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Identification and Characterization of TMEM33 as a Reticulon-binding Protein

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ABSTRACT

Endoplasmic reticulum (ER) is an organelle that has an elaborate and continuous membrane system composed of sheet-like cisternae and a network of interconnected tubules. The ER tubules are shaped by reticulons, a conserved ER membrane protein family. However, how the membrane-shaping activity is regulated remains to be elucidated. To understand the mode of action of reticulons, we isolated TMEM33, a conserved protein harboring three transmembrane domains, as a reticulon 4C-binding protein by affinity chromatography. In addition to reticulon 4C, TMEM33 binds to reticulon 1A, -2B, -3C and a reticulon homology domain-containing protein Arl6IP1. Exogenously expressed TMEM33 localizes at both the ER membrane and the nuclear envelope. Exogenously expressed TMEM33 co-localizes with exogenously expressed reticulon 4C well at the ER sheets and partially at the ER tubules. Exogenously expressed TMEM33 suppresses the exogenously expressed reticulon 4C-induced tubulation of ER. These results suggest that TMEM33 has a potency to suppress the membrane-shaping activity of reticulons, thereby regulating the tubular structure of ER.

INTRODUCTION

The ER (endoplasmic reticulum) is a continuous membrane system extended from nuclear envelope and composed of interconnected sheets and tubules [11,20,21,25,28]. The membrane amount of the ER interconnected sheets and tubules is limited and the ER sheet-to-tubule balance is changed, responding to various cellular statuses. The ER tubules are arranged into a polygonal network and spread throughout the cells. The ER tubule polygonal network is generated by a dynamic process of tubule extension and tubule fusion. The formation and destruction of the ER tubule polygonal network modulates the ER sheet-to-tubule balance.

The ER tubule polygonal network is generated by three distinct membrane protein families conserved from yeasts to mammals, reticulons, REEPs/DP1/Yop1 and atlastins. Reticulons and REEPs/DP1/Yop1 contain two short hairpin transmembrane domains forming a wedge-like shape within the lipid bilayer to allow expansion of the area of the outer leaflet relative to the inner leaflet, thereby generating the high membrane curvature of the ER tubules [12,24,26,29,34]. In particular, the two short hairpin transmembrane domains of reticulons are recognized as the reticulon homology domain (RHD) [8,32,36]. In mammals, there are four members of the reticulon family, reticulon 1-4, containing the RHDs at the C-termini of the proteins [8,32]. Recently, we have identified another RHD-containing protein, ADP-ribosylation factor-like 6 interacting protein 1 (Arl6IP1), also known as ARMER, and shown that Arl6IP1 has a potency to shape high-curvature ER tubules in a reticulon-like fashion [31]. Reticulons and Arl6IP1 bind to atlastins, evolutionally conserved dynamin-like GTPases that mediate membrane fusion of the ER tubules, through the RHDs [13,17,18]. Atlastins localize at the three-way junctions of the ER tubule polygonal network, promote homotypic fusion of ER tubules, and eventually interconnect ER tubules to form the ER tubule polygonal network in cooperation with reticulons and Arl6IP1 [13,31]. As for REEPs/DP1/Yop1, six members of the REEP family, REEP1-6, have been identified as homolog of yeast Yop1p in mammals, where REEP5 is also known as DP1 [22]. REEPs also regulate the tubular ER network in cooperation with atlastins [22]. Of these conserved membrane-shaping proteins, dysfunctions of reticulon 2, atlastin-1 and REEP1 are associated with the hereditary spastic paraplegias [1,15,34,35]. Reticulon 2, atlastin-1 and REEP1 are also known as spastic gait (SPG)12, SPG3A, and SPG31, respectively.

Lunapark family, conserved from yeasts to mammals, contains *N*-myristoylation motif, two transmembrane domains and zinc finger motif [5,16]. Yeast *Saccharomyces cerevisiae* lunapark, Lnp1p, works synergistically with the reticulons and Yop1p, antagonistically with Sey1p (yeast atlastin homolog) [5,6]. Lnp1p localizes at the three-way junctions of the ER tubule polygonal network and interact with the reticulons, Yop1p and Sey1p [5]. Lnp1p competes with Sey1p for the interaction with the reticulons [5]. Lnp1p antagonizes with Sey1p and reduces Sey1p-induced ER tubule fusion [5]. Lnp1p deletion mutant indeed shows the increase in the ER tubule fusion [5]. Protrudin contains Rab-binding domain, three transmembrane domains, FFAT motif and FYVE domain [23]. Dysfunction of protrudin is associated with the hereditary spastic paraplegias [14]. Protrudin is also known as SPG33. Protrudin has long and short hairpin transmembrane domains and interacts with atlastins and REEP family members [4,9]. Protrudin depletion by siRNAs increases in the ER tubule fusion [4]. Two distinct proteins, lunapark and protrudin, are the antagonistic proteins against atlastins in the ER tubule fusion. Taking all of the above mentioned studies together, we are beginning to understand the mechanism how the membrane-shaping function of reticulons, REEPs/DP1/Yop1 and atlastins is regulated. However, there might be as yet unknown regulators for the membrane-shaping activity of reticulons, REEPs/DP1/Yop1 and atlastins in mammalian cells.

TMEM33 family, conserved from yeasts to mammals, has three transmembrane domains [3]. In the budding yeast *Saccharomyces cerevisiae*, there are two TMEM33 homologs, Pom33 and Per33 [3]. Pom33p localizes at nuclear pores and regulates the distribution of nuclear pore complexes, and interact with Rtn1p (a homolog of human reticulons) [3]. Per33p localizes at the ER membrane, but its function is unknown [3]. In the fission yeast *Schizosaccharomyces pombe*, there is a TMEM33 homolog, Tts1p (tetra-spanning protein 1). Tts1p co-localizes with Rtn1p and Yop1p in the peripheral ER and at the nuclear envelope [33]. Tts1p, Rtn1p and Yop1p work together to maintain the ER tubules and regulate cell division plain positioning [33]. Tts1p is supposed to be a member of the membrane-shaping protein similar to Rtn1p and Yop1p [33]. Human TMEM33 gene is selected as one of the pigmentation candidate genes in populations of East Asian ancestry [10]. In addition, melanosome proteomics study reveals that human TMEM33 protein is in melanosomes at various developmental stages of melanogenesis [7]. Thus, evidence is accumulating that TMEM33 is biologically important. However, the functions of TMEM33 still remain to be fully elucidated. In particular, although yeast TMEM33 is involved in the ER tubule network formation similar to the reticulon and REEP family members, the function of TMEM33 as a potential ER membrane shaping protein remains unclear.

In this study, to identify a novel reticulon binding protein, we performed the affinity chromatography from pig brain extract using GST-reticulon 4C as a ligand. We isolated TMEM33, a conserved protein harboring three transmembrane domains, as a reticulons binding protein. Co-immunoprecipitation assay reveals that TMEM33 binds to reticulon 1A, -2B, -3C and Arl6IP1. Exogenously expressed TMEM33 localizes at both the ER membrane and the nuclear envelope in HeLa cells. Exogenously expressed TMEM33 co-localizes with exogenously expressed reticulon 4C at the ER sheets, not at the ER tubules in HeLa cells. Exogenously expressed TMEM33 suppresses the exogenously expressed reticulon 4C-induced tubulation of ER in HeLa cells. Together, we propose that TMEM33 has a potency to suppress the membrane-shaping activity of reticulons, thereby regulating the ER tubule polygonal network.

MATERIALS AND METHODS

This study was approved by the Committee for Safe Handling of Living Modified Organisms in Kobe University (Permission number: 24-86) and carried out according to the guidelines of the committee.

Expression plasmids

To express GST-fused reticulon 4C (Rtn4C) in bacteria, the cDNA encoding human Rtn4C was subcloned into the pGEX-4T vector (GE healthcare). To express TMEM33 with a C-terminal HA tag in mammalian cells, the cDNA encoding human TMEM33 was subcloned into the pCA-HA vector (19). To express reticulon family proteins with an N-terminal FLAG tag in mammalian cells, the cDNAs encoding human Rtn4C, human reticulon 1A (Rtn1A), human reticulon 2B (Rtn2B), mouse reticulon 3C (Rtn3C) and human Arl6IP1 were subcloned into the pCMV-FLAG vector (27).

Affinity chromatography

GST-Rtn4C and GST alone were expressed in *E. coli* harboring pGEX-4T-Rtn4C and pGEX-4T, respectively, and immobilized on glutathione sepharose (GE healthcare). Pig brains were lysed in buffer A containing 20 mM Tris-HCl pH7.5, 150 mM NaCl, 1 mM DTT, 1 mM MgCl₂, 1 mM CaCl₂ and 0.2% TritonX-100 supplemented with protease inhibitor cocktail at 4 °C for 60 min. The lysate was centrifuged at

4,200 × *g* at 4 °C for 50 min, followed by further centrifugation at 100,000 × *g* at 4 °C for 60 min. The supernatant was preabsorbed with glutathione sepharose to remove the native GST, and incubated with GST-Rtn4C immobilized on glutathione sepharose or GST immobilized on glutathione sepharose alone at 4 °C overnight. After being extensively washed with buffer A, the bound proteins were eluted with a buffer containing 20 mM Tris-HCl pH7.5, 2 M NaCl, 5 mM EDTA, 1 mM DTT, and 1% octylglucoside and subjected to SDS-PAGE followed by silver staining. The bands of interest were cut out from the gel and digested with trypsin, followed by mass spectrometry analysis.

Co-immunoprecipitation

Appropriate combinations of pCA-TMEM33-HA, pCMV-FLAG-Rtn4C, pCMV-FLAG-Rtn1A, pCMV-FLAG-Rtn2B, pCMV-FLAG-Rtn3C and pCMV-FLAG-Arl6IP1 were transfected into HEK293 cells with Lipofectamine LTX (Invitrogen) in accordance with the manufacturer's manual. 24 h after transfection, the cells were lysed in a buffer containing 20 mM Tris-HCl pH7.5, 150 mM NaCl, 2 mM MgCl₂, 10 μM APMSF, 10 μg/ml leupeptin, 5 μg/ml aprotinin, and 0.5% TritonX-100 and subjected to ultracentrifugation at 100,000 × *g* at 4 °C for 30 min. The Triton X-100 extracts were incubated with a mouse anti-HA mAb (Covance), followed by immunoprecipitation with Protein G sepharose (GE healthcare). The immunoprecipitates were subjected to SDS-PAGE followed by western blotting with a rat anti-HA mAb (Roche) and a rabbit anti-FLAG pAb (SIGMA).

Immunofluorescence microscopy

Appropriate combinations of pCA-TMEM33-HA, pCMV-FLAG-Rtn4C, and pCMV-FLAG-Rtn3C were transfected into HeLa cells with Effectene (Qiagen) in accordance with the manufacturer's manual. 24 h after transfection, the cells were fixed with 4% paraformaldehyde, followed by permeabilization with 0.2% Triton X-100. After blocking with PBS supplemented with 1% BSA, the samples were incubated with various combinations of the rat anti-HA mAb, the rabbit anti-FLAG pAb, a mouse anti-PDI mAb (Abcam), and a mouse anti-Nup153 mAb (Abcam), followed by incubation with Alexa Fluor-conjugated secondary Abs (Invitrogen). After being washed with PBS, they were embedded and viewed using a confocal imaging system (ZEISS, LSM 510 Meta).

In some instances, the reticulon-mediated membrane constriction was judged by lack of PDI signal in the peripheral ER tubules. To quantify the Rtn4C-mediated membrane constriction, 50 transfected cells were randomly chosen and the number of the cells with decreased PDI signal in the peripheral ER tubules was counted. The transfection was carried out in triplicate and the statistical significance of difference between the cells transfected with FLAG-Rtn4C alone and the cells transfected with both FLAG-Rtn4C and TMEM33-HA was analyzed by the two-tailed Student's *t* test.

RESULTS

Identification of TMEM33 as a reticulon 4C-binding protein.

To understand the molecular mechanism of how the membrane-shaping function of reticulons was regulated, we sought to identify a reticulon-binding protein(s) by affinity chromatography. GST-fused reticulon 4C (GST-Rtn4C) or GST alone was immobilized on glutathione sepharose and incubated with the TritonX-100 extract of pig brains. The bound proteins were subjected to SDS-PAGE followed by silver staining. Two prominent bands at 28 kDa and 25 kDa and three minor bands at 130 kDa, 125 kDa and 100 kDa were specifically detected in the column of GST-Rtn4C (Figure 1A). Mass spectrometry analysis identified the prominent bands at 28 kDa and 25 kDa as transmembrane protein 33 (TMEM33) and ARL1 small GTPase, respectively (Figure 1A). TMEM33 is a three-pass transmembrane protein homologous to yeast Pom33 and Per33, the components of the nuclear pore complex [3]. Interestingly, the previous study suggested that TMEM33 localized at both nuclear envelope and ER membrane [3], although the physiological function of TMEM33 as an ER membrane protein still remains unclear. On the other hand, ARL1 is a Golgi-resident protein that regulates vesicle trafficking [2], and all the other minor bands were not ER membrane proteins (Table I). Therefore, we focused TMEM33 in this study and characterized it in the context of a potential ER membrane protein that binds to reticulons.

We first assessed the binding of Rtn4C to TMEM33 by immunoprecipitation analysis. HA-tagged TMEM33 (TMEM33-HA) was transfected into HEK293 cells along with FLAG-tagged Rtn4C (FLAG-Rtn4C), followed by immunoprecipitation with the anti-HA mAb. FLAG-Rtn4C was co-immunoprecipitated with TMEM33-HA (Figure 1B). This result validates that TMEM33 binds to Rtn4C.

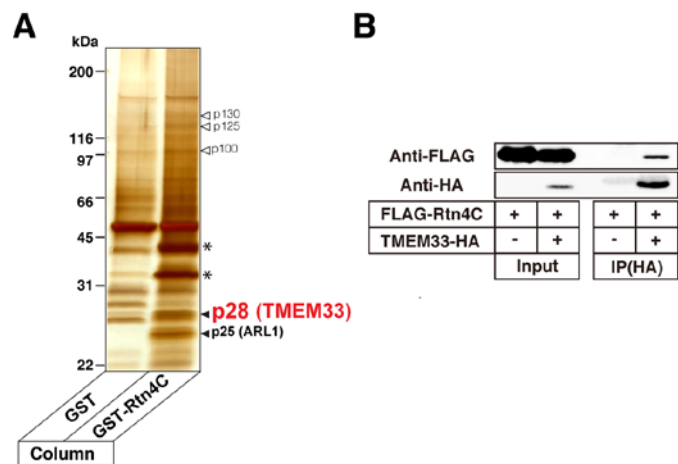


Figure 1. Identification of TMEM33 as a reticulon 4C-binding protein. (A) Purification of TMEM33 by affinity chromatography using GST-Rtn4C as a ligand. GST-Rtn4C and GST alone were immobilized on glutathione sepharose and incubated with the Triton X-100 extracts of pig brains. The bound proteins were eluted with a buffer containing 2 M NaCl and subjected to SDS-PAGE followed by silver staining. The two prominent bands at 28 kDa and 25 kDa indicated by black arrowheads were identified as TMEM33 and ARL1, respectively, by mass spectrometry. Identification of the three minor bands indicated by white arrowheads is listed in Table I. Asterisks indicate the degradation products of GST-Rtn4C.

(B) Co-immunoprecipitation of TMEM33 and Rtn4C. The indicated combinations of TMEM33-HA and FLAG-Rtn4C were transfected into HEK293 cells, followed by immunoprecipitation with the mouse anti-HA mAb. The samples were subjected to SDS-PAGE followed by western blotting with the rabbit anti-FLAG pAb and the rat anti-HA mAb.

Table I. Mass spectrometry identification of the minor bands.

Band	Mass spectrometry identification	Cellular localization
p130	Coatomer subunit alpha	COPI vesicle and Golgi
p125	Neural cell adhesion molecule 1	Plasma membrane
p100	Coatomer subunit beta	COPI vesicle and Golgi

The three minor bands at 130 kDa, 125 kDa and 100 kDa, which were indicated by white arrowheads in Fig. 1A, were identified by mass spectrometry.

TMEM33 binds to reticulon homology domain-containing proteins.

We next examined whether TMEM33 bound to other members of the reticulon family, reticulon 1A (Rtn1A), reticulon 2B (Rtn2B) and reticulon 3C (Rtn3C). Immunoprecipitation analysis was carried out in the same manner as in Figure 1B except that FLAG-tagged reticulon 1A (Rtn1A), FLAG-tagged reticulon 2B (Rtn2B) or FLAG-tagged reticulon 3C (Rtn3C) was used instead of FLAG-Rtn4C. FLAG-Rtn1A, FLAG-Rtn2B and FLAG-Rtn3C were co-immunoprecipitated with TMEM33-HA (Figure 2A), indicating that TMEM33 bound to the broad range of the reticulon family proteins. In addition, we also detected the specific binding of TMEM33 to Arl6IP1, a reticulon-like ER membrane-shaping protein [31], by immunoprecipitation analysis (Figure 2B). These results indicate that TMEM33 has the potency to bind to reticulon homology domain-containing proteins.

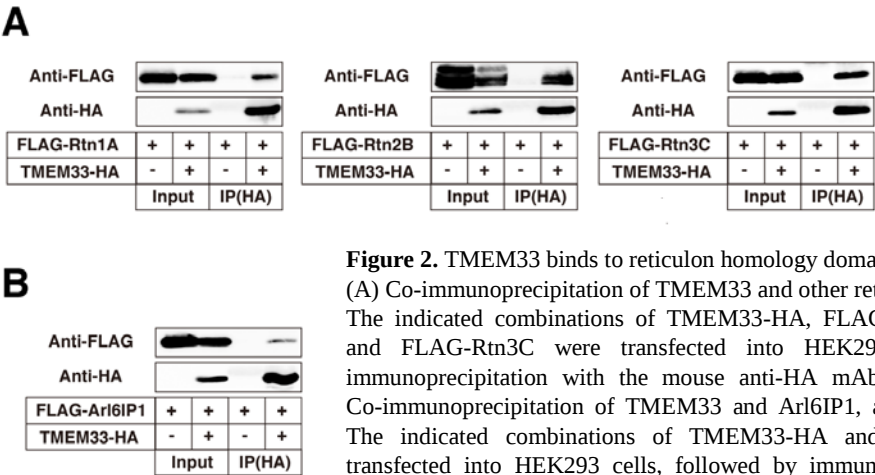


Figure 2. TMEM33 binds to reticulon homology domain-containing proteins. (A) Co-immunoprecipitation of TMEM33 and other reticulon family members. The indicated combinations of TMEM33-HA, FLAG-Rtn1A, FLAG-Rtn2B and FLAG-Rtn3C were transfected into HEK293 cells, followed by immunoprecipitation with the mouse anti-HA mAb as in Fig. 1B. (B) Co-immunoprecipitation of TMEM33 and Arl6IP1, a reticulon-like protein. The indicated combinations of TMEM33-HA and FLAG-Arl6IP1 were transfected into HEK293 cells, followed by immunoprecipitation with the mouse anti-HA mAb as in Fig. 1B.

TMEM33 co-localizes with reticulon 4C at the ER sheets.

The previous preliminary characterization of yeast TMEM33 using the GFP-tagged protein suggested that TMEM33 localized at nuclear envelope and ER membrane [3]. To further confirm the subcellular localization of TMEM33 in mammalian cells, TMEM33-HA was transfected into HeLa cells, followed by immunostaining with the anti-HA mAb and the anti-PDI mAb or the anti-Nup153 mAb. TMEM33-HA was co-localized with PDI, an ER luminal enzyme, and Nup153, a nuclear pore protein (Figure 3A), indicating that TMEM33 was indeed a membrane protein that localized at both the ER membrane and the nuclear envelope.

We next examined whether TMEM33 co-localized with reticulons at the ER membrane. TMEM33-HA and FLAG-Rtn4C were co-transfected into HeLa cells, followed by immunostaining with the anti-HA mAb and the anti-FLAG pAb. We observed the cells moderately expressing FLAG-Rtn4C, because overexpression of FLAG-Rtn4C induced morphological change of the ER as described later. TMEM33-HA and FLAG-Rtn4C showed reticular staining pattern indicative of the ER and co-localized well at the perinuclear region where the ER sheets were enriched (Figure 3B). On the other hand, mosaic arrangement of TMEM33-HA and FLAG-Rtn4C was partially detected at the peripheral region where the ER tubules were enriched (Figure 3B, insets), indicating that TMEM33-HA did not co-localize well with FLAG-Rtn4C at the ER tubules. Given that reticulons preferentially localizes at the ER tubules and the edges of the ER sheets, both of which are characterized by high-membrane curvature [12,24,25,26,29,30,36], these results suggest that TMEM33 localizes at the perinuclear and peripheral ER sheets, apparently not at the peripheral ER tubules, and TMEM33 co-localizes with a subset of Rtn4C at the ER sheets.

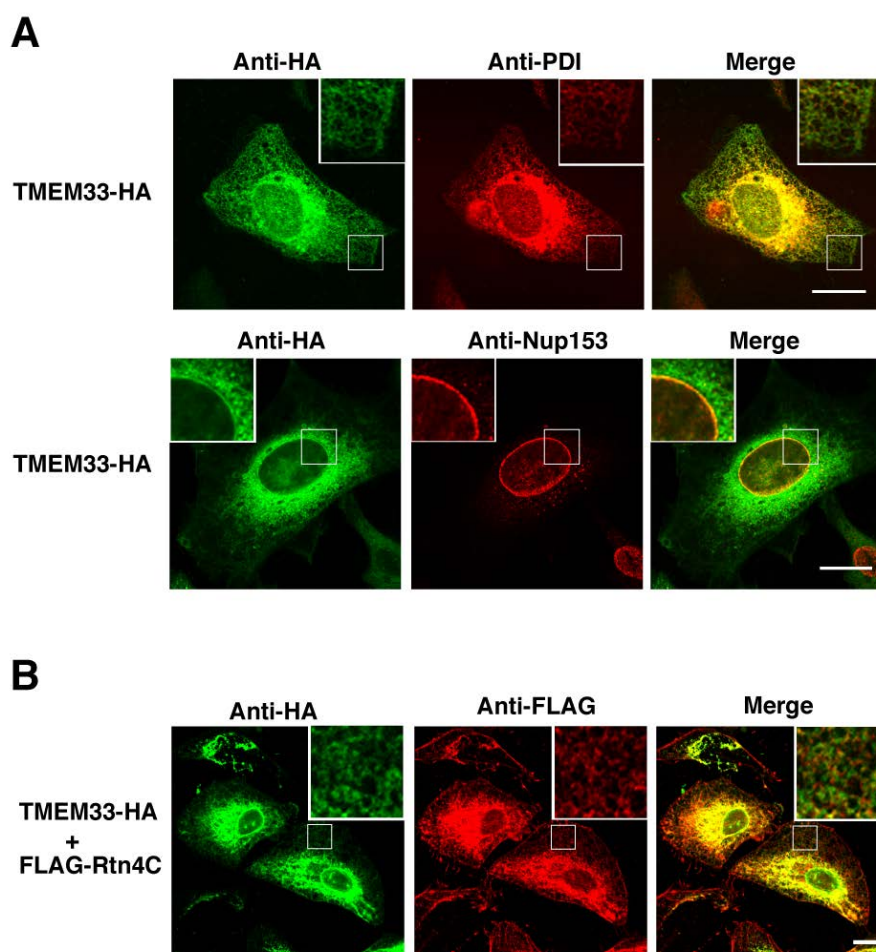


Figure 3. TMEM33 co-localizes with reticulon 4C at the ER sheets.

(A) Localization of TMEM33 at the ER membrane and the nuclear envelope. HeLa cells were transfected with TMEM33-HA, followed by immunostaining with the indicated combinations of the rat anti-HA mAb, the mouse anti-PDI mAb, and the mouse anti-Nup153 mAb. Insets are enlarged images of the boxed area. Scale bars, 20 μm. (B) Partial co-localization of TMEM33 and Rtn4C. HeLa cells were transfected with both TMEM33-HA and FLAG-Rtn4C, followed by immunostaining with the rat anti-HA mAb and the rabbit anti-FLAG pAb. Insets are enlarged images of the boxed area. Scale bar, 20 μm.

Overexpression of TMEM33 suppresses the reticulon 4C-induced tubulation of ER.

We finally examined effect of TMEM33 on the membrane-shaping activity of Rtn4C. HeLa cells were transfected with TMEM33-HA alone, FLAG-Rtn4C alone, or both FLAG-Rtn4C and TMEM33-HA, followed by immunostaining with appropriate combinations of the anti-PDI mAb, the anti-FLAG pAb and the anti-HA mAb. PDI is a luminal ER protein and immunostaining of PDI shows a reticular staining pattern characteristic of the ER sheets and tubules. Reticulon family proteins, such as Rtn4C, have the activity to stabilize the ER tubules. Overexpression of the reticulon family protein induces extensive peripheral ER tubules and significantly decreases PDI staining in the tubules, suggesting that the reticulon family protein constricts the ER tubules and excludes a luminal ER protein PDI from the ER tubules [12,29,31]. Thus, the decreased PDI signal in the peripheral ER tubules represents the effect of the overexpressed reticulon family protein on the ER tubules. Therefore, we counted the cells with decreased PDI signal in the peripheral ER tubules as the cells affected by overexpressed reticulon family protein.

Transfection of TMEM33-HA alone showed the normal ER structure as judged by reticular staining pattern of PDI (Figure 4A). Compared with transfection of TMEM33-HA alone or non-transfection, transfection of FLAG-Rtn4C alone significantly induced the extensive networks of the peripheral ER tubules where PDI staining was decreased (Figures 4A and 4B). These results indicate that, consistent with the previous studies [12,

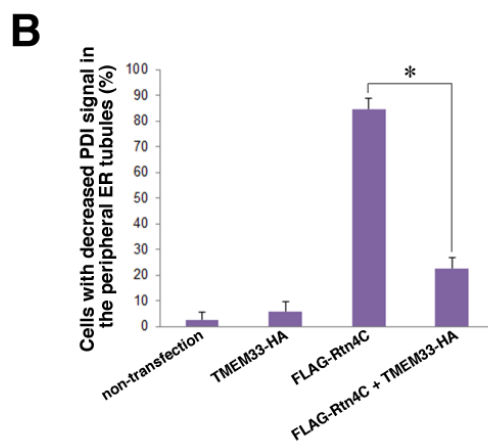
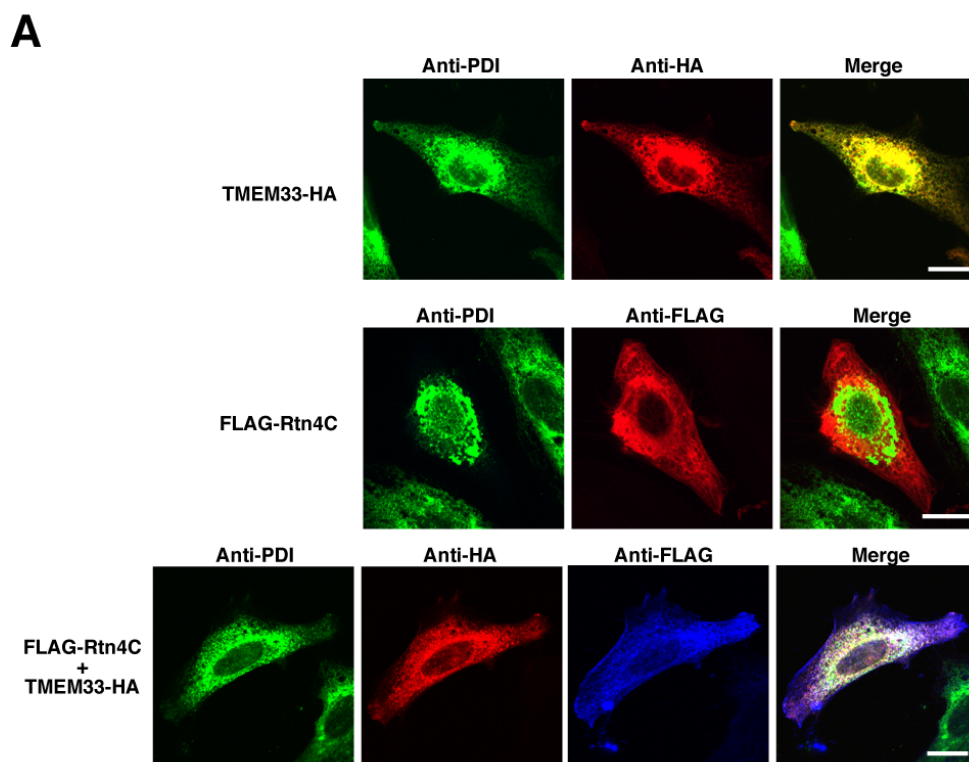


Figure 4. Overexpression of TMEM33 suppresses the reticulon 4C-induced tubulation of ER.

(A) Overexpression of TMEM33 inhibits the Rtn4C-mediated constriction of the ER membrane. HeLa cells were transfected with the indicated combinations of TMEM33-HA and FLAG-Rtn4C, followed by immunostaining with the indicated combinations of the mouse anti-PDI mAb, the rat anti-HA mAb, and the rabbit anti-FLAG pAb. Scale bars, 20 μ m. (B) Quantification of the effect of overexpression of TMEM33 on the Rtn4C-mediated constriction of the ER membrane. The number of cells with decreased PDI signal in the peripheral ER tubules, which was indicative of the potent constriction of the ER tubules, was counted. The error bars represent S.D. Asterisk indicates statistical significance (Student's *t* test; *, $p < 0.01$).

29,31], overexpression of Rtn4C potently constricted the peripheral ER tubules, thereby excluding PDI from the tubules. On the other hand, co-transfection of FLAG-Rtn4C and TMEM33-HA showed the reticular staining of PDI similar to transfection of TMEM33-HA alone (Figure 4A), and significantly decreased the number of the cells showing the potent constriction of the peripheral ER tubules as judged by the decreased PDI staining, relative to transfection of FLAG-Rtn4C alone (Figure 4B). These results indicate that overexpression of TMEM33 decreases the Rtn4C-mediated constriction of the ER tubules. Moreover, we similarly observed that transfection of FLAG-Rtn3C alone showed the potent constriction of the peripheral ER tubules as judged by the decreased PDI staining (data not shown), whereas co-transfection of FLAG-Rtn3C and TMEM33-HA showed the normal ER structure as judged by the reticular staining pattern of PDI (data not shown). Collectively, these results suggest that, upon overexpression, TMEM33 suppresses the membrane-shaping activity of a broad range of the reticulon family proteins.

DISCUSSION

It is well known that reticulons shape the high-curvature domains of the ER by constricting the ER membrane. On the other hand, little is known about the molecular mechanism underlying the regulation of the membrane-shaping activity of reticulons. In order to understand the mechanism, it will be important to probe proteins that bind to reticulons. Indeed, the recent study has demonstrated that two distinct ER membrane proteins, lunapark and protrudin, bind to reticulons and regulate the ER membrane structure [4,5,6,9]. In this study we identified TMEM33 as a novel Rtn4C-binding protein and demonstrated that TMEM33 regulated the membrane-shaping activity of Rtn4C, suggesting that, in addition to lunapark and protrudin, TMEM33 will be a potential regulator for the ER membrane structure, although the functional relationship of TMEM33, lunapark and protrudin remains unknown. Exogenous overexpression of TMEM33 suppressed the ER tubulation induced by exogenous overexpression of Rtn4C as shown in Figure 4. However, this result is not sufficient to conclude whether TMEM33 regulates reticulons positively or negatively, because we have never examined effects of endogenous TMEM33 on the membrane-shaping activity of endogenous reticulons. Further analysis including siRNA knockdown of endogenous TMEM33 will be required to address this concern. We do not yet know the mechanistic insight into the negative effect of TMEM33 on Rtn4C-induced ER shaping activity. Oligomerization of reticulon has been shown to be important for shaping the ER tubules [26,36]. The binding of TMEM33 to Rtn4C may interfere with oligomerization of Rtn4C. However, based on our binding data from affinity chromatography and co-immunoprecipitation assay, we have not established whether the interaction between TMEM33 and Rtn4C is direct or indirect, or whether TMEM33 binds to Rtn4C through its cytoplasmic domain or transmembrane domain. Further analysis using pure recombinant proteins or various deletion mutants will be required to address these concerns.

The ER sheet is composed of two flat membranes connected by highly curved edges. Evidence is accumulating that reticulons themselves are sufficient to generate the high-membrane curvature by inserting their two short hairpin transmembrane domains into the outer leaflet of the ER membrane in a wedge-like fashion [12,24,25,29,30]. Therefore, we are beginning to understand how the highly curved edges of ER sheets are generated. On the other hand, the molecular mechanism underlying formation of the flat membranes of the ER sheets remains unclear. TMEM33 co-localized with a subset of Rtn4C at the ER sheets while TMEM33 did not co-localize well with Rtn4C at the peripheral ER tubules as shown in Figure 3B. These results raise the attractive possibility that, in contrast to reticulons, TMEM33 would preferentially localize at the low-curvature region of the ER membrane, and the binding of TMEM33 to reticulons might be involved in formation of the ER sheets, in particular, the flat membranes of the ER sheets. Co-localization of TMEM33 and Rtn4C as shown in Figure 3B might reflect transformation of the high-curvature region of the ER membrane into the flat membranes of ER sheets.

The yeast *Saccharomyces cerevisiae* has two homologs of TMEM33, Pom33 and Per33 [3]. The previous study revealed that Pom33p is a component of the nuclear pore complex. Interestingly, Pom33p binds to Rtn1p, regulating distribution of the nuclear pores [3]. On the other hand, it remains unknown whether Per33p binds to Rtn1p. Unlike the predominant localization of Pom33p at the nuclear envelope, Per33p localizes at both the nuclear envelope and the ER [3], which is reminiscent of the localization of TMEM33, although the function of Per33p at the ER is unclear. Tts1p, the sole homolog of TMEM33 in the yeast *Schizosaccharomyces pombe*, has been shown to localize at both the nuclear envelope and the ER [33]. Tts1p is supposed to function as an ER membrane-shaping protein together with Rtn1p and Yop1p [33]. More interestingly, Tts1p binds to Rtn1p and Yop1p, thereby maintaining the cortical ER networks toward the proper positioning of the actomyosin ring for cell division [33]. Therefore, apart from the reported physiological roles of the yeast TMEM33 homologs, the binding between TMEM33 and reticulons is likely to be conserved evolutionarily, raising the possibility that

TMEM33 would be an evolutionarily conserved regulator for reticulons. Further studies will be required to address these concerns.

In summary, we showed here that TMEM33 is a potential regulator for the ER membrane structure.

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