Abstract

P₄-ATPases are a family of membrane transporters which have been implicated in the energy-dependent transport of aminophospholipids from the exocyttoplasmic to cytoplasmic surface of biological membranes. This thesis investigation examined the structure-function relationships of ATP8A2, a novel member of the P₄-ATPase family initially discovered in a proteomic study of photoreceptor outer segments. Photoreceptor outer segments are organelles which consist of stacks of membraneous discs containing visual pigment molecules. ATP8A2 is shown to be present in photoreceptor outer segment discs and preferentially transports phosphatidylserine towards the cytosolic leaflet, providing the first direct demonstration of lipid transport by a purified mammalian P₄-ATPase.

CDC50A, the β-subunit of ATP8A2 was discovered using mass spectrometry and Western blotting. Subunit interactions are mediated through the extracellular and membrane domains of CDC50A. The N-terminal domain of CDC50A appears to play a role in pump modulation. ATP8A2 forms a phosphoenzyme intermediate at Asp⁴¹⁶ and phosphatidylserine appears to be transported in a similar manner to that of other cation-transporting P-type ATPases. The phosphoenzyme exists in two distinct conformations: E₁P and E₂P. The E₂P form interacts with aminophospholipids. Lys⁸⁷³ in transmembrane segment M5 located in a region known to be important for cation binding for P-type ATPases is critical for phosphatidylserine binding. Glu¹⁹⁸ in the DGET motif is essential for E₂P dephosphorylation.

ATP8A2 gene expression was disrupted in mice using a neo cassette. Knockout mice develop short outer segments and visual function is impaired. Modification of transbilayer asymmetry and composition appear to be responsible for reduced visual function rather than
structural defects. The decrease in outer segment length suggests that ATP8A2 is involved in vesicular trafficking of proteins to the outer segment by regulating the step of vesicle budding possibly from the trans-Golgi network. We speculate that ATP8A2 plays a similar role in other neuronal cells and thus provide insight into the phenotype of human disorders caused by mutations in \textit{ATP8A2}.

In summary, this study has identified for the first time the transported substrate of a mammalian P$_4$-ATPase, discovered protein-protein interactions regulating function, elucidated the mechanism of lipid transport, and illuminated the function of ATP8A2 in photoreceptor and neuronal biology.
Preface

A version of chapter 2 has been published. [Coleman, J.A.], Kwok, M.C., Molday, R.S., 2009. Localization, purification, and functional reconstitution of the P4-ATPase Atp8a2, a phosphatidylserine flippase in photoreceptor disc membranes. J Biol Chem 284, 32670-32679. The study was initiated by Kwok, M.C., Theresa Hii assisted in generating and screening of hybridoma cells. I performed all other experimental work and wrote the original manuscript. The final copy of the manuscript was edited by Molday, R.S.

A version of chapter 3 has been published. [Coleman, J.A.], Molday, R.S. 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, 17205-17216. Theresa Hii assisted in generating and screening of hybridoma cells. I performed all other experimental work and wrote the original manuscript. The final copy of the manuscript was edited by Molday, R.S.

A version of chapter 4 has been published. [Coleman, J.A.], Vestergaard, A.L., Molday, R.S., Vilsen, B., Peter Andersen, J. Critical role of a transmembrane lysine in aminophospholipid transport by mammalian photoreceptor P4-ATPase ATP8A2. Proc Natl Acad Sci U S A 109, 1449-1454. This study was performed as part of a collaboration with Jens Peter Andersen and Bente Vilsen’s groups at the University of Aarhus, Denmark. Data presented in Figures 4.5, 4.6, and 4.7 were performed by technicians Janne Petersen and Lene Jacobsen, Department of Biomedicine, Aarhus University. Vestergaard, A.L. assisted in data analysis, supervision of technicians, and generated the alignment presented in Figure 4.1C. I
performed all other experimental work at Aarhus University with exception of data presented in Figure 4.2 which was obtained at the University of British Columbia. I wrote the original manuscript. The final copy of the manuscript was edited by Peter Andersen, J., Vilsen, B., and Molday, R.S.

A version of chapter 5 has been presented at the annual ARVO vision meeting in Florida in May 2012. Electron microscopy, electroretinograms, and retinal sectioning were performed with assistance from Laurie Molday, Andrew Metcalfe, and Hidayat Djajadi respectively. All data presented on the \textit{wl/wl} mouse was obtained from Xianjun Zhu and Simon John (Jackson Laboratories). Light micrographs were taken by Dr. Wayne Vogl. I performed all other experimental work and wrote this chapter.

Molecular modeling studies presented in Figures. 4.1 and 6.1 were performed by Stepan Lenevich.

All animal work was performed in accordance with the guidelines of the UBC Animal Care Committee and Canadian Council on Animal Care (Protocol A10-0297).
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
</tr>
<tr>
<td>AD</td>
<td>Autosomal dominant</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>AMD</td>
<td>Age related macular degeneration</td>
</tr>
<tr>
<td>AMP-PNP</td>
<td>Adenylyl-imidodiphosphate</td>
</tr>
<tr>
<td>AR</td>
<td>Autosomal recessive</td>
</tr>
<tr>
<td>ARF</td>
<td>ADP ribosylation factor</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine-5' triphosphate</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>BBSome</td>
<td>Bardet-Biedl Syndrome proteins</td>
</tr>
<tr>
<td>cGMP</td>
<td>Cyclic guanosine monophosphate</td>
</tr>
<tr>
<td>CNGA1</td>
<td>Cyclic nucleotide-gated channel alpha 1</td>
</tr>
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<td>C6 NBD-PC</td>
<td>1-oleoyl-2-{6-[7-nitro-2–1,3-benzoaxadiazol-4-yl)amino]hexanoyl} -&lt;sup&gt;sn&lt;/sup&gt;-glycero-3-phosphocholine</td>
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<td>C12 NBD-PS</td>
<td>1-oleoyl-2-{12-[7-nitro-2–1,3- benzoaxadiazol-4-yl)amino]dodecanoyl} -&lt;sup&gt;sn&lt;/sup&gt;-glycero-3-phosphoserine</td>
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<td>CHAPS</td>
<td>3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid</td>
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<tr>
<td>Chol</td>
<td>Cholesterol</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
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</tr>
<tr>
<td>COS</td>
<td>Cone outer segment</td>
</tr>
<tr>
<td>CRD</td>
<td>Cone-rod dystrophy</td>
</tr>
<tr>
<td>CSNB</td>
<td>Congenital stationary night blindness</td>
</tr>
<tr>
<td>DDM</td>
<td>n-Dodecyl β-D-maltoside</td>
</tr>
<tr>
<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphatidylcholine</td>
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<td>DOPE</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine</td>
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<td>DOPS</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphatidylserine</td>
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<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EM</td>
<td>Electron microscopy</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERG</td>
<td>Electoretinogram</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>GC1</td>
<td>Guanylate cyclase 1</td>
</tr>
<tr>
<td>GCAP</td>
<td>Guanylate cyclase activating protein</td>
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<tr>
<td>GCL</td>
<td>Ganglion cell layer</td>
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<tr>
<td>GEF</td>
<td>Guanine nucleotide exchange factor</td>
</tr>
<tr>
<td>GPCR</td>
<td>G-protein coupled receptor</td>
</tr>
<tr>
<td>GSL</td>
<td>Glycosphingolipid</td>
</tr>
<tr>
<td>GTP</td>
<td>Guanosine-5'-triphosphate</td>
</tr>
<tr>
<td>h</td>
<td>Hour</td>
</tr>
<tr>
<td>HEK</td>
<td>Human embryonic kidney</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>-------------</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>INL</td>
<td>Inner nuclear layer</td>
</tr>
<tr>
<td>IPL</td>
<td>Inner plexiform layer</td>
</tr>
<tr>
<td>IRBP</td>
<td>Interphotoreceptor retinoid-binding protein</td>
</tr>
<tr>
<td>IS</td>
<td>Inner segment</td>
</tr>
<tr>
<td>kDa</td>
<td>Kilodalton</td>
</tr>
<tr>
<td>LC</td>
<td>Liquid chromatography</td>
</tr>
<tr>
<td>LCA</td>
<td>Leber’s congenital amaurosis</td>
</tr>
<tr>
<td>MD</td>
<td>Macular degeneration</td>
</tr>
<tr>
<td>MS</td>
<td>Mass spectrometry</td>
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<td>ONL</td>
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<tr>
<td>OPL</td>
<td>Outer plexiform layer</td>
</tr>
<tr>
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<td>Outer segment</td>
</tr>
<tr>
<td>PA</td>
<td>Phosphatidic acid</td>
</tr>
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<td>Phosphatidylcholine</td>
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<td>Polymerase chain reaction</td>
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<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PFIC</td>
<td>Progressive familial intrahepatic cholestasis</td>
</tr>
<tr>
<td>Abbreviation</td>
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<td>--------------</td>
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</tr>
<tr>
<td>PI</td>
<td>Phosphatidylinositol</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>PS</td>
<td>Phosphatidylserine</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>Rho +/-</td>
<td>Rhodopsin heterozygous mice</td>
</tr>
<tr>
<td>ROS</td>
<td>Rod outer segment</td>
</tr>
<tr>
<td>RP</td>
<td>Retinitis pigmentosa</td>
</tr>
<tr>
<td>RPE</td>
<td>Retinal pigment epithelium</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SM</td>
<td>Sphingomyelin</td>
</tr>
<tr>
<td>SPM</td>
<td>Standard phosphorylation medium</td>
</tr>
<tr>
<td>TGN</td>
<td>Trans-Golgi network</td>
</tr>
<tr>
<td>TLC</td>
<td>Thin layer chromatography</td>
</tr>
<tr>
<td>TNBS</td>
<td>2,4,6-trinitrobenzene sulfonic acid</td>
</tr>
<tr>
<td>XL</td>
<td>X-linked</td>
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<td>wl/wl</td>
<td>Wabbler-lethal</td>
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</table>
Acknowledgements

I would like to thank my supervisor Dr. Robert Molday for the opportunity to study in his laboratory. I feel fortunate to have a mentor that invested so much time in my thesis project and provided invaluable advice which has pointed me in the right direction experimentally and professionally. His keen attention to detail often has steered me clear of potential problems before they arose. I admire his dedication, passion, and integrity for science and education; these are qualities that I will strive to emulate in my own career. Most importantly, the stimulating scientific environment that he has cultivated has taught me how to approach and tackle challenging scientific problems carefully and thoroughly.

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Chapter 1: Introduction

1.1 Visual Perception

Visual perception allows the surrounding environment to be interpreted by processing electromagnetic radiation or visible light. Eyes are sensory organs that detect light, converting it into electrical and chemical signals in neuronal cells. Vision is one of the most important of the five traditionally recognized forms of perception and allows individuals to carry out basic activities necessary for physiological function as well as perform many advanced tasks that improve livelihood and well-being such as reading and writing, exercising, driving, and watching TV.

1.1.1 Anatomy of the Vertebrate Eye

The anterior segment of the eye includes: the cornea, iris, ciliary body, and lens (Fig. 1.1). The cornea forms the transparent anterior part of the eye responsible for two-thirds of the resolving power of the human eye. Light enters the vertebrate eye through the pupil. The iris controls the aperture size of the pupil and thus the amount of light which enters the eye. Unlike the cornea where focus is fixed, the lens can refract light onto the retina by changing curvature and thus the focal distance of the eye, allowing objects at different distances to be accurately resolved. The muscles of the ciliary body are responsible for adjusting the shape of the lens.

The posterior segment of the eye consists of the sclera, vitreous humor, choroid, retina, and optic nerve (McIlwain, 1996, Rodieck, 1998). The eye is protected by the sclera which is continuous with the cornea and forms the opaque, tough, fibrous outer surface of the eye consisting of mainly collagen and elastin. The vitreous humor is a clear gel which occupies the space between the lens and the retina and maintains the shape of the posterior
chamber. The vitreous has multiple points of attachment to the retina. The choroid lies between the sclera and retina and contains vasculature which provides oxygen and nutrients to the photoreceptors. The innermost layer of the eye is the retina which contains light-sensitive neuronal cells and allows visual information to be processed and transmitted by electrical and chemical signaling. Finally, the optic nerve relays visual information to higher visual centers in the brain such as the lateral geniculate nucleus.

1.1.2 The Retina

The retina is composed of three neuronal cell layers (Fig. 1.2): the photoreceptors, containing rods and cones; intermediate neurons, including bipolar, horizontal, and amacrine cells; and the ganglion cells (Cohen, 1963, McIlwain, 1996, Rodieck, 1998). Light must pass through each of these cellular layers to reach the photoreceptor layer.

Unlike many other neurons, photoreceptors, bipolar, and horizontal cells use graded potentials instead of action potentials enabling neurons to distinguish between light of varying intensities. Photoreceptors hyperpolarize in the light and depolarize in the dark. This reduces noise in photoreceptors since many channels need to be closed to hyperpolarize the membrane. Glutamate is released from photoreceptor terminals in the dark, upon illumination, glutamate release is reduced.

Photoreceptor cells synapse with bipolar cells in the outer plexiform layer. Bipolar cells can accept synapses with many neighbouring rods or several nearby cones and are the most numerous interneuron type. Bipolar cells are designating as either rod or cone bipolar cells depending on their synaptic connection. Horizontal cell dendrites span laterally across the retina synapsing with either rods or cones. Horizontal cells depolarize in the dark due to the release of glutamate from photoreceptors and release GABA to adjacent photoreceptors.
GABA inhibits photoreceptors causing hyperpolarization. In the light, release of GABA from horizontal cells is reduced. This allows visual information to be spatially encoded since photoreceptors connected to a horizontal cell depolarize increasing glutamate release to postsynaptic bipolars while central photoreceptors which are only connected to a bipolar cell will hyperpolarize and release less glutamate. Amacrine cell dendrites also span laterally across the retina like horizontal cells, synapsing with bipolar cells and modulating bipolar cell input to ganglion cells.

Retinal ganglion cells are found in the third layer of the retina and form synapses with bipolar cells. There are approximately 100 times fewer ganglion cells than photoreceptors implying that some ganglion cells receive information from hundreds of photoreceptors. Ganglion cells like bipolar cells can also be on-center or off-center which is also important for the transmission of contrast and spatial information. A subtype of ganglion cells is photosensitive and plays a key role in circadian rhythms and pupil reflex. The axons of ganglions cells are myelinated and comprise the tract of the optic nerve.

1.1.3 Photoreceptors and Outer Segments

In the human eye, there are approximately 120 million rod photoreceptor cells and roughly 6 – 7 million cone photoreceptors (Osterberg, 1935). Rod photoreceptor cells are able to respond to a single photon and are responsible for vision in dim light. At high light intensities, rods become bleached and dark adaptation is necessary to return to maximal sensitivity. Rhodopsin, the G protein coupled receptor (GPCR) found in rods absorbs light at 498 nm. Cones are not as sensitive as rods but can respond to many different intensities of light with faster kinetics. Cones are also responsible for colour vision. In humans, there are
three different cones containing opsins which absorb light at 420 (blue), 534 (green), and 564 nm (red) (Nathans, et al., 1986).

Cones are concentrated in the central part of the retina in a region known as the macula while rod photoreceptors are mainly found in the peripheral parts of the macula and retina (McIlwain, 1996, Yamada, 1969). The central part of the macula or the fovea centralis, is devoid of rods and contains densely packed cones. Ganglion cells in the fovea centralis synapse directly with a single cones, making this region responsible for the highest visual acuity in humans.

Rods and cones have similar polarized structures ((Fig. 1.3) and (Rodieck, 1998)). Nevertheless some morphological differences are important for photoreceptor specialization. Rod photoreceptors form long thin structures whereas cones are shorter and thicker. Photoreceptor cells can be divided into five different regions: the outer segment (OS), connecting cilium, inner segment (IS), cell body, and the synapse. The OS is a large specialized organelle which houses the phototransduction proteins. The rod OS (ROS) contains approximately 1000 flattened membraneous discs surrounded by a separate plasma membrane (PM). In the case of cones, OS discs are less numerous and continuous with the PM. The cilium of photoreceptors connects the OS to the IS. There have been two different models proposed for protein trafficking to the OS. Recently, it has been suggested that proteins are trafficked to the OS in vesicles along the cilium, regulated by the phospholipid binding protein SARA (Chuang, et al., 2007). In this model, fusion of vesicles with nascent discs is thought to drive OS morphogenesis. A more widely accepted view is that post-golgi vesicles are targetted and fuse near to the ciliary membrane where they are recognized by a complex of Bardet-Biedl Syndrome proteins (BBSome). Intraflagellar transport machinery
associates with the BBSome and the microtubules promoting anterograde transport of membrane proteins to the cillum (Leroux, 2007). In this model, nascent OS disc morphogenesis occurs through evagination of the PM at the base of the OS.

The IS contains various cellular machinery such as mitochondria, the endoplasmic reticulum (ER), Golgi, endosomes which are necessary for energy production, biosynthesis, and vesicular trafficking. The cell body contains the nucleus. Large numbers of synaptic vesicles are found in synapses tethered to a synaptic ribbon in close proximity to the presynaptic membrane.

### 1.1.4 Phototransduction

Phototransduction is initiated in the OS when a photon of light is absorbed by the covalently bound 11-cis-retinal chromophore of opsin (Molday, 1998, Palczewski, 1994, Pugh and Lamb, 1993). 11-cis-retinal becomes isomerized to all-trans-retinal which results in conformation change of opsin to metarhodopsin II and GPCR activation. All-trans-retinal dissociates from opsin. Rhodopsin activates the heterotrimeric G protein transducin and catalyzes the exchange of GDP for GTP which causes the α-subunit of transducin to dissociate from the βγ-subunits. The α-subunit activates phosphodiesterase which catalyzes the breakdown of cGMP to 5’-GMP. This lowers the cGMP concentration in the OS, resulting in the closure of cGMP gated channels and reducing the influx of Na⁺ and Ca²⁺. The photoreceptor hyperpolarizes and voltage gated calcium channels close, reducing Ca²⁺ concentrations at the synapse and lowering neurotransmitter release.

To return the photoreceptor to the dark state, GTPase activating protein (GAP) interacts with the α-subunit of transducin which hydrolyzes its bound GTP (He, et al., 1998) causing phosphodiesterase to stop hydrolyzing cGMP. Rhodopsin kinase dissociates from
recoverin at low Ca\(^{2+}\) concentrations, allowing it to phosphorylate rhodopsin (Chen, et al., 1995). Phosphorylation of rhodopsin reduces its affinity for transducin. Arrestin binds phosphorylated rhodopsin and completely deactivates it, terminating the phototransduction cascade (Mendez, et al., 2000). The Na\(^{+}/\text{Ca}^{2+}-\text{K}\(^{+}\) exchanger extrudes Ca\(^{2+}\) from the OS causing Ca\(^{2+}\) levels to decrease (Kim, et al., 1998). Guanylate cyclase activating protein (GCAP) becomes activated and binds to guanylate cyclase 1 (GC1) which converts GTP to cGMP (Gorczyca, et al., 1995). As cGMP levels increase, cGMP gated channels open, allowing Na\(^{+}\) and Ca\(^{2+}\) to enter and returning the photoreceptor to the dark state.

1.1.5 Retinal Pigment Epithelium

The retinal pigment epithelium (RPE) is a continuous monolayer of epithelial cells located between the choroid and the OS layer of photoreceptors. The apical surface of the RPE forms long microvilli that extend into the OS layer. The basolateral surface is part of Bruch’s membrane which separates the choroid from the RPE. Photoreceptors rely on the RPE for various metabolic functions which are critical for photoreceptor survival and function. RPE cells absorb nutrients such as glucose, retinol, and fatty acids from the blood, delivering them to the photoreceptors. RPE cells also take up water, ions, and metabolites from the photoreceptors. The RPE contains dense pigment granules of melanin known as melanosomes which absorb stray light not captured by photoreceptors.

The RPE layer also phagocytoses aging OS discs as part of the OS renewal process. Discs are engulfed by the apical processes of the RPE and broken down in lysosomes. Phagocytosis occurs as part of daily cycle that is induced by the onset of light (LaVail, 1976). New discs are constitutively added to the base of the OS near the cilium. It takes approximately 10 days to completely renew OS membranes (Young, 1971).
The RPE also contains key enzymes which are necessary for regeneration of the visual chromophore in a process known as the visual cycle (Travis, et al., 2007). Photoreceptors cannot reisomerize retinal following its absorption of light and dissociation from opsin. Free retinoids are toxic due to their highly reactive nature. All-trans-retinal can react with phosphatidylethanolamine (PE) forming N-retinylidene-PE in the lumen of OS discs (Weng, et al., 1999). ABCA4 removes N-retinylidene-PE from the lumen by flipping it to the cytosolic side of the disc (Illing, et al., 1997, Quazi, et al., 2012). In the cytosol, free all-trans-retinal becomes reduced by retinal dehydrogenase. All-trans-retinol is transported from photoreceptors to the RPE by interphotoreceptor retinoid-binding protein (IRBP) where it is converted by RPE65 and other enzymes to 11-cis-retinal and returned to the photoreceptors.

1.1.6 Inherited Retinal Disorders

Inherited retinal disorders comprise a large group of diseases which either result in retinal cell death or malfunction leading to visual impairment or blindness (Michaelides, et al., 2003). To date, there are 247 genes which have been mapped to loci causing retinal disease including macular degeneration (MD), retinitis pigmentosa (RP), usher syndrome, leber’s congenital amaurosis (LCA), and congenital stationary night blindness (CSNB) (https://sph.uth.tmc.edu/retnet/). MD and RP are the most common forms of inherited blindness. MD affects the central visual field due to photoreceptor damage in the macular region. Age-related macular degeneration (AMD) is the most common cause of blindness in the developed world and is the leading cause of blindness in individuals older than 60. More than 7 million people in North America are at risk of developing AMD and approximately 1.75 million individuals are affected (Friedman, et al., 2004). RP is less prevalent, affecting
approximately 1 in 4000 individuals and is characterized by night blindness and loss of peripheral vision (Haim, 2002). Photoreceptor death usually occurs through the apoptotic pathway (Chang, et al., 1993).

Many genes which encode key proteins required for phototransduction, structure, and the visual cycle are known to cause retinal disorders (Michaelides, et al., 2003). Mutations in phototransduction enzymes such as the cGMP gated channel, phosphodiesterase, and arrestin are known to cause recessive RP. Rhodopsin mutations alone account for more than 30% of cases of autosomal dominant RP with more than 100 distinct mutations having been identified. Rhodopsin gene dosage is a determining factor for OS structure and size since it is the most abundant protein in the OS (Paparmaster and Dreyer, 1974). Mislocalization of rhodopsin in the IS can cause inappropriate GPCR activation and photoreceptor cell death (Hollingsworth and Gross, 2012). Mutations in peripherin-2 and ROM-1 also cause autosomal dominant RP since they form oligomeric complexes which are crucial for OS structure and morphogenesis (Kedzierski, et al., 2001, Loewen and Molday, 2000). Loss of RPE65 causes recessive RP and LCA due to the accumulation of all-trans-retinal derivatives which cannot be isomerized. Apo-opsin is found in the OS of RPE65 -/- mice without a bound 11-cis-retinal (Redmond, et al., 1998). ABCA4 mutations are known to cause Stargardt’s disease, a early onset form of macular degeneration as well as a spectrum of other disorders (Allikmets, et al., 1997). AMD is a complex disorder since many different environmental and genetic factors can increase susceptibility. Mutations in ABCA4 and various complement factor polymorphisms are known to increase the risk of AMD.

It is estimated that approximately 50% of cases of RP are caused by a mutations in genes known to be responsible for RP (Sullivan, et al., 2006). This suggests that defects in
many unknown genes are also responsible for many retinal distrophies. A list of selected
genes associated with various retinal distrophies is shown in Table 1.1.

1.2 Biological Membranes and Lipid Asymmetry

Biological membranes are hydrophobic structures that enclose cells and organelles
and provide a selective barrier to most polar solutes. Biological membranes are composed of
a 30 Å bilayer of lipid containing embedded proteins which surround cells and cellular
compartments such as organelles. According to the fluid mosaic model, hydrophobic
components such as lipids and membrane proteins can freely move laterally within the
membrane (Singer and Nicolson, 1972). However, lipid rafts, the cytoskeleton or the
extracellular matrix can create lipid domains and impose restrictions on lateral mobility.

There are three principle classes of membrane lipids: glycerophospholipids,
spingolipids, and sterols. Glycerophospholipids consist of a polar head group joined through
a phosphodiester linkage to a hydrophobic diacylglycerol containing saturated or cis-
unsaturated fatty acyl chains of differing lengths. Spingosine is the primary backbone of
sphingolipids such as sphingomyelin (SM) and glycosphingolipids (GSL). Sterols such as
cholesterol (Chol) consist of a fused four ring backbone. The major structural lipids of
eukaryotic membranes are glycerophospholipids such as phosphatidylcholine (PC), PE,
phosphatidylserine (PS), and phosphatidylinositol (PI) (van Meer, et al., 2008). Chol, SM,
and GSLs are also major components of the PM. Photoreceptor OS membranes contain
approximately 40% PC, 30% PE, and 10-15% PS (Fliesler and Anderson, 1983, Mason, et
al., 1973). SM, Chol, and PI are minor components of OS membranes. OS disc lipids contain
an unusually high content of polyunsaturated fatty acids such as docosahexaenoic acid which
is important for visual function (Bush, et al., 1994).
Lipids are distributed asymmetrically across biological membranes (Holthuis and Levine, 2005, van Meer, et al., 2008). Aminophospholipids such as PS and PE are found in the cytoplasmic leaflet of the PM while PC, SM, and GSLs are mainly found in the extracellular leaflet of biological membranes. PS is found nearly exclusively in the cytoplasmic leaflet. Approximately 80% of PC and SM are found in the outer leaflet while 80% of PE is found in the inner leaflet. Other minor lipid components of the membrane such as phosphatidic acid (PA) and PI are also enriched in the cytosolic leaflet. The distribution of glycolipids also favors the extracellular leaflet. Chol can form Chol-SM enriched microdomains through preferential interactions with SM providing mechanical strength and reducing permeability. Membranes of intracellular organelles and vesicles also display transbilayer asymmetry (Alder-Baerens, et al., 2006, van Meer, et al., 2008). Several studies have reported that aminophospholipids in the photoreceptor OS are also asymmetric (Miljanich, et al., 1981, Wu and Hubbell, 1993), while other reports have suggested a more symmetrical distribution (Hessel, et al., 2001, Menon, et al., 2011). Further studies are necessary to resolve if OS membranes are asymmetric.

Flip-flop between leaflets is much slower than lateral diffusion which occurs very quickly (Kornberg and McConnell, 1971, Kornberg and McConnell, 1971) and is very energetically unfavourable. In order to generate lipid asymmetry, enzymes are necessary to provide a pathway through the membrane to increase the rate of flip-flop. Lipid transporters can be organized into at least three different protein families (Fig. 1.4): ABC transporters, P₄-ATPases, and scramblases.

Scramblases are energy independent, bi-directional lipid transporters. In general they have broad substrate specificity transporting a wide variety of phospholipids and other
membrane lipids across the lipid bilayer. Scramblases are important for dissipating lipid asymmetry generated during the biosynthesis of lipids in the ER and counteracting lipid asymmetry in other cell membranes to facilitate such processes as phagocytosis, apoptosis, fertilization, and blood coagulation. Scramblases in general are poorly characterized although several studies have reported the cloning and characterization of proteins which appear to function as scramblases (Chang, et al., 2004, Menon, et al., 2011, Suzuki, et al., 2010, Yang, et al., 2012, Zhou, et al., 1997).

P$_4$-ATPases are a class of P-type ATPases which utilize the energy from ATP hydrolysis to transport or flip aminophospholipids from the exocytoplasmic (extracellular/lumen) to the cytoplasmic leaflet of cellular membranes. These phospholipid flippases have been implicated in the generation and maintenance of phospholipid asymmetry found in many cellular membranes and several members have been recently linked to severe human disorders (Bull, et al., 1998, Folmer, et al., 2009, Klomp, et al., 2004, Levano, et al., 2011, Puts and Holthuis, 2009, Yabas, et al., 2011).

ABC transporters comprise a large class of membrane proteins involved in the active transport of a wide variety of compounds across cell membranes. A significant number of ABC proteins are now known to transport phospholipids across cell membranes (Borst, et al., 2000, Kaminski, et al., 2000, Quazi, et al., 2012). Most mammalian ABC transporters function as exporters or floppases transporting substrates from the cytoplasmic leaflet to the exocytoplasmic side of membranes. Recent studies, however, now indicate that some eukaryotic ABC transporters function as importers, flipping phospholipids or other substrates from the exocytoplasmic to the cytoplasmic leaflet of cells (Quazi, et al., 2012). ABC proteins play a crucial role in lipid homeostasis as indicated by the finding that mutations in a
significant number of ABC transporters are known to cause severe human diseases associated with defective phospholipid transport (Coleman, et al., 2012). ABC transporters may also contribute to membrane lipid asymmetry in specific biological membranes.

1.3 The P-type ATPase Superfamily

P-type ATPases comprise a large family of ion and lipid transporters (483 unique sequences, Prosite) which catalyze auto-phosphorylation of aspartic acid of the P-type signature sequence DKTGT. Interconversion of four principle conformational states termed $E_1$, $E_1P$, $E_2P$, $E_2$ (P denotes phosphorylation) drives ion translocation (Kuhlbrandt, 2004). P-type ATPases are found in all kingdoms of life and are classified into five families by ion specificity and sequence (Fig. 1.5). Type IA are bacterial $K^+$ pumps, Type IB are heavy metal pumps, while Type IIA and Type IIB are $Ca^{2+}$ pumps and Type IIC are $Na^+/K^+$ or $H^+/K^+$ pumps. Type IIIA ATPases are specific to $H^+$. Type IV and V ATPases are only found in eukaryotes. Type IV ATPases are thought to transport phospholipids (Tang, et al., 1996). The substrate specificity of Type V ATPases is unknown. An extensive structural account of the reaction sequence for the $Ca^{2+}$ ATPase has described many of the key events which underlie ion pumping (Toyoshima, 2009). Recent structures of the $Na^+,K^+$-ATPase, $H^+$-ATPase, and $Cu^{2+}$-ATPase have advanced our understanding of the similarities and differences between transporters of different ion specificities (Gourdon, et al., 2011, Pedersen, et al., 2007, Toyoshima, et al., 2011) however no structural information is available for the type IV and V ATPases.

1.3.1 Functions of P$_4$-ATPases and Lipid Asymmetry

Transport of phospholipids across cell membranes plays a crucial role in many biological processes. Phospholipid flippases are important in generating lipid imbalance
between the two leaflets which contributes to membrane bending and curvature. Lipid asymmetry and imbalance have been implicated in many biological processes such as phagocytosis, fertilization, membrane budding and vesicle trafficking, modulation of membrane protein function, blood coagulation, cell and organelle shape among others (Puts and Holthuis, 2009). The transport of phospholipids across membranes is also essential for lipid homeostasis. For example, Chol efflux from cells, formation of bile, nutrient transport, removal of toxic compounds from cells, and secretion of pulmonary surfactants all require phospholipid transport. Indeed, the importance of phospholipid transport in these and other processes is evident by the finding that many severe human disorders are caused by defects in lipid transport (Folmer, et al., 2009, Kaminski, et al., 2000, Quazi, et al., 2012).

One of the most widely recognized functions of PS asymmetry occurs in apoptotic cells, where exposure of PS in the outer leaflet of the PM serves as an “eat me” signal for phagocytic cells. In C. elegans, loss of the PS transporter TAT-1 leads to abnormal exposure of PS resulting in the clearance of neuronal and muscle cells (Darland-Ransom, et al., 2008).

Nearly all synthesis of new membranes occurs in the ER. Lipids synthesized in the cytoplasmic leaflet of the ER are rapidly and symmetrically redistributed by the action of scramblases to ensure an even biogenesis of both membrane leaflets (Buton, et al., 1996). Newly synthesized membranes and proteins travel out of the ER by budding of small vesicles which fuse with other membraneous organelles such as the Golgi complex. Lipids are sorted and further processed in the Golgi where lipid asymmetry is first generated by the action of flippases. Proteins are also sorted in the trans-Golgi network (TGN) and are exported to the endosomal/lysosomal pathway or sorted into cargo destined for the exocytic pathway. Clathrin dependent trafficking is a major pathway for vesicle transport from the TGN to
endosomes. ARFs (ADP ribosylation factors) are GTP-binding proteins that in their active GTP-bound form regulate protein trafficking by recruiting coat proteins such as clathrin to sites of vesiculation. ArfGEFs (gaunine nucleotide exchange factors) and ArfGAPs (gaunine nucleotide activating factors) catalyze the exchange of GDP for GTP and increase the rate of Arf GTP hydrolysis respectively.

Accumulating evidence in yeast and other cells supports a role for P₄-ATPases in vesicle transport and budding, regulating many distinct protein trafficking pathways (Hua, et al., 2002, Pomorski, et al., 2003). Loss of TAT-1 in C. elegans results in the accumulation of large intracellular vacuoles derived from the endolysosomal pathway (Ruaud, et al., 2009). Loss of TAT-5, in C. elegans causes large scale shedding of extracellular vesicles disrupting cell adhesion and morphogenesis (Wehman, et al., 2011). P₄-ATPase involvement in vesicle transport in mammalian cells is not as well understood, however, many pathophysiological features suggest that this is a conserved function for P₄-ATPases across evolution. Early studies in yeast, showed that knockout of Drs2p caused accumulation of abnormal membrane structures and an inability to form clathrin-coated vesicles (Chen, et al., 1999, Gall, et al., 2002). Furthermore, genetic interactions with many members of the clathrin-mediated pathway have been described. Drs2p physically interacts with ArfGEF in the Golgi (Chantalat, et al., 2004) and binding of ArfGEF stimulates the flippase activity of Drs2p (Natarajan, et al., 2009). A model has been proposed for vesicle formation whereby ArfGEFs recruit activated Arfs and bind to P₄-ATPases which concentrate lipids on the cytosolic surface. This creates a localized imbalance of phospholipid in the cytosolic leaflet and based on the bilayer couple hypothesis, membrane curvature is generated which drives the formation of a vesicle bud (Sebastian, et al., 2011). Activated-Arfs recruit coat and adaptor
proteins to site of vesiculation which target the newly derived vesicles to their subcellular destinations.

1.3.2 The P₄-ATPase-CDC50 Complex: Domain Structure and Mechanism

P-type ATPases are integral membrane proteins which form an elongated structure distinguished by a large cytoplasmic headpiece. P₄-ATPases likely adopt a similar four domain structure as most other P-type ATPases consisting of cytosolic A (“actuator”), P (phosphorylation), and N (nucleotide binding) domains and an M (membrane) domain made up of ten transmembrane helices M1-10 (Palmgren and Nissen, 2011) and (Fig. 1.6). Short extracellular loops connect each transmembrane helix such that only a small portion of the structure faces the lumenal or extracellular surface. The C-terminal region could be involved in pump regulation or targeting since it is the least similar between pumps of different subfamilies and is often a regulatory region in other P-type ATPases (Enyedi, et al., 1993, Palmgren, et al., 1991). In humans, there are 14 members of the P₄-ATPase family which are organized into five subfamilies based on sequence (Fig. 1.5B, Table 1.2). The CDC50 family consists of only three members: CDC50A, CDC50B, and CDC50C suggesting that many P₄-ATPases can bind the same CDC50 as a β-subunit. In humans and other primates, the CDC50C gene is truncated and may be unfunctional (Osada, et al., 2007).

Structural analysis of the P-ATPase ion pumps indicates that large movements of the cytosolic A-, N-, and P-domains drive the conversion of the enzyme between its four principle conformational states (Palmgren and Nissen, 2011) and (Fig. 1.7). A flexible linker region couples these conformational changes to the M-domain, facilitating ion translocation. The transport cycle is initiated when the N-domain binds ATP in the $E_1$ conformation and rapidly phosphorylates the aspartic acid of the P-domain. Many P-type ATPases require
binding of a transported ion (Na\(^+\) in the case of the Na\(^+\),K\(^+\)-ATPase) to a high affinity cytoplasmic facing site in the \(E_1\) form for phosphorylation by ATP. It is unknown if this is a requirement for P\(_4\)-ATPases. Some P-type ATPases use instead a charged amino acid residue as “built-in” ion (Pedersen, et al., 2007).

The phosphorylated enzyme exists in two distinct conformational states. The \(E_1P\) form can react with ADP producing ATP while the \(E_2P\) form is resistant to ADP. During the transition from \(E_1P\) to \(E_2P\), the A-domain rotates 90° inserting the TGES (DGET in P\(_4\)-ATPases) motif into the space which was occupied by ADP, positioning it near the phosphorylated aspartate (Palmgren and Nissen, 2011). The consequence of A-domain rotation is the conversion of the high affinity cytoplasmic site to a low affinity extracellular facing site allowing exit of the ion. Based on the directionality of transport, it is thought that transported aminophospholipids activate the dephosphorylation of \(E_2P\) for P\(_4\)-ATPases in a manner analogous to the dephosphorylation of the Na\(^+\),K\(^+\)-ATPase by K\(^+\) (Ding, et al., 2000). In the case of the Na\(^+\),K\(^+\)-ATPase, K\(^+\) ions interact with the \(E_2P\) form of the enzyme stabilizing the transition state and accelerating the rate of dephosphorylation. Binding of ions to \(E_2P\), triggers further movement of the glutamic acid of the TGES motif in closer proximity to the P-domain allowing it to catalyze dephosphorylation acting as a base to remove a proton from water which carries out nucleophilic attack on the aspartyl-phosphate (Clausen, et al., 2004). Since K\(^+\) is transported in the same direction as aminophospholipids, it is possible that lipids are transported using a similar mechanism. Dephosphorylation converts the enzyme to the \(E_2\) form changing the high affinity extracellular site to a low affinity cytoplasmic site allowing the K\(^+\) and presumably lipid in the case of P\(_4\)-ATPases to leave. The A-domain moves away from the P-domain, returning the protein to the \(E_1\) conformation.
In most P-type ATPases, transmembrane helices M1-6 are thought to form the core catalytic unit of ion transport while M7-10 support the core catalytic unit and may facilitate interactions with β-subunits. M1-6 are more flexible throughout the transport cycle while M7-10 remain fairly rigid. P₄-ATPases seem to be unique among P-type ATPases in their ability to transport a substrate much larger than a cation. In other P-type ATPases, ion translocating pathways are not large enough to accommodate a “giant substrate” such as a phospholipid (Lenoir, et al., 2007). This leads to an important question: does the lipid translocation pathway resemble the ion translocation pathway in other P-type ATPases? Or are lipids transported through a unique translocation pathway? It has been proposed that other protein partners such a β-subunit could form part of the translocation pathway with P₄-ATPases. It is also thought that only the headgroup may interact with the pathway, leaving the acyl chains free to interact with the rest of the lipid environment. Therefore, lipids could move through the membrane in a concept similar to a credit card reader potentially alleviating the “giant substrate” problem. However, these hypotheses are speculation and are not currently supported by any biochemical or structural evidence.

Most P₄-ATPases form heterologous complexes with the CDC50 family of proteins (Table 1.2). CDC50 proteins are composed of a large glycosylated E (extracellular) domain containing three or more N-linked glycosylations, a membrane domain composed of two transmembrane helices, and two small cytoplasmic N- and C-terminal segments. In the Na⁺,K⁺-ATPase, numerous interactions of the M7 and M10 helices are made with the transmembrane helix of the β-subunit. The extracellular domain forms further interactions with the L7/8 loop of the α-subunit (Toyoshima, et al., 2011). These interactions are clearly necessary for the correct assembly and folding of pumps allowing them to leave the ER
(Paulusma, et al., 2008, Takatsu, et al., 2011) however CDC50 proteins do not seem to be important for subcellular targeting (Lopez-Marques, et al., 2010). For many P-type ATPases, complex interactions of β-subunits exert substantial changes on pump kinetics (Toyoshima, et al., 2011). Further to this, the FXYD family of single spanning membrane proteins modulate the kinetics or affinities for Na⁺ or K⁺ for the Na⁺,K⁺-ATPase in a tissue dependent manner. The CDC50 family has been proposed to behave equivalent to a “fusion” of the FXYD and β-subunits of the Na⁺,K⁺-ATPase (Puts and Holthuis, 2009). In yeast, CDC50 has been shown to bind with higher affinity to the phospholipid-loaded E₂P form of Drs2 suggesting that it could participate directly in lipid flipping (Lenoir, et al., 2009). However, the exact nature of these interactions and the mechanism of pump modulation is not well understood. Experiments in yeast suggest that the interaction surface of the P₄-ATPase-CDC50 complex is extensive and that many of these subunit interactions are necessary for both pump modulation and assembly. The precise role of CDC50 proteins in the reaction cycle requires further structural and biochemical investigations.

1.3.3 ATP8B Subfamily

ATP8B1 was the first P₄-ATPase found to be involved in human disease. Mutations in ATP8B1 cause two forms of intrahepatic cholestasis: progressive familial intrahepatic cholestasis (PFIC) and benign recurrent intrahepatic cholestasis (Bull, et al., 1998). These patients can also experience hearing loss and are predisposed to pneumonia (Ray, et al., 2010, Stapelbroek, et al., 2009). Cholestasis is a condition where the flow of bile from the liver is blocked. Patients often develop the disorder early in life often resulting in cirrhosis within the first decade of life. ATP8B1 mutations can result in a wide spectrum of clinical severity as some patients experience cholestasis intermittently while others do not experience
any symptoms (Klomp, et al., 2004). In hepatocytes, ATP8B1 localizes in the canalicular membrane where its activity is thought to be important for the maintenance of membrane integrity and function of the PC floppase ABCB4 and consequently bile export (Eppens, et al., 2001, Groen, et al., 2011, Paulusma, et al., 2006). ATP8B1 is necessary for apical protein expression and membrane organization and loss of ATP8B1 causes substantial microvilli loss in Caco-2 cells (Verhulst, et al., 2010). Degeneration of stereocilia of cochlear hair cells where ATP8B1 is present is observed in knockout mice (Stapelbroek, et al., 2009). ATP8B1 can bind both CDC50A and CDC50B (Bryde, et al., 2010, Paulusma, et al., 2008, van der Velden, et al., 2010) but in the liver and other tissues, CDC50A is preferentially expressed and likely binds ATP8B1 in situ (Folmer, et al., 2012, Paulusma, et al., 2008). The lipid substrate that is flipped by ATP8B1 is not fully understood. In UPS-1 cells, ATP8B1 has been shown to be involved in PS translocation. In PFIC patients with severe pneumonia, cardiolipin levels were found to be elevated and correlate with higher levels of lung injury. ATP8B1 was found to bind and translocate cardiolipin via a basic residue containing motif (Ray, et al., 2010). Biochemical studies on purified ATP8B1 seemed to indicate that it was trapped in the $E_2P$ conformation and could not complete the reaction cycle (Bryde, et al., 2010). It is unclear if this was due to experimental conditions, regulation, or lack of a suitable substrate. In order to determine which lipid species is transported by this important pump it will be necessary to understand what is required for dephosphorylation and completion of the reaction cycle.

Little is known about ATP8B2 and ATP8B4. ATP8B4 is expressed throughout the brain and may be involved in Alzheimer disease (Li, et al., 2008). Both pumps may be able to interact with CDC50A and CDC50B (Bryde, et al., 2010), although in the case of ATP8B4
experimental conditions may influence interaction with CDC50B (van der Velden, et al., 2010). ATP8B3 and its homolog ATP8B5 in mice are exclusively expressed in the testis where they are known to localize to the acrosomes of spermatids, the organelle which develops in the anterior half of the head in spermatozoa (Gong, et al., 2009, Wang, et al., 2004, Xu, et al., 2009). Knockdown of ATP8B5 has a profound effect on Golgi structure and protein secretion (Xu, et al., 2009). On the basis of cell biology and biochemical studies, ATP8B3 appears to also be a PS pump. ATP8B3 does not seem to interact with either CDC50A or CDC50B (van der Velden, et al., 2010) however it is possible that it could interact with CDC50C based on expression. Interestingly, disruption of ATP8B3 in mice does not affect the morphology of sperm or the rate of fertilization despite sperm-egg interactions being disrupted in vitro (Wang, et al., 2004).

1.3.4 ATP9, ATP10, and ATP11 Subfamilies

Pumps belonging to the ATP9 subfamily are the only members of the P₄-ATPase family which do not interact with CDC50 proteins (Takatsu, et al., 2011) and localize to the trans-Golgi in a CDC50-independent manner. An N-terminal targeting sequence appears to be important for this localization of ATP9B. The substrate transported by these pumps is so far unknown. In yeast, loss of NEO1 an ortholog of the ATP9 members is lethal and it seems to play a role in endosomal membrane trafficking as well as retrograde COPI trafficking to the ER (Hua and Graham, 2003, Wicky, et al., 2004). Further investigation is necessary to understand if the ATP9 subfamily plays a similar role in mammalian cells.

The ATP10 subfamily harbors the least biochemically characterized subfamily of P₄-ATPases. Pumps from this subfamily have a longer C-terminus, the function of which is unknown. CDC50A is required for the ER exit of these ATPases (Takatsu, et al., 2011). The

Recent work from two independent labs has shown that ATP11C is important for the proper development of B-cell lymphocytes in mice (Siggs, et al., 2011, Yabas, et al., 2011). ATP11C seems to be required for efficient flipping of PS in B-cells. Intrahepatic hyperbilirubinemia, an accumulation of bilirubin of hepatocellular origin and high serum levels of cholic acid indicative of cholestasis is also observed in these animals (Siggs, et al., 2011). Not much is known about the other members of the ATP11 subfamily, each member appears to be ubiquitously expressed and only binds CDC50A as a β-subunit (Takatsu, et al., 2011).

1.3.5 ATP8A1

The first evidence that P₄-ATPases transport aminophospholipids came from studies on the yeast Drs2p where notable deficiencies in the uptake of NBD-labeled PS were reported (Tang, et al., 1996). ATP8A1, an ortholog of Drs2p, was the first P₄-ATPase discovered in mammals and is ubiquitously expressed throughout the body ((Mouro, et al., 1999) and (Table 1.2)). Relative to wild type, the brains of ATP8A1 knockout mice appear morphologically normal despite PS-externalization in the hippocampus. Interestingly, these mice display hyperactivity and marked deficiencies in hippocampus-dependent learning (Levano, et al., 2011). Pioneering biochemical studies first isolated a 120 kDa ATPase from red blood cells and the brain by conventional techniques that was robustly activated by aminophospholipids and inhibited by the phosphate analog vanadate and sulfhydryl-reactive reagents (Moriyama and Nelson, 1988, Morrot, et al., 1990, Xie, et al., 1989, Zimmerman
and Daleke, 1993). This ATPase was thought to be responsible for the rapid transport of spin labeled PS and PE observed in both of these systems (Seigneuret and Devaux, 1984, Zachowski, et al., 1989). In red blood cells, phospholipid transport is thought to influence cell shape, inducing the discocyte to echinocyte change (Daleke and Huestis, 1989). Transport stoichiometry was measured to be one ATP molecule per phospholipid (Beleznay, et al., 1993). Reconstitution of the ATPase from red blood cells demonstrated that it was able to catalyze the rapid flipping of PS (Auland, et al., 1994). Despite these efforts, the identity of the red blood cell ATPase still remains uncertain. Western blotting with highly specific antibodies, showed that both red cells and chromaffin granules contain ATP8A1 (Ding, et al., 2000, Soupene and Kuypers, 2006). Heterologously expressed ATP8A1 from insect cells has many similar properties as the ATPase isolated from natural sources (Ding, et al., 2000, Paterson, et al., 2006). ATP8A1 has been reported to bind CDC50A (Bryde, et al., 2010, van der Velden, et al., 2010) although it is possible that it can also bind CDC50B depending on experimental conditions (Bryde, et al., 2010). Splice variants of ATP8A1 differ in their phospholipid selectivity and specific ATPase activity (Ding, et al., 2000). However, markedly different specific ATPase activities were observed in some studies possibly due to the absence of the appropriate CDC50A β-subunit (Paterson, et al., 2006). Isolation and identification of ATP8A1 from natural sources using highly specific reagents and comparison of its enzymatic properties with earlier studies is necessary to clarify the role of ATP8A1 in these tissues.

1.3.6 ATP8A2

ATP8A2 is 67% identical in sequence to ATP8A1 and belongs to the same subfamily of P4-ATPases. Little was known about the biochemical properties and expression of this
pump prior to this investigation. However, recent evidence has suggested that this pump plays an important role in neurophysiology. A patient with severe mental retardation and major hypotonia has been reported carrying a balanced translocation of chromosomes 10 and 13 disrupting the coding sequence of the \textit{ATP8A2} gene (Cacciagli, et al., 2010). Very recently, a family with four individuals carrying a missense mutation in ATP8A2 has been reported (Emre Onat, et al., 2012). These individuals exhibit severe cerebellar ataxia, mental retardation and dysequilibrium syndrome. Recently, wabbler-lethal mice have been reported to carry mutations in the \textit{ATP8A2} gene (Zhu, et al., 2012). These mice develop severe neurological abnormalities including ataxia and body tremors due to distal axonal degeneration in spinal neurons which may be a result of disrupted vesicle transport. Wabbler mice die at approximately one month of age. Loss of both \textit{ATP8A1} and \textit{ATP8A2} results in neonatal lethality suggesting the function of ATP8A1 overlaps with ATP8A2 (Zhu, et al., 2012). ATP8A2 may be involved in vesicular trafficking in neurons providing an explanation for the phenotypes observed in humans and animal models. In PC12 and hippocampal neurons, overexpression of ATP8A2 enhances neurite outgrowth providing support for a role in vesicle formation or transport (Xu, et al., 2012).

The function of P$_4$-ATPases in photoreceptors is unknown. There are no mutations associated with \textit{ATP8A2} that are described to impair visual function. ATP8A2 along with its potential CDC50A β-subunit were detected in photoreceptor OS preparations by mass spectrometry (MS) (Kwok, et al., 2008). No other P$_4$-ATPases were detected in these studies, suggesting that ATP8A2 may be a key player in lipid translocation in the OS.

While the function of P$_4$-ATPases and lipid asymmetry in photoreceptors is unknown, several hypotheses have been proposed. Rhodopsin the major membrane protein of the OS
has recently been shown to act as a phospholipid scramblase (Menon, et al., 2011). The flippase activity of a P₄-ATPase may be necessary to maintain lipid asymmetry necessary for phototransduction in the presence of high rhodopsin concentrations. Flippases may also be involved in OS renewal. Using microarray analysis, ATP8A2 expression was found to be downregulated in Nrl mice, potentially disrupting phospholipid organization (Mustafi, et al., 2011). The retinas of Nrl knockout mice only contain cone photoreceptors and large amounts of abberant phagosomes are present at the RPE-photoreceptor interface. PS is normally localized in the cytoplasmic surface of photoreceptor PM. However, coinciding with the onset of light and phagocytosis, PS becomes exposed to the extracellular surface at the tips of photoreceptors (Ruggiero, et al., 2012). This extracellular PS could then serve as an “eat me” signal for the RPE cells triggering phagocytosis. This would require regulation of PS flippase activity at the apical tips of the photoreceptor. ATP8A2 may also be involved in vesicular trafficking in photoreceptors similar to its proposed role in other neuronal cells.

1.4 Thesis Investigation

In 2007, ATP8A2 a member of the P₄-ATPase was discovered in a proteomic study of photoreceptor OS (Kwok, et al., 2008). Although cell biology and genetic experiments in yeast and mammalian cells suggested that P₄-ATPases were lipid flippases, there was no direct biochemical evidence that P₄-ATPases were lipid transporters and the function of lipid asymmetry in photoreceptors and mammalian cells was unknown. Little was known concerning the cellular localization, expression profile, substrate specificity, protein-protein interactions, mechanism, or role in human disease for ATP8A2 at the start of this project in 2007.
Chapter 2 describes the localization, purification, and functional reconstitution of ATP8A2 from photoreceptors. Using two highly specific monoclonal antibodies as key tools, the localization of ATP8A2 in the mammalian retina and biochemical properties of the transporter were investigated. ATPase activity assays and a newly developed fluorescent-based lipid transport assay were used to define the lipid specificity and transport properties of this pump. This study and work from Todd Graham’s lab (Zhou and Graham, 2009) provided the first direct biochemical evidence that the P₄-ATPase family could transport aminophospholipids.

Chapter 3 delves into protein-protein interactions of ATP8A2 using immunoaffinity chromatography and MS. Some P₄-ATPases are known to associate with the CDC50 family of proteins. This work identifies the CDC50 subunit which binds ATP8A2 in photoreceptors using newly generated anti-CDC50 antibodies. The retinal localization of the CDC50 β-subunit is also demonstrated. A high level expression system in HEK293T cells was developed to explore the role of CDC50 proteins in lipid transport and identify the CDC50 domains which are responsible for P₄-ATPase association. The role of N-linked oligosaccharides in the E-domain of CDC50 proteins was also investigated.

Chapter 4 provides the first insights into the mechanism of lipid transport used by P₄-ATPases. The mechanism of lipid transport was compared to that of other cation transporting P-type ATPases. Using a newly developed phosphorylation assay and mutagenesis, the function of several key residues in the A- and P-domains was elucidated. The ability of different conformations to interact with aminophospholipids and nucleotides was examined, allowing the partial reaction steps of the transport cycle to be understood. The role of different residues in the M-domain was studied to define which residues constitute the lipid
translocation pathway. Finally, different ions were tested for binding to ATP8A2 in order to learn whether other substances could be transported by P$_4$-ATPases.

In chapter 5, the functional importance of ATP8A2 in photoreceptors and other cell types was determined by targeted disruption of the $atp8a2$ gene in mice. A key question is whether loss of lipid asymmetry can disturb photoreceptor structure and function and in turn cause degeneration, blindness, or other disorders. Photoreceptor function was studied using the electroretinogram (ERG). Photoreceptor structure was examined using electron and light microscopy. The abundance, localization, and function of key phototransduction and structural proteins was determined using immunocytochemistry, western blotting, and spectrophotometry. A new procedure was developed to label aminophospholipids in photoreceptor membranes to measure changes in lipid asymmetry. On the basis of these studies, a function for ATP8A2 in photoreceptors and other neurons is proposed.
Table 1.1  Summary of important genes responsible for human retinal dystrophies.

Mutations in approximately 250 different genes are known to be associated with various retinal disorders. Shown here are selected disease-associated genes. A more comprehensive list can be obtained from Retnet (https://sph.uth.tmc.edu/retnet/). Abbreviations used: AD, autosomal dominant; AR, autosomal recessive; XL, x-linked; RP, retinitis pigmentosa; CSNB, congenital stationary night blindness; CRD, cone-rod dystrophy; MD, macular degeneration; AMD, age-related macular degeneration. Modified from (Michaelides, et al., 2003).

<table>
<thead>
<tr>
<th>Disease-Associated Gene</th>
<th>Inheritance Pattern</th>
<th>Retinal Distrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Phototransduction</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rhodopsin</td>
<td>AD</td>
<td>RP, CSNB</td>
</tr>
<tr>
<td>Transducin</td>
<td>AD</td>
<td>CSNB</td>
</tr>
<tr>
<td>Phosphodiesterase α</td>
<td>AR</td>
<td>RP</td>
</tr>
<tr>
<td>Phosphodiesterase β</td>
<td>AR</td>
<td>RP</td>
</tr>
<tr>
<td>Cyclic nucleotide gated channel α</td>
<td>AR</td>
<td>RP</td>
</tr>
<tr>
<td>Cyclic nucleotide gated channel β</td>
<td>AR</td>
<td>RP, CRD</td>
</tr>
<tr>
<td>Ca_{v}1.4 calcium channel</td>
<td>XL</td>
<td>CSNB</td>
</tr>
<tr>
<td><strong>Recovery</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arrestin</td>
<td>AR</td>
<td>RP, CSNB</td>
</tr>
<tr>
<td>Rhodopsin kinase</td>
<td>AR</td>
<td>CSNB</td>
</tr>
<tr>
<td>GCAP</td>
<td>AD</td>
<td>CRD</td>
</tr>
<tr>
<td>Guanylate cyclase</td>
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<td>CRD</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>LCA</td>
</tr>
<tr>
<td><strong>Structural</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Peripherin-2</td>
<td>AD</td>
<td>RP, CRD, MD</td>
</tr>
<tr>
<td>ROM-1</td>
<td>AD</td>
<td>RP</td>
</tr>
<tr>
<td>PROM1</td>
<td>AR</td>
<td>RP, CRD, MD</td>
</tr>
<tr>
<td><strong>Visual cycle</strong></td>
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<td></td>
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<tr>
<td>RLBP1</td>
<td>AR</td>
<td>RP</td>
</tr>
<tr>
<td>RDH5</td>
<td>AR</td>
<td>CRD, CSNB</td>
</tr>
<tr>
<td>RPE65</td>
<td>AR</td>
<td>LCA, RP</td>
</tr>
<tr>
<td>ABCA4</td>
<td>AD</td>
<td>CRD, MD, RP, AMD</td>
</tr>
</tbody>
</table>

Transcription factors
<table>
<thead>
<tr>
<th>Disease-Associated Gene</th>
<th>Inheritance Pattern</th>
<th>Retinal Distrophy</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRX</td>
<td>AD</td>
<td>CRD</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>LCA</td>
</tr>
<tr>
<td>NRL</td>
<td>AD</td>
<td>RP</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>RP</td>
</tr>
<tr>
<td><strong>Miscellaneous</strong></td>
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<td></td>
</tr>
<tr>
<td>ELOVL4</td>
<td>AD</td>
<td>MD</td>
</tr>
<tr>
<td>RP1</td>
<td>AD</td>
<td>RP</td>
</tr>
<tr>
<td></td>
<td>AR</td>
<td>RP</td>
</tr>
<tr>
<td>USH2A</td>
<td>AR</td>
<td>Usher syndrome</td>
</tr>
<tr>
<td>MYO7A</td>
<td>AR</td>
<td>Usher syndrome</td>
</tr>
<tr>
<td>RP2</td>
<td>XL</td>
<td>RP</td>
</tr>
<tr>
<td>RPGR</td>
<td>XL</td>
<td>RP</td>
</tr>
<tr>
<td>RS1</td>
<td>XL</td>
<td>Retinoschisis</td>
</tr>
</tbody>
</table>
Table 1.2    Summary of the properties and associated disorders of the Pγ-ATPase family.

The CDC50 subunit interactions, substrate specificity, expression pattern, and disease association are shown. Disagreement in the literature is indicated using closed brackets and a question mark. When there is no data available on substrate specificity, indicated as unknown. Modified from (Folmer, et al., 2009, Sebastian, et al., 2011).

<table>
<thead>
<tr>
<th>Pγ-ATPase</th>
<th>β-subunit</th>
<th>Substrate</th>
<th>Expression Pattern</th>
<th>Pathophysiology</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP8A1</td>
<td>CDC50A (CDC50B?)</td>
<td>PS, isoforms</td>
<td>Ubiquitous, high in skeletal muscle, thyroid, spinal cord</td>
<td>Defective hippocampus-dependent learning</td>
</tr>
<tr>
<td>ATP8A2</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Unknown</td>
<td>Neurological, spinal, axonal degeneration, mental retardation</td>
</tr>
<tr>
<td>ATP8B1</td>
<td>CDC50A, CDC50B</td>
<td>PS (CL?)</td>
<td>Ubiquitous, high in small intestine, pancreas</td>
<td>Intrahepatic cholestasis, hearing loss, pneumonia</td>
</tr>
<tr>
<td>ATP8B2</td>
<td>CDC50A, CDC50B</td>
<td>Unknown</td>
<td>Ubiquitous</td>
<td>Unknown</td>
</tr>
<tr>
<td>ATP8B3</td>
<td>Unknown</td>
<td>PS</td>
<td>Testis</td>
<td>Defective sperm-egg interactions</td>
</tr>
<tr>
<td>ATP8B4</td>
<td>CDC50A, (CDC50B?)</td>
<td>Unknown</td>
<td>Moderate levels throughout brain</td>
<td>Alzheimer disease?</td>
</tr>
<tr>
<td>ATP9A</td>
<td>Does not bind</td>
<td>Unknown</td>
<td>Ubiquitous, high in brain.</td>
<td>Unknown</td>
</tr>
<tr>
<td>ATP9B</td>
<td>Does not bind</td>
<td>Unknown</td>
<td>Ubiquitous, high in testis</td>
<td>Unknown</td>
</tr>
<tr>
<td>ATP10A</td>
<td>CDC50A for ER exit</td>
<td>Unknown</td>
<td>High in brain, pancreas, kidney, lung</td>
<td>Angelman syndrome, obesity, diabetes</td>
</tr>
<tr>
<td>ATP10B</td>
<td>CDC50A for ER exit</td>
<td>Unknown</td>
<td>Low expression, brain.</td>
<td>Unknown</td>
</tr>
<tr>
<td>ATP10D</td>
<td>CDC50A for ER exit</td>
<td>Unknown</td>
<td>High in placenta, low kidney, undetectable in other major organs</td>
<td>Obesity?</td>
</tr>
<tr>
<td>ATP11A</td>
<td>CDC50A</td>
<td>Unknown</td>
<td>Ubiquitous, moderate levels in liver, skeletal muscle, ovary</td>
<td>Unknown</td>
</tr>
<tr>
<td>$P_4$-ATPase</td>
<td>β-subunit</td>
<td>Substrate</td>
<td>Expression Pattern</td>
<td>Pathophysiology</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
<td>-----------</td>
<td>-----------------------------------------------------------------------------------</td>
<td>-----------------------------------------------------</td>
</tr>
<tr>
<td>ATP11B</td>
<td>CDC50A</td>
<td>Unknown</td>
<td>Ubiquitous, high levels in kidney, testis, ovary</td>
<td>Unknown</td>
</tr>
<tr>
<td>ATP11C</td>
<td>CDC50A</td>
<td>PS</td>
<td>Ubiquitous, high liver, pancreas, heart</td>
<td>Impaired B lymphocyte differentiation, cholestasis</td>
</tr>
</tbody>
</table>
Figure 1.1  The anatomy of the vertebrate eye.

The posterior chamber of the eye is composed of three layers. The outer layer known as the sclera provides shape and prevents damage to the contents of the eye. The choroid layer contains vasculature that provides nourishment to the retina. The retina contains photoreceptors and other neuronal cells involved in the phototransduction of light. Light passes through the anterior chamber containing the cornea, aqueous humour, and lens and through the vitreous humour before reaching the retina. Modified from (http://en.wikipedia.org/wiki/File:Schematic_diagram_of_the_human_eye_en.svg).
Figure 1.2  The organization of the vertebrate retina.

The retina is composed of three layers of neuronal cells: the photoreceptor (PR) cell layer containing rods and cones; the inner layer containing bipolar, horizontal, and amacrine cells; and the ganglion cell layer. Light passes through the ganglion and inner layers before reaching the photoreceptor cell layer. Visual information is transmitted and processed by the bipolar and ganglion cells and propagated along the axons of the ganglion cells which form the optic nerve. Modified with permission from (Bramall, et al., 2010).
Figure 1.3 Rod and cone photoreceptors.

There are two types of photoreceptor cells in the retina: rods and cones. Photoreceptors are highly polarized neurons and can be divided into four different regions: the outer segment, inner segment, cell body, and synaptic terminal. The outer segment contains stacks of light sensitive membraneous discs. In rods, the discs are surrounded by a separate PM and in cones, the PM is continuous with the discs. Adapted from (Molday, 2007).
Figure 1.4 Regulation of lipid asymmetry in biological membranes.

The distribution of lipids in biological membranes is regulated by three distinct families of membrane transporters: ABC transporters, P$_4$-ATPases, and scramblases. ABC transporters catalyze the ATP-dependent transport of lipids from the cytoplasmic leaflet of the bilayer to the extracellular (lumenal) side of the membrane while P$_4$-ATPases transport in the opposite direction. ABC transporters can efflux lipids to lipoproteins such as Apo-A1 in the case of ABCA1. In some special cases, ABC transporters also transport in the same direction as P$_4$-ATPases. Scramblases are energy independent transporters and act to abolish lipid asymmetry by randomizing lipid distributions. Lipids such as PC, SM, and GSLs are found in the extracellular or luminal leaflet while the aminophospholipids PE and PS face the cytosol. Abbreviations used: PC, phosphatidylcholine; SM, sphingomyelin; PE, phosphatidylethanolamine; PS, phosphatidylserine; GSL, glycosphingolipid; Chol, cholesterol.
Figure 1.5  Phylogenetic tree of human P-type and P$_4$-ATPases.

(A) There are 36 different genes which encode for P-type ATPases in humans. Type IB are heavy metal pumps, Type IIA and Type IIB are Ca$^{2+}$ pumps, and Type IIC are Na$^+$/K$^+$ or H$^+$/K$^+$ pumps. Type IV ATPases are thought to transport phospholipids. Type V ATPases have no assigned specificity. Type IA and Type III pumps are not found in humans. (B) There are 14 different Type IV ATPases in humans which are organized into five different subfamilies according to sequence. Trees were generated using ClustalX and visualized using the Interactive Tree of Life online tool (http://itol.embl.de/).
Figure 1.6  Domain structure and organization of P₄-ATPases.

P₄-ATPases adopt a four domain structure consisting of cytosolic A (actuator), P (phosphorylation), and N (nucleotide binding) domains as well as a ten transmembrane segment M (membrane) domain containing the translocation pathway. Absolutely conserved motifs are shown. P-type ATPases are phosphorylated at the aspartic acid of the invariant DKTG motif. The glutamic acid of the DGE motif in the A domain catalyzes dephosphorylation. The CDC50 β-subunit is shown containing four N-linked glycosylations and two disulfide linkages in the large E (extracellular) domain.
Figure 1.7  Proposed mechanism of Pγ-ATPase catalyzed lipid transport.

P-type ATPases exist in four primary conformations. Binding of ATP and phosphorylation of the P-domain in the $E_1$ form converts the enzyme to $E_1P$. During the $E_1P$ to $E_2P$ transition, the A-domain rotates allowing lipids to bind to an extracellular binding site in the M-domain in the $E_2P$ conformation. Dephosphorylation of $E_2P$ drives the translocation of the lipid through the membrane to the cytoplasmic side. The enzyme is converted back to the $E_1$ form when the A-domain moves away from the P-domain. The role of CDC50 (green) is unknown.
Chapter 2: Localization, Purification, and Functional Reconstitution of
the P₄-ATPase ATP8A2, a Phosphatidylserine Flippase in Photoreceptor
Disc Membranes

2.1 Introduction

Mutations in the genes encoding many photoreceptor-specific proteins are known to
cause inherited retinal degenerative diseases, which are a major cause of blindness in the
population (Lev, 2001, Pacione, et al., 2003). However, despite the progress made in
understanding phototransduction and retinal degenerative diseases, we still know relatively
little about proteins involved in photoreceptor OS morphogenesis and structure. Furthermore,
although phospholipids were reported to be asymmetrically distributed across the disc
membrane over 25 years ago, the protein(s) responsible for generating and maintaining this
transbilayer asymmetry have not been identified (Miljanich, et al., 1981, Wu and Hubbell,
1993).

Recently, we have carried out a MS-based proteomic study designed to identify low
abundance membrane and soluble proteins of the photoreceptor OS with the goal of defining
their role in OS structure, function, and morphogenesis (Kwok, et al., 2008). The P₄-ATPase
ATP8A2 was identified as a membrane protein present in photoreceptor OS preparations.
ATP8A2 has been previously reported to be expressed in the testis as a 4.5-kb mRNA with
high levels occurring during early spermatid development (Halleck, et al., 1999). The

1 A version of this chapter has been published.
Coleman, J.A., Kwok, M.C., Molday, R.S., 2009. Localization, purification, and functional reconstitution of the
P₄-ATPase Atp8a2, a phosphatidylserine flippase in photoreceptor disc membranes. J Biol Chem 284, 32670-
32679.
biochemical properties of this member of the P$_4$-ATPase family, however, have not been investigated to date.

To begin to define the role of ATP8A2 in retinal photoreceptors, we have generated several monoclonal antibodies to ATP8A2 and used these immunoreagents to localize, purify, and characterize the functional properties of ATP8A2. Here, we show that ATP8A2 is expressed in the retina as well as testis and is present in the OS disc membranes of rod and cone photoreceptors. Importantly, we have purified ATP8A2 from disc membranes by immunoaffinity chromatography for analysis of its aminophospholipid-dependent ATPase and flippase activities. The ATPase activity of ATP8A2 was activated by PS and to a lesser extent PE. Upon reconstitution into lipid vesicles, ATP8A2 was found to flip fluorescent-labeled PS to the cytoplasmic side of the membrane, confirming the aminophospholipid translocase activity of ATP8A2. This is the first study of a membrane protein linked to phospholipid asymmetry in photoreceptor cells. This report as well as a recent study from Todd Graham’s lab (Zhou and Graham, 2009) are the first demonstrations in which a specific P$_4$-ATPase has been directly shown to display flippase activity by functional reconstitution of the purified protein.

2.2 Methods

2.2.1 Materials

DOPC, DOPE, DOPS, brain polar lipids (porcine), PI (bovine, liver), 1,2-dioleoyl-sn-glycero-3-phosphate, 1,2-dioleoyl-sn-glycero-3-phosphoglycerol, SM (porcine, brain), Chol (ovine, wool), 1-α-phosphatidylcholine (egg, chicken), C6 NBD-PS, C6 NBD-PE, C6 NBD-PC, and C12 NBD-PS were purchased from Avanti Polar Lipids (Alabaster, AL). ATP
and OGP were purchased from Sigma, dithionite was from Fisher, CHAPS was from Anatrace (Maumee, OH), and synthetic 6C11 peptide (Ac-RDRLLKRLS) was purchased from Biomatik (Cambridge, Canada). The Rho 1D4 antibody was obtained from UBC-UlO.

2.2.2 Solutions

The compositions of the buffers were as follows: Buffer A: 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 20 mM CHAPS, 0.5 mg/ml egg PC, complete inhibitor; Buffer B: 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10 mM CHAPS, 0.5 mg/ml egg PC; Buffer C, 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.75% OGP, 0.5 mg/ml egg PC; Buffer D: 50 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% OGP, 5 mg/ml egg PC, 10% sucrose; Buffer E: 10 mM HEPES (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 1% OGP, 5 mg/ml egg PC, 10% sucrose; Buffer F: 50 mM HEPES (pH 7.5), 150 mM NaCl, 12.5 mM MgCl₂, 10 mM CHAPS, and 1 mM DTT; PBS: 10 mM phosphate (pH 7.4), 140 mM NaCl, 3 mM KCl; Solution G: 6% ascorbic acid, 1% ammonium molybdate in 1 N HCl; Solution H: 2% sodium citrate, 2% sodium meta-arsenite, 2% acetic acid.

2.2.3 DNA Constructs

Total bovine, human, and murine retinal RNA was isolated using the guanidinium thiocyanate phenol chloroform method (Chomczynski and Sacchi, 1987). Because the only available sequences for bovine ATP8A2 were incomplete, a 5'-rapid amplification of cDNA ends was performed (Frohman, et al., 1988). Random primed cDNA was prepared using the RT-PCR Master Mix Kit (GE Healthcare). Full-length human, bovine, and mouse atp8a2 were amplified by PCR using Pfu polymerase (Fermentas, Burlington, Canada). Restriction sites were introduced by PCR. Bovine and mouse ATP8A2 were cloned into pcDNA3 and
pCEP4 using the BamHI and NotI restriction sites, and human ATP8A2 was cloned into pcDNA3 using KpnI and NotI. 1D4-tagged ATP8A2 contained a 9-amino acid C-terminal tag (TETSQVAPA). The sequence of bovine ATP8A2 was deposited in GenBank™ (GQ303567). The sequences of human and murine ATP8A2 had previously been deposited (NM_015803.2, NM_016529.4).

2.2.4 In Situ Hybridization

Detection of gene expression using DIG-labeled riboprobes was performed (Grayson, et al., 2000) with modifications. A fragment was isolated from an EcoRI/HindIII digest of murine ATP8A2 and ligated into pcDNA3 (nucleotides 923–1641). Sense and antisense cRNA were made using the DIG RNA labeling kit (Roche Applied Science) with the SP6 and T7 polymerases, respectively. Probes were treated with DNase I (Fermentas) and purified by LiCl precipitation. Eyes from 6-month-old BALB/c mice were fixed for 40 min in 4% paraformaldehyde in PBS, frozen in Tissue-Tek OCT, and cut into 12-μm sections. Probes were diluted to 400 ng/ml and incubated with sections for 16 h at 60 °C. Sections were treated with anti-DIG-alkaline phosphatase (Roche Applied Science) and developed in 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium for 15 h.

2.2.5 Gene Expression by RT-PCR

RNA was isolated from tissues of 6-month-old C57/B6 mice to prepare random primed cDNA. ATP8A2 gene expression was measured using the following forward and reverse primers: 5′-ACGAGGGACGTGCTCATGAAGC-3′ and 5′-CCTCAAGTGTCACCAGCAGGCT-3′. Glyceraldehyde-3-phosphate dehydrogenase was used for comparison employing the following forward and reverse primers: 5′-ATCAAATGGGGTGAGGCCGGTG-3′ and 5′-CGGCATCGAAGGTGGAAGAGTG-3′.
The primers were annealed at 55 °C, and the PCR was run for 25 cycles using Taq polymerase (New England Biolabs).

2.2.6 Generation of Monoclonal Antibodies against ATP8A2

Fragments corresponding to the C-terminal 103 amino acids and the P-domain (amino acids 371–873) of bovine ATP8A2 were cloned in frame with glutathione S-transferase (GST) in the pGEX-4T-1 vector (GE Healthcare). Mice were immunized with GST fusion proteins purified on glutathione-Sepharose 4B (GE Healthcare). Hybridoma cell lines were generated as previously described (MacKenzie and Molday, 1982) and screened for reactivity against ATP8A2 using Western blots of bovine OS. The epitope for the Atp6C11 monoclonal antibody was identified by measuring its immunoreactivity to synthetic overlapping 9-amino acid peptides spanning the C terminus of ATP8A2 as described previously (Illing, et al., 1997).

2.2.7 Expression of ATP8A2 in HEK293T

HEK293T cells (American Type Culture Collection, Manassas, VA) in 10 cm dishes were transfected at 30% confluence with 20 μg of ATP8A2–1D4 in pcDNA3 by the calcium phosphate method (Chen and Okayama, 1987) and harvested 48 h later. For control experiments, cells were transfected with empty pcDNA3. HEK293T membranes were isolated as described (Bungert, et al., 2001).

2.2.8 Immunofluorescence Microscopy

Cryosections of retina tissue from bovine eyes fixed in 4% paraformaldehyde, 100 mM phosphate buffer (PB) (pH 7.4), for 1 h were blocked and permeabilized with 10% normal goat serum and 0.2% Triton X-100 in PB for 30 min. The sections were then labeled overnight at room temperature with Atp2F6 hybridoma culture fluid diluted 1:3 in PB.
containing 2.5% normal goat serum and 0.1% Triton X-100. Sections were washed with PB and labeled for 1 h with Cy3-conjugated goat anti-mouse Ig secondary antibody (diluted 1:1000) and counterstained with 4′,6-diamidino-2-phenylindole nuclear stain. For controls, Atp2F6 was preabsorbed with 20 μg of GST fusion protein for 30 min before the antibody was added to the sections. For double labeling studies, sections were labeled with Atp2F6 and a mixture of cone opsin antibodies (JH 492, JH 455; diluted 1:4000), a kind gift of Jeremy Nathans (Wang, et al., 1992). The sections were labeled with secondary 1:1000 diluted anti-mouse and anti-rabbit antibodies conjugated to Alexa-488 and Alexa-594, respectively. Samples were visualized under a Zeiss LSM 700 confocal microscope.

2.2.9 Isolation of Retina and Outer Segment Membranes

Retina and ROS membranes from frozen bovine retinas were prepared as previously described (Kwok, et al., 2008, Molday and Molday, 1987, Papermaster and Dreyer, 1974). Highly enriched ROS disc membranes were prepared by an immunogold density perturbation procedure (Kwok, et al., 2008).

2.2.10 Purification of ATP8A2

Purified Atp6C11 monoclonal antibody was coupled to CNBr-activated Sepharose at a concentration of 1.5–2 mg of protein/ml of packed beads as described previously (Molday, et al., 1990). ATP8A2 was purified by solubilizing 3.5 mg of ROS in 1 ml of buffer A for 30 min at 4 °C. Insoluble material was removed by centrifugation (100,000 × g for 10 min), and the soluble fraction was incubated with Atp6C11-Sepharose at 4 °C for 2 h. The matrix was washed six times with 500 μl of buffer B and the protein was eluted twice in buffer B containing 0.2 mg/ml of 6C11 peptide at room temperature in 50 μl for 1 h.
2.2.11 **Reconstitution of ATP8A2 into Lipid Vesicles**

ATP8A2 was purified from ROS as described above with the following modifications. Atp6C11-Sepharose was washed in buffer C. Purified protein was mixed 1:1 with buffer D and stirred for 1 h at room temperature. Subsequently, the sample was dialyzed against 1 liter of buffer E for ~20 h with two changes to remove the detergent.

2.2.12 **ATPase Activity Assay**

Typically 25 ng of ATP8A2 was diluted in 25 μl of buffer F containing 5 mM ATP. The concentration of ATP8A2 was determined by comparison with known amounts of bovine serum albumin or with known amounts of ATP8A2 by quantitative Western blots. ATPase activity assays also contained 2.5 mg/ml DOPC and the stimulating lipid at the indicated concentration, in some cases the buffer did not contain CHAPS detergent. Samples were incubated at 37 °C for 15 – 30 min and stopped by the addition of 25 μl of 12% SDS. The concentration of phosphate was determined as described (Gonzalez-Romo, et al., 1992). Briefly, color was developed for 5 min following the addition of 75 μl of Solution G. The reactions were stopped by the addition of 120 μl Solution H and read at 850 nm in a microplate reader. The amount of phosphate released was determined by comparison with known phosphate concentrations. All data points were performed in triplicate, and each experiment was repeated at least three times independently with similar results. Data were analyzed with GraFit 6.0 (Erithacus Software).

2.2.13 **Flippase Assay**

The dithionite NBD-lipid assay was used as described (Eckford and Sharom, 2005, McIntyre and Sleight, 1991, Romsicki and Sharom, 2001) with modifications. ATP8A2 was purified from 3.5 mg of ROS and reconstituted into 250 μl of liposomes at a lipid...
concentration of 2.5 mg/ml containing 2.5% (w/w) NBD-lipid. Transport was initiated by mixing 20 μl of reconstituted ATP8A2 with nucleotide in buffer E to 50 μl (final nucleotide concentration 0.5 mM) and incubated at 23 °C for 2.5 min. In some cases, NEM or sodium orthovanadate were also added at a concentration of 5 and 1 mM, respectively. The sample was then diluted to 1 ml in buffer D, transferred to a cuvette (path length 1 cm), and read in a fluorescence spectrophotometer (Varian, Palo Alto, CA) using excitation and emission wavelengths of 478 and 540 nm, respectively, and a slit width of 5 nm. Sodium dithionite prepared in 1 M Tris (pH 10), was added at a final concentration of 2 mM once a base-line fluorescence was achieved. After a stable base line was reached, Triton X-100 was then added to a final concentration of 1%. The percentage of NBD-lipid that was accessible to dithionite treatment was calculated according to the following formula:

\[
\%\text{NBD}_{\text{out}} = \frac{[F_T - F_D]}{[F_T - F_0]} \times 100
\]

where \(F_T\) is the total fluorescence of the sample before dithionite treatment, \(F_D\) is the fluorescence of the sample after dithionite treatment, and \(F_0\) is the fluorescence after detergent solubilization. The percentage of flipped lipid was calculated from the difference between the transbilayer distribution of NBD lipids with ATP and AMP-PNP treatment. The \%\text{NBD}_{\text{out}} and was used to calculate the extent of NBD-lipid transport using the following equation,

\[
\% \text{ NBD-lipid transport} = (\%\text{NBD}_{\text{out,trial}} - \%\text{NBD}_{\text{out,control}})/( \%\text{NBD}_{\text{out,ATP}} - \%\text{NBD}_{\text{out,control}}) \times 100
\]

where control, trial, and ATP represent liposomes treated with AMP-PNP, various nucleotides or inhibitors, and ATP, respectively. All data points were performed in triplicate, and each experiment was repeated independently at least three times.
2.2.14 SDS-PAGE and Western Blots

Proteins were separated by SDS gel electrophoresis on 9% polyacrylamide gels and either stained with Coomassie Blue or transferred to Immobilon FL membranes (Millipore, Bedford, MA) in buffer containing 25 mM Tris, 192 mM glycine, 10% methanol, pH 8.3. Membranes were blocked with 1% milk in PBS for 30 min, incubated with culture supernatant diluted in PBS for 40 min, washed stringently with PBST (PBS containing 0.05% Tween 20), incubated for 40 min with secondary antibody (goat anti-mouse conjugated with IR dye 680 (LI-COR, Lincoln, NE) diluted 1:20,000 in PBST containing 0.5% milk), and washed with PBST prior to data collection on a LI-COR Odyssey infrared imaging system.

2.3 Results

2.3.1 ATP8A2 mRNA Expression in the Retinal Photoreceptor Cells and Testis

The expression of ATP8A2 mRNA in various tissues of 6-month-old C57/B6 mice was examined by RT-PCR. A 500-bp ATP8A2 fragment was detected in retina and testis but not heart, kidney, spleen, liver, brain, or lung (Fig. 2.1A). Detection in the testis by RT-PCR is in general agreement with earlier studies showing high ATP8A2 expression in the testis by Northern blot analysis (Halleck, et al., 1999).

In situ hybridization using DIG-labeled antisense probes to ATP8A2 was carried out to identify cells in the retina that express ATP8A2. ATP8A2 mRNA expression was detected in photoreceptor cells with the most intense staining observed in the IS and outer nuclear layer (ONL) (Fig. 2.1B). Weak staining was also detected in the inner nuclear layer (INL) and ganglion cell layer (GCL). In control samples, no staining was observed when the sense probe was used.
2.3.2 Monoclonal Antibodies to ATP8A2

Monoclonal antibodies were generated against GST fusion proteins for use as probes to study the biochemical properties and subcellular localization of ATP8A2. The Atp6C11 antibody was obtained from a mouse immunized with a GST fusion protein comprising the C-terminal 103 amino acids of bovine ATP8A2, and the Atp2F6 antibody was produced from a mouse immunized with a GST fusion protein containing amino acids 371–873 of the P-domain of ATP8A2.

The specificity of these antibodies was confirmed on Western blots of expressed bovine 1D4-tagged ATP8A2 (ATP8A2-1D4) and ROS membranes. As shown in Fig. 2.2, both the Atp6C11 and Atp2F6 antibodies labeled a prominent 130-kDa protein in both transfected HEK293T cells and bovine ROS. The size of the protein was consistent with the molecular mass of ~132 kDa determined from the amino acid sequence of bovine ATP8A2. The identity of the 130-kDa protein as ATP8A2 was further confirmed on Western blots of ATP8A2–1D4-transfected cells labeled with the Rho 1D4 antibody (Fig. 2.2). In controls, no labeling was observed in membranes from mock-transfected HEK293T cells. The Atp6C11 antibody against bovine ATP8A2 cross-reacted with both human and mouse ATP8A2, whereas the Atp2F6 antibody only labeled the bovine protein (Fig. 2.2). Mouse ATP8A2 expressed in HEK293T cells appeared less stable than the human and bovine proteins and accordingly showed weaker staining by both the Rho 1D4 and Atp6C11 antibodies.

Synthetic peptides and GST fusion proteins were used to further localize the epitope for these monoclonal antibodies. The epitope for the Atp6C11 antibody was mapped to a 9-amino acid sequence RDRLLKRLS (amino acids 1,118–1,126 of bovine ATP8A2) on the
basis of immunoreactivity to synthetic peptides, whereas the epitope for the Atp2F6 antibody was localized to a region between amino acids 440 and 468 using GST fusion proteins.

2.3.3 Localization of ATP8A2 in the Retina by Immunofluorescence Microscopy

Cryosections of bovine retina were labeled with the Atp2F6 antibody to determine the cellular and subcellular distribution of ATP8A2 within the retina (Fig. 2.3A). Immunolabeling was observed in the OS layer of photoreceptors consisting of mainly ROS but not in other layers of the retina. Double labeling studies using the Atp2F6 monoclonal antibody and cone opsin-specific polyclonal antibodies revealed that cone as well as ROS were stained with the Atp2F6 antibody (Fig. 2.3B). In control samples, immunolabeling was abolished by preabsorbing the Atp2F6 antibody with a GST fusion protein containing the Atp2F6 epitope (Fig. 2.3A). The Atp6C11 antibody could not be used for immunolocalization of ATP8A2 because the epitope was sensitive to paraformaldehyde fixation. Furthermore, additional studies indicated that this antibody cross-reacted with an unidentified soluble protein in retinal extracts by Western blot (Appendix A2).

2.3.4 ATP8A2 Is Present in Outer Segment Disc Membranes

The distribution of ATP8A2 within ROS was further evaluated by subcellular fractionation. Disc membranes, which comprise 95% of total ROS membranes, were separated from the ROS PM by an immunogold density perturbation method (Kwok, et al., 2008, Molday and Molday, 1987). Western blots of crude retinal membranes, isolated ROS membranes, and disc membranes were probed for ATP8A2 for comparison with ABCA4, a marker for the disc membrane, and the cyclic nucleotide-gated channel subunit α-subunit CNGA1, a marker for the PM of ROS (Cook, et al., 1989, Illing, et al., 1997). Fig. 2.4 shows that ATP8A2 was present at similar levels in ROS and disc membranes and at lower amounts
in crude retinal membranes. This profile was similar to that of ABCA4 and distinctly different from CNGA1, which is absent in disc membranes. These results indicate that ATP8A2 is present in disc membranes of ROS.

2.3.5 Purification of ATP8A2 by Immunoaffinity Chromatography

ATP8A2 was purified from detergent-solubilized ROS in order to study its functional properties. Our purification strategy involved 1) solubilization of ATP8A2 from ROS with CHAPS in the presence of PC as a stabilizing lipid; 2) binding of ATP8A2 to an Atp6C11-Sepharose immunoaffinity matrix; 3) removal of unbound protein by extensive washing; and 4) elution of functional ATP8A2 with buffer containing the competing 9-amino acid synthetic 6C11 peptide. The eluted protein identified as ATP8A2 by Western blotting was largely free of other detectable proteins, as shown by Coomassie Blue staining (Fig. 2.5). The remaining ATP8A2 that was not eluted with peptide could be recovered under denaturing conditions by elution with 2% SDS. The purification and enzymatic activity of purified ATP8A2 obtained under nondenaturing conditions is given in Table 2.1. This immunoaffinity purification method resulted in a 51% yield with purification in the specific PS-activated ATPase activity of nearly 2,000-fold.

The abundance and ATPase activity of ATP8A2 were determined in ROS preparations. ATP8A2 comprised less than 0.1% of the total ROS membrane protein, in general agreement with earlier proteomic studies (Kwok, et al., 2008). Despite its low abundance, however, the ATPase activity of ATP8A2 accounted for more than 35% of total ATPase activity in bovine ROS preparations. This was determined by comparing the ATPase activity of solubilized ROS membranes before and after the complete removal of ATP8A2 on the immunoaffinity column under basal conditions for other ATPases (Table 2.1). Additional
studies indicated that phospholipids, such as PC, and reducing agents, such as DTT, were required during purification in order to stabilize ATP8A2 and prevent disulfide bond formation.

2.3.6 Kinetics of ATP Hydrolysis by ATP8A2

Because the ATPase activities of various members of the P_4-ATPase family of proteins have been shown to be stimulated by phospholipids, we studied the effect of different phospholipids on the ATPase activity of detergent-solubilized, immunoaffinity-purified ATP8A2. Fig. 2.6A shows that the ATPase activity of ATP8A2 is strongly stimulated by the addition of 10 mol % brain polar lipids and 10 mol % synthetic PS, resulting in a specific activity of 45–55 μmol of ATP/min/mg of protein. ATP8A2 was only weakly activated by PE and not activated by other lipids, including PC, PI, PA, Chol, and SM.

The effect of increasing PS and PE concentrations on the ATPase activity of ATP8A2 was examined. Michaelis-Menten kinetics was observed for both PS and PE. The $K_m$ for PS and PE was $78 \pm 7$ and $660 \pm 90$ μM, respectively (Table 2.2). The $V_{\text{max}}$ for PS was ~6.5 times higher than for PE. The resulting specificity constant ($K_{\text{cat}}/K_m$) was 60-fold greater for PS compared with PE (Table 2.2).

The effect of ATP concentration on the ATPase activity was also studied at a saturating concentration of PS (Fig. 2.6D). Michaelis-Menten kinetics revealed a $K_m$ for ATP of $704 \pm 7$ μM and a $V_{\text{max}}$ of $57 \pm 2$ μmol of ATP/min/mg of protein. For comparison with other P_4-ATPases purified from natural sources, ATP8A1 from bovine chromaffin granules was reported to have a maximal specific activity of 8 μmol of ATP/min/mg and a $K_m$ of 350 μM (Moriyama and Nelson, 1988), whereas the protein isolated from human erythrocytes
had a specific activity of 790 nmol of ATP/min/mg and a $K_m$ of between 200 and 260 $\mu$M (Morrot, et al., 1990). The kinetic parameters of ATP8A2 are summarized (Table 2.2).

The effect of ATPase inhibitors on the activity of ATP8A2 was also investigated (Appendix A3). The sulfhydryl-modifying reagent NEM at 1 mM and vanadate at 0.1 mM inhibited the ATPase activity of ATP8A2 by over 90%. In contrast, ouabain and azide, inhibitors of Na$^+$,K$^+$-ATPase and mitochondrial ATPases, respectively, had no effect. ATP8A2 was specific for ATP because GTP was not hydrolyzed by ATP8A2 (Appendix A3).

Finally, we have studied the effect of pH on the activity of ATP8A2. Maximum activity was observed between pH 7 and 9 when PS was used as a substrate. In contrast, PE showed a narrow pH optimum at pH 8 with activity dropping to 40% at pH 9 (Appendix A3). This loss in activity above pH 8 may be the result of deprotonation of the primary amino group of PE.

### 2.3.7 Kinetics of Reconstituted ATP8A2

To investigate the activity of ATP8A2 incorporated into a lipid bilayer, ATP8A2 purified from ROS was reconstituted into PC vesicles containing various amounts of PS by a dialysis procedure. These vesicles were unilamellar, as observed by cryoelectron microscopy (data not shown). As in the case of detergent-solubilized enzyme, the ATPase activity of reconstituted ATP8A2 increased, with PS concentration reaching a maximal activity of ~35 $\mu$mol of ATP/min/mg of protein for 5 mol % PS, a value that is ~25% less than ATP8A2 in CHAPS detergent (Appendix A4).

We reasoned that the lower ATPase activity of reconstituted ATP8A2 could arise from an unfavorable orientation of a fraction of ATP8A2 in lipid vesicles, such as to exclude
ATP. The orientation of ATP8A2 was determined by comparing the effect of limited trypsin
digestion of ATP8A2 in hypotonically lysed ROS with that in reconstituted lipid vesicles. As
shown in Appendix A5, the Atp6C11 epitope was rapidly degraded by trypsin in ROS discs,
consistent with the C terminus of ATP8A2 being accessible on the cytoplasmic side of disc
membranes. In contrast, the Atp2F6 epitope was largely resistant to trypsin digestion. In the
case of ATP8A2 reconstituted into lipid vesicles, trypsin had a more limited effect on the
Atp6C11 epitope (Appendix A5). Quantification of the Atp6C11 immunolabeling following
trypsin treatment suggested that 30% of the reconstituted ATP8A2 was inaccessible to
trypsin and correspondingly ATP. Hence, the lower specific activity observed for
reconstituted ATP8A2 relative to the detergent-solubilized protein can be accounted for by
the observation that only ~70% of reconstituted ATP8A2 is accessible to ATP in the lipid
vesicles.

2.3.8 Phospholipid Flippase Activity of ATP8A2

NBD-labeled phospholipids have been previously used to measure lipid flippase
activity in various biological systems by fluorescence measurements (Eckford and Sharom,
2005, McIntyre and Sleight, 1991, Natarajan, et al., 2004). To determine the feasibility of
using this assay for measuring PS flippase activity of ATP8A2, we first determined if NBD-
labeled PS could stimulate the ATPase activity of ATP8A2. As shown in Appendix A4, both
C6 NBD-PS and C12 NBD-PS activated the ATPase activity of ATP8A2 although to a lesser
extent than unlabeled PS at the same lipid concentration. C12 NBD-PS was more effective
than C6 NBD-PS as a substrate for ATP8A2.

Next, the ability of ATP8A2 to flip NBD-labeled PS was studied in reconstituted
liposomes by fluorescence spectroscopy. Reconstituted vesicles containing NBD-labeled PS
were incubated with or without ATP. Dithionite was subsequently added to bleach the fluorescence signal resulting from NBD-labeled lipid on the exposed leaflet of the vesicles, whereas the NBD-lipid on the inside is not affected (Romsicki and Sharom, 2001). Fig. 2.7A shows an example of the fluorescence traces for ATP8A2-reconstituted vesicles containing NBD-labeled PS. In the absence of ATP or in the presence of ATP and the ATPase inhibitor NEM, dithionite resulted a 65% decrease in fluorescence, corresponding to the bleaching of NBD-labeled PS on the outer leaflet of the unilamellar liposomes. ATP8A2-containing vesicles pretreated with ATP in the absence of inhibitor showed a larger reduction in fluorescence upon the addition of dithionite. The difference between the ATP-treated and -untreated is a measure of the extent of flipping of NBD-labeled PS from the inner (lumen) to the outer (cytoplasmic) leaflet of the vesicles. The subsequent addition of Triton X-100 resulted in the complete loss of fluorescence due to the accessibility of all NBD-labeled PS to dithionite. Both C6 NBD-PS and C12 NBD-PS were transported by ATP8A2 in an ATP-dependent manner. In control studies, lipid vesicles devoid of ATP8A2 showed no ATP-dependent difference in fluorescence (Fig. 2.7B). No detectable ATP-dependent flipping of NBD-labeled PC or PE was observed (Fig. 2.7C). The inability to observe NBD-labeled PE flipping may be due to the lack in sensitivity of this assay or the inability of NBD-labeled PE to serve as a substrate for ATP8A2. Finally, the transport of NBD-labeled PS was inhibited by unlabeled PS, supporting the physiological significance of the observed ATP8A2 flippase activity (Fig. 2.7D). The finding that equimolar PS inhibits NBD-PS flippase greater than 75% is consistent with PS being a better substrate than NBD-PS, as found for the PS-activated ATPase activity of ATP8A2 (Appendix A4).
The effect of various nucleotides and ATPase inhibitors on the ATP8A2 flippase activity was also studied. Little or no ATP8A2 flippase activity was observed when AMP-PNP, GDP, GTP, or ADP was used in place of ATP (Fig. 2.7E). The addition of NEM and to a lesser extent vanadate reduced the ATP-dependent flippase activity of ATP8A2.

2.4 Discussion

P4-ATPases constitute a relatively new subfamily of P-type ATPases. Fourteen members of this subfamily have been identified in mammals and five in yeast based on sequence similarities (Folmer, et al., 2009, Halleck, et al., 1999, Wang, et al., 2004). However, despite studies linking a number of P4-ATPases to important cellular processes and inherited human diseases (Folmer, et al., 2009, Puts and Holthuis, 2009), relatively little is known about the biochemical properties of most members of this subfamily of P-type ATPases.

In this study, we have examined the expression, subcellular localization, and biochemical activities of ATP8A2, a member of the P4-ATPase subfamily that shares a 67% identity in amino acid sequence with ATP8A1, the first cloned member of this subfamily. Unlike most other members of the P4-ATPase subfamily, which are widely expressed in different tissues (Folmer, et al., 2009), ATP8A2 shows a restricted tissue distribution in adult mice with high expression in the retina as well as the testis. Within the retina, ATP8A2 mRNA expression was primarily observed in photoreceptor cells, and the protein was localized to disc membranes of the OS of rods and cones, as revealed by immunocytochemical and subcellular fractionation techniques. The presence of ATP8A2 in disc membranes of photoreceptor OS is consistent with our recent proteomic screen in which
ATP8A2 was identified as a low abundant protein in OS disc membrane preparations (Kwok, et al., 2008).

To gain insight into the functional properties of ATP8A2, we have developed a simple and efficient single-step immunoaffinity purification procedure to isolate ATP8A2 from detergent-solubilized ROS membranes. This preparation displayed a single intense 130-kDa protein corresponding to ATP8A2 and was essentially free of other proteins, as analyzed by Coomassie Blue staining of SDS gels. Several other P₄-ATPases have recently been reported to form a heteromeric complex with members of the CDC50/Lem3p family of membrane proteins (Folmer, et al., 2009, Lenoir, et al., 2009, Puts and Holthuis, 2009, Saito, et al., 2004). CDC50 proteins contain two transmembrane segments that are separated by a heavily N-glycosylated exocytoplasmic domain. Recent studies suggest that these proteins play important roles in the stability and trafficking of P₄-ATPases out of the ER as well as in the proper functioning of these transporters (Folmer, et al., 2009, Lenoir, et al., 2009, Puts and Holthuis, 2009). CDC50 proteins have been considered to be the counterpart of the β-subunit of the Na⁺,K⁺-ATPase oligomeric complex. CDC50A was detected in our recent proteomic analysis of OS (Kwok, et al., 2008). It is possible that the CDC50A associates with ATP8A2 and is present in our immunoaffinity-purified preparations but not readily detected by conventional gel staining techniques. Alternatively, ATP8A2 may not require an accessory subunit for expression and activity. The generation of suitable anti-CDC50 antibodies should clarify whether ATP8A2 exists as a heteromeric complex with CDC50A or another member of the CDC50 family of proteins.

The catalytic and lipid transport properties of purified and reconstituted ATP8A2 were examined. The addition of phospholipid together with DTT was required during
detergent solubilization and purification in order to stabilize the protein in its functional state. PC was typically used because this phospholipid does not function as a substrate for ATP8A2. Kinetic studies showed that PS activated the ATPase activity of purified ATP8A2 in detergent and after reconstitution into liposomes. An increase in ATPase activity was also observed for PE, but this aminophospholipid was 60 times less effective than PS as a substrate. In agreement with other P-type ATPases, both NEM and vanadate inhibited the enzymatic activity of ATP8A2. Hence, the substrate specificity and inhibition properties of ATP8A2 resemble those of ATP8A1, although the specific activity of ATP8A2 measured in this study is generally greater than that reported for ATP8A1 (Moriyama and Nelson, 1988).

Although aminophospholipid translocase activity has been inferred for a number of P₄-ATPases from both genetic and PS/PE-dependent ATPase activation studies, the phospholipid flippase activity of purified and reconstituted P₄-ATPases has not been reported previously. In this study, we have used NBD-labeled PS to measure the flippase activity of purified ATP8A2 reconstituted into lipid vesicles. Our studies using a fluorescence-based assay (McIntyre and Sleight, 1991) indicate that ATP8A2 is able to translocate PS from the inner leaflet (equivalent to the exocyttoplasmic side of biological membranes) to the outer leaflet (cytoplasmic side) of vesicles in an ATP-dependent manner. This process requires energy derived from ATP hydrolysis because ADP, AMP-PNP, and GTP were ineffective as nucleotide substrates. Together, these studies provide direct evidence for the role of ATP8A2 as an aminophospholipid translocase with a strong preference for PS as the transported phospholipid substrate.

Previous studies have shown that ROS disc membranes display lipid asymmetry, with PS and PE preferentially localized on the cytoplasmic leaflet (Miljanich, et al., 1981, Wu and
Our studies showing that ATP8A2 is present in disc membranes support the view that this P_4-ATPase functions in the generation and maintenance of transbilayer asymmetry with respect to PS and possibly PE. The specific role of PS/PE asymmetry in disc membranes is not known. However, we speculate that the negatively charged surface of disc membranes contributed by PS may play roles in generating and stabilizing the structure of disc membranes either by inducing non-random lipid distributions within the bilayer or facilitating interactions with cytoskeletal or soluble proteins, thereby inducing the rim region of disc membranes (Manno, et al., 2002). PS asymmetry may also be important in modulating the interaction of enzymes of the visual cascade with disc membranes (Matsuda, et al., 1994). Finally, it is possible that PS asymmetry may play a role in vesicle transport/fusion and phagocytosis of OS discs by RPE cells as part of the OS renewal process.

The importance of ATP8A2 in photoreceptor OS is supported by analysis of ATPase activities of OS. Although ATP8A2 makes up less than 0.1% of the total membrane protein of OS, it is one of the most active ATPases in OS preparations. Analysis of OS ATPase activity before and after removal of ATP8A2 suggests that ATP8A2 is responsible for over 35% of the basal ATPase activity of OS. The Na^+\text{,}K^+-ATPase that is a major contributor to total ATPase activity in most cells is not present in OS preparations (Kwok, et al., 2008, Wetzel, et al., 1999). ABCA4, an ATP transporter implicated in the flipping of N-retinylidene-PE across disc membranes (Beharry, et al., 2004, Molday, et al., 2009, Weng, et al., 1999), is present in OS in greater abundance than ATP8A2 but displays a specific activity that is several orders of magnitude lower than ATP8A2 (Sun, et al., 1999). Hence, ATP8A2 most likely consumes a considerable amount of the ATP of OS to maintain
aminophospholipid asymmetry. ATP8A2 is needed to maintain this lipid asymmetry over the life of the OS, typically 10 days.

It will be important to generate a mouse deficient in ATP8A2 to further evaluate its role in photoreceptor structure and function. Likewise, it is of interest to determine if mutations in atp8a2 are responsible for human retinal degenerative diseases. Although our data strongly suggest an important role for ATP8A2 as an aminophospholipid translocase in photoreceptors, ATP8A2 may also play an important role in reproductive and developmental biology because ATP8A2 is expressed at high levels in the testis and during early sperm development.

In summary, our studies show that ATP8A2 is present in the disc membranes of rod and cone photoreceptors. ATPase and flippase measurements provide direct evidence for the function of ATP8A2 as an aminophospholipid translocase with a high specificity for PS. These studies support the role of this member of the P4-ATPase subfamily in the generation and maintenance of phospholipid asymmetry in photoreceptor disc membranes.
Table 2.1 Purification of ATP8A2 from bovine rod outer segments.

The activity of ATP8A2 was measured in the presence of 5 mM ATP and endogenous lipid concentrations following solubilization for the lysate and unbound fractions, respectively. For the elution fraction, 10% exogenous PS was added to maximally stimulate the ATPase activity.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (µl)</th>
<th>Protein (mg)</th>
<th>Total PS Stimulated Activity (nmol ATP/min)</th>
<th>Specific Activity (nmol ATP/min/mg)</th>
<th>Yield %</th>
<th>Fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysate</td>
<td>490</td>
<td>1.59</td>
<td>0.232</td>
<td>28.67</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>Unbound</td>
<td>490</td>
<td>1.59</td>
<td>0.152</td>
<td>18.74</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Elution</td>
<td>65</td>
<td>4.3x10^-4</td>
<td>1.010</td>
<td>54,087</td>
<td>51</td>
<td>1,887</td>
</tr>
</tbody>
</table>
Table 2.2  The kinetic parameters of the ATPase activity of ATP8A2.

The ATPase activity was measured in 5 mM ATP under varying concentrations of PS and PE to determine the $K_m/V_{max}$ for each lipid. Similarly, varying concentrations of ATP were used in the presence of 10% PS to determine the $K_m/V_{max}$ for ATP.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m$ (µM)</th>
<th>$V_{max}$ (µmol ATP/min/mg)</th>
<th>$K_{cat}$ (s$^{-1}$)</th>
<th>$K_{cat}/K_m$ (s$^{-1}$ µM$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PS</td>
<td>78 ± 7</td>
<td>45.4 ± 0.8</td>
<td>100 ± 2</td>
<td>1.3 ± 0.1</td>
</tr>
<tr>
<td>PE</td>
<td>660 ± 90</td>
<td>6.9 ± 0.4</td>
<td>15.2 ± 0.9</td>
<td>0.023 ± 0.007</td>
</tr>
<tr>
<td>ATP</td>
<td>704 ± 7</td>
<td>57 ± 2</td>
<td>125 ± 4</td>
<td>0.18 ± 0.04</td>
</tr>
</tbody>
</table>
Figure 2.1 Gene expression of *ATP8A2* by RT-PCR and in situ hybridization.

(A) The relative gene expression of *ATP8A2* was measured using gene-specific primers on cDNA prepared from RNA isolated from different mouse tissues. The relative gene expression of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as a loading control. Expression of *ATP8A2* is only detected in the retina and the testis. (B) Labeling of antisense probe and control sense probe to *ATP8A2* in mouse retina. *ATP8A2* mRNA is detected in the outer nuclear layer and inner segment in mouse retina. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. *Bar*, 30 μm.
Figure 2.2 Characterization of ATP8A2 antibodies by Western blotting.

Western blots of membranes (10 μg of protein) from HEK293T cells transfected with human, bovine, or mouse ATP8A2–1D4 vector or empty vector (Mock) together with bovine rod outer segments (ROS) (30 μg of protein) were labeled with the Atp6C11 and Atp2F6 monoclonal antibodies to ATP8A2 and the Rho 1D4 monoclonal antibody to the 1D4 epitope. The same 130-kDa bovine protein was detected in both the HEK293T and ROS membrane samples. The Atp6C11 labeled the human, bovine, and mouse ATP8A2, whereas the Atp2F6 antibody was specific for the bovine protein. No labeling was observed in membranes from mock-transfected cells.
Figure 2.3  Localization of ATP8A2 in the retina by immunofluorescence microscopy.

(A) ATP8A2 was immunolabeled with Atp2F6 antibody (green) and nuclei with 4',6-diamidino-2-phenylindole (blue). In the control sample, Atp2F6 antibody was blocked by excess GST fusion protein prior to immunolabeling. ATP8A2 is localized to the outer segment layer consisting primarily of rod outer segments. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 30 μm. (B) Double labeling of ATP8A2 (green) and cone opsin (red) and merged image showing co-localization of ATP8A2 and cone opsin in the COS (arrows). Bar, 10 μm.
Figure 2.4 ATP8A2 localizes to the disc membranes of outer segments.

Approximately 30 μg of crude retina membranes (Retina), isolated rod outer segments (ROS), and purified disc membranes (Discs) were resolved by SDS gel electrophoresis. The gels were stained with Coomassie Blue, and Western blots were labeled with the Atp6C11 antibody to ATP8A2, PMc 1D1 antibody to CNGA1 as a plasma membrane-specific marker, and the Rim 3F4 to ABCA4 as a disc-specific marker. The profile of Atp6C11 resembled Rim3F4, indicating that ATP8A2 is localized to disc membranes.
Figure 2.5  Purification of ATP8A2 from outer segments.

Rod outer segment membranes solubilized in CHAPS detergent (*Input*) were applied to an Atp6C11-Sepharose column. The flow-through (*Unbound*) and bound Atp6C11 eluted first with the competing 6C11 peptide (*Elution*) and subsequently with SDS were resolved by SDS gel electrophoresis. Gels were stained with Coomassie Blue, and Western blots were labeled with the Atp6C11 antibody to ATP8A2. The following amount of protein was applied to the indicated lanes: input (30 μg), unbound (30 μg), peptide elution (100 ng), and SDS elution (100 ng).
Figure 2.6  Effect of different lipids and ATP on the ATPase activity of ATP8A2.

(A) The velocity of ATP hydrolysis of detergent-solubilized and purified ATP8A2 in the presence of various lipids (10 mol %) and phosphatidylcholine (90 mol %) at 5 mM ATP. BPL, brain polar lipids; PI, phosphatidylinositol; PA, phosphatidic acid; PG, phosphatidylglycerol; Chol, cholesterol; SM, sphingomyelin. (B) The effect of varying PS concentration on the velocity of ATP hydrolysis in the presence of PC and 5 mM ATP. (C) The effect of varying PE concentration on the velocity of ATP hydrolysis of ATP8A2 in the presence of PC and 5 mM ATP. (D) The effect of varying ATP concentration on the velocity of ATP hydrolysis of ATP8A2 in the presence of 10% PS.
Figure 2.7  Transport or flippase activity of ATP8A2.

Loss in NBD fluorescence upon the addition of 2 mM dithionite (+Dithionite) to ATP8A2 proteoliposomes containing C12 NBD-PS pretreated with ATP (solid line) or ATP + NEM (dotted line) (A) and empty liposomes containing C12 NBD-PS pretreated with ATP (solid line) or ATP + NEM (dotted line). (B) The difference in the fluorescence between samples containing ATP and samples containing ATP + NEM (or no ATP) reflects the transport activity. Triton X-100 (1%) was added at the end of the trace to make all C12 NBD-PS accessible to bleaching by dithionite. (C) Transport activity of C6 NBD-PC, -PE, and -PS. (D) Transport activity of C12 NBD-PS in the absence or the presence of an equimolar amount of PS (2.5%). (E) Effect of pretreating ATP8A2 proteoliposomes with various nucleotides (AMP-PNP, GDP, GTP, ADP, and ATP) and inhibitors (NEM and vanadate) on the transport of C12 NBD-PS.
Chapter 3: Critical Role of the Beta-Subunit CDC50A in the Stable Expression, Assembly, Subcellular Localization, and Lipid Transport Activity of the P4-ATPase ATP8A2

3.1 Introduction

A number of yeast, plant, and mammalian P4-ATPases are known to associate with members of the Cdc50 family of proteins (Chen, et al., 2006, Paulusma, et al., 2008, Poulsen, et al., 2008, Saito, et al., 2004). There are three members of this family in yeast (Cdc50p, Crflp, and Lem3/Ros3p) and three in mammals (CDC50A, CDC50B, and CDC50C). These proteins contain two transmembrane segments separated by a relatively large glycosylated exocytoplasmic domain. Several cellular studies indicate that CDC50 proteins play a crucial role in the export of specific P4-ATPases from the ER of cells (Chen, et al., 2006, Lopez-Marques, et al., 2010, Paulusma, et al., 2008, Poulsen, et al., 2008, Saito, et al., 2004, van der Velden, et al., 2010). CDC50 proteins also have been suggested to play a direct role in the reaction cycle of P4-ATPases (Bryde, et al., 2010, Lenoir, et al., 2009). In yeast, the P4-ATPase Drs2p requires Cdc50p for phosphorylation of the key aspartate residue involved in the ATPase catalytic reaction. The affinity of Drs2p for Cdc50p was also found to change during the transport cycle. Additionally, phosphorylation of the catalytically important aspartate residue of the human P4-ATPases, ATP8B1 and ATP8B2, was dependent on the CDC50 subunit. On the basis of these studies, it has been proposed that the CDC50 proteins serve as the β-subunit of P4-ATPases akin to the β-subunit of the Na+/K+ ATPase (Puts and Coleman, J.A., Molday, R.S. 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, 17205-17216.)
Holthuis, 2009). However, although an association between the catalytic subunit of P₄-ATPases and CDC50 proteins has been observed in heterologous cell expression systems, their direct association in mammalian tissues has not been demonstrated, and the domains of CDC50 proteins responsible for their interaction with P₄-ATPases have yet to be determined.

In a proteomic study, unique peptides belonging to CDC50A were detected in photoreceptor OS preparations (Kwok, et al., 2008), but the possible association of CDC50A with ATP8A2 was not investigated. In this study, we show that ATP8A2 is present as a heteromeric complex with CDC50A in photoreceptor OS as well as HEK293T cells expressing ATP8A2. Co-expression of ATP8A2 with CDC50A, but not CDC50B, not only promotes the translocation of ATP8A2 from the ER to the Golgi but also significantly enhances the yield of a functional ATP8A2-CDC50A aminophospholipid transporter. Chimera proteins in which various domains of CDC50B have been replaced with corresponding domains of CDC50A have been used to define regions of CDC50A that are required for the formation of a functionally active ATP8A2-CDC50A complex. Finally, we have investigated the effect of N-linked glycosylation of CDC50A on the stable expression, subunit association, and functional properties of the ATP8A2-CDC50A complex.

3.2 Methods

3.2.1 Materials

The 7F4 peptide Ac-AKDEVDGDP was purchased from Biomatik. Additional materials are described in Chapter 2.2.1.

3.2.2 DNA Constructs

Bovine ATP8A2 containing a 1D4 tag in pcDNA3 was described in Chapter 2. This construct was used as a PCR template to generate each ATP8A2 construct described. The
cDNAs of bovine \textit{CDC50A} (IMAGE: 8284976) and human \textit{CDC50B} (IMAGE: 8322611) were purchased from Open Biosystems (Huntsville, AL). Restriction sites and epitope tags were introduced by PCR. Full-length \textit{ATP8A2} without a tag was PCR-amplified and cloned into pcDNA3 using BamHI and NotI restriction sites. Full-length \textit{CDC50A} with a 1D4 or Myc tag was cloned into pcDNA3 using the HindIII and XhoI restriction sites. \textit{CDC50B} with a 1D4 tag was also cloned into pcDNA3 using HindIII and XhoI sites. The Myc-tagged constructs contained a 10-amino acid C-terminal tag (EQKLISEEDL). Chimeras of \textit{CDC50A} and \textit{CDC50B} were constructed from a 1D4-tagged \textit{CDC50B} construct that had been cloned into a modified pcDNA3 vector by EcoRI and NotI. Silent restriction sites were inserted into \textit{CDC50B} by mutagenesis to facilitate cloning of individual domains. The ECD chimera contained amino acids 1–57 and 301–351 of CDC50B and amino acids 73–308 of CDC50A. The ECD/TM chimera contained amino acids 1–33 and 341–351 of CDC50B and amino acids 49–348 of CDC50A. The ECD/TM/C chimera contained amino acids 1–33 of CDC50B and amino acids 49–361 of CDC50A. The N-terminal chimera contained amino acids 1–47 of CDC50A and amino acids 33–351 of CDC50B. The M1 chimera contained amino acids 1–33 and 55–351 of CDC50B and amino acids 49–69 of CDC50A. The M2 chimera contained amino acids 1–301 and 341–351 of CDC50B and amino acids 310–348 of CDC50A. The M1/M2 chimera contained amino acids 1–33, 55–301, and 341–351 of CDC50B and amino acids 49–69 and 310–348 of CDC50A. GST fusion constructs containing amino acids 1–40 and 194–283 of CDC50A were cloned into pGEX-4T-1 using the BamHI and EcoRI restriction sites. Site-directed mutations were created using the QuikChange mutagenesis kit from Agilent Technologies (Santa Clara, CA). All of the constructs were verified by DNA sequencing (Eurofins MWG Operon, Huntsville, AL).
3.2.3 Gene Expression by RT-PCR

Gene expression was measured according to Chapter 2.2.5. Primer sequences are available in Appendix B1.

3.2.4 Generation of Monoclonal Antibodies against CDC50A

Fragments of bovine CDC50A (amino acids 1–40 and 194–283) were cloned in frame with GST and expressed and purified as described in Chapter 2. Hybridoma cell lines, screening, and epitope identification were performed according to Chapter 2.2.6.

3.2.5 Expression of ATP8A2 and CDC50A in HEK293T and Cos-7 Cells

HEK293T cells were cultured according to Chapter 2.2.7. In some cases, the cells were co-transfected with both ATP8A2 and CDC50A by mixing 10 μg of each plasmid together followed by calcium phosphate precipitation. Cos-7 cells were transfected in six-well plates containing polylysine-treated coverslips with 2.5 μg of each plasmid.

3.2.6 Immunofluorescence Microscopy

Cryosections of bovine retina tissue were labeled according to Chapter 2.2.8. In control studies, Cdc50–7F4 antibody labeling was blocked by preabsorption with 7F4 peptide at a concentration of 0.2 μg/μl for 30 min. Cos-7 cells transfected with 1D4-tagged ATP8A2 or Myc-tagged CDC50A were labeled with either Rho-1D4 hybridoma culture fluid (1:500) or Myc antibody diluted 1:200 (ab10910; Abcam, Cambridge, MA). The cells were also labeled with antibodies against calnexin (Abcam, ab13504) or GM130 (Sigma; G7295) diluted 1:200 and 1:300, respectively. Cos-7 cells transfected with untagged ATP8A2 and 1D4 tagged CDC50A chimeras were labeled with Atp2F6 and GM130 antibodies. Cy3- or an Alexa-488 tagged goat anti-mouse Ig secondary antibody (diluted 1:1000) was used. Double
labeling studies were performed with anti-mouse secondary and Alexa 568 goat anti-rat Ig, Alexa 594 goat anti-rabbit Ig, or Alexa 488 anti-rabbit (diluted 1:1000).

### 3.2.7 Purification and Reconstitution of ATP8A2-CDC50A Complex

The Rho-1D4, Atp6C11, and Cdc50–7F4 immunoaffinity columns were prepared as previously described (Chapter 2.2.10). Purification was performed as described (Chapter 2.2.10). 10-cm dishes of HEK293T cells were lysed in 1 ml of Buffer A. Proteins were eluted with 0.2 mg/ml 6C11 or 1D4 peptides where appropriate. In some cases, 2% SDS was also used. Reconstitution was performed as described (Chapter 2.2.11).

### 3.2.8 Endoglycosidase Treatment of Rod Outer Segments

Approximately 50 μg of ROS were denatured at room temperature and subjected to PNGase F treatment at 37 °C for 1 h according to the manufacturer's instructions (New England Biolabs). The same amount of solubilized ROS in 10 mM CHAPS and 50 mM NaH₂PO₄, pH 5.0, was treated with neuraminidase (Roche Applied Science) for 1 h followed by β-glycosidase (Sigma) for 1 h according to the manufacturer's instructions.

### 3.2.9 Membrane Topology and Glycosylation Site Prediction

Membrane topology prediction was made using the TMHMM server, version 2.0 (Krogh, et al., 2001). Prediction of N-linked and O-linked glycosylation was made using NetNGlyc 1.0 and NetOGlyc 3.1 servers, respectively (Gupta and Brunak, 2002, Julenius, et al., 2005).

### 3.2.10 Tryptic Digestion and Mass Spectrometry

SDS-PAGE gels were divided into several equal slices and digested with trypsin according to established protocol (Shevchenko, et al., 1996). Peptides were concentrated using C₁₈ stop and go extraction (STAGE) tips. The peptides were subjected to LC-MS/MS as
described (Kwok, et al., 2008) using an LTQ-Orbitrap system (ThermoFisher, Bremen, Germany). The peptides were identified using the Mascot search program (Perkins, et al., 1999).

3.2.11 ATPase Activity, Flippase Assay, and SDS-PAGE

ATPase and flippase assays were performed according to Chapter 2.2.12 and 2.2.13. SDS-PAGE was performed according to Chapter 2.2.14.

3.3 Results

3.3.1 Identification of CDC50A as the β-Subunit for ATP8A2

In previous studies, immunoaffinity-purified ATP8A2 from bovine ROS was observed to migrate as a single 130-kDa protein on SDS gels stained with Coomassie Blue (Chapter 2). However, a number of P1-ATPases are known to associate with members of the Cdc50 protein family (Lopez-Marques, et al., 2010, Paulusma, et al., 2008, Saito, et al., 2004). Furthermore, CDC50A was identified as a protein present in photoreceptor OS preparations in a recent proteomic study (Kwok, et al., 2008). On this basis, we speculated that CDC50A may be associated with ATP8A2 but was undetectable in our purified ATP8A2 preparations by conventional protein staining techniques because of its highly glycosylated extracellular domain. To test this hypothesis, we digested immunoaffinity-purified ATP8A2 from photoreceptor membranes with trypsin for analysis by MS. Tryptic peptides from both ATP8A2 and CDC50A were identified with a high level of confidence as shown in (Appendix B2). Several proteins known to be highly abundant in OS preparations were also present including rhodopsin and tubulin. However, these proteins are commonly detected in immunoprecipitated protein samples from photoreceptor OS and therefore may represent residual contaminants in the preparation.
3.3.2 Monoclonal Antibodies to CDC50A

Because highly specific antibodies to CDC50A are not commercially available, we generated anti-Cdc50a monoclonal antibodies for use as probes to study the biochemical properties and localization of CDC50A and its interaction with ATP8A2. Two distinct monoclonal antibodies were obtained from mice immunized with GST fusion proteins comprising the N-terminal domain and part of the nonglycosylated portion of the exocytoplasmic domain of bovine CDC50A. One antibody designated as Cdc50–7F4 recognized the GST fusion protein comprising the N-terminal domain and another monoclonal antibody Cdc50–9C9 bound to the GST fusion protein containing part of the extracellular domain.

The specificity of these antibodies was confirmed on Western blots of CHAPS solubilized bovine ROS and HEK293T cells expressing 1D4 epitope-tagged bovine CDC50A (Fig. 3.1A). Both the Cdc50–7F4 and Cdc50–9C9 antibodies strongly labeled a 50-kDa protein in bovine ROS- and CDC50A-transfected HEK293T cells. The identity of the 50-kDa protein as CDC50A was further confirmed on Western blots of HEK293T cells expressing the 1D4-tagged CDC50A and labeled with the Rho-1D4 antibody. In addition to the 50-kDa protein, a 100-kDa protein was also observed in HEK293T cell extracts labeled with the CDC50A and Rho 1D4 antibodies. This protein most likely represents a CDC50A dimer. CDC50A was not detected in HEK293T cells that had been mock transfected with empty plasmid. This further confirms the specificity of the monoclonal antibodies and further indicates that endogenous CDC50A expression in HEK293T cells is extremely low.

The anti-bovine Cdc50–7F4 antibody cross-reacted with the mouse and human orthologs, whereas the Cdc50–9C9 antibody was specific for the bovine protein (data not
shown). Chemically synthesized peptides were used to map the epitope of the Cdc50–7F4 antibody to a 9-amino acid segment (AKDEVDGGP) encompassing positions 7–15 of bovine CDC50A. Finally, both CDC50A monoclonal antibodies did not label expressed CDC50B (data not shown).

3.3.3 Co-immunoprecipitation of ATP8A2 and CDC50A

To confirm the association of ATP8A2 with CDC50A observed by MS, ATP8A2 was purified from CHAPS solubilized ROS for analysis by Western blotting. Fig. 3.1B shows that the purified ATP8A2 ran as a 130-kDa protein as observed by Coomassie Blue staining and confirmed by Western blotting. The Cdc50–9C9 antibody intensely labeled the 50-kDa CDC50A protein, which was not visible in Coomassie Blue-stained gels. Essentially all of the CDC50A was associated with ATP8A2, because it was largely absent in the unbound fraction from the immunoaffinity column.

The ability of the Cdc50–7F4 antibody to immunoprecipitate the ATP8A2-CDC50A complex from ROS was investigated. As shown in Fig. 3.1B, the eluted fraction showed the 130-kDa Coomassie Blue-stained ATP8A2 protein and a small amount of contaminating antibody. Western blots labeled with the CDC50A and ATP8A2 specific antibodies confirmed the presence of both proteins in the elution fraction from the Cdc50–7F4 immunoaffinity support.

3.3.4 Expression and Localization of CDC50A in the Retina

The cellular and subcellular distribution of CDC50A and ATP8A2 in cryosections of bovine retina were compared by immunofluorescence microscopy. CDC50A labeling with the Cdc50–7F4 antibody was observed in the photoreceptor OS, as well as other retinal layers including a subset of ganglion cells (Fig. 3.2A). In contrast, ATP8A2 labeling with the
Atp2F6 antibody was restricted to the OS of rods and cones as reported previously (Chapter 2). No significant labeling of the Cdc50–7F4 antibody was observed in control samples treated with the competing 7F4 peptide. The presence of CDC50A in the retinal cell layers outside the photoreceptor OS suggests that CDC50A associates with other P₅₋₆-ATPases expressed in photoreceptors and other retinal cells.

To determine the gene expression profile of various members of the CDC50 family, RNA was extracted from retina and other tissues of 6-month-old C57/B6 mice for analysis by RT-PCR. A 600-bp fragment was amplified in all tissues using CDC50A-specific primers (Fig. 3.2B), confirming its ubiquitous tissue expression (Katoh and Katoh, 2004). High levels of expression of CDC50A were detected in the brain, retina, liver, lung, testis, and spleen with lower levels in the heart and kidney. Using CDC50B gene-specific primers, high mRNA levels were observed in the liver, kidney, lung, testis, and spleen but not the brain, retina, and heart (Fig. 3.2B). Finally, CDC50C expression was only seen in the testis, consistent with earlier reports indicating that CDC50C gene expression is restricted to spermatocytes and spermatids (Osada, et al., 2007, Xu and Ding, 2007). On the basis of these studies, CDC50A appears to be the only member of the CDC50 family with detectable expression in adult mammalian retina.

The expression of the CDC50 family in HEK293T cells, a human kidney cell line commonly used for overexpression of mammalian membrane proteins, was also investigated. CDC50A but not CDC50B or CDC50C expression was observed in this cell line as shown in Appendix B3.
3.3.5 ATP8A2 in HEK293T Cells Forms a Functional Complex with CDC50A

Because HEK293T cells express low levels of CDC50A but not ATP8A2, we determined whether endogenous human CDC50A from HEK293T cells interacts with expressed ATP8A2 to form a functional complex. A detergent-solubilized extract from HEK293T cells expressing ATP8A2 was incubated with Atp6C11-Sepharose, and the bound protein was subsequently eluted with the competing 6C11 peptide. A relatively small amount of ATP8A2 was purified from these cells as visualized by Coomassie Blue staining and Western blots (Fig. 3.3A). Western blots labeled with the Cdc50–7F4 antibody showed the presence of the endogenous 50-kDa CDC50A protein in the eluted fraction, which migrated as a broad band. Despite the low yield of purified ATP8A2, the ATPase activity of the protein complex was measured. The ATP8A2-CDC50A complex from HEK293T cells exhibited a high ATPase activity with PS, significantly lower levels with PE, and minimal activity in PC (Fig. 3.3B). The specific activity of $58 \pm 1 \mu$mol/min/mg for expressed ATP8A2 in the presence of PS was similar to the specific activity of $57 \mu$mol/min/mg reported for the ATP8A2 complex isolated from bovine ROS (Chapter 2).

Next, we investigated the effect of co-expression of CDC50A and ATP8A2 in HEK293T cells. In these studies, ATP8A2 was expressed without a tag, and CDC50A was expressed with a 1D4 tag to facilitate the identification and purification of the expressed complex. Significantly higher levels of ATP8A2 were obtained from cells co-expressing ATP8A2 and CDC50A (Fig. 3.3C). A 6-fold increase in ATP8A2 was observed for cells that had been co-transfected with ATP8A2 and CDC50A relative to cells transfected with only ATP8A2 as determined after solubilization in SDS. Importantly, when membranes were
solubilized with the non-denaturing detergent CHAPS, a 15-fold increase in ATP8A2 was obtained from co-transfected cells.

### 3.3.6 ATPase and Lipid Flippase Activity of Purified ATP8A2-CDC50A

A dual immunoaffinity procedure was devised to study the functional activity of the ATP8A2-CDC50A complex from HEK293T cells co-expressing ATP8A2 and CDC50A with a 1D4 tag. A detergent-solubilized cell extract was first subjected to purification on an Atp6C11 immunoaffinity column. After elution with the competing 6C11 peptide, the complex was subjected to a second round of purification on a Rho-1D4 immunoaffinity column. This purification procedure has the potential of removing any free ATP8A2 or ATP8A2 associated with endogenous CDC50A.

Fig. 3.4A compares the purification of the ATP8A2-CDC50A complex on the Atp6C11 column with the complex isolated on the dual Atp6C11 and Rho 1D4 columns. A relatively high yield of the ATP8A2-CDC50A complex was obtained by either procedure as visualized in the Coomassie Blue-stained eluted fractions (Fig. 3.4A). Western blots indicated that the CDC50A-1D4 protein co-purified with ATP8A2 and ran as a broad band.

In a typical experiment, the expressed complex purified only on an Atp6C11 immunoaffinity matrix resulted in a specific activity of $67 \pm 2 \mu \text{mol/min/mg}$, and the complex isolated on the Atp6C11 and Rho1D4 matrix resulted in a slightly higher specific activity of $76 \pm 3 \mu \text{mol/min/mg}$ (Fig. 3.4B). The small increase in activity found after the dual immunoaffinity purification procedure most likely results from the removal of residual nonfunctional forms of ATP8A2 not associated with CDC50A-1D4.

The aminophospholipid flippase activity of the ATP8A2-CDC50A complex expressed in HEK293T cells and isolated by the dual immunoaffinity procedure was
measured using the fluorescence-based NBD-labeled lipid assay (McIntyre and Sleight, 1991, Romsicki and Sharom, 2001). The purified complex reconstituted into liposomes containing NBD-labeled lipid was incubated with ATP or the nonhydrolyzable ATP analog, AMP-PNP as a control. Dithionite was then added to bleach the NBD lipids on the outer surface of the liposomes, resulting in a decrease in the fluorescence signal. The extent of lipid transport or flipping was determined from the difference in fluorescence observed with ATP versus AMP-PNP after dithionite treatment.

A typical fluorescence trace for liposomes reconstituted with ATP8A2-CDC50A and containing NBD-labeled PS is shown in Appendix B4. Liposomes incubated with ATP showed a 6% decrease in fluorescence relative to liposomes incubated with AMP-PNP after treatment with dithionite. This reflects ATP8A2-CDC50A-mediated ATP-dependent transport of NBD-labeled PS from the inner to the outer leaflet of the liposomes. In control experiments, vesicles lacking ATP8A2-CDC50A showed no difference in fluorescence signal with ATP or AMP-PNP after the addition of dithionite.

The flippase activity of the purified and reconstituted ATP8A2-CDC50A complex from co-transfected HEK293T cells was measured for various aminophospholipid substrates (Fig. 3.4C). The complex transported up to 6% of the total NBD-labeled PS, but only 1% of the NBD-labeled PE, and essentially no NBD-labeled PC. In an earlier study, no transport of NBD-PE was observed for ATP8A2 complex purified from ROS (Chapter 2). This was likely due to the lower amounts of protein present in the reconstituted vesicles. As expected, the PS flippase activity of ATP8A2-CDC50A was found to be dependent on the amount of protein reconstituted into the liposomes (Fig. 3.4C).
3.3.7 Functional Characterization of CDC50A/CDC50B Chimera Proteins

The CDC50B variant is 55% identical to CDC50A and displays a similar membrane topology (Fig. 3.5A). However, as previously reported (van der Velden, et al., 2010) and confirmed in this study (Fig. 3.5B), CDC50B, unlike CDC50A, does not interact with ATP8A2. We have taken advantage of this property to begin to define regions of CDC50A that are required for the formation of a functionally active ATP8A2-CDC50A complex. Chimera proteins consisting of a combination of CDC50A and CDC50B domains (Fig. 3.5B) were constructed and co-expressed with ATP8A2 in HEK293T cells. The interaction of ATP8A2 with the CDC50 chimera proteins was determined by co-immunoprecipitation of these proteins on an anti-ATP8A2 immunoaffinity matrix. As shown in Fig. 3.5B, CDC50 chimera proteins in which either the ECD or the N-terminal domain of CDC50B was replaced with corresponding domain of CDC50A failed to interact with ATP8A2. In contrast, the CDC50 chimera protein in which the exocytoplasmic domain and the transmembrane domain consisting of both M1 and M2 (ECD/TM) of CBC50B were replaced with the corresponding CDC50A domains expressed at relatively high levels and co-immunoprecipitated with the ATP8A2. A chimera protein containing the exocytoplasmic domain, the transmembrane domain, and the C-terminal domain (ECD/TM/C) of CDC50A also interacted with ATP8A2. However, chimera proteins in which one or both transmembrane segments (M1/M2) of CDC50B were replaced with the transmembrane segments of CDC50A failed to interact with ATP8A2 (Appendix B5).

The PS-dependent ATPase activities of the purified ATP8A2-CDC50 chimera protein complexes were studied. As shown in Fig. 3.6A, the ECD/TM and ECD/TM/C chimera protein complexes displayed substantially lower PS-dependent ATPase activities compared
with the wild type ATP8A2-CDC50A complex. The wild type ATP8A2-CDC50A complex had a $V_{\text{max}}$ of $107 \pm 8 \mu\text{mol/min/mg}$, whereas the ATP8A2-ECD/TM and ATP8A2-ECD/TM/C complexes had $V_{\text{max}}$ of $40 \pm 5$ and $35 \pm 5 \mu\text{mol/min/mg}$, respectively. The $K_m$ values of the ECD/TM and ECD/TM/C chimera complexes ($K_m$ of $86 \pm 35$ and $73 \pm 32 \mu\text{M}$, respectively), however, were similar to the wild type protein ($K_m$ of $98 \pm 24 \mu\text{M}$).

Vanadate is known to be a potent inhibitor of P-type ATPases and is often used to measure changes in the equilibrium between the $E_1$ and $E_2$ conformational states of ATPases during the reaction cycle. Fig. 3.6A shows that the ATPase activity of purified wild type ATP8A2-CDC50A complex is strongly inhibited by vanadate with $K_i$ of $2.7 \pm 0.3 \mu\text{M}$. The chimera complexes showed a decrease in sensitivity to vanadate exhibiting a $K_i$ for the ECD/TM and ECD/TM/C chimera complexes of $8.7 \pm 0.8$ and $7.3 \pm 0.7 \mu\text{M}$, respectively.

The aminophospholipid flipase activity of equal amounts of purified and reconstituted ATP8A2-CDC50 chimeras was studied (Fig. 3.6B). The wild type complex was able to transport $6.7 \pm 0.3\%$ of total NBD-PS. In contrast, the chimeras were able to transport less NBD-PS consistent with the PS-dependent ATPase studies. The ECD/TM and ECD/TM/C chimeras transported $3.5 \pm 0.4$ and $4.2 \pm 0.5\%$, respectively.

### 3.3.8 Localization of ATP8A2-CDC50 Complexes in Transfected Cos-7 Cells

The subcellular distribution of ATP8A2 and CDC50A individually and co-expressed in Cos-7 cells was investigated in double labeling studies using calnexin as an ER marker and GM130 as a Golgi marker. For these studies, ATP8A2 and CDC50A contained C-terminal 1D4 and Myc tags, respectively, to facilitate double labeling. Immunoprecipitation studies and ATPase assays indicated that the C-terminal tags had no effect on the interaction of ATP8A2 with CDC50A or functional activity of the complex. The majority of ATP8A2
and CDC50A co-localized with calnexin in the ER in singly transfected cells (Appendix B6). In contrast, ATP8A2 and CDC50A co-localized with GM130 in the Golgi of co-transfected cells. This indicates that the association of ATP8A2 with CDC50A resulted in the translocation of the complex from the ER to the Golgi. A similar distribution pattern has been recently reported in cultured U20S cells expressing ATP8A2 and CDC50A (van der Velden, et al., 2010), indicating that this distribution is not specific to Cos-7 cells.

The effect of CDC50 chimera proteins on the localization of ATP8A2 was investigated. When ATP8A2 was co-expressed with the noninteracting CDC50 chimera proteins, ECD or N-terminal domain, ATP8A2 co-localized with calnexin in ER similar to that observed with noninteracting CDC50B (Fig. 3.7). However, when ATP8A2 was co-expressed with the ECD/TM or ECD/TM/C chimera proteins, it co-localized with GM130 to the Golgi similar to that observed for the wild type ATP8A2-CDC50A complex. These results suggest that the ECD/TM and ECD/TM/C chimera proteins promote the folding of ATP8A2 into a native-like conformation, allowing the complex to exit the ER and translocate to the Golgi.

3.3.9  
**N-Linked Glycosylation of CDC50A is Required for the Stable Expression of ATP8A2**

The exocytoplasmic domain of CDC50A contains four consensus sequences for N-linked glycosylation and one possible site for O-linked glycosylation. To determine whether endogenous bovine CDC50A is glycosylated, detergent-solubilized ROS membranes were treated with enzymes known to remove oligosaccharide chains from glycoproteins and analyzed on Western blots labeled with the Cdc50–9C9 antibody. Treatment with PNGase F, which removes N-linked sugars, resulted in a marked decrease in the apparent molecular
mass from 50 to 37 kDa close to the predicted molecular mass of the CDC50A polypeptide (Fig. 3.8A). In contrast, no detectable shift in molecular mass was observed after treatment with neuraminidase alone or in combination with O-glycosidase.

The effect of N-glycosylation of CDC50A on the expression and interaction with ATP8A2 was studied with mutants in which single and multiple consensus sequences for N-glycosylation were abolished. Co-expression of single N-glycosylation defective mutants of CDC50A (T109A, T182A, S192A, and T296A) with ATP8A2 showed a reduction in ATP8A2 expression levels relative to co-expression with wild type CDC50A (Fig. 3.8B). In contrast, the mutation that abolished the putative O-linked glycosylation (T285A) showed no reduction in ATP8A2 expression.

Multiple glycosylation site mutants Δ2 (T109A,T182A), Δ3 (T109A,T182A,S192A), and Δ4 (T109A,T182A,S192A,T296A) showed an inverse additive effect on the ATP8A2 expression levels (Fig. 3.8B). The CDC50A Δ2 mutant resulted in a 60% reduction in ATP8A2 expression relative to wild type CDC50A, whereas the Δ3 mutant showed an 80% reduction, and the Δ4 mutant reduced ATP8A2 expression to levels that were essentially undetectable in cell lysates.

Co-immunoprecipitation studies were carried out to determine whether the N-glycosylation mutants interact with ATP8A2. As shown in Fig. 3.8C, all of the single CDC50A glycosylation mutants co-precipitated with ATP8A2. Western blots of the wild type and mutant CDC50A proteins prior to immunoprecipitation (input) showed an intense compact 50-kDa protein band and a faint diffuse slower migrating band. After immunoprecipitation of ATP8A2, only the slower diffuse migrating band corresponding to the heterogeneous glycosylated form of CDC50A was present in the bound and eluted
fraction. An exception was the S192A CDC50A mutant, which ran as a relatively sharp, intense band migrating just above the 50-kDa band along with a small amount of a slower migrating diffuse band. This suggests that most of the single N-glycosylation mutants interact with ATP8A2 and undergo heterogeneous glycosylation similar to that observed for wild type CDC50A.

Next, we assessed whether the multiple N-glycosylation CDC50A mutants interact with ATP8A2. Fig. 3.8D shows that multiple N-glycosylation mutants (Δ2, Δ3, and Δ4) co-precipitated with ATP8A2. The mutants migrated as sharp multiple bands of decreasing molecular weights with the Δ4 mutant having a molecular mass comparable with that observed for wild type CDC50A treated with PNGase F. Finally, we subjected the glycosylation mutant ATP8A2-CDC50A complexes to the double immunoaffinity purification to remove any endogenous wild type CDC50A bound to ATP8A2 and tested the eluted complexes for PS-dependent ATPase activity. All of the mutants with the exception of T296A, Δ3, and Δ4 were isolated in sufficient quantity for analysis. Each mutant complex exhibited specific ATPase activities comparable with the wild type complex (Appendix B7). The flippase activity for the T109A glycosylation mutant was also found to be similar to wild type (Appendix B7).

3.4 Discussion

Although the interaction of P1-ATPases with CDC50 proteins has been observed in cells co-expressing these proteins, the association of endogenous CDC50 proteins with endogenous P1-ATPases in animal cells has not been reported to date. In the present study, we show that ATP8A2 purified from photoreceptor OS membranes exists as a heteromeric complex with CDC50A by both MS and Western blotting. The purified and reconstituted
complex functions as a PS and to a lesser degree PE lipid transporter as previously reported (Chapter 2). Immunofluorescence labeling studies using the highly specific ATP8A2 monoclonal antibody Atp2F6 and the CDC50A monoclonal antibody Cdc50–7F4 generated as part of this study show that these proteins co-localize to the OS compartment of photoreceptor cells. Although ATP8A2 is restricted to the OS, CDC50A is also present in other retinal cells and other cellular compartments of photoreceptor cells. This suggests that CDC50A also forms a heteromeric complex with other P-ATPases in the retina. It should be possible to use the CDC50A specific monoclonal antibodies in conjunction with immunoprecipitation and MS to identify and characterize the various P-ATPases in the retina and other tissues.

In a recent study, ATP8A2 and other class 1 P-ATPases were found to associate with CDC50 variants when co-expressed in U2OS cells and promote the export of these complexes from the ER (van der Velden, et al., 2010). However, the function of these complexes as phospholipid-dependent ATPases or ATP-dependent lipid transporters was not determined. Here, we have confirmed that ATP8A2 forms a heteromeric complex with CDC50A, but not CDC50B, in HEK293T and Cos-7 cells, and this complex is translocated from the ER to the Golgi. Importantly, we show for the first time that this expressed complex is functionally active as an aminophospholipid transporter when reconstituted into lipid vesicles.

HEK293T cells express low levels of endogeneous CDC50A. When cells are transfected with ATP8A2 alone, a small fraction of the heterologously expressed ATP8A2 interacts with endogenous CDC50A. Although the yield of this ATP8A2-CDC50A complex is low, nonetheless it shows PS- and PE-dependent ATPase activity similar to that previously
observed for ATP8A2-CDC50A complex isolated from photoreceptor OS (Chapter 2). The low quantity of the complex is likely due to the limiting expression of endogenous CDC50A in HEK293T cells because co-expression of ATP8A2 with CDC50A greatly enhances the yield of functionally active complex.

The role of CDC50A in promoting a stable active complex is supported by immunolabeling studies of ATP8A2 and CDC50A expressed in Cos-7 cells. In the absence of expressed CDC50A, the majority of the expressed ATP8A2 is retained in the ER, most likely as a misfolded protein that is poorly soluble in mild detergent. Co-expression of ATP8A2 with CDC50A results in the formation of properly folded heteromeric complex that is transported from the ER and to the Golgi compartment and is readily solubilized in CHAPS detergent as a functionally active PS-dependent ATPase.

CDC50A does not appear to direct the subcellular targeting of P₆-ATPases. It has been previously reported that ATP8B1 associated with CDC50A localizes to the PM of culture cells (Paulusma, et al., 2008), whereas ATP8A2 associated with CDC50A localizes to the Golgi compartment. Hence, the cell targeting signals of most, if not all, mammalian P₆-ATPases appear to reside in the catalytic P₆-ATPase subunit. This is in agreement with studies of Lopez-Marques et al. (Lopez-Marques, et al., 2010), who showed that Arabidopsis P₆-ATPases ALA2 and ALA3 and not CDC50 proteins are responsible for subcellular targeting of the complex.

In HEK293T cells, CDC50A expressed by itself is retained in the ER. It migrates as a relatively compact 50-kDa protein on SDS gels. In contrast, CDC50A associated with ATP8A2 and is present in the Golgi migrates as a diffuse band. This suggests that the N-linked oligosaccharide chains of CDC50A undergo extensive heterogeneous modification.
within the Golgi of HEK293T cells. This highly heterogeneous glycosylation appears to be a characteristic of ATP8A2-CDC50A overexpression in culture cells because CDC50A associated with ATP8A2 in the photoreceptor OS migrates as a more compact band. A similar difference in migration pattern has been reported for the \( N \)-linked glycoprotein rhodopsin. Rhodopsin from the ROS migrates as a relatively tight band on SDS gels, whereas rhodopsin expressed in culture cells migrates as a diffuse band characteristic of heterogeneous \( N \)-linked glycosylation (Doi, et al., 1990). This pattern most likely reflects a difference in the processing of oligosaccharide chains in these distinct cell types.

The four highly conserved \( N \)-linked glycosylation consensus sequences in the exocytoplasmic domain of CDC50A all undergo glycosylation. Analysis of single and multiple glycosylation mutants indicate that each oligosaccharide chain contributes to the optimal, stable expression of the ATP8A2-CDC50A complex. Although the loss of one or more glycosylation sites reduces the yield of the complex, it does not appear to affect the functional activity of the ATP8A2-CDC50A complex as measured by PS-stimulated ATPase and flippase assays.

Glycosylation of CDC50A is likely an important quality control mechanism for protein folding of the ATP8A2-CDC50A complex. During protein translation in the ER, \( N \)-linked glycans are transferred to the Asn-X-Ser/Thr sequence. Glycans are immediately trimmed by glycosidases. If a glycoprotein becomes misfolded, glucose is added to the oligosaccharide chains. Calnexin and calreticulin bind to the terminal glucose of misfolded glycoproteins, preventing their export from the ER as well as acting as chaperones to promote correct protein folding. Terminally misfolded proteins are subjected to disposal by
mannose trimming. Trimmed glycans are recognized and the peptide is translocated to the cytosol where it is degraded by the proteasome (Moremen, et al., 2012).

In this study chimera CDC50A/B proteins were generated and used to investigate the role of the various domains of CDC50A in the binding and functional activity of ATP8A2. Chimera proteins in which either the TM or the ECD domain of CDC50B was replaced with the corresponding CDC50A domain on a CDC50B backbone failed to interact with ATP8A2. In contrast, the chimera protein with both domains (ECD/TM) replaced assembled with ATP8A2 into a functionally active complex capable of actively transporting PS across the lipid bilayer. Like the wild type protein complex, this chimera complex was also exported from the ER to the Golgi. This indicates that both ECD and TM domains of CDC50A are required for the proper folding, subunit assembly, and function of the ATP8A2-CDC50 complex as a PS flippase. A similar situation exists for the Na⁺,K⁺-ATPase. Both the transmembrane and extracellular domains of the β-subunit of Na⁺,K⁺-ATPase have been implicated in its interaction with the catalytic α-subunit and contribute to the transport mechanism (Hasler, et al., 2001, Hasler, et al., 1998, Jaunin, et al., 1993).

The ECD/TM/C chimera protein containing the C-terminal as well as the ECD and TM domains of CDC50A exhibited similar properties as the ECD/TM chimera. This indicates either that the C terminus is not involved in the interaction with ATP8A2 or that the C-terminal domain of CDC50B can effectively substitute for the C-terminal domain of CDC50A in this capacity. Interestingly, although the ECD/TM and ECD/TM/C chimera interact with ATP8A2 to form a functional complex, the PS-dependent ATPase activity and lipid flippase activity are significantly reduced. Likewise, these chimeric complexes have a decreased sensitivity to vanadate, a P-type ATPase inhibitor that interacts with the $E_2$ state.
Collectively, these studies suggest that the N-terminal domain plays a role in the transport cycle of ATP8A2. One possibility is that CDC50A plays a similar role as the β-subunit of the H+/K+ ATPase stabilizing the $E_2P$ state of the enzyme through its N-terminal domain (Abe, et al., 2009). Our finding that CDC50A participates in the ATPase reaction cycle of ATP8A2 is in general agreement with recent studies on the yeast Drs2p-Cdc50p and the ATP8B1-CDC50 P$_e$-ATPases (Bryde, et al., 2010, Lenoir, et al., 2009).

In summary, our studies show that CDC50A is the β-subunit of ATP8A2 in photoreceptor OS membranes and is essential for the proper folding, assembly, and exit of the ATP8A2-CDC50A complex from the ER to the Golgi in culture cells. Importantly, CDC50A is also critical for the formation of a functionally active complex that can catalyze the transport of PS and to a lesser extent PE across cell membranes. Both the transmembrane and exocytoplasmic domains of CDC50A are essential for the formation of a functionally active ATP8A2-CDC50A complex, whereas the N-terminal domain of CDC50A participates in the reaction cycle of ATP8A2, possibly stabilizing the $E_2P$ state. Finally, $N$-linked glycosylation of CDC50A plays an important role in the formation of a stable ATP8A2-CDC50A protein complex.
Figure 3.1  CDC50A antibodies and immunoprecipitation of the ATP8A2-CDC50A complex of rod outer segments.

(A) Western blots of extracts from HEK293T cells transfected with Cdc50a-1D4 plasmid (CDC50A) or empty plasmid (Mock) and bovine rod outer segment (ROS) membranes were labeled with monoclonal antibodies to CDC50A (Cdc50-7F4 and Cdc50-9C9) and Rho 1D4.

(B) The ATP8A2-CDC50A complex was immunoprecipitated (IP) from CHAPS-solubilized ROS membranes (Input) with the ATP8A2 antibody (ATP8A2 IP) or the CDC50A antibody (CDC50A IP) coupled to a Sepharose matrix. The fraction that did not bind to the column (Unbound) and the fraction that was eluted from the column (Elution) using either competing peptide 6C11 peptide (ATP8A2 IP) or 2% SDS (CDC50A IP) were analyzed on SDS gels stained with Coomassie Blue and Western blots labeled with either ATP8A2 or CDC50A specific antibodies. The asterisks indicate the presence of antibody present in the SDS-eluted fraction.
Figure 3.2  Immunofluorescence localization of CDC50A in the retina and gene expression of CDC50 variants in the retina and other tissues.

(A) Retinal cryosections labeled with Atp2F6 or Cdc50–7F4 antibodies to ATP8A2 and CDC50A, respectively (green), and counterstained with the nuclear stain 4′,6-diamidino-2-phenylindole (blue). In the control sample, the CDC50A antibody was treated with excess 7F4 peptide prior to immunolabeling. CDC50A is abundantly localized in the photoreceptor outer segment (OS) and other retinal layers. ATP8A2 is primarily restricted to the photoreceptor OS layer. OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 25 μm. (B) Gene expression of the CDC50 family members in the retina and other tissues of adult mice by RT-PCR. GAPDH was used as a loading control. CDC50A is the only member of the CDC50 family detectable in the retina.
Figure 3.3 ATP8A2 expressed in HEK293T cells interacts with endogenous CDC50A to form a catalytically active ATP8A2-CDC50A complex.

(A) Immunoaffinity purification of ATP8A2-CDC50A complex from HEK293T cells transfected with the ATP8A2 plasmid. HEK293T cell extract (Input) was incubated with the Atp6C11 immunoaffinity matrix, and the unbound fraction (Unbound) and the 6C11 peptide eluted fraction (Elution) were analyzed on SDS gels stained with Coomassie Blue, and Western blots were labeled with antibodies Atp6C11 (ATP8A2) and Cdc50-7F4 (CDC50A).

(B) ATPase activity of the purified ATP8A2-CDC50A complex in 100% phosphatidylcholine (PC) or 10% phosphatidylserine (PS) or 40% phosphatidylethanolamine (PE) each containing the corresponding concentrations of PC.

(C) ATP8A2 and CDC50A containing a 1D4 tag were expressed individually (ATP8A2 or CDC50A) or together (ATP8A2/CDC50A) in HEK293T cells. The amounts of ATP8A2 and CDC50A solubilized with either SDS or CHAPS were measured by Western blotting (left), and the relative quantity of solubilized ATP8A2 was determined (right) (n = 3).
Figure 3.4  ATPase activity and phospholipid flippase activity of the expressed and purified ATP8A2-CDC50A complex.

(A) Coomassie Blue-stained gel and Western blots of the immunoaffinity-purified ATP8A2-CDC50A complex from HEK293T cells co-expressing both ATP8A2 and CDC50A-1D4. The complex was purified on either an Atp6C11 column alone (ATP8A2 IP) or sequentially on Atp6C11 and Rho-1D4 columns (ATP8A2/CDC50A IP).

(B) The ATPase activity of the purified complex was measured in the presence of 100% phosphatidylcholine (PC) or 10% phosphatidylserine (PS) or 40% phosphatidylethanolamine (PE) lipids each containing the appropriate amount of PC.

(C) The ATP8A2-CDC50A complex purified by the dual immunoaffinity procedure was reconstituted into liposomes for phospholipid flippase measurements. The percentage of lipid flipped by the ATP8A2-CDC50A complex was measured in phosphatidylcholine proteoliposomes for 2.5% NBD-labeled PC, PE, or PS. Right panel, the PS flippase activity of the ATP8A2-CDC50A complex was measured for decreasing amounts of reconstituted protein. IP, immunoprecipitation.
Figure 3.5  Interaction of ATP8A2-CDC50 complexes containing CDC50A/B chimera proteins.

(A) Topological model of the ATP8A2-CDC50A complex (left panel). ATP8A2 is shown in green, and CDC50A is in purple. The phosphorylated motif (DKTG) is indicated along with the positions of the N-linked glycosylation sites on CDC50A. Right panel, schematic showing various chimera CDC50A/B proteins used in these studies. (B) Interaction of CDC50A/B chimera proteins containing a 1D4 tag with ATP8A2. Proteins were purified on an ATP8A2 immunoaffinity column and analyzed on SDS gels stained with Coomassie Blue or Western blots labeled with an ATP8A2 antibody (ATP8A2) or Rho-1D4 antibody (CDC50). CDC50A co-purified with ATP8A2, whereas CDC50B did not. Chimera proteins containing only the extracellular domain (ECD) or N-terminal domain (N) of CDC50A did not co-purify with ATP8A2. Chimeras containing the ECD and TM domain consisting of both M1 and M2 segments (ECD/TM) or the ECD, TM, and C-terminal domains (ECD/TM/C) of CDC50A co-purified with ATP8A2.
Figure 3.6  Functional activity of ATP8A2-CDC50 complexes containing CDC50A/B chimera proteins.

(A) ATPase activity of ATP8A2 associated with either wild type CDC50A (WT) or chimera CDC50A proteins (ECD/TM or ECD/TM/C) as a function of phosphatidylserine (PS) concentration in the presence of phosphatidylcholine (PC) (Left panel). Right panel, vanadate inhibition of ATPase activity of ATP8A2-CDC50 complexes in 1000 μM PS in the presence of PC. (B) Flippase activity of ATP8A2-CDC50 complexes containing wild type CDC50A and the ECD/TM and ECD/TM/C chimeras reconstituted in PC in the presence of 2.5% NBD-PS.
Figure 3.7  Immunofluorescence localization of ATP8A2 co-expressed with CDC50A, CDC50B, or chimera CDC50A/B containing a 1D4 tag in Cos-7 cells.

The cells were double labeled for ATP8A2 with the Atp2F6 monoclonal antibody (green) and the GM130 polyclonal antibody as a Golgi marker (red). Co-expression of ATP8A2 with CDC50B or chimeric CDC50A proteins containing the ECD or N-terminal domain (N) domains resulted in a reticular staining pattern of ATP8A2 characteristic of ER localization. Co-expression of ATP8A2 with either wild type CDC50A or chimera proteins containing the ECD/TM or ECD/TM/C domains of CDC50A resulted in localization of ATP8A2 to the Golgi as shown in merged images. Expression of CDC50 proteins was confirmed independently using the Rho 1D4 antibody (data not shown). The nuclei (blue) were labeled with 4’,6-diamidino-2-phenylindole. Bar, 10 μm.
Figure 3.8  \(N\)-Linked glycosylation of CDC50A stabilizes ATP8A2.

(A) Approximately 50 μg of rod outer segment membranes were treated with PNGase F or neuraminidase with or without \(O\)-glycosidase and labeled with the Cdc50–9C9 antibody. (B) Expression levels of ATP8A2 in the presence of CDC50A glycosylation mutants containing a 1D4 tag. ATP8A2 levels were normalized to \(\beta\)-actin as a loading control. (C) Co-immunoprecipitation of single CDC50A glycosylation mutants with ATP8A2 on Atp6C11-Sepharose. ATP8A2 was detected with Atp6C11, and the CDC50A-1D4 mutants were detected with the Rho 1D4 antibody. Solubilized HEK293T cells (Input) were incubated in the column, and the bound proteins (Bound) were eluted with 6C11 peptide. (D) Immunoprecipitation of multiple CDC50A glycosylation mutants with ATP8A2 on Atp6C11-Sepharose.
Chapter 4: Critical Role of a Transmembrane Lysine in Aminophospholipid Transport by Mammalian Photoreceptor P₄-ATPase ATP8A2

4.1 Introduction

P-type ATPases are a large family of membrane pumps believed to be transiently phosphorylated at the conserved aspartate residue of the DKTGT motif during the catalytic cycle. An important challenge is to understand how these proteins couple the utilization of ATP with transport of substances across the membrane. Among the most well characterized P-type ATPases are the sarcoplasmic reticulum Ca²⁺-ATPase and the Na⁺,K⁺-ATPase. During the reaction cycle of these ATPases, the intermediates $E_1$, $E_1P$, $E_2P$, and $E_2$ ($P$ representing phosphorylation) are formed sequentially, involving large movements of the three cytoplasmic domains, N (nucleotide binding), P (phosphorylation), and A (“actuator”), that are coupled through a linker region with the ion translocation occurring in the membrane domain M (Fig. 4.1) (Morth, et al., 2007, Shinoda, et al., 2009, Toyoshima, 2009). For many other P-type ATPases, much less is known regarding the transport mechanism due to lack of structural and biochemical data, and even the existence of a phosphorylated intermediate is a conjecture based on sequence homology.

The amino acid sequence homology suggests that the overall structure and domain topology of P₄-ATPases is similar to that of the catalytic subunits of the Ca²⁺-ATPase and Na⁺,K⁺-ATPase, consisting of cytoplasmic N, P, and A domains and a membrane domain.

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made up of ten transmembrane helices M1-M10. However, there is little information available on the actual mechanism of phospholipid transport. How is ATP hydrolysis coupled with the flipping of lipids? Do flippases form a phosphoenzyme existing in two major conformations, $E_1P$ and $E_2P$? Is lipid flipping toward the cytoplasmic leaflet associated with dephosphorylation of $E_2P$, like the transport of $K^+$ from the exoplasmic to the cytoplasmic side by the Na$^+\text{-}K^+$-ATPase? Is the formation of the phosphoenzyme of flippases activated by a specific substrate being transported, as established for the phosphoenzyme intermediates of Ca$^{2+}$-ATPase and Na$^+\text{-}K^+$-ATPase, which depend on the binding of Ca$^{2+}$ and Na$^+$, respectively?

The expression and purification of ATP8A2 (Chapter 2 and 3) allows detailed mechanistic studies of this flippase, and here we have characterized the phosphoenzyme and its dependence on ions and the transported lipids. We have studied the consequences of key mutations in ATP8A2 located in the A, P, and M domains, and we have identified Lys$^{873}$ of putative transmembrane segment M5 as an essential residue involved in PS-activated dephosphorylation, possibly as a PS interacting residue.

4.2 Methods

4.2.1 Materials

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 ion exchange chromatography to remove contaminant ADP.
4.2.2 DNA Constructs

Bovine CDC50A (Chapter 3) without a tag was cloned into pcDNA3 using the HindIII and XhoI restriction sites. Mutations were introduced into the full length cDNA encoding bovine ATP8A2 (Chapter 2). All constructs were verified by sequencing of the entire coding sequence.

4.2.3 Purification and Functional Analysis of ATP8A2

Expression, Western blotting, ATPase activity, and flippase assays of ATP8A2 were performed as described (Chapter 2 and 3).

4.2.4 Phosphorylation Assay

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 MgCl₂, 1 mM DTT, and 2 µM [γ-³²P]ATP. Studies of phosphorylation and dephosphorylation were carried out at 0°C, except for the rapid kinetic experiments described in Fig. 4.7, Appendix C5, and Appendix C7, where the temperature was 25°C. For experiments at 0°C mixing was manual (Andersen, et al., 1989) or by magnetic stirrer (Vilsen and Andersen, 1998), and for rapid kinetic experiments at 25°C a Bio-Logic QFM-5 quenched-flow module (Bio-Logic Science Instruments, Claix, France) was applied (Sorensen, et al., 2000, Toustrup-Jensen, et al., 2001). Quenching of the phosphorylation reaction was performed with 1 or 2 volumes of 25% (w/v) trichloroacetic acid (TCA) containing 100 mM H₃PO₄. The TCA precipitated enzyme was washed twice with a solution containing 7% TCA and 1 mM H₃PO₄ and dissolved in SDS-PAGE loading buffer (10 mM Na₂HPO₄ (pH 6.0), 10% lithium dodecyl
sulfate (LDS), 2% β-mercaptoethanol (v/v), 0.4% (w/v) bromophenol blue, and 25% (v/v) 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 (Andersen, et al., 1989). 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 non-phosphorylated 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-labelled 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% (Clausen and Andersen, 2003), 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.

4.2.5 Removal of Ions for Determination of Ionic Requirements

To remove alkali metal ions and Cl\textsuperscript{−}, reconstituted ATP8A2 was dialyzed against 3 changes of 1 l of the NMDG medium (10 mM HEPES-Tris pH 7.5, 150 mM NMDG-Acetate, 1 mM Mg-Acetate, 1 mM DTT, and 10% (w/v) sucrose). Following the dialysis the Na\textsuperscript{+} and K\textsuperscript{+} concentrations were less than 25 µM as determined by atomic absorption spectrometry. For functional assays in NMDG medium Cl\textsuperscript{−} was replaced by acetate.

4.2.6 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. Experiments were generally performed at least twice. The average values are reported, except where it was judged relevant to show all the individual data points (stated in legend). Error bars and “±” indicate SEM values.

4.3 Results

4.3.1 ATP8A2 Phosphoenzyme

In the presence of [γ-\textsuperscript{32}P]ATP and Mg\textsuperscript{2+}, purified ATP8A2 reconstituted into PC lipid vesicles formed an acid-stable phosphorylated intermediate detected as an intensely \textsuperscript{32}P-labeled band by phosphorimaging following SDS-PAGE at pH 6.0 (Fig. 4.2A, middle panel). Incubation with hydroxylamine prior to SDS-PAGE abolished labeling, indicating that the phosphoenzyme intermediate is an acyl phosphate (Fig. 4.2A, right panel). The apparent Mg\textsuperscript{2+} affinity for activation of phosphorylation was similar to that of Na\textsuperscript{+},K\textsuperscript{+}-ATPase.
To verify that the phosphorylation site of ATP8A2 is Asp\textsuperscript{416} in the DKTGT P-type ATPase signature sequence, we carried out a similar phosphorylation analysis of the mutant D416N. D416N was expressed at a 10-fold lower level compared to wild type. Nevertheless, when using an amount of D416N similar to wild type, no \textsuperscript{32}P incorporation was observed (Fig. 4.2A, left and middle panels).

4.3.2 Expression and Overall Function of ATP8A2 Mutants

We examined the functional consequences of ATP8A2 mutations D196T, E198Q, K865A, K873A, K873E, K873R, and N874A. Because the glutamate of the A-domain motif TGES in Ca\textsuperscript{2+}-ATPase is a key residue in catalysis of dephosphorylation of the E\textsubscript{2}P phosphoenzyme intermediate (Fig. 4.1B) (Clausen, et al., 2004, Toyoshima, 2009), we selected the corresponding ATP8A2 glutamate for analysis by replacement with glutamine (E198Q). In all P\textsubscript{4}-ATPases, the residue corresponding to threonine in the TGES motif is an aspartate (DGET motif, see Fig. 4.1C). To study the functional impact of this characteristic feature we analyzed the mutant D196T. Furthermore, we selected the highly conserved Lys\textsuperscript{873} and Asn\textsuperscript{874} in the middle of M5 for analysis (Fig. 4.1A), since M5 of Ca\textsuperscript{2+}-ATPase and Na\textsuperscript{+},K\textsuperscript{+}-ATPase is pivotal in the ion translocating events (Blostein, et al., 1997, Clarke, et al., 1989, Morth, et al., 2007, Pedersen, et al., 1998, Shinoda, et al., 2009, Toyoshima, 2009, Vilsen, 1995). Lys\textsuperscript{873} was replaced with alanine, arginine, and glutamate (K873A, K873R, K873E), and Asn\textsuperscript{874} was replaced with alanine (N874A). For comparison, Lys\textsuperscript{865} located near the cytoplasmic border of M5 was also mutated (K865A). These mutants were generally expressed at levels rather similar to the wild type (Appendix C2). Each mutant and the wild type were purified and reconstituted into vesicles consisting of either PC or a mixture of 90% PC and 10% PS (“90PC:10PS”). Both wild type and mutants were largely free of any
contaminant proteins, and the PC and 90PC:10PS preparations contained roughly the same concentration of ATP8A2 (Appendix C2). Comparable levels of CDC50A co-purified with wild type and mutants. Previous studies have indicated that ATP8A2 reconstituted by this method is oriented such that 70% is accessible to trypsin digestion and ATP (ATP binding domain facing outward) (Chapter 2). Hence, in assays of the reconstituted vesicles 70% of the enzyme is expected to be active, and the outer leaflet corresponds functionally to the cytoplasmic leaflet of the cell membrane.

Reconstitution was also carried out in PC together with NBD-PS, which allowed for the determination of flippase activity using fluorescence spectroscopy (Fig. 4.2B and Appendix C2). ATP or the non-hydrolyzable AMP-PNP was added to the reconstituted enzyme, and flipping of NBD-PS was allowed to occur for 2.5 min at 23 °C, followed by dilution to stop the ATP utilization and addition of dithionite to bleach NBD-PS in the outer leaflet, thereby allowing a precise determination of % NBD-PS contained in the inner leaflet (Chapter 2). The difference in inner leaflet % NBD-PS for AMP-PNP versus ATP represents the ATP energized transport to the outer leaflet corresponding to the cytoplasmic side (Appendix C2). D196T, K873R, and N874A were found to transport NBD-PS at about one third to one half of the level transported by wild type, and K873A and K873E about one fifth of wild type. K865A transported NBD-PS at a similar level as wild type. E198Q seemed to catalyze a small net transport of NBD-PS in the opposite direction, from the outer to the inner leaflet (Fig. 4.2B and Appendix C2, see further explanation in legend).

ATP8A2 wild type and mutants reconstituted in 90PC:10PS were assayed for ATPase activity at 37 °C. As seen in Fig. 4.2C,D, ATP utilization was linear over time. Compared with wild type, K873A and K873E displayed ~30- and 70-fold lower specific ATPase
activity (relative to amount of ATP8A2 protein present), respectively. D196T was reduced by 3-fold while K873R and N874A were reduced by 10-fold. K865A exhibited ATPase activity similar to wild type. ATPase activity was not detectable for E198Q or D416N (Fig. 4.2D and Appendix C3).

4.3.3 Phosphorylation and Interaction with Aminophospholipids

Like wild type ATP8A2, the DGET and M5 mutants formed a phosphoenzyme from \([\gamma^{32}\text{P}]\text{ATP}\) (see examples of phosphorylation gels in Fig. 4.3A). The phosphorylation stoichiometry for most mutants was rather similar to that of the wild type (Appendix C4). The apparent affinity for ATP determined for a selected group of mutants was also wild type-like (Appendix C1, \(K_{0.5} \approx 0.1 \mu\text{M}\)). Following chelation of Mg\(^{2+}\) with EDTA, no phosphorylation was observed (Fig. 4.3A). Interestingly, E198Q and K873A exhibited phosphorylation levels in 90PC:10PS of approximately the same magnitude as in PC, whereas the wild type and D196T displayed a much lower steady-state level of phosphorylation in 90PC:10PS than in PC (Fig. 4.3A), as did native ATP8A2 purified from photoreceptor OS (Appendix C2). This consequence of the presence of PS can be explained by assuming that PS activates the dephosphorylation in analogy with the K\(^+\)-induced dephosphorylation of Na\(^+\),K\(^+\)-ATPase, as illustrated in the reaction scheme in Fig. 4.3B. To further address this issue, PC reconstituted wild type and mutants solubilized in CHAPS detergent were phosphorylated followed by addition of PS (Fig. 4.3C). In control experiments in which PC was added instead of PS the phosphoenzyme levels remained almost maximal during 30 s for wild type as well as mutants. Upon addition of PS, the wild type dephosphorylated rapidly, reaching a level less than 10% of the initial level in PC within 5 s. D196T dephosphorylated somewhat slower than wild type, whereas K865A was

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indistinguishable from wild type. N874A dephosphorylated to a level close to 25% that of the wild type. In contrast, the level of phosphoenzyme remained constant at 100% for E198Q even after 30 s incubation with PS, and K873A was only weakly reactive to PS, displaying relatively slow kinetics of dephosphorylation, reaching a constant phosphoenzyme level as high as 70%. This led us to employ the phosphorylation assay to determine the apparent affinity for PS as well as PE by measuring the dephosphorylation at various concentrations of the added aminophospholipid (Fig. 4.4A, Appendix C1 and Appendix C4). The wild type exhibited a $K_{0.5}$ of $\sim 12 \mu M$ for PS and $\sim 140 \mu M$ for PE in agreement with the previously demonstrated selectivity for PS versus PE (Chapter 2 and 3). K865A was rather similar to wild type. Importantly, K873A and K873E displayed marked reductions (7- and 8-fold, respectively) of the apparent affinity for PS, while the PS affinities of K873R and N874A were more moderately reduced (4- and 3-fold, respectively). Using the ATPase activity assay, K873A and K873E also displayed a conspicuous 13- to 16-fold reduction of the apparent affinity for activation by PS, and these mutants were completely insensitive to PE within the concentration range tested. K873R and N874A displayed an intermediate reduction in apparent affinity for PS, and little or no ATPase activation by PE was observed. K865A was again wild type-like (Fig. 4.4B, Appendix C1 and Appendix C4). Similar studies with D196T showed a wild type-like or even enhanced apparent affinity for PS (Appendix C1). These results indicate that Lys$^{873}$ could be a critical lipid interacting residue and that the positive charge of the side chain is important.

4.3.4 Distribution of the Phosphoenzyme between $E_1P$ and $E_2P$ Forms

For Ca$^{2+}$-ATPase and Na$^+$,K$^+$-ATPase it is well known that the phosphoenzyme resides in two major conformational states, $E_1P$ and $E_2P$. $E_1P$ is the phosphoenzyme
intermediate formed by phosphorylation with ATP and is able to donate the phosphoryl group back to ADP. Following the conformational change to $E_2P$ the phosphoenzyme intermediate is insensitive to ADP, and dephosphorylation can only occur by reaction with water, liberating $P_i$. The structural basis is that in $E_2P$ the TGES motif becomes inserted in the catalytic site, thereby partly occupying the space previously taken up by ADP in the $E_1P$ form (Fig 4.1B) (Toyoshima, 2009). To examine whether the phosphoenzyme of ATP8A2 exists in similar ADP-sensitive and -insensitive states, the dephosphorylation upon addition of either ATP or ADP to phosphoenzyme formed in the presence of PC was followed for selected mutants (Fig. 4.5, circles). Two exponential decay phases could be readily distinguished from the ADP dephosphorylation time course, a rapid component corresponding to the reaction of accumulated $E_1P$ with ADP and a slower phase, which may represent the hydrolysis of $E_2P$ and/or back conversion of $E_2P$ to $E_1P$ followed by reaction with ADP. The initial steady-state $E_1P$ and $E_2P$ fractions were estimated by fitting a bi-exponential decay function to the data (Fig. 4.5 and Appendix C3). For wild type, the phosphoenzyme distribution corresponded to 60% $E_2P$, whereas somewhat more $E_2P$ was accumulated for D196T and E198Q but less for K873A. For wild type and D196T in 90PC:10PS, the low steady-state phosphorylation level and rapid PS-induced dephosphorylation precluded a similar determination of the $E_2P$ fraction, but for E198Q and K873A in 90PC:10PS this analysis was possible. For E198Q, the $E_2P$ fraction of the phosphoenzyme increased to ~100% in the presence of PS, whereas for K873A the presence of PS had little effect on the distribution of the phosphoenzyme between $E_1P$ and $E_2P$ (Fig. 4.5, triangles).
4.3.5 Vanadate Binding

Vanadate is known as an analog of the penta-coordinated transition state of $E_2P$ dephosphorylation in P-type ATPases, which binds specifically to the $E_2$ form (Cantley, et al., 1978). Since vanadate is also a potent inhibitor of ATP8A2 (Chapter 3), we studied the changes in apparent vanadate affinity of ATP8A2 induced by the mutations and binding of PS for selected mutants, using a phosphorylation assay that takes advantage of the competition between vanadate and ATP. Wild type and mutants displayed distinct vanadate affinities that depended on the presence of PS (Fig. 4.6 and Appendix C3). In PC, the wild type exhibited a $K_{0.5}$ value for vanadate binding of $\sim 5 \, \mu M$, whereas D196T and E198Q showed $\sim 2$- and $\sim 7$-fold lower apparent affinities, respectively. PC reconstituted K873A displayed a marked $\sim 80$-fold reduction of vanadate affinity relative to wild type. In 90PC:10PS the low phosphorylation levels of wild type and D196T precluded an accurate determination of vanadate affinity by this method, but it was nevertheless clear that a considerable affinity increase occurred as a consequence of the interaction with PS (shown for wild type in Fig. 4.6, $\sim 200$-fold affinity increase). For E198Q and K873A, the apparent affinities for vanadate were respectively 70- and $\sim 25$-fold higher than the corresponding affinities determined in the absence of PS. Hence, the presence of PS increased the vanadate affinity to a lesser extent in K873A as compared with E198Q and wild type.

4.3.6 Dependence of Phosphorylation on Ions

The studies of the phosphoenzyme reported above were carried out in the presence of 150 mM NaCl. In cation-transporting P-type ATPases like Na$^+$.K$^+$.ATPase and Ca$^{2+}$.ATPase, the ion being translocated from the cytoplasmic side toward the exoplasmic side of the membrane activates the reaction of the $E_1$ state with ATP leading to phosphoenzyme
formation. To search for candidate cations or anions required to activate the phosphorylation of ATP8A2, wild type ATP8A2 reconstituted in PC or 90PC:10PS was dialyzed against medium containing 150 mM N-methyl-D-glucamine (NMDG), a cationic sugar that provides for ionic strength but is unable to substitute for Na⁺ in the activation of the Na⁺,K⁺-ATPase. Although the dialysis reduced the concentrations of Na⁺ and K⁺ to less than 25 µM as determined by atomic absorption spectrometry, and Cl⁻ levels were presumably equally low, the phosphorylation rate constant of PC reconstituted ATP8A2 determined in rapid-kinetic measurements was not reduced in the NMDG dialyzed sample (Fig. 4.7 and Appendix C5). Moreover, addition of various salts to this sample slightly reduced the steady-state phosphorylation levels in PC and only slightly increased the ATPase activity in 90PC:10PS. Removal of Ca²⁺ with EGTA had no significant effect either (Appendix C6).

An obvious candidate for ion counter-transport and activation of phosphorylation would be H⁺. Hence, we examined the dependence of the phosphorylation on pH. There was no significant effect of pH on the phosphorylation rate constant of PC reconstituted enzyme in the range of pH 6.5 to pH 9.0 (Appendix C6, compare also with pH 7.5 in Fig. 4.7 and Appendix C5), and over a broad range of pH values, no large differences were observed in the steady-state levels of phosphoenzyme in both PC and PS (Appendix C6), indicating that H⁺ is not required for phosphorylation. This is in agreement with earlier work showing that the ATPase activity remains maximal at pH 9.0 (Chapter 2).

The phosphorylation rate constants of D196T, E198Q, and K873A determined in PC at pH 7.5 in the presence of 150 mM NaCl were similar to that of the expressed wild type ATP8A2 and the native ATP8A2 from photoreceptors reconstituted in PC (Appendix C7 and Appendix C5). The Ca²⁺-ATPase exhibited a 2-fold higher rate constant in medium of
identical composition except for the addition of 100 μM Ca$^{2+}$. Because of the strong inhibition of dephosphorylation in E198Q, rapid kinetic measurements of phosphorylation could be conducted with E198Q in 90PC:10PS, and the rate constant was found 1.6-fold reduced relative to that of E198Q in PC (Appendix C7 and Appendix C5).

4.4 Discussion

The present findings provide insight into the molecular mechanism of phospholipid transport in P$_4$-ATPases, extending our understanding of ion transport to an important but relatively uncharacterized subfamily of mammalian P-type ATPases.

First of all, our results indicate that the phosphoenzyme intermediate of ATP8A2, like that of Ca$^{2+}$-ATPase and Na$^+$,K$^+$-ATPase, is formed by phosphorylation of the aspartic acid of the DKTG signature sequence in the P-domain and exists in ADP-sensitive $E_1$P and ADP-insensitive $E_2$P forms. Comparison of various crystal structures of the Ca$^{2+}$-ATPase has revealed that transformation of $E_1$P to $E_2$P and dephosphorylation of $E_2$P is accomplished by a rotation of the A-domain, allowing the glutamate of the TGES motif to catalyze hydrolysis of the aspartyl phosphoryl bond by acting as a base extracting a proton from the attacking water molecule in the transition state complex (Clausen, et al., 2004, Toyoshima, 2009) and Fig 4.1B. Here we have found that replacement of the corresponding Glu$^{198}$ in ATP8A2 with glutamine leads to block of $E_2$P dephosphorylation with resulting accumulation of $E_2$P (Figs. 4.3 and 4.5), in accordance with a similar mechanism for dephosphorylation in P$_4$-ATPases.

The fact that flipping of PS and to a lesser extent PE is mediated by the purified complex of ATP8A2 with CDC50A provides a clear indication that no other protein is involved (Chapter 2 and 3). The present results demonstrate that PS and PE activate the dephosphorylation of ATP8A2 in analogy with the activating effect of K$^+$ on the
dephosphorylation of Na\textsuperscript{+},K\textsuperscript{+}-ATPase, as depicted in the scheme of Fig. 4.3B. This is deduced both from the much lower steady-state level of phosphoenzyme seen with expressed or purified native wild type ATP8A2 in 90PC:10PS as compared with the enzyme in PC, and from the dephosphorylation induced by addition of PS or PE (Figs. 4.3, 4.4, and Appendix C1). It is therefore conceivable that aminophospholipids interact with the ATP8A2-CDC50A complex in the $E_2P$ form to modify the conformation, such that the transition state complex in the dephosphorylation reaction is stabilized and the rate of dephosphorylation thereby enhanced. In accordance with this hypothesis, vanadate was found to bind much stronger in the presence of PS than only in PC (Fig. 4.6). Because vanadate binds to P-type ATPases in the $E_2$ form and not in $E_1$, a shift of the $E_1-E_2$ equilibrium in favour of $E_2$ could also contribute to the increase of apparent affinity for vanadate induced by PS. Both of these mechanisms would be analogous to the effect of binding of K\textsuperscript{+} to the transport site of the Na\textsuperscript{+},K\textsuperscript{+}-ATPase, suggesting the existence of a PS binding site on the ATP8A2-CDC50A complex. In support of the scheme in Fig. 4.3B we also found that for E198Q, PS shifted the distribution of the phosphoenzyme intermediate between $E_1P$ and $E_2P$ in favour of $E_2P$ (Fig. 4.5).

Our results pinpoint Lys\textsuperscript{873} as a strong candidate for a residue interacting with the transported aminophospholipids. Mutant K873A displayed substantially reduced ATPase and lipid transport activities. K873A was phosphorylated at a rate similar to wild type (Appendix C7), but displayed significantly slower kinetics of PS-activated dephosphorylation and higher steady-state levels of the phosphoenzyme in PS, relative to wild type (Fig. 4.3). Importantly, K873A and K873E exhibited a conspicuous reduction of the affinity for PS activation of dephosphorylation and ATPase activity, relative to wild type, and no response to PE within
the concentration range tested (Fig. 4.4, Appendix C1, Appendix C4). K873A furthermore
displayed a weaker affinity for vanadate than the wild type and the other mutants in PC as
well as in 90PC:10PS, and although the affinity of K873A for vanadate was significantly
higher in the presence of PS than in its absence, the difference was not as large as for E198Q
and wild type, thus supporting a weaker binding of PS to K873A. In addition, PS did not
increase the \( E_2P/E_1P \) ratio of K873A, in contrast to E198Q. Hence, several pieces of evidence
indicate that Lys\(^{873}\) is a key player in the functional interactions with PS and PE. Because
K873R showed less effect on aminophospholipid sensitivity than K873A, despite the
potentially disturbing bulkiness of the arginine side chain, the positive charge of the Lys\(^{873}\)
side chain seems important. Mutation of Asn\(^{874}\) also reduced PS affinity moderately, which
might be an indirect effect of its proximity to Lys\(^{873}\) or reflect an involvement to some extent
of Asn\(^{874}\) in aminophospholipid binding. By contrast, the alanine replacement of Lys\(^{865}\), also
located in M5, but nearer the cytoplasmic boundary than Lys\(^{873}\), did not affect the sensitivity
to aminophospholipid.

The alignment of amino acid sequences depicted in Fig. 4.1C reveals that Lys\(^{873}\) and
Asn\(^{874}\) of ATP8A2 are located corresponding to the cation binding region in the middle of
M5 in Na\(^+\),K\(^+\)-ATPase and Ca\(^{2+}\)-ATPase and are rather well conserved among P\(_4\)-ATPases.
Only ATP9A and ATP9B show a semiconservative replacement of the lysine with arginine.
The Na\(^+\),K\(^+\)-ATPase residue in the corresponding position is a serine, which contributes its
side-chain oxygen to K\(^+\) binding (Blostein, et al., 1997, Morth, et al., 2007, Pedersen, et al.,
1998, Shinoda, et al., 2009). In addition, the asparagine (corresponding to Asn\(^{874}\)) next to the
serine as well as a threonine and a glutamate in M5 also contribute to K\(^+\) binding in Na\(^+\),K\(^+\)-
Furthermore, the corresponding asparagine and glutamate of the Ca\(^{2+}\)-ATPase provide ligands for Ca\(^{2+}\) binding (Clarke, et al., 1989, Toyoshima, 2009). It is also intriguing that the H\(^{+}\),K\(^{+}\)-ATPase has a lysine at the position corresponding to Lys\(^{873}\) of ATP8A2. This lysine seems to help preventing the H\(^{+}\),K\(^{+}\)-ATPase from transporting more than one proton and one K\(^{+}\) ion in each ATP hydrolysis cycle (Burnay, et al., 2003) in contrast to the 3Na\(^{+}\):2K\(^{+}\):1ATP stoichiometry of the Na\(^{+}\),K\(^{+}\)-ATPase. In Na\(^{+}\),K\(^{+}\)-ATPase, replacement of the corresponding serine with alanine reduces the affinity for extracellular K\(^{+}\) 30-fold, and the kinetics indicate a role as a gating residue (Blostein, et al., 1997). Furthermore, the same serine seems to contribute to Na\(^{+}\) binding in the E\(_{1}\) form (Einholm, et al., 2010, Pedersen, et al., 1998) in accordance with an alternating extracellular and cytoplasmic exposure during the transport cycle. Hence, by analogy it may be speculated that during the transport of aminophospholipid by ATP8A2 the lipid head group is bound from the exoplasmic leaflet to Lys\(^{873}\). Because the Lys\(^{873}\) mutations affected PE interaction with the E\(_{2}\)P form of ATP8A2 even more than PS interaction, it is not likely that the lysine binds the carboxylate group specific to PS, but it rather interacts with the head group phosphate. Later in the cycle, following the dephosphorylation, Lys\(^{873}\) might be involved in the ejection of the lipid from ATP8A2 into the cytoplasmic leaflet by repelling the positive charge of the aminophospholipid head group common to both PS and PE. Because the removal of the phospholipid head group from the membrane-water interphase may be energetically more demanding than the movement of the hydrocarbon chains through the membrane, the interaction of the head group with a protein site is likely a crucial part of the flipping mechanism. If the CDC50 subunit plays an essential role in lipid flipping, the alternative to a direct interaction of Lys\(^{873}\) with the lipid head group could be a more indirect engagement in the flipping by tight interaction with CDC50.
Unlike the majority of the P-type ATPase subclasses, the P₄-ATPases possess an aspartate at the position corresponding to the threonine of the TGES motif. Although our results show that Asp¹⁹⁶ is less crucial for the dephosphorylation of $E₂P$ than Glu¹⁹₈, mutation D196T did slow dephosphorylation significantly in the presence of PS (Fig. 4.3C), leading to reduced ATPase activity (Fig. 4.2D). In the Ca²⁺-ATPase and Na⁺,K⁺-ATPase structures, the side chain of the threonine apparently stabilizes the TGES loop through a hydrogen bond formed with the backbone nitrogen of the glutamate, and replacement of the threonine with alanine markedly reduces the rate of dephosphorylation in Na⁺,K⁺-ATPase (Toustrup-Jensen and Vilsen, 2003). The effects observed for the D196T mutation of ATP8A2 are similar although less dramatic, and it is therefore likely that in the P₄-ATPases the aspartate side chain contributes to stabilize the catalytic assembly through additional bond formation(s) that might be under long-distance control by PS binding.

Having realized the similarities between the aminophospholipid transport occurring in relation to dephosphorylation of ATP8A2 and the K⁺ transport by the Na⁺,K⁺-ATPase, one may also wonder how much the mechanism of phosphorylation of the $E₁$ form by ATP resembles that of the archetypical cation transporting P-type ATPases. Apparent ATP and Mg²⁺ affinities determined in the steady-state phosphorylation experiments were quite similar to the values reported for the Na⁺,K⁺-ATPase and Ca²⁺-ATPase under comparable conditions (cf. Appendix C1 and (McIntosh, et al., 1996, Vilsen, 1993)). The rate constant of phosphorylation was somewhat lower for ATP8A2 (~10 s⁻¹) than the rate constants observed for Na⁺,K⁺-ATPase and Ca²⁺-ATPase (20-30 s⁻¹, cf. Appendix C7 and (Toustrup-Jensen and Vilsen, 2003)), but the difference is not dramatic. In accordance with the model in which the A-domain does not interact functionally with the catalytic site of the P-domain until the
transformation of $E_1P$ to $E_2P$ (Fig 4.1B), D196T and E198Q did not significantly affect the rate of phosphorylation by ATP. The less than 2-fold reduction of the phosphorylation rate found for E198Q in 90PC:10PS relative to PC may result from the shift in the $E_1$-$E_2$ equilibrium toward $E_2$ induced by PS binding. A crucial question is whether binding of an ion to the $E_1$ form of the P$_4$-ATPase is required for activation of phosphorylation, but the present data do not support a critical role of Na$^+$, K$^+$, Ca$^{2+}$, Cl$^-$, or H$^+$ ions. Hence, aminophospholipids could be the only species being transported by ATP8A2.
Figure 4.1  Structural modeling of ATP8A2 and location of mutated residues.

(A) ATP8A2 was modeled using the Na⁺,K⁺-ATPase E₂P (2ZXE) structure as a template. Residues mutated in this study are shown as space-filling (carbons, grey; nitrogens, blue; oxygens, red; hydrogens, white). Modeling courtesy of Stepan Lenevich. (B) Depiction of the conformational changes which occur during the reaction sequence. (C) Sequences of bovine ATP8A2 and selected human flippases were aligned with the Na⁺,K⁺-ATPase α₁ isoform, gastric H⁺,K⁺-ATPase, and the sarcoplasmic reticulum Ca²⁺-ATPase (SERCA1). Residues mutated in this study are marked with pink shading, and yellow shading denotes residues of Na⁺,K⁺-ATPase and Ca²⁺-ATPase shown by crystal structures to be implicated in binding of K⁺ or Ca²⁺.
Figure 4.2  Purification and overall function of wild type ATP8A2 and mutants.

(A) Western blot of phosphatidylcholine (PC) reconstituted wild type and D416N mutant with ATP8A2-specific antibody and analysis of \(^{32}\text{P}\)-labeling by SDS-PAGE at pH 6.0 following treatment of \(\gamma^{32}\text{P}\)ATP-incubated enzyme with (+) or without (–) hydroxylamine (HA). (B) Transport of NBD-PS measured using the fluorescence-dithionite assay. A positive value for NBD-PS transport indicates transport from the exoplasmic to the cytoplasmic leaflet, whereas a negative value indicates transport in the opposite direction (see further in Appendix C2). (C, D) ATPase activity of 90PC:10PS reconstituted enzyme. In B, C, and D the activity is calculated relative to the amount of ATP8A2 protein present. See additional information including statistical analysis in Appendix C3.
Figure 4.3  Dephosphorylation induced by phosphatidylserine.

(A) Wild type ATP8A2 and mutants reconstituted in phosphatidylcholine (PC) or 90PC:10PS (PS) were phosphorylated for 10 s or 30 s with [γ^32P]ATP in SPM (for background, EDTA was added). Equal amounts of protein reconstituted in PC and 90PC:10PS were loaded on the gel. (B) Proposed reaction scheme showing various enzyme intermediates and their interaction with nucleotides and PS. (C) Following 10 s phosphorylation of PC reconstituted enzyme with [γ^32P]ATP in SPM containing CHAPS, PS dissolved in CHAPS was added at a final concentration of 55 μM, and quenching was performed at the indicated time intervals for wild type (open circles), D196T (filled circles), E198Q (filled triangle), K865A (filled diamond), K873A (open triangle pointing up), and N874A (open diamond). The time course with PC added instead of PS is shown for wild type (open triangle pointing down) and K873A (open square).
Figure 4.4  Apparent affinities of wild type and mutants for phosphatidylserine.

(A) Following phosphorylation of phosphatidylcholine (PC) reconstituted enzyme at 0°C with [γ-32P]ATP in SPM containing CHAPS, phosphatidylserine (PS) dissolved in CHAPS was added at the indicated concentrations, and dephosphorylation was terminated 5 s later. 

$K_{0.5}$ values for activation by PS (apparent PS affinities) were as follows: Wild type (12 µM), K865A (11 µM), K873A (88 µM), K873E (99 µM), K873R (46 µM), N874A (31 µM).  

(B) ATPase activity of wild type and mutants in the presence of CHAPS and PC with the indicated concentrations of PS. $K_{0.5}$ values for activation by PS were as follows: Wild type (38 µM), K865A (58 µM), K873A (600 µM), K873E (490 µM), K873R (230 µM), N874A (170 µM). Refer to Appendix C4 for statistical analysis.
Figure 4.5  Sensitivity of the phosphoenzyme to ADP.

Following phosphorylation with [$\gamma^{-32}$P]ATP in SPM at 0°C, enzyme reconstituted in phosphatidylcholine (PC) (circles) or 90PC:10PS (triangles) was treated with excess unlabeled ATP (filled symbols) or ADP (open symbols) for the indicated times. The amplitude of the rapid phase of the ADP-induced decay reflects $E_1\text{P}$, whereas the slow phase corresponds to $E_2\text{P}$ ($E_2\text{P}$ fraction indicated in panels). Refer to Appendix C3 for statistical analysis.
Figure 4.6   Vanadate binding determined by inhibition of phosphorylation.

Enzyme reconstituted in phosphatidylcholine (PC) or 90PC:10PS (PS) was incubated at 25°C with the indicated concentration of orthovanadate in SPM containing CHAPS and subsequently phosphorylated with $[^{32}\text{P}]{\text{ATP}}$ at 0°C. The maximal phosphorylation obtained following incubation in the absence of vanadate was taken as 100%. Refer to Appendix C3 for $K_{0.5}$ values and statistical analysis.
Using a quenched-flow module, rapid kinetics of phosphorylation at 25°C was studied with expressed ATP8A2 reconstituted in phosphatidylcholine in SPM (NaCl) or NMDG. All data points are shown. The rate constants extracted from the data are indicated. Refer to Appendix C5 for statistical analysis.
Chapter 5: P₄-ATPase-Mediated Aminophospholipid Asymmetry is Essential for Membrane Trafficking and Phototransduction in Photoreceptors

5.1 Introduction

Mutations in ATP8A2 are known to cause a severe neurological disorder characterized by cerebellar ataxia, mental retardation, and dysequilibrium syndrome (Cacciagli, et al., 2010, Emre Onat, et al., 2012, Zhu, et al., 2012). Recently, it has been shown that wabbler-lethal (wl/wl) mice harbor a mutation in the ATP8A2 which causes distal axonal degeneration in spinal motor neurons and retinal ganglion cells (Zhu, et al., 2012). Knockdown of ATP8A2 in PC12 cells indicates that lipid transport influences neurite length (Xu, et al., 2012). Mutations in other P₄-ATPases are also known to cause other severe disorders (Sebastian, et al., 2011) including the close homolog of ATP8A2, ATP8A1 which is important for the maintenance of PS asymmetry in the hippocampus and hippocampus-dependent learning (Levano, et al., 2011). The function of ATP8A1 seems to overlap with ATP8A2 since loss of both transporters results in neonatal lethality (Zhu, et al., 2012).

Exposure of PS in the outer leaflet of the PM serves as an “eat me” signal for phagocytosis and is thought to be a key event for OS turnover by the RPE (Mustafi, et al., 2011, Ruggiero, et al., 2012). In yeast, P₄-ATPases are thought to regulate vesicle budding by concentrating lipids on the cytosolic surface, inducing membrane bending (Sebastian, et al., 2011). Drs2p, an ortholog of ATP8A2 in yeast is crucial for the formation of clathrin-coated vesicles. Little is known about the role of mammalian P₄-ATPases in vesicle transport,

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4 A version of this chapter has been presented at the ARVO conference in Florida in May 2012.
however the pathophysiology of many of these disorders suggests that this may be a conserved function across evolution (Chapter 1).

The physiological role of P4-ATPases in neurons and other cells, remains largely unknown. Visual function has not been previously studied in humans and mice which carry mutations in ATP8A2. Therefore, we have chosen to study the photoreceptors of \( \text{wl/wl} \) and \( \text{ATP8A2} \) knockout mice using microscopy, electrophysiology, and other biochemical techniques to understand the importance of lipid asymmetry and transport mediated by P4-ATPases. We find that loss of ATP8A2 causes shortening of the OS and dramatically reduces visual function. We propose a dual role for ATP8A2 whereby PS and PE asymmetry and composition is critical for trafficking of proteins to the OS and regulation of membrane-associated proteins involved in phototransduction and speculate that transport of phospholipids by P4-ATPases plays a similar role in other neurons and cells.

5.2 Methods

5.2.1 Animals

Mice were raised in cyclic lighting conditions with a 12 h light-12 h dark cycle. All animal protocols were approved by either the University of British Columbia's Animal Care Committee or the Jackson Laboratory Institutional Animal Care and Use Committee and conform to either the Canadian Council on Animal Care guidelines or the Association for Assessment and Accreditation of Laboratory Animal Care guidelines. Knockout mice were produced by inGenious Targeting Laboratory (Stony Brook, New York). The neomycin resistance cassette replaced 3.86 kb of the gene including exons 11 - 13. Knockout mice were maintained in the C57BL/6 x 129/SvEv background. The \textit{wabbler-lethal} (\textit{wl/wl}) mouse in the
C57BL/6J background has been reported (Zhu, et al., 2012). Mice were genotyped using PCR.

5.2.2 Light and Electron Microscopy

For hematoxylin and eosin staining, eyes were fixed overnight in 1.22% glutaraldehyde and 0.8% paraformaldehyde in 0.08 M phosphate buffer, embedded in Technovit resin, cut in 1.5 µm sections. For electron microscopy (EM), eyes were fixed in 1% gluteraldehyde and 1% paraformaldehyde in phosphate buffer at 4°C for approximately 1 week. Retinas were treated with OsO₄ for 1 h at a concentration of 40 mM in 0.1 M cacodylate buffer (pH 7.4) containing 0.2% sucrose. Retinas were dehydrated with ethanol and embedded in Epon 812-Araldite resin. Ultrathin sections (0.07 µm) were cut and stained with uranyl acetate and lead citrate solution.

5.2.3 Electroretinograms

For electroretinograms (ERGs), a Espion Visual Electrophysiology System (Diagnosis, LLC) was used according to reported procedures (Weber, et al., 2002).

5.2.4 Polyclonal ATP8A2 Antibody

A DNA fragment corresponding to amino acids 369 – 644 of mouse ATP8A2 (NM_016529.4) was cloned in frame with glutathione S-transferase (GST) in the pGEX-4T-1 vector using the EcoRI and Xhol restriction sites. This fragment was subcloned into pMAL-c2 using EcoRI and SalI in frame with maltose binding protein (MBP). ATP8A2 antibodies were raised in rabbits immunized seven times with 500 µg of the GST-fusion protein (YenZym Antibodies, Burlingame, CA). ATP8A2 specific antibodies were purified from 50 ml of serum in PBS on a sepharose 2B column containing the CNBr coupled MBP-fusion protein and eluted with 0.1 M glycine (pH 2.5).
5.2.5 RT-PCR, Western blotting, and Immunofluorescence Microscopy

RT-PCR was performed as described (Chapter 2). Western blotting and immunofluorescence microscopy were performed according to (Chapter 2 and 3) and (Kwok, et al., 2008). Annexin-594 staining of paraformaldehyde-fixed whole-mounted retinas was performed for 1 h in 10 mM HEPES (pH 7.4), 140 mM NaCl, 2.5 mM CaCl2 (Molecular Probes, Eugene, OR).

5.2.6 Measurement of Outer Nuclear and Outer segment Layers

Eyes from wild type of knockout mice were removed, marked on the nasal side for orientation, cut into cryosections, and labeled with DAPI and the 1D4 antibody as described above. The outer nuclear (ONL) and outer segment (OS) layers were defined by DAPI and 1D4 labeling respectively. Three measurements of the ONL and OS layers were taken every 200 µm from the optic nerve and averaged. The optic nerve was defined as 0 µm. In the case of \(wl/wl\) mice, hematoxylin and eosin stained retinas were used to count the rows of photoreceptors in the ONL and measure the OS length.

5.2.7 Mouse Outer Segments and Rhodopsin Quantification

Mouse photoreceptor OS were prepared according to the Optiprep method (Tsang, et al., 1998). For rhodopsin quantification and retinoid analysis, all procedures were conducted under dim red light as described (Liang, et al., 2004). Rhodopsin concentration was determined by the decrease in absorbance at 500 nm following bleaching using a molar extinction coefficient of 40,000 M\(^{-1}\) cm\(^{-1}\). Retinoids were separated by normal phase HPLC and identified by comparison with known standards.
5.2.8 Phagocytosis Assay

The procedure for growing retinal pigment epithelium (RPE) cells on a transwell filter was performed exactly as described (Gibbs, et al., 2003). Briefly, RPE cells were isolated from 10 - 15 day old wild type mice. Eyes were digested using 2% Dispase (Sigma) and sheets of RPE cells were isolated and dispersed into growth media containing (DMEM, 10% bovine FCS, 1% penicillin/strepomycin, 2.5 mM L-glutamine, 1× MEM non-essential amino acids). All cell culture materials were obtained from Invitrogen. RPE cells were grown till confluent at 37°, 5% CO₂. Equal amounts of wild type and knockout OS were added to the RPE cells in growth media and incubated at 37°, 5% CO₂ for 60 min. RPE cells were washed 3× with growth media and incubated for an additional 60 min and then fixed in 4% paraformaldehyde. Bound OS were labeled with the rhodopsin 4D2 antibody followed by an Alexa-488 conjugated goat anti-mouse antibody. RPE cells were permeabilized with 47.5% ethanol. Internalized OS were labeled with the rhodopsin 4D2 antibody and an Alexa-594 conjugated goat anti-mouse antibody.

5.2.9 Quantification and Labeling of Lipids

Lipids were extracted according to the method of Bligh and Dyer (Bligh and Dyer, 1959), separated by thin layer chromatography (TLC) in CHCl₃:MeOH:NH₄OH (65:25:4) followed by a second dimension in CHCl₃:CH₃COOH:MeOH:H₂O (75:25:5:2.2) and quantified by measurement of the phosphorus content (Zhou and Arthur, 1992). Hypotonically lysed OS were labeled for 2 h at 23 °C in 1 ml of 100 mM Borate (pH 8.4), 2 mM 2,4,6-trinitrobenzene sulfonic acid (TNBS). In some cases, 0.1% DDM was added to solubilize the lipids. HCl was added to a concentration of 0.15 M to terminate labeling prior to extraction. TNP-labeled lipids were analyzed by high performance liquid chromatography
(HPLC) with a linear gradient from 0 – 14 min of 100% CHCl₃:MeOH:NH₄OH (80:19.5:0.5) to 100% CHCl₃:MeOH:NH₄OH (60:34:5.5:0.5). TNP-labeled lipids were detected at 340 nm and identified by comparison with known phospholipid standards.

5.3 Results

5.3.1 Morphology of the \textit{wl/wl} Retina

During the generation of \textit{ATP8A2} knockout mice, it was discovered that \textit{wl/wl} mice carry a mutation in the \textit{ATP8A2} gene which causes axonal degeneration (Zhu, et al., 2012). Photoreceptor structure and function had not been studied previously in these mice. Hence, we examined the structure of the \textit{wl/wl} retina using light microscopy to access the importance of ATP8A2 in the mammalian retina. ATP8A2 is expressed in mice as early as 14 days. At 14 days, the \textit{wl/wl} retina was indistinguishable from that of the wild type. The ONL thickness and OS length was comparable to wild type (Fig. 5.1A, Appendix D1). At 30 days of age, the OS length was one-half that of wild type (11.7 ± 0.3 \textit{vs.} 20.4 ± 0.4 µm) and the number of photoreceptor nuclei was reduced by approximately 10%. At 60 days of age, the OS was one-third of wild-type (7 ± 0.3 \textit{vs.} 21.5 ± 0.4 µm) and approximately 30 – 40% of photoreceptor cells had been lost.

Next we studied the ultrastructure of the retina using electron microscopy (EM) at 60 days of age (Fig. 5.1B, Appendix D2). Despite the shorter length of the OS, the structure of the OS appeared remarkably normal. Discs and the PM were well-organized and disc membrane stacks appeared to be correctly aligned. At higher magnification, the connecting cilia did not differ substantially from wild type (Appendix D2).
5.3.2 Generation of \textit{ATP8A2} Mice

Knockout mice were generated by replacing exons 11 - 13 with a \textit{neo} cassette (Fig. 5.2A). Exons 11 – 13 encode transmembrane segment M4 and part of the P-domain and are expected to be crucial for structure and function of the transporter. Knockout mice were identified by PCR genotyping from their wild type and heterozygous littermates (Fig. 5.2B). \textit{ATP8A2} gene expression was not detected by RT-PCR in the retinas of knockout mice (Fig. 5.2C). Western blotting using monoclonal and polyclonal antibodies to ATP8A2 confirmed that ATP8A2 was not present in the retina (Figure 5.2F). The expression level of the β-subunit, CDC50A was also lower. ATP8A2 immunoreactivity in the OS was not detected by immunofluorescence microscopy (Figure 5.2G) nor was CDC50A present in significant amounts in the OS. No substantial differences in CDC50A labeling were observed in the rest of the retina.

Compared to wild type and heterozygous animals, knockout mice were noticeably smaller (Fig. 5.2D), exhibited labored movements, and clasping of hind legs when held by the tail (Fig. 5.2E). The overall phenotype is largely similar to that of \textit{wl/wl} mice and suggests that distal axonal degeneration of the spinal cord also occurs in knockout mice. The mean survival of \textit{ATP8A2} mice was between 1 – 2 months, similar to that of the \textit{wl/wl} mice.

Next we compared the retinal structure of the knockout mouse to that of the wild type and \textit{wl/wl} mouse. ONL thickness at 23 days of age was reduced approximately 15% near the optic nerve (Appendix D3). OS length near the optic nerve was reduced by 60% (7.7 ± 0.6 vs. 18.3 ± 0.9 µm) (Appendix D3). Morphological changes were uniform across the retina and did not differ at 50 days of age. Photoreceptor cell death was detected at 23 days of age using the terminal deoxynucleotidyl transferase dUTP nick end labeling assay but not at 50
days of age. EM showed that structure and morphology is similar to the \(wl/wl\) retina (Appendix D4).

5.3.3 Visual Function of Photoreceptors

To characterize photoreceptor activities, dark and light adapted \(ATP8A2\) mutants were studied using an ERG. In the ERG, the initial negative a-wave reflects photoreceptor activity. Scotopic vision under dim light conditions represents rod function. Photopic vision under light-adapted conditions where rods are light-desensitized reflects cone activity. Large differences were observed in the maximal amplitude of the a-waves for both the knockout and \(wl/wl\) mice under scotopic conditions (Fig. 5.3A). Similar differences were observed for cone vision (Fig. 5.3B). Lower a-wave amplitudes also evoked smaller b-wave amplitudes, representing the function of secondary retinal neurons. The a- and b-wave amplitudes of \(ATP8A2\) mutants were quantified and found to be 4-fold lower than that of wild type and heterozygous mice (Fig. 5.3C,D).

5.3.4 Localization of Outer Segment and Other Photoreceptor Proteins

To study the localization of proteins in the OS, we labeled retinal cryosections with antibodies to various OS proteins (Fig. 5.4). Rhodopsin and CNGA1 were present in the OS of rod photoreceptors. Rhodopsin comprises approximately 80% of the total protein content of OS discs and localizes to both the disc and PMs of photoreceptors (Molday and Molday, 1987, Nir and Papermaster, 1983, Papermaster and Dreyer, 1974). CNGA1 localizes exclusively to the PM of rod photoreceptors (Cook, et al., 1989). Peripherin which localizes exclusively to the rim region of the discs (Molday, et al., 1987) of rods and cones was also correctly localized in the knockout. Cone arrestin labeling showed that like rods, cone OS are also shorter. Similar results were obtained in the \(wl/wl\) retina and by labeling with antibodies.
specific to GC1, ABCA4, transducin, and cone opsin. In the \textit{wl/wl} retina, ATP8A2 appeared to be mislocalized in the IS. No significant mislocalization of any other OS-resident protein was observed. Furthermore, the localization of various IS and synaptic proteins such as GM130 (Golgi), syntaxin 3 (PM), Na\textsuperscript{+},K\textsuperscript{+}-ATPase (PM), Rab11 (endosome), RP1 (cilium), and synaptotagmin (synapse) were found to be similar to that of wild type.

5.3.5 Phagocytosis of Outer Segments

To determine if reduction in OS length is due to increased phagocytosis by the RPE cells, we compared the levels of OS phagocytosis 1 h after the onset of light by toluidine blue staining and light microscopy (Appendix D5). OS phagocytosis peaks approximately 30 min – 1 h after the onset of light and the phagosomes can be stained intensely with toluidine blue (LaVail, 1976). No significant differences in phagocytosis were observed between wild type and knockout mice at 1 and 6 hrs after the onset of light (Appendix D5). However, it was difficult to unambiguously identify phagosomes due to the presence of pigmented melanosomes in the RPE. Therefore, we measured OS phagocytosis in primary cultures of RPE cells isolated from wild type mice by an established assay (Gibbs, et al., 2003). No evidence for an increase in OS phagocytosis of the knockout was found (Appendix D5). Phagocytosis of knockout OS \textit{in vitro} occurred at lower levels than that of wild type.

5.3.6 Analysis of Outer Segments by SDS-PAGE and Spectrophotometry

The abundance of OS proteins in Optiprep preparations (Tsang, et al., 1998) was studied by SDS-PAGE (Fig. 5.5). Coomassie blue stained gels showed only minor proteomic differences between equal amounts of OS isolated from wild type, heterozygous, and knockout mice (Fig. 5.5A). Western blotting with antibodies against ABCA4, GC1, CNGA1, peripherin, and opsin showed that these proteins were present at similar levels in the
knockout mice (Fig. 5.5B). Similar results were obtained with antibodies to transducin and arrestin. The relative amount of opsin was also compared per retina and was found to be reduced by 50% in the knockout (Fig. 5.5C).

Rhodopsin levels can be measured by difference spectrophotometry before and after bleaching. Detergent lysates from knockout mice contained approximately 4-fold lower levels of rhodopsin compared to wild type (Fig. 5.5D). Rhodopsin can also be determined by retinoid analysis since most of 11-cis-retinal is bound to opsin in the eye. A similar result was obtained by retinoid analysis, confirming the lower abundance of rhodopsin in these mice. A representative chromatogram is shown in Fig. 5.5E.

5.3.7 Phospholipid Composition and Asymmetry

Phospholipid composition and asymmetry was investigated using preparations of hypotonically lysed OS membranes containing similar concentrations of opsin (Fig. 5.6A). A representative two-dimensional TLC of OS lipids is shown in Fig. 5.6B. PC, PE, and PS comprise the majority of OS membrane lipids. For wild type, similar compositions of these lipids were found as previously reported for rat, cow, and frog (Boesze-Battaglia, et al., 1994, Mason, et al., 1973, Poincelot and Abrahamson, 1970). The wild type composition of PC : PE : PS was found to be 49 ± 2 : 36 ± 2 : 15 ± 1% (Fig. 5.6C). The knockout contained significantly higher levels of PC and lower amounts of PE and PS (66 ± 4 : 25 ± 2 : 9 ± 2%).

Next, we measured the distribution of PS between the extracellular and cytosolic leaflets of the PM using fluorescent-labeled annexin V, a protein which specifically binds PS (Fig. 5.6D). Annexin V labeled a significant amount of exposed PS in the OS of whole-mounted retinas collected approximately 1 h after light onset, in agreement with the finding that PS becomes exposed at the tips of OS as a signal for RPE phagocytosis (Ruggiero, et
al.). No significant differences were observed in whole-mounted knockout retinas versus wild type (Fig. 5.6D). Furthermore, similar levels of annexin V binding were observed with isolated OS (Appendix D6).

We developed a labeling assay to measure the distribution of aminophospholipids in intracellular membranes utilizing the UV-absorbing, aminoreactive probe TNBS and HPLC. TNBS is charged and reacts only with exposed amino-groups, allowing the cytosolic surface of disc membranes to be labeled (Appendix D7). First, we isolated bovine OS discs by a ficoll procedure which yields osmotically sealed vesicles oriented with the cytosolic leaflet accessible for modification (Appendix D7) and (Clark and Molday, 1979). Discs were labeled in buffer with or without detergent. A representative chromatogram is shown in Appendix D7. TNBS labeling in detergent resulted in complete modification of PS and PE, allowing calculation of cytosol-exposed PS and PE (labeled lipid in buffer without detergent). Rapid labeling of PS and PE was observed reaching a plateau between 1 – 2 h (Appendix D7). We chose to terminate TNBS-labeling at 2 h. Under these conditions, 94 ± 4 and 83 ± 3% of PE and PS respectively were found to residue in the cytosolic leaflet of OS discs (Appendix D7). As a control, we labeled PC:PE (70:30) liposomes in which PE was randomly distributed. Under the same conditions, 52.5 ± 0.5% PE was found to reside in the outer leaflet of the liposomes.

For wild type mouse OS, 91 ± 4 of PE and 82 ± 3% of PS were found to react with TNBS (Fig. 5.6E). In the case of the knockout, lower amounts of PE and PS were found to reside in the cytosolic leaflet; with 82 ± 3 and 76 ± 3% of PE and PS reacting with TNBS.
5.4 Discussion

The present findings provide insight to the importance of P₄-ATPases for vesicular trafficking and lipid asymmetry in neurons and other cells and extend our understanding of many human disorders associated with these pumps.

First of all, the overall phenotype of ATP8A2 knockout mice mirrors that of wl/wl mice (Zhu, et al., 2012). Knockout mice are small, walk with an abnormal gait characteristic of a spinal defect and die at 1 – 2 months of age. OS lengths of wl/wl and ATP8A2 mice are approximately half of wild type. Interestingly, the photoreceptors and rest of the retina appear quite normal. Visual function measured by ERG is 4-fold lower in mutant mice. Loss of visual function may be attributed to significant amounts of nonfunctional rhodopsin, presumably due to changes in the composition and organization of OS membrane lipids.

A recent study has suggested that rhodopsin can act as a scramblase, rapidly redistributing lipids (Menon, et al., 2011). Several studies have reported that PS and PE in the OS are asymmetric (Miljanich, et al., 1981, Wu and Hubbell, 1993), while other reports have suggested a more symmetrical distribution (Hessel, et al., 2001, Menon, et al., 2011). The present findings do not support a role for rhodopsin as a scramblase and argue for an asymmetric lipid distribution. OS discs were depleted of ATP which should allow for rapid redistribution of lipids by rhodopsin. However, lipid asymmetry remained unchanged throughout the experiment. The fact that PS and PE asymmetry was only disrupted by < 10% in the knockout suggests that other mechanisms are also responsible for OS lipid asymmetry (Quazi, et al., 2012). Greater changes were observed for PS and PE composition in the knockout. PS and PE are also present in lower amounts in yeast lacking Drs2p (Pomorski, et al., 2003). Combining the lower degree of PS and PE asymmetry with a smaller contribution
of these lipids to the bilayer, the cytosolic leaflet of knockout discs contains roughly 40% lower levels of these lipids. Lack of PS exposure in the PM suggests that ATP8A2 is not present or is redundant in the PM. No evidence was found for a role of ATP8A2 in phagocytosis.

The retinas of heterozygous rhodopsin mice (Rho+/-) display many structural similarities to wl/wl and ATP8A2 mice (Liang, et al., 2004). Rhodopsin content is the main contributor to OS size due to its high abundance (Papermaster and Dreyer, 1974). Rho+/- mice have an OS length and volume 60% and 40% of wild type. Despite alterations in OS morphology, only minor differences were found by ERG for Rho+/- mice. The maximal a-wave amplitude for Rho+/- mice was similar to wild type. Hence, OS structure of Rho+/- mice is comparable to ATP8A2 mutants and OS length is not a major contributor to defects in photoreceptor function seen in ATP8A2 mutants. Rather, ATP8A2 mutants demonstrate that the environment experienced by membrane and membrane-associated proteins is crucial for proper OS function. Rhodopsin and opsin content is 40% lower in Rho+/- mice whereas ATP8A2 mutants display a reduction of 75% and 50% respectively. Rhodopsin activation is highly dependent on membrane environment. Early studies showed meta II rhodopsin is formed more readily in the presence of PE and PS (Gibson and Brown, 1991). Helix 8 of rhodopsin acts as a membrane switch adopting a helical conformation upon PS binding (Krishna, et al., 2002). Mutagenesis of helix 8 has shown that it is important for folding, 11-cis-retinal binding, and transducin activation (Natochin, et al., 2003). Therefore, phospholipid composition and organization in the OS plays a central role in phototransduction.
A lower rate of rhodopsin-trafficking in \textit{ATP8A2} mutants is likely the main factor that affects OS length and similar phenotypes are observed for other rhodopsin-trafficking proteins (Bachmann-Gagescu, et al., 2011). Depletion of ATP8A2 in PC12 cells, suggests a role for ATP8A2 in polarized vesicle transport in other neurons (Xu, et al., 2012). PS-binding proteins located on surface of transport vesicles may be important for OS targeting (Krappa, et al., 1999, Uchida, et al., 2011). Alternatively, ATP8A2 may play a direct role in vesicle trafficking. The ortholog of ATP8A2 in yeast, Drs2p is a key regulator of the vesicle budding from the trans-Golgi network (Sebastian, et al., 2011). ArfGEF binding to Drs2p stimulates flippase activity (Sebastian, et al., 2011). ArfGEFs recruit activated-Arfs and lipid flipping drives vesicle bud formation. Activated-Arfs recruit adaptors and coat proteins such as clathrin. Rhodopsin-trafficking is known to occur via an Arf4 dependent pathway (Deretic, et al., 2005). Hence, we propose that ATP8A2 is recruited to sites of vesiculation by ArfGEFs and Arf4 and drives formation of rhodopsin-transport carriers. We have been unable to detect ATP8A2 in the IS of photoreceptors due to its high abundance in the OS, yet ATP8A2 has been detected in the Golgi of cultured cells (Chapter 3) similar to the localization of Drs2p. In yeast lacking Drs2p, ribosome levels are downregulated (Sebastian, et al., 2011) presumably to compensate for defective trafficking. In \textit{ATP8A2} mutants, downregulation of rhodopsin expression would explain its lack of mislocalization and suggests that other P$_4$-ATPases such as ATP8A1 are involved in vesicle budding in the IS.
Figure 5.1  Loss of ATP8A2 reduces photoreceptor outer segment length.

(A) The morphology of the *wl/wl* retina is indistinguishable to that of wild type mice at two weeks of age (*P14*; top panels) by light microscopy. Outer segments are shorter at one (*P30*; middle panels) and two months (*P60*; bottom panels) of age. The number of photoreceptor nuclei is reduced in *wl/wl* mice at P30 and P60. *Bar*, 50 µm. (B) Ultrastructural analysis of *wl/wl* retina by electron microscopy. At P60, outer segments (*OS*) are shorter than that of the wild type. The outer segment appears otherwise structurally normal. *Bar*, 2 µm. *RPE*, retinal pigment epithelium; *OS*, outer segment; *IS*, inner segment; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer.
Figure 5.2  Disruption of the ATP8A2 gene in mice.

(A) Scheme showing the targeting strategy for the ATP8A2 gene. (B) Genotyping of mice. PCR of ear punches produced distinct products of 1190 bp and 931 bp for the wild type and knockout mice respectively. Both PCR products were present in the heterozygous mice. (C) RT-PCR analysis of wild type and knockout mice. RT-PCR was performed using primers specific to ATP8A2 and Gapdh, as a loading control (+). As a negative control, the reverse transcriptase was not added (-). (D) Comparison of wild type and knockout mice. Knockout mice (right) are easily distinguished from normal wild type (left) and heterozygous littermates at three weeks due to their runted appearance and clasing of hind limbs (E) when held by the tail. (F) ATP8A2 was detected in photoreceptor outer segment membranes using the monoclonal Atp6C11 antibody (mAb) or a polyclonal antibody (pAb). CDC50A was visualized using the Cdc50-7F4 mAb. β-actin was used as a loading control. (G) Immunofluorescence labeling of cryosections of wild type and knockout retinas using the pAb to ATP8A2 (green) and the CDC50A mAb (red). Nuclei were labeled with 4′,6-diamidino-2-phenylindole (blue). The merged image shows co-localization (yellow). OS, outer segments; IS, inner segments; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Bar, 25 μm.
Figure 5.3  Visual function.

(A) Representative traces of rod (scotopic) and (B) cone (photopic) electroretinogram recordings at one month of age for wild type or heterozygous (black line) and wl/wl (blue line). (C) Quantification of scotopic a-wave and (D) b-wave amplitudes for wild type or heterozygous (n = 9), knockout (n = 6), and wl/wl mice (n = 4). The a- and b-waves are markedly smaller in the mutant mice, approximately 4-fold. Asterisks indicate a statistically significant difference (p-value < 0.05) between wild type and the knockout or wl/wl mice. Error values represent standard error.
Figure 5.4  Trafficking of outer segment proteins.

Outer segment resident-proteins appear to localize correctly in both rod and cone photoreceptors despite the shorter length of the compartment at 23 days of age. Immunolabeling of antibodies (green) specific to rhodopsin, cyclic nucleotide-gated channel alpha 1 (CNGA1), peripherin, and cone arrestin. Nuclei were labeled with 4',6-diamidino-2-phenylindole (blue). OS, outer segments; IS, inner segments; ONL, outer nuclear layer. Bar, 20 μm.
Figure 5.5  SDS-PAGE analysis of photoreceptor outer segment membranes.

(A) Outer segments from wild type (wt), heterozygous (het), and knockout (ko) mice were subjected to SDS-PAGE and stained by Coomassie blue. (B) Western blot analysis (middle) was performed using antibodies specific to outer segment proteins: ABCA4, guanylate cyclase (GC1), cyclic nucleotide-gated channel alpha 1 (CNGA1), and peripherin. Opsin was used as a loading control. (C) Opsin in detergent solubilized lysates was detected by western blot. The relative quantification of opsin per retina is shown (wild type, n = 6; knockout, n = 4). Asterisks indicate a statistically significant difference (p-value < 0.05). Insert, representative western blot of opsin. Opsin levels are reduced 2-fold. (D) Difference spectrum calculated from spectra measured before and after rhodopsin bleaching (wild type, n = 8; knockout, n = 6). (E) Chromatographic separation of retinoids by normal phase high performance liquid chromatography. A representative chromatogram is shown. 1 and 1' represent syn- and anti-11-cis retinal oximes and 2 and 2' represent the syn- and anti-all-trans retinal oximes. Quantification of 11-cis-retinal is shown (wild type, n = 10; knockout, n = 6). Rhodopsin and retinoid levels are reduced 4-fold. Error values represent standard error.
Figure 5.6  Phospholipid organization and composition.

(A) Isolated outer segment discs analyzed by SDS-PAGE. (B) Representative thin layer chromatography of outer segment lipids stained with I₂. (C) Lower phosphatidylethanolamine (PE) and phosphatidylserine (PS) levels were detected in knockout outer segments by phosphate analysis (wild type, n = 13; knockout, n = 5). (D) No difference in the plasma membrane organization of PS was detected by annexin V labeling, 1 hour after the onset of light. Bar, 10 μm. (E) Lower amounts of PE and PS are found in the cytosolic leaflet by trinitrobenzene sulfonic acid (TNBS) labeling (wild type, n = 11; knockout, n = 6). Asterisks indicate a statistically significant difference (two astericks, p-value < 0.05; one asterick, p-value < 0.1) between the wild type and knockout mice. Error values represent standard error.
Chapter 6: Summary and Future Directions

P₄-ATPases comprise a relatively new subfamily of P-type ATPases implicated in the energy-dependent translocation of aminophospholipids across cell membranes. Chapter 2 investigated the localization and functional properties of ATP8A2, a member of the P₄-ATPase subfamily that had not been studied previously. RT-PCR revealed high expression of \textit{ATP8A2} mRNA in the retina and testis. Within the retina, immunofluorescence microscopy and subcellular fractionation studies localized ATP8A2 to OS disc membranes of rod and cone photoreceptor cells. ATP8A2 purified from photoreceptor OS by immunoaffinity chromatography exhibited ATPase activity that was stimulated by PS and to a lesser degree PE but not by PC or other membrane lipids. Purified ATP8A2 was reconstituted into liposomes containing fluorescent-labeled phosphatidylserine to measure the ability of ATP8A2 to flip PS across the lipid bilayer. Fluorescence measurements showed that ATP8A2 flipped fluorescent-labeled PS from the inner leaflet of liposomes (equivalent to the exocyttoplasmic leaflet of cell membranes) to the outer leaflet (equivalent to cytoplasmic leaflet) in an ATP-dependent manner. These studies and a recent study of the yeast P₄-ATPase Drs2p (Zhou and Graham, 2009) provide the first direct biochemical evidence that purified P₄-ATPases can translocate aminophospholipids across membranes and further implicates ATP8A2 in the generation and maintenance of PS asymmetry in photoreceptor disc membranes.

Some P₄-ATPases are known to associate with members of the CDC50 protein family. In chapter 2, it was shown that ATP8A2 purified from photoreceptor membranes and reconstituted into liposomes catalyzes the active transport of PS across membranes. However, it was unclear whether ATP8A2 functioned alone or as a complex with a CDC50
protein. In chapter 3, mass spectrometry and western blotting using newly generated anti-
CDC50A antibodies demonstrated that CDC50A is associated with ATP8A2 purified from
photoreceptor membranes. ATP8A2 expressed in HEK293T cells assembles with
endogenous or expressed CDC50A, but not CDC50B, to generate a heteromeric complex that
actively transports PS and to a lesser extent PE across membranes. Chimera CDC50 proteins
in which various domains of CDC50B were replaced with the corresponding domains of
CDC50A were used to identify domains important in the formation of a functional ATP8A2-
CDC50 complex. These studies indicate that both the transmembrane and exocytoplasmic
domains of CDC50A are required to generate a functionally active complex. The N-terminal
cytoplasmic domain of CDC50A appears to play a direct role in the reaction cycle.

Mutagenesis studies further indicate that the N-linked oligosaccharide chains of CDC50A are
required for stable expression of an active ATP8A2-CDC50A lipid transport complex.
Together, these studies indicate that CDC50A is the β-subunit of ATP8A2 and is crucial for
the correct folding, stable expression, export from ER, and PS flippase activity of ATP8A2.

In chapter 4, the mechanism of lipid transport was examined. It had long been an
enigma whether the mechanism of P₄-ATPases resembles that of the well-characterized
cation-transporting P-type ATPases, and it was unknown whether flippases interacted
directly with lipid and with counterions. This study demonstrates that ATP8A2 forms a
phosphoenzyme intermediate at the conserved aspartate (Asp⁴¹⁶) in the P-type ATPase
signature sequence and exists in E₁P and E₂P forms similar to the archetypical P-type
ATPases. Using the properties of the phosphoenzyme, the partial reaction steps of the
transport cycle were analyzed, and the roles of conserved residues Asp¹⁹⁶, Glu¹⁹⁸, Lys⁸⁷³, and
Asn⁸⁷⁴ in the transport mechanism were elucidated. The former two residues in the A-domain
T/D-G-E-S/T motif are involved in catalysis of $E_2P$ dephosphorylation, the glutamate being essential. Transported aminophospholipids activate the dephosphorylation similar to $K^+$ activation of dephosphorylation in Na$^+$/K$^+$-ATPase. Lys$^{873}$ mutants (particularly K873A and K873E) display a markedly reduced sensitivity to aminophospholipids. Hence, Lys$^{873}$, located in transmembrane segment M5 at a “hot spot” for cation binding in Ca$^{2+}$-ATPase and Na$^+$/K$^+$-ATPase, appears to participate directly in aminophospholipid binding or to mediate a crucial interaction within the ATP8A2-CDC50 complex. By contrast, Lys$^{865}$ is unimportant for aminophospholipid sensitivity. Binding of Na$^+$, H$^+$, K$^+$, Cl$^-$, or Ca$^{2+}$ to the $E_1$ form as a counterion is not required for activation of phosphorylation from ATP. Therefore, phospholipids could be the only substrate transported by ATP8A2.

Finally, in chapter 5 the phenotype of ATP8A2 mutant mice was studied. The physiological and cellular role of these transporters in mammalian cells was largely unclear despite compounding evidence that many of these pumps including ATP8A2 are mutated in severe human disorders. Since the consequence of loss of ATP8A2 function in photoreceptors remains unknown, the retinas of ATP8A2 knockout mice and a naturally-occurring ATP8A2-mutant mouse were studied as an essential step in defining the function of ATP8A2 in photoreceptor and neuronal biology. The results demonstrate that ATP8A2 is crucial for the maintenance of aminophospholipid composition and topology in discs of the OS which is important for the regulation of membrane-associated enzymatic activities and consequently phototransduction. ATP8A2 appears to be largely unimportant for OS phagocytosis and plasma membrane asymmetry. ATP8A2 mutant mice develop short rod and cone OS suggesting a role for ATP8A2 in vesicle trafficking in photoreceptors and other
neuronal cells. These studies are the first to identify a physiological and cellular function for P₄-ATPase-mediated lipid asymmetry in neuronal cells.

Recent biochemical studies now directly support the function of P₄-ATPases as flippases which actively transport aminophospholipids from the exocytoplasmic to the cytosolic leaflet of cell membranes. The contributions of CDC50 β-subunits on modulation of the lipid transport activity of P₄-ATPases, protein stability, and cellular localization are becoming more well-defined. Mutagenesis studies in conjunction with improved protein expression systems and molecular modeling based on other P-type ATPases have identified a number of key residues important in the phospholipid translocation pathway and plausible steps in the catalytic transport cycle.

Many fundamental issues remain to be resolved. Recent studies have identified key residues which form a potential phospholipid translocation pathway (Chapter 4) and (Baldridge and Graham, 2012). In the yeast PC transporter Dnf1, substitution of Phe for Tyr located at the cytosolic membrane interface of M4 alters the specificity of the transporter from PC to PS. Mutation of the equivalent Tyr for Phe in the PS transporter Drs2, essentially eliminates transport. Furthermore, substitution of a Phe in the extracellular loop connecting M3 and M4 reduces PC transport through Dnf1 (Baldridge and Graham, 2012). Based on this work, it has been proposed that phospholipids are translocated through a non-classical pathway located along the groove of M1, M3, and M4 or alternatively M3, M4, and M5. In ATP8A2, mutation of a lysine located in the middle of M5 produces an enzyme which is essentially devoid of PS transport displaying a large reduction in PS affinity relative to wild type and suggesting that lipids are transported through a classical cation pathway (Chapter 4). A recent study based on the Ca²⁺-ATPase structure has compared the two proposed
translocation pathways (Thogersen and Nissen, 2012). However, it is unclear if the pathways are compatible. Further studies are necessary to determine if these proposed pathways are compatible and what additional residues are necessary for phospholipid specificity and transport.

Recent work in our laboratory has produced a molecular model of ATP8A2 using the Na\(^+\),K\(^+\)-ATPase as a template (Fig. 6.1A) (Shinoda, et al., 2009). In this model, Lys\(^{873}\) appears to stabilize transmembrane helix M4 or M6 (Fig. 6.1B). Recent mutagenesis studies have identified a series of important residues in M1 and M4 for PS interaction (Vestergaard, A.L., unpublished data). As shown in Fig. 6.1B, these residues appear to line along the same surface of M4 and may represent a translocation pathway. Mutants of Ile\(^{364}\) and Asn\(^{359}\) dramatically alter PS interactions. Leu\(^{367}\) mutants transform substrate specificity, allowing ATP8A2 to more readily transport PE. Arg\(^{82}\) located in M1 may stabilize the carbonyl of PS. Ile\(^{364}\) aligns with a glutamic acid in other P-type ATPases that is known to be important for cation transport. Interestingly, many charged residues in the M-domain of other P-type ATPases are non-polar or hydrophobic residues in P\(_4\)-ATPases. It is thought that these residues represent an adaptation to accommodate lipids. It would be of interest to study the behavior of additional mutants of non-polar or hydrophobic residues in ATP8A2 which correspond to hydrophilic or polar residues in other P-type ATPases. Molecular modeling has also predicted additional residues which may be important for PS recognition. Further modeling, mutagenesis, and biochemical studies are ongoing to understand the role of these residues and Lys\(^{873}\) as well as determine if the translocation pathway is classical (Chapter 4) or a non-classical pathway (Baldridge and Graham, 2012).
Although sequence analysis suggests that all 14 members of the mammalian P₄-ATPase family may function as phospholipid flippases, to date only one member, ATP8A2, has been purified and shown experimentally to actively flip aminophospholipids and several other members have been implicated in aminophospholipid transport on the basis of genetic and cellular studies. A future challenge is to isolate and characterize other P₄-ATPases to more fully understand their phospholipid substrate specificity, define interactions with CDC50 subunits and other cellular proteins, determine the in vivo tissue distribution and subcellular localization, and elucidate regulatory mechanisms. Additionally, the role of various P₄-ATPases in such cellular processes as vesicle trafficking, bile secretion, fertilization, lipid asymmetry, phagocytosis and membrane protein structure and function needs further study through the generation and characterization of transgenic and knockout animal models, analysis of protein-protein interactions, and functional studies. Although many of the structural properties of P₄-ATPases appear to resemble other P-type ATPases, it will be important to determine the high resolution structure of a P₄-ATPase to more fully define phospholipid recognition and transport mechanisms.

The next decade should witness significant advances in our understanding of phospholipid transport by P₄-ATPases at a molecular and cellular level. This knowledge should provide increased insight into the role of these transporters in physiological processes and facilitate the search for therapeutic treatments for diseases linked to defects in phospholipid transport.
Figure 6.1  Molecular modeling of ATP8A2.

(A) ATP8A2 was modeled using the Na⁺,K⁺-ATPase E₂P (2ZXE) structure as a template. (B) The membrane domain of ATP8A2 viewed from the extracellular surface. Key residues identified in mutagenesis studies are shown in stick representation (carbons, grey; nitrogens, blue; oxygens, red; hydrogens, white). Modeling courtesy of Stepan Lenevich.
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Appendices

Appendix A

A.1 Supplemental Methods

Limited Proteolysis of ATP8A2—Hypotonically lysed ROS were prepared as described (Bungert, et al., 2001). The ROS were resuspended at 4 mg/ml in buffer (20 mM Tris-HCl pH 8, 150 mM NaCl, 0.5 mM EDTA, 10% sucrose). Hypotonically lysed ROS or reconstituted ATP8A2 were then mixed 1:1 with trypsin in the same buffer (final trypsin concentration 4 µg/ml) and incubated on ice for the indicated time. To stop digestion, soybean trypsin inhibitor was added (final concentration 1.67 mg/ml) in buffer E and the samples were loaded onto an SDS-PAGE gel.

A.2 Specificity of Atp6C11 and Atp2F6 Antibodies

Western blot analysis of different sucrose gradient fractions from bovine retina.

Approximately, 30 µg of protein from rod outer segments (ROS), retina membranes (Retina), and soluble retina proteins (Soluble) were labelled with Atp6C11 and Atp2F6. The Atp6C11 antibody non-specifically labels a 75 kDa soluble retina protein which is not present in ROS.
and retina membranes and is therefore this antibody is not suitable for immunocytochemical studies.

**A.3 Effect of Inhibitors, Nucleotides, and pH on ATP8A2**

![Graph A](image1)

Effect of different nucleotides, inhibitors, and pH on ATP8A2 activity. (A) The ATPase inhibitors used were NEM, N-ethylmaleimide at 1 mM; Vanadate at 0.1 mM; Ca\(^{2+}\), calcium at 0.5 mM; Ouabain at 0.2 mM, and Azide at 1 mM. Inhibitors were incubated with purified protein for 30 min prior to assay. (B) The pH dependence of ATPase activation in PS (open circles, solid line) and PE (closed circles, dotted line).

**A.4 ATPase Activity of Reconstituted ATP8A2**

![Graph B](image2)

The effect of PS concentration on the ATPase activity of ATP8A2 reconstituted into liposomes. (A) Purified ATP8A2 was reconstituted into liposomes by detergent dialysis in
the presence of increasing mole % of PS in the presence of PC. In one sample (+CHAPS),
the reconstituted ATP8A2 was solubilized in CHAPS detergent (10 mM) for comparison
with unsolubilized liposomes. The ATPase activity for reconstituted ATP8A2 in the
presence and absence of CHAPS is similar. (B) The ATPase activity of reconstituted
ATP8A2 at 5 mM ATP prepared in the presence of zero PS (0% PS) and 2.5% \textit{C6-NBD-PS},
\textit{C12-NBD-PS}, and \textit{DOPS}.

A.5 Orientation of Reconstituted ATP8A2

Orientation of ATP8A2 in PC liposomes as measured by trypsin accessibility. Rod outer
segments (ROS) and reconstituted liposomes containing ATP8A2 were digested with trypsin
(final concentration of 4 µg/ml) for the indicated times. (A) ROS (30 µg) and (B)
Reconstituted ATP8A2 (100 ng) labelled with Atp6C11 and Atp2F6 antibodies. The
Atp6C11 epitope was rapidly degraded in ROS, but not reconstituted vesicles indicating that
as much as 30% of the epitope of ATP8A2 in reconstituted vesicles was oriented toward the
inside of vesicles.
Appendix B

B.1 Primer Sets used for RT-PCR Gene Expression Analysis

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**probable phospholipid-transporting ATPase IB [Bos taurus]**

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Enzyme : Trypsin
Fixed modifications : Carbamidomethyl (C)
Variable modifications : Oxidation (M)
Mass values : Monoisotopic
Protein Mass : Unrestricted
Peptide Mass Tolerance : ± 1.2 Da
Fragment Mass Tolerance : ± 0.6 Da
Max Missed Cleavages : 1
| Instrument type | : | ESI-QUAD-TOF |
| Number of queries | : | 588 |
B.3 Gene Expression of *CDC50* in HEK293T Cells

Gene expression of each *cdc50* family member in cultured cells by RT-PCR. Only *Cde50a* gene expression is detectable in HEK293T cells.

B.4 Fluorescence Traces of ATP8A2 Flippase Experiments

Lipid flippase or transport activity of the purified and reconstituted ATP8A2-CDC50A complex. ATP8A2-CDC50A complex was purified using the double immunoaffinity purification procedure and reconstituted into phosphatidylcholine vesicles. Arrows indicate loss of NBD-lipid fluorescence in the presence of dithionite (*Dithionite*) and solubilization of vesicles by detergent (*Triton*). (A) Fluorescence trace of NBD-labelled PS in reconstituted ATP8A2-CDC50A vesicles in the presence AMP-PNP (*solid line*) and ATP (*dotted line*). The difference between the ATP and AMP-PNP traces reflects the percentage of lipid transport. (B) Fluorescence trace of NBD-labelled PS in empty liposomes in the presence
AMP-PNP (*solid line*) and ATP (*dotted line*). No difference is observed between the two traces.

**B.5 Immunoprecipitation of Membrane Domain Chimeras**

Immunoprecipitations of CDC50A and M1, M2, and M1/M2 CDC50A 1D4 tagged chimeras with ATP8A2 expressed in HEK293T cells. Solubilized proteins (*Input*) were purified on a ATP6C11 column and eluted from the column using a peptide (*Bound*). ATP8A2 and CDC50A were detected by Coomassie blue staining or western blot using Atp6C11 and Rho-1D4 antibodies respectively. The M1, M2, and M1/M2 chimeras do not immunoprecipitate with ATP8A2.
Expression and localization of ATP8A2 and CDC50A in Cos-7 cells. Immunolabeling of 1D4 tagged ATP8A2 and Myc tagged CDC50A was carried out with the Rho-1D4 monoclonal and Myc polyclonal antibody, respectively. ATP8A2 and CDC50A (green), when singly expressed, colocalized with calnexin (red) to the ER. Coexpression of ATP8A2 and CDC50A resulted in the colocalization of ATP8A2 (red) and CDC50A (green) to the Golgi as shown using GM130 Golgi marker and Calnexin ER marker as revealed in the Merge image. Nuclei (blue) were labelled with 4′,6-diamidino-2-phenylindole. Bar, 20 µm.
B.7 Functional Activity of Glycosylation Mutations

(A) ATPase activity of the purified ATP8A2 in complex with various CDC50A glycosylation mutants expressed in HEK293T cells in the presence of phosphatidylserine (PS), phosphatidylethanolamine (PE), and phosphatidylcholine (PC). (B) Flippase activity of wild type and T109A mutant reconstituted in the presence of NBD-PS.
Appendix C

C.1 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 $\alpha_1$) 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 $\mu$M (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 [$\gamma$-$^{32}$P]ATP concentrations. All data points are shown. The $K_{0.5}$ values extracted from the data are indicated. Refer to Appendix C3 for statistical analysis. (C) Apparent affinity of D196T for PS. The protocols were the same as used for Fig. 4.4. (Left) Following phosphorylation of PC reconstituted D196T at 0°C with [$\gamma$-$^{32}$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 Appendix C4 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. 4.4., but with varying PE instead of PS. (Left) Following phosphorylation of PC reconstituted wild type and K873A at 0°C with [γ-32P]ATP in SPM containing CHAPS, PE dissolved in CHAPS was added at the indicated concentrations, and dephosphorylation was terminated 5 s later. (Right and Bottom) 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 Appendix C4 for statistical analysis.
C.2 Supplemental Data Supporting Figs. 4.2 and 4.3

(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. 4.2B and Appendix C4 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. 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. 4.5), $E_2P$ 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$_i$ (Karlish, et al., 1982). (C) Phosphorylation of native ATP8A2 purified from photoreceptor outer segments. Equal amounts of native ATP8A2 (purified as described in Chapter 2) reconstituted in PC or 90PC:10PS (PS) were phosphorylated at 0°C by $[\gamma$-$^{32}$P]ATP using the same protocol as for Fig. 4.3A.
### C.3 Statistical Analysis of Data in Figs. 4.2, 4.5, 4.6, Appendix C1, C2

<table>
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<tr>
<th>ATP8A2 protein</th>
<th>NBD-PS transport* (% NBD-PS/µg)</th>
<th>ATPase activity† (µmol/min/mg)</th>
<th>$K_{0.5}$(ATP)‡ (µM)</th>
<th>$E_2P$ %§</th>
<th>$K_{0.5}$(vanadate) (µM)¶</th>
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</thead>
<tbody>
<tr>
<td>Wild type</td>
<td>29 ± 1 n = 14</td>
<td>35.4 ± 0.8 n = 30</td>
<td>0.12 ± 0.01 n = 22</td>
<td>60 ± 7 n = 48</td>
<td>n.d.</td>
<td>4.8 ± 0.6 n = 56 n.d. 0.024 ± 0.003 n = 40</td>
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<tr>
<td>D196T</td>
<td>12 ± 3 n = 8</td>
<td>10.4 ± 0.1 n = 15</td>
<td>0.10 ± 0.01 n = 22</td>
<td>75 ± 8 n = 27</td>
<td>n.d.</td>
<td>11 ± 1 n = 1 n.d.</td>
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<td>E198Q</td>
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<td>0.07 ± 0.01 n = 22</td>
<td>76 ± 10 n = 30</td>
<td>≈100</td>
<td>34 ± 10 n = 32 0.50 ± 0.06 n = 32</td>
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<td>K873A</td>
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<td>39 ± 8 n = 18 ≈370 n = 36</td>
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Standard errors and number of data points n, on which the analysis is based, are indicated. n.d., not determined.

*Data corresponding to Fig. 4.2B. 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 Figure 4.2C,D.

‡$K_{0.5}$ values for ATP concentration dependence of phosphorylation were obtained by fitting a single site hyperbolic function to the data in Appendix C1.

§A biexponential decay function was fitted to the data in Fig. 4.5, 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 $EP = E_{P\text{max}} \cdot (1 – [\text{vanadate}]^h/(K_{0.5}^h + [\text{vanadate}]^h))$ to the data in Fig. 4.6, with Hill number $h$ ranging between 0.5 and 1.2.
### C.4 Phosphorylation Stoichiometry and Statistical Analysis of Data in Fig. 4.4 and Appendix C1

<table>
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<tr>
<th>ATP8A2</th>
<th>Phosphorylation stoichiometry (mol (^{32}\text{P}) incorporated/mol protein)*</th>
<th>(K_{0.5}) for activation of dephosphorylation by PS (µM)</th>
<th>(K_{0.5}) for activation of ATP hydrolysis by PS (µM)</th>
<th>(K_{0.5}) for activation of dephosphorylation by PE (µM)</th>
<th>(K_{0.5}) for activation of ATP hydrolysis by PE (µM)</th>
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<tr>
<td>Wild Type</td>
<td>0.20 ± 0.01 n = 25</td>
<td>12 ± 2 n = 20</td>
<td>38 ± 3 n = 160</td>
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<td>E198Q</td>
<td>0.48 ± 0.02 n = 3</td>
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<td>600 ± 100 n = 53</td>
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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. 4.4A).
*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. 4.3C controls in PC without PS added. The reason for the relatively low value for K873E is presently not known.

†n.r., $K_{0.5}$ not relevant, because activation was not statistically significant, see Appendix C1.

‡n.d., $K_{0.5}$ not determined, experiment not performed.
C.5 Statistical Analysis of Data in Fig. 4.7, Appendix C6, C7.

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<th>Rate constant (s(^{-1}))</th>
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<td>8.2 ± 0.9 n = 27</td>
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<td>Wild type ATP8A2 reconstituted in PC, phosphorylation in NMDG, pH 7.5 (Fig. 4.7)</td>
<td>12.4 ± 0.9 n = 54</td>
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<td>Wild type ATP8A2 reconstituted in PC, phosphorylation in NaCl, pH 6.5 (Appendix C6)</td>
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<td>Wild type ATP8A2 reconstituted in PC, phosphorylation in NaCl, pH 9.0 (Appendix C6)</td>
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<td>D196T reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Appendix C7)</td>
<td>8.7 ± 1.1 n = 27</td>
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<td>E198Q reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Appendix C7)</td>
<td>10.5 ± 1.2 n = 27</td>
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<tr>
<td>K873A reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Appendix C7)</td>
<td>8.0 ± 1.3 n = 27</td>
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<td>Native ATP8A2 reconstituted in PC, phosphorylation in NaCl, pH 7.5 (Appendix C7)</td>
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<td>E198Q reconstituted in 90PC:10PS, phosphorylation in NaCl, pH 7.5 (Appendix C7)</td>
<td>6.6 ± 0.6 n = 54</td>
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<tr>
<td>Ca(^{2+})-ATPase (SERCA1a), phosphorylation in NaCl, pH 7.5 (Appendix C7)</td>
<td>20.3 ± 1.7 n = 27</td>
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</table>

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.
C.6 Functional Dependence on Ionic Conditions

(A) Dependence on ionic conditions. ATP8A2 reconstituted in PC or 90PC:10PS was dialyzed as described under 4.2.5 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), or, when indicated by “+”, 50 mM NaCl, 50 mM KCl, 25 mM Na2SO4, 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. (Top) Using the quenched-flow module, PC reconstituted ATP8A2 was
phosphorylated at 25°C in the presence of 2 µM [γ-32P]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 Appendix C5 for statistical analysis. (Bottom) 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.
C.7 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 [$\gamma^{32}$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 Appendix C5 for statistical analysis.
Appendix D

D.1  Morphology of the *wl/wl* Retina

The number of photoreceptor nuclei in the outer nuclear layer (ONL) in wild type (*wt*) and mutant (*wl/wl*) mice was measured. At one month, (*P30*) the number of nuclei in the ONL was reduced from 10 cells per row in wild type to 9 cells per row in mutant animals. At two months (*P60*) the number of cell bodies in the ONL is reduced to 6-7 cells per row. At P30 the outer segment (OS) length was reduced approximately 2-fold from the wild type. At P60 the OS length was approximately one-third of wild type (*n = 4*). Asterisks indicate a statistically significant difference (p-value < 0.05) between the wild type and *wl/wl* mice. Error values represent standard error.

D.2  Ultrastructural Analysis of *wl/wl* Retina

At 60 days of age, the outer segments discs appear normal to that of the wild type control. The connecting cilia (CC) in the wild type (*white arrows*) and *wl/wl* photoreceptors (*black arrows*) did not differ. *Bar*, 500 nm.
D.3 Morphology of the Wild Type and Knockout Retina

(A) The thickness of the outer nuclear layer (ONL) was determined for wild type and knockout at 23 days by labeling with 4′,6-diamidino-2-phenylindole and plotted as a function of distance from the optic nerve (ON) (wild type, n = 6; knockout, n = 7). (B) The length of the photoreceptor outer segment (OS) layer was determined for the wild type and knockout at 23 days of age by labeling of opsin plotted as a function of distance from the optic nerve (wild type, n = 6; knockout, n = 7). (C) Comparison of the ONL and OS at 23 days (P23) and 50 days (P50) taken 400 µm from the optic nerve (n = 4). Photoreceptor apoptosis was detected at P23 using the terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay in knockout retinas but not at P50 (n = 4). Asterisks indicate a statistically significant difference (p-value < 0.05) between the wild type and knockout mice. Error values represent standard error.
D.4 Analysis Photoreceptor Outer Segments by Electron Microscopy

Ultrastructure analysis of knockout photoreceptor outer segments by electron microscopy. Knockout outer segments are characteristically shorter than wild type. Outer segment structure is otherwise normal. Discs and plasma membrane of the knockout also appear normal. RPE, retinal pigment epithelium; OS, outer segment. Bar, 2 µm.
Staining of retinas isolated 1 hour after light onset with toluidine blue. For (A) wild type and knockout retinas, phagosomes can be seen in retinal pigment epithelium (RPE) layer as indicated (arrows). Bar, 10 µm. (B) Quantification of phagosomes 1 hour and 6 hours after light onset in vivo. There is no significant difference in phagocytosis (n = 3). (C) Outer segments were added to polarized RPE primary cultures. Bound (green) and internalized (red) outer segments were visualized using the rhodopsin 4D2 antibody. Nuclei (blue) stained with DAPI. For negative control, no outer segments were added. Bar, 10 µm. (D) Quantification of bound and internalized outer segments in RPE primary cultures. Significantly fewer knockout outer segments are evident in RPE cells (n = 3). Asterisks indicate a statistically significant difference (p-value < 0.05) between the wild type and knockout mice. Error values represent standard error.
Annexin V (red) staining of isolated paraformaldehyde-fixed outer segments. Plasma membranes were visualized using the rhodopsin 4D2 antibody (green). There is no significant difference in annexin V staining of outer segments. Bar, 10 µm.
D.7 Photoreceptor Discs Display Lipid Asymmetry

(A) Chemical structure of trinitrobenzene sulfonic acid (TNBS), an amino reactive, membrane impermeable probe. (B) Isolated bovine outer segment discs analyzed by SDS-PAGE. (C) Representative high performance liquid chromatography traces of TNP-labeled lipids with and without detergent. (D) Timecourse labeling of phosphatidylethanolamine (PE) and phosphatidylserine (PS) (n = 3). (E) PS and PE are found in the cytosolic surface of outer segment discs as determined by TNBS labeling (n = 4). Error values represent standard error.