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PMP2/FABP8 induces PI(4,5)P₂-dependent transbilayer reorganization of sphingomyelin in the plasma membrane

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Summary
Sphingomyelin (SM) is a mammalian lipid mainly distributed in the outer leaflet of the plasma membrane (PM). We show that peripheral myelin protein 2 (PMP2), a member of the fatty acid-binding protein (FABP) family, can localize at the PM and controls the transbilayer distribution of SM. Genetic screening with genome-wide small hairpin RNA libraries identifies PMP2 as a protein involved in the transbilayer movement of SM. A biochemical assay demonstrates that PMP2 is a phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)-binding protein. PMP2 induces the tubulation of model membranes in a PI(4,5)P₂-dependent manner, accompanied by the modification of the transbilayer membrane distribution of lipids. In the PM of the PMP2-overexpressing cells, inner-leaflet SM is increased whereas outer-leaflet SM is reduced. PMP2 is a causative protein of Charcot–Marie–Tooth disease (CMT). A mutation in PMP2 associated with CMT increases its affinity for PI(4,5)P₂ inducing membrane tubulation and the subsequent transbilayer movement of lipids.
Introduction

A characteristic feature of the plasma membrane (PM) in mammalian cells is the transbilayer asymmetry of its lipids. Phosphatidylcholine (PC) is mainly located in the outer leaflet, whereas phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P$_2$) are almost exclusively located in the inner leaflet (Doktorova et al., 2020; Fujimoto and Parmryd, 2016; Kobayashi and Menon, 2018; van Meer, 2011). It has been postulated that the asymmetric distribution of these glycerophospholipids is established and maintained by flippases, floppases, and scramblases (Devaux et al., 2008; Hankins et al., 2015; Holthuis and Menon, 2014; Pomorski and Menon, 2016; Shin and Takatsu, 2019).

Sphingomyelin (SM) is a major sphingolipid, comprising approximately 10% of the total phospholipids in mammalian cells. Several approaches have shown that SM is mainly distributed in the outer leaflet of the PM, creating a barrier to the extracellular environment (Murate et al., 2015; Pomorski and Menon, 2006; van Meer et al., 2008). Bulk SM is synthesized on the luminal side of the Golgi apparatus by SM synthase 1 and transported to the outer leaflet of the PM (Tafesse et al., 2006), whereas local SM is synthesized on the extracellular side of the PM by SM synthase 2 (Mitsutake et al., 2011). Thus, the asymmetric distribution of SM is established by the SM synthases (Huitema et al., 2004; Kumagai and Hanada, 2019). However, the molecular mechanisms underlying the maintenance of the SM distribution are not fully understood, and no protein that catalyzes the transbilayer movement (flip–flop) of SM has been identified (Roland and Graham, 2016).

To understand the organization and dynamics of local SM-rich domains, several approaches to visualize them with fluorescent SM analogs or SM-binding probes have been reported (Hong et al., 2002; Honigmann et al., 2014; Kinoshita et al., 2017; Kiyokawa et al., 2005; Kol et al., 2019). In particular, specific SM-binding proteins have attracted a lot of attention because they can be easily used to label endogenous SM-rich domains in cell membranes, with few artifacts (Kobayashi and Menon, 2018; Ramirez-Carreto et al., 2020). We developed methods for visualizing clusters of SM in vivo using lysenin, a specific SM-binding protein originally isolated from the coelomic fluid of the
earthworm *Eisenia fetida* (Abe and Kobayashi, 2014; Abe et al., 2012; Makino et al., 2015). With this SM-specific probe, we have previously shown that most SM localizes to the outer leaflet, but small amounts of SM are present in the inner leaflet of the PM in nucleated mammalian cells (Murate et al., 2015). Our findings raise the possibility that SM moves from the outer leaflet to the inner leaflet of the PM by an unknown mechanism. In this study, we identified peripheral myelin protein 2 (PMP2) as a protein involved in the transbilayer movement of SM. We also examined the mechanism underlying the movement of this lipid and its effect on membrane organization.
Results

SM moves from the outer leaflet to the inner leaflet

We examined the possibility that the cell surface SM is exposed to the cytoplasm in HeLa cells. To test this, a bacterial sphingomyelinase (SMase; Staphylococcus aureus SMase C) was stably overexpressed in the cytoplasm of HeLa cells. This enzyme is highly active under physiological conditions (37 °C, pH 7.4) (Vandenesch et al., 2012). To visualize the outer-leaflet SM of the PM in the SMase-overexpressing cells, we produced recombinant nontoxic lysenin (NT–Lys) fused with EGFP (EGFP–NT–Lys) in Escherichia coli (Abe et al., 2012), and stained HeLa cells with EGFP–NT–Lys. If SM is transported from the outer leaflet to the cytoplasmic side of the PM, the outer-leaflet SM should be degraded by the cytoplasmic SMase. The outer-leaflet SM labeled with EGFP–NT–Lys decreased dramatically in the SMase-overexpressing cells (Figure 1A). To exclude the possibility that the cytoplasmic SMase was released from inside the cell and degraded the outer-leaflet SM from outside the cell, we co-cultured control and SMase-overexpressing HeLa cells (Figure 1A). The selective labeling of the control cells with EGFP–NT–Lys indicated that no leakage of SMase to the medium from inside the SMase-overexpressing cells occurred. These results suggest that SM does indeed move from the outer leaflet of the PM to the cytoplasmic side.

To examine if the observed phenomenon is protein-mediated, we screened the cellular proteins to identify those responsible for the depletion of the outer-leaflet SM in SMase-overexpressing cells. Previous studies have demonstrated that SM-deficient cells are sensitive to methyl-β-cyclodextrin (MβCD) and detach from the culture dish in its presence (Fukasawa et al., 2000; Hanada et al., 2003). To test whether the SM deficiency in SMase-overexpressing cells also causes high sensitivity to MβCD, SMase-overexpressing cells were subjected to a colony-forming assay after MβCD treatment (Figure 1B). We found that the cells overexpressing SMase were more sensitive to MβCD than the control cells, and no surviving colonies of SMase-overexpressing cells were observed after treatment with 40 mM MβCD (Figure 1B). To identify the factors responsible for the depletion of SM, we screened the genes that, upon silencing, protect SMase-overexpressing cells from MβCD (Figure S1A). We infected HeLa cells
overexpressing SMase with a Human 50K GeneNet shRNA Library that contained 200,000 small hairpin RNAs (shRNAs) targeting 38,500 human genes. The cells were selected with 40 mM MβCD to detect the tolerant cells. After an additional selection with MβCD, 10 target genes of the shRNAs were identified in the tolerant cells. To test whether the knockdown of each gene restored the reduction in SM in the SMase-overexpressing cells, we synthesized small interfering RNAs (siRNAs), transfected the cells individually with each siRNA, and stained the outer-leaflet SM with exogenous EGFP–NT–Lys (Figure S1B). The knockdown of PMP2, SLC40A1, or GGA1 in the SMase-overexpressing cells resulted in robust outer-leaflet labeling with EGFP–NT–Lys (Figure S1B). Silencing of ASAP2, RAB6A, and OR51E2 restored partly outer-leaflet SM in the SMase-overexpressing cells and will be described elsewhere. We made knockout cells with the CRISPR-Cas9 system in the SMase-overexpressing cells and stained the outer-leaflet SM (Figure 1C, left). Measurement of fluorescence intensity of EGFP–NT–Lys indicates that the knockout of PMP2, SLC40A1, or GGA1 in the SMase-overexpressing cells also increased significantly the outer-leaflet SM (Figure 1C, right). We verified that there was an only small difference in the expression levels of SMase between the cells and such expression level of SMase was sufficient to abolish EGFP–NT–Lys labeling in control cells. These results suggest that the identified factors are candidate proteins for the exposure of the outer-leaflet SM to the cytoplasm.

**PMP2 is responsible for the endocytosis-independent transport of fluorescent SM from outer to the inner leaflet of the PM**

To examine the fate of the outer-leaflet SM in SMase-overexpressing cells, we used the fluorescent SM analogue N-[6-[(7-nitro-2,1,3-benzoxadiazol-4-yl)amino]hexanoyl]-sphingosine-1-phosphocholine (C6-NBD-SM). C6- and C12-NBD-phospholipid analogues are spontaneously inserted into the outer leaflets of model and biological membranes and are widely used to measure the transbilayer movements of lipids in membranes (John et al., 2002; Kobayashi and Arakawa, 1991; Koval and Pagano, 1989; McIntyre and Sleight, 1991). Due to the hydrophilic nature of NBD fluorophore, the physical properties of NBD lipids differ from those of their natural
counterparts (Chattopadhyay, 1990). However, these lipid analogues are useful in
examining flippase activity (Martin and Pagano, 1987; Shin and Takatsu, 2019). We
examined the transbilayer-movement-dependent metabolism of C6-NBD-SM in SMase-
expressing HeLa cells. The cells were labeled with C6-NBD-SM for 30 min on ice,
washed, and incubated at 15 °C for 2 h (Figure 2A). Under these conditions, fluid-phase
endocytosis is severely slowed down (Wolkers et al., 2003). In the SMase-overexpressing
cells, C6-NBD-ceramide (Cer) and C6-NBD-glucosylceramide (GlcCer) were detected
on high-performance thin-layer chromatography (HPTLC) plates in addition to C6-NBD-
SM. The results suggested that the C6-NBD-SM inserted in the outer leaflet was moved
to the inner leaflet of the PM and was degraded by the cytoplasmic SMase to C6-NBD-
Cer. C6-NBD-Cer was then spontaneously transported to the Golgi apparatus (Martin et
al., 1993) and was used to synthesize C6-NBD-GlcCer. In the SMase-overexpressing
cells, 39.9 ± 3.1% of total NBD fluorescence (mean ± SEM, n = 3) was detected as C6-
NBD-Cer. In contrast, the knockout of PMP2 significantly reduced the levels of C6-
NBD-Cer in the SMase-overexpressing cells to 4.2 ± 1.5% (mean ± SEM, n = 3) (Figure
2A and B). The knockout of SLC40A1 had only a moderate effect on the SMase-
overexpressing cells, reducing C6-NBD-Cer content to 28.5 ± 11.4% (n = 3). Knockdown
of GGA1 did not affect the production of C6-NBD-Cer. These results suggest that outer-
leaflet C6-NBD-SM was transported to the inner leaflet of the PM in the control cells,
whereas this transport was inhibited in the PMP2-knockout cells.

We also examined the incorporation of C6-NBD-SM in the SMase-nonexpressing
cells. After labeling with C6-NBD-SM at 15 °C for 2 h, the cells were treated with or
without dithionite. Dithionite is a chemical reductant that slowly permeates membranes
and irreversibly renders the NBD fluorophore nonfluorescent, so it preferentially reduces
outer-leaflet fluorescence (Angeletti and Nichols, 1998; John et al., 2002; Kobayashi et
al., 1992; McIntyre and Sleight, 1991). As mentioned above, fluid-phase endocytosis is
reported to be severely slowed under these conditions (Wolkers et al., 2003). In contrast
to the SMase-overexpressing cells, no significant metabolism of C6-NBD-SM was
observed in cells not overexpressing SMase (Figure 2C). Similar to control, the
SLC40A1- and PMP2-knockout cells were labeled with C6-NBD-SM (Figure 2C, right
three lanes), suggesting that the insertion of C6-NBD-SM to the outer leaflet was not inhibited in the knockout cells. To normalize the difference of C6-NBD-SM insensitive to dithionite among the cells, we measured the ratio of the fluorescence intensity of C6-NBD-SM after dithionite treatment to the intensity before the treatment. After dithionite treatment, a small but significant amount of C6-NBD-SM remained in the control (12.5 ± 0.7%, mean ± SEM, n = 3) and SLC40A1-knockout cells (13.3 ± 2.1%, n = 3), indicating that a portion of C6-NBD-SM was transferred to the cytoplasmic leaflet (Figure 2D). In the PMP2-knockout cells, a smaller amount of C6-NBD-SM remained after dithionite treatment (6.9 ± 0.5%, n = 3, p < 0.05). These results suggest that PMP2 was responsible for the transport of C6-NBD-SM from the outer leaflet to the inner leaflet in both the SMase-overexpressing (Figures 2A and 2B) and control cells (Figures 2C and 2D).

Next, we tested the incorporation of other lipids in the SMase-nonexpressing HeLa cells. After labeling the cells with 2-[12-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]dodecanoyl-1-hexadecanoyl-sn-glycero-3-phosphocholine (C12-NBD-PC; Figure S2A) or 1-oleoyl-2-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sn-glycero-3-phosphoethanolamine (C12-NBD-PE; Figure S2C) at 15 °C for 2 h, we measured the fluorescence remaining in the inner leaflets of the PM. In contrast to C6-NBD-SM, we did not see a significant difference in the fluorescence remaining between control and PMP2-knockout cells (Figures S2B and S2D). These results suggest that PMP2 preferentially transports C6-NBD-SM from the outer leaflet to the inner leaflet of the PM in living cells. Because PMP2 displayed a clear phenotype compared to SLC40 and GGA1, we focused on this protein in the subsequent experiments.

We then examined the effect of PMP2 on the distribution of endogenous SM in the PM (Figure 3A). HeLa cells displayed heterogeneous staining with EGFP–NT–Lys within a cell population (Figure 1A). Therefore, we used Madin–Darby canine kidney (MDCK) cells, which were homogeneously labeled with EGFP–NT–Lys (Makino et al., 2015). We generated PMP2-knockout and PMP2-stably-overexpressing MDCK cells and measured SM content in the cells (Figure S3A). Quantification of SM fluorescence after lipid separation on HPTLC plates and primuline staining indicates that SM contents were not significantly different among the control, PMP2-knockout, and PMP2-overexpressing cells, suggesting that PMP2 does not significantly affect the distribution of endogenous SM in the PM.
MDCK cells (Figure S3B) ($p > 0.1$ on Dunnett’s multiple comparisons test). Mass spectrometry analysis indicated that SM contents (Figure S3C) and SM species (Figure S3D) were not changed in PMP2-knockout and PMP2-overexpressing MDCK cells. 

Next, control, PMP2-knockout, and PMP2-overexpressing MDCK cells were treated with SDS-digested freeze–fracture replica labeling (SDS-FRL) to examine the distribution of SM in the outer and inner leaflets of the PM separately (Figure 3A). These cells did not overexpress SMase. SM was labeled with lysenin, followed by an anti-lysenin antibody and a secondary antibody conjugated with 10 nm colloidal gold. The density of colloidal gold in the outer leaflet of the PM was not significantly different between the control cells ($403.5 \pm 47.4$ /µm$^2$; mean $\pm$ SEM, $n = 17$) and the PMP2-knockout cells ($325.6 \pm 51.2$ /µm$^2$, $n = 12$) ($p > 0.1$ on Mann–Whitney’s $U$ test). However, the density of colloidal gold decreased significantly to $29.4 \pm 6.7$ /µm$^2$ ($n = 7$) in the PMP2-overexpressing cells compared to the control cells, indicating that the outer-leaflet SM was dramatically reduced ($p < 0.01$ on Mann–Whitney’s $U$ test) in these cells. This is consistent with the observation that PMP2 is involved in the transbilayer movement of C6-NBD-SM from the outer to the inner leaflet of the PM. In contrast to the outer-leaflet SM, the density of the inner-leaflet SM was similar in all three cell groups: $2.3 \pm 0.2$ /µm$^2$ ($n = 9$) in the control cells, $5.9 \pm 0.4$ /µm$^2$ ($n = 13$) in the PMP2-knockout cells, and $2.3 \pm 0.3$ /µm$^2$ ($n = 5$) in the PMP2-overexpressing cells. We found small circular structures on the P-face of the PM in the PMP2-overexpressing cells (red arrows in Figure 3A). Ultrathin-section electron micrographs showed the accumulation of small (<100 nm) electron-lucent elliptical areas on the PM of PMP2-overexpressing cells (red arrowheads in Figure 3B). Because the electron-lucent structures were elliptical and were located 200–500 nm beneath the PM, they may have been deep invaginations of the PM induced by the overexpression of PMP2 and thus, could not be detected with lysenin by SDS-FRL.

The results described above suggested that we had underestimated the density of the inner-leaflet SM in the PMP2-overexpressing cells because it might have accumulated into the deep invaginations, which were hidden under the stubs observed on the P-face of the membrane in the PMP2-overexpressing MDCK cells (arrows in Figure 3A). Total
internal reflection (TIRF) microscopy is a suitable method for imaging the PM on or near the coverslip. Therefore, we expressed NT–Lys in the cytoplasm, labeled it with the spontaneously blinking fluorophore HMSiR, and observed the NT–Lys-labeled inner-leaflet SM on the adherent basal PM with TIRF-based stochastic optical reconstruction microscopy (STORM; Figure 3C). The localization density of NT–Lys labeled with HMSiR was significantly increased in the PMP2-overexpressing cells (249.5 ± 26.7 /µm², mean ± SEM, n = 28) compared with that in the PMP2-knockout cells (35.6 ± 7.2 /µm², n = 20), indicating that PMP2 overexpression increased the SM-rich domains seven-fold in the inner leaflet of the PM. When we stained outer-leaflet SM with Alexa647–NT–Lys, the fluorescence intensity of Alexa647–NT–Lys measured with TIRF microscopy was decreased in the PMP2-overexpressing cells to one-fifth of that in the PMP2-knockout cells (Figure 3D), confirming the SDS-FRL results shown in Figure 3A.

To test the possibility that SM flipped to the inner leaflet of the PM is transported to the intracellular organelles in the PMP2-overexpressing cells, we permeabilized the cells and stained intracellular SM with EGFP–NT–Lys (Figure S4A). Although SM was accumulated in late endosomes/lysosomes in Niemann-Pick type A fibroblast (Figure S4A, right) as described (Kiyokawa et al., 2005), we could not find significant intracellular EGFP–NT–Lys staining in the PMP2-overexpressing (Figure S4A, middle) as well as PMP2-knockout (Figure S4A, left) cells. The HPTLC, SDS-FRL, TIRF, and STORM results suggested that the outer-leaflet SM was decreased and the inner-leaflet SM was increased in the PM, whereas total SM content was unchanged in PMP2-overexpressing cells. These results support the idea that the inner-leaflet SM localizes in the deep invaginations of the PM and suggest that PMP2 could induce transbilayer movement of SM from outer to inner leaflet of the PM in a membrane deformation-dependent manner. TIRF and intracellular microscopy experiments suggest that SM stays in the inner leaflet of the PM and is not transported to a specific organelle under our experimental conditions.

We next examined whether PMP2 stimulates transbilayer movement of lipids from the inner leaflet to the outer leaflet of the PM. SDS-FRL with anti-PI(4,5)P₂ antibody detected PI(4,5)P₂ in the inner leaflet (P-face) but not in the outer leaflet (E-
face) of the PM both in the PMP2-knockout and the PMP2-overexpressing cells (Figure S4B). This could be due to the interaction of the lipid with PMP2. We then examined the exposure of another inner leaflet lipid, PS. PS-specific probe, EGFP–Lact–C2 (Fairn et al., 2011; Yeung et al., 2008), did not stain the PM from outside of the PMP2-knockout as well as PMP2-overexpressing cells (Figure S4C). When lipid scrambling was induced in these cells by ionomycin (Fairn et al., 2011), EGFP–Lact–C2 stained PS in the outer leaflet of the PM to the almost same level (Figure S4C, right) in both cells, suggesting that PS content in the PM is not different between the PMP2-knockout and the PMP2-overexpressing cells. These results suggest that PMP2 does not influence the transbilayer distribution of well-known inner-leaflet lipids such as PI(4,5)P$_2$ and PS in the PM.

PMP2 induces the tubulation of model membranes

PMP2, also known as P2, FABP8, or M-FABP, is a major cytosolic protein constituent of myelin in the peripheral nervous system, although the protein tissue distribution is ubiquitous (Ruskamo et al., 2020). PMP2 is also a member of the fatty acid-binding protein (FABP) family expressed in mammalian cells. The endogenous lipid substrate of PMP2 has not yet been identified. To understand the PMP2-dependent mechanism that controls the transport of SM, we performed the following experiments. First, we examined whether PMP2 is associated with the inner leaflet of the PM in MDCK cells. Although GFP–PMP2 is mainly located in the nucleus and the cytoplasm with several tubule-like structures (Figure 4A, arrows indicate tubule-like structures), TIRF images indicated that the Halo–PMP2 fusion protein also distributed on the PM (Figure 4B). We quantified the mean fluorescence intensity in the TIRF images and compared the intensities of the Halo–PMP2 fusion protein with those of the Halo protein. Since cells displayed heterogeneous expression of the Halo–PMP2 fusion protein or the Halo protein, the fluorescence intensity of the TIRF images was normalized with that of the epifluorescence images. As shown in Figure 4C, the normalized fluorescence intensity of the Halo–PMP2 fusion protein was significantly greater (19.5 ± 0.6 x 10$^{-3}$, mean ± SEM, n = 20) than that of the Halo protein (8.7 ± 0.5 x 10$^{-3}$, mean ± SEM, n =
(p < 0.001 on Welch’s t-test). These results indicate that PMP2 associates with the
PM.

Since PMP2 belongs to the FABP family, PMP2 likely binds PM through binding
of lipid(s). We used model membranes to identify the targeted lipid in the PM that binds
to PMP2. We prepared liposomes composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine
(DOPC) containing 5 mol % of one of the major lipids of the PM and measured the
binding of PMP2 to the liposomes (Figure 5A). PMP2 bound to the PI(4,5)P₂-containing
liposomes, but did not bind to the DOPC liposomes containing PS, PE, SM, or PI.

Although the binding of PMP2 to cholesterol (Chol) has been suggested (Majava et al.,
2010), PMP2 did not bind Chol, ceramide, or galactosylceramide (cerebrosides, GalCer)
in our solid-phase assay (data not shown). We then examined whether other members of
the FABP family bind PI(4,5)P₂. Figure 5B shows that FABP3 (62.4% identity with
PMP2) and FABP4 (66.7% identity with PMP2) did not bind PI(4,5)P₂, suggesting that
binding to PI(4,5)P₂ is property to PMP2. We tested the ability of PMP2 to bind several
acidic phospholipids using protein-lipid blot overlay assays displaying similar amounts of
phospholipid species (Figure 5C). PMP2 strongly bound PI(4,5)P₂ and PI(3,5)P₂, but
weakly bound other acidic phospholipids including PS spotted on the nitrocellulose
membrane. However, liposome flotation assay indicated that the binding of PMP2 to
liposomes containing 5 mol% PS is below the detectable level under such binding
conditions (Figure 5A). We measured the dissociation constant (K_D) of the interaction
between PMP2 with LUVs composed of DOPC:Chol:PI(4,5)P₂ (49:49:2) or
DOPC:Chol:PI(3,5)P₂ (49:49:2). Surface plasmon resonance (SPR) analysis indicated
that K_D for DOPC:Chol:PI(4,5)P₂ and DOPC:Chol:PI(3,5)P₂ was 1.19 ± 0.04 µM (mean
± SEM, n = 3) and 0.94 ± 0.02 µM (n = 3), respectively.

It has been shown that the amount of PI(4,5)P₂ is decreased in the cells
overexpressing synaptojanin (Field et al., 2005). TIRF images demonstrated that
overexpression of synaptojanin significantly reduced the fluorescence intensity of the
Halo–PMP2 (Figures 4B and 4C), suggesting that PMP2 is associated with the inner
leaflet of the PM in a PI(4,5)P₂-dependent manner. These results suggest that PMP2 binds
PI(4,5)P₂ both in vitro and in vivo. Moreover, overexpression of synaptojanin decreases
the inner-leaflet SM staining and increases the outer-leaflet SM at the PM in the PMP2 overexpressing cells (Figures 3C and 3D). These results support the idea that PI(4,5)P2-dependent binding of PMP2 is involved in the transbilayer movement of SM in the PM.

We next examined the effect of PMP2 on the shape of Φ100-nm large unilamellar vesicles (LUVs) composed of DOPC:Chol:PI(4,5)P2 (49:49:2) using negatively stained electron microscopy (Figure 5D). In the absence of PMP2, the liposomes were round and the diameter was ~100 nm. However, in the presence of PMP2, most of the liposomes developed tubule-like structures (red arrowheads in Figure 5D). The length of the tubule-like structure is several hundred nm. We measured the apparent size of liposomes with tunable resistive pulse sensing (TRPS), a high-throughput method of estimating the size distribution of single particles by measuring the blockade magnitude, which depends on the apparent size, not the exact diameter (Vogel et al., 2011). The apparent diameter of the PMP2-treated liposomes increased significantly in a time-dependent manner (Figure 5E, left and middle), consistent with our morphological observations (Figure 5D). In contrast, PMP2 did not increase liposome diameter in liposomes lacking PI(4,5)P2 (Figure 5E, right). These results indicate that PMP2 induces the PI(4,5)P2-dependent tubulation of liposomes.

We then examined whether the tubulation of liposomes was accompanied by a change in the transbilayer distribution of lipids. LUVs composed of DOPC:Chol: N-[12-[(7-nitro-2-1,3-benzoxadiazol-4-yl)amino]dodecanoyl]-sphingosine-1-phosphocholine (C12-NBD-SM) (49.5:49.5:1) or DOPC:Chol:PI(4,5)P2:C12-NBD-SM (48.5:48.5:2:1) were incubated in the presence or absence of recombinant PMP2 at 37 °C for 60 min. In these LUVs, C12-NBD-SM was incorporated into both the outer and inner leaflets. The preparation was a mixture of unilamellar and oligolamellar vesicles. After treatment with dithionite to reduce the outer-leaflet NBD fluorescence, the remaining NBD fluorescence was measured (Figure 6A). In the absence of PMP2, the relative fluorescence intensity of C12-NBD-SM in the LUVs containing PI(4,5)P2 was 0.759 ± 0.010 (mean ± SEM, n = 3) at 5 min after the addition of dithionite (gray circles, Figure 6B). In the presence of PMP2, the fluorescence of the remaining NBD was reduced to 0.698 ± 0.003 (mean ± SEM, n = 4) (blue triangles, Figure 6B). Fluorescence decreased in a PMP2-dose-
dependent manner (Figure S5A), suggesting that PMP2 enhances the movement of C12-NBD-SM to the same side of the membrane where PMP2 resides. This reduction was not detected in LUVs without PI(4,5)P$_2$ (Figure 6C), indicating that the movement of C12-NBD-SM depends on both PMP2 and PI(4,5)P$_2$. Although the fluorescence remaining in C12-NBD-PC (Figure S5B)- and C12-NBD-PE (Figure S5C)-containing liposomes slightly decreased in the presence of PMP2, the decrease of fluorescence was smaller than that of C12-NBD-SM. These results suggest that PMP2-induced tubulation is responsible for the movement of SM from the inner leaflet to the outer leaflet of the tubules in model membranes.

Pathogenic mutant PMP2 binds more strongly to PI(4,5)P$_2$

PMP2 is a causative protein of Charcot–Marie–Tooth (CMT) disease, a hereditary motor and sensory neuropathy. CMT is classified into two major types: demyelinating (CMT1, CMT3, and CMT4) and axon-defective (CMT2) (Pareyson and Marchesi, 2009; Zuchner and Vance, 2006). A point mutation (I43N) in PMP2 has been suggested as the potential pathogenic mutation in a family with autosomal dominant CMT1 (Gonzaga-Jauregui et al., 2015; Hong et al., 2016). Figure 7A compares the binding of wild-type PMP2, PMP2(I43N), and PMP2(R127Q) to DOPC:PI(4,5)P$_2$ (95:5) liposomes. PMP2(R127Q), which has a mutation in the conserved Arg residue involved in the interaction with fatty acids (Hofer et al., 2015; Jones et al., 1988), exhibited a low affinity for PI(4,5)P$_2$. In contrast, PMP2(I43N) showed a higher affinity for PI(4,5)P$_2$ than wild-type PMP2 (8.7 ± 1.9-fold, mean ± SEM, n = 4) (Figure 7A). We measured $K_D$ between the recombinant proteins of PMP2 variants and LUVs composed of DOPC:Chol:PI(4,5)P$_2$ (49:49:2) (Table S1). SPR analysis demonstrated that PMP2(R127Q) has a higher $K_D$ (4.26 ± 0.79 µM, mean ± SEM, n = 3), whereas PMP2(I43N) possesses a lower $K_D$ (0.26 ± 0.02 µM, n = 3) than wild-type PMP2 (1.19 ± 0.04 µM, n = 3), which are consistent with the lipid-binding results (Figure 7A). Wild-type PMP2 increased the apparent size of the LUVs and the transbilayer movement of C12-NBD-SM (Figures 5E and 6B, respectively). These effects were enhanced when wild-type PMP2 was replaced with PMP2(I43N) (Figures 7B and 7C). These results
suggest that I43N is a gain-of-function mutation, increasing the function of PMP2 in membranes containing PI(4,5)P$_2$.

PI(4,5)P$_2$ directly inhibits the activity of the cation channel TRPV4 (Takahashi et al., 2014). The overexpression of the phospholipase C-δ1 pleckstrin homology domain (PLCδ-PH) sequesters PI(4,5)P$_2$, leading to an increase in TRPV4 activity (Takahashi et al., 2014). Single-molecule tracking with TIRF microscopy indicated that the mean squared distance (MSD) of PMP2(I43N) molecules (0.42 ± 0.02 μm$^2$ at $\Delta t = 0.3$ s, mean ± SEM, $n = 25$) was similar to that of PLCδ-PH molecules (0.34 ± 0.02 μm$^2$ at $\Delta t = 0.3$ s, $n = 19$) in HeLa cells (Figures S6A-S6C). MSD corresponds to the average squared distance that a molecule travels from its starting point within a certain time interval (Yanagawa and Sako, 2021). Our finding suggests that PMP2(I43N), like the PLCδ-PH domain, may sequester PI(4,5)P$_2$ from TRPV4 and thus, might increase its activity. To test this, we measured the TRPV4 activity in PMP2(I43N)-overexpressing cells (Figure 7D). If PMP2(I43N) sequesters PI(4,5)P$_2$ from TRPV4, TRPV4 activity should be higher in cells overexpressing PMP2(I43N), as observed in cells overexpressing PLCδ-PH (Takahashi et al., 2014). The addition of GSK1016790A, an agonist of TRPV4, induced Ca$^{2+}$ influx into HeLa cells expressing TRPV4 ($V_{\text{max}} = 0.24 \pm 0.02$, mean ± SEM, $n = 6$). In HeLa cells overexpressing PMP2, the rate of Ca$^{2+}$ influx ($V_{\text{max}} = 0.28 \pm 0.03$, $n = 6$) was slightly higher than that in the control cells. The overexpression of PMP2(I43N) further increased the rate of Ca$^{2+}$ influx ($V_{\text{max}} = 0.33 \pm 0.02$, $n = 6$). These results suggest that the pathogenic I43N mutation of PMP2 efficiently sequesters PI(4,5)P$_2$ from TRPV4, thereby activating TRPV4 and leading to Ca$^{2+}$ overload.
Discussion

In this study, we identified PMP2 as a protein involved in the transbilayer distribution of SM. The knockdown of PMP2 restored the sensitivity of the cells to MβCD and the SM deficiency on the cell surface in SMase-overexpressing cells. The knockout of PMP2 reduced the transport of C6-NBD-SM from the outer leaflet to the inner leaflet in both the SMase-overexpressing and control cells. Although the total SM content was unchanged in the PMP2-overexpressing cells, the amount of inner-leaflet SM was increased, whereas the amount of outer-leaflet SM was reduced in the PM. The transbilayer distribution of SM in the PM was both PMP2- and PI(4,5)P₂-dependent. Recombinant PMP2 increased the movement of C12-NBD-SM to the same side of the membrane, where PMP2 resides in model membranes, in a PI(4,5)P₂-dependent manner. From these results, we concluded that PMP2 induces the transbilayer movement of SM from the outer to the inner leaflet of the PM in a PI(4,5)P₂-dependent manner.

SM is moved from the extracellular leaflet to the cytoplasmic leaflet

SM is synthesized on the lumenal side of the Golgi apparatus and transported to the extracellular leaflet of the PM (Tafesse et al., 2006), whereas local SM is synthesized on the extracellular side of the PM (Mitsutake et al., 2011). We found that overexpression of bacterial SMase in the cytoplasm dramatically decreased cell-surface staining by EGFP–NT–Lys (Figure 1A), which binds SM clusters and is a sensitive probe for membrane SM concentrations (Abe et al., 2012; Kiyokawa et al., 2005; Makino et al., 2015). This result suggests that SM can move from one leaflet to another. Although 10% of C6-NBD-SM was internalized in the control cells under our experimental conditions (Figure 2D), large amounts of endogenous SM were still located to the outer leaflet under steady-state conditions (Figure 3A), implying that there are additional mechanism(s) to maintain SM on the cell surface. We still found inner-leaflet SM in the PMP2-knockout cells (Figure 3A), suggesting that there are several factors involved in the transbilayer movement of SM in addition to PMP2. Cell-surface SM is continuously endocytosed and either recycled back to the PM or degraded in late endosomes/lysosomes (Slotte, 2013). The transbilayer movement of SM in the PM as well as the interconnection with the
Golgi apparatus/transport vesicles and recycling endosomes/transport vesicles could decrease SM on the extracellular leaflet of the PM. Previously, the exposure of SM to the cytosolic side of the cis-Golgi membrane (Bakrac et al., 2010) and of the endosome/lysosome membrane (Ellison et al., 2020) has been reported. The present study revealed that PMP2 is involved in the transbilayer movement of SM in the PM. In addition to PMP2, our screening identified several proteins involved in membrane trafficking (GGA1 (Puertollano et al., 2001), RAB6A (Shibata et al., 2019), and ASAP2 (Turner et al., 2001)). It would be interesting to examine whether these proteins are involved in the transbilayer movement of SM in the membranes of intracellular organelles.

**Tubulation is accompanied by the transbilayer movement of SM**

Our experiments using cell membranes and model membranes indicate that PMP2 induces a tubular structure in membranes in a PI(4,5)P$_2$-dependent manner. Tubulation/invagination is accompanied by the tubule interleaflet area increasing in the cytoplasmic leaflet and decreasing in the extracellular leaflet of the tubules. These changes lead to a shortage of lipids in the cytoplasmic leaflet of the tubules. In a 20 nm diameter vesicle composed of PC, the outer leaflet and the inner leaflet contain ~1700 molecules and ~800 molecules of PC, respectively (Huang and Mason, 1978). Thus, the invagination of the PM should be accompanied by the exposure of the extracellular leaflet lipids to the cytoplasmic leaflet. Analogous to our observations, Takada et al. (Takada et al., 2018) showed that the transbilayer lipid movement from the outer to the inner leaflet of the PM is accompanied by membrane tubulation. Because PMP2 did not bind SM, it is unlikely that the binding of PMP2 to SM induces lipid movement and tubulation. Overexpression of PMP2 did not induce the outer leaflet exposure of PI(4,5)P$_2$ or PS, indicating that PMP2 induces vectorial lipid transport from the outer to the inner leaflet of the PM where PMP2 is located. We were surprised by the selectivity of the lipid transport from the outer leaflet to the inner leaflet; the fluorescent SM analog was preferentially transported compared with the PC and PE analogs. Both SM (Slotte, 2016) and PI(4,5)P$_2$ (Kooijman et al., 2009) form inter-lipid hydrogen bonding. Previously, it
was suggested that SM inhibits the phospholipase C-δ1-catalyzed hydrolysis of PI(4,5)P_2 by increasing inter-lipid hydrogen bonding (Scarlata et al., 1996). We speculate that the inter-lipid hydrogen bonding between SM and PI(4,5)P_2 and between SM and SM is important for trapping SM on the cytosolic side of the invaginations. PMP2 may be involved in the stabilization of the SM/PI(4,5)P_2 domains.

In addition to the formation of hydrogen bonding, interbilayer colocalization between the outer leaflet and the inner leaflet may contribute to the selectivity of lipids in the PM. Previously, we showed that clustered SM-rich domains in the extracellular leaflet are colocalized just above the PI(4,5)P_2-rich domains in the cytoplasmic leaflet of the PMs in mammalian cells (Abe et al., 2012; Makino et al., 2017) (Figure 7E, left). Although the mechanism of this interbilayer colocalization is unclear, one explanation is the restricted localization of phosphatidylinositol 4-phosphate 5-kinase β (PIP5Kβ), which produces PI(4,5)P_2 from phosphatidylinositol 4-phosphate (Abe et al., 2012). The SM/Chol and PI(4,5)P_2-rich lipid domains are rigid structures and their lateral diffusion is slow (Eggeling et al., 2009; Makino et al., 2017) (MSD of PLCδ-PH molecules is 0.34 ± 0.02 μm^2; Figure S6C). Therefore, the shortage of lipids in the area around the PI(4,5)P_2-rich domain on the cytosolic side of the invaginations may be preferentially compensated with SM that is flipped from the extracellular leaflet of the PM (Figure 7E, right).

One possible mechanism for tubulation is that PMP2 may produce positive curvature, as observed with BAR domain proteins. Several F-BAR domain proteins, N-BAR domain proteins, and PX-BAR domain proteins form positive curvature in a phosphoinositide-dependent manner (Itoh and De Camilli, 2006; Suetsugu, 2010). The large headgroup in PI(4,5)P_2 has an inverted conical shape; thus, clustering of PI(4,5)P_2 in membranes tends to bend the membrane into a positive curvature (Zimmerberg and Kozlov, 2006). Indeed, N-BAR domain protein BIN1, which is involved in T-tubule biogenesis in muscle cells, clusters PI(4,5)P_2 in model and cell membranes (Picas et al., 2014). Our electron microscope images of PMP2-overexpressed cells suggest that PMP2 induces the formation of a high curvature structure in the cytoplasmic surface of the PM. Although PMP2 does not contain a coiled-coil motif that is involved in BAR domain-lipid interaction (Majava et al., 2010), PMP2 may induce positive curvature if PMP2
causes local PI(4,5)P₂ clustering. The affinity of PX-BAR domain protein SNX9 for phosphoinositides is similar to that of PMP2 (Schöneberg et al., 2017), suggesting that PMP2 binds sufficiently to the phosphoinositides. Structure studies of the PMP2 and PI(4,5)P₂ complex are needed to elucidate the exact molecular mechanisms.

**PMP2 as a causative protein of CMT**

The molecular mechanism of the effect of the pathogenic I43N mutation of PMP2 on the clinical symptoms of CMT1 is not clear. Demyelination is the pathological and physiological hallmark of CMT1 (Gonzaga-Jauregui et al., 2015; Hong et al., 2016). In this study, we showed that the pathogenic mutant PMP2 binds more strongly to PI(4,5)P₂ and sequesters PI(4,5)P₂ in HeLa cells. A possible explanation of this etiology of CMT1 is that the sequestration of PI(4,5)P₂ by the PMP2(I43N) protein leads to defects in Schwann cell myelination. PI(4,5)P₂ is required for the electrostatic association of myelin basic protein (MBP) with myelin-forming membranes, a crucial step in myelination (Nawaz et al., 2009). PI(4,5)P₂ also regulates the dynamics of actin and ion channel activity (Balla, 2013), both of which are necessary for myelination (Herbert and Monk, 2017). The overexpression of PMP2 also affects myelination in transgenic mice (Hong et al., 2016). Based on these data, we speculate that the PMP2(I43N) protein sequesters PI(4,5)P₂ from several proteins required for myelination, leading to demyelination, which in turn causes the slow transmission of nerve signals, which is a physical characteristic of CMT1.

It has been proposed that impaired SM metabolism contributes to the myelination defect in Schwann cells (Alvarez-Prats et al., 2018). In this study, we have shown that the outer-leaflet SM is significantly reduced in PMP2-overexpressing cells. Thus, another possible explanation is that a defect in outer-leaflet SM in Schwann cells affects their myelination. Interestingly, PMP2-deficient mice displayed changes in the lipid profile of myelin from the sciatic nerves as the amounts of SM and PC species decreased (Zenker et al., 2014).

In the present study, we found that PMP2 can bind both PI(4,5)P₂ and PI(3,5)P₂. PI(4,5)P₂, the most abundant phosphoinositide in most mammalian cells (Lorent et al.,
2020), is localized mainly to the cytoplasmic leaflet of the PM (Hullin-Matsuda et al., 2014; Tsuji et al., 2019). In contrast, PI(3,5)P₂, which is a less abundant phosphoinositide (Guerrero-Valero et al., 2021), is found only in endosomes/lysosomes, not in the PM (Hullin-Matsuda et al., 2014; Tsuji et al., 2019). Because PI(4,5)P₂ and PI(3,5)P₂ are involved in other subtypes of CMT, PMP2 may intersect with multiple other forms of CMT. PI(4,5)P₂ directly inhibits the activity of TRPV4 (Takahashi et al., 2014). In addition, CMT2C-associated TRPV4 mutations abolish PI(4,5)P₂ binding and increase the channel activity of TRPV4 (Takahashi et al., 2014), leading to increased Ca²⁺ influx and subsequent Ca²⁺ overload, which are thought to cause the CMT2C phenotype (Landoure et al., 2010). The common CMT4J-associated lipid phosphatase FIG4 mutation reduces cellular PI(3,5)P₂ levels (Chow et al., 2007), accompanied by the defects in lysosome/endosome trafficking (Zhang et al., 2008). These findings suggest that the mutations in PMP2 may also lead to dysfunction in TRPV4 through PI(4,5)P₂ changes or defect in lysosome/endosome trafficking through PI(3,5)P₂ changes, thus contributing to the pathophysiology of CMT. Further experiments are required to understand the pathological role of PMP2 in this context.

**Limitations of the study**

In this study, we used NT–Lys as a probe to visualize SM in the PM. NT–Lys selectively binds to clustered SM-rich lipid domains. We did not examine the behavior of other SM pools in the PM. Our results suggest that several proteins are involved in the exposure of outer-leaflet SM to the cytoplasmic side. This study elucidated one of the mechanisms. We examined the functions of PMP2 in HeLa and MDCK cells. The role of PMP2 in Schwann cells has not yet been clarified. Although the physiological functions of PMP2 are presumably conserved, its roles in the PM may differ between cell types. Negatively stained electron microscopy revealed that LUVs develop very thin tubule-like structures in the presence of PMP2 (Figure 5D). By contrast, transmission electron microscopy detected electron-lucent vesicle-like structures instead of tubule-like structures in the PM (Figure 3B). Future studies using state-of-the-art microscopic...
methods, such as 3D focused ion beam scanning electron microscopy, may reveal the tubule-like structures in the PM.
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Author Contributions
M.A., Y.S., and T.K. designed the experiments and analyzed the data; M.A. performed genetic screening, TIRF imaging, STORM imaging, and SPR; A.M. and F.H.M. performed lipid analyses; M.M. performed electron microscopy; M.Y. performed SMT analysis, STORM analysis, and calcium assay; M.A. and T.K. wrote the manuscript with input from all co-authors.

Declaration of interests
The authors declare no competing interests.
Figure legends

Figure 1 Identification of PMP2 as a factor involved in SM movement from the outer leaflet to the inner leaflet of the PM.

(A) Cell-surface SM. Staining of outer-leaflet SM with EGFP–NT–Lys (green) is abolished in HeLa cells overexpressing SMase (magenta), but not in the control non-overexpressing cells grown in the same dish.

(B) Colony-forming assay after MβCD treatment.

(C) Cell-surface SM in knockout HeLa cells expressing SMase. Left, images of EGFP–NT–Lys (green) obtained under the same condition. Right, the fluorescence intensity of EGFP–NT–Lys. Data are means ± SEM of at least 20 cells. P values were determined by Welch's t-test.

Figure 2 Metabolism and incorporation of C6-NBD-SM in knockout HeLa cells.

(A and B) Metabolism of C6-NBD-SM in SMase-expressing cells. HPTLC analysis (A), the fluorescence intensity of NBD-Cer (B). Data are shown as a ratio of the fluorescence intensity of NBD-Cer to the intensity of total NBD (NBD-Cer, NBD-GlcCer, and NBD-SM). Data are means ± SEM of three independent experiments. P values were determined by Dunnett’s test. NS, not significant.

(C and D) Incorporation of C6-NBD-SM in cells not overexpressing SMase. HPTLC analysis (C), the fluorescence intensity of NBD-SM (D). Data are means ± SEM of three independent experiments. P values were determined by Dunnett’s test.

Figure 3 Reduction of SM in the outer leaflet of the PM and appearance of electron-lucent structures in MDCK cells overexpressing PMP2.

(A) Upper: E-face (outer leaflet) of the PM of SDS-FRL cells labeled with lysenin, showing clustered SM. Lower: P-face (inner leaflet) of SDS-FRL cells labeled with lysenin. Red arrows indicate stub-like structures.

(B) Transmission electron microscopy. Red arrowheads indicate electron-lucent vesicle-like structures.
(C) Super-resolution images of inner-leaflet SM in the PM. Left, inner-leaflet SM stained with HMSiR–NT–Lys was visualized with STORM. Right, the localization density of HMSiR–NT–Lys. Data are means ± SEM of at least 20 images. P values were determined by Welch's t-test.

(D) TIRF images of outer-leaflet SM in the PM. Left, outer-leaflet SM was stained with Alexa647–NT–Lys. Right, the fluorescence intensity of Alexa647–NT–Lys in the PM. Data are means ± SEM of at least 38 images. P values were determined by Welch's t-test.

**Figure 4 Binding of PMP2 to the inner leaflet of the basal PM in a PI(4,5)P₂ dependent manner.**

(A) Fluorescence image of PMP2-AcGFP in living cells. Arrows indicate tubule-like structures.

(B) TIRF images of Halo-PMP2. HeLa cells transiently expressing Halo (left), Halo-PMP2 (middle), or Halo-PMP2 and mCherry-synaptojanin (right) were stained with HaloTag Oregon Green Ligand. Upper, TIRF images; middle and lower, epifluorescence images.

(C) Fluorescence intensity of Oregon Green-PMP2. Mean fluorescence intensity was quantified in the cell region in the TIRF images and the epifluorescence images. The mean fluorescence intensity of the TIRF images was normalized with that of the epifluorescence images. Data are means ± SEM of 20 images. P values were determined by Welch's t-test.

**Figure 5 PMP2 preferentially binds PI(4,5)P₂ and induces membrane tubulation.**

(A) The binding of PMP2 to liposomes containing 95% DOPC and 5% of one of the indicated lipids.

(B) The binding of FABPs to liposomes containing PI(4,5)P₂.

(C) The binding of PMP2 to different phospholipids in protein-lipid blot overlay assays.

(D) Negatively stained electron micrographs of liposomes (DOPC:Chol:PI(4,5)P₂ (49:49:2)) incubated with (right) and without PMP2 (left).
Figure 6 PMP2 induces the transbilayer movement of lipids to the side of PMP2.

(A) Schema of the experiments. In LUVs, NBD (green)-labeled lipid was incorporated into both the outer and inner leaflets. LUVs were incubated in the presence or absence of recombinant PMP2 at 37 °C for 60 min. After dithionite had reduced the outer-leaflet NBD, the remaining NBD fluorescence was measured.

(B and C) Non-quenched C12-NBD-SM after dithionite treatment in LUVs composed of DOPC:Chol:PI(4,5)P_2 (B) or DOPC:Chol (C). Data are means ± SEM of at least 3.

Figure 7 CMT-associated mutation in PMP2 enhances its affinity for PI(4,5)P_2, the tubulation of model membranes, and the transbilayer movement of lipids.

(A) Binding of mutant PMP2 to liposomes containing 5% PI(4,5)P_2. Data are means ± SEM, n = 4. P values were determined by Dunnett’s test.

(B) Apparent diameters of liposomes (DOPC:Chol:PI(4,5)P_2 (49:49:2)) measured with TRPS.

(C) Increase in the transbilayer movement of C12-NBD-SM to the side of PMP2(I43N) protein. Data are means ± SEM, n = 4.

(D) Increase in Ca^{2+} influx in PMP2(I43N)-overexpressing cells. Data are means ± SEM, n = 6.

(E) Model of the transbilayer movement of SM in the PM. In the PM, SM (green)-rich domains in the extracellular leaflet colocalize with PI(4,5)P_2 (magenta)-rich domains in the cytoplasmic leaflet. PMP2 (blue) binds the PI(4,5)P_2-rich membrane domains (left), induces membrane tubulation where the lipid density of the cytoplasmic leaflet is lower than that of the extracellular leaflet, leading to the exposure of extracellular-leaflet SM to the inner leaflet (right). After flipping of SM from the extracellular leaflet, inter-lipid hydrogen bonding may stabilize SM and PI(4,5)P_2 in the cytoplasmic leaflet, as well as between SM and SM.
STAR★methods

Resource availability

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mitsuhiro Abe (abemitsu@riken.jp).

Materials availability
Plasmids generated in this study have been deposited to RIKEN BRC Gene Bank.
Catalog numbers are listed in the Key Resources Table.

Data and Code Availability

- Fluorescence images, luminescence images, and analyzed data have been deposited at Mendeley Data and are publicly available as of the date of publication. DOIs are listed in the key resources table. Electron micrographs reported in this paper will be shared by the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

Experimental model and subject details

Cultured cell lines
HeLa and MDCK cells were maintained at 37 °C in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum. Niemann-Pick type A cells were grown at 37 °C in Ham F-10 nutrient mix supplemented with 10% fetal bovine serum as described (Makino et al., 2015).

Method details

Genetic screening
HeLa cells were transfected with the pTet-DualOFF vector (TaKaRa Bio, Shiga, Japan), and the stable cell line was cloned. cDNA of Staphylococcus aureus SMase C was synthesized and cloned into the pTRE-Dual2 vector (TaKaRa Bio). HeLa cells were
transfected with the plasmid and the stable cell line expressing *S. aureus* SMase C was cloned. These cells were infected with the Human 50K Plasmid shRNA Library (System Biosciences, Palo Alto, CA) and grown for 1 week (Figure S1A). The cells were grown in 40 mM methyl-β-cyclodextrin (MβCD; Cyclolab, Budapest, Hungary) for 2 h, washed, and grown in fresh medium for 1 week. The viable cells were treated again with 40 mM MβCD for 2 h, and grown in fresh medium for 1 week. The viable cells were selected and total RNA was isolated with the GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). After the cDNA was produced with SuperScript III reverse transcriptase (Thermo Fisher Scientific), the siRNA inserts were amplified from the cDNA by two-step PCR. The sense strand was removed by treatment with lambda exonuclease (NEB, Ipswich, MA), and the integrated siRNA was determined with an Affymetrix GeneChip array (Thermo Fisher Scientific).

**Colony-forming assay**

Cells were grown in MβCD for 2 h, washed, and grown in fresh medium for 1 week. Crystal violet (0.1%) in 19% methanol was added to the wells to fix and stain the cells.

**Cell surface labeling of SM**

To stain the SM-rich domain in the PM for confocal microscopy, cells were incubated in DMEM supplemented with 10% lipoprotein-deficient serum containing 10 μg/mL EGFP–NT–Lys for 10 min, followed by fixation with 4% paraformaldehyde and 0.2% glutaraldehyde, as previously described (Abe et al., 2012). Confocal images were obtained with a confocal microscope (FV 1000, Olympus, Tokyo, Japan) equipped with a 60 × 1.1 NA objective lens (Plan Apo, Mitutoyo, Kawasaki, Japan). Images were captured with the FV10-ASW software (Olympus). To stain the SM-rich domain in the PM for TIRF microscopy, cells were incubated in DMEM supplemented with 10% lipoprotein-deficient serum containing 10 μg/mL Alexa647–NT–Lys for 10 min, followed by fixation with 4% paraformaldehyde and 0.2% glutaraldehyde, as previously described (Abe et al., 2012). Alexa647–NT–Lys on the basal PM were illuminated with a 637-nm, 140-mW laser (OBIS 637, Coherent) and observed with total internal reflection illumination with an inverted fluorescence microscope (TiE, Nikon). Quantifications were performed with ImageJ software (National Institutes of Health).
Intracellular labeling of SM

To label intracellular membranes, cells were permeabilized with 50 μg/ml digitonin after fixation with 4% paraformaldehyde and 0.2% glutaraldehyde, followed by labeling with 200 μg/mL EGFP-NT-Lys.

Cell surface labeling of PS

Lipid scrambling was induced with 10 μM ionomycin in the presence of 2.5 mM Ca^{2+}, as described previously (Fairn et al., 2011). To stain PS in the PM, cells were incubated in DMEM supplemented with 10% lipoprotein-deficient serum containing 40 μg/mL EGFP-tagged C2 domain of lactadherin (EGFP–Lact–C2) for 5 min, followed by fixation with 4% paraformaldehyde and 0.2% glutaraldehyde.

Knockdown of genes by synthesized siRNA

The siRNA was designed with the online program at http://rna.co.jp/lsci/about.html. The sequences were shown in Table S2. HeLa cells were transfected with the synthesized siRNA using Lipofectamine RNAiMAX (Thermo Fisher Scientific).

Generation of PMP2-knockout cells with CRISPR/Cas9 gene editing

To construct the gene-editing plasmids, oligomers (PMP2-top1 and PMP2-bottom1 for HeLa cells, PMP2-top2 and PMP2-bottom2 for MDCK cells, see Key Resources Table) were synthesized, annealed, and cloned into the PX459 vector (#48139, Addgene) at the BbsI site, as previously described (Ran et al., 2013). HeLa cells were transfected with the resultant plasmid using Lipofectamine 3000 (Thermo Fisher Scientific). Single colonies were picked after selection with 0.2 μg/ml puromycin for 3 days. The genomic DNAs were extracted from the clones with the GenElute™ Mammalian Genomic DNA Miniprep Kit (Sigma-Aldrich). The genome sequences were examined with PCR using the following primers: PMP2-F1 and PMP2-R1 for HeLa cells, or PMP2-F2 and PMP2-R2 for MDCK cells. Sequence analysis revealed that the HeLa PMP2 knockout had a 1-bp insertion and a 2-bp deletion next to the protospacer adjacent motif (PAM) sequence, and that the MDCK PMP2 knockout had a 1-bp and a 2-bp insertion adjacent to the PAM sequence.

Generation of PMP2-overexpressing MDCK cells
MDCK cells were transfected with the pTet-DualOFF vector (TaKaRa Bio), and the stable cell line was cloned. cDNA of human PMP2 was cloned into the pTRE-Dual vector (TaKaRa Bio). MDCK cells were transfected with the plasmid and the stable cell line overexpressing PMP2 was cloned.

**Incorporation and metabolism of NBD-lipids in HeLa cells**

Cells were grown in six-well plates. To examine the metabolism of C6-NBD-SM in SMase-expressing cells, the cells were washed with cold phosphate-buffered saline (PBS) and labeled with 1 µM C6-NBD-SM for 30 min on ice. The cells were then washed with cold PBS and incubated in DMEM/F-12 medium supplemented with 0.1% bovine serum albumin (BSA) at 15 °C for 2 h. After incubation, lipids were extracted (Bligh and Dyer, 1959) from the cells and applied to HPTLC plates (Merck KGaA, Darmstadt, Germany). The plates were developed with chloroform, methanol, and water (65:25:4, v/v) as solvent. After HPTLC plates were visualized by their fluorescence with ImageQuant LAS 4000 (Cytiva, Marlborough, MA), the fluorescence of NBD-Cer, NBD-GlcCer, and NBD-SM was quantified with ImageJ software (National Institutes of Health). The statistical significance of differences between datasets was determined by Dunnett’s multiple comparisons test. To examine the incorporation of C6-NBD-SM, C12-NBD-PC, or C12-NBD-PE in SMase-nonexpressing cells, the cells were washed with cold PBS and labeled with 1 µM C6-NBD-SM, C12-NBD-PC, or C12-NBD-PE in DMEM/F-12 medium supplemented with 0.1% BSA at 15 °C for 2 h, after which dithionite (25 mM) was added. The fluorescence insensitive to dithionite was measured with HPTLC, as described above.

**SDS-FRL**

SDS-FRL transmission electron microscopy was performed as previously described (Murate et al., 2015). Lysenin was purchased from the Peptide Institute Inc. (Osaka, Japan). The anti-PI(4,5)P_2 antibody was a gift from Dr. Masato Umeda (Miyazawa et al., 1988). Specimens were examined under a transmission electron microscope (JEM1230, JEOL, Tokyo, Japan) with the help of the Materials Characterization Team at the RIKEN Advanced Technology Support Division (Wako, Japan).

**Negatively stained EM and ultrathin-section EM**
A liposome suspension was incubated in the presence or absence of PMP2 at 37 °C for 1 h, and then the liposomes were negatively stained as reported previously (Inaba et al., 2016). The ultrastructures of MDCK cells were observed as reported previously (Inaba et al., 2016).

**STORM imaging**

PMP2-knockout cells or PMP2-overexpressing cells were transfected with pHalo–NT–Lys expressing NT–Lys fused with HaloTag by Neon Transfection System (Thermo Fisher Scientific). After 1 day, the cells were incubated with 10 nM HaloTag HMSiR Ligand (GORYO Chemical, Inc. Sapporo, Japan) for 16 h. Cells were washed with fresh medium and further incubated for 3 h. Cells were fixed with 4% paraformaldehyde and 0.2% glutaraldehyde for 60 min and washed with PBS. To calibrate the drift, TetraSpeck™ Microspheres (Thermo Fisher Scientific) was added before observation. HMSiR labeled NT–Lys on the basal PM were illuminated with a 637-nm, 140-mW laser (OBIS 637, Coherent) and observed with total internal reflection illumination with an inverted fluorescence microscope (TiE, Nikon). 1000 frames of the fluorescent images were acquired with the MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices) at a frame rate of 50 ms. The positions of individual fluorescence spots were determined with the Auto Analysis System (Zido, Osaka, Japan). Image reconstruction was performed with an in-house computer program and the density of probes (molecular density) was calculated in the reconstructed image.

**Recombinant protein**

To generate PMP2 tagged with His, the cDNA of human PMP2 was cloned into the pET-28b vector (Merck KGaA, Darmstadt, Germany). Mutant PMP2 was constructed with site-directed mutagenesis. The cDNAs of human FABP3 and FABP4 were cloned into the pET-28b vector. EGFP–Lact–C2 was cloned into the pET-28b vector. The recombinant protein was expressed in *Escherichia coli* strain BL21(DE3) and purified with HisTrap FF crude columns (Cytiva), as described previously (Abe and Kobayashi, 2017).

**Liposome flotation assay**
Multilamellar vesicles (MLVs) were prepared by hydrating a lipid film containing N-(lissamine rhodamine B sulfonyl)-dioleoylphosphatidylethanolamine (N-LRh-PE) with 10 mM HEPES–150 mM NaCl (pH 7.5) and vortex mixing (final concentration of lipids: 1 mM). 0.1 ml of the MLVs were incubated with or without 20 µM protein at 37 °C for 1 h. An aliquot of this suspension was mixed with 1 ml of 2.1 M sucrose in 10 mM HEPES (pH 7.5), loaded into the bottom of an ultracentrifuge tube (MLS50; Beckman Coulter, Brea, CA), and overlaid with 1.5 ml of 1.2 M sucrose and 2 ml of 0.8 M sucrose. The sample was centrifuged for 1 h at 35,608 × g at 4 °C in a Optima MAX-E ultracentrifuge (Beckman Coulter, Brea, CA). The top fraction (100–200 µl) was collected. The recovery yield of the MLVs after flotation was normalized with rhodamine B fluorescence. The normalized fraction was subjected to SDS-PAGE and Western blotting with an anti-His antibody (Qiagen, Hilden, Germany). Lipid phosphate was measured to monitor the concentration of liposomes in the gradient.

**Protein-lipid blot overlay assays**

Lipid-binding assays were performed with PIP Strips™ containing similar amount of lipid per spot (Echelon Biosciences, Salt Lake City, UT) according to the manufacturer’s instruction.

**Preparation of LUVs**

MLVs were subjected to three freeze–thaw cycles by alternately placing the sample vial in a liquid nitrogen bath and a warm water bath. The MLVs were passed through polycarbonate filters with a 100 nm pore size (cat. # 800309, Cytiva) at least 20 times with an extruder (Mini Extruder, Avanti Polar Lipids).

**Measurement of LUV size**

LUVs (0.1 mM) were incubated with 2 µM protein at 37 °C for 30 min. The apparent sizes of the LUVs were measured with TRPS and qNano Gold (Izon Science, Christchurch, New Zealand), according to the manufacturer’s instructions. At least 1000 particles were recorded in each experiment. Nanopore NP150 (Izon Science) was used as the nanopore.

**Measurement of NBD–lipid remaining in the inner leaflets of LUVs**
LUVs (50 µM) containing 1% C12-NBD-lipid analogues in PBS were incubated in the presence or absence of 1 µM recombinant protein at 37 °C for 60 min in a 96-well plate. Each sample was then applied to a microplate reader (FlexStation, Molecular Devices, San Jose, CA). Dithionite (25 mM) was added and the fluorescence of the C12-NBD remained was measured.

**Measurement of changes in the intracellular calcium concentration ([Ca^{2+}]_i)**

Human TRPV4 cDNA was cloned into the pTRE-Dual vector (TaKaRa Bio). Cells were transfected with pTRE-TRPV4, and grown for 1 day. The cells (4.0 \times 10^4) were plated in a 96-well culture microplate (Greiner Bio-One GmbH, Frickenhausen, Germany, cat. #675986) and incubated overnight at 37 °C under 5% CO₂ in 40 µl of culture medium. Changes in [Ca^{2+}] were measured with the FLIPR® Calcium 6 Assay Explorer Kit (Molecular Devices). After addition of 40 µl of loading buffer to each well, the cells were incubated at 37 °C for 2 h. The cells were incubated at room temperature for 30 min and fluorescence was measured with a FlexStation® instrument (Molecular Devices). During measurement, 6.25 nM GSK1016790A (Cayman Chemical, Ann Arbor, MI) was added.

**Phospholipid analysis.**

MDCK cells were washed twice with cold PBS and then scraped on ice in 2 mM EDTA. Aliquots of cell extract were taken for protein quantification and lipid extraction performed according to Bligh and Dyer (Bligh and Dyer, 1959). Lipids were separated on HPTLC plates with a solvent mixture of methyl acetate/propanol/chloroform/methanol/0.25% KCl (25:25:25:10:9, v/v) (Yasuda et al., 2001). The HPTLC plate was sprayed with primuline reagent until it became wet, and then, the plate was air-dried. Lipid bands were visualized with ImageQuant LAS 500 (Cytiva, Marlborough, MA). The fluorescence was quantified with ImageJ software (National Institutes of Health). The statistical significance of differences between datasets was determined by Dunnett’s multiple comparisons test.

**TIRF and single-molecule tracking (SMT) analysis**

TIRF images were obtained as described previously (Yanagawa et al., 2018). After HeLa cells were transfected with pHalo–PMP2 expressing PMP2 fused with HaloTag, the cells were stained with 30 nM HaloTag Oregon Green Ligand (Promega, Madison, WI) for 30
min and fixed with 4% paraformaldehyde before observation. The fluorescently labeled proteins on the basal PM were illuminated with a 488-nm laser (Sapphire 488-200, Coherent) and observed with total internal reflection illumination with an inverted fluorescence microscope (TiE, Nikon, Tokyo, Japan). The fluorescent images were recorded with the MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices) and quantified with ImageJ software (National Institutes of Health). An SMT analysis was performed as described previously (Yanagawa et al., 2018). After HeLa cells were transfected with pHalo–PMP (I43N) or pHalo–PLCδ–PH, the cells were stained with 30 nM HaloTag TMR Ligand (Promega, Madison, WI). Single-molecule imaging in living cells was performed at room temperature (25 °C) 30 min after the addition of the ligand. The fluorescently labeled proteins were observed with the above microscope (TiE, Nikon, Tokyo, Japan). The cells were illuminated with a 532-nm, 100-mW laser (Compass 315M-100) with an ND50 filter for TMR. The fluorescent images were recorded with the MetaMorph Microscopy Automation and Image Analysis Software (Molecular Devices), with an exposure time of 30.5 ms. The single-molecule images were analyzed as described (Yanagawa and Sako, 2021). The SMT analysis was performed with the Auto Analysis System (Zido) based on a two-dimensional Gaussian-fitting algorithm. The time-averaged MSD within time $n\Delta t$ of each trajectory was calculated with the function

$$\text{MSD}(n\Delta t) = \frac{1}{N-1-n} \sum_{j=1}^{N-1-n} [(x(j\Delta t + n\Delta t) - x(j\Delta t))^2 + (y(j\Delta t + n\Delta t) - y(j\Delta t))^2],$$

where $n$ is the length of the frame, $\Delta t$ is the frame rate, and $N$ is the total frame number of the trajectory.

SPR experiments were carried out in a Biacore X 100 Plus Package (Cytiva) in a single-cycle kinetic assay mode at 25 °C. The L1 sensor chip (Cytiva) was used since the chip can maintain lipid bilayer structure. Before use, the chip was rinsed with two injections of 40 mM n-Octyl-β-D-glucoside. LUVs composed of DOPC:Chol:PI(4,5)P$_2$ (49:49:2) was immobilized onto the L1 sensor chip by injecting 1 mM LUVs in running buffer (PBS) for 60 s. After the sensor chip was washed, 5 series of different concentrations of the recombinant proteins were injected for 60 s and solutes were allowed to dissociate for
300 s. The sensor chip surface regeneration was performed with sequential injections of 40 mM n-Octyl-β-D-glucoside. The sensorgrams were analyzed with the BIA evaluation software (Cytiva) using the 1:1 kinetic binding model to generate $k_{on}$, $k_{off}$, and $K_D$. The evaluation software also generates chi-square value to determine the accuracy of these fitted parameters.

**Quantification and statistical analysis**

Statistical analyses were performed using Igor Pro software (WaveMetrics, Inc, Lake Oswego, OR) or Microsoft Excel (Microsoft, Redmond, WA). Experiments were repeated for at least three times as described in the figures and corresponding figure legends. The results were expressed as the mean ± SEM. Statistical significance was determined using Dunnett’s multiple comparisons test, Mann–Whitney’s $U$ test, or Welch's $t$-test between groups.
References


37


control cells

expressing SMase

outer-leaflet SM

C

cells expressing SMase

SLC40 KO expressing SMase

PMP2 KO expressing SMase

GGA1 KO expressing SMase

outer-leaflet SM

20 µm

SCC

expression

SMase

MβCD

40 mM 10 mM 0 mM

cells expressing SMase

control cells

cells expressing SMase

expression

SMase

p < 0.001

p < 0.01

p < 0.001

SM fluorescence intensity (AU × 10^4)

20

15

10

5

0

cells expressing SMase

SLC40 KO expressing SMase

PMP2 KO expressing SMase

GGA1 KO expressing SMase

20 µm
A. 

B. 

C. 

D. 

剩余 NBD-SM

剩余 NBD-SM

剩余 NBD-SM

剩余 NBD-SM

控制

SLC40 KO

PMP2 KO

GGA1 KO

控制

SLC40 KO

PMP2 KO

NBD-Cer

NBD-GlcCer

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-Cer

NBD-GlcCer

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM

NBD-SM
A) 

E-face (outer leaflet) 

control  | PMP2 KO | PMP2 overexpression
--- | --- | ---
403.5 ± 47.4 / µm² | 325.6 ± 51.2 / µm² | 29.4 ± 6.7 / µm²

P-face (inner leaflet) 

2.3 ± 0.2 / µm² | 5.9 ± 0.4 / µm² | 2.3 ± 0.3 / µm²

B) 

control | PMP2 KO | PMP2 overexpression
--- | --- | ---

C) 

PMP2 KO | PMP2 overexpression
--- | ---

STORM

inner-leaflet SM | inner-leaflet SM | inner-leaflet SM

epi | synaptojanin

1.0 µm

D) 

PMP2 KO | PMP2 overexpression
--- | ---

TIRF

outer-leaflet SM | outer-leaflet SM | outer-leaflet SM

epi | synaptojanin

5.0 µm

fluorescence intensity (AU x 10⁴ / µm²)

p < 0.001

p < 0.001
A

PMP2-AcGFP

5.0 µm

B

Halo
–

Halo-PMP2
–
synaptojanin

TIRF

epi

epi

C

TIRF intensity / epifluorescence intensity

Halo
–

Halo-PMP2
–

Halo-PMP2
synaptojanin

p < 0.001

p < 0.01

PMP2-AcGFP

5.0 µm
**A**

<table>
<thead>
<tr>
<th>PC</th>
<th>PI(4,5)P</th>
<th>PS</th>
<th>PE</th>
<th>SM</th>
<th>PI</th>
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<tbody>
<tr>
<td>PMP2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**B**

- **input**
- **bound to PI(4,5)P**

**C**

- PC
- PI(3,4)P
- PI(3,5)P
- PI(4,5)P
- PI(3,4,5)P
- PA
- PS
- Blank

**D**

- control
- + PMP2

**E**

- DOPC / Chol / PI(4,5)P
- DOPC / Chol / PI(4,5)P
- DOPC / Chol

**Legend:**
- % population
- Apparent diameter (nm)
- control
- + PMP2, 30 min
- + PMP2, 60 min
A

+ PMP2
37°C 60 min
+ dithionite

PMP2

B

[Graph showing relative fluorescence intensity over time for DOPC/Chol/PI(4,5)P₂/NBD-SM with control and + PMP2 conditions.]

C

[Graph showing relative fluorescence intensity over time for DOPC/Chol/NBD-SM with control and + PMP2 conditions.]

37ºC 60 min