Supporting Information

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SI Materials and Methods

Materials. PE (1,2-dioleoyl-sn-glycero-3-phospho-ethanolamine), PS (1,2-dioleoyl-sn-glycero-3-phospho-serine), PC (L-α-phosphatidylcholine, egg lecithin from chicken), and C6-NBD-PS were purchased from Avanti Polar Lipids. ATP, ADP, AMP-PNP, sodium orthovanadate, and n-octyl-β-d-glucopyranoside (OGP) were purchased from Sigma; sodium dithionite was from Fisher; CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid) was from Anatrace; and synthetic 1D4 (Ac-TETSQVAPA) and 6C11 (Ac-RDRLLKRLS) peptides were from Biomatik. The stock solution of sodium orthovanadate was adjusted to pH 10 with NaOH and boiled for 2 min to ensure the absence of polyvanadates. For use in dephosphorylation experiments, ATP was purified by ionic exchange chromatography to remove contaminant ADP. Complete Protease Inhibitor was from Roche. The Rho 1D4 antibody was obtained from UBC-UILO. Restriction enzymes were from New England Biolabs. Primers were purchased from Integrated DNA Technologies.

DNA Constructs. Bovine CDC50A (IMAGE: 8284976) without a tag was cloned into pcDNA3 using the HindIII and XhoI restriction sites. Restriction sites were introduced by PCR. Mutations were introduced into the full-length cDNA encoding bovine ATP8A2 (GQ303567) (1) containing a C-terminal 1D4 epitope tag in the pcDNA3 vector (Invitrogen) using the QuikChange mutagenesis kit (Stratagene). All constructs were verified by sequencing of the entire coding sequence.

Expression and Reconstitution of ATP8A2. Expression of ATP8A2 and CDC50A was performed as described (2). HEK293T cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% bovine growth serum, 100 units/mL penicillin, 100 μg/mL streptomycin, 2 mM L-glutamine, and 1.25 μg/mL fungizone. HEK293T cells grown in 10-cm dishes were cotransfected at 30% confluence with 10 μg of pcDNA3 containing ATP8A2 with a C-terminal 1D4 tag and 10 μg of pcDNA3 containing CDC50A by the calcium phosphate method (3). Cells were harvested 48 h after transfection. Each 10-cm dish was lysed in 0.5 mL of 50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 20 mM CHAPS, 0.5 mg/mL PC, and Complete Protease Inhibitor used according to the manufacturer's instructions for 30 min at 4 °C with stirring. After solubilization, insoluble material was removed by ultracentrifugation at 100,000 × *g* for 10 min. The detergent solubilized proteins from approximately ten plates were added to approximately 50 μL of packed 1D4 immunoaffinity matrix that had been preequilibrated in lysis buffer. After 1.5 h incubation, the matrix was washed 6× with 500 μL of 50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.75% OGP, 0.5 mg/mL PC. ATP8A2 was eluted from the matrix with 100 μL of 0.2 mg/mL 1D4 peptide in 50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 0.75% OGP, 0.5 mg/mL PC. Elutions were performed twice, 30 min each. Following elution, 5–10 μg of purified ATP8A2 was mixed in 1 mL of 50 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 1% OGP, 6.25–10% sucrose, and varying concentrations of lipid with stirring at 23 °C for 1 h. ATP8A2 was reconstituted in either 100% (wt/wt) PC ("PC"), 90% PC and 10% PS ("90PC:10PS"), or 97.5% PC and 2.5% NBD-PS lipids. The total lipid concentration was in most cases 2.5 mg/mL. Following incubation, ATP8A2 in mixed detergent lipid micelles was dialyzed overnight at 4 °C against at least 1 L of 10 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10% sucrose. Reconstituted ATP8A2 in PC or 90PC:10PS was kept at −20 °C until use. A similar procedure was undertaken for native ATP8A2 purified from photoreceptors using an immunoaffinity matrix specific to untagged ATP8A2 as described (1).

SDS-PAGE and Western Blotting. ATP8A2 was separated by SDS gel electrophoresis in 9% polyacrylamide gels and stained with either Coomassie blue or transferred onto Immobilon FL membranes (Millipore) in 25 mM Tris (pH 8.3), 192 mM glycine, 10% methanol. Membranes were blocked with 1% milk in PBS for 30 min and incubated 40 min with either Atp6C11 (specific to ATP8A2) or Cdc50-7F4 (specific to CDC50A) monoclonal antibody supernatants diluted 1:20 and 1:10, respectively. Membranes were washed with PBST (PBS, 0.05% Tween-20) and incubated with goat anti-mouse antibody conjugated with IR dye 680 (LI-COR) diluted 1:20,000 in PBST with 0.5% milk. Following washing, data was collected using a LI-COR Odyssey infrared imaging system.

ATPase Assay. ATPase activity was measured as described (1). Reconstituted ATP8A2 in 90PC:10PS was assayed in 50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 12.5 mM $MgCl_2$, 1 mM DTT, and 5 mM ATP at 37 °C for the indicated times. For studies of apparent lipid affinity, the ATPase activity was determined in the same reaction medium containing 10 mM CHAPS together with PC and PS or PE at varying concentrations, the total lipid concentration being 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 (4).

Flippase Assay. Flippase assays were performed as described (1, 2). Typically, 0.2 μg of reconstituted ATP8A2 in 97.5% PC and 2.5% NBD-PS proteoliposomes was mixed with either ATP or AMP-PNP (for background activity) at a final concentration of 0.5 mM in 50 μL of 50 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, and 10% sucrose, and incubated at 23 °C for 2.5 min. Subsequently, the sample was diluted 20-fold in the same medium without nucleotide, and the sample was transferred to a cuvette and read in a fluorescence spectrophotometer (Varian) using excitation and emission wavelengths of 478 and 540 nm. After another 2.5 min, 500 mM dithionite in 1 M Tris (pH 10) was added to a final concentration of 2 mM, and the loss of NBD fluorescence was monitored for an additional 7.5 min. At 10 min, Triton X-100 was added to a final concentration of 1% and the fluorescence was read for additional 1.5 min. The percentage of NBD-PS contained in the outer leaflet of the liposomes was defined as the percentage of NBD-PS that had reacted with dithionite at 10 min. Transport of NBD-PS was normalized to the amount of ATP8A2 contained in the proteoliposomes and was defined as the % NBD-PS flipped upon addition of ATP per μg of ATP8A2 (% NBD-PS Flipped/μg).

Phosphorylation Assays. Typically, 10–50 ng purified and reconstituted wild-type or mutant ATP8A2 was phosphorylated in 25–200 μL of the standard phosphorylation medium (SPM) consisting of 50 mM HEPES-NaOH (pH 7.5), 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 2 μM [γ-^{32}P]ATP. Studies of phosphorylation and dephosphorylation were carried out at 0 °C, except for the rapid kinetic experiments described in Fig. 6 and Figs. S4, and S5, where the temperature was 25 °C. For experiments at 0 °C

mixing was manual (5) or by magnetic stirrer (6), and for rapid kinetic experiments at 25 °C a BioLogic QFM-5 quenched-flow module (BioLogic Science Instruments) was applied (7, 8). Quenching of the phosphorylation reaction was performed with 1 or 2 volumes of 25% (wt/vol) trichloroacetic acid (TCA) containing 100 mM H_3PO_4. The TCA precipitated enzyme was washed twice with a solution containing 7% TCA and 1 mM H_3PO_4 and dissolved in SDS-PAGE loading buffer (10 mM Na_2HPO_4 (pH 6.0), 10% lithium dodecyl sulfate (LDS), 2% β-mercaptoethanol (vol/vol), 0.4% (wt/vol) bromophenol blue, and 25% (vol/vol) glycerol). In some cases, quenching was performed by addition of an equal volume of the SDS-PAGE loading buffer, and the sample was subjected directly to SDS-PAGE without washing. SDS-PAGE was performed in 5.8% gels at pH 6.0 for 2–4 h (5). The gels were fixed in 10% acetic acid for 10 min and dried under vacuum. The ^{32}P-labeled radioactive band corresponding to the phosphorylated flippase was quantified by phosphorimaging using the Packard Cyclone™ storage phosphor system. Blanks corresponding to nonphosphorylated enzyme (20 mM EDTA replacing $MgCl_2$ in the phosphorylation medium) were subtracted before further processing of the data. The sensitivity to hydroxylamine was examined by incubating the ^{32}P-labeled enzyme with 60 mM hydroxylamine at 23 °C for 15 min. For vanadate binding studies, orthovanadate at various concentrations was allowed to bind to ATP8A2 for 30 min at 25 °C in the presence of 50 mM HEPES-NaOH pH 7.5, 150 mM NaCl, 1 mM $MgCl_2$, 1 mM DTT, and 10 mM CHAPS. For each vanadate concentration, the fraction of enzyme without bound vanadate was determined by phosphorylation at 0 °C with 2 μM [γ-^{32}P]ATP for 10 s, taking the level obtained after incubation in the absence of vanadate as 100% (9), and the vanadate-bound fractions were calculated by subtraction of the free enzyme fractions from 100%. To examine the ADP sensitivity of the phosphoenzyme, 1 mM ATP or ADP was added at 0 °C, following 10 s phosphorylation in SPM, and quenching was performed after various time intervals. To study the sensitivity of the phosphoenzyme to PS or PE, phosphorylation of PC reconstituted enzyme was carried out for 10 or 20 s in SPM to which 10 mM CHAPS had been added. A mixture of PS or PE together with PC solubilized in 10 mM CHAPS was then added at 0 °C at the indicated final concentration of the aminophospholipid, giving a total concentration of added lipid of 0.1 mg/mL, followed by quenching after 5 s or the indicated time intervals.

Removal of Ions for Determination of Ionic Requirements. To remove alkali metal ions and Cl^-, reconstituted ATP8A2 was dialyzed against three changes of 1 L of the NMDG medium described in the *Materials and Methods* section of the main text. Following the dialysis the Na^+ and K^+ concentrations were less than 25 μM as determined by atomic absorption spectrometry. For functional assays in NMDG medium Cl^- was replaced by acetate.

Curve Fitting and Statistical Analysis. Data was analyzed by nonlinear regression using the SigmaPlot program (SPSS, Inc.). Errors are reported as standard errors, and the value of n represents the number of experimental data points on which the analysis is based.

1. Coleman JA, Kwok MC, Molday RS (2009) Localization, purification, and functional reconstitution of the P_4-ATPase Atp8a2, a phosphatidylserine flippase in photoreceptor disc membranes. *J Biol Chem* 284:32670–32679.
2. Coleman JA, Molday RS (2011) Critical role of the β-subunit CDC50A in the stable expression, assembly, subcellular localization and lipid transport activity of the P_4-ATPase ATP8A2. *J Biol Chem* 286:17205–17216.
3. Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. *Mol Cell Biol* 7:2745–2752.
4. Gonzalez-Romo P, Sanchez-Nieto S, Gavilanes-Ruiz M (1992) A modified colorimetric method for the determination of orthophosphate in the presence of high ATP concentrations. *Anal Biochem* 200:235–238.
5. Andersen JP, Vilsen B, Leberer E, MacLennan DH (1989) Functional consequences of mutations in the β-strand sector of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J Biol Chem* 264:21018–21023.
6. Vilsen B, Andersen JP (1998) Mutation to the glutamate in the fourth membrane segment of Na^+, K^+-ATPase and Ca^{2+}-ATPase affects cation binding from both sides of the membrane and destabilizes the occluded enzyme forms. *Biochemistry* 37:10961–10971.
7. Sorensen TL, Dupont Y, Vilsen B, Andersen JP (2000) Fast kinetic analysis of conformational changes in mutants of the Ca^{2+}-ATPase of sarcoplasmic reticulum. *J Biol Chem* 275:5400–5408.
8. Toustrup-Jensen M, Hauge M, Vilsen B (2001) Mutational effects on conformational changes of the dephospho- and phospho-forms of the Na^+, K^+-ATPase. *Biochemistry* 40:5521–5532.
9. Clausen JD, Andersen JP (2003) Roles of Leu249, Lys252, and Leu253 in membrane segment M3 of sarcoplasmic reticulum Ca^{2+}-ATPase in control of Ca^{2+} migration and long-range intramolecular communication. *Biochemistry* 42:2585–2594.

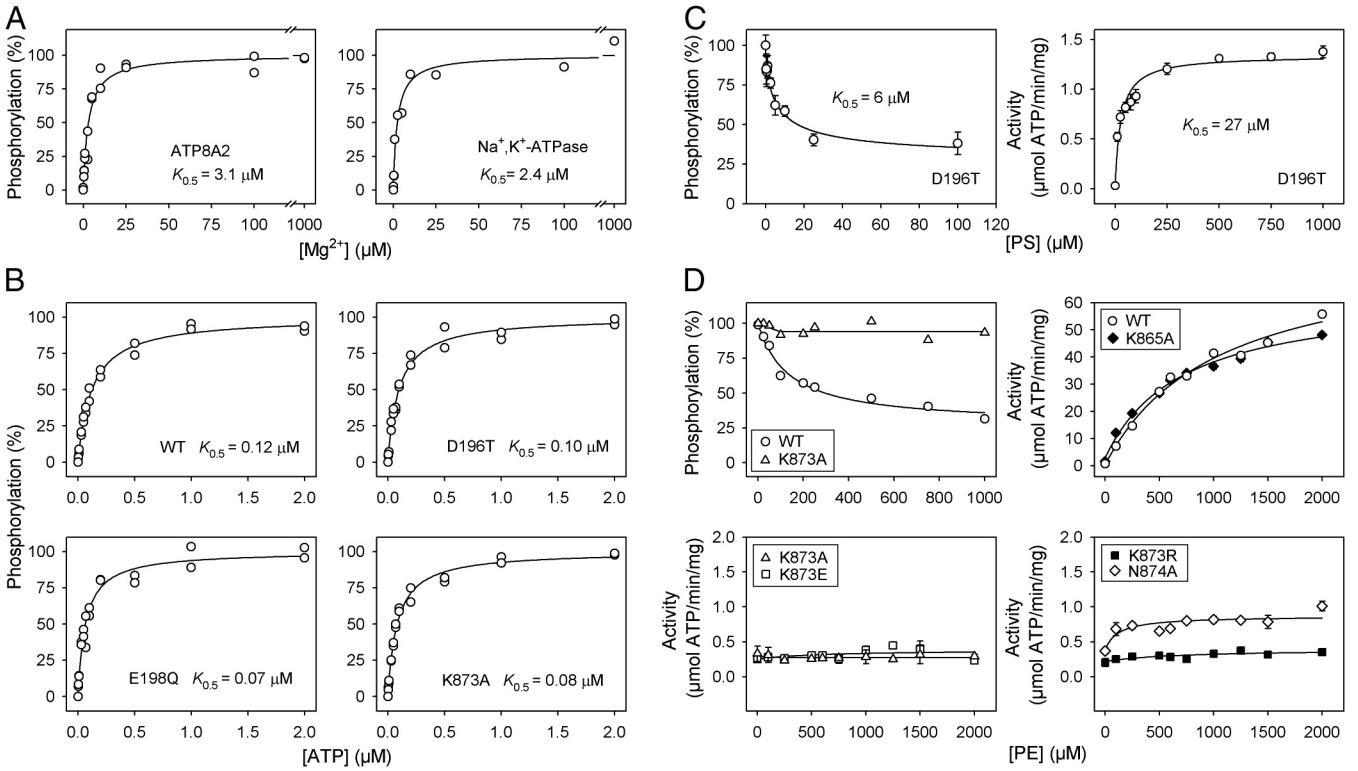

Fig. S1. Supplemental characterization of Mg^{2+}, ATP, and aminophospholipid dependence. (*A*) Apparent affinity of ATP8A2 and the Na^+,K^+-ATPase for Mg^{2+}. Reconstituted ATP8A2 in PC vesicles and the Na^+,K^+-ATPase (rat α1) expressed in COS cells were phosphorylated at 0 °C in SPM with 20 mM EDTA and varying additions of $MgCl_2$ giving the indicated concentrations of free Mg^{2+}. All data points are shown. The $K_{0.5}$ values extracted from the data are 3.1 ± 0.4 μM ($n = 21$) and 2.4 ± 0.5 ($n = 11$) for ATP8A2 and Na^+,K^+-ATPase, respectively. (*B*) Apparent affinity for ATP. Reconstituted wild-type and mutant ATP8A2 in PC was phosphorylated at 0 °C in SPM with the indicated [γ-^{32}P]ATP concentrations. All data points are shown. The $K_{0.5}$ values extracted from the data are indicated. Refer to Table S1 for statistical analysis. (*C*) Apparent affinity of D196T for PS. The protocols were the same as used for Fig. 3. (*Left*) Following phosphorylation of PC reconstituted D196T at 0 °C with [γ-^{32}P]ATP in SPM containing CHAPS, PS dissolved in CHAPS was added at the indicated concentrations, and dephosphorylation was terminated 5 s later. (*Right*) ATPase activity of the D196T mutant in the presence of CHAPS and PC with the indicated concentrations of PS. The $K_{0.5}$ values extracted from the data are indicated. Refer to Table S2 for statistical analysis. (*D*) Sensitivity of wild type, K865A, K873A, K873E, K873R, and N874A to PE. The protocols were the same as used for Fig. 3, but with varying PE instead of PS. (*Left*) Following phosphorylation of PC reconstituted wild type and K873A at 0 °C with [γ-^{32}P]ATP in SPM containing CHAPS, PE dissolved in CHAPS was added at the indicated concentrations, and dephosphorylation was terminated 5 s later. (*Right and Lower*) ATPase activity of wild type, K865A, K873A, K873E, K873R, and N874A in the presence of CHAPS and PC with the indicated concentrations of PE. Average values with standard errors are shown. Refer to Table S2 for statistical analysis.

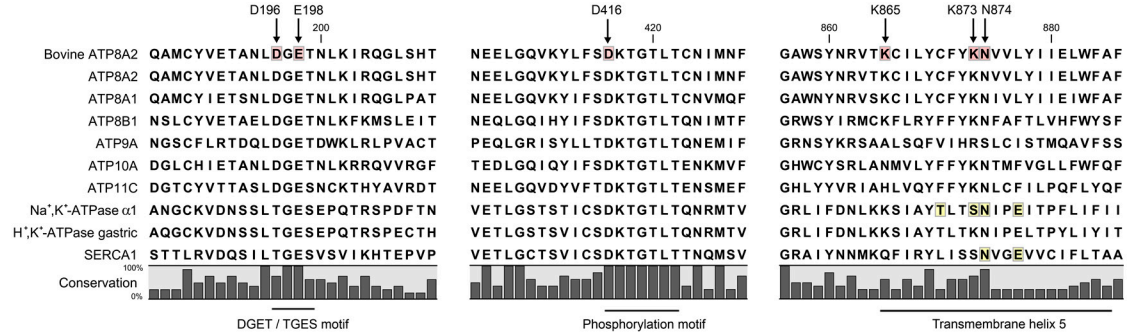

Fig. S2. Alignment of selected P-type ATPase protein sequences. Protein sequences of the bovine ATP8A2 studied here and selected human flippases were aligned with the human Na^+,K^+-ATPase α1 isoform, gastric H^+,K^+-ATPase, and the sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA1) based on the ClustalW algorithm and on structural alignment of the crystal structures of Na^+,K^+-ATPase and SERCA. Numbers denote the residue numbers of bovine ATP8A2. The conservation in % is shown below as dark gray columns, residues mutated in this study are marked with pink shading, and yellow shading denotes residues of Na^+,K^+-ATPase and Ca^{2+}-ATPase shown by crystal structures to be implicated in binding of K^+ or Ca^{2+}. Conserved motifs are indicated below, and the position of transmembrane segment M5 is indicated based on the known crystal structures of the Na^+,K^+-ATPase and SERCA (see, e.g., ref. 1).

1 Shinoda T, Ogawa H, Cornelius F, Toyoshima C (2009) Crystal structure of the sodium-potassium pump at 2.4 Å resolution. *Nature* 459:446–450.

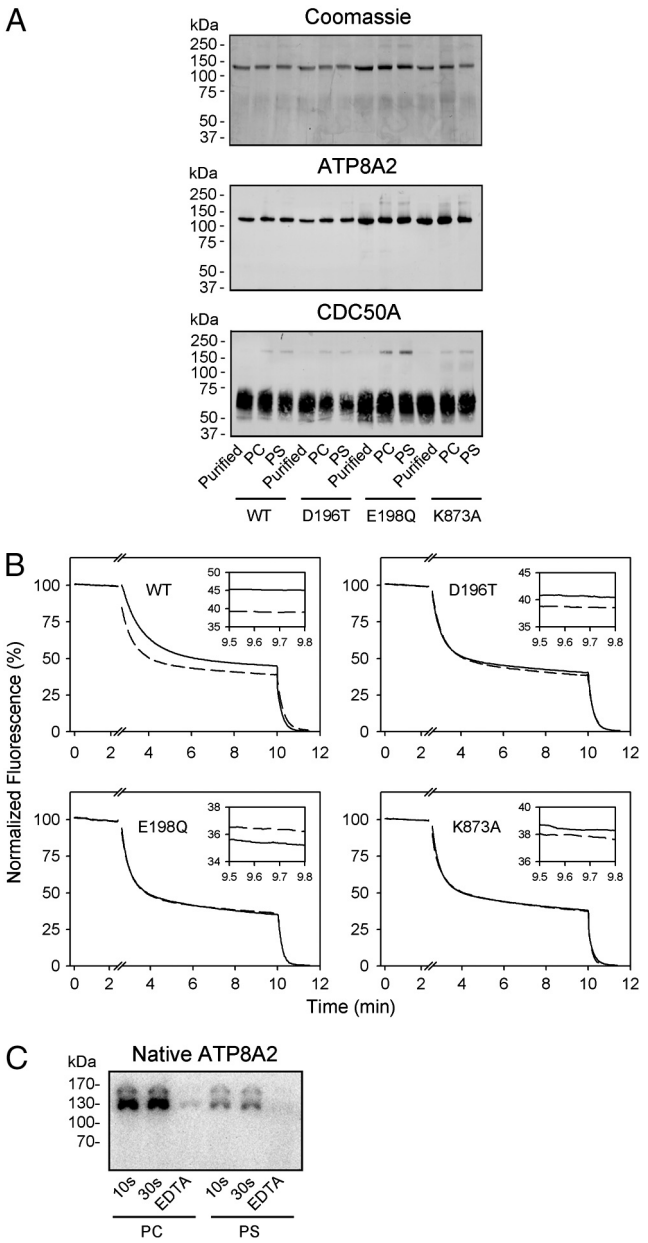

Fig. S3. Supplemental data supporting Figs. 1 and 2. (*A*) Characterization of purified and reconstituted wild-type (WT) and mutant ATP8A2 expressed in HEK293T cells by SDS-PAGE. Purified ATP8A2 wild type and mutants were reconstituted by detergent dialysis into lipid vesicles containing either phosphatidylcholine ("PC") or 90PC:10PS ("PS"). The reconstituted protein was subjected to SDS-PAGE followed by Coomassie blue staining or Western blotting with monoclonal antibodies with the indicated specificity to ATP8A2 (antibody Atp6C11) or CDC50A (antibody Cdc50-7F4). (*B*) Examples of determination of lipid transport ("flippase") activity (see Fig. 1*B* and Table S1 for representation of all data). ATP8A2 wild type and mutants were purified and reconstituted into vesicles containing 97.5% phosphatidylcholine and 2.5% NBD-PS. Fluorescence traces of NBD-PS obtained in the presence of AMP-PNP (solid line) and ATP (broken line) are shown. Breaks at 2.5 min indicate addition of 2 mM dithionite to bleach the fluorescence from NBD-PS in the outer leaflet such that remaining fluorescence represents only NBD-PS present in the inner leaflet (see further in *SI Materials and Methods*). Solubilization of vesicles was accomplished by addition of Triton-X100 detergent at 10 min. Following bleaching the fluorescence trace corresponding to AMP-PNP indicates the initial distribution of NBD-PS (55:45%/% in the outer leaflet versus inner leaflet for wild type). The difference between the AMP-PNP and ATP traces reflects the percentage of NBD-PS that has been transported from the inner to the outer leaflet during the incubation prior to bleaching (corresponding to transport toward the cytoplasmic side). The inset shows a close-up of the traces from 9.5 to 9.8 min. Note that for E198Q the trace corresponding to ATP shows higher fluorescence than that corresponding to AMP-PNP, contrary to the situation for the wild type and the other mutants. This indicates that E198Q supports net transport from the outer to the inner leaflet, which may occur down the concentration gradient of NBD-PS, as a relatively higher fraction of NBD-PS is initially present in the outer leaflet for E198Q compared with wild type (65:35%/% in the outer leaflet versus inner leaflet for E198Q, read from the AMP-PNP traces). Because for E198Q E_2P accumulates almost to 100% in the presence of PS (Fig. 4), E_2P seems to allow alternating exposure of the lipid toward the two membrane sides in this mutant, a possible consequence of the destabilization of the transition state of E_2P dephosphorylation. This appears equivalent to the observation that the Na^+,K^+-ATPase in E_2 form can transport Rb^+ passively from the cytoplasmic to the extracellular surface stimulated by phosphorylation of E_2 by P_i (1). (*C*) Phosphorylation of native ATP8A2 purified from photoreceptor outer segments. Equal amounts of native ATP8A2 (purified as described in ref. 2) reconstituted in PC or 90PC:10PS ("PS") were phosphorylated at 0 °C by [γ-^{32}P]ATP using the same protocol as for Fig. 2*A*.

1 Karlish SJD, Lieb WR, Stein WD (1982) Combined effects of ATP and phosphate on rubidium exchange mediated by Na-K-ATPase reconstituted into phospholipid vesicles.*J Physiol* 328:333–350.

2 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* 284:32670–32679.

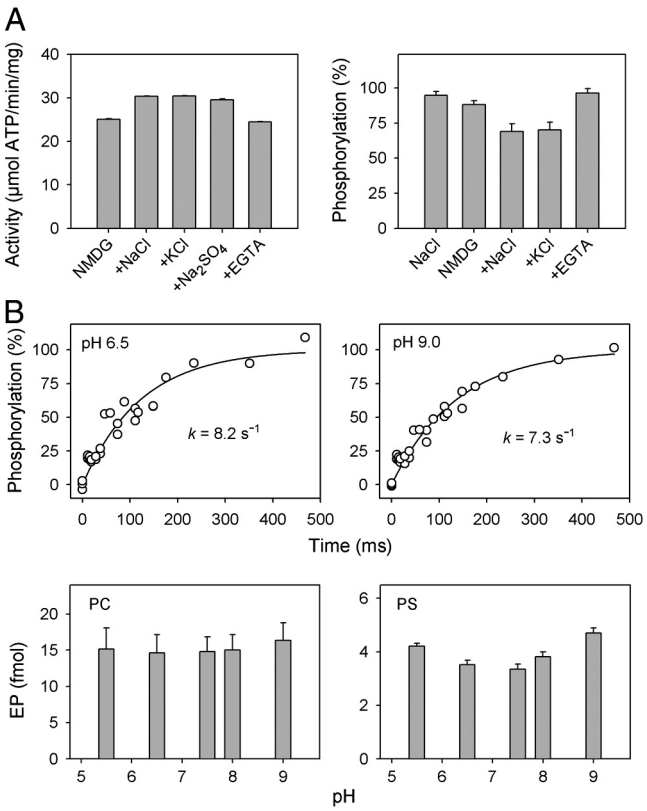

Fig. S4. Dependence of ATPase activity and phosphorylation on ionic conditions and pH. (*A*) Dependence on ionic conditions. ATP8A2 reconstituted in PC or 90PC:10PS was dialyzed as described in *Materials and Methods* to remove alkali metal ions and Cl^-. ATPase activity of the 90PC:10PS reconstituted enzyme was measured at 37 °C in the absence of Na^+ or K^+ and presence of 150 mM NMDG ("NMDG"; see *Materials and Methods*), or, when indicated by "+," 50 mM NaCl, 50 mM KCl, 25 mM Na_2SO_4, or 1 mM EGTA was also included. Phosphorylation of the PC reconstituted enzyme was carried out at 0 °C in the presence of 150 mM Na^+ and Cl^- in SPM ("NaCl") or with the dialyzed sample in the presence of 150 mM NMDG ("NMDG"). When indicated by "+," 50 mM NaCl, 50 mM KCl, or 1 mM EGTA was added to the NMDG medium. Average values with standard errors are shown. (*B*) Dependence of phosphorylation on pH. (*Upper*) Using the quenched-flow module, PC reconstituted ATP8A2 was phosphorylated at 25 °C in the presence of 2 μM [γ-^{32}P]ATP for the indicated times at either pH 6.5 or pH 9.0 (buffered with 50 mM MES-Tris and 50 mM Tris-HCl, respectively, other medium components being identical to SPM). All data points are shown. The rate constants extracted from the data are indicated. Refer to Table S3 for statistical analysis. (*Lower*) The steady-state levels of phosphoenzyme for ATP8A2 reconstituted in either PC or 90PC:10PS ("PS") was analyzed at 0 °C and pH 5.5, 6.5, 7.5, 8.0, or 9.0. The respective buffers used were 50 mM MES-Tris at pH 5.5 or 6.5, 50 mM HEPES-Tris at pH 7.5, 50 mM TES-HCl at pH 8.0, and 50 mM Tris-HCl at pH 9.0, other medium components being identical to SPM. Average values with standard errors are shown.

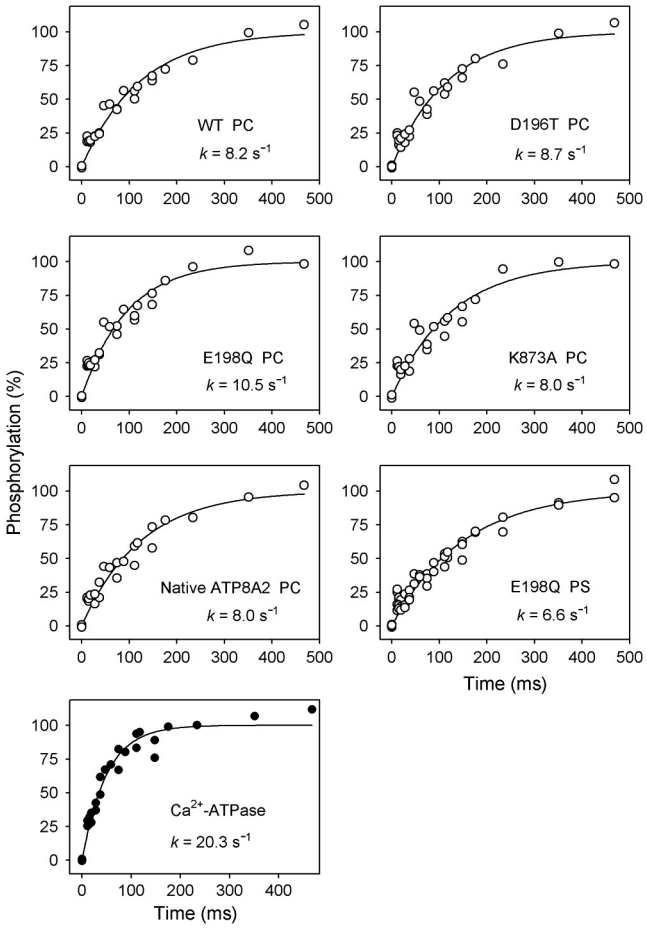

Fig. S5. Effect of ATP8A2 mutations on phosphorylation rate constant. For comparison the phosphorylation time course of the Ca^{2+}-ATPase (SERCA1a expressed in COS cells) is also shown. Phosphorylation was performed at 25 °C in the presence of 2 μM [γ-^{32}P]ATP in SPM (with 100 μM $CaCl_2$ added for Ca^{2+}-ATPase) for the indicated times, using the quenched-flow module. All data points are shown. The rate constants extracted from the data are indicated. Refer to Table S3 for statistical analysis.

Table S1. Statistical analysis of data in Figs. 1, 4, 5, S1*B*, and S3*B*

ATP8A2 protein	NBD-PS transport*, % NBD-PS/μg	ATPase activity†, μmol · min^{-1} · mg^{-1}	$K_{0.5}$ (ATP)‡, μM	E_2P %§ PC	E_2P %§ 90PC:10PS	$K_{0.5}$ (vanadate), μM¶ PC	$K_{0.5}$ (vanadate), μM¶ 90PC:10PS
Wild type	29 ± 1 $n = 14$	35.4 ± 0.8 $n = 30$	0.12 ± 0.01 $n = 22$	60 ± 7 $n = 48$	n.d.	4.8 ± 0.6 $n = 56$	0.024 ± 0.003 $n = 40$
D196T	12 ± 3 $n = 8$	10.4 ± 0.1 $n = 15$	0.10 ± 0.01 $n = 22$	75 ± 8 $n = 27$	n.d.	11 ± 1 $n = 32$	n.d.
E198Q	–8 ± 2 $n = 8$	0.02 ± 0.01 $n = 15$	0.07 ± 0.01 $n = 22$	76 ± 10 $n = 30$	≈100 $n = 18$	34 ± 10 $n = 32$	0.50 ± 0.06 $n = 32$
D416N	n.d.	0.03 ± 0.1 $n = 9$	n.d.	n.d.	n.d.	n.d.	n.d.
K865A	27 ± 2 $n = 6$	28.7 ± 0.5 $n = 15$	n.d.	n.d.	n.d.	n.d.	n.d.
K873A	6 ± 2 $n = 8$	1.34 ± 0.04 $n = 15$	0.08 ± 0.01 $n = 22$	47 ± 9 $n = 30$	39 ± 8 $n = 18$	≈370 $n = 36$	14.8 ± 0.8 $n = 16$
K873E	6 ± 3 $n = 6$	0.5 ± 0.2 $n = 15$	n.d.	n.d.	n.d.	n.d.	n.d.
K873R	14 ± 1 $n = 6$	3.8 ± 0.5 $n = 15$	n.d.	n.d.	n.d.	n.d.	n.d.
N874A	16 ± 2 $n = 6$	4.3 ± 0.2 $n = 15$	n.d.	n.d.	n.d.	n.d.	n.d.

Standard errors and number of data points *n*, on which the analysis is based, are indicated. n.d., not determined.

*Data corresponding to Fig. 1*B*. A positive value for NBD-PS transport indicates transport from the exoplasmic to the cytoplasmic leaflet (i.e., from inner to outer leaflet in the reconstituted vesicles), whereas a negative value indicates transport in the opposite direction.

†Specific ATPase activities of 90PC:10PS reconstituted enzyme were calculated by fitting a straight line to the data presented in Fig. 1 *C* and *D*.

‡$K_{0.5}$ values for ATP concentration dependence of phosphorylation were obtained by fitting a single site hyperbolic function to the data in Fig. S1*B*.

§A biexponential decay function was fitted to the data in Fig. 4, and the amplitude of the slow phase reflecting the initial amount of E_2P is indicated for enzyme reconstituted in PC or in 90PC:10PS.

¶$K_{0.5}$ values were obtained by fitting a Hill ligand binding equation $EP = EP_{max} \cdot (1 - [\text{vanadate}]^h/(K_{0.5}{}^h + [\text{vanadate}]^h))$ to the data in Fig. 5, with Hill number *h* ranging between 0.5 and 1.2.

Table S2. Phosphorylation stoichiometry and statistical analysis of data in Fig. 3 and Fig. S1 *C* and *D*

ATP8A2	Phosphorylation stoichiometry, mol^{32}P incorporated/mol protein*	$K_{0.5}$ for activation of dephosphorylation by PS, μM	$K_{0.5}$ for activation of ATP hydrolysis by PS, μM	$K_{0.5}$ for activation of dephosphorylation by PE, μM	$K_{0.5}$ for activation of ATP hydrolysis by PE, μM
Wild type	0.20 ± 0.01 $n = 25$	12 ± 2 $n = 20$	38 ± 3 $n = 160$	140 ± 60 $n = 18$	1,100 ± 100 $n = 30$
D196T	0.34 ± 0.04 $n = 3$	6 ± 4 $n = 30$	27 ± 4 $n = 60$	n.d.†	n.d.†
E198Q	0.48 ± 0.02 $n = 3$	n.r.‡	n.r.‡	n.d.†	n.d.†
K865A	0.23 ± 0.01 $n = 15$	11 ± 1 $n = 31$	58 ± 4 $n = 140$	n.d.†	710 ± 70 $n = 30$
K873A	0.22 ± 0.04 $n = 15$	88 ± 19 $n = 29$	600 ± 100 $n = 53$	n.r.‡ $n = 18$	n.r.‡ $n = 30$
K873E	0.06 ± 0.02 $n = 25$	99 ± 16 $n = 49$	490 ± 90 $n = 60$	n.d.†	n.r.‡ $n = 30$
K873R	0.16 ± 0.04 $n = 15$	46 ± 5 $n = 38$	230 ± 20 $n = 80$	n.d.†	n.r.‡ $n = 30$
N874A	0.18 ± 0.04 $n = 14$	31 ± 2 $n = 60$	170 ± 20 $n = 80$	n.d.†	n.r.‡ $n = 30$

Standard errors and number of data points *n*, on which the analysis is based, are indicated.

To determine the $K_{0.5}$ values, dephosphorylation and ATPase activity data were fitted by a single site hyperbolic function plus a constant (subtracted from data points shown in Fig. 3*A*).

*Estimated by relating the ^{32}P-labeling of the bands on the gel to the amount of ATP8A2 protein applied to the gel for experiments carried out corresponding to Fig. 2*C* controls in PC without PS added. The reason for the relatively low value for K873E is presently not known.

†n.d., $K_{0.5}$ not determined, experiment not performed.

‡n.r., $K_{0.5}$ not relevant, because activation was not statistically significant, see Fig. 2C and Fig. S1*D*.

Table S3. Statistical analysis of data in Fig. 6, Fig. S4, and Fig. S5

Protein/condition	Rate constant, s^{-1}
Wild-type ATP8A2 reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Fig. 6 and Fig. S5)	8.2 ± 0.9 $n = 27$
Wild-type ATP8A2 reconstituted in PC, phosphorylation in NMDG, pH 7.5 (Fig. 6)	12.4 ± 0.9 $n = 54$
Wild-type ATP8A2 reconstituted in PC, phosphorylation in NaCl, pH 6.5 (Fig. S4)	8.2 ± 1.2 $n = 27$
Wild-type ATP8A2 reconstituted in PC, phosphorylation in NaCl, pH 9.0 (Fig. S4)	7.3 ± 0.8 $n = 27$
D196T reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Fig. S5)	8.7 ± 1.1 $n = 27$
E198Q reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Fig. S5)	10.5 ± 1.2 $n = 27$
K873A reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Fig. S5)	8.0 ± 1.3 $n = 27$
Native ATP8A2 reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Fig. S5)	8.0 ± 1.0 $n = 27$
E198Q reconstituted in 90PC:10PS, phosphorylation in NaCl, pH 7.5 (Fig. S5)	6.6 ± 0.6 $n = 54$
Ca^{2+}-ATPase (SERCA1a), phosphorylation in NaCl, pH 7.5 (Fig. S5)	20.3 ± 1.7 $n = 27$

The rate constants were obtained by fitting a monoexponential "rise to max" function to the data. Standard errors and number of data points n, on which the analysis is based, are indicated.