THE STUDY OF P4-ATPASE, ATP8A2, INVESTIGATING DISEASE-CAUSING MUTANTS AND METHODS FOR HIGH THROUGHPUT PRODUCTION AND PURIFICATION

by

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submitted by Hanbin Choi in partial fulfillment of the requirements for

the degree of Master of Science

in Biochemistry and Molecular Biology

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Abstract

Human diseases have been linked to genetic mutations, causing the inability of proteins to function properly. Of these proteins, membrane proteins are crucial for the function of a cell; thus, a mutation affecting these classes of proteins causes extensive harm. This study was focused on the phospholipid transporter \( P_4 \)-ATPase, ATP8A2, investigating the effects of mutations associated with human diseases. Six associated mutants have been studied: I376M, K429M, K429N, A544P, R625W, and W702R. The study allowed us to differentiate between two classes of mutants: proteins which fold in a native-like conformation allowing export from endoplasmic reticulum (ER), but lacking activity; and proteins that are highly misfolded, retained in the ER, and are rapidly degraded by proteasomes.

The well-studied \( P_2 \)-ATPases transport small ion substrates, whereas \( P_4 \)-ATPases transport much larger substrates across the plasma membrane (PM). ATP8A2 was further studied with its \( \beta \)-subunit, CDC50A, to develop a high expression system for protein structural studies. The use of 20 mM CHAPS and 0.04\% GDN was the best combination of detergents to solubilize HEK 293F cells with sonication and stirring at 4°C for 45 minutes for maximal purification. Samples analyzed under a transmission electron microscope (TEM) were monodispersed and non-aggregated; however, the same peptidisc-reconstituted sample was aggregated by cryo-electron microscopy (cryo-EM). Further studies are required to prevent aggregation during freezing of ATP8A2-CDC50A for analysis by cryo-EM.
Lay Summary

Cerebellar ataxia-intellectual disability-dysequilibrium syndrome (CAMRQ), encephalopathy, intellectual disability, psychomotor delay, chorea, and optic atrophy, classified as clinical heterogeneous disorders (Onat et al. 2013) and severe hypotonia and hyperkinetic movement disorder (McMillan et al., 2018) have been linked to genetic mutations on membrane protein ATP8A2. How these mutations affect the cells in which ATP8A2 is expressed is the question dealt in this research.

To further elucidate ATP8A2, the study of protein structure is important to determine the mechanism of transport. Such information will allow scientists to understand how to approach these mutations to better address patients who have been diagnosed with these diseases and provide a step forward toward developing a treatment for these diseases.
Preface

Chapter 1 figure 1.3 is used with permission from applicable sources.

A version of chapter 2 was submitted. [Choi, H., Anderson, J.P., and Molday, R.S. (2019) Expression and functional characterization of missense mutations in ATP8A2 linked to severe neurological disorders]. The experiments conducted in this thesis were designed through discussions between myself and my supervisor, Robert Molday. Design of oligonucleotides and preparation of materials used in the experiments were done by me except those described below. Common laboratory reagents, such as DH5α competent cells, were prepared by members of the Molday lab. ATP8A2 with 1D4 tag and CDC50A were cloned into pcDNA3 vector by Jonathan Coleman. Rho 1D4 and CDC50A-7F4 monoclonal antibodies were prepared by Theresa Hii. Expression and functional characterization of missense mutations in ATP8A2 linked to severe neurological disorders and the analysis of data were done by Robert Molday and I. I wrote the original manuscript and Robert Molday and Jens Anderson edited and contributed to discussion.

Chapter 3 describes studies on the expression and characterization of ATP8A2-CDC50A for the structural analysis, all experiments were completed by me except experimental procedures dealing with reconstitution of the ATP8A2-CDC50A in peptidisc were done by Harveer Dhupar.

I was the lead investigator for the projects in Chapter 2 and 3 and I wrote this thesis, the thesis was revised by Robert Molday and committee members.
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<tr>
<td>ABC</td>
<td>ATP-binding cassette</td>
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<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
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<tr>
<td>ATP</td>
<td>Adenosine-5′-triphosphate</td>
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<tr>
<td>BGS</td>
<td>Bovine growth serum</td>
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<tr>
<td>CAMRQ</td>
<td>Cerebellar ataxia, mental retardation, and disequilibrium syndrome</td>
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<tr>
<td>CHAPS</td>
<td>3-[(3-Cholamidopropyl)dimethylammonio]propanesulfonic acid</td>
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<tr>
<td>Chol</td>
<td>Cholestrol</td>
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<td>CNS</td>
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<td>Delbecco’s modified Eagle medium</td>
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<td>DOPC</td>
<td>1,2-dioleoyl-sn-glycero-3-phosphatidylcholine</td>
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<td>kDa</td>
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<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
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<td>PBS</td>
<td>Phosphate buffered saline</td>
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<td>PC</td>
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<td>PCR</td>
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<td>PE</td>
<td>Phosphatidylethanolamine</td>
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<tr>
<td>PM</td>
<td>Plasma membrane</td>
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<td>PS</td>
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<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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Chapter 1: Introduction

1.1 Biological cells and their membrane

Cells are what constitutes a biological organism. Biochemical processes that make up the fundamental qualities resulting in life occur within a cell. The cell membrane was first found to consist of a lipid bilayer by extracting lipids from red blood cells (Gorter & Grendel, 1925). For an ordered and localized reaction to occur, the organelles and the cell’s internal solutes are entrapped within the boundaries of a cell by a cellular membrane. To account for the observation that the membrane had a much lower surface tension than a water-lipid interface, proteins were proposed to be on either side of lipid bilayer (Danielli & Davson, 1935). Defined by the fluid mosaic model: the biological membrane is composed of amphipathic molecules, which freely move laterally within the membrane (Singer & Nicolson, 1972). The membrane consists of three classes of membrane lipids: glycerophospholipids, sphingolipids, and sterols. The glycerophospholipids consist of a polar head group linked to a hydrophobic diacylglycerol backbone by a phosphodiester linkage; the hydrophobic region is comprised of either saturated or cis-unsaturated fatty acyl chains that can vary in length. Sphingosine is the primary backbone of sphingolipids such as sphingomyelin (SM) and glycosphingolipids (GSL). Sterols are made up of four fused rings. The four major glycerophospholipids of eukaryotes are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI).

These individual lipid classes are distributed asymmetrically across all biological plasma membranes (Holthuis & Levine, 2005; van Meer, Voelker, & Feigenson, 2008). For example, PS and PE are found in the cytoplasmic leaflet and PC, SM, and GSL are found primarily in extracellular leaflet of cell membranes of eukaryotes. Other enrichment components such as phosphatidic acid (PA) and PI are present in the cytosolic leaflet (Lenoir, Williamson, & Holthuis,
Cholesterol (Chol), under the sterol family, has strong interactions with SM and can form a Chol-SM rich microdomains, known as lipid rafts, due to the mechanical strength provided by SM. This in turn reduces permeability. Many, but not all, cellular organelles and vesicles also have lipid asymmetry (Alder-Baerens, Lisman, Luong, Pomorski, & Holthuis, 2006; van Meer et al., 2008).

The lipids in the plasma membrane (PM) originate in the ER, the location where most of the lipids are synthesized. Initially, lipids are synthesized in the ER and randomly distributed by scramblases to both sides of the membrane (Buton, Morrot, Fellmann, & Seigneuret, 1996). The lipids are exported as budding vesicles with proteins synthesized in the ER. These vesicles fuse with other membranous organelles such as the Golgi. Lateral diffusion of lipids is easily achieved whereas the transverse movement of lipids is much harder to accomplish, due to their amphiphilic nature. Within the Golgi, lipids are sorted by flippases, generating lipid asymmetry. Proteins that have been exported from the ER are also sorted in the trans-Golgi network (TGN) and either remain in the Golgi or are further exported via the exocytic pathway.

1.2 P-type ATPase superfamily

The P-type ATPase family transports variety of substrates across the impermeable lipid membranes of a cell. 483 unique amino acid sequences are within in this family (Prosite). All these transporters have a signature DKTGT motif that allows the auto-phosphorylation of aspartic acid during its four-principle conformational states: $E_1$, $E_1P$, $E_2P$, and $E_2$ (P denotes phosphorylation, Kühlbrandt 2004). This family of ATPases is found in all kingdoms of life and is subdivided into five subfamilies according to their substrate specificity, sequence identity, and localization. The five subfamilies from the P-type ATPases are: $P_1$, $P_2$, $P_3$, $P_4$, and $P_3$-ATPase (Fig. 1.1). $P_1$-ATPase is subdivided to $P_1A$ and $P_1B$, a potassium pump and heavy metal pump, respectively. $P_2$-ATPase
is subdivided into $P_2A$ (calcium pump), $P_2B$, $P_2C$ (sodium/potassium and proton/potassium pumps) and $P_2D$ (sodium pumps). $P_3$-ATPase consists of $P_3A$ (proton pump) and $P_3B$ (magnesium pump). The $P_4$-ATPase are flippases involved in transportation of phospholipids. The $P_5$-ATPase is subdivided into $P_5A$ and $P_5B$, and its substrate specificity is not yet known. However, these transporters seem to be involved in regulation of homeostasis in the ER (Sørensen, Holen, Holemans, Vangheluwe, & Palmgren, 2015) and are found in the lysosomal membrane of animals (Ramirez et al., 2006). Further, $P_5$-ATPases have been linked to Parkinson’s disease (De La Hera, Corradi, Adamo, & De Tezanos Pinto, 2013; Di Fonzo et al., 2007; Ramírez-Ruiz, Junqué, Martí, Valldeoriola, & Tolosa, 2006). $P_2$-ATPases have been well studied, structurally and mechanistically. The $P_4$-ATPases are different from other P-type ATPases as they transport a much larger substrate, a phospholipid.

![Figure 1.1 Phylogenetic tree of P-type ATPases. A) The rooted tree of subfamilies of P-type ATPases. B) Phylogenetic tree with real branch length of P$_4$-ATPases.](image-url)
P₄-ATPases have four domains as part of their structure: the cytosolic A (actuator) domain, the P (phosphorylation) domain, the N (nucleotide binding) domain, and the M (membrane) domain, which consists of up to ten transmembrane segments (Palmgren & Nissen, 2011). The Ca²⁺ and Na⁺/K⁺ transporters have been structurally analyzed. Some valuable insights have been gained by using structural data to anticipate a large movement of cytosolic A, N, and P domains to drive the four-principle conformational states (Palmgren & Nissen, 2011). The transport cycle is initiated by the binding of ATP to the N-domain which facilitates the phosphorylation of an aspartic residue in the P-domain during the $E_1$ conformation. These transporters require binding of their specific molecule at their substrate site. Upon binding, a 90° rotation of the A-domain results in the insertion of the TGES motif (DGET in P₄-ATPases) into a space previously occupied by ADP; this positions the TGES near the phosphorylated aspartic residue during transition from $E_1P$ to $E_2P$ (Palmgren & Nissen, 2011). Consequently, the high affinity cytoplasmic site converts to a low affinity extracellular facing site to facilitate the exit of the ion. As the ion exits, the positively charged ion dephosphorylates the ATPase by stabilizing the transition state. Although the mechanism is specific to the Na⁺/K⁺-ATPase, it is possible that a similar mechanism of dephosphorylation may occur in other P-type ATPases (Ding et al., 2000). Binding of ions to $E_2P$ state further triggers the TGES motif to meet the P-domain to catalyze the dephosphorylation step by utilizing a proton from a water molecule to impose a nucleophilic attack on the aspartyl-phosphate (Clausen, Vilsen, McIntosh, Einholm, & Andersen, 2004). This dephosphorylation step converts the protein into $E_2$ state causing the substrate binding site to go from a high affinity to a low affinity conformation. Once the A-domain moves away from the P-domain, the P-type ATPase returns to the $E_1$ conformation. Since the direction of transport is the same between the Na⁺/K⁺-ATPase, such as ATP1A1, and P₄-ATPases, the mode of transport may be similar.
1.2.1 Functions of the P₄-ATPases and lipid asymmetry

The transport of phospholipids across the membrane bilayer to maintain a specific lipid asymmetry on each side is vital to maintain cell shape and cell physiology as well as to regulate intracellular and extracellular signaling (Bretscher, 1972; Op den Kamp, 1979). Furthermore, lipid asymmetry is also known to play roles in membrane protein regulation, membrane stability, impermeability, apoptosis, and fertilization, among others. Phospholipids are mixed in the exoplasmic and cytoplasmic leaflets by scramblases (Bratton et al., 1997). PS is a type of phospholipid, primarily located in the cytoplasmic leaflet (Daleke, 2003; Devaux, 1992). Exposure of PS on the external leaflet can induce blood coagulation, cell-cell fusion, and apoptosis (Fadeel & Xue, 2009). Additionally, PS exposure can result in an “eat-me” signal during phagocytosis (Fadok, Bratton, Guthrie, & Henson, 2001; Kagan et al., 2002). Many human diseases have been linked to lipid transporters, highlighting their importance within a cell (Folmer, van der Mark, Ho-Mok, Oude Elferink, & Paulusma, 2009; Kaminski et al., 2000; Quazi, Lenevich, & Molday, 2012).

P₄-ATPases have ten predicted transmembrane segments. The helices M1-6 are flexible and form the catalytic core of the transporter whilst the rigid M7-10 support the former and may interact with their respective β-subunit. Drs2p was the first P₄-ATPases which the function was studied, reporting deficiencies in the uptake of NBD-labeled PS (Tang, Halleck, Schlegel, & Williamson, 1996). Other studies found that knocking out Drs2p caused accumulation of abnormal membrane structures and an inability to form clathrin-coated vesicles (Chen et al. 1999; Gall et al. 2002).

The P₄-ATPases are recognized by their signature ATP binding motif DKTGT. Among mammalian P₄-ATPases, PS flipping by ATP8A1 is needed for recruitment of EHD1 to recycling endosomes (Lee et al., 2015). Depletion of ATP9A delayed transferrin recycling from endosomes
to the plasma membrane (Tanaka et al., 2016). Recently ATP9A was found to be crucial for exosome biogenesis by regulating phospholipid transport (Naik et al., 2019). Further, the P₄-ATPases have been shown to play a role in vesicle transport and budding for regulation of protein trafficking (Hua, Fatheddin, & Graham, 2002; Pomorski et al., 2003) and membrane deformation (Takada et al., 2018). Caspase cleavage of ATP11C has been shown to expose PS and trigger apoptosis (Segawa et al., 2014) and anemia (Liou, Molday, Wang, Andersen, & Molday, 2019). Similarly, ATP8A1 or CDC50A deficient cells lacked endocytosis or recycling endosomes (Kato et al., 2013).

1.2.2 ATP8A2

ATP8A2 is one of the phospholipid transporters to sustain asymmetric distribution of lipids across the cellular membrane. ATP8A2 is a P₄-ATPase under class I, member 2 of type 8A membrane proteins. It functions to transport phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the exoplasmic to cytoplasmic leaflet, thereby acting as a flippase. The well characterized P₂-ATPases, which transport ions such as calcium, potassium, and sodium, have relatable similarity in sequence and structure (Andersen et al. 2016). Therefore, platform theories were based on P₂-ATPase structure and transport mechanisms. These insights will serve as a guide in determining the structure, mechanism, regulation, and sub-unit interactions of ATP8A2.

The ATP8A2 transporter is a large 129 kDa protein consisting of five domains: the actuator domain, the transmembrane domain, the nucleotide binding domain, the phosphorylation domain (Vestergaard et al., 2014), and the regulatory domain (Chalat, Moleschi, & Molday, 2017) (Fig. 1.2). These five major domains are essential for flippase activity, but, a subsequent beta subunit, CDC50A, is also vital for ATP8A2 functionality (Coleman et al. 2012; Coleman, Kwok, and...
Molday 2009; Coleman and Molday 2011). ATP8A2 and CDC50A must interact for the flippase to fold into its tertiary structure and leave the ER. The complex is subsequently transported to the Golgi, where a small fraction is further exported to the plasma membrane.

ATP8A2 functions as a phosphatidylserine flippase in the brain, retina, and testes in human. Mutations in ATP8A2 have been linked to the cerebellar ataxia, mental retardation, and disequilibrium syndrome (CAMRQ) and optic atrophy in humans and have been shown to cause motor neuron degeneration in mice (Coleman et al. 2014; Onat et al. 2013; Zhu et al. 2012).

![Figure 1.2](image)

**Figure 1.2** Schematic of ATP8A2-CDC50A complex showing various domains and disease-causing point mutation sites. Topological model of ATP8A2, with a membrane domain containing 10 transmembrane segments, an actuator domain (A), a phosphorylation domain (P), and a nucleotide-binding domain (N), interacting with its glycosylated β-subunit, CDC50A. Locations of the six disease-causing mutations examined in this thesis are labelled.

In *C. elegans*, the P₄-ATPase TAT-1 localizes to the plasma membrane, like its orthologs Drs2p and ATP8A1. TAT-1 is required for endocytosis and for lysosome biogenesis, due to its role in PS asymmetry at the plasma membrane (Chen et al. 2010; Darland-Ransom et al. 2008; Ruaud et al. 2009). The CDC50 that interacts with TAT-1 is CHAT-1. The complex-mediated translocation of aminophospholipids may create an imbalance of phospholipids between the two
leaflets, causing membrane deformation into a highly curved structure, further driving membrane tubulation and vesicle formation (Chen et al. 2010). The importance of the PISL motif was previously shown in bovine ATP8A2 through point mutations at I364, the equivalent amino acid is I376 in humans (Vestergaards et al., 2014). Recently, the importance of the PISL and DKTGT motif in TAT-1 has been noticed (Chen et al. 2019), highlighting the need for investigation of the same motifs in human ATP8A2, especially for point mutations that have been linked to human diseases.

1.2.3 β-subunits of P4-ATPases

The P4-ATPases, apart from ATP9A and ATP9B, utilize a β-subunit belonging to the CDC50 family. This β-subunit family is composed of CDC50A, CDC50B, and CDC50C. Together, they form the CDC50/LEM3 family, which is conserved in all eukaryotes (Katoh & Katoh, 2004; Saito et al., 2004). Different P4-ATPases associate with the same CDC50 proteins to form a heterodimer complex (Bryde et al., 2010). Specific interactions of P4-ATPases have been studied by heterologous protein co-expression and co-immunoprecipitation (Coleman and Molday 2011; Naito et al. 2015; Paulusma et al. 2007; Takatsu et al. 2011, 2014; van der Velden et al. 2010). There are no clear reasons to explain multiple specificity. ATP8A2 is found to interact with CDC50A in the photoreceptors in the retina. This interaction has been further shown by immunoprecipitation studies (Coleman, Kwok, and Molday 2009; Coleman and Molday 2011).

CDC50A has two transmembrane domains connected by an extracellular domain and cytosolic N and C terminal segments. The extracellular domain contains three or more N-linked glycosylation sites which contribute to the stability of the protein (Coleman and Molday 2011; García-Sánchez et al. 2014). The structure is further supported by four conserved cysteine residues in the extracellular loop which form disulfide bridges (Puts et al., 2012).
The Na⁺/K⁺-ATPase α-subunit intertwines with its β-subunit through the M7-10 helices of the α-subunit. The extracellular domains of the β-subunit further interacts with the L7/8 loop of the α-subunit (Toyoshima, Kanai, & Cornelius, 2011). The interaction between the α- and β-subunits is necessary for correct protein folding and export from ER (Paulusma et al., 2007; Takatsu et al., 2011). The interaction of many P-type ATPases with their β-subunit has been shown to impose a significant factor in activity (Toyoshima et al., 2011). To export out of the ER, ATP8A2 and CDC50A must interact. CDC50A is retained in the ER if no P₄-ATPases are present (Coleman and Molday 2011; López-Marqués et al. 2010; Puts et al. 2012). However, further effects of the CDC50A subunit on ATP8A2 remain unknown (Coleman and Molday 2011; López-Marqués et al. 2010). Thus, subsequent studies need to be conducted to understand the full relation of CDC50A to ATP8A2.

A recent study identified specific mutations in the transmembrane domain of ATP8A2 that lead to a decrease in the glycosylation of CDC50; furthermore, this study showed that the α-subunit remained active and suggest the transmembrane helices M1, M3, and M4 of ATP8A2 may directly interact with CDC50A (Vestergaard et al., 2015).

In conclusion, the CDC50 accessory proteins may interact with P₄-ATPases at multiple sites to promote correct protein folding, exit of the P₄-ATPase complex from the ER, and formation of a functionally active flippase complex. However, whether the β-subunit participates during the activity of phospholipid translocation has yet to be determined. Experiments with the reconstituted yeast orthologs, Drs2p and Dnf1p, suggest that the α-subunit plays the dominant role in substrate specificity and translocation of the phospholipid (Baldridge & Graham, 2012; X. Zhou & Graham, 2009).
1.2.4 Model mechanisms for ATP8A2

The P-type ATPases that have been most studied are ion transporters. However, P₄-ATPases transport a bulky aminophospholipid, questioning the mode of transport across a lipid membrane. The translocation pathways of ion transporters are not large enough to accommodate such large substrates. This leads to the “giant substrate” problem. The structure and the mechanism by which a P₄-ATPase, specifically ATP8A2, transports PS has yet to be determined. Four models of transport for ATP8A2 have been proposed and modelled from the structurally similar Ca²⁺-ATPase and Na⁺/K⁺-ATPase. These four mechanisms are: the ‘credit-card,’ the ‘non-canonical,’ the ‘hydrophobic gate peripheral pathway,’ and the ‘merge of structures’ models.

The ‘credit-card’ model considers the large size of the substrate, which has a hydrophilic head group and two hydrophobic acyl chains. Since transporting a huge amphipathic molecule is difficult, this model envisions the hydrophilic head group of phospholipids interacting with ATP8A2 while the acyl chains remain within the hydrophobic lipid bilayer region (Fig. 1.3A). Like the interaction of a credit card terminal interacting with the magnetic strip of a credit card, the hydrophilic head group is the only segment of the phospholipid interacting with ATP8A2.

The ‘non-canonical model’ is adapted from the observations made on yeast P₄-ATPases, Dnf1p and Drs2p, which transports PC and PS, respectively. The yeast structural models were generated from the crystal structure of a Na⁺/K⁺-ATPase (PDB: 2ZXE) (Baldridge & Graham, 2012, 2013). These two P₄-ATPases have been found to have specificity for PC and PS recognized by critical M1 residues (including but not limited to Q95 and Q96), M3 N550, and M4 Y618 (Baldridge & Graham, 2012, 2013), to suggest how the ATPase initiates the interaction with its substrate to start transport like a ‘credit-card.’ These residues, initially thought to be important for
specificity, are part of translocation process of the phospholipid head group in the non-canonical pathway (Fig. 1.3B).

A model centralized around the I376 residue, within M4, has led to the ‘hydrophobic gate peripheral pathway model’ (Vestergaard et al., 2014). The Ile within the PISL motif is conserved in P4-ATPases, in transmembrane segment four. This residue corresponds to the Glu in the PEGL motif of the Ca\(^{2+}\)-ATPase and Na\(^+\)/K\(^+\)-ATPases. The PEGL motif acts as a pump to deliver sodium or calcium ions vertically into exoplasmic side (Vilsen and Andersen, 1998). This model shows how PS and PE may get transported from extracellular to cytoplasmic side of the membrane by regulating water traffic within the protein groove (Fig. 1.3B). The movement of water molecules past I376 residue aids with the flipping of phospholipid head groups since water molecules interact with the hydrophilic headgroup (Vestergaard et al., 2014). Previously, I376M mutation has been shown to have reduced ATPase activity in vitro (Vestergaard et al., 2014). The hydrophobic gate pathway also considers the hydrophobic residue I125 along with I376 to regulate water movement (Vestergaard et al., 2014) and the N371 residue is thought to recognize its respective substrate (Andersen et al., 2016).

The last model is the ‘merge of structures’ model, which integrates studies done for ATP8A2 and the yeast flippase and relates their similarities. This model highlights the I376 as part of the hydrophobic gate pathway but also considers Q95 and Q96 residues, which were thought to be important in yeast models (Baldridge & Graham, 2012, 2013). The ‘merge of structures’ model considers L376, A119, and E123 residues for substrate specificity in conjunction with the hydrophobic gate model. A combination of these models is likely how ATP8A2 translocates phospholipids (Andersen et al., 2016). Only a defined structure and molecular dynamic simulations will allow conclusions pertaining to these models.
Figure 1.3 Overview of the proposed models of transport mechanism for ATP8A2. A) A sketch of the ‘credit card’ model transporting phospholipid from exoplasmic to cytoplasmic leaflet. B) The ‘hydrophobic gate’ and ‘non-canonical’ pathway, the bovine ATP8A2 modeled from SERCA. Selected residues in licorice representation are orange for hydrophobic residues and in yellow for polar/charged residues. (Taken from Andersen et al., 2016. Permission to use is given by author.)

1.2.5 Human diseases associated with P₄-ATPases

Many human diseases arise from genetic mutations of key proteins involved in crucial intracellular pathways. Whole exome sequencing (WES) can be used to uncover such mutations from genomic DNA of patients, using exome kits and PCR amplification in combination with sequencing instruments such as Illumnia (McMillan et al., 2018). Sequence results are analyzed and “filtered based on inheritance patterns, gene lists of interest, phenotype, and population frequencies as appropriate” (McMillan et al., 2018). The difference in genetic sequences is confirmed with healthy family members for genetically inherited diseases, linking genetic mutation to a particular disease.

Channels and transporters are critical to maintain homeostasis within the cell. Mutations in a few P₄-ATPases have been linked to genetic disorders. ATP8B1 mutations are linked to two
related liver diseases: benign recurrent intrahepatic cholestasis type 1 (BRIC1) and progressive familial intrahepatic cholestasis type 1 (PFIC1) (Bull et al., 1998; Klomp et al., 2004; Paulusma et al., 2006). A genetic defect in ATP8A2 is linked to CAMRQ in a consanguineous family from Turkey (Onat et al. 2013). These P4-ATPases have been associated with PS translocation (Coleman and Molday 2011; Paulusma et al. 2007). Genetic analysis of patients showed homozygous or compound heterozygous mutations in the aminophospholipid transporter, class I, type 8A, member 2 gene (ATP8A2) in Turkish and Spanish patients and a de novo–balanced translocation, t(10;13), which disrupted the ATP8A2 gene (Cacciagli et al., 2010). CAMRQ is a heterogeneous group of genetic disorders, transmitted by autosomal recessive mechanisms. Further studies on ATP8A2 have shown a broader spectrum of clinical diseases: CAMRQ, encephalopathy, central hypotonia, psychomotor delay, chorea, and optic atrophy, classified as clinical heterogeneous disorders (Cacciagli et al., 2010; J. A. Coleman et al., 2014; Emre Onat et al., 2013; Zhu et al., 2012).

1.2.6 Understanding misfolded proteins

A few degradation pathways exist within the cell to deal with over accumulation of proteins that are not needed any longer, or misfolded proteins. Much of membrane protein synthesis occurs within the ER, as in the case of ATP8A2. For misfolded proteins within the ER, a common pathway for degradation is the use of proteasomes. Previously, different proteasome inhibitors have been developed and used. This initially started with the discovery of the natural product lactacystin (Fenteany et al., 1995). One type of proteasome inhibitor, MG132 (carbobenzyoxyl-L-leucyl-L-leucyl-L-leucinal), was shown to inhibit calpain and proteasome activities between 20 nM and 10 μM for PC12 cells (Tsubuki, Saito, Tomioka, Ito, & Kawashima, 1996). A proteasome inhibition study on a P4-ATPase, ATP8B1, human disease-causing mutant was done at 10 μM for 6 hours on UPS-1 cells (Folmer et al., 2009). Poorly expressed mutants of ATP8A2 need to be analyzed, how
these are being treated after translation is of question. Proteasomes may be a degradation pathway which these mutational proteins are being disposed.

1.3 Microscopic analysis of protein structures

Electron microscopes (EM) have been used and developed for specific analysis of protein structure as an alternative to the gold-standard of protein structure determination, X-ray crystallography. The variable properties of individual proteins require optimization of protein solution to allow crystallization. The use of EM brings many advantages to gain protein structural information. Once the difficulties to obtaining a protein structure are overcome, one can gain mechanistic information among other information.

1.3.1 Structural studies on P-Type ATPases and P4-ATPases

The first P-type ATPase studied was a Na⁺-K⁺-ATPase by Jens C. Skou. Intrigued by how anesthetics affect nerves, Skou started by looking at ATPase activity in crab nerves. Skou found that the ATPase activity was at its highest when exposed to optimal concentrations of sodium, potassium, and magnesium ions (Skou, 1957). To understand how the ATP was hydrolyzed at differing concentrations of ions, he thought that a highly active enzyme was required to account for experimental results.

The first P-type ATPase to be crystallized was a sarco(endo)plasmic reticulum Ca²⁺-ATPase from a fast twitch muscle in rabbit (Toyoshima, Nakasako, Nomura, & Ogawa, 2000). The structure is currently the representative molecule for the P-type ATPases. From this discovery, the characteristic P-, N-, A-, and T- domains, which are critical for substrate transportation were found. The structure of multiple solute transporters have since been solved: heavy metal transporter (P1-ATPase; Meng, Bruschweiler-Li, Zhang, & Brüschweiler, 2015), sodium-
potassium transporters (P$_2$-ATPase; Morth et al., 2007), and proton pumps (P$_3$-ATPase; Pedersen, Buch-Pedersen, Preben Morth, Palmgren, & Nissen, 2007).

The in-depth study of P$_2$-ATPases provide valuable data for the analysis of other P-type ATPases. However, since P$_4$-ATPases are different from P$_2$-ATPases, due to their large substrate problem, only a limited amount of structural and mechanistic information can be extrapolated. In particular, the P$_4$-ATPases subfamily, ATP8A2, is of interest due to its implications in severe human diseases.

1.3.2 Peptidisc for membrane proteins

The isolation of membrane proteins for biochemical analysis is known to be extremely difficult due to their amphipathic nature. Membrane proteins are embedded in the anisotropic environment of lipid bilayer with a hydrophobic core to shield the hydrophobic surface of membrane proteins from the aqueous environment. To purify a membrane protein, in a stable form, small detergents, lipid-like amphipols, bicelles, and membrane scaffold proteins (MSP) have been used in the past. Detergent micelles are widely used to extract proteins from the cell membrane by interaction between the hydrophobic area of the detergent and the hydrophobic patches of membrane proteins. However, micelles are not effective due to increased susceptibility to protein denaturation from the dynamic replacement of detergent molecules, the inability of micelles to accurately reflect a lipid bilayer and different physiochemical properties (Bordag & Keller, 2010; Zhou & Cross, 2013). Detergents may also be problematic for functional studies of membrane proteins even after optimization of detergents (Chae et al., 2010; Frotscher et al., 2015). Amphipols, a class of amphipathic polymers, have been designed as a replacement for detergents, increasing membrane stability for some membrane proteins (Zoonens & Popot, 2014). However, the lack of a lipid bilayer like environment makes this polymer unfavorable to work with and alterations of
the polymers are needed to increase stability between the target protein and amphipols. Maintaining the structure, function, and stability is kept by retaining a lipid bilayer-like environment (Zhou and Cross 2013). Thus, detergents and phospholipids have been used together to create a bicelle. Such mixture generates 8-50 nm diameter bicelles (Prosser, Hwang, & Vold, 1998). However, lipid composition and stability are issues faced by bicelles. A method that uses an amphipathic protein, known as MSP, wrap around membrane proteins and form a small lipid bilayer (Bayburt, Grinkova, & Sligar, 2002, 2006). MSP is a helical amphipathic protein developed from the human apolipoprotein A-1. It can entrap membrane proteins in their native oligomeric state (Boldog, Grimme, Li, Sligar, & Hazelbauer, 2006). However, the challenge comes from having to optimize the size of MSP for different membrane proteins (Grinkova, Denisov, & Sligar, 2010; Hagn, Etzkorn, Raschle, & Wagner, 2013; Wang, Mu, Li, Bi, & Wang, 2015).

Moreover, isolation of membrane proteins in their functional oligomeric state, yet maintaining a stable state, is challenging. The reconstitution of a membrane protein in a way “that is cost-efficient, rapid, unhindered by issues of solubility” is possible with the use of peptidisc (Carlson et al., 2018). Peptidiscs (NSP) consists of a modified amphipathic bi-helical peptide that can wrap around membrane proteins. NSP, due to its ability to capture membrane proteins by direct contact with hydrophobic areas, forms a soluble protein without additional lipids surrounding the solubilized protein. This increases the homogeneous composition of purified membrane proteins and facilitates structural studies.

1.4 Thesis Investigation

The study of human disease-causing mutants and the structural analysis of the protein ATP8A2, are of interest to biomedical research because ATP8A2 has been associated with the following clinical symptoms: CAMRQ, encephalopathy, central hypotonia, psychomotor delay,
chorea, and optic atrophy, classified as clinical heterogeneous disorders. Several genetic mutations in ATP8A2 have been linked to clinical disorders including I376M (Onat et al. 2013); K429N, A544P, and R625W (Martín-Hernández et al., 2016), and K429M and W702R (Alsahli, Alrifai, Al Tala, Mutairi, & Alfadhel, 2018). The effects of these critical point mutations on ATP8A2 structure and function were unknown. Thus, investigation at the biochemical level was of interest. Further, it is of interest to elucidate the structure of ATP8A2-CDC50A to understand the transport mechanism of this lipid transporter.

Chapter 2 describes the biochemical effects that disease-causing mutations have on the function and trafficking of ATP8A2. Two different classes of mutants have been elucidated in this study. One class is composed of mutant proteins that can export out of the ER, and thus, are likely to be correctly folded yet have attenuated activity levels, and the second class consists of mutant proteins that are poorly expressed and are retained in the ER as misfolded proteins.

Chapter 3 deals with the scale-up of ATP8A2-CDC50A complex for structural analysis. Few optimizations have been tested for purifying the membrane protein. The analysis of the structure has been attempted with negatively stained grids analyzed by transmission electron microscopy (TEM) for low resolution analysis and cryo-electron microscopy (cyro-EM) for high resolution protein analysis.
Chapter 2: Defining disease causing mutants of P4-ATPase, ATP8A2

2.1 Introduction

Studies on ATP8A2 are of interest to biomedical research because mutations in ATP8A2 are associated with the following clinical diseases: CAMRQ, encephalopathy, intellectual disability, psychomotor delay, chorea, and optic atrophy, classified as clinical heterogeneous disorders (Onat et al. 2013) and severe hypotonia and hyperkinetic movement disorder (McMillan et al., 2018). The first missense mutation discovered was the I376M located in the transmembrane domain of ATP8A2 (Onat et al. 2013). Subsequently, the K429N, A544P, and R625W (Martín-Hernández et al., 2016) and K429M and W702R mutations (Alsahl et al., 2018) have been discovered and linked to these clinical disorders.

CAMRQ is a rare genetically heterogeneous disorder characterized by cerebellar ataxia with mental retardation and dysarthric speech, with or without quadrupedal gait. Since the first mapping of the gene locus on chromosome 17p13, two additional loci on chromosomes 9p24 and 8q12 have been reported, and causative mutations have been identified in VLDLR, CA8 and WDR81 (Gulsuner et al., 2011; Ozcelik et al., 2008; Turkmen et al., 2005; Türkmen et al., 2009).

ATP8A2 is involved in the transport of aminophospholipids toward the cytoplasmic leaflet in brain cells, retinal photoreceptors and testis (Coleman et al., 2009). In humans, ATP8A2 is mainly expressed in brain tissues, with highest levels in cerebellum, as well as in retina and testis (Cacciagli et al., 2010). Cerebellum is a crucial regulatory organ for motor coordination and this expression pattern is consistent with CAMRQ. The fact that CAMRQ-associated genes have retinal expression (Coleman et al., 2009) raises the possibility that eye abnormalities may be an additional clinical feature of the phenotype. Strabismus has been observed in almost all affected
individuals in all the families reported (Gulsuner et al., 2011; Ozcelik et al., 2008; Tan, 2006; Turkmen et al., 2005; Türkmen et al., 2009). I performed the expression assays, ATPase activity assays, immunofluorescence studies, and proteasome inhibition studies to analyze the six individual point mutations in this study.

2.2 Methods

2.2.1 Materials

1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1, 2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), and 1,2-dioleoyl-sn-glycero-3-phosphoserine (DOPS) were purchased from Avanti Polar Lipids (Alabaster, AL). ATP was purchased from Sigma, dithionite was from Fisher, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) was from Anatrace (Maumee, OH), Protease Arrest from G-Biosciences (St. Louis, MO), and the 1D4 peptide from Celtek Peptides (Franklin, TN). Rho 1D4 antibody used for preparation of immunoaffinity columns was generated in-house (Hodges et al., 1988; Quazi and Molday, 2013) and purchased from UBC through Flintbox (www.rho1d4.com/); primary antibodies against calnexin, actin, and tubulin were from Abcam; fluorescent-tagged secondary antibodies for immunofluorescence imaging were from Molecular Probes; and anti-Cdc50-7F4 (CDC50A) primary antibodies used in Western blots and immunofluorescence analysis were raised in-house (Coleman and Molday, 2011). Restriction enzymes, Instant sticky-end ligase, Antarctic Phosphatase, Phusion polymerase, and Q5 DNA polymerase were procured from New England Biolabs.

2.2.2 Solutions

The buffer compositions are as follows: Buffer A: 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl2, 1 mM
dithiothreitol (DTT), 20 mM CHAPS, 0.5 mg/ml DOPC, 1× Protease Arrest; Buffer B: 50 mM HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10 mM CHAPS, 0.5 mg/ml DOPC; Buffer C: 50 mM HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10 mM CHAPS, 0.5 mg/ml DOPC, 0.4 mg/ mL 1D4 peptide; Solution D: 6% ascorbic acid, 1% ammonium molybdate in 1 N HCl; Solution E: 2% sodium citrate, 2% sodium meta-arsenite, 2% acetic acid.

2.2.3 DNA constructs

ATP8A2 in pcDNA3 and CDC50A in pcDNA3 were available. Using ATP8A2 plasmid with 1D4 tag, mutations were introduced by using the Q5 site-directed mutagenesis kit (NEB E0552S). Primers were synthesized by IDT, specific to each point mutation and mutated constructs were verified by sequencing of the entire coding sequence by Genewiz. The purification of ATP8A2 with its associated CDC50A protein was facilitated by the C-terminal nine amino acid 1D4 tag on ATP8A2. This allowed for the application of immunoaffinity chromatography using the Rho 1D4 antibody.

2.2.4 Expression of ATP8A2 in HEK 293T

HEK293T cells (American Type Culture Collection, Manassas, VA) were transfected in 100 mm dishes and COS7 cells were transfected in 6 well plates, transfected at 30% and 50% confluence, respectively. Co-transfection of cells was carried out with 2.5 μg of ATP8A2-1D4 in pcDNA3 and 2.5 μg CDC50A in pcDNA3 using 15 μg of polyethylenimine (PEI) as transfection reagent. Transfection of COS7 cells for confocal microscopy was carried out using the calcium phosphate method with 1.25 μg of each plasmid. The cells were harvested 48 h post-transfection for analysis of the overexpressed protein. Cells were maintained in DMEM (Sigma-Aldrich)
supplemented with 8% bovine growth serum (Thermo Scientific), 100 IU/ml penicillin, 100 μg/ml streptomycin, 0.25 μg/ml amphotericin B (Corning), and 2 mM L-glutamine.

2.2.5 Purification of ATP8A2

Methods are adopted from detailed procedure by Coleman and colleagues (2009). Purified 1D4 monoclonal antibody was coupled to CNBr-activated Sepharose at a concentration of 2 mg of protein/ mL of beads as described previously (Molday & MacKenzie, 1983). Cells co-overexpressing ATP8A2-1D4 and CDC50A were harvested from culture plates and resuspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM dithiothreitol (DTT), 20 mM CHAPS, 0.5 mg/ml DOPC, and 1× Protease Arrest (lysis buffer). Resuspended cells were lysed by stirring at 4°C for 30 min. After lysis, the CHAPS-insoluble fraction was removed by centrifugation at 40,000 × g<sub>av</sub> for 12 min. The CHAPS-soluble fraction was then loaded on an anti-Rho 1D4 immunoaffinity column preequilibrated with 10 column volumes of lysis buffer. After binding for 30 min at 4°C, the column was washed six times with 500 μl of buffer containing 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 10 mM CHAPS, and 0.5 mg/ml ePC (column wash buffer). Bound protein was then eluted from column with 1D4 elution buffer (column wash buffer and 0.4 mg/ml 1D4 peptide) in two iterations of 30 min each with agitation at 18°C. The concentration of purified protein was measured on a Coomassie blue–stained SDS–PAGE gel with bovine serum albumin of known concentration as standards.

2.2.6 MG132 treatment of HEK 293T cells.

The cells were grown for 48 hours after transfection and media was refreshed with new media. 10 μM MG132 was added per plate, 15 minutes after refreshing cells with new media. After 5 hours of incubation at 37°C and 5% CO₂, the cells were harvested.

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2.2.7 SDS-solubilization

For total protein content, the cells were solubilized with 4% SDS. Cells co-overexpressing ATP8A2-1D4 and CDC50A were harvested from culture plates and resuspended in 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH, pH 7.5, 150 mM NaCl, 5 mM MgCl$_2$, 1 mM dithiothreitol (DTT), 4% SDS, and 1× Protease Arrest (lysis buffer). Resuspended cells were lysed by stirring at room temperature for 30 min. After lysis, the insoluble fraction was removed by centrifugation at 40,000 × g$_{av}$ for 12 min. Supernatant was analyzed on SDS-PAGE and transferred to Western blots.

2.2.8 ATPase activity assay

The ATPase activity of immunopurified WT and mutant ATP8A2 was measured as previously described using a colorimetric method (Coleman, Kwok, and Molday 2009) developed from earlier method (González-Romo, Sánchez-Nieto, & Gavilanes-Ruíz, 1992). Briefly, ~1 ng of purified protein was mixed with 5 mM ATP and 2.5 mg/ml lipid (a mixture of DOPC and DOPS combined at different predetermined ratios) to obtain a final volume of 25 μl. Both ATP and lipids were prepared in ATPase assay buffer containing 50 mM HEPES/NaOH, pH 7.5, 150 mM NaCl, 12.5 mM MgCl$_2$, 1 mM DTT, and 10 mM CHAPS. The reaction was carried out at 37°C for 20 min and terminated by addition of 25 μl of 12% SDS. The phosphate released from ATP hydrolysis was measured by treating the reaction mix with 75 μl of solution D for 5 min, followed by 120 μl of solution E. The intensity of the resultant color was measured from the absorbance at 850 nm in a microplate reader. This measured intensity was compared with those of known phosphate concentrations on a standard curve to calculate the reaction velocity (micromoles of phosphate released/minute/milligram) for each PS concentration used in the assay. The resultant data were fitted to a Michaelis–Menten equation to calculate the maximum reaction velocity (Vmax) and PS-
activation constant \( (K_A) \) for the WT and mutant ATP8A2 alleles using GraphPad 7. Each measurement was recorded in triplicate, and every experiment was repeated at least four times independently.

2.2.9 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 PVDF 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 45 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.

2.2.10 Immunofluorescence microscopy

Cells grown on glass coverslips were fixed with 4% paraformaldehyde for 15 min. After permeabilization for 1 h with 10% normal goat serum (NGS) and 0.2% Triton X-100, cells were treated for 2 h with primary antibody solution containing 10% NGS, 0.1% Triton X-100, mouse Rho1D4 monoclonal antibody (mAb) or Cdc50-7F4 mAb and rabbit polyclonal anti-calnexin antibody or anti-GM130 antibody. The coverslips were washed and labeled with 4′,6-diamidino-2-phenylindole (DAPI) and goat Alexa 488 anti-mouse and goat Alexa 594 anti-rabbit secondary antibodies for 0.5 h. Coverslips were washed and mounted on glass slides for imaging with Mowiol mounting medium. The reagents for fixing, permeabilizing, and labeling were prepared in 100 mM phosphate buffer (pH 7.4), and all antibodies were used at 1:1000 dilution. Fluorescence images were acquired at room temperature using a 40× objective with a numerical aperture of 0.8 on a
Zeiss LSM 700 confocal microscope equipped with Zen Image analysis software. Composite figures were prepared using ImageJ software. To analyze the colocalization of ATP8A2 (WT and mutants) to the ER and Golgi complex, the Manders colocalization coefficient was calculated using ImageJ. Cells that were not labeled by the antibody were not considered for analysis.

2.3 Results

2.3.1 Mutants associated with ATP8A2

Missense and non-sense mutations in the gene encoding ATP8A2 have been shown to cause severe neurological diseases. Six disease-causing missense mutations have been studied here including I376M (Onat et al. 2013); K429N, A544P, and R625W (Martín-Hernández et al., 2016); and K429M and W702R (Alsahli et al., 2018). Alignments performed using ClustalW show that in general there is a high degree of conservation of residues within the locations where the mutations are present (Fig. 2.1B). Thus, a drastic change of residue that has different biophysical properties will likely have a critical effect on the structure and function of ATP8A2 and consequently on the patients harboring these mutations.
Figure 2.1. Multiple sequence alignment of ATP8A2 and P2-ATPases. The amino acids highlighted in yellow are the disease-causing mutants of ATP8A2. A) Multiple sequence alignment of the disease mutation sites, highlighted, from five different species [human (Hu), bovine (Bo), chicken, mice, and yeast (Drs2)] and P2-ATPase family, showing the high degree of conservation. B) Multiple sequence alignment of the disease mutation sites, highlighted, for all members of the P2-ATPase family. The consensus symbols below the alignments are as follows: * is highly conserved residues, : is conservation of residues of strongly similar properties (score greater than 0.5 in Gonnet PAM 250 matrix), and . indicates residue conservation of weakly similar properties (score less than or equal to 0.5 in Gonnet PAM 250 matrix).

To determine the effect of these mutations on total ATP8A2 expression, cell lysates from transfected HEK293T cells were directly solubilized in 4% SDS and analyzed on Western blots labeled for ATP8A2 with the Rho1D4 antibody. Figure 2.2 shows that the overall expression level of I376M and K429M was comparable to wild-type (WT) protein. To estimate the amount of protein that could be solubilized in a mild detergent, cell lysates were treated with CHAPS detergent, and the solubilized fraction obtained after removal of aggregated protein by centrifugation was analyzed by Western blotting. The intensity of protein bands from Western were quantified relative to WT ATP8A2. The low amount of A544P, R625W, and W702R prevented an extensive characterization of these mutants at a biochemical level.
Figure 2.2. The expression assay of ATP8A2-CDC50A and disease-causing mutants. Co-transfected HEK 293T cells, solubilized in 20 mM CHAPS or 4% SDS were resolved on a 9% SDS-PAGE gel. Western blots were labeled for ATP8A2 variants; β-tubulin was used as a loading control. Insoluble contents were removed by high-speed centrifugation at 40 k RPM. Western blotting (upper panel) were imaged on a Licor Odyssey imager (lower panel). Data is a mean ± SD for n = 10.

Quantitative representations of total protein expression of the various ATP8A2 mutants are shown in the lower panel of Figure 2.2. To better understand the localization of disease-causing mutants, the ATP8A2 co-expressed with CDC50A in HEK 293T cells were solubilized in 4% SDS (Fig. 2.2). The mutant A544P was barely detectable even when cells were solubilized in SDS, solubilizing the ER membranes. Higher levels of expression of cells solubilized in SDS are observed for the remaining mutants poorly expressed in CHAPS: K429N, R625W, and W702R.
2.3.2 ATPase activity assay

For analysis of ATPase activity, ATP8A2 mutants co-expressed with its accessory subunit CDC50A were purified by affinity chromatography on a Rho-1D4 column. Figure 2.3A shows the input and eluted sample fractions from the immunoaffinity column. The wild-type, I376M, K429M, and K429N expressed at levels suitable for measuring activities. The expression of A544P, R625W, and W702R variants was too low for ATPase activity determination. The eluted protein sample was used for activity assay to test against PS and PE. The purified ATP8A2 protein was collected (Fig. 2.3A) and used to measure the ATPase activities expressed as μmol ATP hydrolyzed per minute per mg of protein at 37°C. The three variants were tested in presence of increasing concentrations of PS or PE using PC as the base phospholipid. The mutant I364M (bovine counterpart to I376M) was previously reported to have no activity by Coleman and colleagues (2014) and adjacent residues to the I364M were also analyzed (Vestergaard et al., 2014). Our studies confirmed the absence of phosphatidylserine and phosphatidylethanolamine stimulated ATPase activity for I376M, K429M, and K429N mutations in human ATP8A2.
Figure 2.3. The ATPase activity of ATP8A2 and disease-causing mutants. ATP8A2 and disease-causing mutants were co-expressed with CDC50A, purified on Rho1D4 immunoaffinity matrix, for analysis by SDS-PAGE gel electrophoresis and ATPase activity assays. A) Coomassie blue stained gel of cell lysate (input) and purified ATP8A2 variants. B) Western blot labelled for ATP8A2 with Rho1D4 antibody and CDC50A with Cdc50A-7F4 antibody, confirming protein identity. C) The ATPase activity analysis of WT, I376M, K429M, and K429N as a function of PS concentration. Curves were fitted with Michalis-Menten kinetics. Measurements were done in quintuplicates. D) The ATPase activity analysis of WT, I376M, K429M, and K429N as a function of PE concentration.

2.3.3 Localization of ATP8A2 to Golgi apparatus and endoplasmic reticulum

To supplement findings from expression assays (Fig. 2.2), the disease-causing mutants were expressed in COS-7 cells for immunofluorescence studies. The highly-expressed mutants:
I376M and K429M like WT ATP8A2 co-localized mainly with GM130. In contrast, the poorly expressed mutants: K429N, R625W, and W702R mutants co-localized with the ER marker, calnexin (Fig. 2.4).

Figure 2.4. Immunofluorescence image of disease-causing ATP8A2 variants. Representative COS-7 cell immunofluorescence images of human disease-causing mutants of ATP8A2, co-expressed with CDC50A by calcium phosphate transfection protocol. Left panel: Cells were labelled for ATP8A2 and its variants (green) and Golgi marker GM130 (red) and counter-stained with nuclear stain DAPI (blue) for confocal fluorescence microscopy. Right panel: Cells were labelled for ATP8A2 and its variants (green) and ER marker calnexin (red) and counter-stained with nuclear stain DAPI (blue). WT, I376M, and K429M showed higher degree of colocalization with GM130 compared to K429N, R625W, and W702R mutants.
2.3.4 Analysis of K429 mutants

ATP8A2 has two highly conserved domains, one of which is DKTGT motif, conserved across all P₄-ATPase species. The lysine residue, part of this conserved motif, upon mutation to a methionine or asparagine (K429M and K429N) is a human disease-causing mutation. These two mutants have been shown to display differing expression patterns. Thus, three additional mutants have been made: alanine, to examine the effect of the non-bulky and chemically inert methyl group; leucine, to examine if a hydrophobicity is crucial; and arginine, to examine implications of a positive charge. The K429M and K429A mutants expressed near wild-type levels whereas, K429N, K429L, and K429R expressed at much lower levels (Fig. 2.5). When the activity assays were carried out as a function of PS concentration, only wild-type displayed a dependence of activity with PS (Fig. 2.6).

Figure 2.5. The expression of K429 variants. Upper panel: expression of ATP8A2 variants in HEK 293T cells as shown by western blotting. Lower panel: expression levels of ATP8A2 variants relative to WT ATP8A2 as analyzed on western blots. Data is the mean ± SD for n = 5.
2.3.5 Degradation of misfolded proteins

Most of the human disease-causing mutants of ATP8A2 analyzed here, specifically K429N, A544P, R625W, and W702R, expressed poorly suggesting that they may undergo rapid degradation (Fig. 2.2). This was investigated by examining the expression levels of the disease-associated variants in the presence and absence of MG132, a proteasome inhibitor (Fig. 2.7). For this analysis, cells were solubilized in CHAPS (Fig. 2.7A) and SDS (Fig. 2.7B) to show the degree of expression. The relative expression values normalized to the β-tubulin were calculated for each mutant in the presence and absence of inhibitor. The relative expression levels are shown in Figure 2.7B and C. In general, a two-fold increase in expression was observed for the SDS-solubilized mutants from HEK 293T cells, grown in the presence of MG132. No increase was observed for the mutants solubilized in CHAPS.
Figure 2.7. Effect of MG132 on the degradation of ATP8A2 variants expressed in HEK 293T cells. (A) Western blots of ATP8A2 variants in the presence and absence of MG132, solubilized HEK 293T cells in 4% SDS. (B) Show the quantification from western blot of SDS solubilization. (C) Show the quantification from western blot of 20 mM CHAPS solubilization. Data shows mean ± SD for \( n = 5 \). P values for ATP8A2 variants ± GM132 are: * K429N = 0.027; ** A544P = 0.0008; *** R625W = 0.002; and **** W702R = 0.0029.

2.4 Discussion

The focus of this study was to analyze the implications of mutations on the expression and activity of ATP8A2 to begin to define the molecular basis for the neurological diseases associated with mutations in ATP8A2. Western blot analysis and immunofluorescence analysis complement each other: the relative percent expression of colocalization calculated from Golgi to calnexin are comparable to the relative percent expression found through 20 mM CHAPS solubilized expression (Table 2.1). The immunofluorescence colocalization was calculated by ImageJ plugin.
Colocalization was calculated for 1D4 and GM130 labeled cells and 1D4 and calnexin labeled cells, each giving separate values.

Table 2.1. The colocalization between 1D4 and GM130 or Calnexin. Immunofluorescence images were taken from Cos-7 cells co-expressing disease-causing mutants of ATP8A2 and CDC50A. 15 images were averaged to give colocalization values, calculated by Pearson’s coefficient.

<table>
<thead>
<tr>
<th>Strain</th>
<th>GM130</th>
<th>Calnexin</th>
<th>GM/Cal</th>
<th>% Relative Expression</th>
<th>Immuno-blot % expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>28.30 ± 4.2</td>
<td>48.90 ± 6.6</td>
<td>0.58</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>I376M</td>
<td>37.90 ± 7.5</td>
<td>57.40 ± 4.1</td>
<td>0.66</td>
<td>114.1</td>
<td>111.6</td>
</tr>
<tr>
<td>K429M</td>
<td>30.60 ± 9.4</td>
<td>58.20 ± 7.3</td>
<td>0.53</td>
<td>90.8</td>
<td>98.9</td>
</tr>
<tr>
<td>K429N</td>
<td>6.30 ± 2.7</td>
<td>79.80 ± 5.0</td>
<td>0.08</td>
<td>13.6</td>
<td>11.4</td>
</tr>
<tr>
<td>R625W</td>
<td>1.30 ± 1.9</td>
<td>83.80 ± 9.5</td>
<td>0.02</td>
<td>2.7</td>
<td>1.5</td>
</tr>
<tr>
<td>W702R</td>
<td>0.95 ± 1.2</td>
<td>78.10 ± 7.1</td>
<td>0.01</td>
<td>2.1</td>
<td>0.6</td>
</tr>
</tbody>
</table>

The mutant A544P was not part of immunofluorescence studies due to its exceedingly low expression as visualized by western blotting of SDS solubilized cell lysates (Fig. 2.1). The ATPase activity was also not conducted for A544P, R625W, and W702R due to their low level of expression. Whereas, the I376M and K429M mutants expressed at or near WT ATP8A2 levels. Like WT ATP8A2, these variants preferentially localized to the Golgi/recycling endosomal system in cells indicating that these amino acid substitutions did not affect the global folding or stability of the protein. However, these mutants were devoid of functional activity as measured by PS and PE stimulated ATPase activity (Fig. 2.3). The I376M has been shown previously to be devoid of ATP-dependent phospholipid flippase activity as well as PS and PE stimulated ATPase activity (Vestergaard et al, 2014; Lee et al, 2015). The N917D disease-causing mutation (N905D in the bovine orthologue) can be added to this category since it had been reported to express at WT-like levels but lacked ATPase activity (Mikkelsen et al, 2019). Although these two categories of mutations display significant differences in levels of protein expression, they both can be considered as loss-of-function mutations since neither category displayed phospholipid activated ATPase activity. This is in agreement with clinical studies in which patients with these different
missense mutations all show similar severe neurological phenotypes and the same disorders as patients homozygous for premature stop codons and frameshift mutations in the ATP8A2 gene (Alsahli et al., 2018; Martin-Hernandez et al., 2016; McMillan et al., 2018; Onat et al., 2012). Although not all the phenotypes are fully characterized and were limited to the number of patients with same genotype, the following have been described by different groups (Table 2.2).

Table 2.2 Clinical characteristics of individuals with ATP8A2 mutations. (Data from Tan, 2010 [I376M]; Herendez et al., 2016, McMillan et al., 2018 [K492N, K429M, A544P/R625W, and W702R]; and Alsahli, et al., 2018 [N917D]). ‘Y’ is displays phenotype and ‘N’ is does not display phenotype and ‘n.d’ were phenotypes that were not recorded in study, values listed as percentage are the percent of patients affected with phenotypes with the point mutation.

<table>
<thead>
<tr>
<th>Phenotypes</th>
<th>Mutation</th>
<th>I376M</th>
<th>K429M</th>
<th>K429N</th>
<th>A544P/R625W</th>
<th>W702R</th>
<th>N917D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypotonia Onset</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chorea</td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Dystonia</td>
<td></td>
<td>n.d</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>n.d</td>
</tr>
<tr>
<td>Hearing loss</td>
<td></td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Grasping Objects</td>
<td></td>
<td>Weak</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Unable</td>
<td>Unable</td>
</tr>
<tr>
<td>Encephalopathy</td>
<td></td>
<td>Y</td>
<td>n.d</td>
<td>Y</td>
<td>Y</td>
<td>n.d</td>
<td>40%</td>
</tr>
<tr>
<td>Cerebral atrophy</td>
<td></td>
<td>Gyral</td>
<td>N</td>
<td>Mild</td>
<td>Y</td>
<td>Y</td>
<td>40%</td>
</tr>
<tr>
<td>Optic Atrophy</td>
<td></td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Ptosis</td>
<td></td>
<td>n.d</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Feeding Issue</td>
<td></td>
<td>n.d</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Sleep disturbance</td>
<td></td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>Y</td>
<td>n.d</td>
<td>n.d</td>
</tr>
<tr>
<td>Facial dyskinesia</td>
<td></td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
<td>n.d</td>
</tr>
</tbody>
</table>

The disease-causing missense mutations except for A544P are located in highly conserved regions of ATP8A2. The I376M and N917D mutations are present in transmembrane segments 4 and 6, respectively. Mutational studies together with molecular modeling and molecular simulation indicate that isoleucine 376 may be part of a hydrophobic gate that controls the translocation of PS and PE through a groove formed by transmembrane segments M1, M2, M4
and M6 of ATP8A2 (Andersen et al, 2016; Vestergaard et al, 2014). Asparagine 917, on the other hand, plays a crucial role in phospholipid induced dephosphorylation of ATP8A2 during phospholipid translocation (Mikkelsen et al, 2019). The K429M, K429N, A544P, R625W, and W702R are located within domains important for the catalytic cycle.

Lysine 429, in particular, is part of the highly conserved DK^{429}TGTLT motif within the P-domain and adjacent to aspartic acid 428 which undergoes transient phosphorylation during the ATPase catalytic cycle. Replacement of lysine 429 with amino acids varying in side chains including methionine, asparagine, alanine, leucine and arginine all resulted in proteins devoid of ATPase activity (Fig. 2.6) indicating that the lysine residue plays a crucial role in the function of ATP8A2 as a phospholipid transporter. Interestingly, only alanine and methionine substitutions yielded proteins with WT-like expression levels. Furthermore, the length of the residue R-group decides proper folding and the positive charge is important for functionality of ATP8A2.

Previously, D454 in the highly conserved DKTGT motif has been studied in ATP8B1, corresponding to D428 in human ATP8A2. D454A of ATP8B1 destabilized the protein, which was only recoverable using proteasome inhibitors (Folmer et al., 2009). The last motif of the highly conserved region was studied in TAT-1 of C. elegans, the T391I corresponding to T432 of ATP8A2 displayed protein misfolding. TAT-1 was further studied to show wild-type-like expression of P335L, yet lacked activity (Chen et al. 2019), similar to the I376M mutant (Fig. 2.2). The N917D was not part of this study because N905D was extensively studied in bovine ATP8A2 and shown to lack ATPase activity and insensitive to lipid substrate (Mikkelsen et al., 2019).

Variation of phenotypes are displayed in individual patients, even amongst patients with same point mutation. One possibility is that other P_{4}-ATPases with similar substrate specificities as ATP8A2 compensate for the loss in function of ATP8A2 in selective tissues. ATP8A1, ATP11A,
ATP11B and ATP11C like ATP8A2 actively flip PS and PE across cell membranes (Takatsu et al., 2014; Wang et al., 2018). Of these P4-ATPases, ATP8A1 is the most likely candidate since it is 67% identical in sequence to ATP8A2 and ubiquitously expressed (Wang et al., 2018). Furthermore, it has been shown that ATP8A2 can rescue endosomal defects including the EHD1 binding as part of membrane fission process in ATP8A1 depleted cells (Lee et al., 2015). The overlapping roles of ATP8A1 and ATP8A2 may also explain differences between mice and humans. In mice ATP8A2 deficiency causes a significant decrease in visual function and photoreceptor survival whereas loss in vision and photoreceptors have not been reported in patients deficient in ATP8A2 (Coleman et al., 2014; McMillan et al., 2018; Zhu et al., 2012). It is possible that ATP8A1 can fully compensate for the loss in ATP8A2 in humans but not mice. Finally, the overlapping function of ATP8A2 and ATP8A1 is inferred in the analysis of knockout mice. ATP8A1 deficient mice appear generally normal except for some abnormal behavior traits and ATP8A2 deficient mice can live up to 6 months. However, loss of both P4-ATPases results in neonatal lethality in mice (Zhu et al., 2012).

Although ATP8A2 is known to transport PS and PE to generate membrane lipid asymmetry (Coleman et al., 2009), its role in neuronal cells is not well understood. However, previous studies have shown that ATP8A2 promotes axonal elongation in PC12 cells and hippocampal neurons (Xu et al., 2012). Furthermore, trafficking of transferrin receptors to the plasma membrane of neurons isolated from Atp8a2 knockout mice is significantly reduced (Lee et al., 2015). These studies suggest that ATP8A2 may function in the trafficking of key neuronal cell surface proteins via the endosomal recycling system. Interestingly, mutations in very low-density lipoprotein receptor (VLDLR) found on the surface of neuronal cells, like mutations in ATP8A2, are known to cause cerebellar ataxia, mental retardation, and disequilibrium (CAMRQ) syndrome (Moheb et al., 2008).
Hence ATP8A2 may play a key role in trafficking of VLDLR to cell surfaces such that either reduction in function of ATP8A2 or VLDLR may result in similar disease phenotypes. Further studies are required to determine if ATP8A2 and VLDLR are linked through the same cellular trafficking pathway in neuronal cells.

One of the intracellular pathways for protein degradation utilizes proteasomes, a multicatalytic protease. Most proteins which are recognized by proteasomes are ubiquitinated, to mark for degradation. MG132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal, Z-LLL-CHO) is a reversible inhibitor peptide that can inhibit different types of proteases, including calpain (Tsubuki et al., 1996). Prolonged exposure to and high concentrations of MG132 will result in cell death. Accordingly, the duration and the concentration of the proteasome inhibitor must be examined to minimize cell death. These conditions will vary for different cell lines. Different amounts of MG132 have been applied to HEK 293T cells for an allotted time ranging from 10 µM MG132 incubated for 1 hour, 40-hour post transfection (Dueber et al., 2011) and 50 µM MG132 incubated between 4 to 7 hours, 24-hour post transfection (Noels et al., 2009). For the mutant constructs K429N, A544P, R625W, and W702R which are poorly expressed as analyzed by CHAPS and SDS solubilization, the concentration and duration of MG132 was investigated (Fig. 2.7). We found that the cell culture kept for 5 hours in the presence of 10 µM MG132 was optimal to prevent cell death yet giving enough time to inhibit proteasome degradation of misfolded proteins. The findings from this study show the proteins which are poorly expressed are indeed being misfolded and are being degraded by proteasomes, due to significant recovery of proteins in presence of MG132 (Fig. 2.7).
Chapter 3: Purification of scaled up production of ATP8A2-CDC50A complex and microscopic analysis of protein system.

3.1 Introduction

The focus of this chapter was to purify a scaled up preparation of the P₄-ATPase membrane protein, ATP8A2 co-expressed with CDC50A. Previously, ATP8A2 has been well expressed in HEK 293T cells (Coleman, Kwok, and Molday 2009; Kwok et al. 2008). However, the membrane protein complex could only be recovered in nanogram quantities. Scaling up ATP8A2-CDC50A complex production and optimizing the purification procedure of the complex had to be investigated for protein structural analysis.

Different organisms can be used for protein production, such as yeast, bacteria, insect, and mammalian cell lines. The complexity of the organism determine the in-house post-translational modification machineries readily available for the protein of interest. The human variant of ATP8A2 with its respective β-subunit, CDC50A, was studied here. The use of higher-order organisms was better suited for proper post-translational modifications, such as glycosylation.

An in-depth investigation of purifying protein is crucial. Contaminants in the isolated sample will cause artifacts in activity assays and would also be observed during microscopic analysis of a protein sample. Contaminants can be introduced starting from solubilization of the cells. Misfolded proteins can also be present after solubilization in some detergents. The use of detergents is required to disrupt the amphiphilic lipid bilayer and extract membrane proteins. Detergent also cover hydrophobic patches on membrane proteins thereby preventing aggregation. The CMC (critical micelle concentration) is extremely important for optimal purification of protein of interest.
3.2 Methods

3.2.1 Solutions

The buffers listed in Chapter 2 is used here. In short: Buffer A: 50 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol (DTT), 20 mM CHAPS, 0.5 mg/ml DOPC, 1× Protease Arrest; Buffer B: 50 mM HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM DTT, 10 mM CHAPS, 0.5 mg/ml DOPC; Buffer C: 50 mM HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM DTT, 10 mM CHAPS, 0.5 mg/ml DOPC, 0.4 mg/ mL 1D4 peptide; Solution D: 6% ascorbic acid, 1% ammonium molybdate in 1 N HCl; Solution E: 2% sodium citrate, 2% sodium meta-arsenite, 2% acetic acid; Buffer D (GDN buffer): HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol (DTT), 0.04% GDN (2 CMC), and 1× Protease Arrest; Buffer E: HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol (DTT), 0.04% GDN; Buffer F (GC buffer): HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol (DTT), 20 mM CHAPS, 0.04% GDN, and 1× Protease Arrest; Buffer G: HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol (DTT), 0.04% GDN, 0.4 mg/ mL 1D4 peptide; Buffer H: HEPES/NaOH (pH 7.5), 150 mM NaCl, 5 mM MgCl\(_2\), 1 mM dithiothreitol (DTT).

3.2.2 DNA constructs

The genes ATP8A2 and CDC50A were in pcDNA3. The purification of ATP8A2 with its associated CDC50A protein was facilitated by the C-terminal nine amino acid 1D4 tag on ATP8A2 allowing immunoaffinity chromatography using the Rho-1D4 antibody. For insertion of ATP8A2 and CDC50A in pfastBac Dual plasmid, ATP8A2 and the plasmid was initially cleaved with NheI and KpnI. Instant sticky-end ligase master mix (NEB) was used to ligate the plasmid and insert the sequence following the protocol given by NEB for instant ligase. The ligated product was
transformed into *E. coli* DH5α cells for DNA extraction by E.Z.N.A. mini-prep (Omega Bio-tek). Upon successful insertion, checked by analysis on agarose gels, *CDC50A* and pfastBac Dual-*ATP8A2* was cleaved with BssHII and XbaI. Again, ligated product was transformed into *E. coli* DH5α cells for DNA extraction by E.Z.N.A. mini-prep (Omega Bio-tek).

The HEK F cells were co-transfected with *ATP8A2* and *CDC50A* in pcDNA3 plasmid using PEI-MAX as transfecting agent.

### 3.2.3 Cell growth and transfection

#### 3.2.3.1 Sf9 cells

Procedure given by ThermoFischer for Cellfectin II was used. Briefly, Sf9 cells were cultured at 27°C in the dark. Cells were counted once cell density reached 1.5 – 2.5 X 10^6 cells/mL. Approximately, 8 X 10^6 cells were plated on 6 well plate with 2 mL Grace’s Insect Medium. Eight µL of Cellfectin II was mixed with 100 µL of unsupplemented Grace’s Insect Medium. One µg bacmid DNA added to 100 µL of unsupplemented Grace’s Insect Medium by gentle pipetting. The solution was then incubated for 20 minutes at room temperature. The DNA-lipid mixture was added dropwise to the cells in each well at 27°C for 5 hours. The Grace’s Insect Medium and DNA mixture was replaced with fresh supplemented Grace’s Insect Medium and cells were incubated at 27°C for 72 hours. Passage 1 is designated at the end of this cycle. The baculovirus must be isolated from the medium by centrifuging at 500 X g for 5 minutes. The supernatant is stored at 4°C, protected from light. To amplify, or increase titer, P1 is used to infect 2 X 10^6 cells/ well.

#### 3.2.3.2 HEK F cell and co-transfection

The HEK F cells were cultured and split to 0.1 X 10^6 cells/ mL and 0.5 X 10^6 cells/ mL for transfection. For 100 mL of cell culture 100 µg of DNA (co-transfection of *ATP8A2* and *CDC50A*) and 200 µL PEI-MAX (1 mg/ mL) were mixed together and diluted with 10 mL of PBS (0.137 M
NaCl, 2.7 mM KCl, 0.01 M Na₂HPO₄, 1.8 mM KH₂PO₄) for 20 minutes at room temperature. The cell density was 1 X 10⁶ cells/mL.

3.2.3.3 HEK F cell growth

HEK F cells were grown in HEK Freestyle media grown in sterile volumetric flasks. Each flask had a volume between 0.2 and 0.32 of the total volume the flask. The cells were incubated at 37°C with shaking at 125 RPM. The HEK F cells were cultured and split to 0.1 X 10⁶ cells/mL and 0.5 X 10⁶ cells/mL for transfection.

3.2.4 Purification of ATP8A2

The expression, western blotting, and ATPase activity assays of ATP8A2-CDC50A were performed as described in Chapter 2.

The 48 h grown HEK F cells were harvested and counted using hemocytometer. The cells were pelleted at 1.5k RPM for 5 minutes and supernatant was aspirated. The cell pellet was quickly weighted and resuspended in Buffer H, at 1 mL of buffer/ 1 g of cell pellet. 10 mL solubilization buffer (buffer A, D, or F)/ 1 g of cell pellet was added. The cell mixture was sonicated on ice for 30 seconds and was further solubilized for 45 minutes at 4°C with stirring. The mixture was spun down at 40k RPM for 12 minutes at 4°C with a Beckman ultracentrifuge. The supernatant was applied to an immunoaffinity column consisting of the Rho-1D4 antibody coupled to Sepharose. The column was rotated at 4°C for 45 minutes. The column was subsequently washed with buffer B six times, 3 times the volume of beads, to remove unbound cellular content. The beads incubated with 500 μL of buffer G to elute bound proteins from the 1D4-column. Eluted sample was analyzed on a SDS-PAGE gel to measure protein concentration against BSA standards and to initially screen for contaminant bands. For samples solubilized in buffer D or F, the immunoaffinity column was washed with buffer E and eluted with buffer G, by the same protocol listed above.
3.2.5 Reconstitution of ATP8A2-CDC50A with NSP

A 10-20% continuous sucrose gradient was prepared. Differing concentrations of sucrose mixed with NSP was layered in a spin column and kept horizontally for 3 hours. ATP8A2-CDC50A complex in the respective detergent was spun on a 10-20% continuous sucrose gradient at 4°C for 15 hours at 37k RPM using SW41 rotor. 500 µL aliquots were removed from top to bottom of the tube and run on clear native-PAGE gels. The gel was analyzed on a Typhoon imager at 494 nm to detect the presence of fluorescein, present on each NSP, as a marker for the tertiary ATP8A2-CDC50A complex. Positive tubes are pooled together for activity assays and microscopic analysis of protein structure.

3.2.6 Activity assay

The purified ATP8A2-CDC50A complexes in buffer C, buffer G, and NSP reconstituted sample were analyzed by a previously described ATPase activity assay using a colorimetric method (Coleman, Kwok, and Molday 2009) developed from earlier method (González-Romo et al., 1992). Briefly, ~1 ng of purified protein was mixed with 5 mM ATP and 2.5 mg/ml lipid (a mixture of DOPC and DOPS combined at different predetermined ratios) to obtain a final volume of 25 µl. Both ATP and lipids were prepared in buffer B for samples eluted in buffer containing CHAPS and buffer E for samples eluted in buffer G and samples reconstituted in NSP. The reaction was carried out at 37°C for 30 min and terminated by addition of 25 µl of 12% SDS. The phosphate released from ATP hydrolysis was measured by treating the reaction mix with 75 µl of solution A for 5 min, followed by 120 µl of solution B. The intensity of the resultant color was measured from the absorbance at 850 nm in a SpectraMax M3 microplate reader (Molecular Devices). The measured intensity was compared with those of known phosphate concentrations on a standard curve to calculate the reaction velocity (µmoles of Pi released/minute/milligram of protein) for
each PS concentration used in the assay. The resultant data were fitted to a Michaelis–Menten equation to calculate the maximum reaction velocity ($V_{\text{max}}$) and PS-activation constant ($K_A$). Each measurement was recorded in triplicate, and every experiment was repeated at least four times independently.

3.2.7 TEM imaging

The purified protein in detergent was kept for imaging as well as NSP samples after reconstitution, after buffer exchange, and after concentration. The four differing samples were analyzed at 30 µg of protein/ 4 µL/ grid. The copper 400 mesh carbon films (Electron Microscopy Sciences) were used for TEM imaging. The grids were glow discharged by PELCO easiGlow system at 15 mA plasma current and 0.39 mBar pressure for 10 seconds. The grids were then quickly incubated with 4 µL of protein sample for 30 seconds and blot dried at the sides of the grid, preventing introduction of paper fibers from blotting paper. The grid is then washed with 50 µL water droplets twice and once with uranyl acetate, each time blotted dry. The grid is then plunged in 50 µL of 70.9 mM (pH 4.5) uranyl acetate for 30 seconds to again dry with blotting paper and then vacuum is used to evaporate most of excess liquid. The prepared grid is stored in darkness until screened on Tecnai G2 Twin TEM (FEI). The Tecnai G2 was used for imaging with 200 kV LaB$_6$ filament imaged by AMT 2K side mount CCD camera.

3.2.8 Cryo-EM imaging

The purified and concentrated protein was frozen onto Quantifoil R 2/2, 200 mesh copper grids (Electron Microscopy Sciences), glow discharged by PELCO easiGlow system at 15 mA plasma current and 0.39 mBar pressure for 10 seconds. The Vitrobot Mark VI (FEI) using parameters listed in Table 3.1 to plunge freeze protein sample onto grid. The plunge frozen grids were quickly moved to liquid nitrogen and were stored in cryo grid boxes with lids (Electron
Microscopy Sciences), stored in a liquid nitrogen dewar until ready to screen. The grids were analyzed with a Krios Titan cryo-TEM (FEI) equipped with a Falcon 3EC Direct Electron Detector (FEI) to check for concentration, aggregation, and orientation of protein sample on each grid.

Table 3.1. The Vitrobot parameters used for cryo-EM grid preparations. Vitrobot was constantly at 4°C and 70% humidity. All settings are adjustable on Vitrobot interface.

<table>
<thead>
<tr>
<th>Date</th>
<th>Grid Type</th>
<th>Blot Force</th>
<th>Blot Time (seconds)</th>
<th>Concentration (mg/mL)</th>
<th>Aggregation</th>
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</thead>
<tbody>
<tr>
<td>2018 12 21</td>
<td>QF 2X2</td>
<td>-10</td>
<td>2</td>
<td>0.15</td>
<td>Y</td>
</tr>
<tr>
<td>2019 01 21</td>
<td>QF 2X2</td>
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<td>2</td>
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<td>Y</td>
</tr>
<tr>
<td>2019 02 13</td>
<td>QF 2X2</td>
<td>25</td>
<td>0.5</td>
<td>0.45</td>
<td>N/A</td>
</tr>
</tbody>
</table>

3.3 Results

3.3.1 Choosing HEK F cells for production of ATP8A2

The study initially started by using Sf9 cells. Sf9 cells are insect cells, which can be transiently transfected to express the protein of interest, given the appropriate promoter regions within the plasmid. Since ATP8A2 and CDC50A must be co-expressed within a single cell for export out of the ER (Coleman et al., 2009), a plasmid that can accommodate two separate genes was required. The plasmid pFastBac Dual was selected because it has two baculovirus promoters, PH and p10. To transpose the two genes into bacterial plasmid, DH10 EMBacY cells were used. The advantage of using DH10EMBacY cells is the inclusion of a YFP reporter gene for easy monitoring of virus performance and protein production after transfection of insect cells. Two trials of ATP8A2 and CDC50A expression were attempted; however, no fluorescent signal from YFP expression was observed under a confocal microscope. Due to difficulties in transfection and infection of Sf9 cells, HEK F cells were chosen as an alternate high expression system for co-expressing ATP8A2 and CDC50A.
3.3.2 Optimizing Solubilizing Buffer Content

ATP8A2 and CDC50A both are membranous proteins, and thus have hydrophobic regions in their tertiary structure. Therefore, solubilizing cell membranes and keeping the target protein as a protein-lipid-detergent complex is important to keep it in its active-tertiary state. The ATP8A2-CDC50A complex has been solubilized in 20 mM CHAPS from HEK 293T cells as previously reported (Chalat, Moleschi, and Molday 2017; Coleman, Kwok, and Molday 2009) and described in Chapter 2. However, since an aim of this study was to develop a high expression system for membrane proteins to recover milligram amounts of protein, optimization of protein purification had to be further investigated. One condition tested was the optimization of the solubilization buffer. GDN detergent was selected for protein microscopic analysis by TEM. Thus, the solubilization of cells at 0.1% GDN was of interest. As well, the mixture of CHAPS and GDN together was also tested.

Solubilization of HEK F cells exclusively in GDN did not offer high solubilization from the HEK cells. However, solubilization of HEK F cells in 20 mM CHAPS and 0.04% GDN (GC buffer) gave marginal difference between cells that were solubilized exclusively in 20 mM CHAPS and 0.1% GDN (Fig. 3.1).
Figure 3.1. Coomassie blue stain of ATP8A2-CDC50A, purified in differing detergents. The expression and purified ATP8A2 and CDC50A from HEK F cells. The CHAPS sample is solubilized in buffer A (20 mM CHAPS) and eluted in buffer E (10 mM CHAPS). The GDN sample is solubilized in buffer B (0.1% GDN) and eluted in buffer F (0.04% GDN). The GC sample is solubilized in buffer C (20 mM CHAPS and 0.04% GDN) and eluted in buffer F (0.04% GDN). Solubilization of HEK F cells occurred for 45 minutes after 30 second sonication on ice, spun at 4°C and was purified on Rho-1D4 immunoaffinity column.

3.3.3 Activity for ATP8A2-CDC50A in CHAPS and GDN

The purified ATP8A2-CDC50A complex in buffer G, buffer H, and NSP reconstituted sample were analyzed following the protocol described in Chapter 2. The assays were conducted so that proteins were analyzed in same detergent as used for eluting (Fig. 3.2).
Figure 3.2. The ATPase activity of purified ATP8A2-CDC50A complex, solubilized and purified in different detergents. The activity of the samples was measured as a function of PS concentration in presence of 5 mM ATP. The CHAPS sample is solubilized in buffer A and eluted in buffer G. The GDN sample is solubilized in buffer B and eluted in buffer H. The GC sample is solubilized in buffer C and eluted in buffer H. NSP sample is solubilized in buffer C and eluted in buffer H and reconstituted in NSP. The ATPase activity of ATP8A2-CDC50A complex in different solutions and reconstitution titrated against increasing concentrations of PS in the presence of 5 mM ATP, fitted to Michalis-Menten equation to calculate maximal reaction rate ($V_{\text{max}}$) and half-maximum activation constant ($K_A$). Measurements were done in triplicates.

The activity of the ATP8A2-CDC50A complex solubilized in GC buffer (buffer C) had the highest $V_{\text{max}}$. To question whether the detergent affected the activity of the protein, cells solubilized in 20 mM CHAPS were tested in either 10 mM CHAPS or 0.04% GDN (Fig. 3.3). The ATPase activity assay done in CHAPS detergent and GDN detergent displayed a similar $V_{\text{max}}$ (Fig. 3.3). Furthermore, the detergent used to solubilize the HEK F cells determined the $V_{\text{max}}$ of the protein sample and the detergent which the ATPase activity assay was conducted in did not affect $V_{\text{max}}$ nor $K_A$. 

<table>
<thead>
<tr>
<th>Detergent</th>
<th>$V_{\text{max}}$ (mol ATP/min/mg)</th>
<th>$K_A$ (µM)</th>
<th>N=</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPS</td>
<td>64.38 ± 2.24</td>
<td>24.87 ± 3.22</td>
<td>10</td>
</tr>
<tr>
<td>GDN</td>
<td>38.46 ± 6.00</td>
<td>2.51 ± 4.07</td>
<td>3</td>
</tr>
<tr>
<td>GC</td>
<td>140.7 ± 9.2</td>
<td>16.65 ± 4.48</td>
<td>3</td>
</tr>
<tr>
<td>NSP</td>
<td>13.39 ± 1.92</td>
<td>2.13 ± 3.57</td>
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</table>
Figure 3.3. The ATPase activity assay of ATP8A2 solubilized and tested in different detergents. The ATPase activity of purified ATP8A2-CDC50A complex measured as a function of PS concentration in presence of 5 mM ATP. The ATPase activity of ATP8A2-CDC50A complex in different solutions and measured against increasing concentrations of PS in the presence of 5 mM ATP. The data were fitted to Michalis-Menten equation to calculate maximal reaction rate ($V_{\text{max}}$) and half-maximum activation constant ($K_{A}$). Measurements were done in triplicates.

3.3.4 Imaging ATP8A2-CDC50A under TEM and cryo-TEM

An aliquot of the purified ATP8A2-CDC50A sample was stained for imaging proteins purified in buffer E and F by TEM. Subsequent samples were collected for TEM imaging: NSP reconstituted ATP8A2-CDC50A complex, NSP reconstituted ATP8A2-CDC50A complex after buffer exchange, and concentrated NSP reconstituted sample. The complex in CHAPS (Fig 3.4A) was poorly resolved compared to those in GDN (Fig 3.4B, C) because of the nature of the detergent under TEM. Protein samples in GDN displayed better contrast under the TEM microscope (Fig 3.4B, C). However, the NSP-reconstituted ATP8A2-CDC50A, having no detergent, had better contrasted image (Fig 3.4D).
The sample lysed in 0.1% GDN and eluted in 0.04% GDN is shown in Figure 3.4B. Compared to other images, this sample has spherical objects; the other images showed molecules which are more elongated and tapered in one direction (Fig. 3.4D). The spherical objects may be aggregated ATP8A2 which has 1D4 tag at its C-terminus to elute from column. This observation may be explained by the fact that a lower concentration of ATP8A2-CDC50A was eluted, compared to other solubilization mixtures (Fig. 3.1) in which a significant fraction of the protein was lost due to aggregation. Only those that are aggregated but expose the 1D4 at the surface are purified. The assumption that the 0.1% GDN solubilized sample is aggregated is also further supported by the fact that this sample had low activity (Fig. 3.2). Moreover, the use of a suitable detergent to lyse cells and to expose membrane proteins into lipid-detergent environment is important.

Samples that did not aggregate and were at a relatively high concentration after purification were examined by cryo-EM (Fig. 3.5). Screening of the frozen grids showed that the particles were not well dispersed and were aggregated near the edge of holes. Further studies are needed to express and purify more protein and limit aggregation for data collection.
Figure 3.4. Raw-TEM images of differently prepared ATP8A2-CDC50A sample. TEM images of ATP8A2-CDC50A complexes in different solutions: A) 0.01 M CHAPS sample solubilized in 0.02 M CHAPS, B) 0.04% GDN sample solubilized in 0.1% GDN, C) 0.04% GDN sample solubilized in 0.02 M CHAPS and 0.04% GDN, and D) NSP reconstituted sample solubilized in 0.02 M CHAPS and 0.04% GDN. Images were taken using FEI TECNAI G20.
Figure 3.5. Raw-cryo-EM images of NSP reconstituted ATP8A2-CDC50A during screening. Samples were reconstituted by NSP after purification by detergent. Scale bar: 100 nm. A) The reconstituted sample was frozen on 2018 12 21 at a concentration of 0.15 mg/ mL. B) The reconstituted sample was frozen on 2019 01 21 at a concentration of 0.3 mg/ mL.

3.4 Discussion

The Na\(^+\)/K\(^+\)-ATPase and Ca\(^{2+}\)-ATPase (Toyoshima et al, 2000) are two out of a handful of P-type ATPases which have been structurally elucidated in the past. The information from P\(_2\)-ATPases can be used to theorize structure and mechanism of P\(_4\)-ATPases due to the predicted domain structure and conserved motifs for phosphorylation and dephosphorylation. However, it is challenging to understand the mechanism of ATP8A2 activity, due to its specificity for a large substrate. Therefore, ‘credit-card,’ the ‘non-canonical,’ the ‘hydrophobic gate peripheral pathway,’ and the ‘merge of structures’ models have been theorized (Andersen et al, 2016).

To understand how ATP8A2 transports such a large substrate, structural analysis of the P\(_4\)-ATPase is required. For structural analysis of proteins, X-ray crystallography was widely used for multiple decades. Microscopy has been developed to be able to take images of proteins using TEM for structural determinations. A milligram quantity of protein is still required for protein structure
analysis. The development of methods to harvest the P$_4$-ATPase, ATP8A2-CDC50A, in a scaled-up preparation was the focus of this study.

The study initiated by using Sf9 insect cells as a high expression system to produce the ATP8A2-CDC50A complex. However, after multiple attempts to transflect and infect insect cells, a different cell line was used to grow the complex. The adherent cell line, HEK 293T cells was previously used to grow ATP8A2-CDC50A complex (Coleman, Kwok, and Molday 2009; Kwok et al. 2008). An alternative cell line is the HEK 293 F cells, which are suspension cells. Adherent cell lines are limited to growth within the surface of the area on an artificial substrate. However, suspension cell growth is limited to concentration of cells in the medium. Therefore, more transfected cells can grow within a given volume.

The ATP8A2-CDC50A complex, since a membrane protein, is difficult to solubilize from cell membrane whilst retaining its integrity as a functional protein. Different buffers have been tested for optimal cell lysis. CHAPS was previously used for HEK 293T cells (Coleman, Kwok, and Molday 2009), thus, CHAPS was tested for optimization. Three different detergent mixtures have been prepared: buffer A (20 mM CHAPS), buffer B (0.1% GDN), and buffer C (20 mM CHAPS and 0.04% GDN). Buffer C resulted in a higher recovery of ATP8A2 (Fig. 3.1). After cell lysis and purification, ATP8A2-CDC50A complexes have been checked using Coomassie blue stained SDS-PAGE gels, Western blots, and activity assay prior to analysis by microscopy. The reconstitution of the membrane protein complex in NSP was done in collaboration with Dr. Franck Duong’s lab.

Although recovery may be higher, the protein may no longer be functional. Therefore, the ATPase activity assay of protein samples was conducted. Samples lysed in buffer A and buffer C displayed similar activity values (Fig. 3.2). However, samples reconstituted in NSP and samples
lysed in buffer B displayed poor ATPase activity. NSP reconstituted samples likely had no ATPase activity due to NSP peptides limiting from normal protein movement.

The difference of $V_{\text{max}}$ between the CHAPS and GC samples was of interest (Fig. 3.2). Two possibilities to result in the differences was anticipated: first, the detergent may alter the tertiary structure to result in higher catalysis of enzyme reaction. Second, the detergent may be affecting how the protein interacts with its substrates to cause the samples in GDN to be hyperactive compared to those in CHAPS. The ATPase activity of a protein sample extracted from cells using buffer A and purified and eluted in buffer E was tested in either 10 mM CHAPS or 0.04% GDN. Similar levels of $V_{\text{max}}$ and $K_A$ resulted (Fig. 3.3). Therefore, the allosteric binding of either ATP or phospholipid may have been altered to result in a hyperactive enzyme, yet not affecting its affinity for its substrates.

The purity and activity of each protein sample was analyzed. Those samples which have passed these stringent tests were then analyzed under the TEM. Cells lysed in different detergents have been analyzed under TEM, which the structure displays a globular ring and a tail (Fig. 3.4). For further structural studies of ATP8A2-CDC50A complexes to be conducted in detergent should use GDN medium (Fig. 3.4B and C) for best contrast, compared to CHAPS (Fig. 3.4A). Whereas, samples without any detergent in its medium was best in terms of contrast and clarity (Fig. 3.4D). Which is one of the benefits of utilizing peptidisc reconstitution, isolating proteins free from detergents. The cells lysed in 0.1% GDN and eluted in 0.04% GDN is shown in Figure 3.4B. This sample lacks the ring and tail, observed in other samples. Therefore, aggregation may have occurred to render a non-functional protein, also may be the reason for lower expression levels (Fig. 3.1). Therefore, the low degree of ATPase activity observed for sample in Figure 3.4B (Fig. 3.2) is likely because most proteins eluted is aggregated. However, there is also the possibility of
observing lipid micelles from having too high of a CMC in the buffer. Therefore, ATP8A2-CDC50A complex is sensitive to the detergent used for cell lysis.

Protein samples stained on TEM grids which are monodisperse (Fig. 3.4) were found to be aggregated under cryo-EM (Fig. 3.5). The samples on each cryo-EM grid were between concentrations of 0.15 – 0.40 mg/mL (Table 3.1). Samples that were not aggregated on TEM grids aggregated on cryo-EM grids. Particles were visible but aggregated towards the edge of holes. A few possible adjustments can be made if one were to pursue structural studies with NSP reconstitution. These include varying the Vitrobot settings for freezing grids, type of grids used, and settings for glow discharge of grids. Also, one can further analyze different detergents for cell lysis, to optimize cell lysis and protein recovery.
Chapter 4: Summary and future directions

The P4-ATPase, ATP8A2, was studied. Severe neurological phenotypes including hypotonia, cognitive impairment, and hyperkinetic movement disorders have been observed in all patients with mutations in ATP8A2, some phenotypic traits such as progressive optic atrophy, hearing loss, dystonia, and cerebral and cerebellar atrophy were only observed in some patients (McMillan et al, 2018). In chapter 2, six human disease-causing mutants of ATP8A2 have been characterized. An expression assay using CHAPS and SDS solubilization was carried out to determine relative expression of each disease-causing mutant. The mutants K429N, A544P, R625W, and W702R expressed poorly. Immunofluorescence studies were undertaken to determine the localization of each mutant ATP8A2. As well, an extensive study on the K429 amino acid was performed showing the importance of the length and charge of the residue for proper folding and activity of ATP8A2. The activity assay was completed for disease-causing mutants which expressed near wild-type levels. These assays together distinguished between membranous protein exportable from ER but lacking in the ability to hydrolyze ATP and another class of proteins which are unable to export out of the ER due to misfolding.

Variation in these clinical features does are not appear to be associated with differences in activity of the ATP8A2 variants since all mutations result in a loss-in-function. Instead, clinical variations appear to be associated with other genetic differences in patients. One possibility is that other P4-ATPases with similar substrate specificities as ATP8A2 compensate for the loss in function of ATP8A2 in selective tissues. ATP8A1, ATP11A, ATP11B and ATP11C like ATP8A2 actively flip PS and PE across cell membranes (Takatsu et al, 2014). Interestingly, ATP8A1 and ATP8A2 share the same substrate, 67% identical in sequence, and ubiquitously expressed. The overlapping roles of ATP8A1 and ATP8A2 may also explain differences between mice and
humans. In mice ATP8A2 deficiency causes a significant decrease in visual function and photoreceptor survival, whereas loss in vision and photoreceptors has not been reported in patients deficient in ATP8A2 (Coleman et al, 2014; McMillan et al, 2018; Zhu et al, 2012). It is possible that ATP8A1 can fully compensate for the loss in ATP8A2 in human retina but not mice retina. Finally, the overlapping function of ATP8A2 and ATP8A1 is implicated in the analysis of knockout mice. ATP8A1 deficient mice appear generally normal except for some abnormal behavior traits and ATP8A2 deficient mice can live up to 6 months. However, loss of both P4-ATPases in mice results in neonatal lethality (Zhu et al, 2012).

Although ATP8A2 is known to transport PS and PE to generate membrane lipid asymmetry (Coleman et al, 2009), its role in neuronal cells is not well understood. However, previous studies have shown that ATP8A2 promotes axonal elongation in PC12 cells and hippocampal neurons (Xu et al, 2012). Furthermore, trafficking of transferrin receptors to the plasma membrane of neurons isolated from Atp8a2 knockout mice is significantly reduced (Lee et al, 2015). These studies suggest that ATP8A2 may function in the trafficking of key neuronal cell surface proteins via the endosomal recycling system. Interestingly, mutations in very low-density lipoprotein receptor (VLDLR) found on the surface of neuronal cells, like mutations in ATP8A2, are known to cause cerebellar ataxia, mental retardation, and disequilibrium (CAMRQ) syndrome (Moheb et al, 2008). Hence ATP8A2 may play a key role in trafficking of VLDLR to cell surfaces such that either reduction in function of ATP8A2 or VLDLR may result in similar disease phenotypes. Further studies are required to determine if ATP8A2 and VLDLR are linked through the same cellular trafficking pathway in neuronal cells.

Chapter 3 comprised of the investigation of high throughput production of co-expression of ATP8A2 and CDC50A for structural studies. This study focused on optimizations that should
be implemented to increase recovery of this purified protein. We found using 20 mM CHAPS with 0.04% GDN to lyse cells was most efficient. After NSP, reconstitution the ATP8A2-CDC50A was concentrated to 0.45 mg/ mL. Although samples looked monodisperse and well contrasted under TEM, aggregated proteins were only observable towards the edges of grid holes. To proceed with further attempts to image the ATP8A2-CDC50A complex, more proteins should be synthesized by scaling up suspension media to liter quantities, since this study only covered up to 400 mL of cell media. Another important aspect is to resolve the aggregation issue on cryo-EM grids. Proteins may be aggregating due to freezing but other causes such as precipitation of proteins at high concentration could be other factors to prevent a monodisperse sample on the cryo-EM grids.

The characteristics of ATP8A2 have been studied previously (Coleman, 2012). However, what is still lacking is the therapy that would be needed to treat patients who are diagnosed with diseases such as CAMRQ. However, in order to start answering this question, the structure of ATP8A2-CDC50A must be resolved and, further, the substrate recognition and mechanism of transport of this protein must be understood. To understand the pivotal residue which inhibits phospholipid transportation, other P₄-ATPases need to be characterized. A recent study of ATP11C deals with disease-causing mutants which show lower expression and activity levels resulting in PS exposure and anemia (Liou et al., 2019). Together, this wealth of knowledge will not only be valuable for the development of the therapeutic treatment of diseases linked to ATP8A2 but also for diseases linked to mutations in large substrate transporters.
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