Current Topics

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Review

Regulatory Roles of N- and C-Terminal Cytoplasmic Regions of P4-ATPases

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Received January 24, 2022

P4-ATPases, which are subfamily members of P-type ATPase superfamily, translocate membrane lipids from the exoplasmic/luminal leaflet to the cytoplasmic leaflet, thus regulating *trans*-bilayer lipid asymmetry. Mammalian P4-ATPases localize to the specific subcellular organelles or the plasma membrane where they translocate the specific lipids. Although recent advances in the structural analysis of P4-ATPases have improved our understanding of lipid transporting machinery, the mechanism of substrate specificity and the regulatory mechanism of the enzymes remain largely unknown. Recent studies have uncovered several specific localization and regulatory mechanisms of P4-ATPases. Here, we review the current understanding of the regulatory mechanism of P4-ATPase activity and localization in mammalian cells.

Key words lipid bilayer; lipid asymmetry; P4-ATPase; flippase

1. Introduction

Cellular membranes exhibit transbilayer lipid asymmetry. In the plasma membrane of mammalian cells, the aminophospholipids phosphatidylserine (PS) and phosphatidylethanolamine (PE) are restricted to the cytoplasmic leaflet, whereas phosphatidylcholine (PC), sphingomyelin, and glycosphingolipids are enriched in the exoplasmic leaflet.[1–5] This transbilayer lipid asymmetry is generated and regulated by lipid transporters, such as flippases, floppases, and scramblases. Flippases and floppases translocate membrane lipids vectorially from the exoplasmic/luminal to the cytosolic leaflet and in the opposite direction, respectively, in an ATP-dependent manner. Scramblases transport lipids both directions of the bilayers in an ATP-independent manner.[6–8)]

Members of the P4-ATPase family—a subfamily of P-type ATPase superfamily— translocate (flip) membrane lipids from the exoplasmic or luminal leaflet to the cytosolic leaflet of cellular membranes, whereas other P-type ATPases mostly transport cations that regulate cation gradients across membranes.[7–10] Recently, it has been reported that P5B-ATPase (ATP13A2) transports polyamines across lysosomal membranes, and P5A-ATPase (ATP13A1) can dislocate tail-anchored proteins in the endoplasmic reticulum (ER).[11,12] Among P4-ATPases, ATP8A1 and ATP8A2 preferentially translocate PS[13,14] (Table 1). ATP8B1, ATP8B2, and ATP10A preferentially flip PC at the plasma membrane,[15,16] and ATP8B1 is

Table 1. Substrates of Human P4-ATPases

Clade	Mammalian P4-ATPase	Substrate				Ref
		ATPase assay	Microsome or liposome-based flippase assay	Cell-based flippase assay		
				Transported	Failed to transport	
P4A	ATP8A1	PS >> PE	PS			13)
	ATP8A2	PS	PS			14,46,90)
	ATP8B1			PC, (PS?)	PS, PE, SM, GlcCer	15,17)
	ATP8B2			PC	PS, PE, SM, GlcCer	15)
	ATP8B3					
	ATP8B4				PS, PE, PC, SM, GlcCer	15,19)
	ATP10A			PC >> GlcCer	PS, PE, SM	16)
	ATP10B	PC, GlcCer	PC, GlcCer			20)
	ATP10D			GlcCer	PS, PE, PC, SM	19)
	ATP11A	PS > PE	PS >> PE	PS, PE	PC, SM, GlcCer	15,21,46)
	ATP11B	PS > PE	PS >> PE			21)
	ATP11C	PS > PE	PS > PE	PS > PE	PC, SM, GlcCer	15,21,46)
P4B	ATP9A	Unknown	Unknown	Unknown		
	ATP9B	Unknown	Unknown	Unknown		

PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin; GlcCer, Glucosylceramide.

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also shown to flip PS[17] and cardiolipin.[18] ATP10D specifically flips a glycosphingolipid, glucosylceramide (GlcCer),[19] and ATP10B has been shown to flip PC and GlcCer.[20] ATP10A is also able to flip GlcCer but to a much lesser extent compared with PC.[19] ATP11A, ATP11B, and ATP11C flip PS and PE[15,21] (Table 1).

P4-ATPases are composed of ten transmembrane domains and harbor three cytoplasmic domains [A (actuator), P (phosphorylation), and N (nucleotide binding)] which are crucial for their ATPase activity. The N- and C-terminal regions of P4-ATPases face cytosol[22,23] and those regions are recently shown to play an important regulatory role in their activities and localizations. In this review, we focus on the regulatory roles of the N- and C-terminal cytoplasmic regions of P4-ATPases.

2. ATPase Reaction Cycle

The P4-ATPase transport process takes place according to the Post-Albers cycle, similarly to other P-type ATPase proteins, such as P2-ATPases (Na^+/K^+-ATPase, Ca^{2+}-ATPase, and H^+/K^+-ATPase),[24–26] which involve inward-directed E1, E1P, and outward directed E2P and E2 intermediates (Fig. 1). Although P2-ATPases transport two different substrates (ions) across membranes during the ATPase cycle (Fig. 1A), P4-ATPases seem to transport a substrate (lipid) from the extracellular/luminal to the cytosolic leaflet[27,28] (Fig. 1B). Three cytoplasmic domains, actuator (A), phosphorylation (P), and nucleotide binding (N) domains (Figs. 1, 2), are reorganized during the conformation changes among E1, E1P, E2P, and E2 states, and these conformation changes allow the transport of substrates across membranes through the transmembrane domains (TMs) (Fig. 1). The N domain binds and positions ATP for phosphorylation of the aspartic acid (Asp) residue of a universally conserved DKTGT motif in the P domain, thus forming a high-energy intermediate (Figs. 2A, B). Placement of a water molecule by the glutamic acid (Glu) residue of a conserved [D/T]GE[S/T] motif in the A domain leads to self-dephosphorylation of the P domain (Figs. 2A, B). The TM1-TM6 helices mainly contribute to substrate transport (Figs. 2B, C, orange), and the TM7-TM10 helices form a supporting domain (gray).

3. Exit from the ER

In the human genome, fourteen P4-ATPases are encoded, whereas *Saccharomyces cerevisiae* includes five P4-ATPases (Drs2p, Dnf1p, Dnf2p, Dnf3p, and Neo1p). P4-ATPases are divided into three clades, P4A, P4B, and P4C.[10] (Table 1) P4A ATPases require interaction with chaperone-like CDC50 proteins to exit from the ER and target the appropriate cellular compartments and the plasma membrane[8,29–31] (Figs. 2B, C). There are two CDC50 proteins in humans, CDC50A and B, and three in yeast, Cdc50p, Lem3p, and Crf1p.[32] On the other hand, P4B ATPases (ATP9A, ATP9B, and *sc*Neo1p) do not interact with CDC50 proteins and do not require the

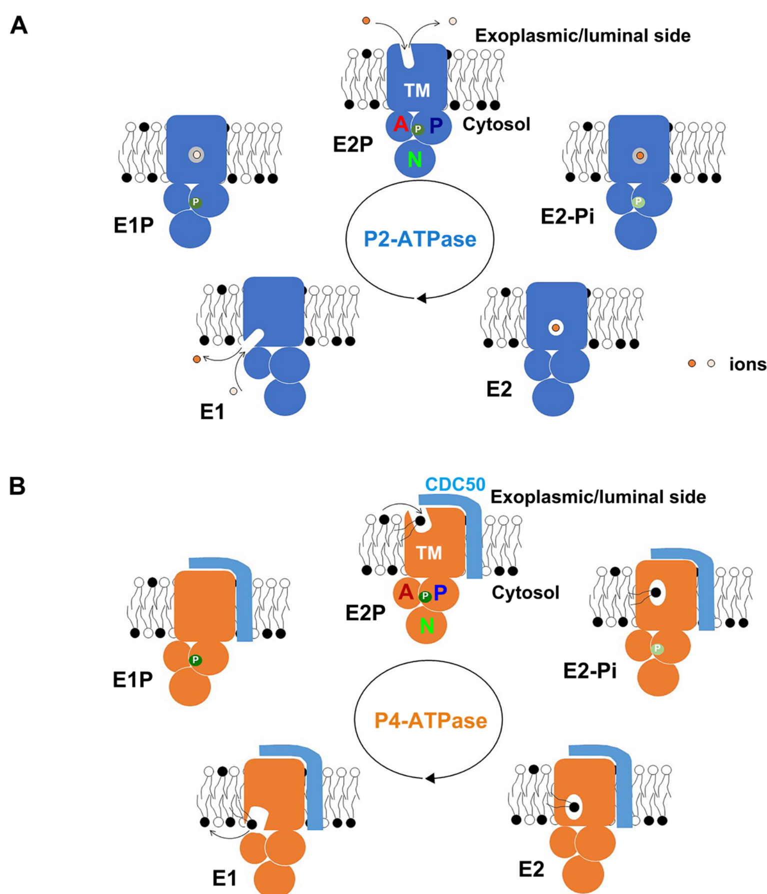

Fig. 1. ATPase Reaction Cycle of P2-ATPase and P4-ATPase

(A) P2-ATPases transport two different substrates (ions) across membranes during the ATPase reaction cycle. **(B)** P4-ATPases seem to transport a substrate (lipid) from the extracellular/luminal to the cytosolic leaflet. A, actuator domain; P, phosphorylation domain; N, nucleotide binding domain; TM, transmembrane domain.

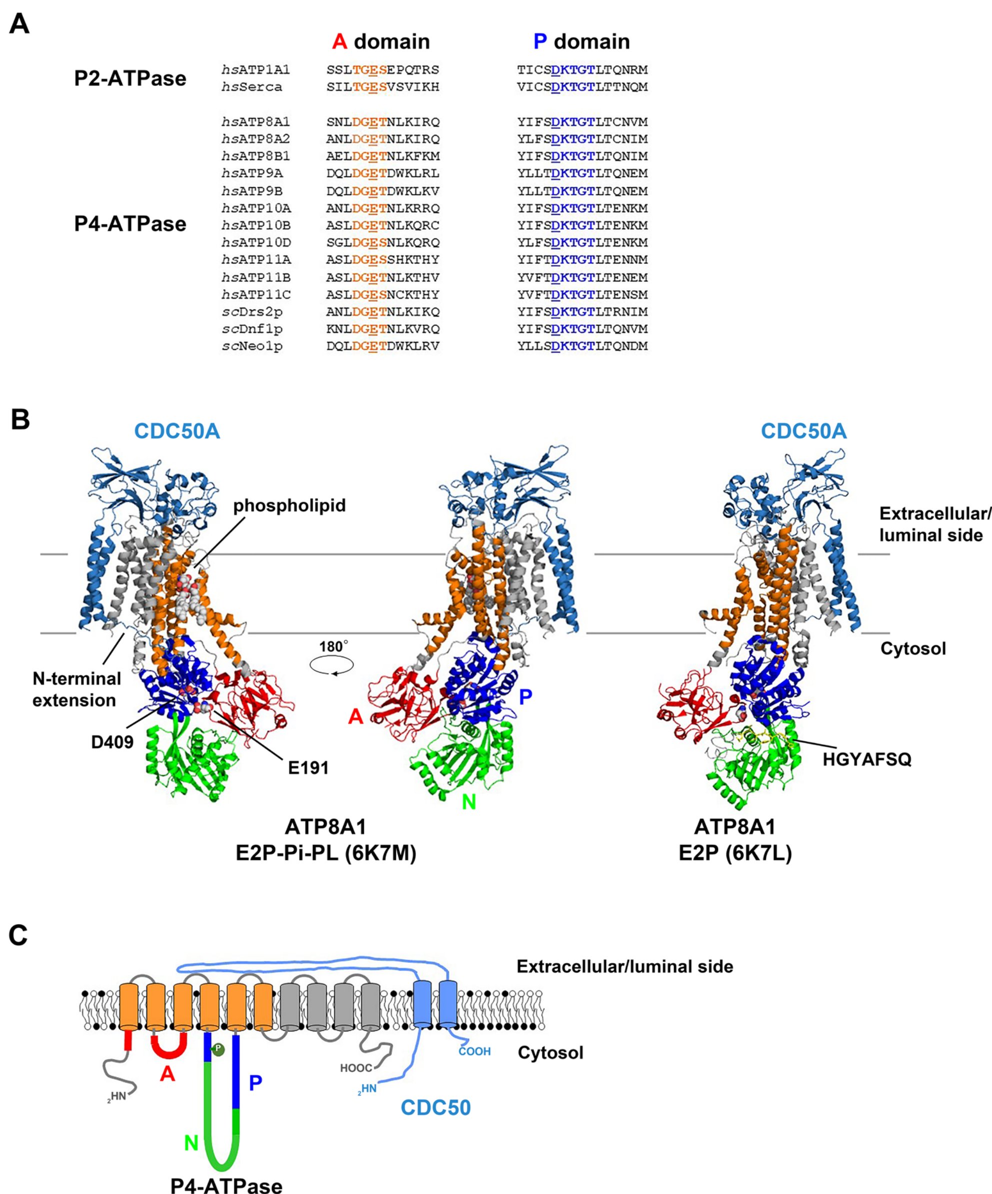

Fig. 2. Structure of P4-ATPase

(A) Conserved motifs of P-type ATPases in A domain ([T/D]GE[S/T], orange) and P domain (DKTGT, blue) are shown. The Glu residue in A domain and the Asp residue in P domain are underlined. **(B, C)** TM1-TM6 helices (orange) mainly contribute to a substrate transport and the TM7-TM10 helices (gray) form a supporting domain. A, actuator domain (red); P, phosphorylation domain (blue); N, nucleotide binding domain (green). **(B)** Cryo-EM structures of P4-ATPase (human ATP8A1, PDB: 6k7m and 6k7l) with CDC50A (light blue).[23] N-Terminal extension of CDC50A is indicated. A phospholipid is shown in E2P-Pi state and the C-terminal regulatory residues are shown in E2P state (HGYFSQ, yellow). Asp residue in P domain, which can be phosphorylated, and Glu residue in A domain, which is critical for dephosphorylation, are indicated. **(C)** Schematic domain structure of P4-ATPase and CDC50 protein.

interaction for their appropriate cellular localizations, such as the Golgi complex and endosomes[31,33] (Table 2). Based on structural analyses performed using crystallography and cryo-electron microscopy (cryo-EM), it would seem that CDC50A tightly binds to not only the transmembrane regions but also the extracellular regions among TM3–TM10 of P4-ATPases (Figs. 2B, C). The N-terminal cytoplasmic regions of CDC50 proteins are largely disordered, but some ordered residues were extended to the P domain of P4-ATPases[22,23,34] (Fig. 2B). However, the role of extended residues is currently unknown. In contrast, the N-terminal cytoplasmic region of *sc*Lem3p is ordered and extends to the A domain of Dnf1p; this region may contribute to substrate binding.[35]

The results of biochemical studies indicate that CDC50A hardly interacts with E1-mimicking D-to-N mutants of the P domain but does interact with E2P-mimicking E-to-Q mutants of the A domain,[36,37] and the D-to-N mutants cannot exit from the ER.[31,36] Accordingly, it was initially predicted that E1-mimicking P4-ATPases cannot exit from the ER, due to lacking interaction with CDC50. However, structural analyses show that CDC50 proteins seem to tightly interact with P4-ATPases with any intermediate[22,23,34] (Fig. 2B). Moreover, E1-mimicking D-to-N mutants of ATP9A and ATP9B also cannot exit from the ER[36] suggesting that an unknown quality control mechanism may bring about the removal of the phosphorylation-deficient P4-ATPases at the ER.

4. C-Terminal Regions of ATP8s

ATP8A1 was firstly cloned from bovine chromaffin granules[38] and has been confirmed to flip PS and to a lesser

Table 2. Subcellular Localization of P4-ATPases and Their Chimeric Proteins

WT	NT substitution	CT substitution	Localization
ATP9A			EE/RE, TGN
ATP9B			TGN
	9B-ATP9A		TGN
	9A-ATP9B		ER*
ATP10A		ATP10A-10B	PM
ATP10B		ATP10B-10A, ATP10B-10D	LE/lysosomes
ATP10D		ATP10D-10B	PM
	10B-ATP10A		LE/lysosomes
	10A-ATP10B, 10D-ATP10B		ER*/PM
	10B-ATP10D		LE/lysosomes
ATP11A	11B-ATP11A		PM
ATP11B	11A-ATP11B, 11C-ATP11B		EE/RE
ATP11C	11B-ATP11C		PM
		ATP11A-11B	EE/RE
		ATP11B-11A, ATP11B-11C	PM
		ATP11C-11B	EE/RE

*Those chimeras may not be properly folded and thus remain in the ER. NT, N-terminus; CT, C-terminus; EE, early endosome; RE, recycling endosome; TGN, *trans*-Golgi network; ER, endoplasmic reticulum; LE, late endosome; PM, plasma membrane.

Fig. 3. Sequence Alignments of N- and C-Terminal Cytoplasmic Regions of P4-ATPases

(A) Autoinhibitory residues of *sc*Drs2p and consensus sequences of ATP8 members are shown in pink and underlined. The C-terminal di-leucine motif of ATP8A1 (shown in light blue) is required for targeting lysosome-related organelles (LROs). The arrow and arrowhead indicate truncation site of ATP8A2 mutant analysis.[44] **(B)** N-Terminal sequences shown in purple are important for Golgi targeting of ATP9B. **(C)** N-Terminal di-leucine motif of ATP10B (shown in light blue) is critical for targeting the late endosomes/lysosomes. **(D)** Signal dependent di-leucine motif of ATP11C-a, which is critical for the downregulation, is shown in light blue. The C-terminal LLxY motif of ATP11C-b, which is critical for polarized localization and interaction with ezrin, is shown in orange.

extent PE across membranes in proteoliposomes[13] (Table 1). An exogenously expressed ATP8A1 exhibits various cellular localizations. For example, ATP8A1 mainly localizes to late endosomes/lysosomes in HeLa cells,[31] recycling endosomes in COS-1 cells[13] and the Golgi and the plasma membrane in HeLa and U2OS cells.[29,30] Recently, ATP8A1 has been found in intracellular organelles but not in the plasma membrane in platelets,[39] and in lysosome-related organelles (LROs) in alveolar type 2 cells.[40] Therefore, ATP8A1 localization appears to vary across cell types. The C-terminal region of ATP8A1 includes a di-leucine motif ([D/E]xxxL[L/I], ERAQLL), which serves as a targeting signal to the LROs in alveolar type 2 cells[40] (Fig. 3A). The di-leucine motif is conserved in ATP8A1 orthologs across mammalian species but not in the yeast ortholog Drs2p (Fig. 3A). Many transmembrane cargoes harbor the conserved acidic di-leucine motif [D/E]xxxL[L/I] in their cytoplasmic regions.[41] The di-leucine motif functions as a protein sorting signal by interacting with cytosolic adaptor protein (AP)-complexes AP-1, AP-2, and AP-3. Kook *et al.* showed that AP-3-dependent ATP8A1 trafficking is required for its targeting to the LROs.[40] The C-terminal mutant of ATP8A1, in which two leucines of the di-leucine motif are replaced with alanines, does not localize to LROs anymore but localizes to recycling endosomes. These results suggest that ATP8A1 targets LROs from recycling endosomes in an AP-3 dependent manner[40] (Fig. 4A).

The C-terminal cytosolic region of Drs2p exhibits autoinhibitory effects, and this inhibition can be released by interaction with phosphatidylinositol-4-phosphate (PI4P) *via* its C-terminal region.[42,43] Indeed, the C-terminal cytoplasmic region (Fig. 3) interact with the A, N, and P domains (the GFAFS motif interacts with the N domain) in E2P state as a result of cryo-EM analysis, and this interaction inhibits the enzymatic activity of Drs2p.[22,34,42,43] Moreover, the C-

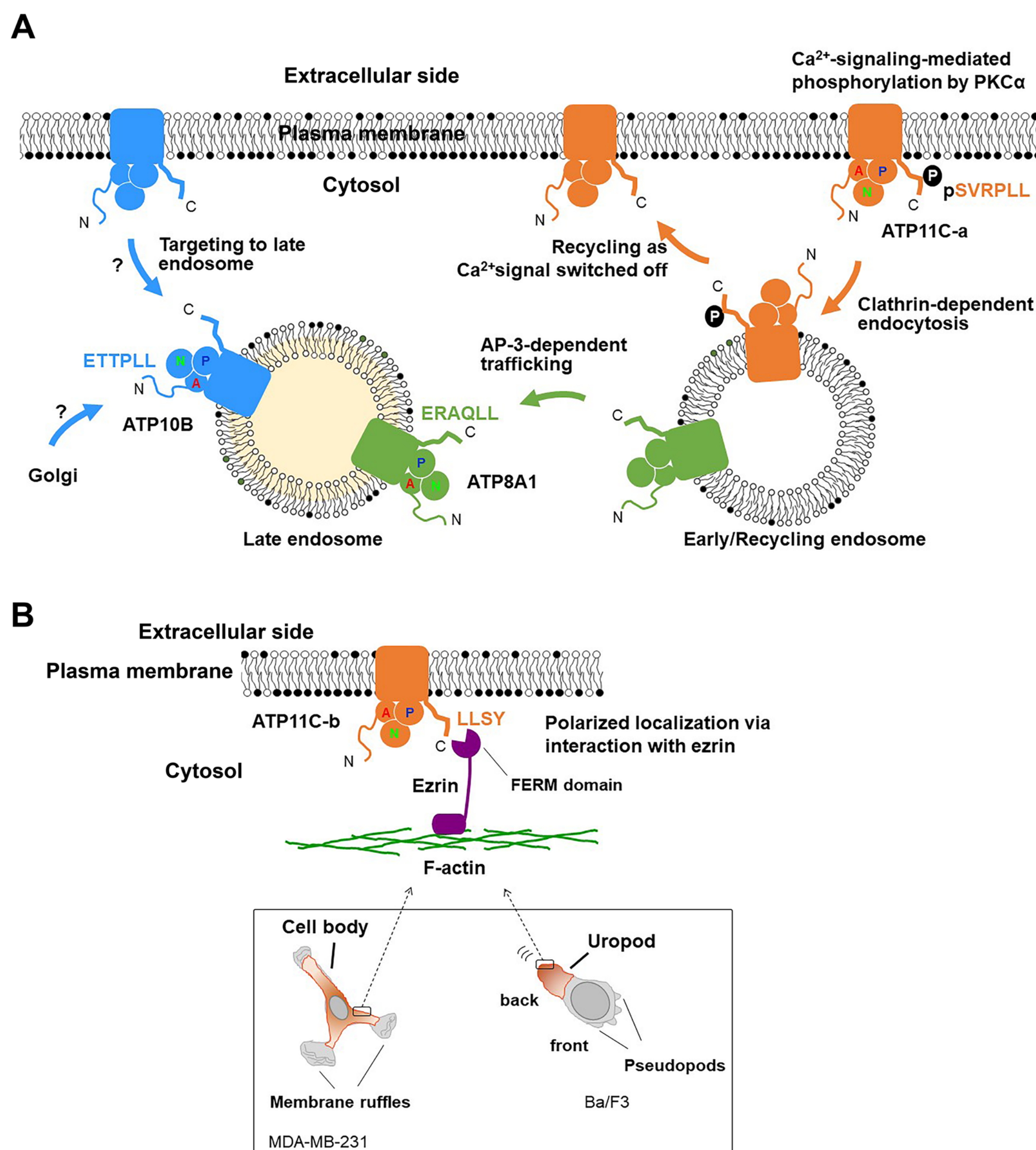

Fig. 4. Schematic Illustration of P4-ATPases Trafficking Regulated by Their N- and C-Terminal Motifs

(A) The N-terminal di-leucine motif (ETTPLL) of ATP10B serves as a targeting signal to late endosomes from the plasma membrane and/or the Golgi complex. The C-terminal di-leucine motif (ERAQLL) of ATP8A1 serves as a trafficking signal to late endosomes from early/recycling endosomes *via* interacting with AP-3. The C-terminal SVRPLL sequence of ATP11C-a serves as an endocytic di-leucine motif only after the Ser is phosphorylated by Ca^{2+} signaling-mediated PKC*α* activation (pSVRPLL). Sequestered ATP11C-a from the plasma membrane can cycle back when the Ca^{2+} signal is switched off. (B) The C-terminal LLxY (LLSY) motif of ATP11C-b is required for its polarized localization (such as cell body region of the plasma membrane in MDA-MB-231 cells and uropod in Ba/F3 cells) and interaction with ezrin. ATP11C-b does not localize to membrane ruffles and pseudopods where phalloidin-positive F-actin is enriched. Ezrin may crosslink ATP11C-b and membrane proximal F-actin at the restricted region of the plasma membrane.

terminal region of Drs2p is disordered in the E2-Pi state and disengaged from the cytoplasmic domains in the presence of PI4P[22,34)] supporting that interaction with PI4P relieves the autoinhibition. The GFAFS motif, which plays a role in autoinhibition, of Drs2p is conserved in mammalian ATP8 members, including ATP8A1, an ortholog of Drs2p (Fig. 3A, [G/A][F/Y]AFS). The C-terminal region of ATP8A1 including the GYAFS motif is ordered and shown to interact with the N domain in the E2P state (Fig. 2B, HGYAFSQ residues in yellow) but is disordered in other states, such as E1-ATP, E1P, and E2-Pi.[22)] Although it is currently unknown whether the C-terminal region of ATP8A1 plays an autoinhibitory role similar to Drs2p, the GYAFS motif is likely to play a regulatory role. ATP8A2 also includes the C-terminal conserved residues (Fig. 3A), but the regulation seems to be rather complicated.[44)] The partial truncation of the short C-terminal regions (Fig. 3A, arrowhead) in which the GYAFS motif is removed, resulting in decreased ATPase activity of ATP8A2, whereas the decreased activity was recovered with the truncation of the longer C-terminal regions[44)] (Fig. 3, arrow). Therefore, the G[F/Y]AFS motifs of ATP8A2 and Drs2p may differently regulate their activities.

ATP8A2 expresses mainly in eye, testis, and brain.[45,46)] An Ile mutation of ATP8A2 in TM4, which is found in cerebellar ataxia, mental retardation, and dysequilibrium syndrome,[47)] dramatically decreased PS-flip activity,[48)] indicating the importance of the ATP8A2 activity in brain function.

Recently, two groups reported that the N- and C-terminal

regions of ATP8B1 play an autoinhibitory role.[49,50] The truncation of N- and C-terminal regions results in full activation of ATP8B1. Notably, ATP8B1 also harbors [G/A][F/Y]AFS motif in its C-terminal region (Fig. 3A). Therefore, the [G/A][F/Y]AFS motif, which is conserved in Drs2p and the ATP8 family, appears to play a regulatory role and/or an autoinhibitory role. The two groups show distinct substrate preferences of ATP8B1; one shows preference for PC and PE, the other for PS. Given that we have previously shown that ATP8B1 flips NBD-PC but not NBD-PS or NBD-PE using cell-based flippase activity assay[15] (Table 1), we think that ATP8B1 prefers PC at the plasma membrane. On one hand, the flipping activity of ATP8B1 toward NBD-PC was much lower than that of ATP10A, another PC-flippase,[16] suggesting the possibility that the flippase activity of ATP8B1 is partially autoinhibited by intramolecular interactions.

ATP8B1 is associated with a genetic disease, progressive familial intrahepatic cholestasis (PFIC) 1. Some mutations derived from PFIC1 patients decreased flippase activity toward NBD-PC,[15,51–53] indicating the importance of PC-flippase activity of ATP8B1 for the homeostasis of bile acid secretion in hepatocytes.

5. N-Terminal Region of ATP9B

ATP9A and ATP9B, initially assigned to class 2 P4-ATPase, are now classified into the P4B clade (Table 1). *sc*Neo1p is also included in this clade, but the P4B clade does not contain any plant proteins.[10] ATP9A and ATP9B, as well as Neo1p, do not require interaction with CDC50s for ER exit and cellular localization.[31,33] ATP9A is mainly localized to the *trans*-Golgi network (TGN) and early/recycling endosomes (EE/RE), and some are found in the plasma membrane, indicating the intracellular trafficking of ATP9A in steady state.[31] ATP9B is mainly localized to the TGN but not to endosomal compartments[31] (Table 2).

Notably, the N-terminal cytoplasmic region of ATP9B alone is sufficient for localization to the TGN, and replacement of the N-terminal region of ATP9A by that of ATP9B (designated as 9B-ATP9A) restricts ATP9A localization to the TGN[31] (Table 2). The N-terminal region, highlighted in purple of ATP9B (Fig. 3B), is important for TGN targeting and retention.[31] However, the actual TGN targeting and retention mechanisms remain to be elucidated. Another P-type ATPase, ATP13A2, which transports polyamines at late endosomes/lysosomes,[11] contains an N-terminal hydrophobic region, which is sufficient for late endosomal/lysosomal targeting *via* interaction with phospholipids, phosphatidic acid, and phosphatidylinositol 3,5-bisphosphate.[54] Therefore, membrane lipids, as well as proteins, might function as the interacting partner of the N-terminal region of ATP9B to target the TGN.

Another P4B ATPase, *sc*Neo1p, also does not interact with CDC50 proteins.[33] Phosphoenzyme intermediate structures of Neo1p were uncovered by cryo-EM analyses[55] and appear to be similar to those of P4A clade proteins, such as Drs2p, Dnf1p, ATP8A1, and ATP11C.[22,23,34,35,55–57] Unlike in the case of P2-ATPases, a tight interaction between P4-ATPases and CDC50 proteins is thought to be a reason for the limited movement of each domain of P4-ATPases during the ATPase cycle. However, Neo1p has features similar to other P4A ATPases suggesting that the limited domain movement of P4-ATPases may not be due to the interaction with CDC50.

6. N-Terminal Regions of ATP10s

ATP10D is ubiquitously expressed in human tissues and many cell types, whereas ATP10A and ATP10B are enriched in specific tissues, such as brain and intestine, and are expressed in limited cell types (Human Protein Atlas, https://www.proteinatlas.org/).[58] C57BL/6 mouse strain carries a stop codon in the *Atp10d* exon[59]; accordingly, an expression profile of Atp10d in mouse data base should be carefully considered. ATP10A shows flippase activity toward NBD-PC and, to a much lesser extent, NBD-GlcCer, and ATP10D was found to specifically flip NBD-GlcCer in cell-based flippase assay[16,19] (Table 1). The ATPase activity of ATP10B is promoted by GlcCer and PC and ATP10B specifically flips NBD-GlcCer and NBD-PC in microsomal membrane fractions. Moreover, some mutations of ATP10B associated with Parkinson's disease have been shown to decrease the ATPase activity.[20] Therefore, ATP10A and ATP10B play important roles in some specific tissues by flipping PC and/or GlcCer.

ATP10D single nucleotide polymorphisms (SNPs) are associated with an increase of plasma GlcCer.[60] Moreover, the Atp10d transgenic C57BL/6 mouse exhibits decreased hexosylceramide in plasma and liver and reduced weight when fed a high-fat diet, compared with wild type C57BL/6 mice, which do not express Atp10d.[61] These findings suggest that ATP10D activity may contribute to hepatic and plasma hexosylceramide homeostasis and reduction of high-fat diet induced obesity.

ATP10A, B, and D require CDC50A for their exit from the ER and are localized to the plasma membrane (ATP10A, ATP10D) and late endosomes/lysosomes (ATP10B).[16] The N-terminal cytoplasmic region of ATP10s is critical for their cellular localization.[62] When the N-terminal cytoplasmic region of ATP10s was exchanged with that of other ATP10 members, it was evident that ATP10B harboring the N-terminal region of ATP10A or ATP10D (designated as 10A-ATP10B and 10D-ATP10B, respectively in Table 2), localizes to the plasma membrane—although some remained in the ER, probably due to misfolding of overexpressed chimeric proteins. In contrast, ATP10A or ATP10D harboring the N-terminal region of ATP10B (10B-ATP10A or 10B-ATP10D), localizes to late endosomes/lysosomes.[62] Therefore, the divergent N-terminal cytoplasmic region among ATP10 members (Fig. 3C) is able to determine their subcellular localization. Notably, the N-terminal region of ATP10B possesses a di-leucine motif (ETTPLL) (Fig. 3C), and indeed the two leucines are important for late endosomal targeting of ATP10B,[62] probably by interacting with AP complexes (Fig. 4A). It is not yet clear whether the motif serves as a lysosomal targeting signal at the TGN during biosynthetic pathway and/or at the plasma membrane during endocytosis (Fig. 4A). Although microsomal ATP10B is able to flip NBD-GlcCer and NBD-PC,[20] plasma membrane targeted ATP10B chimera (10D-ATP10B) did not exhibit a flippase activity toward either NBD-PC or NBD-GlcCer (our unpublished data), assuming that ATP10B may require an appropriate lipid environment for its activity.

7. C-Terminal Regions of ATP11s

The expression of ATP11A, B, and C is ubiquitous, and ATP11C is highly expressed in the liver.[21,46,63] ATP11A and ATP11C shows specific flippase activity toward NBD-PS and NBD-PE in cell-based flippase assay. The ATPase activities of ATP11A, ATP11B, and ATP11C are promoted by the pres-

ence of PS and PE, although PE activates them to a much lesser extent[15,21,46] (Table 1). ATP11C appears to be a major PS-flippase at the plasma membrane in some cell types and tissues, and ATP11C-deficiency has been shown to result in defects in B cell development, altered erythrocytes shape, and cholestasis.[21,64–69] A genetic mutation in *ATP11C* found in a male patient caused congenital hemolytic anemia with X-linked recessive inheritance and the PS-flip activity of the mutant dramatically decreased.[70] Recently, a *de novo* heterozygous point mutation of ATP11A was found in a patient with neurological defects, and the mutant flipped not only PS but also PC indicating the physiological importance of substrate specificity of P4-ATPases.[71]

ATP11A, B, and C require CDC50A for their exit from the ER and mainly localize to the plasma membrane (ATP11A, ATP11C) and early/recycling endosomes (ATP11B) (Table 1), although some ATP11A and ATP11B are found in endosomal structures and the plasma membrane, respectively, suggesting intracellular trafficking in steady state.[15] The C-terminal cytoplasmic regions of ATP11s play important roles in their cellular localization.[62] When the C-terminal cytoplasmic region of ATP11s was exchanged with that of other ATP11 members, ATP11B harboring the C-terminal region of ATP11A or ATP11C (designated as ATP11B-11A and ATP11B-11C, respectively) mostly localizes to the plasma membrane, whereas ATP11A or ATP11C harboring the C-terminal region of ATP11B (ATP11A-11B or ATP11C-11B) mainly localizes to early/recycling endosomes[62] (Table 2). Therefore, the divergent C-terminal cytoplasmic regions among ATP11 members (Fig. 3D) determine the cellular localization of these P4-ATPases.

Notably, ATP11C includes C-terminal splice variants (Fig. 3D, ATP11C-a and ATP11C-b), and the C-terminal regions of ATP11C variants are important for signal dependent downregulation and polarized distribution at the plasma membrane[63,72,73] (Fig. 4). ATP11C-a and ATP11C-b variants expresses almost ubiquitously, although proportions of the expression levels are varied in different cell types and tissues.[63] The C-terminal region of ATP11C-a includes the SVRPLL sequence (Fig. 3D), and the serine (Ser) residue can be phosphorylated by PKCα activation upon cytosolic Ca^{2+} increase, including Gq-coupled GPCR-mediated Ca^{2+} increase.[71] Upon phosphorylation of the Ser residue, the SVRPLL forms a dileucine-like motif ([D/E]xxxL[L/I]), which can serve as an endocytic sorting signal (Fig. 4A). Consequently, the phosphorylated pSVRPLL motif leads sequestration of ATP11C-a from the plasma membrane and downregulates ATP11C-a *via* clathrin-dependent endocytosis.[73] Indeed, the NBD-PS flipping activity at the plasma membrane decreases upon PKCα activation. Moreover, the endocytosed ATP11C-a can be recycled back to the plasma membrane when the Ca^{2+} signal shuts down.[73] The endocytosis (downregulation) and recycling of ATP11C-a by Ca^{2+}-signaling may allow reversible PS exposure in living cells. The mechanism of irreversible PS exposure in cells destined to die, such as apoptotic cells and activated platelets, is relatively well understood.[67,74,75] ATP11C as well as ATP11A, are cleaved by caspases 3 and 7, and inactivated in apoptotic cells.[67] At the same time, scramblases are cleaved by caspases 3 and 7 for activation.[76,77] Therefore, caspase-dependent activation of scramblase and inactivation of flippase leads to irreversible PS exposure in apoptotic cells.[5] However, the mechanism and physiological relevance of reversible PS exposure in living cells are poorly understood, despite the fact that transient PS exposure is found in many important cellular activities, such as neuronal degeneration and regeneration, myotube formation, osteoclast differentiation, and so on.[78–83] A more detailed explanation of the physiological relevance of PS exposure can be found in a recent review.[32]

Another splice variant ATP11C-b includes an LLxY motif (Fig. 3D), which has been recognized a critical motif for polarized localization in motile cells, such as breast cancer cells (MDA-MB-231) and Pro B cells (Ba/F3).[63,72] Notably, the C-terminal region of ATP11C-b specifically interacts with ezrin, ERM (ezrin/radixin/moesin) proteins, and the LLxY motif is required for the interaction with ezrin as well as polarized localization[72] (Fig. 4B). Active ezrin links the actin cytoskeleton and the plasma membrane through its C-terminal actin binding region and N-terminal FERM domain which interacts with membrane proteins and phospholipids, and plays a critical role in cell polarity, actin cytoskeleton reorganization, and cell migration *etc*.[84–89] ATP11C-b colocalized with the active ezrin and the membrane proximal F-actin at the restricted plasma membrane regions such as the cell body region in MDA-MB-231 cells and the uropod (cell posterior) in Ba/F3 cells (Fig. 4B, *inset*). By contrast, it did not colocalize with the phalloidin-positive F-actin, which was enriched in membrane ruffles and pseudopods.[72] Ezrin and ATP11C-b are likely to be stabilized interdependently at the restricted plasma membrane regions, probably being crosslinked to the membrane proximal F-actin.[72]

8. Concluding Remarks

It is becoming clear that the N- and C-terminal cytoplasmic regions of P4-ATPases, which are the most divergent among the P4-ATPase family members, play a key role in regulation of ATPase activity and cellular trafficking. However, the mechanisms driving regulation of enzymatic activity, the targeting to specific membranes, and intracellular trafficking of P4-ATPases remain to be addressed. Moreover, PC is enriched in the extracellular/luminal leaflet of the plasma membrane, indicating that PC-flippase activity should be properly regulated. Advances in the structural analysis of P4-ATPase has greatly improved our understanding of the lipid transport mechanism. However, the molecular mechanism behind substrate specificity still remains to be elucidated. The N- and C-terminal cytoplasmic regions of P4-ATPases tend to be disordered in structural analyses suggesting flexibility in their structure, and this might allow intra- or inter-molecular interactions during ATPase cycle. Further biochemical and cell biological analyses are essential to understand P4-ATPases function at the molecular and cellular level, as well as in the pathophysiological conditions.

Acknowledgments The work was supported by JSPS KAKENHI Grant Numbers JP20H03209 (to H.-W.S.) and JP20K07325 (to H.T.); the Takeda Science Foundation (to H.-W.S.); and the Uehara Memorial Foundation (to H.-W.S.).

Conflict of Interest The authors declare no conflict of interest.

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