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CELLULAR TRANSLOCATION AND FUNCTIONS OF FXYD1
(PHOSPHOLEMMAN) AND FXYD7

Advisor:
Prof. Haim Garty

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To my family for their endless support and encouragement.
And to Ori, who put-up with everything… and reminded me there is a world outside of the lab
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## List of abbreviation

<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
</tr>
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<tbody>
<tr>
<td>7PP</td>
<td>Chimera in which N terminus of FXYD7 is fused to the transmembrane and cytoplasmic domain of phospholemman.</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BGN</td>
<td>Benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside</td>
</tr>
<tr>
<td>C₁₂E₁₀</td>
<td>Polyoxyethylene 10 lauryl ether</td>
</tr>
<tr>
<td>CFP</td>
<td>Cyan fluorescent protein</td>
</tr>
<tr>
<td>Co-IP</td>
<td>Co-immunoprecipitation</td>
</tr>
<tr>
<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>Ethylene glycol tetraacetic acid</td>
</tr>
<tr>
<td>ENaC</td>
<td>Epithelial sodium channel</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FRAP</td>
<td>Fluorescence recovery after photobleaching</td>
</tr>
<tr>
<td>FXYD</td>
<td>Phenylalanine-X-Tyrosine-Aspartate</td>
</tr>
<tr>
<td>GAPDH</td>
<td>Glyceraldehyde 3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>H1299</td>
<td>Human non small cell lung carcinoma cell line</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin A</td>
</tr>
<tr>
<td>HEPES</td>
<td>4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>MW</td>
<td>Molecular weight</td>
</tr>
<tr>
<td>M1</td>
<td>M1-CCD cell line (mouse cortical collecting duct)</td>
</tr>
<tr>
<td>NaK</td>
<td>Na⁺/K⁺ ATPase</td>
</tr>
<tr>
<td>NCX1</td>
<td>Na⁺/Ca²⁺ exchanger isoform 1.1</td>
</tr>
<tr>
<td>PAGE</td>
<td>Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLM</td>
<td>Phospholemman</td>
</tr>
<tr>
<td>PMSF</td>
<td>Phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>Polyvinylidene fluoride</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PP7</td>
<td>Chimera in which the N terminus and transmembrane domains of phospholemman are fused to the C terminus of FXYD7</td>
</tr>
<tr>
<td>ROI</td>
<td>Region of interest</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
<tr>
<td>YFP</td>
<td>Yellow fluorescent protein</td>
</tr>
</tbody>
</table>
Abstract

FXYD proteins are a group of seven short single-span transmembrane proteins named after an invariant FXYD motif. They all interact with the Na⁺/K⁺ ATPase and modulate its kinetic properties. The current study characterized intracellular trafficking of two family members FXYD1 (phospholemman, PLM) and FXYD7. Our initial observations were that surface expression of PLM in Xenopus oocytes requires co-expression with the Na⁺/K⁺ ATPase. On the other hand the Na⁺/Ca²⁺ exchanger, another PLM interacting protein could not drive it to the cell surface. The Na⁺/K⁺ ATPase dependent surface expression of PLM could be facilitated by either phosphorylation mimicking mutation of T69 or by truncating three terminal arginine residues, suggesting a role of charged residues in the C-tail of PLM in the interaction with Na⁺/K⁺ ATPase. Next, we have showed that unlike PLM, FXYD7 can translocate to the cell surface of xenopus oocytes independently of the pump. Studying the differences between the two FXYD proteins demonstrated that the ecto-domain of FXYD7 is responsible for the Na⁺/K⁺ ATPase-independent membrane translocation. This domain has three conserved threonine residues (T3, T5 and T9) which are thought to undergo O-glycosylation. Mutating these residues to alanine, abolished surface expression of FXYD7, indicating the importance of O-glycosylation in membrane trafficking of this protein. Subsequent experiments in mammalian cells confirmed the role of T3, T5 and T9 in surface expression and demonstrated that FXYD7 protein in which these residues were mutated into A is trapped in the Golgi apparatus. Different approaches have been employed to explore the possibility that in mammalian cells too FXYD7 resides in the plasma membrane not associated with the Na⁺/K⁺ ATPase. Data did not support this possibility, yet evidences were obtained for FXYD7 functions in addition to modulation of the pump kinetics.
Introduction

FXYD proteins

FXYD is a family of seven single-span transmembrane proteins termed after the invariant extracellular motif Phe-Xxx-Tyr-Asp (Sweedner and Rael, 2000). They all were shown to specifically interact with the Na⁺/K⁺ ATPase and modulate its kinetic properties (for review see (Garty and Karlish, 2006; Geering, 2006). Thus, a common notion is that FXYD proteins are tissue specific regulators, or auxiliary subunits of the Na⁺/K⁺ ATPase, whose role is to adjust its kinetic properties to the needs of a specific tissue or physiological state without affecting it elsewhere. Table 1 summarizes the known effects of FXYD proteins on the Na⁺/K⁺ ATPase kinetics. In addition to their effects on the pump kinetics FXYD protein where shown to largely stabilize the active conformation of the Na⁺/K⁺ ATPase against heat inactivation and presumably other physiological challenges (Lifshitz et al., 2007; Mishra et al., 2011). Other Na⁺/K⁺ ATPase independent functions of FXYD proteins such as forming or modulating ionic channels and participating in cell adhesion, were suggested as well but are less characterized (Garty and Karlish, 2006).

Figure 1 sequence alignment of rat FXYD proteins.

The conserved FXYD motif is marked by an arrow. The transmembrane domain is marked by a horizontal black line. The cleavage sites of the signal peptide in FXYD1 and FXYD4 are marked by vertical red lines. FXYD5 has additional 100 amino acids not shown in the figure and the protein segment shown in this alignment starts at position 101. Alignment was done using PRALINE (Simossis and Heringa, 2005).
<table>
<thead>
<tr>
<th>FXYD</th>
<th>Experimental system</th>
<th>Kinetic parameter</th>
<th>Fold effect of FXYD protein as compared to control</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLM</td>
<td>Xenopus oocytes$^a$</td>
<td>$K_{1/2}^{Na^+}$</td>
<td>1.8 ($\alpha 1$), 1.51 ($\alpha 2$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{K^+}$</td>
<td>$\leq 1.36$ ($\alpha 1$), 1.42 ($\alpha 2$)</td>
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<tr>
<td></td>
<td></td>
<td>$V_{max}$</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td>Choroid plexus, neutralizing Ab</td>
<td>$K_{1/2}^{Na^+}$</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V_{max}$</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>KO mouse-cardiac SLM</td>
<td>$K_{1/2}^{Na^+}$</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$V_{max}$</td>
<td>2.14</td>
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<tr>
<td></td>
<td>Cardiac SLM</td>
<td>$V_{max}$</td>
<td>2.8 ($P$-PLM)</td>
</tr>
<tr>
<td></td>
<td>Cardiac myocytes</td>
<td>$V_{max}$</td>
<td>1.36 ($\alpha 1$) ($P$-PLM)</td>
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<tr>
<td>PLMS</td>
<td>Proteolytic digestion</td>
<td>$V_{max}$</td>
<td>1.4</td>
</tr>
<tr>
<td>$\gamma$</td>
<td>Xenopus oocytes</td>
<td>$K_{1/2}^{K^+}$</td>
<td>$\leq 0.7-0.8$ ($\gamma a$ or $\gamma b$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{Na^+}$</td>
<td>No change</td>
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<td>Renal NaK neutralizing Ab</td>
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<tr>
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<td>$K_{1/2}^{ATP}$</td>
<td>0.5 ($\gamma a$ or $\gamma b$)</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{ATP}$</td>
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<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{Na^+}$</td>
<td>1.48 ($\gamma a$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{K^+}$</td>
<td>1.56 ($\gamma a$)</td>
</tr>
<tr>
<td></td>
<td></td>
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<td>1.58 ($\gamma b$)</td>
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<td></td>
<td>1.61 ($\gamma a$)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>No change ($\gamma a$ or $\gamma b$)</td>
</tr>
<tr>
<td>Mammalian cells</td>
<td>$K_{1/2}^{ATP}$</td>
<td>0.78 ($\gamma b$)</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{K^+}$</td>
<td>0.64</td>
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<tr>
<td>Reconstituation with $\gamma$ peptide</td>
<td>$K_{1/2}^{Na^+}$</td>
<td>2-3</td>
<td></td>
</tr>
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<td>KO mouse. Kidney membranes</td>
<td>$K_{1/2}^{Na^+}$</td>
<td>1.33</td>
<td></td>
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<td>$K_{1/2}^{K^+}$</td>
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</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{ATP}$</td>
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<td>Xenopus oocytes</td>
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<td>0.64</td>
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<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{K^+}$</td>
<td>1.55</td>
</tr>
<tr>
<td>Mammalian cells</td>
<td>$K_{1/2}^{Na^+}$</td>
<td>0.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{K^+}$</td>
<td>No change</td>
</tr>
<tr>
<td>RIC$^c$</td>
<td>Xenopus oocytes</td>
<td>$V_{max}$</td>
<td>$\geq 2$</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{K^+}$</td>
<td>No change</td>
</tr>
<tr>
<td>FXYD7</td>
<td>Xenopus oocytes</td>
<td>$K_{1/2}^{Na^+}$</td>
<td>No change</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{K^+}$</td>
<td>1.3-1.9</td>
</tr>
<tr>
<td>Mat-8</td>
<td>Xenopus oocytes</td>
<td>$K_{1/2}^{Na^+}$</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>$K_{1/2}^{K^+}$</td>
<td>1.15-1.4</td>
</tr>
</tbody>
</table>

**Table 1.** Effect of FXYD proteins on Na$^+$/K$^+$ ATPase activity. Adapted from (Garty and Karlish, 2006)

$K_{1/2}^{K^+}$ refers to extracellular K$^+$ and $K_{1/2}^{Na^+}$ to intracellular Na$^+$.

$^a$Figures quoted for $K_{1/2}^{K^+}$ measured in *Xenopus* oocytes are in the presence of extracellular Na$^+$. The effect of PLM and FXYD7 also depend on the $\alpha$ isoform used.

$^b$In this study, the $\gamma$ subunit was expressed as a doublet in some experiments, which showed effects on both $K_{1/2}^{Na^+}$ and $K_{1/2}^{K^+}$, or as a single band in other experiments, which showed only the $K_{1/2}^{K^+}$ effect.

$^c$. (Lubarski et al., 2007)
**PLM**

PLM is primarily expressed in cardiac myocytes and skeletal muscle (Chen et al., 1997; Palmer et al., 1991). It has been identified as the major plasma membrane PKA and PKC substrate in these tissues and hence, named phospholemman. In *Xenopus* oocytes PLM specifically interacts with the Na⁺/K⁺ ATPase and lowers its affinity towards cell Na⁺ (Crambert et al., 2002). Such a decrease will increase intracellular Na⁺ leading to an increase of cytoplasmic Ca²⁺ by lowering the driving force for Na⁺/Ca²⁺ exchange across the plasma membrane, and hence increase contractility. In addition, PLM directly interacts with the cardiac Na⁺/Ca²⁺ exchanger (NCX1) and inhibits its function (Ahlers et al., 2005; Zhang et al., 2006; Zhang et al., 2009). Hearts of PLM knockout mice exhibit a depressed contractile function associated with a mild cardiac hypertrophy. There is also an increase in Na⁺/K⁺ ATPase activity (Bell et al., 2008). The intracellular carboxy tail of PLM is phosphorylated by both PKA and PKC on at least three residues: S63, S68 and S/T69. Phosphorylation of these residues mediates effects of β adrenergic agonists and plays a role in the structural and functional interactions with both Na⁺/K⁺ ATPase and NCX1 (Despa et al., 2005; Fuller et al., 2009; Han et al., 2006; Silverman et al., 2005).

**FXYD7**

FXYD7 is a brain specific FXYD protein expressed in astrocytes and possibly neurons (Beguin et al., 2002; Peng et al., 2010). In *Xenopus* oocyte it doubles K₁/₂ to extracellular K⁺ with no apparent effect on the Na⁺ affinity of the pump (Beguin et al., 2002; Crambert et al., 2004). A decreased affinity to extracellular K⁺ enables efficient clearance of excess potassium from the extracellular milieu, while decreasing intracellular Na⁺, following neuronal excitation. FXYD7 undergoes post-translational modification, presumably O-glycosylation, on three extracellular N-terminal threonine residues. In has been reported that these modifications play a role in protein stability but not on its surface expression and interaction with the Na⁺/K⁺ ATPase (Beguin et al., 2002; Crambert et al., 2004).
The Na⁺/K⁺ ATPase

The Na⁺/K⁺ ATPase is an integral membrane protein crucial for maintenance of the high Na⁺ and K⁺ gradients across the plasma membrane, by pumping three Na⁺ ions out and two K⁺ ions into the cell, a process that involves hydrolysis of one ATP molecule per cycle. The Na⁺ gradient across the cells membrane provides a driving force secondary transporters such as Na⁺/H⁺ and Na⁺/Ca²⁺ exchange or Na⁺-glucose co-transport. The minimal functional unit is a αβ heterodimer. Four α and three β isoforms expressed in a tissue specific manners have been identified in humans. The catalytic α subunit has 10 transmembrane domains (M1-M10), with both its N and C termini located in the cytoplasm, and is responsible for ion and ATP hydrolysis. The β subunit is a heavily glycosylated, single transmembrane protein. The β subunit act as a chaperone, and is crucial for the correct folding of the α subunit as well as facilitating the transport of the αβ complex to the cell surface (Geering, 2001; Geering, 2008). The β subunit also modulates some of the functional properties of the Na⁺/K⁺ ATPase, such as affecting the apparent K⁺ affinity and K⁺ occlusion (Geering, 2001; Lutsenko and Kaplan, 1993). As mentioned above, FXYD proteins act as tissue specific regulators of the Na⁺/K⁺ ATPase. Recently, the crystal structure of the Na⁺/K⁺ ATPase has been elucidated, and light was shed on the interaction between the pump and the FXYD protein family (Shinoda et al., 2009). It has been found that the extracellular FXYD motif interacts with both the α and β subunits, thereby stabilizing the αβ complex. The transmembrane segment of FXYD interacts exclusively with M9 of the Na⁺/K⁺ ATPase. It is yet not fully understood how different FXYD proteins affect the kinetic properties of the Na⁺/K⁺ ATPase.

The Na⁺/Ca²⁺ exchanger

NCX1 is a membrane protein with 9 transmembrane segments, and a large intracellular look located between the 5th and 6th transmembrane domains. Segments 2,3 and 7 of the protein are involved in ion transport, while the intracellular loop contains the Ca²⁺ binding domain (Philipson and Nicoll, 2000). The exchanger transports three Na⁺ ions in exchange to a single Ca²⁺ ion. The the transport process is bi-directional; it can either extrude Ca²⁺ or bring Ca²⁺ into the cell. Yet, under
physiological conditions, it mainly removes \( \text{Ca}^{2+} \) from the cells (Bers, 2002). Regulation of the exchanger by PLM is mediated by interactions between the cytoplasmic tail of PLM and two regions within the intracellular loop of NCX (residues 238-270 and 300-328 of the exchanger) (Zhang et al., 2009).

**Regulation of the \( \text{Na}^+/\text{K}^+ \) ATPase and \( \text{Na}^+/\text{Ca}^{2+} \) exchanger by PLM**

Unlike other members of the FXYD family, PLM has three phosphorylation sites at its cytoplasmic domain (S63, S68, S/T69). These phosphorylation sites have been shown to be crucial for the ability of PLM to modulate the kinetics of the pump. Unphosphorylated PLM inhibit the \( \text{Na}^+/\text{K}^+ \) ATPase by reducing its apparent affinity towards intracellular \( \text{Na}^+ \) in cardiac myocytes, and also reduces the apparent affinity to extracellular \( \text{K}^+ \) when expressed in oocytes. Phosphorylation of PLM by PKA (S68) or PKC (S63, S68, S/T 69) relieves the inhibitory effect of PLM on the pump. Recently, PLM has been shown to undergo palmitoylation at C40 and C42. Increased palmitoylation was observed following PKA phosphorylation at S68. It was further showed that inhibition of PLM palmitoylation abolished the inhibitory effect of PLM on the \( \text{Na}^+/\text{K}^+ \) ATPase (Tulloch et al., 2011).

PLM, phosphorylated at S68, interacts with the intracellular loop of NCX and inhibits the activity of the exchanger. Cheung et al (Cheung et al., 2010) suggests that PKA phosphorylation of PLM, during \( \beta \)-adrenergic stimulation of the heart, activates the \( \text{Na}^+/\text{K}^+ \) ATPase, thus leading to increased clearance of \( \text{Na}^+ \). This, in turn, minimizes the risk of cardiac arrhythmia. Simultaneous inhibition of the NCX, prevents extrusion of \( \text{Ca}^{2+} \) and preserves the ionotropy, which is necessary during the stress response (Cheung et al., 2010).
In my thesis I have characterized intracellular trafficking and cell surface expression in *Xenopus* oocytes and mammalian cells of both PLM and FXYD7. Initial oocyte studies have demonstrated that PLM interacts with the Na$^+/K^+$ ATPase intracellularly and can not reach the plasma membrane unless co-expressed with the Na$^+/K^+$ ATPase. FXYD7 on the other hand can traffic equally well to the cell surface with and without expression of Na$^+/K^+$ ATPase. Domains and residues involved in the intracellular trafficking of the two proteins were identified and studied both in oocytes and mammalian cells. Finally, characterization of FXYD7 expressed in the epithelial cell line M1-CCD suggested that this protein has roles in addition to modulating the kinetics of the Na$^+/K^+$ ATPase.
Research aims

The aim of my thesis was to study trafficking of FXYD proteins to the plasma membrane, and identify domains and residues that control their intracellular trafficking and cell surface expression. The work focused on two family members FXYD1 (phospholemman or PLM) and FXYD7.
Materials and Methods

cDNA clones and cRNA. N-terminally Hemeaglutin A (HA) tagged rat PLM (HA-PLM) was prepared by replacing its signal peptide with an HA epitope. HA-PLM served as a template for several HA-PLM mutants. The mutations introduced were: S63E/S68E, T69D, S63E/S68E/T69D and truncating the three terminal arginines (Δ3R). Mutations were created by a single PCR step, using PfuUltra High fidelity DNA polymerase (Stratagene, La Jolla, CA). HA-FXYD7 was constructed by inserting an HA epitope upstream the FXYD7 open reading frame (deleting the first ATG). HA-FXYD7 served as a template for the following HA-FXYD7 mutants: T3A, T5A, T9A, T3A/T5A and T3A/T5A/T9A. In addition a construct in which the conserved extracellular F, Y and D residues comprising the FXYD motif were changed into A was created. All constructs were verified by sequencing. For expression in Xenopus oocytes, the open reading frames of various cDNAs were subcloned between 5’ and 3’ sequences of Xenopus β-globin in pGEM or pBluescript-derived vectors. These include: the above FXYD constructs, NCX1.1 (a kind gift from Jonathan Lytton, University of Calgary), rat α1 and 6XHis tagged pig β1 Na⁺/K⁺ ATPase. The Xenopus β globin non-coding sequences are known to improve protein expression level in oocytes by increasing cRNA stability (Liman et al., 1992). cRNAs were synthesized from the T7 promoter of linearized plasmids, using T7 mMESSAGE mMACHINE (Ambion).

For expression in mammalian cells, HA-PLM and HA-FXYD7 were cloned into pIRES-hyg expression vector (clontech). HA-PLM, HA-FXYD7 and HA-FXYD7 T3/5/9A tagged at their C-terminus with CFP, were created by replacing the stop codon with an appropriate restriction site, and sub-cloning into pECFP-N1 vector. The CFP tagged constructs, were excised and inserted into pIRES-hyg. PLM/FXYD7 chimera in which the extracellular and intracellular domains of PLM (residues 1-37 and 59-92) were replaced by the corresponding FXYD7 regions (M1-K26 and V49-V80) were prepared using overlapping oligonucleotides. The ER marker, pDSRED2-ER was purchased from clontech.
**Antibodies.** A rabbit polyclonal antibody directed to the C-terminal sequence CRSSIRRLSTRRR of PLM was described previously (Crambert et al., 2002). A rabbit polyclonal antibody against the C-terminal region (R52–V80) of FXYD7, described in (Beguin et al., 2002), was a gift from Prof. Kathi Geering (University of Lausanne, Switzerland). A monoclonal antibody recognizing the N-terminus of the α subunit of the Na⁺/K⁺ ATPase (6H) was kindly provided by Dr. MJ. Caplan, Yale University School of Medicine. A mouse monoclonal anti HA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). A monoclonal antibody against β-tubulin was purchased from Sigma. A rabbit polyclonal antibody recognizing total ERK was a gift from Prof. Rony Seger, Weizmann institute of science, Israel). Rabbit polyclonal antibody recognizing Golgi reassembly stacking protein (GRASP65) was from Abcam (Cambridge, MA). Mouse monoclonal antibody directed at the intracellular loop of NCX1 (R3F1) was purchased from Swant (Bellinzona, Switzerland). Cy3 coupled anti rabbit and Cy5 coupled anti mouse secondary antibodies were from Jackson Immuno Research Laboratories (West Grove, PA).

**Cell culture and transfection.** H1299 and M1-CCD cells were cultured under standard conditions. Transfections were carried out using ICAFectin®441 (Eurogentec) or JetPei (Polyplus Transfection, France) according to manufacturers instructions. Stable clones were selected and isolated using 150 μg/ml hygromycin B (M1-CCD) or 500 μg/ml hygromycin B (H1299).

**Total and surface expression of FXYD proteins in Xenopus oocytes.** Stage V-VI oocytes were injected with 50 nl of cRNA mixtures corresponding to various FXYD constructs ± α1 and β1 subunits of the Na⁺/K⁺ ATPase or NCX1. Three days after injection, oocytes were washed three times with ND96 (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 5 mM HEPES pH 7.6, 1.8 mM CaCl₂, 2.5 mM sodium-pyruvate) and fixed with 4% formaldehyde for 20 minutes at 4°C. They were washed three more times with cold ND96, and blocked with 1% BSA in ND96 for 6-7 hours. Monoclonal mouse anti HA antibody was added to the blocking media (1:5000) and the oocytes were further incubated for overnight at 4°C. Next, they were washed extensively and a secondary, horseradish peroxidase (HRP) conjugated goat anti mouse IgG (1:5000),
was added for 2 hours at 4°C. Oocytes were washed extensively and divided among the wells of a 96 well plate (one oocytes per well). Enhanced chemiluminescence (ECL) substrate solution was added (100 µl/well) and cell surface expression was quantified using Fluor-S MAX MultiImager.

Total protein expression was determined in microsomes prepared from groups of 10 oocytes. Cells were homogenized in a buffer containing 83 mM NaCl, 1 mM MgCl₂ and 10 mM HEPES pH 7.9 supplemented with the protease inhibitors cocktail. Homogenates were centrifuged twice at 1000g for 10 minutes at 4°C to pellet the yolk and pigment, and the supernatant was collected. The supernatant was further centrifuged at 10,000g for 20 minutes at 4°C and the pellet was suspended in a buffer containing 30 mM histidine, 18 mM Tris and 5 mM EDTA, pH 7.4. Samples were resolved on Tricine gel and blotted onto PVDF membrane. Membranes were cut to low, medium and high MW region and hybridized with different antibodies according to the experimental design. The ECL signal was quantified using Fluor-S MAX MultiImager. In all cases the values obtained were within the linear detection range. Surface expressions of PLM were normalized to small changes of the total amount of PLM expressed (usually 10-15%). This is justified since under the experimental conditions used the surface expression is limited by the amount of PLM and not the amount of Na⁺/K⁺ ATPase or the ‘trafficking capacity’ of the cell (cf. Fig 2B). In the case of FXYD7 this was more problematic because this protein runs as multiple bands which may differ in their cellular location. However, all data reported are valid at least qualitatively without normalization to total expression. i.e. inhibition of FXYD7 or PLM by a particular treatment was apparent also under condition that this protein was expressed more than the control and vice versa.

**Tyrosine biotinylation of PLM expressed in *Xenopus* oocytes.** Three days after injecting the appropriate cRNA mixture, oocytes were washed three times with ND96. Surfacingly expressed proteins were labelled with p-diazobenzoyl biocytin (DBB), reactive towards the phenolic group of tyrosines and the imidazole group of histidines (Wilchek et al., 1986). DBB was synthesized shortly before the biotinylation as follows: Five mg of p-aminobenzoyl biocytin were mixed with 40 µl of DMSO followed by addition of 107 µl of ice cold 1 M HCl and allowed to dissolve for 30 min. One hundred forty seven microliters of ice-cold NaNO₂ (7.7 mg NaNO₂/ml
distilled water) were added and incubated for 5 min at 4°C. One hundred twenty-six microliters of 1 M NaOH were added to stop the reaction, after which 100 µl of 0.2 M sodium borate pH 8.4 were added. DBB was added to the oocytes at a final concentration of 0.5 mg/ml. Oocytes were incubated with the DBB for 1 hour at room temperature on a rotator. Unbound biotin was quenched using cold quenching buffer (0.1% BSA, in ND96), followed by 6 washes with ND96. Oocytes were homogenized on ice in 10 µl of oocytes homogenization buffer (20 mM Tris pH-7.4, 5 mM MgCl2, 5 mM Na2HPO4, 1 mM EDTA, 80 mM sucrose with protease inhibitors cocktail), by passing 10 times though a 27 G needle. The homogenate was centrifuged for 5 min at 200 g at 4°C to pellet the yolk. The resulting supernatant (yolk free homogenate) was centrifuged for 20 min at 14,000 g at 4°C to pellet the membrane fraction. The pellet was resuspended in T-BST (150 mM NaCl, 10 mM Tris pH-7.5, 1% Triton X-100) at 10 µl per egg, and incubated on ice for 40 min. The suspension was then centrifuged for 20 min at 14,000 g at 4°C and the supernatant collected. Streptavidin beads were washed 3 times with T-BST after which 30 µl of packed streptavidin beads were added to each sample for 1 hour at 4°C on a rotator. Then, the streptavidin beads were washed 3 times with T-BST, SDS-PAGE sample buffer was added and the samples were incubated at 85°C for 15 min.

**Tyrosine biotinylation of surface proteins in M1-CCD cells.** M1-CCD (0.2x10^6) cells were seeded on 12mm Transwell Permeable Support (Costar, pore size 0.4 μm). The cells were grown for 7 days with daily changes of medium until confluent high electrical resistance monolayers were formed. DBB was prepared immediately before use as described above and applied at a final concentration of 0.5 mg/ml. The cells were washed 3 times with ice cold PBS containing Ca^{2+} and Mg^{2+} (PBS++) to remove all traces of serum proteins. DBB was added to either the basolateral surface (lower compartment) or the apical surface (upper compartment) for 30 min at 4°C on a shaker. Free biotin was removed by incubating the cells 4 times for 2 minutes in an ice-cold quenching buffer (0.1% BSA, 100mM glycine in PBS++), followed by two washes in cold PBS++. The permeable supports were excised and cells scraped and lysed by a 25 min incubation in a lysis buffer (Tris, pH 7.4, 2 mM EDTA, 2 mM EGTA, 0.1% SDS and 1% triton x-100) s. Cell lysates were centrifuged at 14,000 rpm for 10 minutes to remove unbroken cells and nuclei, and the supernatant was collected. Protein concentration was determined by BCA assay (Thermo).
Streptavidin agarose beads were washed 3 times with lysis buffer, after which 30 µl of packed streptavidin beads were added to each sample for 1 hour at 4°C on a rotator. The streptavidin beads were washed 2 times with low salt buffer (50 mM Tris pH 7.4, 5 mM EDTA, 50 mM NaCl, 0.1% triton x-100), twice with high salt buffer (20 mM Tris pH 7.4, 500 mM LiCl and 0.1% triton x-100) and 2 times with buffer containing 10mM Tris pH 7.4. Bound proteins were eluted in x2 SDS-PAGE sample buffer containing 100 mM DTT for 20 min at 80°C. Proteins were resolved on 10% tricine gel and transferred to PVDF membrane. A similar protocol have been used for surface biotinylation of H1299 grown in 10 cm culture dishes by 1.5mg/ml NHS-SS-biotin.

**Confocal microscopy:** H1299 cells expressing YFP tagged α1 subunit of the Na⁺/K⁺ ATPase and various CFP-tagged FXYD constructs were seeded on Lab-Tek Chamber coverglass (Nunc). Twenty-four hours later, cells were visualized using scanning confocal microscope (Olympus FV1000) through a x60, oil immersion objective. M1-CCD cells were seeded in 12mm Transwell Permeable Support inserts (Costar, pore size 0.4 µm, 0.2x10⁶ cells/insert), and grown for one week. Cells were fixed for 30 minutes at room temperature in PLP buffer (2% paraformaldehyde, 75 mM L-lysine, 10mM Sodium-metaperiodate (McLean and Nakane, 1974)) and washed 4 times with PBS. Blocking and permeabilization was done in PBS containing 5% BSA + 0.05% saponin for 1 hour. The transwell permeable support was cut out and stained with the sequential application of: Rabbit anti FXYD7 (2 hours incubation, diluted 1:40), Cy3 coupled anti rabbit secondary antibody (1 hour incubation, 1:400,), mouse anti 6H (2 hours incubation, diluted 1:50) and Cy5 coupled anti mouse secondary antibody (1 hour incubation 1:400). Labelled samples were mounted onto slides using Immuno-Mount (Thermo-Shandon, Pittsburgh, PA) and visualized under the microscope. Cy3 was excited using 488 nm laser (emission 520) and Cy5 was excited using 635 nm laser (emission 670 nm)

**Fluorescence recovery after photo-bleaching (FRAP):** In FRAP experiments, cells were seeded on Lab-Tek chamber cover-glass 24 hours prior to the experiment. The imaging stage was pre-warmed to 37°C and CO₂ was supplied. Cells were imaged in the confocal microscope in complete medium. YFP and CFP were bleached in a tornado scan mode using 440 nm laser at full intensity for 0.3 sec. Images were
acquired every second for the first 10 seconds after bleaching and then every 30
seconds for a period of 5 minutes at which a new steady-state was reached. YFP was
excited at 515 nm and CFP at 440nm. Emissions were collected at 527 nm and 476
nm, respectively.

**FRAP analysis:** Kinetic analysis of fluorescence recovery has been done as follows.
Data from 10 kinetic measurements in two different cultures were averaged. The
recovered steady state fluorescence (usually ~80% of the original fluorescence) was
set to 1 and the fractional fluorescence changes were plotted against time. Data were
fitted to various kinetic models and the best fits were obtained for sum of two first
order reactions giving rise to two t_{1/2} values (R>0.99). Diffusion constants (D) were
calculated from t_{1/2} using the following equation (Woda et al., 1980):

\[ D = \frac{W^2}{4x(t_{1/2})} \]

Where D is the diffusion coefficient (in μm^2/sec), W is the radius of the bleached area
(3.105 μm) and t_{1/2} half recovery time (in sec).

**Cell adhesion assay:** M1-CCD cells were trypsinized and seeded on Lab-Tek
chamber cover-glass. After settling cells were visualized using a scanning confocal
microscope (Olympus FV1000) through a x20 objective. Imaging stage was pre-
warmed to 37°C and CO₂ was supplied. Images were taken every hour for 14 hours.
Quantification was preformed using ImageJ.

**Cell proliferation assay:** Cell proliferation rate was quantified by reduction of 2,3-
bis-(2-methoxy -4- nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT)
(biological industries). M1-CCD cells were seeded in 96 well plates. Forty-eight
hours after seeding, 50μl XTT reaction solution were added to half of the plates. Cells
were incubated with the reaction solution for 4 hours, after which absorbance was
measured at 450 nm. Non specific background was measured at 650 nm. A second
XTT assay was preformed on the remaining plates 72 hours after seeding. Relative
proliferation rates were calculated as OD_{450 nm} at 72h/OD_{450 nm} at 48h.
Transepithelial electrical resistance and voltage measurements: M1-CCD cells were seeded on 6 mm Millicell cell culture inserts (Millipore, Bedford, MA), and grown on PC-1 medium supplemented with 10% FCS and 5 µM Dexamethasone. Medium was changed daily and transepithelial resistance (R_{TE}) and voltage (V_{TE}) were measured right before the medium change using Epithelial Volt-ohmmeter (World Precision Instruments, Sarasota, FL). Short circuit current (I_{sc}) was calculated as the V_{TE}/R_{TE} ratio. At the end of the experiment, 10 µM amiloride were added to the apical side, in order to dissect contributions of the transcellular and paracellular pathways to R_{TE}.

Data analysis
Statistical analysis was preformed using paired or unpaired Student’s T test as justified.
Results

Surface expression of PLM and FXYD7 in *Xenopus* oocytes

Surface expression of FXYD proteins was measured in *Xenopus* oocytes injected with cRNA coding for PLM or FXYD7 tagged in their extracellular domain by an HA epitope. Intact oocytes were incubated with a monoclonal anti-HA antibody and the amount of specifically bound antibody was quantified by enhanced chemiluminescence.

**Figure 2.** Cell surface expression of FXYD proteins in *Xenopus* oocytes.

Groups of oocytes were either non injected (NI) or injected with 0.5ng or 2ng HA tagged FXYD cRNA ± 10ng α1 and 7ng β1 Na⁺/K⁺ ATPase (NaK) cRNA. Three days later cells were fixed, divided into individual wells of 96 well plate (single oocyte/well), and assayed for cell surface expression of the HA epitope by chemiluminescence. A. A representative experiment showing the ECL signal in oocytes injected with various cRNA mixtures. Bottom: Western blot of oocyte microsomes with anti α1 Na⁺/K⁺ ATPase and anti HA antibodies. FXYD7 migrates as at least three species that presumably correspond to different degrees of O-glycosylation (Beguin et al., 2002). B. Quantification of FXYD surface expression. Means ± SEM of 30-32 oocytes from 3 different frogs are depicted. Data were normalized to the mean chemiluminescence measured in oocytes from the same frog injected with PLM + Na⁺/K⁺ ATPase (=1).
Large difference in the amount of surfacely expressed PLM was apparent between oocytes injected with and without Na⁺/K⁺ ATPase (Fig. 2A). This difference is not likely to be due to rapid degradation of the FXYD protein expressed in the absence of the Na⁺/K⁺ ATPase. Western blotting with the anti HA antibody demonstrates that the steady state abundance of PLM is even higher in oocytes that do not express exogenous Na⁺/K⁺ ATPase. In contrast to the large effect of the Na⁺/K⁺ ATPase on PLM surface expression, FXYD7 was expressed on the oocyte surface at similar levels irrespective of the co-expression of exogenous Na⁺/K⁺ ATPase. The above observation was further established by averaging 30-32 oocytes from three different frogs in each group (Fig. 2B). Since the same antibody is being used to detect surface expression of PLM and FXYD7, the data are comparable and indicate that the amount of FXYD7 protein that reaches the membrane without exogenous Na⁺/K⁺ ATPase is similar to the amount of surface PLM expressed with the pump. The experiment also demonstrates that increasing the amount of injected cRNA from 0.5 ng to 2.0 ng proportionally increases surface expression of PLM. Thus, under the experimental conditions used, surface expression of this protein is limited by the amount of its cRNA and not by that of Na⁺/K⁺ ATPase or the cell’s trafficking capacity.

Since all FXYD proteins were shown to specifically interact with the Na⁺/K⁺ ATPase it is not surprising that the level of PLM surface expression strongly depends on co-expression of the Na⁺/K⁺ ATPase. It is well established that multimeric protein complexes assemble intracellularly and the individual subunits are usually unable reach the cell surface alone (Lee et al., 2004). Similar results were reported before for FXYD2 (γ) (Beguin et al., 1997), and also observed in our laboratory for FXYD4 and FXYD5 (unpublished data). The ability of FXYD7 to be transported to the cell surface equally well with and without Na⁺/K⁺ ATPase is somewhat surprising and may suggest other functions of this FXYD protein.

In the above experiment surface expression of FXYD proteins was measured as binding of antibody to extracellular HA epitope. In the case of PLM this involved eliminating the signal peptide, a manipulation that may affect intracellular trafficking. Therefore, we wanted to confirm the data of Fig. 2 by a second, independent approach using non-modified PLM. This has been done by surface biotinylation of intact oocytes and detecting the amount of PLM that could be precipitated by streptavidin
attached to agarose beads. The commonly used N-hydroxysuccinimide esters derivatives of biotin are reactive towards primary amino groups and will react with the ε-amino group of lysine residues and N terminal α amine. Since the extracellular domain of PLM lacks Lysines (human PLM contains one lysine residue in the extracellular domain, while the rest of mammalians PLM have no lysines), we were unable to significantly label surfacey expressed PLM with these biotinylating reagents. We could achieve some labelling of non-modified PLM using the Tyrosine and Histidine specific biotinylating reagent p-Diazobenzoyl biocytin (DBB) (Wilchek et al., 1986). DBB is a diazonium reagent containing biotin, no longer available commercially, which was kindly synthesized for us by Prof. Meir Wilchek (Weizmann Institute of Science, Rehovot, Israel). Diazonium salts react with phenol and imidazole groups and can be used to label tyrosines and histidines in proteins (Fig. 3). It has to be prepared shortly before use form its precursor p-aminobenzoyl biocytin, using HCl and sodium nitrite.

![DBB labeling of tyrosines and histidines](image)

**Figure 3.** DBB labeling of tyrosines and histidines. From reference (Wilchek et al., 1986).
Figure 4. Cell surface biotinylation of PLM.

Oocytes were injected with 2ng of HA-PLM or unmodified PLM ± 10 ng α1 and 7 ng β1 Na+/K+ ATPase (NaK). Three days letters oocytes were divided into two equal groups that were treated as follows (A) Oocytes were lysed, proteins resolved electrophoretically and blotted with an antibody to the carboxy tail of PLM. (a) HA-PLM, (b) HA-PLM+ NaK, (c) WT-PLM, (d) WT-PLM+ NaK. (B) Oocytes were biotinylated by a 60 min incubation with 0.5 mg/ml DBB at room temperature. They were lysed and biotinylated proteins were precipitated on streptavidin beads as described under Materials and Methods. Pulled down biotinylated proteins were then blotted with antibody to the carboxy tail of PLM. Arrows indicate positions of HA-PLM and PLM. The differences in size are due to the extra 9 amino acids of the HA epitope and different amounts of bound biotin. The streptavidin pulled down PLM is of slightly higher molecular weight due to the biotin moiety attached to it. The proteins in the microsomal fractions are of non-biotinylated oocytes.

As can be seen in Fig. 4, even with DBB the labelling was relatively weak since the ecto domain of rat PLM has only two Tyrosines and a single Histidine all located very close to the membrane surface. Nevertheless, a marked difference in the surface expression of PLM injected ± Na+/K+ ATPase was apparent. Thus, the large effect of Na+/K+ ATPase on surface expression of PLM is confirmed by biotinylation of unmodified PLM. In the case of HA-PLM labelling was much more intense since the HA epitope has three additional tyrosines located at a relatively larger distance from the membrane surface. DBB is cell impermeable, and does not label intracellular proteins, as demonstrated in subsequent experiment using M1 cells (see below).
Factors affecting PLM surface expression

Several studies have demonstrated inhibition of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (NCX1) by a direct interaction with PLM (Ahlers et al., 2005; Zhang et al., 2009). We therefore tested whether NCX1 can too drive PLM to the oocyte surface. However, unlike the Na\(^+\)/K\(^+\) ATPase co-expressing NCX1 with PLM was without effect on the surface expression of the FXYD protein (Fig. 5). Experiments in transfected cells and PLM knockout mice have demonstrated that inhibition of NCX1 requires phosphorylation of S68 and possibly S63 in the carboxy tail of PLM (Song et al., 2005; Zhang et al., 2006).

![Figure 5. Effects of NCX1 and phosphorylation mimicking mutations on surface expression of PLM.](image)

Oocytes were either non injected (NI) or injected with 2ng of either HA-PLM or HA-PLM construct in which S63 and S68 were mutated into glutamic acid (S63/68E). Part of the oocytes were injected in addition with cRNA for the α1 (10 ng) and β1 (7ng) subunits of the Na\(^+\)/K\(^+\) ATPase (NaK) or the 1.1 isoform of the cardiac Na\(^+\)/Ca\(^{2+}\) exchanger (NCX, 5ng). Total and surface expression of the various proteins was measured three days later. Right: microsomal proteins were resolved electrophoretically and the blot was cut to low, medium and high MW regions. These were blotted with antibodies to HA, β tubulin, and either the α subunit of Na\(^+\)/K\(^+\) ATPase or NCX1, respectively. Left: surface expression of the HA epitope in the same groups of oocytes. Data were normalized for small differences in the total expression of PLM and β tubulin measured in the above Western blot. Means ± SEM of data from 12 oocytes are depicted and expressed as fraction of the mean value in oocytes injected with PLM + NaK.

Since PLM expressed in oocytes is mostly unphosphorylated at these residues (Bibert et al., 2008), we tested expression of PLM mutant in which both serine residues were...
replaced by glutamic acid. These mutations were shown to mimic effects of PKA and PKC on the interaction of PLM with NCX1 (Zhang et al., 2006). However, they were without effect on surface expression of PLM in oocytes with or without NCX1 or Na⁺/K⁺ ATPase (Fig. 5).

To further explore possible PLM-NCX interactions we looked at the possible co-immunoprecipitation of the two proteins. Unlike the Na⁺/K⁺ ATPase, which can be co-immunoprecipitated (co-IP) with PLM from oocytes and cardiac myocytes (Crambert et al., 2002), we could not get co-IP of PLM with NCX1 in xenopus oocytes. Co-IP of all three proteins was observed in cardiac myocytes (Fig 6,7).

**Figure 6. Co-immunoprecipitation of PLM, NCX and the Na⁺/K⁺ ATPase from rat heart microsomes.**

Rat heart microsomes were solubilised in C₁₂E₁₀. (A) anti NCX or anti α (6H) antibodies were used to co-IP PLM. Proteins were visualized using anti NCX (upper panel) or anti PLM antibodies (lower panel). (B) Anti NCX and anti 6H antibodies were used to co-IP the α subunit of the Na⁺/K⁺ ATPase. The α subunit was visualized using anti α antibodies (anti KETYY).

**Figure 7. Co-immunoprecipitation of PLM and NCX expressed in Xenopus oocytes.**

(A) PLM and NCX were expressed in oocytes, Microsomes were prepared and subjected to Western blotting. (B) Following solubilization of the membranes, the sample was divided to two aliquots, and proteins were immunoprecipitated with either anti PLM or anti NCX (as indicated in the figure). Upper panel: WB using an anti NCX antibody. Lower panel: WB using anti PLM antibody.
A possible explanation may be that the association between PLM and NCX1 is mediated by either the Na⁺/K⁺ ATPase or Ankyrin-B. It has been shown that Ankyrin-B forms a complex with the Na⁺/K⁺ ATPase and NCX1 (Bers and Despa, 2009; Mohler et al., 2005). PLM could be part of this complex by virtue of its association with the Na⁺/K⁺ ATPase with no direct interaction with NCX1. Such complex may not be formed in oocytes injected with PLM and NCX1 due to the absence of sufficient amount of either Ankyrin-B or Na⁺/K⁺ ATPase.

Lansbery et al. (Lansbery et al., 2006) reported that in transfected MDCK cells PLM resides primarily in the ER and a shift in its localization towards the plasma membrane is achieved either by activating PKC, or by mutating S63, 68 and 69 into aspartic acid. Such translocation was also achieved by deleting three terminal arginine residues, which may act as an ER retention signal (Scott et al., 2003). We have tested whether any of these modifications can elevate plasma membrane localization of PLM expressed with or without Na⁺/K⁺ ATPase. Accordingly, oocytes were injected with cRNA mixtures containing various PLM constructs ± αβ Na⁺/K⁺ ATPase (Fig. 8). The double mutant S63/68E was without effect on the surface expression of PLM. However an additional mutation of T69 into aspartic acid or truncating the three C-tail arginine residues (Δ3R) resulted in significant increase in surface expression. This increase was apparent only in oocytes injected with the mutated PLM constructs + αβ Na⁺/K⁺ ATPase, and not in oocytes injected with PLM alone.

Next, we wanted to determine whether phosphorylation at T69 alone is enough for membrane localization of PLM, or whether the three negative charges (S63E/S68E/T69D) are required for elevated surface expression of PLM. To this end, we created a PLM with a single mutation, T69D. As shown in fig. 9, this mutation alone is sufficient to induce higher surface expression of PLM injected with the αβ Na⁺/K⁺ ATPase. Possible interpretations of this observation are described in the Discussion.
**Figure 8.** Effect of phosphorylation mimicking mutations and deletion of C tail Arginine residues on surface expression PLM injected with but not without Na$^+$/K$^+$ ATPase.

Oocytes were either non injected (NI) or injected with cRNA mixtures coding for HA tagged PLM constructs (2ng) ±α1 (10ng) and β1 (7ng) Na$^+$/K$^+$ ATPase (NaK). The PLM mutations tested were: S63/68E, S63/68E+T69D, T69D, and truncated construct lacking the three arginine residues at positions 70-72 (Δ3R). **Bar graphs** surface expression of the HA epitope under the different conditions. The chemiluminescence signal was normalized to the total PLM protein expressed. Data are expressed as fractions of the values in oocytes injected with PLM+ NaK and means ± SEM of 16 oocytes are depicted. Statistical significance was calculated by unpaired t-test. **Image:** Western blots of the same oocytes with antibodies to HA, β tubulin, and the α subunit of Na$^+$/K$^+$ ATPase. The different lanes represent oocytes injected with the following cRNA mixtures: (1) non injected, (2) PLM, (3) S63/68E, (4) S63/68E+T69D, (5) Δ3R, (6-9) as (2-5) together with the α and β subunits of Na$^+$/K$^+$ ATPase. **Box:** Sequence alignment of the cytoplasmic domain of PLM from different species. The conserved residues at positions 63,68,69, and 71-72 are framed.
Figure 9. Effect of phosphorylation mimicking mutation on surface expression of PLM injected with but not without Na⁺/K⁺ ATPase.  

Oocytes were either non injected (NI) or injected with cRNA mixtures coding for HA tagged PLM constructs (2ng) ± α1 (10ng) and β1 (7ng) Na⁺/K⁺ ATPase (NaK). The PLM mutations tested were: S63/68E ± T69D. Bar graphs surface expression of the HA epitope under the different conditions. The chemiluminescence signal was normalized to the total PLM protein expressed. Data are expressed as fractions of the values in oocytes injected with PLM+ NaK and means ± SEM of 16 oocytes are depicted. Statistical significance was calculated by unpaired t-test. Image: Western blot of the same oocytes with antibodies to HA, β tubulin, and the α subunit of Na⁺/K⁺ ATPase. The different lanes represent oocytes injected with the following cRNA mixtures: (1) non injected, (2) PLM, (3) PLM+ NaK, (4) S63/68E+T69D, (5) S63/68E+T69D+NaK, (6) T69D, (7) T69D+NaK.
Features enabling FXYD7 to be transported to the surface without Na\(^+\)/K\(^+\) ATPase.

The finding that co-expression of the \(\alpha_1\) and \(\beta_1\) subunits of the Na\(^+\)/K\(^+\) ATPase elevates surface expression of PLM is evidence that interaction of the two proteins in the ER or Golgi apparatus is required in order to achieve exit of PLM from the intracellular compartment and trafficking to the membrane surface. The amount of PLM that is expressed on the oocyte surface in the absence of exogenous Na\(^+\)/K\(^+\) ATPase may be transported by a ‘bulk flow’ mechanism (Lee et al., 2004) or assembled with endogenous oocyte Na\(^+\)/K\(^+\) ATPase. Surface expression of FXYD7 in the absence of exogenous Na\(^+\)/K\(^+\) ATPase suggests the presence of an ‘exit signal’ that allows it to be transported to the oocyte surface without \(\alpha\beta\). To identify the domain(s) and residues involved, we injected oocytes with a number of PLM/FXYD7 chimera ± Na\(^+\)/K\(^+\) ATPase and measured their surface expression. Previously, it was reported that a C terminal Valine in FXYD7 (V80) functions as an ER exit signal and its deletion delays its cell surface expression (Crambert et al., 2004). This effect was however a temporary one and no significant difference between wild type and mutated FXYD7 were apparent following a 48 hours incubation. In agreement, we found that replacing the C-tail domain of PLM by the corresponding FXYD7 region (chimera PP7) slightly increased surface expression relative to PLM (Fig. 10). However, exchanging the extracellular N terminal had a much bigger effect and surface expression of chimera 7PP injected without \(\alpha\beta\) was not significantly smaller than that of PLM co-injected with Na\(^+\)/K\(^+\) ATPase. Thus, the extracellular domain of FXYD7 appears to be the protein segment that enables it to be efficiently transported to the cell surface without \(\alpha\beta\).
Oocytes were either not injected (NI) or injected with cRNA mixtures coding for HA tagged PLM or FXYD7 (2ng) ± α1 (10 ng) and β1 (7ng) Na⁺/K⁺ ATPase (NaK). Additional groups of oocytes were injected with 2 ng of two PLM/FXYD7 chimera. One had the extracellular domain of HA-FXYD7 but the transmembrane and intracellular segments of PLM (7PP). The other had the extracellular and transmembrane domains of HA-PLM and the intracellular segment of FXYD7 (PP7). Surface expression of the HA tag was measured three days later and the figure depicts Means ± SEM of 24 oocytes. Data are expressed as a fraction of the mean chemiluminescence signal in oocytes expressing HA-FXYD7. On the right, a schematic representation of the FXYD7/PLM chimera. TM-transmembrane.

The extracellular N terminal of FXYD7 contains three, well conserved threonine residues predicted to be mucin-type N-acetylgalactosamine acceptor sites. Their mutation affects the electrophoretic mobility of multiple FXYD7 bands and a similar effect is achieved also by extensive treatment with neuraminidase plus O-glycosidase, suggesting that at least some of them are O-glycosylated (Beguin et al., 2002). In few previous cases O-glycosylation was involved in the control of membrane translocation and apical targeting in epithelial cells (Huet et al., 1998; Monlauzeur et al., 1998; Slimane et al., 2000). We therefore examined whether mutating these residues will affect surface expression. Mutating each of the three threonine residues into Alanine evoked a small but significant decrease in FXYD7 surface expression (Fig. 11). Double or triple mutation (i.e. T3/5A and T3/5/9A, respectively) largely inhibited surface expression to a similar level seen for PLM expressed without Na⁺/K⁺ ATPase.
To further explore interactions between FXYD7 and the Na\(^+\)/K\(^+\) ATPase, wild type and mutated FXYD7 constructs were expressed with and without Na\(^+\)/K\(^+\) ATPase. Again double and triple mutants of these residues lowered surface expression of FXYD7 by two and four folds, respectively. Significantly, co-expression of the Na\(^+\)/K\(^+\) ATPase could not bypass the inhibitory effect of the above mutations (Fig. 12). A similar, Na\(^+\)/K\(^+\) ATPase insensitive, inhibition of FXYD7 surface expression was observed by mutating the FXYD motif conserved among all family members. This mutation (AFAA) is without effect on FXYD7 O-glycosylation (Western blot at the bottom of Fig. 12 and (Crambert et al., 2004) but nevertheless inhibited surface expression to the same level as the triple T/A mutant. This result is somewhat surprising since the FXYD domain is involved in the interaction of the FXYD proteins with the Na\(^+\)/K\(^+\) ATPase and is not supposed to affect trafficking of FXYD7 expressed without the pump (Shinoda et al., 2009).

As reported in (Beguin et al., 2002) and can also be seen at the bottom of Fig. 11, FXYD7 runs as multiple bands whose pattern is affected by the above T/A mutations. Therefore, these are likely to represent differentially glycosylated forms of the protein. It also appears that expression level of the triple mutant T3/5/9A is considerably lower than that of wild type and partly mutated constructs. However, subsequent experiments have demonstrated inhibited surface expression of the T3/5/9A mutant also under conditions where this protein was expressed at a higher level than unmodified FXYD7 (Fig. 12). Inhibition of protein glycosylation has been shown to induce ER accumulation and degradation (Yeo et al., 1989). Again, the fact that that the mutated protein is expressed at a level that is even higher than the wild type FXYD7 argues against this option.
Figure 11. Effect of Mutating O-glycosylation sites on surface expression of FXYD7.

Oocytes were injected with the above amounts of cRNA of either FXYD7 ± Na⁺/K⁺ ATPase or FXYD7 constructs in which threonine residues at position 3, 5 and 9 were mutated into alanine, alone or in combination. Number of oocytes averaged is indicated in brackets. All three individual mutations had a significant effect on surface expression * P<0.01; ** P<0.05. Box: Sequence alignment of the extracellular domain of FXYD7 from different species. The conserved T residues are indicated by red boxes.
Figure 12. Expression of Na⁺/K⁺ ATPase does not rescue FXYD7 from inhibition by extracellular mutations.

Oocyte were injected with the regular amounts of cRNA coding for wild type and mutated HA tagged FXYD7 constructs ± the α₁ and β₁ subunit of Na⁺/K⁺ ATPase. AFAA is an FXYD7 mutant in which the conserved F, Y and D of the FXYD motif were replaced by Ala. Surface expression was measured three days later and means ± SEM of 8 oocytes are depicted. Bottom: Western blots with anti-HA and anti-α₁. Since it is not known which of the partly O-glycosylated species reaches the surface, surface expressions were not normalized to variations in the amount of protein expressed.

To further confirm the role of these Threonine residues in surface translocation of FXYD7 we introduced the same mutation in the chimera 7PP and tested its effect on surface expression. As for FXYD7 the triple T/A mutant largely inhibited translocation of the FXYD chimera to the cell surface, confirming its role in surface expression (Fig. 13).

It is likely that the above mutations inhibit surface translocation of FXYD7 and 7PP by preventing O-glycosylation of these sites. Yet, other possibilities, such as mutation
induced conformational changes cannot be ruled out. To further examine this issue, oocytes were incubated with Benzyl 2-acetamido-2-deoxy-α-D-galactopyranoside (BGN), a permeable competitive inhibitor for elaboration of the core GalNAc residue, found in mucin-type O-linked glycans (Prescher and Bertozzi, 2006). To rule out lack of BGN effect due to restricted permeability, the blocker (4 mM) was included in the cRNA injection mixture and also present in the medium throughout the incubation. In order to exclude the possibility that BGN affects FXYD7 trafficking by influencing other processes (e.g. the cell translocation machinery), the oocytes were also injected with PLM and the Na⁺/K⁺ ATPase, proteins that do not have O-glycosylation sites, and therefore their surface expression should not be affected by the inhibitor. We could not see any effect of the inhibitor on the band pattern or surface expression of neither PLM nor FXYD7 in oocytes (Fig. 14). This implies glycosylation type that is different than the BGN sensitive mucin-type O-glycosylation.

Figure 13. Comparison of the effect of the triple T/A mutant on FXYD7 and FXYD7/PLM chimera.

The experimental protocol was the same as in Fig. 6, mean ± SEM of 16 oocytes are depicted.
Figure 14. Effect of the O-glycosylation inhibitor α-benzyl GalNAc on surface expression of FXYD7.

Oocytes were injected with the regular amounts of cRNA of either PLM or FXYD7 ± Na⁺/K⁺ ATPase in the presence or absence of BGN. In BGN treated oocytes, the inhibitor was injected together with the cRNA (50 nl of 4mM) and added to the medium to a final concentration of 4mM. Control oocytes received an equal volume of DMSO (final 0.5%). Three days later, surface expression of the HA tagged proteins was measured. (A) surface expression of the HA epitope under different conditions. (B) microsomal proteins were resolved electrophoretically and blotted with the indicated antibodies. 1,2 non injected oocytes. 3. PLM. 4,5 PLM+NaK. 6,7 FXYD7
Surface expression of FXYD proteins in mammalian cells

The ability of FXYD7 to reach the cell surface alone when expressed in *Xenopus* oocytes and its dependence on O-glycosylation is surprising and may have mechanistic implications. We therefore aimed to determine whether these phenomena are valid in mammalian cells too. In the first stage we tested whether mutating the extracellular putative O-glycosylated residues affects surface expression of FXYD7 in mammalian cells too. Two different approaches have been used to assess this issue. The first was to image mammalian cells expressing PLM and FXYD7 tagged in their carboxy tail with CFP. The second was surface biotinylation of FXYD proteins in such cells.

H1299 cells were selected for this study because of the availability of a cell clone that already expresses YFP tagged Na⁺/K⁺ ATPase under its regular promoter. This clone was identified in the Library of Annotated Reporter Cell-clones (LARC) available from the lab of Prof. Uri Alon at the Weizmann Institute. LARC was constructed by random integration of YFP flanked by splice acceptor and splice donor sites into the H1299 genome (Sigal et al., 2007). In one of the cell clones identified, YFP was incorporated into the first intron of the α1 subunit of Na⁺/K⁺ ATPase. The resulting protein has an YFP insert between the 5th and 6th amino acids in the N-terminal of α1. This provides a functional, fluorescently tagged α1 that is expressed under normal transcriptional control from one of the two α1 alleles.

To study interactions with FXYD proteins we stably transfected the YFP-α1 cells with HA-FXYD7, FXYD7 T3/5/9A and HA-PLM. The FXYD proteins were tagged by CFP in their C-tail a position shown before not to have effect on their function (Bossuyt et al., 2006). This system enables detection of surface and intracellular location of FXYD7 and PLM as well as their co-localization with the Na⁺/K⁺ ATPase.

Fig. 15 depicts expression of two representative cell clones stably expressing HA-FXYD7-CFP (lanes 2,3) and two cell clones expressing T3/5/9A-CFP (lanes 4,5). Adding the fluorescent tag increased the MW to the protein to about 43 kDa In

Reference:

1 Sigal et al., 2007 (http://www.weizmann.ac.il/mcb/UriAlon/DynamProt/).
addition, the T/A mutation resulted in a small decrease in size attributed to the lack of O-glycosylation.

**Figure 15. Expression of HA-FXYD7-CFP and HA-FXYD7 T3/5/9A-CFP in H1299 cells.**

H1299 cells were either non-transfected (lane 1) or stably transfected with HA-FXYD7-CFP (lane 2,3) or HA-FXYD7 T3/5/9A-CFP (lanes 4,5). Membranes were prepared and blotted with anti XFP antibody. All cell clones express also endogenous YFP tagged α1 Na+/K+ ATPase. However, since this protein runs as ~130 kDa band it is not visualized on this gel.

Confocal images of these cells are depicted in Fig. 16. In all cell clones YFP-α Na+/K+ ATPase is localized primarily in the plasma membrane (green label in all panels of Fig. 16). HA-FXYD7-CFP and HA-PLM-CFP too appeared to reside mainly in the plasma membrane but had also much intracellular localization that did not co-localize with α1 (Fig. 16, two middle rows). It is possible that the intracellular location reflects an overexpression of the FXYD protein from the strong plasmid promoter. Unlike HA-FXYD7 and HA-PLM, the triple mutant, HA-FXYD7-T3/5/9, is found almost exclusively inside the cell (Fig. 16, lowest row).

ER and Golgi labels were added in order to examine the intracellular location of the mutated FXYD7. In the first case cells were transiently transfected with the ER marker plasmid pDSRed2-ER. This plasmid encodes the ER targeting sequence of calreticulin and the ER retention sequence, KDEL, fused to the N and C termini of DSRed2, respectively. Thus, it translates a fluorescently red protein that is restricted to the ER. In order to stain for the Golgi, we used an antibody recognizing the Golgi
reassembly stacking protein 1 (GRASP65). Labeling cells for ER and Golgi demonstrates localization of FXYD7 in both compartments (Fig. 17). In the case of WT-FXYD7 most protein resides in the plasma membrane and therefore the intracellular localization may be contributed by protein ‘en route’ to the cell surface (Fig. 17A). This may be an outcome of overexpression driven by the strong plasmid promoter. In the T3/5/9A mutant however, the protein reaches the Golgi apparatus (Green+blue=pink staining in Fig. 17B) but does not advance to the cell surface. This result supports O-glycosylation of the FXYD7, as this post-translational modification takes place in the Golgi (Hanisch, 2001).

**Figure 16.** Cellular localization of YFP-α1, PLM and FXYD7 or its mutant in H1299 cells.

H1299 cells were grown in LabTek chamber cover slides. Confocal images were taken 24 hours after seeding.
Figure 17. Co-localization of FXYD7 and T3/5/9A-CFP with ER and Golgi markers.

(A) Fluorescence microscopy images of H1299 (YFP-α1) stably expressing FXYD7-CFP (upper panel) or T3/5/9A-CFP (lower panel). In addition, cells were transiently transfected with the ER marker pDSRed2-ER (a plasmid encoding a the ER targeting sequence of calreticulin and the ER retention sequence, KDEL, fused to the N and C termini of DSRed2, respectively). (B) H1299 (YFP-α1) cells stably expressing FXYD7 T3/5/9A-CFP were probed with anti GRASP65 followed by Cy3 conjugated secondary antibody.

Another approach used to establish intracellular location of the 3T/A mutant was surface biotinylation. In H1299 cells, unlike in *xenopus* oocytes and M1 cells (see below), Sulfo-NHS-SS biotin effectively labelled the FXYD proteins. This may be due to some structural difference such as lack of heavy glycosylation of neighbouring proteins, which makes the N-terminal NH$_2$ more accessible to the biotinylating
reagent. The results obtained in this experiment, confirm the data obtained by the confocal images. Namely, PLM and FXYD7 could be effectively biotinylated indicating that most of the protein was expressed in the membrane surface (Fig. 18). The triple mutant on the other hand could not be biotinylated at all indicating that it resides within the cell only. Another interesting observation in the experiment of Fig. 18 is that FXYD7 runs as doublet but only the upper band is surface biotinylated (this is better seen in 18B). The lower band however corresponds in size to the triple T/A mutant of FXYD7. We therefore suggest that the slower migrating band represents the mature, glycosylated form that reaches the plasma membrane, while the faster one, is the immature, unglycosylated form that remains inside the cell. These results further confirm that O-glycosylation of the N-terminal of FXYD7 is essential in order for this protein to leave the ER and reach the plasma membrane.

The fact that PLM effectively expresses in the plasma membrane of H1299 can be explained in two possible ways. (1) These cells expresses sufficient Na\(^+\)/K\(^+\) ATPase to “drive” PLM to the membrane (2) In H1299, unlike *Xenopus* oocytes PLM is effectively phosphorylated. PLM phosphorylation was shown to be important factor in its cellular trafficking (Lansbery et al., 2006).

![Figure 18. Surface biotinylation of H1299 cells.](image)

(A) H1299 cells expressing HA-PLM-CFP, HA-FXYD7-CFP or HA-FXYD7 T3/5/9A (3T/A) were biotinylated using sulfo-NHS-SS biotin. After biotinylation, the cells were lysed and subjected to streptavidin pull-down. 5% of the total cell lysate (T) and the whole streptavidin bound material (PD) were resolved on SDS-PAGE gel and blotted with anti HA and anti α1 (6H) antibodies. (B) Another experiment with the same protocol which better demonstrate the existence of two FXYD7 bands only one of which is surfacely expressed.
Co-localization of FXYD7 and Na\(^+\)/K\(^+\) ATPase in the plasma membrane of mammalian cells.

Next we addressed the question whether FXYD7 can reside in the plasma membrane without being co-localized with \(\alpha1\). One approach was to compare diffusion of \(\alpha1\) and FXYD7 or PLM, by measuring recovery of YFP and CFP fluorescence following photo bleaching (FRAP). In FRAP experiments, a specific area in the cell is irreversibly photo bleached using a high intensity laser, and the movement of unbleached fluorescent molecules into the bleached region is monitored (Snapp et al., 2003). There is a large difference in the MW of \(\alpha1\) Na\(^+\)/K\(^+\) ATPase and FXYD protein and in particular in the membrane embedded region (10 transmembrane domains in \(\alpha1\) vs. one in FXYD). In addition \(\alpha1\) is known to interact with the cytoskeleton while FXYD proteins are not (Liu et al., 2008; Mohler and Bennett, 2005; Stabach et al., 2008) Thus, if expressed alone the two proteins are expected to diffuse at a very different rates in the plasma membrane. If however the dominant structure in the membrane is an \(\alpha1\)-FXYD complex the CFP and YFP fluorescence recovery rates will be identical. The experimental protocol used was to bleach a small membranal region of interest (ROI), and follow the kinetics of fluorescence recovery. Bleaching of both YFP and CFP at the cell surface was done with 440 nm laser. Recovery of YFP and CFP signals was monitored during 5 minutes. Recovery was not complete, and reaches saturation at about 80% of the original signal. This is a common behaviour likely to indicate the existence of an immobile fraction of the protein that is not replaced after bleaching.

Fig. 19 summarizes experiments in which small area of plasma membrane in H1299 cells expressing \(\alpha1\)-YFP and either HA-FXYD7-CFP or HA-PLM-CFP was bleached. Typical images collected before, right after and 5 minutes after the bleaching are depicted in Fig. 19A. Recovery curves obtained by averaging values of 10 different cells from 2 independent experiments are shown and analysed in Fig. 19B-D. The data could not be fitted to a single exponent but was in good agreement with two time constants representing fast \((t_{1/2} \sim 30\text{sec})\) and slow \((t_{1/2} \sim 1\text{ hr})\) components. It is likely that the fast recovery represents lateral diffusion in the membrane. The slow process may be intracellular trafficking of new fluorescently...
tagged proteins, or exchange with a different more immobile pool that does not recover within 5 min (e.g. protein in lipid caveoli). Most significantly, the recovery of α1 and FXYD7 fluorescence occurred at a very similar rates indicating that the two proteins move into and within the membrane as a single complex. Surprisingly, PLM had a significantly slower recovery rate compared to the Na⁺/K⁺ ATPase. We currently do not have an explanation for this observation. While a t₁/₂ value is relevant to a particular experimental settings, the Na⁺/K⁺ ATPase t₁/₂ (~30 sec) is similar to the value reported by Jesaitis and Yguerabide in sub-confluent MDCK cells (26 sec) (Jesaitis and Yguerabide, 1986). There are however many parameters affecting the t₁/₂ of the recovery, and these will be elaborated in the discussion.

Figure 19A. Fluorescence recovery after photo bleaching of the plasma membrane.

(A) Confocal fluorescence images of H1299 expressing YFP-α1 and either HA-FXYD7-CFP (upper panel) or HA-PLM-CFP (lower panel) before and after photo bleaching. Cells were seeded in LabTek chamber slides. Twenty-four hours later, FRAP was measured by bleaching a region of interest in the membrane (marked by a white circle) using 440 nm laser at full intensity. Pictures were taken every second for the first 10 seconds after bleaching, and every 30 seconds for 5 minutes thereafter. The pictures shown in the figure are merges of the two channels used: YFP-α1 is green, and FXYD7/PLM-CFP in red. The yellow staining indicates co-localization of the two proteins.
Figure 19B-D. Fluorescence recovery after photo bleaching of the plasma membrane.

(B) Average fluorescence recovery curves of YFP-α1, HA-FXYD7-CFP and HA-PLM-CFP. The initial intensity was set to 1, and the intensity of the image collected immediately after bleaching was set to 0. Values represent means of 10 ROIs from two independent experiments. (C) Kinetic analysis of fluorescence recovery. Means ± sem of data collected in 10 ROI are presented. The continuous lines are the best fits of these data to two time constants. (D) A summary of the kinetic parameters obtained by the above fitting of experimental data to two time constants. Diffusion coefficient (D) was calculated using T_{1/2} of the fast recovery rate.

Another approach utilized in order to test possible existence of plasma membrane FXYD7 not in a complex with Na^+/K^+ ATPase was to quantify the ouabain-induced
internalization of the two proteins. Liu et al (Liu et al., 2005) have demonstrated that adding ouabain at a concentration that does not substantially inhibit the Na⁺/K⁺ ATPase activity to LLC-PK1 cells induces endocytosis of the protein. Such ouabain-induced internalization of αβ Na⁺/K⁺ ATPase should not cause internalization of FXYD7 unless the two proteins interact. Accordingly, we applied ouabain to H1299 cells and quantified the amount of Na⁺/K⁺ ATPase and FXYD7 internalized. As seen in Figure 20, addition of 100 nM ouabain to FXYD7 expressing H1299 cells caused a ~55% decrease in the membrane abundance of both the Na⁺/K⁺ ATPase and FXYD7. This result demonstrates that FXYD7 is affected by the binding of ouabain to the Na⁺/K⁺ ATPase to the same extent as the Na⁺/K⁺ ATPase itself. This result is consistent with the results obtained by FRAP experiments, indicating that that FXYD7 and the Na⁺/K⁺ ATPase form a complex in mammalian cells.

**Figure 20. Effect of ouabain on surface expression of the Na⁺/K⁺ ATPase and FXYD7.**

H1299 cells were treated with 100 nM ouabain or diluent for 16 hours. Surface proteins were biotinylated, and pulled down using streptavidin beads. (A) The eluted proteins were resolved on SDS-PAGE and subjected to western blotting using anti 6H (α1 Na⁺/K⁺ ATPase) or HA (FXYD7). The higher molecular weight of both α1 and FXYD7 are due to the attached XFP protein. (B) quantification of blot in A. Data are shown as fraction of total protein expressed.
**Functional effects of FXYD7 in M1 cells.**

While the above data argue against the possibility that FXYD7 resides in the plasma membrane of mammalian cells not associated with the Na\(^+\)/K\(^+\) ATPase, it does not exclude functions other than modulation of the pump kinetics. Such additional functions, which are not independent of the interaction with the Na\(^+\)/K\(^+\) ATPase, have been reported recently for FXYD5. This member of the FXYD family has well-established effects on cell adhesion and motility and was recently shown to impair epithelial tight junctions (Ino et al., 2002; Lubarski et al., 2011; Miller and Davis, 2008; Shimamura et al., 2004; Tsuiji et al., 2003). The molecular mechanism suggested was association of the extracellular domain of FXYD5 with β1 Na\(^+\)/K\(^+\) ATPase, inhibiting β1 N-glycosylation and thereby impairing intercellular β1-β1 interactions essential for maintaining cell-cell contact (Lubarski et al., 2011). Effects on the paracellular permeability were also reported for FXYD3, another family member overexpressed in cancer cells (Bibert et al., 2009). This effect is in the opposite direction to the one described for FXYD5 and may, therefore, reflect a general role of FXYD proteins in cell-cell contact.

To assess for possible effects of FXYD7 on cell-cell contact and tight junction permeability we turned to M1 cells, used before to study effects of FXYD5. This is a mouse cortical collecting duct cell line that forms polarized, high electrical resistance epithelium expressing the amiloride-blockable epithelial sodium channel (ENaC) at the apical surface, and the Na\(^+\)/K\(^+\) ATPase to the basolateral side (Stoos et al., 1991). First, we generated M1 cell clones that stably express HA-FXYD7. Figure 21 demonstrates high level expression of HA-FXYD7 in the antibiotic resistance clones, tested by Western blotting with specific antibodies. In M1 cells, FXYD7 runs as a single band, corresponding to the fully glycosylated protein. This is in contrast to *Xenopus* oocytes in which 3 bands of FXYD7 can be detected, representing different glycosylation stages, and unlike H1299 cells, in which 2 bands of FXYD7 could be detected, corresponding to the glycosylated and unglycosylated form.
Figure 21. Expression of HA-PLM and HA-FXYD7 in M1 cells.

M1 cells lysates (25 μg) were resolved on SDS-PAGE, and subjected to western blotting. The membranes were cut to high, medium and low molecular weight regions and probed with antibodies against α subunit of the Na⁺/K⁺ ATPase (6H), β-tubulin and FXYD7.

Next, we studied the effect of FXYD7 on the transepithelial resistance of M1 cells. When cultivated on permeable support, M1 monolayers develop a transepithelial electric potential (V_{TE}) reflecting an apical to basolateral active Na⁺ transport. The current that has to be passed in order to nullify this potential “the short circuit current” (I_{sc}) is equal to the Na⁺ flux generated. To test for possible effects of FXYD7, wild-type and FXYD7 transfected cells were plated on Millicell cell culture inserts. Transepithelial electrical resistance (R_{TE}) and voltage were recorded daily. I_{sc} was then calculated as the ratio of the two parameters. After 8 days the experiment was terminated by the addition 10 μM amiloride which fully blocks ENaC mediated Na⁺ flux.

Fig. 22 averages results of 4 independent experiments. Two differences between the FXYD7 containing and FXYD7 lacking cells were noted. The first is a faster formation of cell-cell contacts and tight junctions in the FXYD7 transfected cells, as evident by the faster increase and higher R_{TE} which can not be explained by reduced ENaC activity (see below). This may, in principle, be the outcome of a faster rate of cell proliferation in the FXYD7 transfected culture. Effects of FXYD proteins on cell proliferation has been reported before for both FXYD2 and FXYD3 (Arystarkhova et al., 2007; Kayed et al., 2006; Yamamoto et al., 2009). We have therefore studied the effect of FXYD7 on cell attachment and proliferation rate of transfected M1 cells. Imaging cells during the first 14 hours after seeding demonstrated that FXYD7
transfected cells attach to the plate at a significantly slower rate, and tend to form aggregates (While 75-80% of WT cells are adhered to the plate at 14 hours after seeding, 15-30% of FXYD7 transfected cells are adhered. The large variability in FXYD7 adhesion is a result of cells aggregates which are difficult to quantify) (Fig. 23A). Similar observation has been made before in FXYD5 transfected cells and H1299 cells in which FXYD5 has been silenced (Lubarski et al. unpublished). This may indicate effect of FXYD7 on cell adhesion, as reported previously for FXYD5. No difference in the proliferation rate was noted between wild type and FXYD7 transfected cells (Fig. 23B). Once the FXYD7 expressing cells adhere to the support, they proliferate at the same rate as the non-transfected cells.

![Graph A](image1)

![Graph B](image2)

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<td>FXYD7</td>
<td>1.5±0.2</td>
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**Figure 22. Transepithelial resistance and short-circuit current of WT-M1 and FXYD7 transfected cells.**

(A) M1 cells (0.1x10^6) were seeded on millicell cell culture inserts, and incubated in PC-1 medium. Transepithelial resistance (RTE) and potentials were measured daily as described under Material and Methods. After measurements were made at day 8, 10 μM amiloride were added to the incubating solutions, and measurements were taken again. Means ±SEM of 9-11 filters of each group from 4 different experiments are depicted. P (FXYD7± amiloride)>0.05, P (WT-M1± amiloride) <0.05. (B) Short circuit current (Isc) at day 8 before and after the addition of amiloride, P (FXYD7± amiloride) >0.05. P (WT-M1± amiloride) <0.05. P value was calculated by paired t-test. Table: RTE and Isc values measured at day 8 before and after addition of amiloride.
Figure 23A. Adhesion assay of WT and FXYD7 transfected cells.

WT and FXYD7 transfected cells were trypsinized and seeded in LabTek chamber cover slides. Confocal images were taken after allowing the cells to settle. Cells were imaged for 14 hours. 75-80% of WT and 15-30% of FXYD7 transfected cells are adhered after 14 hours.
Figure 23B. Proliferation rate of transfected and non-transfected M1 cells.

M1 cells were plated in 96 well plates at the indicated densities. Proliferation of the cells was assessed by XTT-assay at 48 and 72 hours after plating. Shown is the relative proliferation of cells (OD 450nm at 72h/ OD 450nm at 48h) ±SD. There is no difference in the proliferation rate of the transfected and non-transfected M1 cells.

A more significant, and in fact striking difference between the two populations was noted in the amiloride blockable Isc. While wild type cells developed a typical channel activity of ~10 μA/cm², no significant Na⁺ current was apparent in the FXYD7 transfected cells. Studies in Xenopus oocytes have demonstrated that FXYD7 inhibits the Na⁺/K⁺ ATPase activity by decreasing its apparent affinity to K⁺ and lowered Isc by 50% (Beguin et al., 2002). This may in principle account for at least part of the observed effect of FXYD7 on Isc in the above experiment. Yet, the observed effect is unexpectedly large compared to the previously reported values. Also, Isc is thought to be limited by the apical Na⁺ entry and not the basolateral pumping. One option is that an FXYD7 induced inhibition of the Na⁺/K⁺ ATPase increases cell Na⁺ and “feedback” to inhibit ENaC (Macrobbie and Ussing, 1961). However, it is also possible that FXYD7 affects either the activity or expression level of ENaC. The first effect will require co-localization in the apical surface. It was therefore of interest to measure the cellular distribution of FXYD7 and determine whether it can be found in the apical ENaC containing surface or only in the basolateral side with the Na⁺/K⁺ ATPase. Such measurements were done by both surface biotinylation and imaging FXYD7 in transfected cells.
For surface biotinylation confluent cell monolayers which express high $R_{TE}$ were biotinylated by adding DBB to either the upper (apical) or lower (basolateral) compartment. Cells were lysed, biotinylated proteins isolated on streptavidin beads and analysed by Western blotting. Fig. 24 depicts a representative experiment. In general, the basolateral labelling efficiency was low, since DBB has to cross the permeable support. Nevertheless, the data clearly indicates that it is possible to label FXYD7 when the reagent is added to the basolateral but not the apical side. Thus, FXYD7 seem to be expressed in the basolateral side only. Validity of the approach is demonstrated by the observation that the $Na^+/K^+$ ATPase too is labelled from the basolateral but not apical side (upper panel in Fig. 24). Also, ERK failed to be labelled by DBB indicating that the biotinylating reagent does not penetrate cells.

The above conclusion is confirmed by immunofluorescence of M1 cells. Confocal images of transfected cells stained by anti FXYD7 antibody indicate basolateral labelling that was co-localized with the $Na^+/K^+$ ATPase. This is evident from the vertical stains seen in the XZ plain (Fig. 25).

**Figure 24. Surface biotinylation of M1 cells.**

M1 cells ($0.2 \times 10^6$) cells were seeded on 12 well transwell inserts and grown for 7 days. DBB (0.5 mg/ml) was added either to the basolateral (Bl) or the apical (Ap) side of the membrane. After biotinylation and quenching of excess reagent, cells were scraped into lysis buffer. Streptavidin beads were added and the biotinylated proteins were pulled-down (PD). Five percent of the lysate (total protein (T)) and the whole volume of the pulled down material were run on SDS-PAGE and transferred to membranes. The membrane was cut to high, medium and low molecular weight regions and probed with the indicated antibodies.
Figure 25. Confocal images of Na⁺/K⁺ ATPase, FXYD7 and PLM in M1 cells.

M1 cells were grown for 7 days on transwell inserts. The cells were fixed and stained with 6H (anti α1 Na⁺/K⁺ ATPase-green), anti FXYD7 (red). Shown are XY and XZ sections, indicating that FXYD7 and α1 Na⁺/K⁺ ATPase co-localize on the lateral membrane.

Thus, the data does not indicate significant apical location of FXYD7 in M1 cells. This however is another proof that the protein does not traffic to the plasma membrane independently of the Na⁺/K⁺ ATPase.

To study possible effect of FXYD7 on ENaC expression, mRNA level of the three channel subunits was quantified by real-time PCR using GAPDH as control. A preliminary experiment indicated a two fold decrease in the expression level of αENaC and some increase in βENaC (not shown). While the effect of FXYD7 on I_{sc} and ENaC expression level is of interest and could suggest additional functions of this protein, time limitation did not permit to pursue this direction which is out of the scope of the thesis.
Discussion

Many studies have establish that FXYD proteins function as auxiliary subunits or regulators of the Na⁺/K⁺ ATPase and alter its kinetic properties in tissue and physiological state specific manner (for review see (Garty and Karlish, 2006; Geering, 2006)). Additional functions such as interactions with the Na⁺/Ca²⁺ exchanger, formation or regulation of ion channels and involvement in cell adhesion, have been postulated as well (Attali et al., 1995; Ino et al., 2002; Minor et al., 1998; Moorman et al., 1992; Song et al., 2005; Zhang et al., 2006). In this study we have characterized the intracellular trafficking and surface expression in Xenopus oocytes of two FXYD proteins PLM and FXYD7. The data suggested that unlike PLM which associates with the Na⁺/K⁺ ATPase intracellularly FXYD7 traffic to the plasma membrane not associated with αβ Na⁺/K⁺ ATPase. Since this observation may suggest additional, Na⁺/K⁺ ATPase independent, function of FXYD7 it was further explored in mammalian cells. In this case however, no evidence for lack of association between FXYD7 and the Na⁺/K⁺ ATPase could be obtained using different approaches. We therefore suggest that while FXYD7 has the capability to traffic “alone” from the ER/Golgi to the plasma membrane, it first choice will be to associate with the pump. Other experiments have established the critical role of O-glycosylation of FXYD7 in Golgi exit and plasma membrane localization.

Xenopus oocytes have been used as a model system to study intracellular trafficking of FXYD proteins for two reasons (I) The ease with which proteins can be heterologously expressed and the ability to co-express several proteins by co-injecting cRNA mixtures. (II) The very low level and slow turnover of endogenous Na⁺/K⁺ ATPase which enables to conveniently characterize effects of Na⁺/K⁺ ATPase on FXYD surface expression by co-injection. A key observation of this set of experiments was that translocation of PLM to the plasma membrane strongly depends on co-expression of the Na⁺/K⁺ ATPase while FXYD7 reaches the cell surface equally well with and without Na⁺/K⁺ ATPase. The result obtained for PLM is not surprising. It is well established that multimeric proteins assemble intracellularly and such assembly is required for trafficking to the plasma membrane (Lee et al., 2004). The small but significant plasma membrane PLM obtained in the absence of
exogenously expressed α and β Na⁺/K⁺ ATPase may reflect either passive ‘bulk flow’ that does not involve receptor mediated concentration of cargo proteins in transport vesicles, or association with endogenous Na⁺/K⁺ ATPase subunits.

Unlike the Na⁺/K⁺ ATPase, NCX1 shown to be inhibited by PLM in a phosphorylation dependent manner (Song et al., 2005; Zhang et al., 2006) had no effect on the surface expression of PLM. This was the case for either wild type or mutated PLM construct in which serine residues 63 and 68 were mutated into Glutamic acid. Such phosphorylation mimicking mutations were shown to enhance inhibition of NCX1 mediated current by PLM (Zhang et al., 2006). Also, we could not demonstrate co-immunoprecipitation of PLM and NCX from oocytes microsome. Such co-immunoprecipitation could be obtained in heart microsome, suggesting that the detergent solubilization protocol we used preserves the putative PLM-NCX1 interaction. Also, it has been previously reported that fluorescence resonance energy transfer (FRET) can be demonstrated between PLM and the Na⁺/K⁺ ATPase, it was not between PLM and NCX1 (Bossuyt et al., 2006). A possible reason to this difference could be that interaction of PLM with NCX1 is (Moorman et al., 1992) secondary to its association with Na⁺/K⁺ ATPase. Mohler et al. (Mohler et al., 2006) reported the formation of a complex between Ankyrin-B, Na⁺/K⁺ ATPase, NCX and InsP3 receptor in cardiac T-tubule/SR microdomain. Such complex is not likely to be formed at significant amounts in oocytes not injected with Na⁺/K⁺ ATPase and/or ankyrin-B.

Lansbery et al. (Lansbery et al., 2006) reported that in transfected MDCK cells, PLM resides primarily in the ER and translocation to the plasma membrane is achieved either by the phosphorylation mimicking mutations of S63, S68 and T69 or by truncating the three terminal arginine residues R⁷⁰-⁷². In agreement, we found that both the triple 63, 68, 69 mutations and the C-tail truncation significantly elevated plasma membrane expression of PLM in oocytes that co-expressed the Na⁺/K⁺ ATPase. However the double mutant S63/68E was without effect, and the single mutant T69D was sufficient to produce the full augmentation. Thus, T69 appears to be the key residue in enhancing the translocation of PLM. T69 was identified as another PKC site whose phosphorylation augments the pump mediated current in
cardiac myocytes beyond the level achieved by phosphorylating S63 and S68 (Fuller et al., 2009). Sildenafil, an inhibitor of phosphodiesterase type 5 which protects against ischemia reperfusion injury (Elrod et al., 2007), was found to stimulate phosphorylation of T69. This mechanism may underlie the protective effect of sildenafil (Madhani et al., 2010).

While mutating the C-tail of PLM nearly doubled its surface expression in oocytes that co-express αβ Na⁺/K⁺ ATPase it was without effect in oocytes injected with PLM alone (Fig. 8). One interpretation of this observation is that phosphorylation and arginine truncation enhance interaction with the Na⁺/K⁺ ATPase rather than disruption an ER retention signal. This could be an outcome of a decrease in the positive charge of the wild type C-tail. No structural information on such cytoplasmic interactions is available and this region is not resolved in the crystal structure of the shark Na⁺/K⁺ ATPase (Shinoda et al., 2009). However, previous study of our group demonstrated chemical crosslinking of the carboxy tail of FXYD2 with the S4 stalk segment of α1 Na⁺/K⁺ ATPase both in native membranes and transfected cells (Fuzesi et al., 2005). Alternatively, it is possible that the mutations do facilitate ER exit of PLM but plasma membrane expression is limited by interaction with the Na⁺/K⁺ ATPase at a post ER site e.g. the Golgi apparatus. Such interaction is required in order for PLM to exit the Golgi and reach plasma membrane. In such scenario the above mutations will increase the Golgi pool of PLM available for interaction with the Na⁺/K⁺ ATPase and thereby its plasma membrane abundance.

Unlike PLM, FXYD7 surface expression was high, irrespective of the co-expression of Na⁺/K⁺ ATPase. Independent transport to the cell surface and assembly in the plasma membrane is an unusual mechanism for the construction of a multimeric protein complex. Therefore the data may indicate additional, Na⁺/K⁺ ATPase independent role of FXYD7. Indications for functions that do not involve association with Na⁺/K⁺ ATPase or NCX have been proposed for a number of FXYD proteins. e.g. the expression of PLM, FXYD2 (γ), FXYD3 (Mat-8), and FXYD4 (CHIF) in *Xenopus* oocytes was shown to evoke ionic conductance(Attali et al., 1995; Minor et al., 1998; Moorman et al., 1992; Morrison et al., 1995). Such channel activity however could not be detected for FXYD7 (Beguin et al., 2002). In addition, certain
epithelia express FXYD3 and FXYD5 (RIC or dysadherin) in the apical surface that lacks Na⁺/K⁺ ATPase (Crambert et al., 2005; Lubarski et al., 2005). Alternatively, it is possible that FXYD7 is not involved in the constitutive regulation of Na⁺/K⁺ ATPase and equilibrium exists in the plasma membrane between FXYD7 that is and is not at complex with Na⁺/K⁺ ATPase. Evidence for such equilibrium involving PLM oligomerization have been suggested by demonstrating fluorescence energy transfer between YFP and CFP tagged PLM (Bossuyt et al., 2006).

To test the above possibility we have attempted to determine whether in mammalian cells too FXYD7 resides in the plasma membrane not associated with the Na⁺/K⁺ ATPase. Two experimental protocols were employed. In the first, FRAP of YFP tagged α1 and CFP tagged PLM and FXYD7 were compared in transfected H1299 cells. As discussed under Results, similar diffusion rates of the YFP and CFP tagged proteins into the bleached area are likely only if the two proteins are part of a single complex. This indeed seems to be the case. The rate constant of the diffusion of YFP-α1 into the bleached are was similar to values reported before (Jesaitis and Yguerabide, 1986). FXYD7 diffused at a practically identical rate even though it is a much smaller protein and unlike α1 are not attached to the cytoskeleton. PLM, however, had a significantly slower diffusion rate compared to YFP-α1. This result is surprising and currently unexplained, as PLM and the Na⁺/K⁺ ATPase associate intracellularly and reside in close proximity (Bossuyt et al., 2006). A Possible mechanism is that in the case of PLM FRAP picks up a complex that includes in addition to Na⁺/K⁺ ATPase also ankyrin and NCX. Such a complex is expected to move slower than Na⁺/K⁺ ATPase-FXYD7 complex. Yet, the lack of such slower component in the analysis of Na⁺/K⁺ ATPase-FXYD7 kinetics will imply that PLM plays an important role in its formation.

A second protocol attempted to examine Na⁺/K⁺ ATPase-FXYD7 association was to induce internalization of Na⁺/K⁺ ATPase and determine whether this will also evoke a similar internalization of FXYD7. Xie and co-workers (Liu et al., 2005; Liu et al., 2000) characterized ouabain-induced internalization of a specific pool of caveolin associated Na⁺/K⁺ ATPase. The underlying mechanism is yet unknown and this process is presumably involved in the signalling function of cardiac glycosides. We
could obtain a substantial ouabain-induced internalization of Na⁺/K⁺ ATPase in H1299 too, measured by a decrease in the fraction of biotinylated pumps. A very similar decrease in the surface expression of FXYD7 was noted (Fig. 20). Namely, internalization of α1 does not “leave behind” FXYD7 in the plasma membrane. Since the α1 pool internalized resides in specific membrane region the data can not be explained by a similar, but even distribution of the two proteins in the cell surface. It suggests that α1 and FXYD7 are in a closed physical proximity and internalize jointly.

The apparent difference between *Xenopus* oocytes and mammalian cells can be accounted for by assuming that FXYD7 can in principle, traffic and resides in the plasma membrane not associated with the Na⁺/K⁺ ATPase. However, in the presence of sufficient amount of pump units it will favour intracellular association with the Na⁺/K⁺ ATPase. This issue can be tested by over-expression of FXYD7 to levels that are much higher than those of Na⁺/K⁺ ATPase. Such over-expression is not physiologically significant and may lead to a non-specific cellular localization. It is also possible that the behaviour of FXYD7 in mammalian cells is affected by attaching a CFP tag to the short C-tail of the protein. Yet, experiments testing apical localization of FXYD7 in M1 cells were carried out with unmodified FXYD7 and as discussed above its absence from the apical surface argues against Na⁺/K⁺ ATPase-independent trafficking.

Measuring surface expression of PLM/FXYD7 chimera and mutants in *Xenopus* oocytes demonstrated that that surface expression of FXYD7 critically depends on at least two of the three N-terminal threonine residues, which presumably undergo O-glycosylation (Beguin et al., 2002). A number of previous studies have suggested a role for O-glycosylation in the stability and sorting of membrane proteins. O-glycans were shown to act as sorting signals that direct proteins to the apical surface (for review see (Potter et al., 2006). In other cases cytoplasmic O-glycosylation inhibited proteins from reaching the cell surface and dictate their intracellular location (Kanno and Fukuda, 2008; Zhu et al., 2001). In principle, inhibition of surface expression of T/A mutants could be secondary to a rapid degradation of this protein relative to wild type FXYD7 reported in (Beguin et al., 2002). Such mechanism may in principle also
account for the limited surface expression of PLM in oocytes that do not co-express Na\(^+\)/K\(^+\) ATPase. However, the Western blots shown in figures 2, 12 and 14 demonstrate that lack of surface expression of either PLM or mutated FXYD7 do not correlate with a decrease in the steady state amount of these proteins in the cell, on the contrary their abundance seem to increase in the absence of exogenous Na\(^+\)/K\(^+\) ATPase. In addition, the rapid degradation of the triple T/A mutant reported in (Crambert et al., 2003) was prevented by the co-expression of \(\alpha\) and \(\beta\) Na\(^+\)/K\(^+\) ATPase. In our experiments the co-expression of Na\(^+\)/K\(^+\) ATPase did not reverse inhibition of surface expression by the T/A mutation (Figures 12, 13).

Interestingly, marked inhibition of FXYD7 surface expression in *Xenopus* oocytes, was also apparent by mutating the FXYD motif common to all members of the family. Mutating the conserved F,Y and D residues into A had no effect on O-glycosylation of FXYD7. This is evident from the electrophoretic mobility of the mutated protein (un-shown data and (Crambert et al., 2004)). The crystal structure of shark rectal gland Na\(^+\)/K\(^+\) ATPase demonstrates interactions of this motif with both \(\alpha\) and \(\beta\) suggesting a structural role (Shinoda et al., 2009). The fact that mutating this domain has a marked effect on the translocation of FXYD7 to the plasma membrane even when the protein is expressed without \(\alpha\beta\) Na\(^+\)/K\(^+\) ATPase, suggests an additional yet unknown role of this motif in the intracellular trafficking of FXYD proteins. The above data differs from that reported in (Crambert et al., 2004) were the kinetic effects of FXYD7 on the Na\(^+\)/K\(^+\) ATPase was not affected by the AFAA nor the 3T/A mutations. It is possible that under the experimental conditions of this study sufficient mutated protein reaches the oocyte membrane to form 1:1 complex with the amount of Na\(^+\)/K\(^+\) ATPase expressed by them.

Similarly to *xenopus* oocytes, expression of T3/5/9A in H1299 cells showed that the protein is unable to reach the plasma membrane, and reaches only as far as the Golgi apparatus. Cellular retention of the glycosylation defective FXYD7 in H1299 cells was also demonstrated by surface biotinylation of the cells. In this assay, it is clearly shown that while the cells express both glycosylated (high MW) and unglycosylated proteins, only the mature form is expressed at the cell surface. These results are consistent with O-glycosylation of the FXYD7, as the various glycosyltransferases,
mediating the O-glycosylation process, reside in the Golgi (Van den Steen et al., 1998). The results from both oocytes and mammalian cells, suggest that surface expression of FXYD7 and its regulatory role are independent of one another, and that O-glycosylation of FXYD7 is important for membrane targeting rather than for the Na⁺/K⁺ ATPase modulation.

In an attempt to examine whether FXYD7 indeed undergoes O-glycosylation, we treated FXYD7 injected oocytes with BGN. BGN is a structural analogue of GalNAc-α-1-O-serine/threonine, the first metabolite of the O-glycosylation process. Inside cells, it is metabolized to benzyl disaccharide Galβ1-3GalNAca-O-bn, which acts as a competitive inhibitor of the synthesis of the glycosyltransferase ST3Gal-I (Gouyer et al., 2001). The lack of effect of BGN on FXYD7 expressed in *xenopus* oocytes may in principle be accounted for by inability to achieve a sufficiently high intracellular localization due to limited permeabilization. We attempted to circumvent such limitation by injecting BGN into the oocytes. This however, does not exclude lack of effect due to compartmentalization within the oocytes filled with yolk granules. Yet, lack of effect of BGN were observed also in FXYD7 transfected H1299 and HEK293 cells (data not shown). Gouyer et al. have found that different cell types express different set of glycosyl transferases and as a result, the same protein may be modified differently in different cells, and exhibit different sensitivities to the inhibitor (Gouyer et al., 2001). This may account for the lack of effect of BGN on FXYD7 in mammalian cells. Another possibility is that FXYD7 is not O-glycosylated and the multiple sizes affected by the T to A mutations reflect some another post translational modification. We consider such a possibility highly unlikely.

The above study demonstrates a role of N-terminal O-glycosylation as well as C-terminal positive charges in the surface expression of FXYD7 and PLM respectively. Yet, the ability of FXYD7 to reach the cell surface of oocytes without association with the Na⁺/K⁺ ATPase, raises the possibility that FXYD7 has a cellular role other than modulating the Na⁺/K⁺ ATPase kinetics This hypothesis was examined by expressing FXYD7 in M1 cells. These cells have been previously used by our group to characterize the functional effect of FXYD5 on the paracellular permeability (Lubarski et al., 2011). Expressed in M1 cells, FXYD7 increased the transepithelial resistance compared to WT cells (P<0.01). Also, a faster increase of the
transepithelial resistance suggests a faster formation of cell-cell contact. These effects are not attributed to a faster proliferation rate of the transfected cells, as indicated by cell proliferation assay. However, FXYD7 transfected cells adhere at a slower rate to the plate, and tend to aggregate. A similar effect of FXYD5 on the adhesion of M1 cells was previously reported (Lubarski et al. unpublished data).

A surprising result was that FXYD7 transfected cells fail to develop Isc. This effect may either result from direct effect on ENaC or secondary to inhibition of the Na⁺/K⁺ ATPase, and intracellular Na⁺ accumulation (Macrobbie and Ussing, 1961). Selective biotinylation of the basolateral and apical surfaces of M1 cells and immunocytochemistry of polarized M1 cells, demonstrated that FXYD7 is expressed exclusively at the basolateral surface of the cells, and thus a direct effect on ENaC, which is localized to the apical membrane can be excluded. This result provides an additional evidence that FXYD7 does not resides in the plasma membrane independent of the Na⁺/K⁺ ATPase. As discussed in the introduction, FXYD7 is expressed in non-epithelial brain cells. As such it is not expected to have signals that will target it exclusively to the basolateral pole in transfected epithelial cells. The fact that in M1 cells it is found in the basolateral but not apical membrane is best described by intracellular association with the Na⁺/K⁺ ATPase which does have such targeting signals. Real-time PCR analysis of ENaC expression level showed an altered expression of α and β ENaC. Additional genes have been shown to be regulated by FXYD5 (Lubarski et al unpublished). Thus, we suspect that these reflect some general effect of FXYD7 on gene expression, reported before for FXYD5 and FXYD3. While the observed effects of FXYD7 on the paracellular resistance, cell adhesion, aggregation and down regulation of αENaC mRNA are far from being understood, they do suggest additional roles of this FXYD protein as reported for FXYD3 and FXYD5. These however are outside the scope of this thesis.

In summary, the current study characterizes intracellular trafficking of PLM and FXYD7 in Xenopus oocytes and mammalian cells and identified motifs and residues determining their efficiency. It demonstrated that unlike PLM, FXYD7 can travel to the plasma membrane independent of the Na⁺/K⁺ ATPase but it appears that this is not
the case in mammalian cells under normal expression levels. We did however obtained evidence for a function other than modulation the pump kinetics. Such effects may be secondary to interactions with the Na\(^+/\)K\(^+\) ATPase and its effects on cell adhesion.
References


The FXYD family is composed of seven small membrane-associated proteins, named FXYD, based on the presence of two conserved glutamate residues in all family members. Each member of the family binds specifically to a specific transporter, and the FXYD7 homolog, xenopus, is involved in the transport of ions between the cytoplasm and the extracellular space.

In this study, the factors influencing the transmembrane movement from RE and igloG to the cytoplasmic membrane of two family members, FXYD1 (phospholemman, PLM) and FXYD7, were investigated. Preliminary results obtained using Xenopus laevis expression systems showed that the PLM membrane requires a prior interaction with the carrier. However, the ion transporters are not essential for the PLM expression. It was also found that the arrival of FXYD1 depends on the phosphorylation of the cytoplasmic end of the protein, or by the removal of three arginines at the C-terminal end of the protein.

In contrast, FXYD7 was able to reach the membrane without interaction with the carrier, suggesting additional functions for this protein that are not related to the kinetics of the transporter.

An important additional finding was that the transmembrane expression of FXYD7 is dependent on O-glycosylation of at least two of the three serine residues conserved in the extracellular domain of the protein. In continuation, we sought to extend the conclusions to animal expression systems. It was found that the expression of FXYD7 in animal cells is blocked by the presence of a carrier, and that FXYD7 is not capable of reaching the membrane in the absence of the carrier, but shows additional roles.

The findings suggest new roles for FXYD7 in the transport of ions and other molecules, as well as new functions for FXYD1 in the transport of ions.