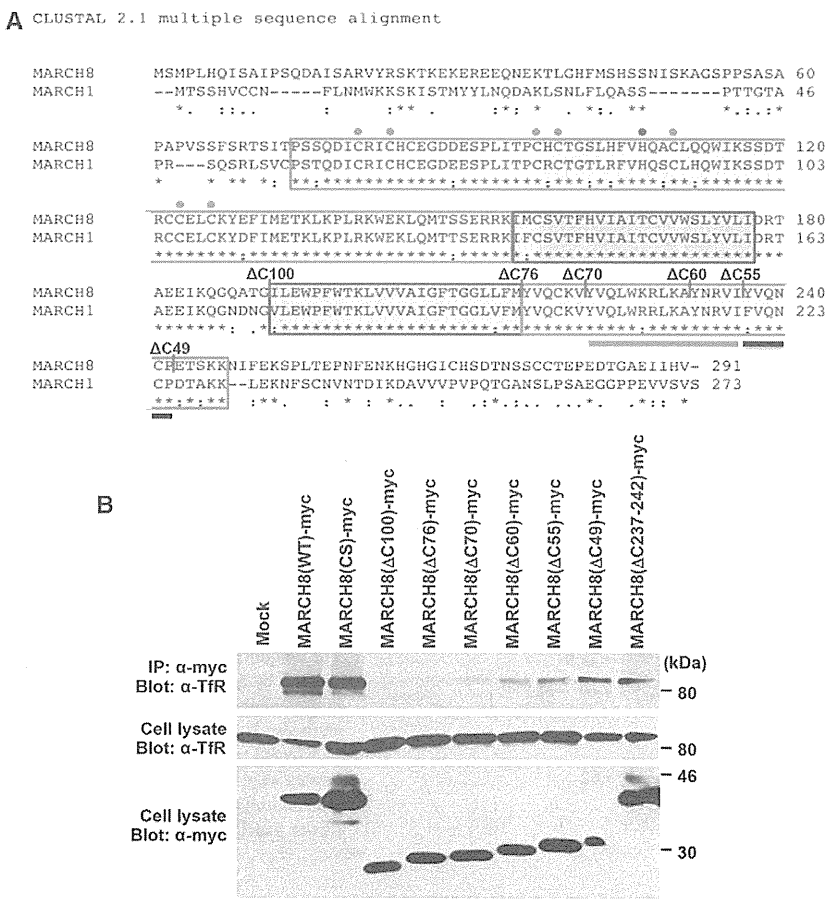

Fig. 7. The C-terminal membrane-proximal region of MARCH8 interacts with TfR. (**A**) Sequence alignment of MARCH8 and MARCH1 was performed using CLUSTAL 2.0.11. The blue boxes indicate the TM domains of MARCH8 and MARCH1. The red boxes indicate the highly homologous regions located in both N- and C-terminal 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 (ΔC100, ΔC76, ΔC70, ΔC60, ΔC55, and ΔC49). 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).

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 ΔC100, ΔC76, and ΔC70 mutants showed no or little association with TfR, whereas the ΔC60, ΔC55, and ΔC49 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

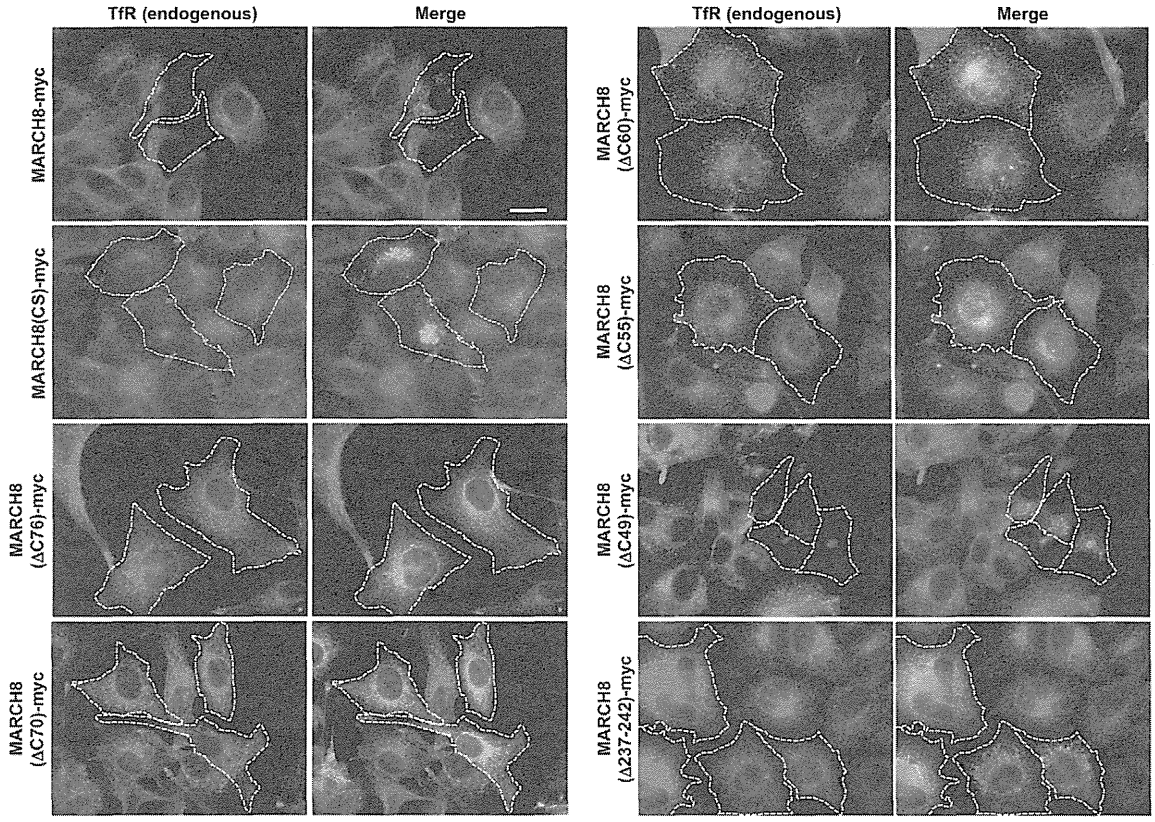

Fig. 8. A highly conserved six-amino-acid region in the C-terminal CT domain of MARCH8 is responsible for its ability to downregulate TfR. HeLa cells transfected with plasmids encoding the C-terminal deletion mutants of MARCH8–myc chimeras were processed for IF analysis with anti-myc and anti-TfR antibodies. 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 μm.

WT and the CS mutant of MATCH8-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 (ΔC100, ΔC76, and ΔC70) that showed no or little association with TfR were unable to downregulate TfR (Fig. 8, third and bottom panels, left columns; ΔC100; 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 ΔC60 and ΔC55 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 ΔC49 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 ΔC60 and ΔC55 mutants with TfR was insufficient to downregulate TfR, because these mutants lack the six-amino-acid region present in the ΔC49 mutant. Finally, we examined an internal deletion mutant (Δ237–242) 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 ΔC49 mutant, it failed to downregulate TfR (Fig. 8, bottom panel, right columns). In agreement with this, the internal deletion mutant (Δ237–242) 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

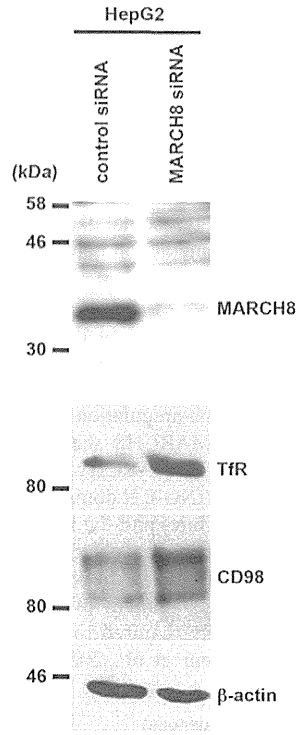

Fig. 9. Effect of the siRNA-mediated knockdown of endogenous MARCH8 on the expression levels of MARCH8 substrates, TfR and CD98, in HepG2 cells. HepG2 cells transfected with either control siRNA or siRNA directed against MARCH8 were cultured for 72 hours. Equivalent amounts of protein extracted from 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′-atagaattcatgaccagcagccacgtttg-3′, 5′-ataggatcccatgagactgatacaacttcagggg-3′); MARCH2 (5′-atagaattcatgacgacgggtgactgctgc-3′, 5′-ataggatcccatactggtgtctcctctgccacc-3′); MARCH3 (5′-atagaattcatgacaaccagccgctgcagtcacc-3′, 5′-ataggatcccaaacaactgtctcctttgagttcctcttg-3′); and MARCH8 (5′-atagaattcatgagcatgccactgcatcag-3′, 5′-ataggatcccagacgtgaatgatttctgctcc-3′). The mutants of MARCH8 were constructed with the following sets of primers: CS mutant (5′-agccacagcacaggaagcctccacttcgtgcaccaggccagc-3′, 5′-gctggcctggtgcacgaagtggaggcttcctgtgctgtggct-3′, 5′-gacatcagcaggatcagccactgtg-3′, 5′-cacagtggctgatcctgctgacgtc-3′, 5′-cgctgcagcgagctcagcaagtatg-3′, 5′-catacttgctgagctcgctgcagcg-3′), ΔC100 (5′-ataggatcccatcctgttgcctgcccctgcttgatc-3′), ΔC76 (5′-ataggatcccacataaaaagaagtcctccggtgaag-3′), ΔC70 (5′-ataggatcccacactttacactgaacatacataaaaag-3′), ΔC60 (5′-ataggatcccaggccttgagtctcttccacaattgc-3′), ΔC55 (5′-ataggatcccagatcactctattataggccttgag-3′), ΔC49 (5′-ataggatcccatggacagttttgaacatagatcac-3′), and ΔC237–242 (5′-cctataatagagtgatcgaaacaagcaaaaag-3′, 5′-ctttttgcttgtttcgatcactctattatagg-3′). The plasmids encoding the BST-2/TfR chimeric proteins were described previously (Iwabu et al., 2009). The plasmids encoding 3× 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℃. 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℃ with the appropriate antibodies. The total cell lysate was incubated with the antibody-coupled Sepharose for 20 hours at 4℃, 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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Fig. S1

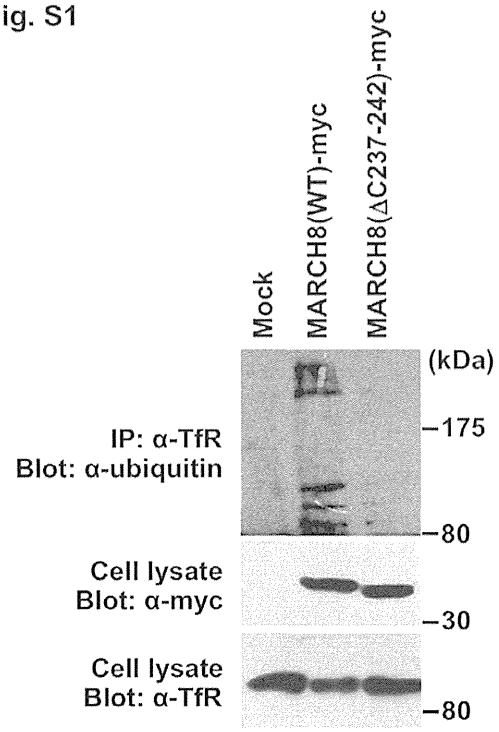

Fig. S1. The ΔC237-242 mutant lacked the ability to ubiquitylate TfR. COS7 cells transfected with plasmids encoding MARCH8-myc (WT or ΔC237-242 mutant) for 24 h were processed for IP with an anti-TfR antibody followed by immunoblot analysis with a ubiquitin antibody. To confirm the expression of TfR and MARCH8-myc proteins, aliquots of cell lysates were subjected to immunoblot analysis with anti-myc (middle panel) and anti-TfR (lower panel) antibodies, respectively.

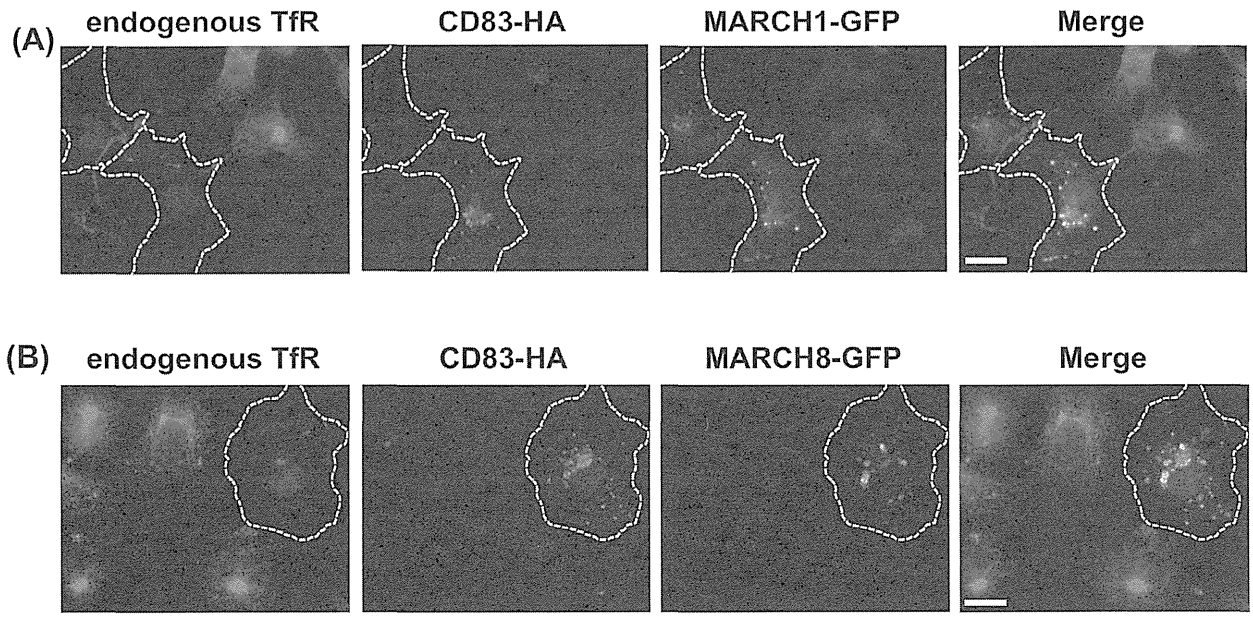

Fig. S2. Effects of CD83 expression on the downregulation of TfR induced by MARCH1 or MARCH8. The plasmids encoding CD83-HA, together with either WT MARCH1-GFP (A) or MARCH8-GFP (B), were transfected into COS7 cells. After 24 h, the cells were processed for IF with anti-HA (blue) and anti-TfR (red) antibodies. The cells expressing CD83-HA and MARCH8-GFP (green) are indicated by the yellow dotted lines. Scale bar: 20 μm.

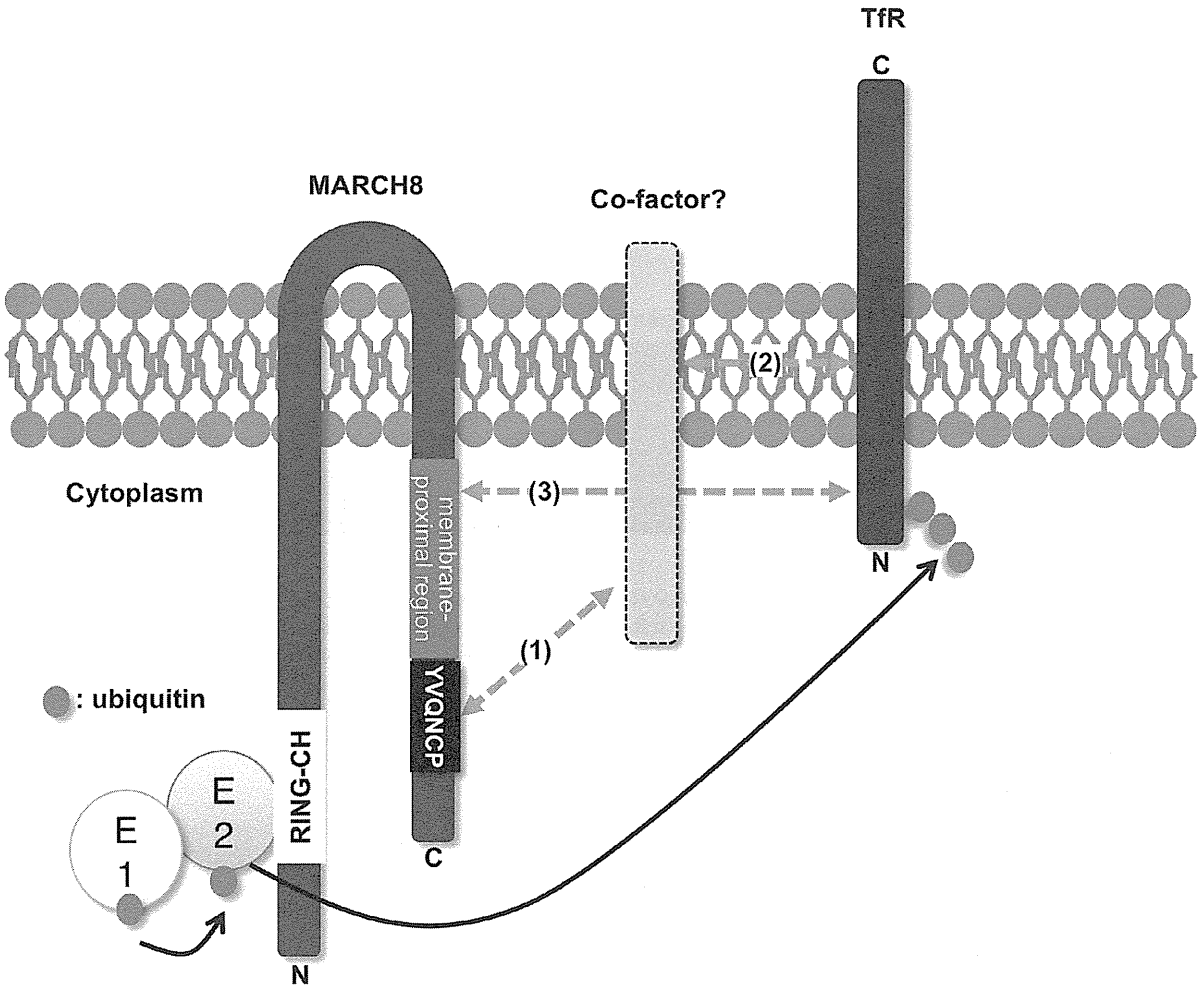

Fig. S3. Proposed model for the MARCH8-induced ubiquitylation of TfR. The RING-CH domain (yellow box) of MARCH8 (purple hairpin) associates with the E1/E2 complex and transfers ubiquitin (red circle) to the lysine residues located in the CT domain of TfR (dark blue). The lipid bilayer is depicted in gray. (1): the six-amino-acid region of the C-terminal CT domain of MARCH8 (black box), which is required for the downregulation of TfR, may interact with the unknown co-factor (light blue box). (2): the TM domain of the unknown co-factor may recognize and interact with the TM domain of TfR. (3): the membrane-proximal region of the C-terminal CT domain of MARCH8 (red box) may be required for both its association with TfR and exit from the ER.