博士論文

A phosphatidylserine flippase regulates intracellular membrane traffic through recycling endosomes

（ホスファチジルセリンフリッパーゼによる

細胞内膜輸送の制御機構）

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Contents

- Main text

Chapter 1

Introduction ---------- 2

Results ---------- 5

Discussion ---------- 18

Chapter 2

Introduction ---------- 24

Results ---------- 25

Discussion ---------- 30

- Materials and Methods

Chapter 1 ---------- 33

Chapter 2 ---------- 44

- References ---------- 51

- Figures ---------- 60

Chapter 1

Membrane traffic through recycling endosomes requires EHD1 recruitment by phosphatidylserine flippases, ATP8A1 and ATP8A2:

insight into a flippase-associcated disease

Introduction

Membrane-bound organelles of eukaryotic cells have distinct lipid compositions that influence the function of organelles (Behnia and Munro, 2005). Membrane lipids not only serve as a physical barrier, but also interact with a wide variety of integral and peripheral membrane proteins to regulate their localization and activity. Moreover, phospholipids can be distributed asymmetrically between the two leaflets of the lipid bilayer (Devaux, 1992), which provides two distinct surfaces for protein interactions. For example, the cytosolic leaflet of the plasma membrane (PM) is enriched in the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE), whereas the extracellular leaflet of the PM is enriched in sphingolipids. A low PS concentration in the extracellular leaflet of the PM is crucial for cell life, since phagocytes recognize and engulf cells by the binding to the PS in the surface of target cells (Balasubramanian and Schroit, 2003). A high PS concentration in the cytosolic leaflet of the PM is essential to facilitate various signaling events through membrane translocation and activation of various kinases, such as protein kinase C (Newton and Keranen, 1994) and Akt kinase (Huang *et al.*, 2011). In addition to the PM, the membranes of endocytic organelles have asymmetric transbilayer distributions of PS (Fairn *et al.*, 2011).

The transbilayer asymmetry of phospholipids is generated, in part, by the selective transport of phospholipids across the membranes (Graham, 2004). The P4 subfamily of P-type ATPases (P_4-ATPases) are responsible for the flipping of aminophospholipids to the cytosolic

leaflet (Coleman *et al.*, 2012b; Sebastian *et al.*, 2012). The human genome contains 14 P_4-ATPases and mutations in some P_4-ATPases cause inherited genetic diseases. Mutations in ATP8B1 are associated with familial intrahepatic cholestasis type 1 (Bull *et al.*, 1998) and mutations in ATP8A2 are associated with a severe neurological disorder characterized by cerebellar ataxia, mental retardation, and dysequilibrium syndrome (CAMRQ) (Emre Onat *et al.*, 2012). In mice, mutations in ATP8A2 cause axonal degeneration (Zhu *et al.*, 2012) and mutations in ATP11C cause B cell–deficiency syndrome (Siggs *et al.*, 2011; Yabas *et al.*, 2011). Despite the mounting evidence showing the physiological importance of P_4-ATPases, how their dysfunction causes diseases remains unclear.

Recycling endosomes (REs) function in several membrane transport pathways (Mellman, 1996; Maxfield and McGraw, 2004; Taguchi, 2013): the recycling of internalized molecules back to the PM, the exocytic trafficking of several cargoes, and the retrograde transport of internalized molecules to the Golgi. REs also serve as a source of membranes for autophagosomes (Longatti *et al.*, 2012; Knaevelsrud *et al.*, 2013; Puri *et al.*, 2013). We previously showed that evectin-2, an RE protein with a pleckstrin homology (PH) domain, is essential for retrograde traffic (Uchida *et al.*, 2011). The PH domain of evectin-2 selectively binds PS *in vitro* and is required for the localization of evectin-2 to REs. The PH domain of evectin-2 faces the cytosol, indicating that PS is present in the cytosolic leaflet of RE membranes. The C2 domain of lactadherin tagged with GFP (GFP-lact-C2) is a widely used PS probe (Yeung *et al.*, 2008; Fairn *et al.*, 2011). When GFP-lact-C2 was overexpressed in the cytosol, it accumulated at the REs and suppressed the retrograde traffic from REs to the Golgi, indicating that PS at cytosolic leaflet has a critical role in endosomal membrane traffic (Uchida *et al.*, 2011). How PS is enriched in the cytosolic leaflet of RE membranes remains unknown.

Eps15 homology domain-containing protein 1 (EHD1) belongs to the EHD or

receptor-mediated endocytosis (RME)-1 family, whose members are highly conserved eukaryotic ATPases that have been implicated in membrane remodeling in the endosomal system (Daumke *et al.*, 2007; Grant and Caplan, 2008). The human genome contains four EHDs (EHD1-4), one of which (EHD1) is essential for recycling membrane traffic from REs to the PM (Grant *et al.*, 2001; Lin *et al.*, 2001). EHD1 is also required for the retrograde membrane transport of Shiga toxin from REs to the Golgi (McKenzie *et al.*, 2012). *In vitro*, EHD1 facilitates tubulation or fission of liposomes that contain anionic phospholipids, suggesting that EHD1 functions in the formation of membrane carriers from REs (Pant *et al.*, 2009).

Here we show that a P_4-ATPase (ATP8A1) localizes at REs, regulates the transbilayer asymmetry of PS in the RE membranes, and is essential for the RE localization of EHD1. Together with the observations that EHD1 binds PS *in vitro* and the membrane localization of EHD1 is lost in cells that are defective in PS synthesis, we propose that the PS flipped to the cytosolic leaflet by ATP8A1 is essential for the EHD1 recruitment to REs, thereby regulating the membrane traffic through REs. ATP8A2 is a tissue-specific ATP8A1 paralogue and is associated with a neurodegenerative disease CAMRQ (Emre Onat *et al.*, 2012). ATP8A2, but not the disease-causative ATP8A2 mutant, rescued the endosomal defects in ATP8A1-depleted cells. Primary neurons from *Atp8a2*$^{-/-}$ mice had nearly the same abundance of TfnR as wild-type (WT) neurons, but showed a lower expression of TfnR at the PM. This suggests that ATP8A2 in neurons is essential for recycling membrane traffic and defects in this pathway provide a molecular mechanism for CAMRQ.

Results

ATP8A1 is essential for membrane traffic through REs

Four P_4-ATPases (ATP8A1, ATP9A, ATP11A, and ATP11B) are ubiquitously expressed and localize at early endosomes (EEs) and/or REs (Takatsu *et al.*, 2011; Kato *et al.*, 2012). Because the ATPase activity of ATP8A1 is strongly activated by PS (Ding *et al.*, 2000; Paterson *et al.*, 2006; Soupene *et al.*, 2008) and because Drs2 (the yeast homologue of ATP8A1) can translocate PS (Zhou and Graham, 2009), we reasoned that ATP8A1 is involved in the enrichment of PS in the cytosolic leaflet of RE membranes.

We first examined the subcellular localization of ATP8A1 in COS-1 cells. COS-1 cells are a cell line with distinctly separate organelles, therefore are suitable for the precise localization of endosomal proteins (Misaki *et al.*, 2007) (Figure 1A). ATP8A1 forms a heteromeric complex with CDC50A, a protein that is essential for the transport of newly synthesized ATP8A1 from the endoplasmic reticulum (ER) (van der Velden *et al.*, 2010). When co-expressed with CDC50A, ATP8A1 co-localized with the RE markers, Rab11 and TfnR, but did not co-localize with other organelle markers [TGN46 (trans-Golgi network), GM130 (cis-Golgi), VPS26 (EEs), CD63 (late endosomes), and LAMP2 (lysosomes)] (Figure 1B). These results showed that ATP8A1 is predominantly localized at REs.

We examined whether ATP8A1 is involved in two membrane transport pathways through REs: the recycling pathway that transports cargoes, such as Tfn/TfnR, from REs to the PM and the retrograde pathway that transports cargoes, such as cholera toxin B subunit (CTxB), from REs to the Golgi. Among three siRNAs designed against human ATP8A1 (siRNA1-3), siRNA1 was the most effective at suppressing ATP8A1 expression (Figure 2A), and was used for the following experiments. For the recycling assay, cells were first pulsed with Alexa 594-Tfn and then chased in media without Alexa 594-Tfn. During the chase, cells

were imaged every 6 min for 1 h. After the pulse, Alexa 594-Tfn accumulated at REs in both control and siRNA-treated cells. In control cells, the fluorescence intensity of Alexa 594-Tfn in REs decreased by 50% after a 30-min chase and decreased by 70% after a 50-min chase (Figures 2B and 2C). In contrast, in ATP8A1-depleted cells, the fluorescence intensity of Alexa 594-Tfn in REs decreased by 10% after a 30-min chase and decreased by 20% after a 50-min chase, suggesting that ATP8A1 is required for efficient recycling of Tfn from REs to the PM.

To determine whether ATP8A1 activity is required for Tfn recycling, we generated an activity-deficient ATP8A1 mutant. We mutated Glu191 in human ATP8A1 to Gln because the corresponding Glu198 in bovine ATP8A2, a paralogue of ATP8A1, was found to be essential for its ATPase and flippase activities (Coleman *et al.*, 2012a). The recombinant Glu191Gln (E191Q) protein complexed with CDC50A was purified to apparent homogeneity (Figure 3A). The purified wild-type (WT) ATP8A1 showed ATPase activity in a PS-concentration-dependent manner and flippase activity toward NBD-labeled PS and PE. In contrast, the E191Q mutant showed only marginal ATPase and flippase activity (Figures 3B and 3C). We then performed the rescue experiment by the expression of an siRNA-resistant WT or E191Q mutant ATP8A1. As shown in Figures 2B and 2D, Tfn recycling was restored by the expression of WT ATP8A1 (not significant versus "Control" in Figure 2C by Student's *t*-test), but not by the expression of E191Q mutant. These results suggest that Tfn recycling depend on the flippase activity of ATP8A1.

For the retrograde assay, cells were pulsed with Alexa 594-CTxB and then chased for 15 or 60 min. As was shown previously (Uchida *et al.*, 2011), CTxB reached REs after a 15-min chase (Figure 4A), and then reached the Golgi after a 60-min chase. In ATP8A1 knockdown cells, CTxB reached REs after a 15-min chase as in control cells, but CTxB did not reach the Golgi

after a 60-min chase. The colocalization between CTxB and GM130 after a 60-min chase was significantly lower in ATP8A1 knockdown cells than in control cells (Figure 4B). Thus, ATP8A1 is required for the retrograde traffic of CTxB from REs to the Golgi.

Epidermal growth factor (EGF) binds its receptor (EGFR) on the PM and undergoes endocytosis, followed by their endosomal traffic to lysosomes and degradation. This pathway does not pass through REs (Mellman, 1996). We asked if ATP8A1 knockdown affected this process. In control cells, EGF stimulation resulted in gradual disapperance of EGFR, showing EGF/EGFR complex is delivered to lysosomes and degraded. In contrast, in ATP8A1 knockdown cells, EGF stimulation did not affect EGFR expression level, showing that EGFR degradation is impaired (Figure 5). Interestingly, EGFR expression level immediately after EGF stimulation was two-fold higher than in control cells. This result indicates that either endosomal traffic to lysosomes or lysosomal degradation is impaired in ATP8A1-depleted cells. We therefore examined EGF traffic to lysosomes. Cells were pulsed with Alexa 555-EGF and chased. After a 60-min chase of EGF, a portion of EGF co-localized with LAMP1 in control and ATP8A1-depleted cells, and no significant difference in the Pearson's coefficient between LAMP1 and EGF was observed (Figures 6A and 6B). Thus, ATP8A1 knockdown does not affect EGF/EGFR traffic to lysosomes. Together, these data indicate that the degradation process inside the lysosomes is impaired by ATP8A1 knockdown.

Depletion of ATP8A1 induced the formation of aberrant endosomal tubules

Tfn is recycled as a complex with TfnR (Yamashiro *et al.*, 1984). The impaired recycling traffic of Tfn in ATP8A1-depleted cells led us to examine the steady-state distribution of TfnR. In control cells, an endogenous TfnR stain showed a bright perinuclear structure and a number of dispersed puncta, which correspond to REs and EEs, respectively (Misaki *et al.*, 2007)

(Figure 7A). In ATP8A1-depleted cells, although the RE localization of TfnR was basically maintained, TfnR also localized at an extensive array of fine tubules that emanate radially from REs. The tubules were even more obvious in the intensified image. The lengths of some TfnR-positive tubules exceeded 20 μm, nearly reaching the PM. The number of cells that had TfnR-positive tubules was reduced by the expression of the siRNA-resistant WT ATP8A1 but not by the expression of the siRNA-resistant E191Q mutant (Figure 7B). The treatment of ATP8A1-depleted cells with nocodazole, a microtubule-depolymerizing agent, disassembled the tubules, indicating that the emergence of TfnR-positive tubules depends on microtubules (Figure 8). The staining pattern of VPS26 (EEs), CD63 (late endosomes), or syntaxin 5 (Golgi) was indistinguishable between control and ATP8A1-depleted cells (Figure 7C), suggesting that this tubulation of membranes occurred specifically at REs.

The distribution of TfnR was examined in live cells with the expression of TfnR tagged with GFP. In control cells, TfnR-GFP was mainly found at the mixture of small vesicles and short tubules at REs (Figure 9A). In contrast, in ATP8A1-depleted cells, TfnR-GFP localized at a number of aberrant tubules, as expected from the observation with endogenous TfnR. Using time-lapse imaging, the dynamics of the TfnR tubules were monitored. The tubule marked with a red asterisk retracted over the time monitored and was finally absorbed into REs (Figure 9B). The tubule marked with a green asterisk remained associated with REs. At the time of 0.075 min, small stub with a black asterisk budded from the middle of the tubule but was absorbed by the time of 0.084 min. Few fission events that released TfnR-positive tubules from REs into the cytoplasm were observed.

Together, these results suggest that normal RE distribution of TfnR and Tfn recycling depend on the activity of ATP8A1.

The RE localization of EHD1 depends on ATP8A1

The emergence of aberrant TfnR-positive tubules that appear resistant to fission suggests that endosomal membrane fission machinery is dysfunctional in ATP8A1-depleted cells. EHD1 regulates membrane traffic through REs (Grant and Caplan, 2008) and facilitates tubulation or fission of liposomes that contain the anionic phospholipids, PS and phosphoinositides (PIPs) (Pant *et al.*, 2009). We therefore reasoned that ATP8A1 knockdown would impair EHD1 function.

We first examined the effect of EHD1 knockdown on membrane traffic through REs. siRNA1 designed against EHD1 effectively suppressed EHD1 expression (Figure 10A), and was used for the knockdown experiments. EHD1 knockdown significantly suppressed the recycling of Tfn from REs to the PM (Figures 10B and 10C) and the retrograde transport of CTxB from REs to the Golgi (Figures 10D and 10E). Strikingly, EHD1 knockdown, like ATP8A1 knockdown, resulted in the emergence of aberrant TfnR-positive tubules from REs (Figure 11A). Time-lapse images of EHD1-depleted cells that express TfnR-GFP also showed aberrant TfnR-positive tubules, which appear resistant to fission (Figure 11B). In summary, EHD1 knockdown phenocopied the ATP8A1 knockdown, in that both impair membrane traffic through REs and generate aberrant TfnR-positive tubules.

We next examined the effect of ATP8A1 knockdown on the subcellular localization of EHD1. Endogenous EHD1 mainly localized perinuclearly and co-localized with GFP-ATP8A1 (Figure 12A), confirming the RE localization of EHD1 as previously described (Lin *et al.*, 2001). In ATP8A1-depleted cells, EHD1 was not localized at the REs but diffusively distributed in the cytoplasm. The RE localization of EHD1 in ATP8A1-depleted cells was restored by the expression of siRNA-resistant WT ATP8A1, but not by the expression of siRNA-resistant E191Q mutant (Figure 12B). These results suggest that the

activity of ATP8A1 is required for the RE localization of EHD1.

Cellular PS level is critical for EHD1 localization

We reasoned that EHD1 is recruited to REs by phospholipids that are flipped by ATP8A1. The ATPase activity of ATP8A1 is most strongly activated by PS among the various phospholipids [(Ding *et al.*, 2000) and Figure 3B]. ATP8A1 is suggested to flip PS in isolated chromaffin granules (Zachowski *et al.*, 1989; Tang *et al.*, 1996), whereas flipping of PE, but not PS, at the PM is impaired in ATP8A1-depleted cells (Kato *et al.*, 2012). Consistent with these reports, we showed that ATP8A1 could flip NBD-labeled PS and PE in a reconstituted system (Figure 3C).

We therefore examined whether the cellular levels of PS and/or PE affect EHD1 localization. In mammals, PS is synthesized by two distinct base-exchange enzymes, PS synthase-I (PSS-I) and PS synthase-II (PSS-II) (Kuge and Nishijima, 1997). PSS-I exchanges serine for the choline of PC, whereas PSS-II exchanges serine for the ethanolamine (Etn) of PE (Figure 13B). A PS-auxotrophic mutant of CHO cells, PSA-3, lacks the activity of PSS-I (Kuge *et al.*, 1986). When PSA-3 cells are cultured with dialyzed fetal bovine serum (FBS), which is depleted of Etn, cellular levels of PS and PE decrease by around 30% compared to PSA-3 cells maintained in FBS plus Etn (Lee *et al.*, 2012). Under these conditions, the cytoplasmic PS levels drastically decrease, in particular, in REs (Lee *et al.*, 2012). EHD1 localization was examined in PSA-3 cells under PS/PE-deficient or -rich conditions. Since anti-EHD1 antibody, which was used to stain EHD1 in COS-1 cells, did not stain endogenous EHD1 in CHO cells, GFP-EHD1 was transiently expressed. Unlike in COS-1 cells, GFP-EHD1 expressed in PSA-3 cells under PS/PE-rich conditions localized at discrete tubular compartments as well as perinuclear REs (Figure 13C). In CHO cells, from which PSA-3 cells

were derived, EHD1 localized at perinuclear REs and some peripheral tubules (Lin *et al.*, 2001). The tubular compartments resemble "EHD1-positive tubular compartments" that were observed in HeLa cells (Caplan *et al.*, 2002; Sharma *et al.*, 2009). The EHD1-positive tubular compartments are suggested to be a way station for recycling cargoes from the perinuclear REs to the PM (Grant and Donaldson, 2009). The different morphologies of EHD1-positive compartments among cell lines may be due to how cells develop post-RE compartments. Despite these differences, under PS/PE-deficient conditions, GFP-EHD1 was evenly distributed into the cytoplasm and not localized to the tubules and perinuclear REs (Figure 13C). These results suggest that the decrease of PS and/or PE levels in cells influences the membrane localization of EHD1.

To dissect the roles of PS and PE in EHD1 localization, another CHO mutant cell line (R-41) was exploited. R-41 cells are defective in the synthesis of PE from PS (Emoto and Umeda, 2000). When R-41 cells are cultured with dialyzed FBS, in a condition where PE synthesis from Etn is suppressed, PE levels decrease by around 40%, whereas PS levels are unchanged (Emoto and Umeda, 2000; Lee *et al.*, 2012). Between the two culture conditions: dialyzed FBS plus Etn (PS/PE-rich) or dialyzed FBS only (PS-rich, PE-deficient), the tubular and perinuclear RE localization of EHD1 was indistinguishable (Figure 13D). These results suggest that PE is not an essential determinant for the membrane localization of EHD1. These findings, together with the findings utilizing PSA-3 cells, indicate that PS is critical for the membrane localization of EHD1.

EHD1 binds PS-containing liposomes

To clarify the roles of PS in EHD1 binding, we performed the binding assay with PC-based liposomes that contain different amounts of PS. In brief, His-tagged EHD1 was mixed with

liposomes for 15 min, and the mixture was spun at 100,000 *g* for 30 min. The resultant supernatant and pellet were subjected to SDS-PAGE and the gels were then stained with Coomassie blue for the presence of EHD1 and phospholipids (Boucrot *et al.*, 2012). The recombinant EHD1 did not bind to liposomes that do not contain PS (Figures 14A and 14B). When the PS concentration in liposomes increased, the binding of EHD1 increased sigmoidally (Figure 14C). A sharp increase of the binding of EHD1 was observed from 40 to 70% PS with half-maximal binding at 53% PS. EHD1 did not show significant binding to liposomes that contain 60% PE.

We next examined the effect of liposome curvature on EHD1 binding. Liposomes with three different diameters were prepared (Figure 15). As shown in Figure 16, when the curvature of liposomes increased (*i.e.*, the diameter decreased), more EHD1 bound to liposomes at three PS concentrations examined (20%, 40%, and 60%). These results show that the membrane curvature influences the EHD1 binding to the membranes. The PS concentration required for EHD1 binding to membranes could be lowered by the membrane curvature.

We then aimed to indentify the amino acid residues in EHD1 responsible for its binding to PS. Members in EHD family contain an amino-terminal ATP-binding G-domain, followed by a helical domain and a carboxy-terminal EH-domain [(Grant and Caplan, 2008) and Figure 17A]. EH domain interacts with NPF motif-containing proteins, such as MICAL-L1 (Sharma *et al.*, 2009) and Pacsin (Giridharan *et al.*, 2013), and this interaction is required for the recruitment of EHD1 to tubular endosomal membrane (Sharma *et al.*, 2009). The central helical domain contains a polybasic region and is proposed to interact with membrane based on the crystal structure of EHD2 (Daumke *et al.*, 2007), a paralogue of EHD1. Mutations in this region of EHD2 (F322A, K324D, K327D, K328D, or K329D) results in the loss of

membrane localization of EHD2, showing the critical role of these amino acids in the helical domain in membrane localization of EHD2 (Daumke *et al.*, 2007). We mutated the corresponding residues in EHD1 and examined the localizations of the mutants. In COS-1 cells, GFP-EHD1 (WT) localized at perinuclear REs and cytoplasmic tubular structures (Figure 17B). EHD1 (F322A, K324A, or K324D) lost its membrane localization in COS-1 cells similarly to EHD2 mutants (Figure 17B). In contrast, EHD1 that lacks EH-domain (ΔEH) still localized at perinuclear REs, but not at tubular endosomal membrane (Figure 17B), consistent with the previous report (Caplan *et al.*, 2002). We then asked if these mutants could bind to PS-liposomes *in vitro*. EHD1 (K324D) did not bind to PS-liposomes (Figure 18), whereas EHD1 (ΔEH) mutant bound to PS-liposomes *in vitro* (Figure 18). Together, these data suggest that the polybasic region in the central helical domain of EHD1 binds to PS-containing RE membrane.

EHD1 is shown to bind to liposomes that contain 10% of PIPs and 10% PS (Blume *et al.*, 2007; Pant *et al.*, 2009). As Blume *et al.* reported, EHD1 bound to liposomes [35% PC, 35% PE, 10% PS, 10% cholesterol, and 10% PI(4)P or PI(4,5)P_2] under our experimental conditions (Figure 19). We also examined the binding of EHD1 to liposomes that lack PS [45% PC, 35% PE, 0% PS, 10% cholesterol, and 10% PI(4)P or PI(4,5)P_2] and found that EHD1 did not bind to these membranes (Figure 19). These results indicate that PS is required for EHD1 binding to the membranes. Given that EHD1 did not bind to liposomes with a low concentration of PS in the absence of PIPs (Figure 14), other lipid factors may affect the EHD1 binding to the membrane containing PS. As has been reported (Behnia and Munro, 2005; Uchida *et al.*, 2011), PIPs were not identified in the RE membrane (Figure 20), which suggests that the contribution of PIPs for the EHD1 recruitment to the RE membranes may be minimal.

ATP8A1 knockdown increases PS levels in the luminal leaflet of RE membranes

We hypothesized that ATP8A1 controls the transbilayer distribution of PS in RE membranes. To test this hypothesis, we generated a new PS-specific probe based on the PH domain of evectin-2. The PH domain of evectin-2 specifically binds PS, but with low affinity (Uchida *et al.*, 2011). Two evectin-2 PH domains were tandemly connected, hereafter named 2xPH, expressed in *Escherichia coli* and purified. The recombinant 2xPH retained the PS specificity and had a high affinity for PS (Figure 21A). More than 95% of 2xPH was sedimented with liposomes containing 5% PS, whereas about only 40% of single PH was sedimented with liposomes containing even 20% PS (Uchida *et al.*, 2011). Recombinant 2xPH was then applied to cells that had been fixed and treated with 0.1% saponin. Saponin was used to permeabilize RE membranes based on the observations that RE membranes are enriched in cholesterol (Gagescu *et al.*, 2000; Mondal *et al.*, 2009). Under these conditions, the antibody that recognizes the luminal part of TfnR stained REs, ensuring the permeabilization of RE membranes (Figure 22A). 2xPH primarily stained a perinuclear area that co-localized with Tfn (Figure 21B), indicating that RE membranes are enriched in PS compared with other intracellular organelles. It should be noted that the stain of 2xPH under these conditions reflects the presence of PS in both the cytosolic and luminal leaflets of the membranes.

The C2 domain of lactadherin tagged with GFP (GFP-lact-C2) is a widely used PS probe (Yeung *et al.*, 2008). GFP-lact-C2 that is expressed in the cytosol binds PS in the cytosolic leaflet, but not in the luminal leaflet of the membranes. As was shown previously (Uchida *et al.*, 2011), the cytosolic expression of GFP-lact-C2 strongly stained REs (Figure 21C), indicating that the cytosolic leaflet of the RE membranes is the most enriched with PS.

Using these two PS-specific probes, we examined how PS is distributed between the two leaflets of the RE membranes. Cells were transiently transfected with the GFP-lact-C2 plasmid,

then fixed with PFA, permeabilized with saponin, washed, and stained by 2xPH. The image (Figure 23A) showed two representative cells, one that did not express GFP-lact-C2 in the cytosol (indicated by arrowhead) and the other that expressed it (indicated by asterisk). In a cell that did not express GFP-lact-C2, the post-stain by 2xPH clearly visualized REs, as shown above (Figure 23A). In contrast, in a cell that expressed GFP-lact-C2, the post-stain by 2xPH was much fainter than that in a cell that did not express GFP-lact-C2. The intensity of 2xPH at REs in cells with GFP-lact-C2 expression decreased to about 30% of that in cells without GFP-lact-C2 expression (Figure 23B). As mentioned above, GFP-lact-C2, which is expressed in the cytosol, binds PS in the cytosolic leaflet, but not in the luminal leaflet of the membranes. Given that GFP-lact-C2 in the cytosol masks all of the PS in the cytosolic leaflet of the membranes in fixed cells and the fluorescence of the post-stain with 2xPH represents the amount of unmasked PS in the luminal leaflet (Figure 22B), this result suggests that 70% of the PS in REs localized in the cytosolic leaflet and the other 30% localized in the luminal leaflet. If any PS in the cytosolic leaflet could not be masked by GFP-lact-C2 and was post-stained by 2xPH, the amount of PS in the cytosolic leaflet should be higher than 70%.

We then examined if ATP8A1 depletion affects the post-stain by 2xPH at REs. In this experiment, we used COS-1 cells that stably express GFP-lact-C2 in the cytosol. The stain of 2xPH at REs in control cells was as faint as the stain in cells that transiently expressed GFP-lact-C2 (Figure 23C), whereas the stain of 2xPH in ATP8A1-depleted cells was bright with 1.5-fold increase in the fluorescence intensity (Figures 23C and 23D). The ratio of the fluorescence intensity of 2xPH to that of GFP-lact-C2 was significantly higher in ATP8A1-depleted cells than in control cells (Figure 23E). This result suggests that ATP8A1 flips PS from the luminal to the cytosolic leaflet in RE membranes. On the other hand, EHD1 knockdown did not increase the intensity of post-stain of 2xPH, suggesting that EHD1 did not

affect the transbilayer distribution of PS in REs.

ATP8A2 can compensate for the loss of ATP8A1

ATP8A2, a tissue-specific paralogue of ATP8A1, is highly expressed in brain, testis, and retina (Zhu *et al.*, 2012). ATP8A2 is the causative gene of wabbler-lethal mice, which show axonal degeneration (Zhu *et al.*, 2012). A missense variant of the human ATP8A2 (Ile376Met) has recently been shown to be associated with neurodegenerative disease CAMRQ (Emre Onat *et al.*, 2012). Single knockout mice of either ATP8A1 or ATP8A2 are viable, but double knockout mice of ATP8A1 and ATP8A2 are neonatal lethal (Zhu *et al.*, 2012), suggesting that the two flippases have redundant roles. We reasoned that ATP8A2, like ATP8A1, is a regulator of endosomal membrane traffic, and examined if ATP8A2 can compensate for the loss of ATP8A1 in COS-1 cells in which no ATP8A2 expression is observed.

GFP-ATP8A2 co-expressed with myc-CDC50A co-localized with TfnR in the perinuclear region (Figure 24), showing the RE localization of ATP8A2. Like bovine ATP8A2 (Coleman *et al.*, 2009), the recombinant human ATP8A2 displayed ATPase activity that was induced by PS (Figure 25A) and flippase activity toward NBD-labeled PS (Figure 25B). In contrast, the disease-associated I376M ATP8A2 mutant displayed little ATPase or flippase activity, consistent with the previous study using the corresponding bovine mutant (I364M) (Vestergaard *et al.*, 2014). We confirmed that ATP8A2 (I376M) localized at REs in a CDC50A-dependent manner like WT ATP8A2 (Figure 25C). We then performed a rescue experiment in ATP8A1-depleted cells with the expression of three types of human ATP8A2 (WT, I376M, and E210Q), the latter corresponding to flippase-deficient E198Q of bovine ATP8A2. Only the WT restored EHD1 localization to REs and resulted in the disappearance of aberrant TfnR-positive tubules (Figures 25D and 25E). Thus, ATP8A2 can compensate for

the loss of ATP8A1 function in a flippase-dependent manner, suggesting that ATP8A2 has a role in the transbilayer distribution of PS in RE membranes.

We then asked whether endogenous ATP8A2 participates in the localization of recycling cargoes such as TfnR. Since ATP8A2 is not ubiquitously expressed (Coleman *et al.*, 2009) but expressed in brain (Zhu *et al.*, 2012), we used primary cortical neurons that were prepared from *Atp8a2*$^{+/+}$ or *Atp8a2*$^{-/-}$ mice (Coleman *et al.*, 2014). Although the total amounts of TfnR in *Atp8a2*$^{-/-}$ and *Atp8a2*$^{+/+}$ neurons were comparable, the ratio of surface TfnR to total TfnR was dramatically reduced in *Atp8a2*$^{-/-}$ neurons (Figures 26A and 26B). These results imply that ATP8A2 functions in recycling membrane traffic to the PM in neurons.

Discussion

In the present study, we showed in mammalian cells that a P_4-ATPase ATP8A1 translocates PS from the luminal leaflet to the cytosolic leaflet in the RE membranes. We also found that a membrane fission protein EHD1, which binds PS *in vitro*, is dispersed from REs to the cytoplasm by ATP8A1 depletion. Cells depleted of ATP8A1 generate extensively tubulated membranes from REs, which appear resistant to fission. Cells depleted of EHD1 also generate tubular membranes from REs. From these results, we propose that the flipping of PS to the cytosolic leaflet by a P_4-ATPase is critical for recruiting a membrane fission protein to the RE membranes and generating transport vesicles from REs. Studies in *Saccharomyces cerevisiae* have shown that a P_4-ATPase Drs2, which flips PS and to a lesser extent PE, is essential for membrane traffic between the late Golgi compartment and endosomes (Sebastian *et al.*, 2012) and that other P_4-ATPases Dnf1 and Dnf2, which flip phospholipids such as PC and PE, participate in endocytic vesicle formation (Pomorski *et al.*, 2003). In *Caenorhabditis elegans*, a P_4-ATPase TAT-1 is most closely related to mammalian ATP8A1. The loss of *tat-1* leads to the generation of abnormal endo-lysosomal compartments, suggesting that endocytic cargo sorting and recycling are impaired (Ruaud *et al.*, 2009; Chen *et al.*, 2010). In *Arabidopsis thaliana*, a P_4-ATPase ALA3 that localizes at the Golgi, is essential for the formation of secretory vesicles (Poulsen *et al.*, 2008). Thus, these studies, together with the present finding in mammalian cells, indicate that the function of phospholipid flippases in vesicular membrane traffic is evolutionally conserved from lower eukaryotes to mammals. In the *C. elegans tat-1* mutant, an EHD1 homologue, RME-1, still localized at abnormal endo-lysosomes (Chen *et al.*, 2010). We cannot explain at the present time the apparent discrepancy on the localization of EHD1/RME-1 in the loss of ATP8A1/TAT-1.

One hypothesis for the mechanism by which P_4-ATPases function in the vesicular

membrane traffic is that P_4-ATPases generate membrane curvature through unidirectional movement of phospholipids, which facilitates membrane budding to form transport vesicles together with budding machinery (Sebastian *et al.*, 2012). Recently, *S. cerevisiae* Gcs1, which is an Arf GTPase-activating protein and regulates retrograde transport from endosomes to the late Golgi, was shown to have an +ALPS-motif that binds highly curved membranes with anionic phospholipids including PS (Xu *et al.*, 2013). Importantly, the localization of Gcs1 to the late Golgi/endosomes is regulated by Drs2. Our study in mammalian cells provides another mechanistic insight into the site of action of P_4-ATPases: PS flipped by ATP8A1 to the cytosolic leaflet recruits a membrane fission protein EHD1 that have an ability to bind PS, thereby regulating the membrane fission process.

PS comprises 5 - 10% of the total phospholipids in mammalian cells (Vance and Steenbergen, 2005; Lee *et al.*, 2012). PS is 5-fold more abundant in the purified RE fraction than in the cell lysate (Gagescu *et al.*, 2000), suggesting that PS accounts for 25 - 50% of the total phospholipids in RE membranes. Using two PS-specific probes, we roughly estimated that 70% and 30% of PS are localized in the cytosolic and the luminal leaflets of RE membranes, respectively. Taking this PS asymmetry into consideration, PS may comprise 35% (= 25 x 2 x 0.7) - 70% (= 50 x 2 x 0.7) of total phospholipids in the cytosolic leaflet of RE membranes. PS is also enriched and asymmetrically distributed in the cytosolic leaflet of the PM (Devaux, 1992). A study using red blood cells, which lack intracellular organelles, showed that PS comprises 15% of total phospholipids (Dodge and Phillips, 1967), suggesting that PS comprises about 30% of the total phospholipids in the cytosolic leaflet of the PM. Therefore, the abundance of PS in the cytosolic leaflet of RE membranes could be comparable or even higher than that in the PM. This high concentration of PS in the cytosolic leaflet of RE membranes, which is produced by a P_4-ATPase ATP8A1, might be enough to recruit EHD1

specifically to REs, since the half-maximal binding of EHD1 to PS was observed at ~50% PS in the liposome co-sedimentation assay.

Despite the increase of PS in the luminal leaflet in ATP8A1-depleted cells, PS was still present in the cytosolic leaflet of RE membranes as the PS-probe expressed in the cytosol significantly stained REs in ATP8A1-depleted cells. The presence of PS in the cytosolic leaflet of RE membranes was also supported by the observation that evectin-2, a protein that requires PS in the cytosolic leaflet for its RE localization (Uchida *et al.*, 2011), localized at REs in ATP8A1-depleted cells (Figure 27). Thus, although ATP8A1 contributes to the enrichment of PS in the cytosolic leaflet of RE membranes, its knockdown does not result in the complete disappearance of PS at the cytosolic leaflet. Scramblases that mediate bidirectional transbilayer movement of phospholipids or other P_4-ATPases may participate in the exposure of PS at the cytosolic leaflet in ATP8A1-depleted cells. PS is synthesized in the cytosolic leaflet of the ER and likely moves via non-vesicular routes (Maeda *et al.*, 2013) and/or vesicular routes to the PM and other organelles. These processes (Holthuis and Menon, 2014) may also contribute to the localization of PS in the cytosolic leaflet of the RE membranes.

Recent studies have revealed the significance of PS in the cytosolic leaflet of endosomes [reviewed in (Bigay and Antonny, 2012; Holthuis and Menon, 2014)]. As mentioned above, *S. cerevisiae* Gcs1 has a +ALPS-motif that binds highly curved membranes with anionic phospholipids that include PS (Xu *et al.*, 2013). Gcs1 localizes at the late Golgi/endosomes and its localization is regulated by a P_4-ATPase Drs2, suggesting that PS flipped by Drs2 is essential for Gcs1 recruitment. K-Ras is a major proto-oncogene product (Hancock, 2003). K-Ras mainly localizes at the PM, but some of it localizes at endosomes (Lu *et al.*, 2009; Gelabert-Baldrich *et al.*, 2014). K-Ras has a polybasic stretch at the carboxy-terminus that electrostatically interacts with PS (Yeung *et al.*, 2008). This interaction was recently shown to

be essential for K-Ras trapping at REs, from where vesicular transport delivers K-Ras to the PM (Schmick *et al.*, 2014). Evectin-2 that localizes at REs has a PH domain that binds PS but not PIPs. This PH domain is essential for the RE localization of evectin-2 and the regulation of the retrograde traffic from REs to the Golgi (Uchida *et al.*, 2011). These findings, together with the present study, indicate that PS in REs has multifaceted functions in membrane traffic, such as cargo collection, membrane budding, and membrane fission. Other membrane systems, including PM, EEs, LEs, and the Golgi, exploit PIPs to exert their own functions through a variety of PIP effectors (Behnia and Munro, 2005; Di Paolo and De Camilli, 2006). Importantly, the levels of PIPs are tightly regulated by phosphorylation and dephosphorylation. As far as we know, the metabolic conversion of PS is confined to the ER and/or mitochondria in mammalian cells (Vance and Steenbergen, 2005). Therefore, in other cellular membranes, cells may exploit the lipid flipping system by P_4-ATPases to regulate the local levels of cytosolic PS. It is also noted that Drs2p in the trans-Golgi network is activated by PI(4)P (Natarajan *et al.*, 2009), suggesting that PIPs could control the PS content in the cytosolic leaflet of the membranes.

ATP8A2 is a paralogue of ATP8A1 and mutations of ATP8A2 cause axonal degeneration in mice (wabbler-lethal mice) (Zhu *et al.*, 2012) and are associated with neurodegenerative disease CAMRQ in humans (Cacciagli *et al.*, 2010; Emre Onat *et al.*, 2012). However, cellular mechanism of ATP8A2 has been unclear. The present study showed that ATP8A2 localized at REs and could compensate for the loss of ATP8A1 when expressed in COS-1 cells. In contrast, the disease-causative human ATP8A2 mutant (I376M), which lacked ATPase and flippase activities, could not rescue the loss of ATP8A1. Furthermore, primary cortical neurons isolated from *Atp8a2*$^{-/-}$ mice showed a drastic reduction of TfnR present in the PM, with the total amount of TfnR being comparable to that in primary cortical neurons

isolated from *Atp8a2*$^{+/+}$ mice. These findings suggest that ATP8A2 functions in endosomal membrane traffic through REs. Of note, ATP8A1 protein was expessed in both *Atp8a2*$^{+/+}$ and *Atp8a2*$^{-/-}$ neuron culture (Figure 26C). It is currently unknown whether ATP8A1 and ATP8A2 are expressed in the same type of neuron, or ATP8A1 and ATP8A2 is expressed in different types of neurons. If the former is correct, it is tempting to speculate that ATP8A1 and ATP8A2 plays a role in the different type of REs and thereby ATP8A1 cannot compensate for the loss of ATP8A2. Indeed, REs in neurons are proposed to localize seperately at axon, dendrite and cell soma (Sann *et al.*, 2009).

ATP8A2 in photoreceptor cells is localized both in the perinuclear compartment of the inner segment and in the disc membranes of the outer segment (Coleman *et al.*, 2009, 2014). ATP8A2 in the perinuclear compartment may produce the lipid asymmetry in the bilayer to facilitate vesicle trafficking to the disc membrane, which could explain a drastic reduction in the length of outer segments in ATP8A2-deficient mice (Coleman *et al.*, 2014). Since disc membranes have a symmetrical distribution of PE and PS (Hessel *et al.*, 2000), presumably due to significant scramblase activity of rhodopsin (Menon *et al.*, 2011), ATP8A2 does not appear to play a role in generating global lipid asymmetry in disc membranes. Some portion of ATP8A2 may be transported to the disc membranes as a result of vesicle trafficking from the perinuclear compartment.

Mutations in the very low-density lipoprotein receptor (VLDLR) is also linked to CAMRQ (Ozcelik *et al.*, 2008; Turkmen *et al.*, 2008). VLDLR is a receptor for Reelin, an extracellular protein that guides neuronal migration in the cerebral cortex and cerebellum (Herz and Chen, 2006) and circulates between the PM and endosomes by endocytic recycling (Duit *et al.*, 2010). Therefore, recycling traffic of VLDLR to the PM might be impaired in neurons without functional ATP8A2. Available antibodies failed to detect VLDLR protein in

primary cortical neurons used in this study. Further studies are required to address this issue.

Chapter 2

Phosphorylation of ATP8A1 regulates its activity and membrane traffic through recycling endosomes

Introduction

Recycling endosomes (REs) function mainly in two intracellular membrane traffic pathways: recycling traffic from REs to the plasma membrane (PM) and retrograde traffic from the PM to the Golgi (Taguchi, 2013). Although recycling traffic of transferrin (Tfn) from REs is regarded constitutively active under basal culture condition (Maxfield and McGraw, 2004), its kinetics might be affected by growth factors (Davis and Czech, 1986) or serum/amino acid starvation (Puri *et al.*, 2013). Furthermore, recycling traffic of cell adhesion molecules or receptors (e.g., β1-integrin and AMPA receptor) from REs is activated by extracellular signals (Park *et al.*, 2004; Powelka *et al.*, 2004). Despite these findings, how signaling pathways ultimately target the machinery molecules in REs is not fully understood.

In chapter 1, we reported that a phospholipid flippase ATP8A1 regulates recycling traffic by recruiting a membrane fission protein EHD1 (Lee *et al.*, 2015). ATP8A1 regulates the transbilayer asymmetry of PS in RE membranes, and EHD1 is recruited to RE membrane by binding to PS on the cytoplasmic leaflet, thereby mediating membrane fission process. Because membrane fission is the rate-limiting step in membrane carrier biogenesis (Anitei and Hoflack, 2012), we hypothesized that ATP8A1 activity and EHD1 recruitment could be tightly regulated by an upstream signaling pathway. In yeast, flippase activity is activated by interaction with small GTPase proteins and phosphatidylinositol-4-phosphate (PI4P) (Natarajan *et al.*, 2009), or by kinase-mediated phosphorylation (Nakano *et al.*, 2008; Roelants *et al.*, 2010). However, how the activities of mammalian flippases are regulated is completely

unknown.

Here we show that ATP8A1 activity and membrane traffic at REs is regulated by kinase-mediated phosphorylation. We found that ATP8A1 was phosphorylated at Ser25/Ser29 in the N terminus, and mutation of these serines inhibited ATP8A1 activity *in vitro* and impaired its function at REs. We also show that inhibition of casein kinase 1 (CK1) by a specific inhibitor decreased phosphorylation of ATP8A1. Furthermore, knockdown of casein kinase 1α (CK1α) decreased the amount of cytosolic PS at REs and resulted in aberrant Tfn receptor (TfnR) tubular localization. Thus, we propose that kinase-mediated phosphorylation and activation of ATP8A1 regulates membrane traffic through REs.

Results

Phosphoproteome studies have indentified multiple phosphorylation sites in mice ATP8A1 (Munton *et al.*, 2007; Villen *et al.*, 2007; Trost *et al.*, 2009). Since these sites are conserved in human ATP8A1, we first examined whether ATP8A1 is phosphorylated in cultured human cells. HEK293A cells were transiently transfected with GFP-ATP8A1 (WT) and myc-CDC50A and labeled with [^{32}P]orthophosphate for 4 h. GFP-ATP8A1 was immunoprecipitated with anti-GFP antibody. Westernblotting with anti-ATP8A1 antibody and autoradiography detected ^{32}P-labeled protein band co-migrating with the GFP-ATP8A1 (WT) (Figure 28A). This result indicates that ATP8A1 is phosphorylated in cells cultured under basal condition.

We then aimed to indentify the phosphorylated site. Of the phosphorylated sites reported in the previous studies (Munton *et al.*, 2007; Villen *et al.*, 2007; Trost *et al.*, 2009) and in PhosphoSitePlus database (http://www.phosphosite.org/), phosphorylations at Ser25 and Ser29 were most frequently detected. Furthermore, NetPhos 2.0 (Blom *et al.*, 1999) revealed that

Ser25 and Ser29 were the most likely phosphorylated sites (Table 1). We therefore mutated these Ser to Ala and examined the phosphorylation level. ^{32}P labeling in GFP-ATP8A1 was reduced by 20% in S25A mutant, and by 60% in S29A and S25A/S29A mutants (Figures 28A and 28B). The ^{32}P incorporation was not completely abolished in these mutants, suggesting that there remain other phosphorylated sites. Together, Ser25 and/or Ser29 is one of the phosphorylated sites in ATP8A1.

P-type ATPases including flippases undergo phosphorylation at a conserved Asp residue during the catalytic cycle, hence the designation of "P"-type (Lopez-Marques *et al.*, 2011). Mutation in DGET motif of ATP8A2 (E198Q) was shown to block the dephosphorylation step of the Asp (Coleman *et al.*, 2012a). We obsereved no difference in the ^{32}P incorporation level of the DGET motif mutant ATP8A1 (E191Q) and wild-type (Figure 29), indicating that ^{32}P incorporation obsereved in our experiments does not reflect the phosphorylation at the conserved Asp.

We then developed an anti-phosphospecific antiserum against phospho-Ser29 in ATP8A1. We validated the antisera by immunoblot for GFP-ATP8A1 (WT or S29A) expressed in HEK293A cells. The antisera detected about 130 kDa band corresponding to the band detected by anti-ATP8A1 in GFP-ATP8A1 (WT) expressing cells, but not in GFP-ATP8A1 (S29A) cells (Figure 30). Strikingly, phosphatase treatment abolished tha band in cells expressing GFP-ATP8A1 (WT). Thus, we conclude that this antiserum detects phosphorylated Ser29 in ATP8A1.

We then examined whether the phosphorylation at S25/S29 modulates the activity of ATP8A1. ATP8A1 mutant (S29A or S25A/S29A) with CDC50A was expressed in HEK293 cells and purified. ATP hydrolysis by purified ATP8A1 was measured in the presence of PC and varying concentrations of PS. ATP8A1 (WT) displayed ATPase activity in a

PS-concentration-dependent manner (Figure 31A), and had a V_{max} of 160 ± 26 μmol/min/mg, which is comparable with the V_{max} value for purified ATP8A2 (Vestergaard *et al.*, 2014). Both S29A and S25A/S29A mutants showed ATPase activity in a PS-concentration-dependent manner, but the V_{max} was reduced by around 40% compared with wild-type (117 ± 23 μmol/min/mg for S29A, and 115 ± 26 μmol/min/mg for S25A/S29A). Furthermore, the PS concentration at half V_{max} ($K_{0.5}$) was significantly increased in S25A/S29A (Figure 31B), showing reduction of the apparent affinity for PS. These data indicate that phosphorylation at Ser25 and Ser29 modulates ATP8A1 activity.

We then asked if phosphorylation at S25/S29 of ATP8A1 is required for its function at REs. ATP8A1 (S25A, S29A or S25A/S29A) localized at REs, indicating that ATP8A1 phosphorylation is not required for its exit from ER and localization to REs (Figure 32). Then, rescue experiments for ATP8A1 knockdown with these mutants were performed. The number of cells with aberrant TfnR-tubulation was reduced by WT ATP8A1, but not by S29A and S25A/S29A mutants (Figure 33). Of note, a phosphomimetic mutant ATP8A1 (S25D/S29D) did not rescue ATP8A1 knockdown (Figure 33), despite its normal localization to REs (Figure 32). This may result from the fact that the negative charge of phosphomimetic residues does not match that of phosphorylated serine (Dephoure *et al.*, 2013), or may imply that a phosphorylation/dephosphorylation cycle is indispensable for ATP8A1 function in cells. Together, these results suggest that phosphorylation at S25/S29 are required for the role of ATP8A1 in recycling traffic.

What is the kinase responsible for phosphorylation of ATP8A1? We noted that Ser29 in ATP8A1 lies in a D/E-X-X-S/T motif, which is a non-canonical sequence recognized by casein kinase 1 (CK1) (Pulgar *et al.*, 1999). CK1 is a family of evolutionally conserved Ser/Thr kinases that are involved in various biological processes such as circadian rhythms,

Wnt pathway and cancer (Knippschild *et al.*, 2005; Cheong and Virshup, 2011). To examine whether CK1 is involved in phosphorylation of ATP8A1, we utilized a CK1-selective inhibitor D4476 (Rena *et al.*, 2004). HEK293A cells expressing GFP-ATP8A1 and myc-CDC50A were treated with 25 or 100 μM D4476 for 1 h, and then labeled with [^{32}P]orthophosphate for 4 h in the presence of D4476. ^{32}P incorporation into GFP-ATP8A1 was decreased by around 50% by 100 μM D4476 treatment (Figure 34), suggesting that CK1 is involved in phosphorylation of ATP8A1. Furthermore, D4476 treatment resulted in emergence of aberrant TfnR tubules (Figure 35), similar to ATP8A1 knockdown (Figure 7). Together, CK1 family kinases are likely candidates for phosphorylation and proper function of ATP8A1.

We then aimed to determine which of CK1 family members is responsible for phosphorylation of ATP8A1. Human CK1 family consists of 6 isoforms: α, β, γ1-3, δ and ε (Knippschild *et al.*, 2005). We depleted each isoform using specific siRNA and examined its effect on TfnR localization in COS-1 cells. We found that CK1α RNAi resulted in aberrant TfnR tubule formation (Figure 36), which phenocopied ATP8A1 or EHD1 knockdown (Figures 7A and 11A). CK1γ1, CK1γ2 or CK1γ3 RNAi did not affect TfnR localization. Interestingly, CK1δ or CK1ε RNAi resulted in dispersal of TfnR from perinuclear region (Figure 36). CK1δ and CK1ε are known to regulate endocytosis of Tfn from the PM (Peng *et al.*, 2015). Thus, TfnR dispersal upon CK1δ or CK1ε RNAi might be attributed to impaired traffic of Tfn/TfnR complex from the PM to REs. To confirm the effect of CK1α RNAi on TfnR localization, we used 4 more siRNAs against CK1α. The genome of primates has CK1α-like as well as CK1α, and they share high homology (84% homology in coding region DNA sequence). CK1α siRNA1, 2 and CK1α siRNA3, 4 were designed based on mRNA sequence of CK1α and CK1α-like, respectively (see Materials and Methods). Due to the sequence homology, the siRNAs against CK1α (siRNA1 and 2) have only 1 or 2 mismatches

to CK1α-like mRNA, and vice versa. Therefore, we used CK1α siRNA1-4 for knockdown of both CK1α and CK1α-like, and we hereafter refer to CK1α and CK1α-like as "CK1α". CK1α siRNA1, 2 and 4 effectively depleted CK1α protein expression and resulted in TfnR tubule formation, ruling out the possibility of off-target effects (Figure 37). The staining pattern of VPS26 (early endosomes) or syntaxin 5 (Golgi) was indistinguishable between control and CK1α-depleted cells (Figure 38), suggesting that CK1α knockdown does not affect the membrane traffic through these organelles. Taken together, CK1α is the best candidate for the kinase responsible for ATP8A1 phosphorylation and activation.

We then asked if CK1α knockdown affects PS localization on the cytoplasmic leaflet of REs. To do this, we developed a PS-specific probe based on EHD1. As shown in chapter 1, the helical domain of EHD1 is indispensable for EHD1 binding to PS, whereas EH domain is dispensable (Figure 18). The mutant EHD1 (ΔEH) retained its binding specificity to PS *in vitro* (Figure 39A), and its membrane localization was completely abolished in PSA-3 cells cultured in PS-depleting condition (Figure 40). When the PS concentration in liposomes increased, the binding of EHD1 (ΔEH) increased sigmoidally (Figure 39B), as shown for WT EHD1 (Figure 14C). Of note, the half-maximal binding of EHD1 (ΔEH) to PS was observed at 74% PS, whereas that of WT EHD1 was observed at 58% PS. Thus, the concentration range in which EHD1 (ΔEH) can sense decrease of PS would be higher than that of WT EHD1. To examine the effect of CK1α knockdown on the localization of EHD1 (ΔEH), we established COS-1 cells stably expressing GFP-EHD1 (ΔEH). In control cells, GFP-EHD1 (ΔEH) localized specifically at perinuclear REs with TfnR, showing GFP-EHD1 (ΔEH) labeled PS in the cytoplasmic leaflet in REs. In contrast, in ATP8A1-depleted cells, GFP-EHD1 (ΔEH) lost RE localization and was dispersed throughout the cytoplasm (Figure 41). Since GFP-EHD1 (ΔEH) proteins were sparsely distributed in the cytoplasm, they are invisible in the

representative image. Strikingly, CK1α knockdown resulted in dispersal of GFP-EHD1 (ΔEH) (Figure 41). CK1α knockdown did not obviously affect RE localization of GFP-lact-C2 (Figure 42), which binds to liposomes containing 20% concentration of PS (Yeung *et al.*, 2008), suggesting CK1α knockdown does not result in complete disappearance of PS from REs. Together, these data indicate that CK1α is required for the localization of PS in the cytoplasmic leaflet of REs.

CK1α has been shown to localize at the cytoplasm and nucleus (Fu *et al.*, 2001; Burzio *et al.*, 2002). To confirm these previous results, we obtained cDNA of two splicing isoforms (Fu *et al.*, 2001) of human CK1α: CK1αS and CK1αLS (Figure 43A). Notably, CK1αLS harbors a nuclear localization signal in the L-insert. We expressed these isoforms tagged with GFP in COS-1 cells. Consistent with the previous report (Fu *et al.*, 2001), GFP-CK1αS localized in the cytoplasm and a centrosome-like structure and to a lesser extent in nucleus, whereas GFP-CK1αLS localized mainly in nucleus and a centrosome-like structure and to a lesser extent in the cytoplasm (Figure 43B). All of the CK1α siRNAs used above match both CK1αS and CK1αLS.

Discussion

In the present study, we showed that ATP8A1 is phosphorylated at S25/S29 under basal condition, and that these phosphorylations modulate ATP8A1 activity and function at REs. Inhibition of CK1 by a selective inhibitor resulted in reduced phosphorylation of ATP8A1 and TfnR tubular localization. Importantly, CK1α knockdown resulted in dispersal of EHD1 (ΔEH) from REs and emergence of aberrant TfnR tubules. These findings suggest that CK1α regulates PS localization at REs and recycling traffic via phosphorylation of ATP8A1. Further experiments are needed to determine whether CK1α directly phophorylates ATP8A1 at Ser29.

Although S29A or S25A/S29A ATP8A1 mutant did not rescue ATP8A1 knockdown (Figure 32), their ATPase activities *in vitro* were decreased but retained (Figure 31), in contrast to the activity-deficient mutant E191Q (Figure 3). These results suggest that PS flipping rate must be above a certain level in order to induce the membrane fission process mediated by EHD1. Crystal structure analysis of EHD2 indicated that EHD proteins bind to membrane as an oligomer (Daumke *et al.*, 2007). In the present study, EHD1 bound PS with a Hill coefficient of 7.1 (> 1.0) (Figure 14), showing a positive cooperativity and indicating that EHD1 forms an oligomer. We speculate that PS must be clustered in order to recruit EHD1 oligomer. ATP8A1 S25A/S29A mutant may not be able to recruit EHD1 oligomer because their PS flipping rate is slow and the flipped PS undergoes lateral diffusion or flopping to the luminal leaflet before clustered.

How does the phosphorylation regulate ATP8A1 activity? In *S. cerevisiae,* Drs2p, the closest homologue of ATP8A1, is autoinihibited by its C-terminal tail and this autoinhibition is released by interaction with PI4P (Zhou *et al.*, 2013). Dnf1p and Dnf2p are known to be activated by kinase-mediated phosphorylation on their C terminus (Nakano *et al.*, 2008; Roelants *et al.*, 2010), but the precise mechanism of the activation remains unknown. P_3-ATPase family, which comprises plasma membrane proton pumps in fungi and plants, has autoinihbitory regions in its C terminus and this autoinhibition is released by phosphorylation and subsequent binding to 14-3-3 proteins (Ekberg *et al.*, 2010). Although we failed to observe the interaction between 14-3-3 and ATP8A1 in our experimental condition where the interaction with CDC50A was observed (Figure 44), further experiments are needed to examine whether ATP8A1 utilizes a similar activation mechanism.

Although members of CK1 family are regarded as constitutively active kinases, several factors are known to be able to modulate CK1 activity. Stimulation by insulin or growth

factors such as EGF leads to elevated CK1 activity (Cobb and Rosen, 1983; Rena *et al.*, 2002). CK1α activity is reduced by phosphatidylinositol-4,5-biphosphate (PI(4,5)P_2) in neuronal cells (Gross *et al.*, 1995). Recently, it was reported that CK1α activity is inhibited by autophosphorylation and is elevated with phosphatase treatment (Bedri *et al.*, 2007). Given that insulin or growth factors stimulate membrane traffic of Tfn to the plasma membrane (Davis and Czech, 1986), we propose that an extracellular signal-regulated kinase/phosphatase cascade modulates membrane traffic from REs via activation of ATP8A1. We are currently addressing this issue.

Materials and Methods

Chapter 1

Antibodies

The following antibodies were purchased from the manufacturers as noted: rabbit anti-ATP8A1 and rabbit anti-ARF1 (Proteintech); mouse anti-1D4 (Millipore); mouse anti-α-tubulin, rabbit anti-GM130, and mouse anti-myc (9E10) (Sigma); mouse anti-PI(4)P (Echelon); mouse anti-GM130 and mouse anti-LAMP1 (H4A3) (BD Biosciences); mouse anti-human TfnR (H68.4; Zymed Laboratories); rabbit anti-EHD1 (epitomics); mouse anti-LAMP2 (Santa Cruz); mouse anti-CD63 (Cymbus Biotechnology); rabbit anti-myc (Upstate); goat anti-VPS26 (Everest biotech); mouse anti-6xHis (Wako); rabbit anti-syntaxin 5 (Synaptic Systems); mouse anti-TfnR antibody (13E4) (Abcam); Alexa 488-, 594-, or 647-conjugated secondary antibodies (Invitrogen); sheep anti-mouse IgG antibody-HRP and donkey anti-rabbit IgG antibody-HRP (GE Healthcare).

Reagents

The following reagents were purchased from the manufacturers as noted: cholesterol (C8503), sulfatide, PA, ethanolamine, ATP and n-octyl-β-D-glucopyranoside (Sigma); and PI (Echelon); di16:0-PIPs (CellSignals); PS, PE, and PC (porcine brain) for liposome cosedimentation assay, 1,2-dioleoyl-PC, 1,2-dioleoyl-PE, 1,2-dioleoyl-PS, 1,2-dioleoyl-PI, 1,2-dioleoyl-PG, 1,2-dioleoyl-PA, brain polar lipids (porcine), sphingomyelin (porcine brain), cholesterol (ovine wool), C6-NBD-PS and C6-NBD-PE (Avanti Polar Lipids); dithionite (Fisher); CHAPS (Anatrace); Alexa 594-Tfn, Alexa 594-CTxB, Alexa 555-EGF (Invitrogen). To prepare Alexa 488-Tfn, human holo-Tfn (Sigma) was conjugated with Alexa 488 using Alexa Flour succinimidyl ester (Invitrogen) and then purified by PD-10 desalting columns (GE

Healthcare).

Plasmids

The following genes were amplified by polymerase chain reaction (PCR) with cDNA derived from HeLa cells using the following primers: 5'-AGCCGCCTCGAGCATGCCCACCATGCGGAGGAC-3' (ATP8A1; sense primer, XhoI site is underlined) and 5'-AGGCTCCCCGGGTCACCATTCGTCGGGCCTC-3' (ATP8A1; antisense primer, SmaI site is underlined); 5'-GCCTCGAGATGTTCAGCTGGGTCAGC-3' (EHD1; sense primer, XhoI site is underlined) and 5'-GCGAATTCTCACTCATGTCTGCGCTTG-3' (EHD1; antisense primer, EcoRI site is underlined); 5'-TGGCAGGAATTCATGATGGATCAAGCTAGATC-3' (TfnR; sense primer, EcoRI site is underlined) and 5'-GGTATCGGATCCCAAACTCATTGTCAATGTCCC-3' (TfnR; antisense primer, BamHI site is underlined); 5'-GGAAGACCTGGAATTCATGGCGATGAACTATAACG-3' (CDC50A; sense primer, EcoRI site is underlined) and 5'-TAGATGCATGCTCGAGTTAAATGGTAATGTCAGC-3' (CDC50A; antisense primer, XhoI site is underlined); 5'-GCAGATCTCATGGGCACCCGCGACGACGA-3' (Rab11; sense primer, BglII site is underlined) and 5'-GCGTCGACTTAGATGTTCTGACAGCACT-3' (Rab11; antisense primer). Only one isoform (NM_001105529) of ATP8A1 was amplified, which corresponds to bovine isoform β1 (Ding *et al.*, 2000). The products encoding ATP8A1 and EHD1 were introduced into pEGFP-C3 and pEGFP-C2 (Clontech), respectively, to generate N-terminal GFP-tagged constructs. The product encoding TfnR was introduced into pEGFP-N2 (Clontech) to generate a C-terminal GFP-tagged construct. The product encoding CDC50A was introduced into pcDNA with myc-tag (Invitrogen) to generate an N-terminal myc-tagged

construct. The product encoding Rab11 was introduced into pmCherry-C3 (generated by the replacement of the EGFP sequence in pEGFP-C3 with an mCherry sequence) to generate an N-terminal mCherry-tagged construct. For the preparation of siRNA-resistant N-terminal GFP-tagged ATP8A1 expression vector, six silent mutations were introduced by PCR in the ATP8A1 siRNA1 target sequence (see below) using 5'-GCACCTCGAATCTGAATGAGGAACT-3'(sense primer) and 5'-GCGCCATCGCCGCAGTGTCTGTGGG-3' (antisense primer). ATP8A1 E191Q mutant was constructed by site-directed mutagenesis of siRNA-resistant wild-type ATP8A1. Human ATP8A2 (Coleman *et al.*, 2009) was introduced into pEGFP-C2 using In-Fusion HD Cloning Kit (TAKARA). ATP8A2 (E210Q or I376M) and EHD1 (K324D) were constructed by site-directed mutagenesis of wild-type ATP8A2 and EHD1, respectively. GFP-EHD1 ΔEH (1-438) was generated using 5'-GCCTCGAGATGTTCAGCTGGGTCAGC-3' (sense primer, XhoI site is underlined) and GCGAATTCTCACTCCACGTCGTCGATGC (antisense primer, EcoRI site is underlined).

Cell culture

COS-1 cells were maintained in DMEM with 10% FBS. PSA-3 and R-41 cells were cultured in Ham's F-12 medium as previously described (Lee *et al.*, 2012).

Plasmid transfection

Cells were transiently transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA interference

siRNA duplex oligomers were designed (Nippon EGT) as follows: GCUAUGGCUCGAACAUCUA (ATP8A1 siRNA1), CUCUGACUACUCCAUAGCU (ATP8A1 siRNA2), GGUGUAUAGGUCUCUAUAA (ATP8A1 siRNA3), GUUUCCUCGCCUCUCGAAA (EHD1 siRNA1), and CCGUCACUCCAUACAGUAU (EHD1 siRNA2). A total of 20 nM siRNA was introduced to COS-1 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction. After 4 h, the medium was replaced by DMEM with 10% FBS and cells were further incubated for 72 h for subsequent experiments. Cells treated only with Lipofectamine RNAiMAX are defined as control cells.

Immunocytochemistry

Immunocytochemisty was performed as described previously (Uchida *et al.*, 2011). TfnR staining was performed using anti-TfnR antibody (H68.4) unless indicated otherwise. For the stain of endogenous EHD1, cells were fixed with pre-warmed 2% paraformaldehyde in PBS at room temperature for 10 min, quenched with 50 mM NH_4Cl, and permeabilized with 0.1% Triton X-100 in PBS at room temperature for 5 min. After blocking with 3% BSA in PBS, cells were incubated with rabbit anti-EHD1 antibody. For ARF1 staining, fixed and permeabilized cells were incubated with the primary antibody in Can Get Signal immunostain solution A (TOYOBO). For LAMP1 and PI4P staining, fixed cells were permeabilized with 20 μM digitonin in PBS for 5 min at room temperature before blocking. Rab11 staining was performed as previously described (Lee *et al.*, 2012).

Establishment of COS-1 cells stably expressing GFP-lact-C2

GFP-lact-C2 was amplified from lact-C2 in pEGFP-C2 vector (Uchida *et al.*, 2011) using 5'-

GCAGACAAGCTTCGCCACCATGGTGAGCAAGG-3' (sense primer) and 5'-GCAGACATCGATCTAACAGCCCAGCAGCTCC-3' (antisense primer), and subsequently cloned into pLHCX (Clontech) at HindIII/ClaI. Recombinant retrovirus harboring GFP-lact-C2 was prepared using a retrovirus packaging kit ampho (Takara-bio) according to the manufacturer's instructions. In brief, pLHCX/GFP-lact-C2 was transfected into HEK293T cells in combination with pGP (encoding gag and pol) and pE-ampho (encoding amphotropic env). The conditioned medium containing recombinant retrovirus particle was collected, then filtrated with 0.4 μm membrane filter to remove cell debris, and stored at 4°C before use. COS-1 cells were infected with EGFP-lact-C2 retrovirus in DMEM with 10% FBS and 8 μg/ml polybrene, and cells were subjected to drug-selection with 200 μg/ml hygromycin for 2 weeks. The hygromycin-resistant colonies were then picked up.

Visualization of PS at the luminal leaflet

COS-1 cells that were transiently or stably expressing GFP-lact-C2 were fixed with 3.7% formaldehyde in PBS at room temperature for 15 min and permeabilized with 0.1% saponin in PBS at room temperature for 10 min. After blocking with 3% BSA in PBS, cells were treated with recombinant 2xPH-6xHis (2 μg/ml), mouse anti-6xHis tag (1:1000), and rabbit anti-GM130 at 4°C overnight. After washing with PBS, cells were incubated with anti-mouse IgG conjugated with Alexa 594 and anti-rabbit IgG conjugated with Alexa 647 at room temperature for 1 h, washed with PBS, and then mounted. Intensity of recombinant 2xPH-6xHis in RE area was quantified with ImageJ (NIH).

Streptolysin O treatment

Cells were fixed with 2% PFA in PBS and incubated at 4°C for 15 min with 200 units/ml

Streptolysin O (Sigma) in ice-cold PBS containing 10 mM DTT. Cells were then washed with ice-cold PBS containing 10 mM DTT twice, incubated in PBS containing 10 mM DTT at 37°C for 20 min., and washed with PBS.

Confocal microscopy

Confocal microscopy was performed using a LSM510 META (Carl Zeiss Microimaging) with a 63 x 1.4 Plan-Apochromat oil immersion lens, or a TCS SP8 (Leica) with a 63 x 1.2 Plan-Apochromat water immersion lens. With a TCS SP8, excitation was done with a 65 mW Argon laser emitting at 488 nm. Emissions were collected from 500 to 600 nm using Spectral detector.

Tfn recycling assay

COS-1 cells were first incubated in serum-free medium for 30 min at 37°C and subsequently in medium containing 200 µg/ml Alexa 488 or 594-Tfn for 60 min. After washing with ice-cold PBS, cells were incubated in "acid-wash buffer" (20 mM sodium-acetate buffer, 1mM $CaCl_2$, 150 mM NaCl, pH 4.8) on ice for 5 min to remove surface-bound transferrin. Cells were washed with ice-cold PBS, and chased in phenol red-free DMEM containing 400 µg/ml unlabeled human holo-Tfn at 37 °C with 5% CO2. During the chase, cells were imaged every 3 or 6 min for 1 h with a TCS SP8. The perinuclear fluorescence intensity of Alexa 488 or 594 minus the background fluorescence intensity was quantified with ImageJ.

Live imaging for TfnR-GFP in xyt mode

COS-1 cells expressing TfnR-GFP were placed in phenol red-free DMEM/10% FBS. Cells were imaged in xyt mode at 37°C with 5% CO_2 using a TCS SP8. After the photobleach with

100% power of 488 nm laser for 10 sec exposure, the images were collected every 0.5 sec for 1-2 min.

Epifluorescence microscopy imaging system based on an inverted microscope (IX81-ZDC; Olympus), 150× NA 1.45 TIRFM objective (Olympus), motorized filter wheel (Olympus), electron multiplying charge coupled device camera (iXon; Andor), 488 nm laser line and a MetaMorph software (MDS Analytical Technologies). The emission filter 531/46 nm with 488 nm laser for acquisition of EGFP-streaming images was used.

Expression and purification of ATP8A1 and ATP8A2

Expression, purification and reconstitution of ATP8A1 (WT and E191Q) and ATP8A2 (WT and I376M) containing the nine amino acid 1D4 C-terminal tag were performed as described previously (Coleman *et al.*, 2009).

ATPase assay

ATPase activity was measured as described (Coleman *et al.*, 2009). In brief, immunoaffinity purified ATP8A1 or ATP8A2 in PC with increasing amounts of PS was assayed in 50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 12.5 mM $MgCl_2$, 1 mM DTT, 5 mM ATP, 10 mM CHAPS at 37°C. The total lipid concentration was kept constant at 2.5 mg/ml. Assays were terminated by the addition of 6% (wt/vol) SDS. The release of phosphate from ATP was determined using the colorimetric microplate method.

Flippase Assay

Flippase assays were performed as described (Coleman *et al.*, 2009; Coleman and Molday, 2011) with ATP8A1 or ATP8A2 reconstituted in liposome consisting of 97.5% PC and 2.5%

fluorescent-labeled (NBD) PS or PE.

Expression and purification of recombinant proteins

WT or mutant EHD1 cDNA was subcloned into pET-45b (Novagen), and the N-terminal His-tagged protein was expressed in E. coli Arctic Express (DE3) RP cells (Agilent Technologies). Bacterial cultures in LB medium were induced with 1 mM IPTG, and grown overnight at 13°C. The protein was purified using a HisTrap HP column (GE Healthcare) according to the manufacturer's instructions, and then dialyzed against 20 mM HEPES-NaOH (pH 7.5) containing 300 mM NaCl, 1 mM $MgCl_2$, and 1mM DTT.

The sequence encoding a tandem fusion of evt-2 PH domain (2xPH) was introduced into pET-21a (Novagen), and the C-terminal His-tagged protein was expressed in E. coli BL21 (DE3) cells. Bacterial cultures in LB medium were induced with 1 mM IPTG, and grown overnight at 20°C. The protein was purified using a HisTrap HP column, and then dialyzed against PBS.

Liposome co-sedimentation assay

The liposome co-sedimentaion assay using His-tagged 2xPH was performed as previously described (Uchida *et al.*, 2011) with modifications. Lipid mixtures were dried under nitrogen gas and hydrated in 25 mM HEPES-NaOH (pH 7.5) containing 100 mM NaCl and 0.5 mM EDTA for 90 min at 37°C, and vortexed briefly. To remove protein aggregates, the protein solution was subjected to centrifugation at 100,000 *g* for 30 min at 4°C. 10 μg of proteins were incubated with 65 nmol liposomes in the buffer (50 μl) for 15 min at room temperature and the mixture was centrifuged at 100,000 *g* for 30 min at 20°C. The resultant supernatant and pellet were subjected to SDS-PAGE, and the proteins and lipids were stained with Coomassie blue.

Lipid stain was observed just below bromophenol blue. As a negative control, a sample without liposomes was subjected to the same procedure.

The assay using His-tagged EHD1 was performed as described above with the following modifications: (i) dried lipid mixtures were hydrated in 20 mM HEPES-NaOH (pH 7.5) containing 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT; (ii) the protein solution was subjected to centrifugation at 100,000 *g* for 15 min at 4°C to remove aggregates; (iii) 7 μg of proteins were incubated with 50 nmol liposomes.

The intensities of individual bands were quantified with ImageJ. The band intensity in the pellet fraction of the sample without liposomes (a) was subtracted from that of the sample with liposomes (Ippt) to calculate the amount of bound protein (Ippt - a). The percentage of the bound protein was obtained by the following equation: %bound = 100 × (Ippt - a) / [Isup + (Ippt - a)], where Isup represents the band intensity in the supernatant fraction. The binding data from two independent experiments were analyzed by fitting to the 'one-site specific binding with Hill slope' equation in GraphPad Prism 5 (GraphPad Software). Y = Bmax × Xh / (kh + Xh), where k and h represent the concentration of PS resulting in half-maximal binding and the Hill coefficient, respectively.

To prepare liposomes of decreasing size, dried lipid mixtures containing 1% (mol/mol) rhodamine-labelled PE (Avanti) were hydrated in 20 mM HEPES-NaOH (pH 7.5) containing 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT and then dipped into liquid nitrogen for rapid cooling. After completely frozen, the lipids were transferred to a bath at 37°C for thawing. After completely thawed, the lipids were frozen again. This freeze-thaw operation was repeated three times. The lipids were then extruded through 400 nm or 100 nm polycarbonate flilters (Whatman) at room temperature using Avanti mini-extruder, and stored on ice for further experiments. After centrifugation with His-EHD1, the fluorescence of each supernatant

was measured, and the amount of precipitated liposomes was calculated. The percentage of precipitated liposomes was used to calculate the percentage of liposome-bound His-EHD1 in Fig 5D and E. Approximately 70-95% of liposomes were precipitated.

Liposome size measurement by dynamic light scattering (DLS)

The liposome size was measured using a Zetasizer Nano ZS (Malvern) equipped with a 4 mW He-Ne laser (633 nm) and disposable cuvettes (ZEN0112, Malvern) at 25°C. The z-averaged diameter of the particles was calculated from the intensity of the scattered light using the Malvern Zetasizer software. The data of all samples met quality criteria assuming monomodal size distributions.

Western blotting

Western blotting was performed as described previously (Uchida *et al.*, 2011).

CTxB retrograde traffic assay

CTxB retrograde traffic assay was performed as described previously (Uchida *et al.*, 2011).

EGF traffic assay

Cells were preincubated in serum-free DMEM for 30 min at 37°C, and then pulsed with 2 μg/ml Alexa 555-EGF in serum-free DMEM for 5 min at 37°C. After washing, cells were chased in DMEM containing 10% FBS for 60 min at 37°C, fixed and stained for LAMP1. Pearson's coefficient between Alexa 555-EGF and LAMP1 was determined with ImageJ.

Cortical neuron culture

Cortical neurons were obtained from *Atp8a2*$^{+/+}$ or *Atp8a2*$^{-/-}$ mice (embryonic day 15-17) as previously described (Koie *et al.*, 2014). In brief, 6-well plate dishes were coated with 0.01% poly-L-lysine hydrobromide (Sigma) in PBS for overnight at 37°C and subsequently washed four times with water. Cerebral cortices were trypsinized for 5 min at 37°C, treated with 2 U/µl DNase I (Invitrogen), and dissociated by triturating with fire-polished Pasteur pipet. The neurons were cultued in Neurobasal medium (Invitrogen) containing 2% B27 supplement (Invitrogen), 2 mM Glutamax (Invitrogen) and antibiotics for 4 days.

Biotinylation assay

For surface biotinylation, cortical neurons (4 DIV) were cooled on ice, washed three times with ice-cold PBS containing 1 mM $CaCl_2$ and 0.5 mM $MgCl_2$, and then incubated with PBS containing 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, and 1 mg/ml EZ-link Sulfo-NHS-SS-Biotin (Thermo) for 40 min at 4°C. Unreacted biotin was quenched by washing cells with ice-cold PBS containing 100 mM glycine, and then cells were washed twice with ice-cold PBS. Cells were harvested in lysis buffer (20 mM Tris-HCl pH7.4, 5 mM EDTA, 100 mM NaCl, 1% Triton X-100, 0.1% SDS) with complete protease inhibitor cocktail (Roche). Homogenates were centrifuged at 13,000 rpm for 10 min at 4°C. 10% of the resulting supernatant was stored as the total fraction. The remaining 85% of the supernatant was rotated for 1 h at 4°C with NeutrAvidin agarose resin (Thermo) and centrifuged at 500 *g* for 1 min at 4°C. Precipitates were washed three times with Wash buffer (25 mM HEPES, 150 mM NaCl, 5mM EDTA, 0.1% Triton X-100) and stored as the surface fraction. Both total and surface protein samples were boiled for 5 min at 95°C. Western blotting was performed and band intensities were quantified with ImageJ. The ratio of surface to total TfnR or tubulin was calculated, and that of tubulin was subtracted from that of TfnR to exclude the amount of cytoplasmic protein in the

surface fraction. The calculated value was then normalized to that of *Atp8a2*$^{+/+}$.

Statistical analysis

Statistical comparisons of means were made using two-tailed Student's *t*-test or one-way ANOVA. $P < 0.05$ was used as the criterion for statistical significance.

Chapter 2

Antibodies

The following antibodies were purchased from the manufacturers as noted: rabbit anti-CK1α antibody and rabbit anti-ATP8A1 antibody (Proteintech); mouse anti-α-tubulin and mouse anti-myc (9E10) (Sigma); mouse anti-human TfnR (H68.4; Zymed Laboratories); rabbit anti-EHD1 (epitomics); goat anti-VPS26 (Everest biotech); rabbit anti-syntaxin 5 (Synaptic Systems). Mouse anti-pan-14-3-3 (Santa Cruz, sc-1657) was kindly provided by Dr. Komada (Tokyo Institute of Technology). Alexa 488-, 594-, or 647-conjugated secondary antibodies (Invitrogen); sheep anti-mouse IgG antibody-HRP and donkey anti-rabbit IgG antibody-HRP (GE Healthcare).

Reagents

The following reagents were purchased from the manufacturers as noted: D4476, ethanolamine, ATP and n-octyl-β-D-glucopyranoside (Sigma); di16:0-PIPs (CellSignals); PS and PC (porcine brain) for liposome cosedimentation assay, 1,2-dioleoyl-PC, 1,2-dioleoyl-PS, C6-NBD-PS and C6-NBD-PE (Avanti Polar Lipids); dithionite (Fisher); CHAPS (Anatrace).

Plasmids

CK1αS (NM_001892.5) and CK1αLS (NM_001025105.2) cDNA were amplified by PCR with cDNA derived from HeLa cells using the following primers: 5'-CCGCTCGAGCATGGCGAGTAGCAGCGGC -3' (sense primer, XhoI site is underlined) and 5'- TCCCCCGGGTTAGAAACCTTTCATGTT -3' (antisense primer, SmaI site is underlined). The products encoding CK1αS and CK1αLS were introduced into pEGFP-C2 (Clontech) to generate N-terminal GFP-tagged constructs. To construct the expression vector for siRNA-resistant ATP8A1 without a tag, siRNA-resistant ATP8A1 was amplified from the vector described previously (Lee *et al.*, 2015) and introduced into pEGFP-N3. Since there is a stop codon between ATP8A1 and EGFP, the expressed siRNA-resisntant ATP8A1 has no tag. siRNA-resistant ATP8A1 mutants (S25A, S29A, S25D or S25A/S29A) with GFP or without a tag were constructed by site-directed mutagenesis of siRNA-resistant wild-type ATP8A1 using the primers listed below. Then, siRNA-resistant ATP8A1 mutant (S25D/S29D) was constructed by site-directed mutagenesis of siRNA-resistant ATP8A1 (S25D) using the primers listed below.

Number		Fwd	Rvs
1	human CK1α	CCGCTCGAGCATGGCGAGTAGCAGCGGC	TCCCCCGGGTTAGAAACCTTTCATGTT
2	human ATP8A1 (S25A)	GCTGACCAGGAGGAAGTAAGGACTA	CAGTGAGGTCTTCTCTGCAACATCA
3	human ATP8A1 (S29A)		CAGTGCGGTCTTCTCTGAAACATCA
4	human ATP8A1 (S25D)		CAGTGAGGTCTTCTCATCAACATCA
5	human ATP8A1 (S25A/S29A)		CAGTGCGGTCTTCTCTGCAACATCA
6	human ATP8A1 (S25D/S29D)		CAGATCGGTCTTCTCATCAACATCA

Cell culture

COS-1 cells were maintained in DMEM with 10% FBS. PSA-3 cells were cultured in Ham's

F-12 medium as previously described (Lee *et al.*, 2012).

Plasmid transfection

Cells were transiently transfected with plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

RNA interference

ATP8A1 siRNA1 was designed (Nippon EGT) as described (Lee *et al.*, 2015). To perform RNAi screening for CK1 family members, siRNAs targeting each isoform were picked out from siGENOME siRNA library Human Druggable Subsets (G-004675, Dharmacon). For CK1α RNAi, silencer select validated siRNA (Applied Biosystems) was used. In this study, CSNK1A1 siRNA (s3624), CSNK1A1 siRNA (s3625), CSNK1A1L siRNA (s42512) and CSNK1A1L siRNA (s42513) were referred to as CK1α siRNA1, siRNA2, siRNA3 and siRNA4, resepctively. Because CSNK1A1 siRNA has only 1- or 2-nt mismatches to CSNK1A1L mRNA and vice versa, all the CK1α siRNA likely target both CSNK1A1 and CSNK1A1L mRNA. A total of 20 nM siRNA was introduced to COS-1 cells using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's instruction. After 4 h, the medium was replaced by DMEM with 10% FBS and cells were further incubated for 72 h for subsequent experiments. Cells treated only with Lipofectamine RNAiMAX are defined as control cells.

Metabolic labeling

HEK293A cells were transfected with GFP-ATP8A1 and myc-CDC50A. Twenty-four hour after transfection, cells were washed three times with HBSS (GIBCO) and then labeled with 1

mCi/ml [^{32}P]orthophosphate (PerkinElmer) in phosphate-free DMEM (Invitrogen) containing 1% Pen/Strep/L-Glu (Invitrogen) for 4 h.

Immunoprecipitation and autoradiography

Cells were washed with HBSS and lysed with IP buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing 1x c0mplete, EDTA-free protease inhibitor (Roche) and phosphatase inhibitors (8 mM NaF, 12 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1.2 mM Na_2MoO_4, 5 μM Cantharidin, 2 mM Imidazole). Cell lysate was cetrifuged at 12,000 rpm for 10 min at 4℃. The resulting supernatants were incubated with 50% slurry Protein G sepharose 4 fast flow (GE healthcare) for 30 min at 4℃ and centrifuged at 3,000 rpm for 1 min at 4℃. 5% of the supernatants was stored as "input". The remaining 90% of the supernatants was incubated with mouse anti-GFP monoclonal antibody (clone 3E6) and 50% slurry Protein G sepharose 4 fast flow for 150 min at 4℃ and then centrifuged at 3,000 rpm for 1 min at 4℃. The precipitates was washed three times with IP wash buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% NP-40) and stored as "IP". The proteins were resolved by SDS-PAGE and transferred to PVDF membrane (Millipore), followed by autoradiography using Typhoon FLA 9000 (GE healthcare).

Generation of anti phosphospecifc ATP8A1 pS29 antisera

Rabbits were immunized 6 times with the human KLH peptide C+VSEKT(pS)LADQE. Five week after first immunization, rabbit sera were collected. All procedures were performed by Eurofins genomics.

Cell lysate preperation for phosphorylated ATP8A1 detection

For immunoblot with anti-ATP8A1 pS29 antisera, cells were lysed with IP buffer (20 mM HEPES pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% NP-40) containing 1x c0mplete, EDTA-free protease inhibitor (Roche) and phosphatase inhibitors (8 mM NaF, 12 mM beta-glycerophosphate, 1 mM Na_3VO_4, 1.2 mM Na_2MoO_4, 5 μM Cantharidin, 2 mM Imidazole).

For phosphatase treatment, cells were lysed with buffer containing containing 20 mM HEPES pH 7.4, 150 mM NaCl, 1% NP-40 and 1 mM $MgCl_2$ and without EDTA. Then 1 μL of 30 U/μL calf intestine alkaline phosphatase (2250A, TAKARA) was added to 100 μL cell lysate and incubated at 37°C for 1 h.

Western blotting

Proteins were seperated in polyacrylamide gel and then transferred to PVDF membrane (Millipore). After blocking with 1% BSA in PBS for 30 min at room temperature, membranes were incubated with primary antibodies or antisera diluted in 1% BSA in PBS, followed by secondary antibodies conjugated to peroxidase. The proteins were visualized by enhanced chemiluminescence using LAS 4000 (GE Healthcare).

Immunocytochemistry

Immunocytochemisty was performed as described previously (Uchida *et al.*, 2011).

Establishment of COS-1 cells stably expressing GFP-EHD1 (ΔEH)

GFP-EHD1 (ΔEH) (1-438) (Lee *et al.*, 2015) was amplified using 5'-CCGGAATTCATGTTCAGCTGGGTCAGCAAGGATGC -3' (sense primer) and 5'-TTTATAGCGGCCGCTCACTCCACGTCGTCGATGCC -3' (antisense primer), and

subsequently cloned into pMRX-IRES-Puro at EcoRI/NotI. To prepare recombinant retrovirus harboring GFP-EHD1 (ΔEH), GFP-EHD1 (ΔEH) in pMRX-IRES-Puro was transfected into HEK293T cells in combination with pCG-VSV-G and pCG-gag-pol. The conditioned medium containing recombinant retrovirus particle was collected, centrifuged at 2,000 rpm for 5 min, filtrated with 0.45 μm membrane filter to remove cell debris, and then stored at 4°C before use. COS-1 cells were infected with GFP-EHD1 (ΔEH) retrovirus in DMEM with 10% FBS and 8 μg/ml polybrene for 24 h. Cells were then subjected to drug-selection with 2.5 μg/ml puromycin for 2 days. The puromycin-resistant cells were maintained in DMEM with 10% FBS and 2 μg/ml puromycin.

Confocal microscopy

Confocal microscopy was performed using a TCS SP8 (Leica) with a 63 x 1.2 Plan-Apochromat water immersion lens.

Expression and purification of ATP8A1

Expression, purification and reconstitution of ATP8A1 (WT and mutants) containing the nine amino acid 1D4 C-terminal tag were performed as described previously (Coleman *et al.*, 2009).

ATPase assay

ATPase activity was measured as described (Coleman *et al.*, 2009; Lee *et al.*, 2015).

Liposome co-sedimentation assay

Preparation of recombinant His-tagged EHD1 proteins and the liposome co-sedimentaion

assay were performed as previously described (Lee *et al.*, 2015). Briefly, lipid mixtures were dried under nitrogen gas and hydrated in 20 mM HEPES-NaOH (pH 7.5) containing 100 mM NaCl, 1 mM $MgCl_2$, and 1 mM DTT for 90 min at 37°C, and vortexed briefly. To remove protein aggregates, the protein solution was subjected to centrifugation at 100,000 *g* for 30 min at 4°C. 7 μg of proteins were incubated with 50 nmol liposomes in the buffer (50 μl) for 15 min at room temperature and the mixture was centrifuged at 100,000 *g* for 15 min at 4°C. The resultant supernatant and pellet were subjected to SDS-PAGE, and the proteins and lipids were stained with Coomassie blue. Lipid stain was observed just below bromophenol blue. As a negative control, a sample without liposomes was subjected to the same procedure.

The intensities of individual bands were quantified with ImageJ. The band intensity in the pellet fraction of the sample without liposomes (a) was subtracted from that of the sample with liposomes (Ippt) to calculate the amount of bound protein (Ippt - a). The percentage of the bound protein was obtained by the following equation: %bound = 100 × (Ippt - a) / [Isup + (Ippt - a)], where Isup represents the band intensity in the supernatant fraction. The binding data from two independent experiments were analyzed by fitting to the 'one-site specific binding with Hill slope' equation in GraphPad Prism 6 (GraphPad Software). Y = Bmax × Xh / (kh + Xh), where k and h represent the concentration of PS resulting in half-maximal binding and the Hill coefficient, respectively.

References

Anitei M, Hoflack B (2012) Bridging membrane and cytoskeleton dynamics in the secretory and endocytic pathways. *Nat Cell Biol*, **14:** 11–9

Balasubramanian K, Schroit AJ (2003) Aminophospholipid asymmetry: A matter of life and death. *Annu Rev Physiol*, **65:** 701–34

Bedri S, Cizek SM, Rastarhuyeva I, Stone JR (2007) Regulation of protein kinase CK1alphaLS by dephosphorylation in response to hydrogen peroxide. *Arch Biochem Biophys*, **466:** 242–49

Behnia R, Munro S (2005) Organelle identity and the signposts for membrane traffic. *Nature*, **438:** 597–04

Bigay J, Antonny B (2012) Curvature, Lipid Packing, and Electrostatics of Membrane Organelles: Defining Cellular Territories in Determining Specificity. *Developmental Cell*, **23:** 886–95

Blom N, Gammeltoft S, Brunak S (1999) Sequence and structure-based prediction of eukaryotic protein phosphorylation sites. *J Mol Biol*, **294:** 1351–362

Blume JJ, Halbach A, Behrendt D, Paulsson M, Plomann M (2007) EHD proteins are associated with tubular and vesicular compartments and interact with specific phospholipids. *Exp Cell Res*, **313:** 219–31

Boucrot E, Pick A, Camdere G, Liska N, Evergren E, McMahon HT, Kozlov MM (2012) Membrane fission is promoted by insertion of amphipathic helices and is restricted by crescent BAR domains. *Cell*, **149:** 124–36

Bull LN, van Eijk MJ, Pawlikowska L, DeYoung JA, Juijn JA, Liao M, Klomp LW, Lomri N, Berger R, Scharschmidt BF, Knisely AS, Houwen RH, Freimer NB (1998) A gene encoding a P-type ATPase mutated in two forms of hereditary cholestasis. *Nat Genet*, **18:** 219–24

Burzio V, Antonelli M, Allende CC, Allende JE (2002) Biochemical and cellular characteristics of the four splice variants of protein kinase CK1alpha from zebrafish (Danio rerio). *J Cell Biochem*, **86:** 805–14

Cacciagli P, Haddad MR, Mignon-Ravix C, El-Waly B, Moncla A, Missirian C, Chabrol B, Villard L (2010) Disruption of the ATP8A2 gene in a patient with a t(10;13) de novo balanced translocation and a severe neurological phenotype. *Eur J Hum Genet*, **18:** 1360–363

Caplan S, Naslavsky N, Hartnell LM, Lodge R, Polishchuk RS, Donaldson JG, Bonifacino JS (2002) A tubular EHD1-containing compartment involved in the recycling of major

histocompatibility complex class I molecules to the plasma membrane. *EMBO J*, **21:** 2557–567

Chen B, Jiang Y, Zeng S, Yan J, Li X, Zhang Y, Zou W, Wang X (2010) Endocytic sorting and recycling require membrane phosphatidylserine asymmetry maintained by TAT-1/CHAT-1. *PLoS Genet*, **6:** e1001235

Cheong JK, Virshup DM (2011) Casein kinase 1: Complexity in the family. *Int J Biochem Cell Biol*, **43:** 465–69

Cobb MH, Rosen OM (1983) Description of a protein kinase derived from insulin-treated 3T3-L1 cells that catalyzes the phosphorylation of ribosomal protein S6 and casein. *J Biol Chem*, **258:** 12472–2481

Coleman JA, Vestergaard AL, Molday RS, Vilsen B, Andersen JP (2012a) Critical role of a transmembrane lysine in aminophospholipid transport by mammalian photoreceptor P4-ATPase ATP8A2. *Proceedings of the National Academy of Sciences*, **109:** 1449–454

Coleman JA, Kwok MC, Molday RS (2009) Localization, purification, and functional reconstitution of the P4-ATPase Atp8a2, a phosphatidylserine flippase in photoreceptor disc membranes. *J Biol Chem*, **284:** 32670–2679

Coleman JA, Molday RS (2011) Critical role of the beta-subunit CDC50A in the stable expression, assembly, subcellular localization, and lipid transport activity of the P4-ATPase ATP8A2. *J Biol Chem*, **286:** 17205–7216

Coleman JA, Quazi F, Molday RS (2012b) Mammalian P(4)-ATPases and ABC transporters and their role in phospholipid transport. *Biochim Biophys Acta*,

Coleman JA, Zhu X, Djajadi HR, Molday LL, Smith RS, Libby RT, John SW, Molday RS (2014) Phospholipid flippase ATP8A2 is required for normal visual and auditory function and photoreceptor and spiral ganglion cell survival. *J Cell Sci*, **127:** 1138–149

Daumke O, Lundmark R, Vallis Y, Martens S, Butler PJ, McMahon HT (2007) Architectural and mechanistic insights into an EHD ATPase involved in membrane remodelling. *Nature*, **449:** 923–27

Davis RJ, Czech MP (1986) Regulation of transferrin receptor expression at the cell surface by insulin-like growth factors, epidermal growth factor and platelet-derived growth factor. *EMBO J*, **5:** 653–58

Dephoure N, Gould KL, Gygi SP, Kellogg DR (2013) Mapping and analysis of phosphorylation sites: a quick guide for cell biologists. *Mol Biol Cell*, **24:** 535–42

Devaux PF (1992) Protein involvement in transmembrane lipid asymmetry. *Annu Rev Biophys Biomol Struct*, **21:** 417–39

Di Paolo G, De Camilli P (2006) Phosphoinositides in cell regulation and membrane dynamics. *Nature*, **443:** 651–57

Ding J, Wu Z, Crider BP, Ma Y, Li X, Slaughter C, Gong L, Xie XS (2000) Identification and functional expression of four isoforms of ATPase II, the putative aminophospholipid translocase. Effect of isoform variation on the ATPase activity and phospholipid specificity. *J Biol Chem*, **275:** 23378–3386

Dodge JT, Phillips GB (1967) Composition of phospholipids and of phospholipid fatty acids and aldehydes in human red cells. *J Lipid Res*, **8:** 667–75

Duit S, Mayer H, Blake SM, Schneider WJ, Nimpf J (2010) Differential functions of ApoER2 and very low density lipoprotein receptor in Reelin signaling depend on differential sorting of the receptors. *J Biol Chem*, **285:** 4896–908

Ekberg K, Palmgren MG, Veierskov B, Buch-Pedersen MJ (2010) A novel mechanism of P-type ATPase autoinhibition involving both termini of the protein. *J Biol Chem*, **285:** 7344–350

Emoto K, Umeda M (2000) An essential role for a membrane lipid in cytokinesis. Regulation of contractile ring disassembly by redistribution of phosphatidylethanolamine. *J Cell Biol*, **149:** 1215–224

Emre Onat O, Gulsuner S, Bilguvar K, Nazli Basak A, Topaloglu H, Tan M, Tan U, Gunel M, Ozcelik T (2012) Missense mutation in the ATPase, aminophospholipid transporter protein ATP8A2 is associated with cerebellar atrophy and quadrupedal locomotion. *Eur J Hum Genet*,

Fairn GD, Schieber NL, Ariotti N, Murphy S, Kuerschner L, Webb RI, Grinstein S, Parton RG (2011) High-resolution mapping reveals topologically distinct cellular pools of phosphatidylserine. *J Cell Biol*, **194:** 257–75

Fu Z, Chakraborti T, Morse S, Bennett GS, Shaw G (2001) Four casein kinase I isoforms are differentially partitioned between nucleus and cytoplasm. *Exp Cell Res*, **269:** 275–86

Gagescu R, Demaurex N, Parton RG, Hunziker W, Huber LA, Gruenberg J (2000) The recycling endosome of Madin-Darby canine kidney cells is a mildly acidic compartment rich in raft components. *Mol Biol Cell*, **11:** 2775–791

Gelabert-Baldrich M, Soriano-Castell D, Calvo M, Lu A, Vina-Vilaseca A, Rentero C, Pol A, Grinstein S, Enrich C, Tebar F (2014) Dynamics of KRas on endosomes: involvement of acidic phospholipids in its association. *FASEB J*, **28:** 3023–037

Giridharan SS, Cai B, Vitale N, Naslavsky N, Caplan S (2013) Cooperation of MICAL-L1, syndapin2, and phosphatidic acid in tubular recycling endosome biogenesis. *Mol Biol Cell*, **24:** 1776–0, S1-15

Graham TR (2004) Flippases and vesicle-mediated protein transport. *Trends Cell Biol*, **14:** 670–77

Grant B, Zhang Y, Paupard MC, Lin SX, Hall DH, Hirsh D (2001) Evidence that RME-1, a

conserved C. elegans EH-domain protein, functions in endocytic recycling. *Nat Cell Biol*, **3:** 573–79

Grant BD, Caplan S (2008) Mechanisms of EHD/RME-1 protein function in endocytic transport. *Traffic*, **9:** 2043–052

Grant BD, Donaldson JG (2009) Pathways and mechanisms of endocytic recycling. *Nat Rev Mol Cell Biol*, **10:** 597–08

Gross SD, Hoffman DP, Fisette PL, Baas P, Anderson RA (1995) A phosphatidylinositol 4,5-bisphosphate-sensitive casein kinase I alpha associates with synaptic vesicles and phosphorylates a subset of vesicle proteins. *J Cell Biol*, **130:** 711–24

Hancock JF (2003) Ras proteins: different signals from different locations. *Nat Rev Mol Cell Biol*, **4:** 373–84

Herz J, Chen Y (2006) Reelin, lipoprotein receptors and synaptic plasticity. *Nat Rev Neurosci*, **7:** 850–59

Hessel E, Herrmann A, Muller P, Schnetkamp PP, Hofmann KP (2000) The transbilayer distribution of phospholipids in disc membranes is a dynamic equilibrium evidence for rapid flip and flop movement. *Eur J Biochem*, **267:** 1473–483

Holthuis JC, Menon AK (2014) Lipid landscapes and pipelines in membrane homeostasis. *Nature*, **510:** 48–7

Huang BX, Akbar M, Kevala K, Kim HY (2011) Phosphatidylserine is a critical modulator for Akt activation. *J Cell Biol*, **192:** 979–92

Kato U, Inadome H, Yamamoto M, Emoto K, Kobayashi T, Umeda M (2012) Role for phospholipid flippase complex of ATP8A1 and CDC50A in cell migration. *J Biol Chem*,

Knaevelsrud H, Soreng K, Raiborg C, Haberg K, Rasmuson F, Brech A, Liestol K, Rusten TE, Stenmark H, Neufeld TP, Carlsson SR, Simonsen A (2013) Membrane remodeling by the PX-BAR protein SNX18 promotes autophagosome formation. *The Journal of Cell Biology*, **202:** 331–49

Knippschild U, Gocht A, Wolff S, Huber N, Lohler J, Stoter M (2005) The casein kinase 1 family: participation in multiple cellular processes in eukaryotes. *Cell Signal*, **17:** 675–89

Koie M, Okumura K, Hisanaga A, Kamei T, Sasaki K, Deng M, Baba A, Kohno T, Hattori M (2014) Cleavage within Reelin repeat 3 regulates the duration and range of the signaling activity of Reelin protein. *J Biol Chem*, **289:** 12922–2930

Kuge O, Nishijima M (1997) Phosphatidylserine synthase I and II of mammalian cells. *Biochim Biophys Acta*, **1348:** 151–56

Kuge O, Nishijima M, Akamatsu Y (1986) Phosphatidylserine biosynthesis in cultured Chinese hamster ovary cells. II. Isolation and characterization of phosphatidylserine auxotrophs. *J Biol Chem*, **261:** 5790–794

Lee S, Uchida Y, Emoto K, Umeda M, Kuge O, Taguchi T, Arai H (2012) Impaired retrograde membrane traffic through endosomes in a mutant CHO cell defective in phosphatidylserine synthesis. *Genes Cells*, **17:** 728–36

Lee S, Uchida Y, Wang J, Matsudaira T, Nakagawa T, Kishimoto T, Mukai K, Inaba T, Kobayashi T, Molday RS, Taguchi T, Arai H (2015) Transport through recycling endosomes requires EHD1 recruitment by a phosphatidylserine translocase. *EMBO J*, **34:** 669–88

Lin SX, Grant B, Hirsh D, Maxfield FR (2001) Rme-1 regulates the distribution and function of the endocytic recycling compartment in mammalian cells. *Nat Cell Biol*, **3:** 567–72

Longatti A, Lamb CA, Razi M, Yoshimura S, Barr FA, Tooze SA (2012) TBC1D14 regulates autophagosome formation via Rab11- and ULK1-positive recycling endosomes. *J Cell Biol*, **197:** 659–75

Lopez-Marques RL, Holthuis JC, Pomorski TG (2011) Pumping lipids with P4-ATPases. *Biol Chem*, **392:** 67–6

Lu A, Tebar F, Alvarez-Moya B, Lopez-Alcala C, Calvo M, Enrich C, Agell N, Nakamura T, Matsuda M, Bachs O (2009) A clathrin-dependent pathway leads to KRas signaling on late endosomes en route to lysosomes. *J Cell Biol*, **184:** 863–79

Maeda K, Anand K, Chiapparino A, Kumar A, Poletto M, Kaksonen M, Gavin AC (2013) Interactome map uncovers phosphatidylserine transport by oxysterol-binding proteins. *Nature*,

Maxfield FR, McGraw TE (2004) Endocytic recycling. *Nat Rev Mol Cell Biol*, **5:** 121–32

McKenzie JE, Raisley B, Zhou X, Naslavsky N, Taguchi T, Caplan S, Sheff D (2012) Retromer Guides STxB and CD8-M6PR from Early to Recycling Endosomes, EHD1 Guides STxB from Recycling Endosome to Golgi. *Traffic*,

Mellman I (1996) Endocytosis and molecular sorting. *Annu Rev Cell Dev Biol*, **12:** 575–25

Menon I, Huber T, Sanyal S, Banerjee S, Barre P, Canis S, Warren JD, Hwa J, Sakmar TP, Menon AK (2011) Opsin is a phospholipid flippase. *Curr Biol*, **21:** 149–53

Misaki R, Nakagawa T, Fukuda M, Taniguchi N, Taguchi T (2007) Spatial segregation of degradation- and recycling-trafficking pathways in COS-1 cells. *Biochem Biophys Res Commun*, **360:** 580–85

Mondal M, Mesmin B, Mukherjee S, Maxfield FR (2009) Sterols are mainly in the cytoplasmic leaflet of the plasma membrane and the endocytic recycling compartment in CHO cells. *Mol Biol Cell*, **20:** 581–88

Munton RP, Tweedie-Cullen R, Livingstone-Zatchej M, Weinandy F, Waidelich M, Longo D, Gehrig P, Potthast F, Rutishauser D, Gerrits B, Panse C, Schlapbach R, Mansuy IM (2007) Qualitative and quantitative analyses of protein phosphorylation in naive and

stimulated mouse synaptosomal preparations. *Mol Cell Proteomics*, **6:** 283–93

Nakano K, Yamamoto T, Kishimoto T, Noji T, Tanaka K (2008) Protein kinases Fpk1p and Fpk2p are novel regulators of phospholipid asymmetry. *Mol Biol Cell*, **19:** 1783–797

Natarajan P, Liu K, Patil DV, Sciorra VA, Jackson CL, Graham TR (2009) Regulation of a Golgi flippase by phosphoinositides and an ArfGEF. *Nat Cell Biol*, **11:** 1421–426

Newton AC, Keranen LM (1994) Phosphatidyl-L-serine is necessary for protein kinase C's high-affinity interaction with diacylglycerol-containing membranes. *Biochemistry*, **33:** 6651–658

Ozcelik T, Akarsu N, Uz E, Caglayan S, Gulsuner S, Onat OE, Tan M, Tan U (2008) Mutations in the very low-density lipoprotein receptor VLDLR cause cerebellar hypoplasia and quadrupedal locomotion in humans. *Proc Natl Acad Sci U S A*, **105:** 4232–236

Pant S, Sharma M, Patel K, Caplan S, Carr CM, Grant BD (2009) AMPH-1/Amphiphysin/Bin1 functions with RME-1/Ehd1 in endocytic recycling. *Nat Cell Biol*, **11:** 1399–410

Park M, Penick EC, Edwards JG, Kauer JA, Ehlers MD (2004) Recycling endosomes supply AMPA receptors for LTP. *Science*, **305:** 1972–975

Paterson JK, Renkema K, Burden L, Halleck MS, Schlegel RA, Williamson P, Daleke DL (2006) Lipid specific activation of the murine P4-ATPase Atp8a1 (ATPase II). *Biochemistry*, **45:** 5367–376

Peng Y, Grassart A, Lu R, Wong CC, Yates Jr, Barnes G, Drubin DG (2015) Casein kinase 1 promotes initiation of clathrin-mediated endocytosis. *Dev Cell*, **32:** 231–40

Pomorski T, Lombardi R, Riezman H, Devaux PF, van Meer G, Holthuis JC (2003) Drs2p-related P-type ATPases Dnf1p and Dnf2p are required for phospholipid translocation across the yeast plasma membrane and serve a role in endocytosis. *Mol Biol Cell*, **14:** 1240–254

Poulsen LR, Lopez-Marques RL, McDowell SC, Okkeri J, Licht D, Schulz A, Pomorski T, Harper JF, Palmgren MG (2008) The Arabidopsis P4-ATPase ALA3 localizes to the golgi and requires a beta-subunit to function in lipid translocation and secretory vesicle formation. *Plant Cell*, **20:** 658–76

Powelka AM, Sun J, Li J, Gao M, Shaw LM, Sonnenberg A, Hsu VW (2004) Stimulation-dependent recycling of integrin beta1 regulated by ARF6 and Rab11. *Traffic*, **5:** 20–6

Pulgar V, Marin O, Meggio F, Allende CC, Allende JE, Pinna LA (1999) Optimal sequences for non-phosphate-directed phosphorylation by protein kinase CK1 (casein kinase-1)--a re-evaluation. *Eur J Biochem*, **260:** 520–26

Puri C, Renna M, Bento C, Moreau K, Rubinsztein D (2013) Diverse Autophagosome Membrane Sources Coalesce in Recycling Endosomes. *Cell*, **154:** 1285–299

Rena G, Bain J, Elliott M, Cohen P (2004) D4476, a cell-permeant inhibitor of CK1, suppresses the site-specific phosphorylation and nuclear exclusion of FOXO1a. *EMBO Rep*, **5:** 60–5

Rena G, Woods YL, Prescott AR, Peggie M, Unterman TG, Williams MR, Cohen P (2002) Two novel phosphorylation sites on FKHR that are critical for its nuclear exclusion. *EMBO J*, **21:** 2263–271

Roelants FM, Baltz AG, Trott AE, Fereres S, Thorner J (2010) A protein kinase network regulates the function of aminophospholipid flippases. *Proc Natl Acad Sci U S A*, **107:** 34–9

Ruaud AF, Nilsson L, Richard F, Larsen MK, Bessereau JL, Tuck S (2009) The C. elegans P4-ATPase TAT-1 regulates lysosome biogenesis and endocytosis. *Traffic*, **10:** 88–00

Sann S, Wang Z, Brown H, Jin Y (2009) Roles of endosomal trafficking in neurite outgrowth and guidance. *Trends Cell Biol*, **19:** 317–24

Schmick M, Vartak N, Papke B, Kovacevic M, Truxius DC, Rossmannek L, Bastiaens PI (2014) KRas Localizes to the Plasma Membrane by Spatial Cycles of Solubilization, Trapping and Vesicular Transport. *Cell*, **157:** 459–71

Sebastian TT, Baldridge RD, Xu P, Graham TR (2012) Phospholipid flippases: building asymmetric membranes and transport vesicles. *Biochim Biophys Acta*, **1821:** 1068–077

Sharma M, Giridharan SS, Rahajeng J, Naslavsky N, Caplan S (2009) MICAL-L1 links EHD1 to tubular recycling endosomes and regulates receptor recycling. *Mol Biol Cell*, **20:** 5181–194

Siggs OM, Arnold CN, Huber C, Pirie E, Xia Y, Lin P, Nemazee D, Beutler B (2011) The P4-type ATPase ATP11C is essential for B lymphopoiesis in adult bone marrow. *Nat Immunol*, **12:** 434–40

Soupene E, Kemaladewi DU, Kuypers FA (2008) ATP8A1 activity and phosphatidylserine transbilayer movement. *J Receptor Ligand Channel Res*, **1:** 1–0

Taguchi T (2013) Emerging roles of recycling endosomes. *J Biochem*, **153:** 505–10

Takatsu H, Baba K, Shima T, Umino H, Kato U, Umeda M, Nakayama K, Shin HW (2011) ATP9B, a P4-ATPase (a putative aminophospholipid translocase), localizes to the trans-Golgi network in a CDC50 protein-independent manner. *J Biol Chem*, **286:** 38159–8167

Tang X, Halleck MS, Schlegel RA, Williamson P (1996) A subfamily of P-type ATPases with aminophospholipid transporting activity. *Science*, **272:** 1495–497

Trost M, English L, Lemieux S, Courcelles M, Desjardins M, Thibault P (2009) The

phagosomal proteome in interferon-gamma-activated macrophages. *Immunity*, **30:** 143–54

Turkmen S, Hoffmann K, Demirhan O, Aruoba D, Humphrey N, Mundlos S (2008) Cerebellar hypoplasia, with quadrupedal locomotion, caused by mutations in the very low-density lipoprotein receptor gene. *Eur J Hum Genet*, **16:** 1070–074

Uchida Y, Hasegawa J, Chinnapen D, Inoue T, Okazaki S, Kato R, Wakatsuki S, Misaki R, Koike M, Uchiyama Y, Iemura S, Natsume T, Kuwahara R, Nakagawa T, Nishikawa K, Mukai K, Miyoshi E, Taniguchi N, Sheff D, Lencer WI, Taguchi T, Arai H (2011) Intracellular phosphatidylserine is essential for retrograde membrane traffic through endosomes. *Proc Natl Acad Sci U S A*, **108:** 15846–5851

van der Velden LM, Wichers CG, van Breevoort AE, Coleman JA, Molday RS, Berger R, Klomp LW, van de Graaf SF (2010) Heteromeric interactions required for abundance and subcellular localization of human CDC50 proteins and class 1 P4-ATPases. *J Biol Chem*, **285:** 40088–0096

Vance JE, Steenbergen R (2005) Metabolism and functions of phosphatidylserine. *Prog Lipid Res*, **44:** 207–34

Vestergaard AL, Coleman JA, Lemmin T, Mikkelsen SA, Molday LL, Vilsen B, Molday RS, Dal Peraro M, Andersen JP (2014) Critical roles of isoleucine-364 and adjacent residues in a hydrophobic gate control of phospholipid transport by the mammalian P4-ATPase ATP8A2. *Proc Natl Acad Sci U S A*, **111:** E1334–3

Villen J, Beausoleil SA, Gerber SA, Gygi SP (2007) Large-scale phosphorylation analysis of mouse liver. *Proc Natl Acad Sci U S A*, **104:** 1488–493

Xu P, Baldridge RD, Chi RJ, Burd CG, Graham TR (2013) Phosphatidylserine flipping enhances membrane curvature and negative charge required for vesicular transport. *The Journal of Cell Biology*, 679

Yabas M, Teh CE, Frankenreiter S, Lal D, Roots CM, Whittle B, Andrews DT, Zhang Y, Teoh NC, Sprent J, Tze LE, Kucharska EM, Kofler J, Farell GC, Broer S, Goodnow CC, Enders A (2011) ATP11C is critical for the internalization of phosphatidylserine and differentiation of B lymphocytes. *Nat Immunol*, **12:** 441–49

Yamashiro DJ, Tycko B, Fluss SR, Maxfield FR (1984) Segregation of transferrin to a mildly acidic (pH 6.5) para-Golgi compartment in the recycling pathway. *Cell*, **37:** 789–00

Yeung T, Gilbert GE, Shi J, Silvius J, Kapus A, Grinstein S (2008) Membrane phosphatidylserine regulates surface charge and protein localization. *Science*, **319:** 210–13

Zachowski A, Henry JP, Devaux PF (1989) Control of transmembrane lipid asymmetry in chromaffin granules by an ATP-dependent protein. *Nature*, **340:** 75–6

Zhou X, Graham TR (2009) Reconstitution of phospholipid translocase activity with purified Drs2p, a type-IV P-type ATPase from budding yeast. *Proc Natl Acad Sci U S A*, **106:** 16586–6591

Zhou X, Sebastian TT, Graham TR (2013) Auto-Inhibition of Drs2p, a Yeast Phospholipid Flippase, by its Carboxyl-Terminal Tail. *J Biol Chem*,

Zhu X, Libby RT, de Vries WN, Smith RS, Wright DL, Bronson RT, Seburn KL, John SW (2012) Mutations in a p-type ATPase gene cause axonal degeneration. *PLoS Genet*, **8:** e1002853

Figure 1

Figure 1. ATP8A1 localizes at REs. (A) Schematic illustration of organelle distribution in COS-1 cells. The Golgi aligns in circle. REs are spatially confined within the Golgi circle, whereas early endosomes (EEs), late endosomes (LEs), and lysosomes are excluded.
(B) COS-1 cells co-transfected with GFP-ATP8A1 and myc-CDC50A were stained for myc-tag and TfnR, TGN46, GM130, VPS26, CD63 or LAMP2. mCherry-Rab11 was expressed in a cell in the top row. Nuclei were stained with DAPI (in blue). Pearson's coefficient between GFP-ATP8A1 and each organelle marker is shown in the merged image (Mean ± SD, n > 20 cells from two experiments). Scale bars, 10 µm.

Figure 2

Figure 2. Flippase activity of ATP8A1 is required for Tfn recycling.
(A) COS-1 cells were treated with ATP8A1 siRNAs for 72 h. Cell lysates were prepared and then immunoblotted with anti-ATP8A1 antibody. α-tubulin was used as a loading control.
(B-D) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h. siRNA-resistant ATP8A1 (WT or E191Q) tagged with GFP and myc-CDC50A were transfected 48 h after siRNA transfection. Cells were pulsed with Alexa 594-Tfn and chased in medium with unlabeled Tfn. During the chase, cells were imaged every 6 min for 54 min. In (C) and (D), the fluorescence intensity of Alexa 594-Tfn is shown as percentage of that at 0 min (Mean ± SEM, n = 4-6; ***P < 0.001; Student's t-test). Scale bars, 10 μm.

Figure 3

Figure 3. ***In vitro*** **ATPase activity and flippase activity of ATP8A1 (WT and E191Q mutant).**
(A) Purification of the ATP8A1-CDC50A complex from HEK293T cells. Wild-type (WT) ATP8A1 and the E191Q mutant each containing a 9 amino acid 1D4 tag were co-expressed with CDC50A in HEK293T cells. The CHAPS-solubilized cell lysate was applied to a Rho1D4-Sepharose matrix. After extensively washing the matrix to remove unbound protein, the ATP8A1-CDC50A complex was eluted with the 1D4 peptide for the experiments in Fig 2D, 4A and 4B. The cell lysate (input) and peptide eluted fraction (elution) were analyzed on SDS gels stained with Coomassie blue or by Western blots with anti-Rho1D4 antibody (ATP8A1) and anti-CDC50A antibody (CDC50A). Both the WT and E191Q mutant were expressed and co-purified with CDC50A at similar levels. Please be noted that CDC50A is not readily detected by Coomassie Blue stain because of it highly glycosylated extracellular domain (Coleman & Molday, 2011).
(B) The effect of increasing levels of 1,2-dioleoyl-PS on the ATPase activity of purifed ATP8A1 (WT and E191Q)-CDC50A complex. The $K_{0.5}$ and Vmax for the WT complex were 59.1 ± 3.5 μM and 38.7 ± 0.8 μmol ATP/min/mg protein, respectively.
(C) NBD-labeled PS or PE transport (flippase) activity of ATP8A1 (WT and E191Q)-CDC50A complex. The percentage of NBD-lipid flipped was shown.

Figure 4

Figure 4. CTxB retrograde traffic from REs to Golgi is impaired upon ATP8A1 knockdown. (A, B) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h. Cells were pulsed with Alexa 594-CTxB for 5 min and chased for 15 or 60 min. Cells were then fixed and stained for GM130. In (A), representative images are shown. In (B), the Pearson's coefficient between CTxB and GM130 after 60-min chase is shown. Data represent mean ± SD (n > 26 cells from three independent experiments). ***P < 0.001; two-tailed Student's t-test.
Scale bars, 10 µm.

Figure 5

Figure 5. EGF-dependent EGFR degradation is blocked upon ATP8A1 knockdown.
(A) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h. Cells were stimulated with 100 ng/mL EGF for 5 min, washed, and chased for the indicated time. Cell lysates were prepared and then immunoblotted with anti-EGFR antibody. α-tubulin was used as a loading control.
(B) The ratio of EGFR blot to α-tubuin blot was quantified and shown as percentage of that of control siRNA (0 min chase). Values are mean of two independent experiments.

Figure 6

Figure 6. ATP8A1 knockdown does not affect EGF traffic to lysosomes.
(A) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h. Cells were pulsed with Alexa 555-EGF for 5 min, washed, and chased for 60 min. Cells were then fixed, permeabilized, and stained for LAMP1. Magnified images of boxed regions were shown in the bottom row. Scale bars, 10 µm.
(B) Pearson coefficient between EGF and LAMP1 after 60 min chase is shown as mean ± SD (n = 29 cells from two independent experiments). P value was determined with Student's two-tailed t-test.

Figure 7

Figure 7. Flippase activity of ATP8A1 is essential for fission of TfnR-positive membrane carriers.

(A) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h and stained for TfnR. Red dashed lines and arrowheads indicate nuclei and TfnR puncta, respectively.

(B) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h. siRNA-resistant ATP8A1 (WT or E191Q) and myc-CDC50A were transfected 48 h after siRNA transfection. Cells were then fixed and stained for TfnR. The graph shows percentages of cells showing aberrant TfnR-positive tubules as mean ± SD of three independent experiments. At least 50 cells were counted per experiment.

(C) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h and stained for the indicated proteins. Scale bars, 10 μm.

Figure 8

Figure 8. TfnR-positive tubules in ATP8A1-depleted cells depends on the integrity of microtubules. COS-1 cells were treated with ATP8A1 siRNA1 for 72 h. Cells were then incubated in medium with 10 μM nocodazole for 5 min, washed, fixed, and stained for TfnR and GM130. The images represent the maximum intensity z-projection of four confocal slices. Scale bars, 10 μm.

Figure 9

Figure 9. TfnR-GFP in live cells.
(A and B) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h. TfnR-GFP was transfected 48 h after siRNA transfection. Cells were then imaged with an epifluorescent microscope. In (B), selected stills are shown and the asterisks indicate the tips of the tubules of interest.
Scale bars, 10 µm (A); 5 µm (B).

Figure 10

Figure 10. Recycling traffic and retrograde traffic is impaired upon EHD1 knockdown.
(A) COS-1 cells were treated with EHD1 siRNAs for 72 h. Cell lysates were prepared and then immunoblotted with anti-EHD1 antibody.
(B, C) Cells were treated with EHD1 siRNA1 for 72 h. Cells were pulsed with Alexa 488-Tfn and chased in medium with unlabeled Tfn. In (C), the fluorescence intensity of Alexa 488-Tfn is shown as percentage of that at 0 min (Mean ± SEM, $n > 3$ cells from three independent experiments). ***$P < 0.001$; two-tailed Student's *t*-test.
(D, E) COS-1 cells were treated with EHD1 siRNA1 for 72 h. Cells were pulsed with Alexa 594-CTxB for 5 min and chased for 60 min. Cells were then fixed and stained for GM130. In (D), the Pearson's coefficient between CTxB and GM130 after 60-min chase is shown. Data represent mean ± SD (n > 26 cells from three independent experiments). ***$P < 0.001$; two-tailed Student's t-test.
Scale bars, 10 µm.

Figure 11

Figure 11. EHD1 knockdown causes the formation of aberrant TfnR-positive tubules.
(A) COS-1 cells were treated with EHD1 siRNA1 or siRNA2 for 72 h. Cells were then fixed and stained for TfnR. The graph shows percentages of cells showing aberrant TfnR-positive tubules as mean ± SD of three independent experiments. At least 20 cells were counted per experiment. Scale bars, 10 µm.
(B) COS-1 cells were transfected with EHD1 siRNA1 and TfnR-GFP. Seventy-two hours after transfection, cells were imaged with an epifluorescent microscopy. Selected stills are shown. The tubule indicated by red asterisks lingered over 10 sec. Scale bars, 5 µm.

Figure 12

Figure 12. EHD1 localizes at REs in an ATP8A1-activity dependent manner .
(A) COS-1 cells co-transfected with GFP-ATP8A1 and myc-CDC50A were stained for EHD1.
(B) COS-1 cells were treated with ATP8A1 siRNA1 for 72 h and stained for EHD1. For the rescue experiments, siRNA-resistant GFP-ATP8A1 (WT or E191Q) and myc-CDC50A were transfected 48 h after siRNA transfection. Asterisks indicate cells with GFP-ATP8A1 expression. The graph represents percentage of cells with perinuclear localization of EHD1 as mean ± SD of three independent experiments (at least 141 cells were counted for each condition).
Scale bars, 10 µm.

Figure 13

Figure 13. EHD1 localization is dependent on cellular PS levels.
(A) The effect of various lipids on the ATPase activity of the purified ATP8A1-CDC50A complex. The ATPase activity was measured in the presence of 5 mM ATP and 10 mol % of one of the following lipids: 1,2-dioleoyl-phosphatidycholine (PC), 1,2-dioleoyl-PS, 1,2-dioleoyl-PE, 1,2-dioleoyl-phosphatidylglycerol (PG), 1,2-dioleoyl-phosphatidylinositol (PI), 1,2-dioleoyl-phosphatidic acid (PA), sphingomyelin (SM), cholesterol (Chol), or brain polar lipid (BPL) and 90% PC.
(B) Schematic of the biosynthetic pathway of PS in mammalian cells. The impaired pathways in PSA-3 and R-41 cells are indicated.
(C, D) PSA-3 and R-41 cells were transiently transfected with GFP-EHD1 and then cultured under the indicated conditions for 72 h. Cells were then fixed and the nuclei was strained with DAPI. Arrowheads indicate the perinuclear RE localization of GFP-EHD1. Scale bars, 10 μm.

Figure 14

Figure 14. EHD1 binds PS *in vitro*.
(A) His-tagged EHD1 was mixed with PC-based liposomes containing the indicated % (mol/mol) of PS or PE. After 15 min, the mixture was spun at 100,000 g for 30 min, and the resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The proteins and lipids were then stained with Coomassie blue.
(B, C) The intensities of individual bands in (A) were quantified with ImageJ, and the percentage of bound proteins was calculated. In (B), the data represent the mean ± SD of n = 3 independent experiments, and were analyzed with one-way analysis of variance (ANOVA) followed by Tukey-Kramer post-hoc test. *P < 0.01; NS, not significant. In (C), each point represents the mean of two independent experiments with an error bar showing the range of values obtained. The sigmoidal fitting was applied.

Figure 15

Figure 15. Liposome size determined by dynamic light scattering (DLS).
PC-based liposomes containing 20, 40, or 60% PS were extruded through 400 or 100 nm filters or stored at 4°C without extrusion (pre-extrusion). Liposome size was then determined by DLS. In (A), representative data of size distribution of liposomes based on intensity are shown. In (B), the average size of liposomes is shown (mean of two independent experiments).

Figure 16

Figure 16. EHD1 binding to PS is dependent on liposome membrane curvature.
His-tagged EHD1 was mixed with PC-based liposomes (20, 40, or 60% PS) of decreasing size produced by extrusion through filter pores of the size indicated. After 15 min, the mixture was spun at 100,000 g for 30 min, and the resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The proteins and lipids were then stained with Coomassie blue. The graph shows the intensities of individual bands (mean ± SEM of three independent experiments)

Figure 17

Figure 17. F322 and K324 in helical domain are required for EHD1 localization to REs, but EH domain is not.

(A) Schematic diagram of EHD1 protein domain architecture.

(B) COS-1 cells were transiently transfected with GFP-EHD1 (WT or mutants) and were then fixed. Magnified images of the boxed regions are shown in the bottom row. Arrowheads indicate GFP-EHD1-positive tubular structures. Scale bars, 10 μm.

Figure 18

Figure 18. EHD1 (K324D) does not bind PS *in vitro*.
(A) Recombinant His-tagged EHD1 (WT, K324D, or ΔEH) was mixed with liposomes containing the indicated % (mol/mol) of lipids. After 15 min, the mixture was spun at 100,000 g for 30 min, and the resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The proteins and lipids were then stained with Coomassie blue.
(B) The intensities of individual bands in (A) were quantified with ImageJ, and the percentage of bound proteins was calculated. Data represent mean of two independent experiments.

Figure 19

Figure 19. PI(4)P or PI(4,5)P_2 affects EHD1 binding to liposomes containing 10% PS.
(A and B) His-tagged EHD1 was mixed with liposomes containing the indicated % (mol/mol) of lipids. After 15 min, the mixture was spun at 100,000 g for 30 min, and the resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The proteins and lipids were then stained with Coomassie blue. In (A), "60% PI(4,5)P_2" liposomes consist of 40% PC and 60% PI(4,5)P_2. In (B), "10% PI(4)P or PI(4,5)P_2, 10% PS" liposomes consist of 35% PC, 35% PE, 10% PS, 10% cholesterol, and 10% PI(4)P or PI(4,5)P_2, respectively. "10% PI(4)P or PI(4,5)P_2, 0% PS" liposomes consist of 45% PC, 35% PE, 0% PS, 10% cholesterol, and 10% PI(4)P or PI(4,5)P_2, respectively.
(C) The intensities of individual bands in (B) were quantified with ImageJ, and the percentage of bound proteins was calculated. Data represent the mean ± SD of $n = 3$ independent experiments, and were analyzed with Student's t-test. $^{**}P < 0.01$; $^{***}P < 0.001$; NS, not significant.

Figure 20

Figure 20. Subcellular localization of several PIPs.
(A) GFP-FYVE (EEA1), GFP-PH (OSBP), GFP-PH (PLCδ), and GFP-PH (GRP1) were transiently expressed in COS-1 cells for the detection of PI(3)P, PI(4)P, PI(4,5)P_2, and PI(3,4,5)P_3, respectively. Cells were then fixed and stained for GM130 (*cis*-Golgi) or ARF1 (*trans*-Golgi). Magnified images around the REs/Golgi region are shown in the right column. Arrowheads indicate the signals at membrane ruffles.
(B) COS-1 cells were fixed and stained with anti-PI(4)P and anti-syntaxin 5 (Golgi) antibodies. A magnified image is shown in the right panel. Scale bars, 10 μm.

Figure 21

Figure 21. 2xPH (evt-2) binds specifically to PS and stains REs.
(A) His-tagged 2xPH was mixed with liposomes that were composed of 75% (mol/mol) PE, 20% (mol/mol) PC, and 5% (mol/mol) of PS, PA, PI, PIPs, or sulfatide. In the case of PE/PC, liposomes of 80% (mol/mol) PE and 20% (mol/mol) PC were used. After 15 min, the mixture was spun at 100,000 g for 30 min, and the resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The gels were stained with Coomassie blue.
(B) COS-1 cells were incubated in medium containing Alexa 488-Tfn for 40 min, washed, fixed, and stained with recombinant 2xPH. Magnified image is shown in the right panel.
(C) COS-1 cells were transiently transfected with GFP-lact-C2 and stained for Rab11.
Scale bars, 10 µm.

Figure 22

Figure 22. An assay to distinguish PS in both leaflets of endomembrane.
(A) COS-1 cells were fixed, permeabilized with streptolysin O or saponin, and stained with anti-TfnR (13E4) and anti-syntaxin 5 antibodies. Anti-syntaxin 5 antibody recognizes the cytosolic region of syntaxin 5. Anti-TfnR antibody was raised against the soluble TfnR, thus recognizes the luminal region of TfnR. In cells treated with streptolysin O, syntaxin5, but not TfnR was stained at the perinuclear region, showing that streptolysin O permeabilized PM, but not RE membranes. In contrast, in cells treated with saponin, perinuclear RE staining of TfnR was observed, showing that saponin permeabilized RE membranes. Scale bars, 10 µm.
(B) Schematic of the assay to distinguish PS in both leaflets of endomembrane. GFP-lact-C2 expressed in the cytosol binds PS in the cytosolic leaflet, but not in the luminal leaflet. Cells were then fixed, permeabilized with saponin, and treated with recombinant 2xPH-His. The stain with 2xPH-His represents PS in the luminal leaflet.

Figure 23

Figure 23. ATP8A1 knockdown causes an increase in RE luminal PS Levels.
(A, B) COS-1 cells were transiently transfected with GFP-lact-C2 and stained with recombinant 2xPH and anti-GM130 antibody. The asterisk indicates a cell expressing GFP–lact-C2, and the arrowhead indicates a non-expressing cell. The graph in (B) shows fluorescence intensity per pixel of recombinant 2xPH stain in RE area, which was delimited by GM130. Data are normalized to the 2xPH intensity in GFP-lact-C2 (-) cells (Mean ± SD, n = 13 cells for each condition).
(C-E) COS-1 cells stably expressing GFP-lact-C2 were treated with ATP8A1 siRNA1 or EHD1 siRNA1 for 72 h and stained with recombinant 2xPH and anti-GM130 antibody. The intensities of 2xPH staining and GFP-lact-C2 in RE area were measured. The intensity of 2xPH (D) and the ratio of 2xPH to GFP-lact-C2 intensities (E) are shown. In (E), data are normalized to that of control cells. Data represent mean ± SD, and were analyzed with two-tailed Student's t test (n > 26 cells from three independent experiments). **P < 0.01; ***P < 0.001; NS, not significant. Scale bars, 10 μm.

Figure 24

Figure 24. ATP8A2 localizes at REs in a CDC50A-dependent manner.
COS-1 cells transfected with GFP-ATP8A2 and with or without myc-CDC50A were stained for myc-tag and TfnR. Scale bars, 10 µm.

Figure 25

Figure 25. Wild-type ATP8A2, but not a disease-associated mutant (I376M), can compensate for the loss of ATP8A1 function.
(A) The effect of increasing levels of PS on the ATPase activity of ATP8A2 (WT and I376M)-CDC50A complex.
(B) NBD-labeled PS transport (flippase) activity of ATP8A2 (WT and I376M)-CDC50A complex. The percentage of NBD-lipid flipped was shown.
(C) COS-1 cells transfected with GFP-ATP8A2 (I376M) and with or without myc-CDC50A were stained for myc-tag and TfnR. Scale bars, 10 μm.
(D) Cells were treated with ATP8A1 siRNA1 for 72 h. ATP8A2-1D4 (WT or mutants) and myc-CDC50A were transfected 48 h after siRNA transfection. The cells were then stained for EHD1 and 1D4-tag. The graph shows percentages of cells with perinuclear EHD1 localization. Data represent mean ± SD of three or four independent experiments. At least 20 cells were counted per experiment.
(E) Cells were treated with ATP8A1 siRNA for 72 h. GFP-ATP8A2 (WT or mutant) and myc-CDC50A were transfected 48 h after siRNA transfection. The cells were then stained for TfnR. The graph shows percentages of cells showing aberrant TfnR-positive tubules as mean ± SD of three or four independent experiments. At least 20 cells were counted per experiment.

Figure 26

Figure 26. Cell surface localization of TfnR is impaired in *Atp8a2-/-* cortical neurons.
(A, B) Surface biotinylation assay with *Atp8a2+/+* or *Atp8a2-/-* cortical neurons. The ratio of surface to total TfnR was quantified and normalized to *Atp8a2+/+*. Data represent mean value (2 mice for each genotype).
(C) Whole cell lysate from *Atp8a2+/+* or *Atp8a2-/-* cortical neurons were prepared and immunoblotted with anti-ATP8A1 antibody and anti-α-tubulin antibody.

Figure 27

Figure 27. ATP8A1 knockdown does not affect RE localization of evectin-2.
COS-1 cells were transfected with ATP8A1 siRNA1 and GFP-evectin-2. Cells were fixed and stained for TfnR. Scale bars, 10 µm.

Figure 28

Figure 28. ATP8A1 is phosphorylated at S25/S29.
(A) HEK293A cells were transfected with myc-CDC50A alone (GFP-ATP8A1(-)) or with GFP-ATP8A1 (WT or mutants) and myc-CDC50A. Cells were then labeled with [^{32}P]orthophosphate for 4 h. Cell lysates were immunoprecipitated with anti-GFP antibody folllowed by autoradiography and immunoblot with anti-ATP8A1 antibody.
(B) The ratio of ^{32}P intensity to ATP8A1 immunoblot intensity in IP samples in (A) were quantified and shown as a percantage of GFP-ATP8A1 (WT).

Table 1

Serine

Position	Sequence	Score	Phosphoproteome
9	RRTV**S**EIRS	0.996	Yes
13	SEIR**S**RAEG	0.757	
25	TDDV**S**EKTS	0.992	Yes
29	SEKT**S**LADQ	0.993	Yes
173	LISL**S**SSEP	0.907	
174	ISLS**S**SEPQ	0.816	
175	SLSS**S**EPQA	0.699	
186	YIET**S**NLDG	0.649	
204	LPAT**S**DIKD	0.77	
211	KDVD**S**LMRI	0.932	
216	LMRI**S**GRIE	0.895	
223	IECE**S**PNRH	0.958	
394	MART**S**NLNE	0.918	
459	EKTF**S**DSSL	0.835	
462	FSDS**S**LLEN	0.962	
505	YQAA**S**PDEG	0.996	
529	RTPD**S**VIID	0.666	
557	RKRM**S**VIVR	0.978	
564	VRTP**S**GKLR	0.977	Yes
586	LAET**S**KYKE	0.751	
615	VAEI**S**ESDF	0.935	
630	YQRA**S**TSVQ	0.715	
643	KLEE**S**YELI	0.786	
698	NIGH**S**CKLL	0.946	
724	RETL**S**RHCT	0.976	
777	CCRV**S**PLQK	0.905	
1091	LEAK**S**QDPG	0.844	
1102	VLGK**S**LTER	0.562	
1126	YRSE**S**LQQN	0.529	Yes
1146	NGIV**S**QSEV	0.736	

Table 1. Phosphorylated sites in ATP8A1 cytoplasmic domains predicted by NetPhos 2.0.
Phosphorylation site in the cytoplasmic domains of ATP8A1 was predicted by NetPhos 2.0 with a threshold score >0.5. The phosphorylated residue is shown in bold.
In "Phosphoproteome" column, "Yes" indicates that phosphorylation at the site has been reported in PhosphoSitePlus database.

Threonine

Position	Sequence	Score	Phosphoproteome
3	MP**T**MRRT	0.753	
7	TMRR**T**VSEI	0.56	Yes
387	EPTD**T**AAMA	0.577	Yes
523	NFVF**T**GRTP	0.52	
526	FTGR**T**PDSV	0.796	
562	VIVR**T**PSGK	0.519	
585	RLAE**T**SKYK	0.608	
592	YKEI**T**LKHL	0.805	
631	QRAS**T**SVQN	0.805	
673	ETIE**T**LMKA	0.68	
722	GTRE**T**LSRH	0.637	
728	SRHC**T**TLGD	0.587	
729	RHCT**T**LGDA	0.74	
811	SMIQ**T**AHVG	0.599	Yes
1079	TAFKTLVDE	0.676	
1156	RAYDTTKQR	0.958	

Tyrosine

Position	Sequence	Score	Phosphoproteome
18	RAEG**Y**EKTD	0.961	Yes
59	STAK**Y**NIIT	0.72	
229	NRHL**Y**DFVG	0.513	
440	EPED**Y**GCSP	0.976	
541	QEER**Y**ELLN	0.967	
579	DTVI**Y**DRLA	0.587	
588	ETSK**Y**KEIT	0.955	
644	LEES**Y**ELIE	0.917	
832	NSSD**Y**SIAQ	0.852	
923	NMLK**Y**PELY	0.701	
927	YPEL**Y**KTSQ	0.78	

Figure 29

Figure 29. Phosphorylation level of ATP8A1 (E191Q) is comparable with that of WT ATP8A1 .
HEK293A cells were transfected with myc-CDC50A alone (GFP-ATP8A1(-)) or with GFP-ATP8A1 (WT or mutants) and myc-CDC50A. Cells were then labeled with [^{32}P]orthophosphate for 4 h. Cell lysates were immunoprecipitated with anti-GFP antibody, folllowed by autoradiography and immunoblot with anti-ATP8A1 antibody.

Figure 30

Figure 30. Anti-phosphospecific ATP8A1 pS29 antiserum detected phosphorylation of ATP8A1 at Ser29.
HEK293A cells were transfected with GFP-ATP8A1 (WT or S29A) and myc-CDC50A and whole cell lysates were prepared. For phosphatase treatment, cell lysates were incubated at 37°C for 1 h in the presence of alkaline phosphatase. Cell lysates were then immunoblotted with anti-ATP8A1 antibody and anti-ATP8A1 pS29 antiserum. Arrowheads indicate the band that corresponds to phosphorylated GFP-ATP8A1 WT.

Figure 31

Figure 31. ***In vitro*** **ATPase acivity of ATP8A1 (S29A and S25A/S29A).**
(A) The effect of increasing levels of 1,2-dioleoyl-PS on the ATPase activity of purifed ATP8A1 (WT and mutants)-CDC50A complex. Values are mean ± SD of three independent experiments.
(B) $K_{0.5}$ values (concentration at half Vmax) for activation of ATPase activity were determined from the rising phase of the PS concentration dependence. Values are mean ± SD of three independent experiments. *$P < 0.05$ (Student's *t*-test).

Figure 32

Figure 32. ATP8A1 (S25A and S29A) mutants localize at REs in a CDC50A-dependent manner. COS-1 cells transfected with ATP8A1 (WT or mutants) and with or without myc-CDC50A were stained with anti-ATP8A1 antibody and anti-TfnR antibody. Scale bars, 10 µm.

Figure 33

Figure 33. ATP8A1 (S25A and S29A) cannnot rescue the knockdwon of ATP8A1. COS-1 cells were treated with ATP8A1 siRNA for 72 h. ATP8A1 (WT or mutant) and myc-CDC50A were transfected 48 h after siRNA transfection. The cells were then stained for TfnR. Data represent mean ± SD of three independent experiments. At least 50 cells were counted per experiment.

Figure 34

Figure 34. Phosphorylation of ATP8A1 is inhibited by CK1 inhibitor D4476.
HEK293A cells were transfected with myc-CDC50A alone (GFP-ATP8A1(-)) or with GFP-ATP8A1 (WT or mutants) and myc-CDC50A. Cells were treated with 25 or 100 μM D4476 for 1 h. Cells were then labeled with [^{32}P]orthophosphate in the presence 25 or 100 μM D4476 of for 4 h. Cell lysates were immunoprecipitated with anti-GFP antibody folllowed by autoradiography and immunoblot with anti-ATP8A1 antibody. The graph shows the ratio of ^{32}P intensity to ATP8A1 immunoblot intensity in IP samples as a percantage of GFP-ATP8A1 (WT). Values are mean of two independent experiments.

Figure 35

Figure 35. D4476 treatment causes aberrant TfnR tubule formation.
COS-1 cells were treated with DMSO or 100 μM D4476 for 20 min and stained for TfnR. Scale bars, 10 μm.

Figure 36

Figure 36. RNAi screening to explore a candicate for the kinase responsible for ATP8A1 phosphorylation.
COS-1 cells were treated with indicated siRNA and stained for TfnR. Arrowheads indicate aberrant tubular localizations of TfnR.
Scale bars, 10 μm.

Figure 37

Figure 37. CK1α knockdown causes aberrant TfnR tubule formation.
(A) COS-1 cells were treated with indicated siRNA and cell lysate was immunoblotted with anti-CK1α antibody.
(B) COS-1 cells were treated with indicated siRNA and stained for TfnR. Arrowheads indicate abnormal tubular localization of TfnR.
Scale bars, 10 μm.

Figure 38

Figure 38. CK1α knockdown does not affect localizations of GFP-ATP8A1, VPS26 (EEs) and syntaxin 5 (Golgi).
(A) COS-1 cells were treated with CK1α siRNA1 and transfected with GFP-ATP8A1 and myc-CDC50A (48 h after siRNA transfection), and stained with TfnR.
(B, C) COS-1 cells were treated with CK1α siRNA1 and stained with VPS26, TfnR and syntaxin 5.
Scale bars, 10 μm.

Figure 39

Figure 39. EHD1 (ΔEH) binds PS *in vitro*. (Perfomed by S. Sugahara in Arai lab)
(A) His-tagged EHD1 (WT or ΔEH mutant) was mixed with PC-based liposomes containing the indicated % (mol/mol) of PS or PIPs. After 15 min, the mixture was spun at 100,000 g for 30 min, and the resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The proteins and lipids were then stained with Coomassie blue. The graph shows the percentage of bound proteins calculated with ImageJ.
(B) His-tagged EHD1 (WT or ΔEH mutant) was mixed with PC-based liposomes containing the indicated % (mol/mol) of PS. After 15 min, the mixture was spun at 100,000 g for 30 min, and the resultant supernatant (S) and pellet (P) were subjected to SDS-PAGE. The proteins and lipids were then stained with Coomassie blue. The graph shows the percentage of bound proteins calculated with ImageJ. Each point represents the mean of two independent experiments with an error bar showing the range of values obtained. The sigmoidal fitting was applied.

Figure 40

Figure 40. Membrane localization of EHD1 (ΔEH) depends on cellular PS levels. PSA-3 cells were transiently transfected with GFP-EHD1 (ΔEH) and then cultured under the indicated conditions for 72 h. Cells were then fixed and the nuclei was strained with DAPI. Scale bars, 10 µm.

Figure 41

Figure 41. RE localization of EHD1 (ΔEH) is abolished upon CK1α knockdown.
COS-1 cells stably expressing GFP-EHD1 (ΔEH) were treated with indicated siRNA and stained for TfnR. Scale bars, 10 µm.

Figure 42

Figure 42. RE localization of GFP-lact-C2 is not obviously affected by CK1α knockdwon. COS-1 cells stably expressing GFP-lact-C2 were treated with CK1α siRNA1 and stained with DAPI. Scale bars, 10 μm.

Figure 43

Figure 43. Subcellular localizations of GFP-CK1α.
(A) Schematic diagram of GFP-tagged CK1αS and CK1αLS. Note that CK1αLS has a nuclear localization signal in L insert.
(B) COS-1 cells were transfected with GFP-CK1αS or GFP-CK1αLS and stained with TfnR. Arrowheads in the magnified images indicate the perinuclear accumulation of GFP-CK1α, which seems to be a centrosomal localization. Scale bars, 10 μm.

Figure 44

Figure 44. 14-3-3 proteins were not co-immunoprecipitated with GFP-ATP8A1.
HEK293A cells were transfected with myc-CDC50A alone (GFP-ATP8A1(-)) or with GFP-ATP8A1 (WT or mutants) and myc-CDC50A. Cells lysates were immunoprecipitated with anti-GFP and immunoblotted with anti-ATP8A1, anti-myc, or anti-pan-14-3-3 antibody.