Regulation of the cardiac Na^+/K^+-ATPase by phospholemman

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Abstract

Phospholemman (PLM) is a regulatory subunit of the cardiac Na^+/K^+-ATPase (NKA), but exists also as an independent tetramer. The membrane spanning protein consists of 72 amino acid residues and is the first member of the FXYD motif-containing family of tissue specific NKA regulatory subunits (FXYD1). A comparative model of the human PLM/NKA complex shows the interactions between NKA and the extracellular FXYD motif as well as the transmembrane helix-helix interactions. A variety of intracellular posttranslational modifications points to a highly dynamic picture of interactions between NKA and the intracellular part of PLM. Posttranslational modifications of PLM include NKA-activating phosphorylation, inhibiting palmitoylation and activating glutathionylation. PLM gene expression has the potential for posttranscriptional regulation by the formation of potassium-ion stabilised G-quadruplex structures in pre-splicing mRNA. The overall physiological role of cardiac PLM is to protect the heart under conditions of increased heart rate and oxidative stress avoiding calcium overload of the cytoplasm and arrhythmias. The PLM tetramer possibly exists as a storage pool in order for the heart to react quickly to changing conditions.

Key words: fxyd1, cardiac protein, transmembrane protein, protein-protein interactions, posttranslational modification, oligomerisation

1 Introduction

The Na^+/K^+-ATPase (NKA) is a P-type ATPase originally discovered by Skou [1] and responsible for the export of three sodium ions to the outside of the cell and the import of two potassium ions. This ion transport against the concentration gradient is driven by the hydrolysis of ATP. The ion gradient established by NKA is essential to many membrane transport processes including the generation of action potentials in nerve and muscle cells. NKA is composed of a minimum of two subunits, namely the catalytically active α subunit with a molecular mass of approximately 100 kDa and the β subunit with approximately 33 kDa (in humans), which is required for intracellular transport of NKA to the plasma membrane [2]. At least four isoforms of α subunits and three β isoforms are known, while in cardiac muscle $\alpha_1\beta_1$ and $\alpha_2\beta_1$ are mainly expressed. With ouabain-based photoaffinity labels a third γ subunit (also known as FXYD2) was discovered in the porcine kidney [3] and later it was reported that NKA is generally associated with a third subunit. Whether this association is permanent or transient remains a matter of investigation. The identity of the third subunit varies in different tissues, but the FXYD (phenylalanine-X-tyrosine-aspartate) sequence motif is common to the third subunit in all tissues [4]. In cardiac muscle tissue the third subunit is termed phospholemman [5] or FXYD1, which is also used as the name of the gene encoding for the protein phospholemman (PLM).

While the structure and function of NKA has been reviewed in chapter 1 of this volume, this chapter focuses on the structural and biochemical aspects of the NKA regulation by PLM and its physiological consequences. First, the protein structure of PLM is discussed with reference to various sites of posttranslational modification, followed by the structure of the human FXYD1 gene and its potential for regulation at the level of transcription and mRNA processing, and finally the physiological consequences of PLM-NKA interaction are reviewed. Many excellent reviews about PLM and its physiological role in particular have appeared in the literature [6-9] and should be consulted by the interested reader in addition to the present chapter.

2 Phospholemman sequence and structure

The protein phospholemman (PLM) was originally characterised as a major plasma membrane substrate of protein kinase A (PKA) and C (PKC) that consisted of 72 amino acid residues in the mature protein and a cleavable 20-residue N-terminal signal sequence [10]. PLM is expressed in the canine heart, skeletal muscle, smooth muscle and liver, while it is absent from the brain and the kidney. Later, as outlined above, PLM was classified as the first member of the FXYD class of proteins [4]. The phosphorylation sites have been identified in later studies as Ser63 (PKC) and Ser68 (PKA and PKC) and Thr69 (PKC) [11]. Palmitoylation occurs at Cys40 and Cys42 [12] and glutathionylation at Cys42 [13]. Due to the increasing number of genome sequencing projects we can, at the time of writing, identify 25 sequences of FXYD1 from UniProt [14]. Except the *Ophiophagus hannah* (King cobra) FXYD1, all other 24 organisms FXYD1 sequence show >50% sequence identity. The multiple sequence

alignment of FXYD1 protein sequences without signal sequence is shown in figure 1 colour coded by conservation with a blue-red gradient. It can be seen that there are very few variations to the FXYD motif. Other regions of high conservation are the transmembrane domain as well as the intracellular C-terminal domain between Cys40 and Arg66 with some exceptions. Note that PLM follows the positive-inside rule for transmembrane proteins [15] showing a higher number of positively charged residues on the intracellular part of the protein as well as aromatic residues Tyr11 and Tyr13 at the membrane water interface.

2.1 The phospholemman monomer

The 3D-structure of the PLM monomer was determined by solution-state NMR spectroscopy (PDB-ID: 2JO1) in SDS micelles at pH 5 [16]. It reveals, in addition to a long helix from Gln14 to Phe44 encompassing the transmembrane domain, short helical segments in the N-terminal part and a longer helical segment from Phe60 to Thr69 in the C-terminal part. The angle of the helical axes between the two main helical segments is approximately 90° giving rise to the characteristic L-shape of the PLM monomer. The C-terminal part including the helix is very basic containing altogether six Arg residues between Phe60 and Arg72, thus it is positively charged at physiological pH. This explained the association with the negatively charged micelle surface in the NMR study. Teriete et al. suggested that negative charges introduced by phosphorylation could lead to a reorientation of the C-terminal segment facilitated by the flexible linker region between Asp45 and Thr59. Interestingly there is a high level of conservation in the linker region among FXYD1 orthologues (fig. 1).

2.2 The phospholemman tetramer

Gel-electrophoresis of synthetic PLM transmembrane peptides with perfluoro-octaneoate, a detergent that keeps transmembrane protein complexes intact, has shown that PLM forms tetramers [17]. In the same study it was shown by attenuated total reflection Fourier transform infrared spectroscopy (ATR-FTIR) that the lipid-membrane embedded tetramers are α-helical and have a predominantly transmembrane orientation similar to the monomer structure obtained in SDS micelles. A solid state magic angle spinning ^{17}O-NMR study of the lipid-membrane embedded PLM transmembrane domain indicated that the tetramer is not completely symmetric, but may have a C_2 (dimer of dimers) or C_1 rotational symmetry along the bilayer normal [18]. An atomic structural model of the tetrameric PLM transmembrane domain was obtained by a combination of orientational constraints derived from site-specific infrared dichroism [reviewed in 19] and a systematic conformational search based on molecular dynamics simulation of a transmembrane helical bundle protein [20]. The PLM transmembrane helical bundle reveals an average helix tilt angle of 7.3° in line with the previous solution state NMR-study of the monomer. The tetramer is closely packed and does not show any indication of an open pore in the centre of the tetramer. However, the helix-helix packing shows the unusual feature that some small residues, such as Gly19 and Gly20, point to the outside of the helical bundle (figure 2a), which supported the hypothesis that a PLM tetramer may occur in vivo as a storage form that readily

interacts with NKA [21]. A two-stage model of PLM-NKA interaction was proposed that involves a slow interaction of the PLM tetramer with NKA leading to the abstraction of one PLM monomer (figure 2b). In the second stage the remaining PLM trimer interacts fast with other NKA molecules. The existence of PLM tetramers in human embryonic kidney cells has been confirmed by fluorescence resonance energy transfer (FRET) and it was shown that the tetramer is stabilised by phosphorylation [22]. More recently the existence of PLM homo-oligomers was reported in cardiac muscle [23]. In this carefully conducted study a significant proportion of PLM (>50%) was identified that was not associated with NKA but formed a separate pool of multimeric PLM. The stoichiometry of multimeric PLM was not clearly identified, but using formaldehyde crosslinking without denaturation prior to electrophoresis resulted in a band consistent with a tetramer that was detected by Western-blotting probed with a PLM-phospho-Ser68 specific antibody. The phosphorylation pattern of NKA-associated and multimeric PLM is different, with NKA associated PLM being unphosphorylated or phosphorylated at Ser68, while multimeric PLM shows Ser63 phosphorylation in addition to phosphorylation at other sites. It was suggested that phosphorylation does not change the distribution between multimeric and NKA-associated PLM, but the protein phosphatase PP2A associated with NKA leads to a dephosphorylation of PLM-Ser63 [23]. The PLM multimer in cardiac muscle cells located in the same membrane compartment as NKA does not seem to interact with other proteins, thus it was suggested that it acts as storage pool for PLM confirming the postulation made in earlier work [21]. There is a potential parallel to phospholamban (PLB) that regulates the sarco/endoplasmatic reticulum Ca-ATPase (SERCA). Although not related by sequence similarity, PLB is structurally similar to PLM with 52-amino acid residues and one transmembrane domain. It associates with and regulates SERCA via phosphorylation and exists as a pentamer in its non-associated form [reviewed in 24]. Since PLB shows measureable ion channel activity, the function of the PLB pentamer is still under discussion [25,26], while based on the structure of the PLM tetramer transmembrane domain, no potential for ion conduction was found [21], thus the hypothesis of the PLM tetramer as a storage pool for regulation of NKA seems the most likely one.

2.3 The structure of the phospholemman-NKA complex

The atomic structure of the cardiac human phospholemman-NKA complex is not known, but a number of potassium-bound homologue structures are available in the Protein Data Bank, namely the pig renal NKA with parts of FXYD2 at 3.5 Å resolution (PDB-ID: 3B8E) [27], a shark NKA model in the E2 state with FXYD10 at 2.4 Å resolution (PDB-ID: 2ZXE) [28] and a sodium bound form from pig kidney at 4.2 Å resolution (PDB-ID: 4HQJ) [29]. In particular the high resolution shark NKA model shows a large portion of the FXYD10 subunit including the FXYD motif. The sequence identity between the shark and the human NKA subunits is 88% for the α-subunit, 65% for the β-subunit and 35% between FXYD10 and PLM, while a 30% sequence identity is considered as the lower limit for comparative modelling [30]. For

this review we have prepared a comparative model of the human NKA/PLM complex using the shark 2ZXE and the human PLM monomer structure (2JO1, 100% sequence identity) as templates with the explicit inclusion of three potassium ions, one Mg^{2+} and one phosphate analogue MgF_4^{2-} ion found in the 2ZXE template. The structure of this comparative model shown in figure 3 is discussed in the following text. The PLM backbone structure is coloured according to sequence conservation showing a tendency towards lower conservation of residues facing away from NKA. The arrangement of subunits is shown in figure 4a, revealing the details of the interactions of the FXYD1 motif with NKA (figure 4b), namely Phe9 is interacting with residues Val183, Gln69 Val72, Ala73, Phe186 of the β-subunit, and Tyr11 is interacting with Tyr68, Asp70 (β-subunit), Lys984, Pro985 (α-subunit), while Asp10 is not involved in close interactions with its side chain pointing towards the solvent.

The palmitoylation sites Cys40 and Cys42 are located at the C-terminal end of the long helix and face to opposite sites of the helix. Cys40 points towards the outside (fig. 4c), while Cys42 (hidden in fig. 4c) points towards the α-subunit with the closest contact being the guanidinium group of Arg353 at a distance of 5.8 Å from the cysteine sulphur atom. The positive Arg328 could stabilise the thiolate anion of Cys42 at physiological pH, which may explain the susceptibility of Cys42 to glutathionylation (see section 4.3). The thiolate anion is much more reactive towards forming disulphide bonds than the protonated form. Some information about the structural consequences of phosphorylation comes from fluorescence resonance energy transfer (FRET) experiments between cyan fluorescent protein labelled NKA and yellow fluorescent protein labelled PLM. Upon phosphorylation of Ser63 and Ser68, the amount of FRET decreased indicating that the intermolecular distance between PLM and NKA was increased [31]. It is possible that phosphorylation increases the flexibility of the PLM C-terminus and releases it from NKA binding sites. Another possibility is that the phosphorylated PLM C-terminus shifts to other phospho-specific NKA binding sites as indicated by experiments with PLM knockout mice. Upon addition of a synthetic Ser68 phosphopeptide (PLM residues 54-72), NKA activation was observed [32]. A FRET study that investigated alanine mutations on the α1-subunit of NKA at Phe956, Glu960, Leu964 and Phe967 identified the interaction between Glu960 and Phe28 of PLM to be among the critical interaction sites between PLM and NKA. The comparative model of NKA/PLM developed for this review identifies a close interaction between Glu960 and Phe28 as shown in figure 5, although this information was not included in the modelling protocol. The structure of the short C-terminal helix containing the phosphorylation sites is entirely based on the solution-state NMR structure of the PLM monomer, thus the orientation and any interactions shown are not indicative of the native PLM-NKA complex. In the absence of structural detail, even available through comparative modelling, molecular dynamics simulations of the PLM-NKA complex in realistic lipid bilayers [33] could be carried out to assess the molecular details of this interaction.

3 The phospholemman/FXYD1 gene

The human FXYD1 gene is located on the q arm of chromosome 19 from bases 35,138,789 - 35,143,055 (assembly GRCh38) amounting to 4286 bases in the full transcript. There are in total eight exons including one 5′ and one 3′ untranslated exon. As outlined above the PLM protein is a target of significant posttranslational modification. Another way to regulate proteins is via the regulation of gene expression. As part of the ENCODE project transcription factors associated with genes were identified with ChIP-Seq (Chromatin Immuno-Precipitation Sequencing) experiments [34]. Using the ENCODE ChIP-Seq Significance Tool [35] a number of potential transcription factors were found around the transcription start site of FXYD1 (shown in table 1). Among general transcription factors, such as TAF required for the DNA transcription activity, there are a number of transcription factors involved in cell differentiation, such as BHLHE40 and EGR1. P300 is the only cardiac-related transcription factor implicated in the enlargement of cardiac myocytes. This may reflect the variety of cell types analysed in the ENCODE project with underrepresentation of cardiac myocytes due to the difficulties of obtaining functional cardiac myocytes in cell culture. It can, however, be concluded that the FXYD1 gene is under the control of transcription factors.

The initial pre-mRNA transcript is subjected to the process of splicing and eleven transcripts are reported in Ensembl Human Release 77 [36]. Nine of those are supported by at least one expressed sequence tag (transcript support level 1 to 3). Most transcripts differ in the position of the transcription start site and the length and or existence of the last non-protein coding exon. Transcript FXYD1-008 encodes for a 115 residue protein that contains a 27 residue insert after the transmembrane domain (after LIVLS in figure 1). The 115 residue protein appears to have a 12-residue shortened signal peptide, but a 5′ truncation in the transcript evidence prevented the complete assignment of the coding sequence, thus the transcript may indeed contain the full 20-residue signal peptide. Two of the well supported transcripts, FXYD1-004 and FXYD1-010 have a retained intron but do not contain any protein coding region as annotated by the HAVANA team [37].

A computational analysis of the *Homo sapiens* FXYD1 pre-mRNA alongside sixteen orthologues revealed that the FXYD1 pre-mRNA contains sequences capable of folding into higher-order intramolecular RNA structures called G-quadruplexes and that this feature is conserved in evolution [38]. G-quadruplexes are formed by square planar arrangements of four guanine bases (G-tetrads) stabilised by hydrogen bonds. At least two G-tetrads stack together stabilised by sandwiched potassium ions [39]. In FXYD1 and orthologues it was found that stretches of three Gs were conserved indicating that three G-tetrads could form a stable G-quadruplex structure. Using synthetic oligonucleotides with sequences taken from human and bovine FXYD1 pre-mRNA, the formation of G-quadruplexes was shown *in vitro* using fluorescence spectroscopy and native polyacrylamide gel electrophoresis [38]. It is interesting to note in the context of the sodium-potassium exchanging NKA that the required potassium ion concentration for stable G-quadruplexes to form is in the region of 100

mM, which is similar to the intracellular potassium ion concentration of 120 mM. Considering the evolutionary conservation of G-quadruplex forming sequences it was suggested that G-quadruplex formation of FXYD1 pre-mRNA may control the splicing (either through inhibition or favouring alternative spliced products) and thus the expression of the phospholemman protein product [38] (see figure 6). However, these *in-vitro* results await further experimental confirmation in particular in cardiomyocytes.

4 Physiology of NKA regulation by phospholemman

While the presence of PLM is not essential for survival as PLM knockout experiments have shown [40], PLM may have a protective role for the heart under conditions such as increased heart rate and oxidative stress. PLM knockout mice showed slightly depressed cardiac contractile function as well as a mild cardiac hypertrophy. Under the conditions of increased stimulation frequency and β-adrenoreceptor activation PLM knockout mice showed a larger increase in intracellular sodium concentration, a larger calcium load of the sarcoplasmic reticulum and a larger calcium transient leading to more arrhythmias compared to wild type mice [40]. Therefore it was postulated that the physiological role of PLM "may be to limit the rise in intracellular Na^+ during sympathetic stimulation and thereby preventing Ca^{2+} overload and triggered arrhythmias in the heart" [8].

4.1 Phospholemman phosphorylation and dephosphorylation

PLM is phosphorylated by protein kinase A (PKA) at Ser68 [41] and at Ser63, Ser68 and Thr69 by protein kinase C [11]. The biochemical consequences of PKA phosphorylation is an increase in sodium affinity [42], while PKC phosphorylation increases v_{max} [43]. Overall PLM phosphorylation stimulates NKA, while unphosphorylated PLM inhibits NKA activity [44,32] explaining the physiological role of PLM mentioned above.

The regulation of PLM by phosphatases has been investigated to a much lesser extent than kinases, but recently it was shown that the Ser/Thr phosphatase 1 (PP-1) acts on Ser68 [45], but not on Thr69 under physiological conditions as the reported EC_{50} of 2.7 μM for Thr69 was too high. Ser63 is dephosphorylated by Ser/Thr protein phosphatase 2A (PP-2A) [45], which was shown to be associated with the NKA complex [46]. As outlined above the PLM Ser63 dephosphorylation occurs most likely, when PLM is associated with NKA, but not in the PLM tetramer complex [23]. Taken together PLM phosphorylation exerts a protective effect on the heart muscle [47]. In the absence of PLM an increased heart rate and β-adrenergic stimulation would lead to a higher intracellular sodium concentration and a larger calcium content of the sarcoplasmic reticulum leading to more arrhythmias. NKA activation via phosphorylation of PLM prevents this.

4.2 Phospholemman palmitoylation

As outlined in section two, PLM is palmitoylated at Cys40 and Cys42 [12]. The effect of palmitoylation is an increase of the half-life of PLM and a decrease of NKA activity. Furthermore, PLM phosphorylation at Ser68 increased its palmitoylation [12], which is surprising as phosphorylation and palmitoylation events are causing opposite effects. At this point the physiological significance of PLM palmitoylation has not been established. The enzymes responsible for palmitoylation of proteins are palmitoyl-S-transferase enzymes (known as DHHC proteins) and depalmitoylation is catalysed by thioesterase enzymes. It was shown in human fibroblast-derived cardiomyocytes that overexpression of DHCC5 decreased NKA pump currents by 55%, while siRNA knockdown of DHCC5 increased NKA pump currents by 38% [48]. Thus, DHCC5 contributes to palmitoylation of PLM leading to a subsequent decrease in NKA activity. In the same study it was postulated that the damage caused by reoxygenation of cardiac tissue after an ischemic event may be related to PLM palmitoylation. Upon reperfusion massive endocytosis of cardiac cell membrane including the NKA/PLM complex occurs that limits the recovery after an ischemic event. Several observations linked PLM palmitoylation to this event, namely internalised PLM was palmitoylated to a higher level than cell-surface bound PLM and the extent of massive endocytosis was reduced in hearts lacking PLM or DHHC5. Thus reperfusion damage may be reduced by inhibition of palmitoylation [48]. The normal physiological function of PLM palmitoylation may be to regulate NKA under conditions of metabolic stress or to contribute to NKA/PLM turnover.

4.3 Phospholemman glutathionylation

The sensitivity of NKA to oxidative stress and to the cellular sulfhydryl redox status is already known since a long time [49,50] and linked to glutathionylation of the α [51] and β1 subunit [52], which both have inhibitory effects. PLM has been shown to reverse glutathionylation of the β1 subunit by becoming itself glutathionylated at Cys42, thus exerting an activating effect on NKA [13]. However, the physiological role of PLM glutathionylation is complicated, as it is linked with phosphorylation by PKA and palmitoylation that also occurs at Cys42. Palmitoylation of PLM, which has an inhibitory effect, is promoted by PKA phosphorylation at Ser68 [12], and to further complicate matters, oxidative stress can activate PKA [53]. Possibly electrophysiological modelling of cardiomyocyte activity taking explicit account of PLM could explain some of the complexities. Models of heart failure that highlight the importance of sodium currents and NKA activity have been recently presented [54], albeit modulation of NKA by PLM was not included.

5 Conclusions

PLM has emerged as an important modulator of NKA in particular under conditions of stress, such as increased heart rate or metabolic stress. A number of posttranslational modifications of PLM have been characterised and their

physiological consequences have been described. Further research is needed to understand the interplay between different posttranslational modifications and how they relate to overall cardiac physiology. Additionally, PLM regulation may occur at the level of gene expression, involving transcription, processing of mRNA, regulating the stability of mRNA and the rate of transcription. Gene regulation with regards to PLM is largely unexplored, but the exciting possibility of potassium ion-stabilised higher-order G-quadruplex structures of PLM pre-mRNA warrants further investigations. Progress has been made in the area of structural biology with the determination of high-resolution human homologue NKA/PLM structures, the structure of a PLM monomer and a tetramer complex. These structures can be used for comparative modelling of the human NKA/PLM complex as it was attempted for this review. However, experimentally determined protein structures and comparative models provide only a static picture, while the NKA-PLM interactions modulated by posttranslational modifications are surely dynamic. Most experimental methods investigating this dynamic interaction will be confounded by the heterogeneity of PLM posttranslational modifications in cardiomyocytes, thus single-molecule based experimental methods together with complementary *in silico* simulation methods may be required to resolve this heterogeneity.

6 References

1. Skou JC (1957) The influence of some cations on an adenosine triphosphatase from peripheral nerves. Biochem Biophys Acta 23:394-401.

2. Geering K (1991) The functional role of the beta-subunit in the maturation and intracellular transport of Na,K-ATPase. FEBS Lett 285 (2):189-193.

3. Forbush B, 3rd, Kaplan JH, Hoffman JF (1978) Characterization of a new photoaffinity derivative of ouabain: labeling of the large polypeptide and of a proteolipid component of the Na, K-ATPase. Biochemistry 17 (17):3667-3676.

4. Sweadner KJ, Rael E (2000) The FXYD gene family of small ion transport regulators or channels: cDNA sequence, protein signature sequence, and expression. Genomics 68 (1):41-56.

5. Crambert G, Fuzesi M, Garty H et al. (2002) Phospholemman (FXYD1) associates with Na,K-ATPase and regulates its transport properties. Proceedings of the National Academy of Sciences of the United States of America 99 (17):11476-11481.

6. Fuller W, Tulloch LB, Shattock MJ et al. (2013) Regulation of the cardiac sodium pump. Cell Mol Life Sci 70 (8):1357-1380.

7. Shattock MJ (2009) Phospholemman: its role in normal cardiac physiology and potential as a drugable target in disease. Current Opinion in Pharmacology 9 (2):160-166.

8. Pavlovic D, Fuller W, Shattock MJ (2013) Novel regulation of cardiac Na pump via phospholemman. J Mol Cell Cardiol 61:83-93.

9. Cheung JY, Zhang XQ, Song J et al. (2010) Phospholemman: a novel cardiac stress protein. Clin Transl Sci 3 (4):189-196.

10. Palmer CJ, Scott BT, Jones LR (1991) Purification and complete sequence determination of the major plasma membrane substrate for cAMP-dependent protein kinase and protein kinase C in myocardium. J Biol Chem 266 (17):11126-11130.

11. Fuller W, Howie J, McLatchie LM et al. (2009) FXYD1 phosphorylation in vitro and in adult rat cardiac myocytes: threonine 69 is a novel substrate for protein kinase C. American Journal of Physiology-Cell Physiology 296 (6):C1346-C1355.

12. Tulloch LB, Howie J, Wypijewski KJ et al. (2011) The inhibitory effect of phospholemman on the sodium pump requires its palmitoylation. J Biol Chem 286 (41):36020-36031.

13. Bibert S, Liu CC, Figtree GA et al. (2011) FXYD proteins reverse inhibition of the Na+-K+ pump mediated by glutathionylation of its beta1 subunit. J Biol Chem 286 (21):18562-18572.

14. TheUniProtConsortium (2014) Update on activities at the Universal Protein Resource (UniProt). Nucleic Acids Res 42 (Database issue):D191-198.

15. Heijne G (1986) The distribution of positively charged residues in bacterial inner membrane proteins correlates with the trans-membrane topology. EMBO J 5 (11):3021-3027.

16. Teriete P, Franzin CM, Choi J et al. (2007) Structure of the Na,K-ATPase regulatory protein FXYD1 in Micelles. Biochemistry 46 (23):6774-6783.

17. Beevers AJ, Kukol A (2006) Secondary structure, orientation, and oligomerization of phospholemman, a cardiac transmembrane protein. Protein Science 15 (5):1127-1132.

18. Wong A, Beevers AJ, Kukol A et al. (2008) Solid-state O-17 NMR spectroscopy of a phospholemman transmembrane domain protein: Implications for the limits of detecting dilute 170 sites in biomaterials. Solid State Nuclear Magnetic Resonance 33 (4):72-75.

19. Kukol A (2005) Site-specific IR spectroscopy and molecular modelling combined towards solving transmembrane protein structure. Spectroscopy 19:1-16.

20. Beevers AJ, Kukol A (2008) Transmembrane protein models based on high-throughput molecular dynamics simulations with experimental constraints. Methods Mol Biol 443:213-227.

21. Beevers AJ, Kukol A (2007) Phospholemman transmembrane structure reveals potential interactions with Na+/K+-ATPase. Journal of Biological Chemistry 282 (45):32742-32748.

22. Song QJ, Pallikkuth S, Bossuyt J et al. (2011) Phosphomimetic Mutations Enhance Oligomerization of Phospholemman and Modulate Its Interaction with the Na/K-ATPase. Journal of Biological Chemistry 286 (11):9120-9126.

23. Wypijewski KJ, Howie J, Reilly L et al. (2013) A separate pool of cardiac phospholemman that does not regulate or associate with the sodium pump: multimers of phospholemman in ventricular muscle. J Biol Chem 288 (19):13808-13820.

24. Kranias EG, Hajjar RJ (2012) Modulation of cardiac contractility by the phospholamban/SERCA2a regulatome. Circ Res 110 (12):1646-1660.

25. Becucci L, Cembran A, Karim CB et al. (2009) On the function of pentameric phospholamban: ion channel or storage form? Biophys J 96 (10):L60-62.

26. Smeazzetto S, Saponaro A, Young HS et al. (2013) Structure-function relation of phospholamban: modulation of channel activity as a potential regulator of SERCA activity. PLoS One 8 (1):e52744.

27. Morth JP, Pedersen BP, Toustrup-Jensen MS et al. (2007) Crystal structure of the sodium-potassium pump. Nature 450 (7172):1043-U1046.

28. Shinoda T, Ogawa H, Cornelius F et al. (2009) Crystal structure of the sodium-potassium pump at 2.4 A resolution. Nature 459 (7245):446-450.

29. Nyblom M, Poulsen H, Gourdon P et al. (2013) Crystal structure of Na+, K(+)-ATPase in the Na(+)-bound state. Science 342 (6154):123-127.

30. Rost B (1999) Twilight zone of protein sequence alignments. Protein Eng 12 (2):85-94.

31. Bossuyt J, Despa S, Martin JL et al. (2006) Phospholemman phosphorylation alters its fluorescence resonance energy transfer with the Na/K-ATPase pump. Journal of Biological Chemistry 281 (43):32765-32773.

32. Pavlovic D, Fuller W, Shattock MJ (2007) The intracellular region of FXYD1 is sufficient to regulate cardiac Na/K ATPase. FASEB J 21 (7):1539-1546.

33. Kukol A (2009) Lipid models for united-atom molecular dynamics simulations of proteins. Journal of Chemical Theory and Computation 5 (3):615-626.

34. Consortium TEP (2012) An integrated encyclopedia of DNA elements in the human genome. Nature 489:57-74.

35. Auerbach R (2014) ENCODE ChIP-Seq Significance Tool. Leland Stanford Junior University. http://encodeqt.simple-encode.org/. Accessed 22/11/2014 2014

36. Flicek P, Amode MR, Barrell D et al. (2014) Ensembl 2014. Nucleic Acids Res 42 (Database issue):D749-755.

37. Djebali S, Davis CA, Merkel A et al. (2012) Landscape of transcription in human cells. Nature 489 (7414):101-108.

38. Dhayan H, Baydoun AR, Kukol A (2014) G-quadruplex formation of FXYD1 pre-mRNA indicates the possibility of regulating expression of its protein product. Arch Biochem Biophys 560:52-58.

39. Franceschin M (2009) G-Quadruplex DNA Structures and Organic Chemistry: More Than One Connection. European Journal of Organic Chemistry (14):2225-2238.

40. Bell JR, Kennington E, Fuller W et al. (2008) Characterization of the phospholemman knockout mouse heart: depressed left ventricular function with increased Na-K-ATPase activity. Am J Physiol Heart Circ Physiol 294 (2):H613-621.

41. Walaas SI, Czernik AJ, Olstad OK et al. (1994) Protein kinase C and cyclic AMP-dependent protein kinase phosphorylate phospholemman, an insulin and adrenaline-regulated membrane phosphoprotein, at specific sites in the carboxy terminal domain. Biochem J 304 (Pt 2):635-640.

42. Bibert S, Roy S, Schaer D et al. (2008) Phosphorylation of phospholemman (FXYD1) by protein kinases A and C modulates distinct Na,K-ATPase isozymes. J Biol Chem 283 (1):476-486.

43. Han F, Bossuyt J, Despa S et al. (2006) Phospholemman phosphorylation mediates the protein kinase C-dependent effects on Na+/K+ pump function in cardiac myocytes. Circulation Research 99 (12):1376-1383.

44. Crambert G, Fuzesi M, Garty H et al. (2002) Phospholemman (FXYD1) associates with Na,K-ATPase and regulates its transport properties. Proc Natl Acad Sci U S A 99 (17):11476-11481.

45. El-Armouche A, Wittkopper K, Fuller W et al. (2011) Phospholemman-dependent regulation of the cardiac Na/K-ATPase activity is modulated by inhibitor-1 sensitive type-1 phosphatase. FASEB J 25 (12):4467-4475.

46. Kimura T, Han W, Pagel P et al. (2011) Protein phosphatase 2A interacts with the Na,K-ATPase and modulates its trafficking by inhibition of its association with arrestin. PLoS One 6 (12):e29269.

47. Despa S, Tucker AL, Bers DM (2008) Phospholemman-mediated activation of Na/K-ATPase limits [Na]i and inotropic state during beta-adrenergic stimulation in mouse ventricular myocytes. Circulation 117 (14):1849-1855.

48. Lin MJ, Fine M, Lu JY et al. (2013) Massive palmitoylation-dependent endocytosis during reoxygenation of anoxic cardiac muscle. Elife 2:e01295.

49. Shattock MJ, Matsuura H (1993) Measurement of Na(+)-K+ pump current in isolated rabbit ventricular myocytes using the whole-cell voltage-clamp technique. Inhibition of the pump by oxidant stress. Circ Res 72 (1):91-101.

50. Haddock PS, Shattock MJ, Hearse DJ (1995) Modulation of cardiac Na(+)-K+ pump current: role of protein and nonprotein sulfhydryl redox status. Am J Physiol 269 (1 Pt 2):H297-307.

51. Petrushanko IY, Yakushev S, Mitkevich VA et al. (2012) S-glutathionylation of the Na,K-ATPase catalytic alpha subunit is a determinant of the enzyme redox sensitivity. J Biol Chem 287 (38):32195-32205.

52. Figtree GA, Liu CC, Bibert S et al. (2009) Reversible oxidative modification: a key mechanism of Na+-K+ pump regulation. Circ Res 105 (2):185-193.

53. Brennan JP, Bardswell SC, Burgoyne JR et al. (2006) Oxidant-induced activation of type I protein kinase A is mediated by RI subunit interprotein disulfide bond formation. J Biol Chem 281 (31):21827-21836.

54. Trenor B, Cardona K, Gomez JF et al. (2012) Simulation and mechanistic investigation of the arrhythmogenic role of the late sodium current in human heart failure. PLoS One 7 (3):e32659.

55. Waterhouse AM, Procter JB, Martin DM et al. (2009) Jalview Version 2--a multiple sequence alignment editor and analysis workbench. Bioinformatics 25 (9):1189-1191.

56. Bernstein HJ (2000) Recent changes to RasMol, recombining the variants. Trends Biochem Sci 25 (9):453-455.

57. Webb B, Sali A (2014) Protein structure modeling with MODELLER. Methods Mol Biol 1137:1-15.

Table 1: Fxyd1 transcription factors detected in the ENCODE project based on ChIP-Seq (minimum false discovery rate = 0.05). A regions from 2500 bases upstream to 500 bases downstream of the transcription start site was included.

Transcription factor[1]	Function[2]
BHLHE40	Circadian control and cell differentiation.
CEBPB	Transcriptional activator involved in immune and inflammatory responses.
CMYC	A proto-oncogene involved in cell division (negative regulation), apoptosis.
EGR1	Transcription activator of genes required for mitogenesis and differentiation.
FOXA1	Embryonic development, establishment of tissue-specific gene expression, modulates transcriptional activity of nuclear hormone receptors, cell-cycle regulation, regulation of apoptosis, glucose homeostasis.
GABP	Transcription activator, necessary for the expression of the adenovirus E4 gene.
HNF4A HNF4G	Required for the transcription of alpha 1-antitrypsin, apolipoprotein CIII, transthyretin genes and HNF1-alpha, may be essential for development of the liver, kidney and intestine.
MAX	Acts in complex with CMYC as repressor and in complex with MAD as activator.
MBD4	DNA N-glycosylase involved in DNA repair.
MXI1	Competes with CMYC for binding to MAX, thus antagonises CMYC function.
P300	Histone acetyltransferase P300, regulates transcription via chromatin remodelling, promotes cardiac myocyte enlargement, participates in circadian rythms.
POL2	Subunit of RNA polymerase II, that synthesises mRNA precursors
RXRA	Retinoic acid receptor, regulates gene expression in complex with other nuclear receptors, regulates various biological processes
SP1	Regulates expression of a large number of genes involved in cell growth, apoptosis, differentiation and immune response.
TAF	General transcription factor required for transcription of genes
USF1	Upstream stimulatory factor one, binds to the promotor of a variety of genes
ZBTB7A	Transcription repressor, possibly involved in the development of B-cells among other roles.

1) Factors in bold are supported by the strongest level of evidence.

2) Function obtained from UniprotKB [14]

Figures:

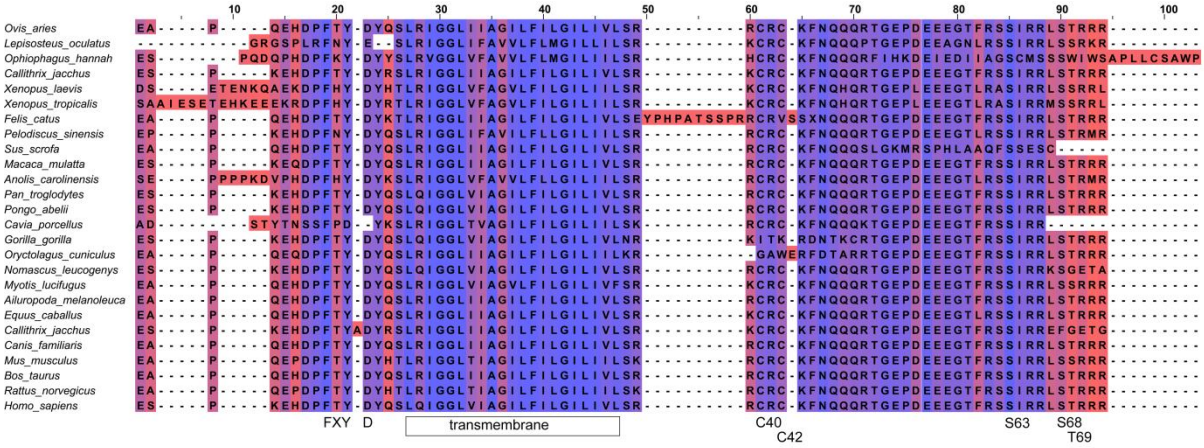

Figure 1: Multiple sequence alignment of FXYD1 orthologues. The alignment is coloured according to alignment quality (= conservation) with a red (low conservation) to blue (high conservation) gradient. Important functional sites of the human sequence discussed in the text are indicated. The figure was prepared with JalView [55].

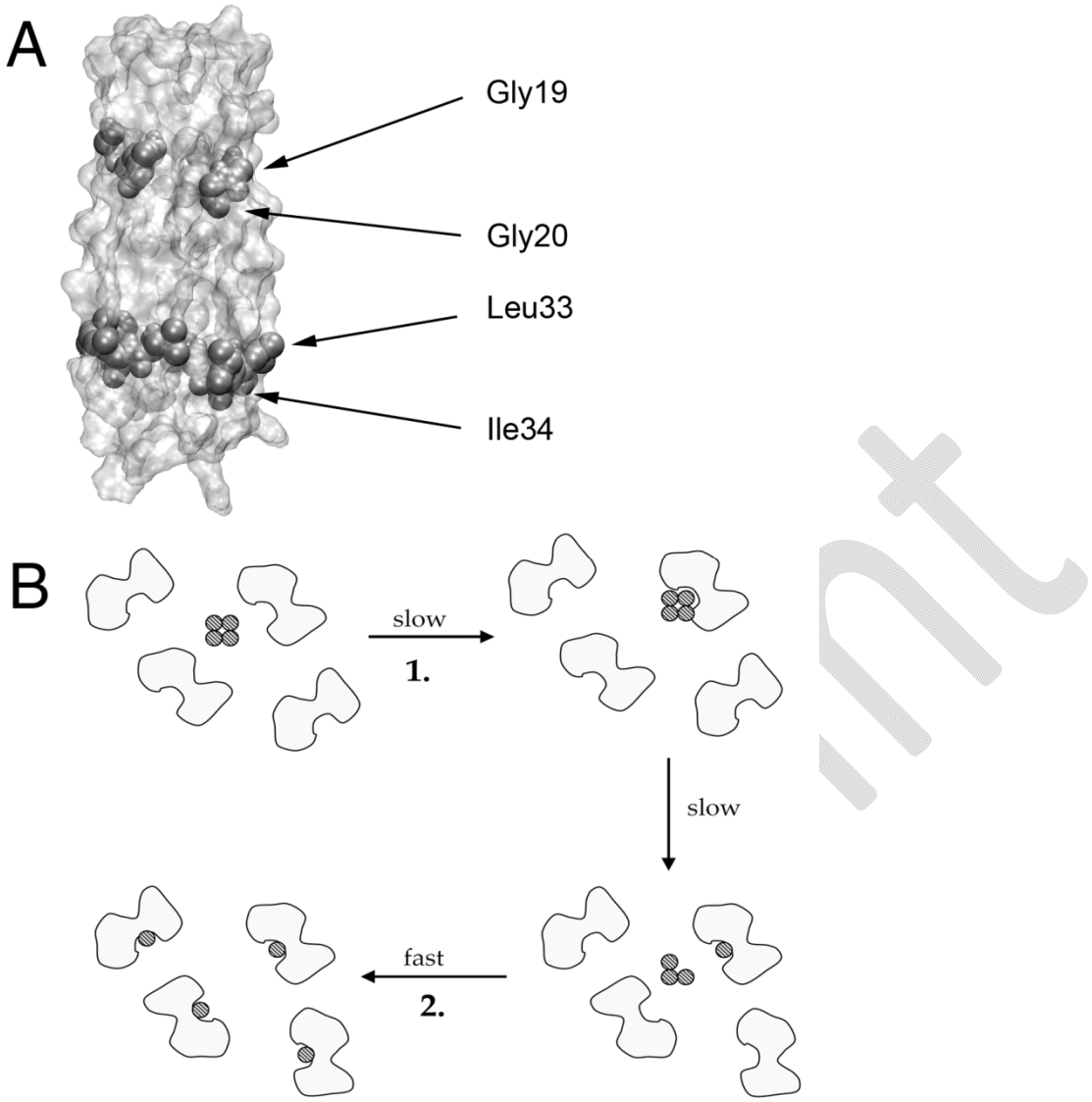

Figure 2: A) The structure of the PLM transmembrane domain tetramer showing the protein solvent-accessible surface. Residues in dark shade have been used to derive orientational constraints with site-specific infrared dichroim. B) A putative model of the PLM tetramer – NKA interaction utilising the tetramer as a storage form of PLM.

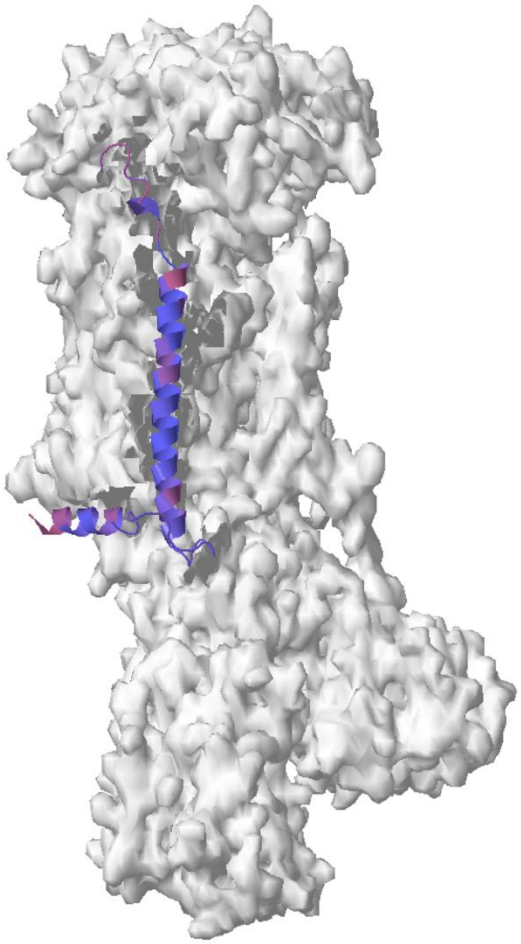

Figure 3: A protein surface model of NKA with PLM shown as ribbons colour coded by conservation calculated from the alignment shown in figure 1. Protein structure displays were made with Rasmol [56].

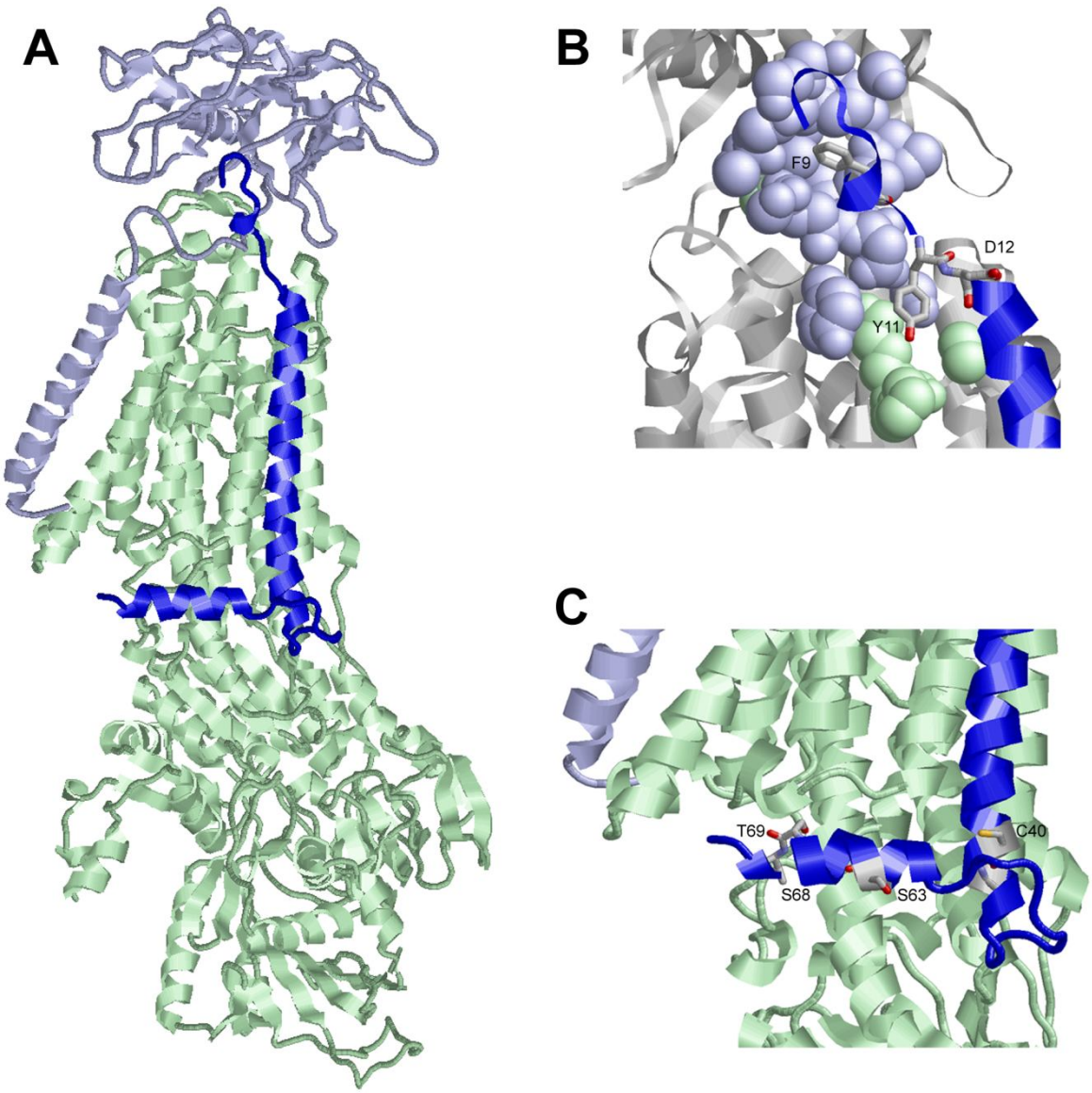

Figure 4: A) A backbone ribbon display of the NKA/PLM complex based on comparative modelling with Modeller 9v6 [57] as explained in the text. B) Details of the interaction of PLM (blue) with NKA α1 (green tint) and β1 (blue tint) subunits. The amino acid residues from NKA interacting with PLM are shown in spacefill representation, while PLM residues are shown as sticks. C) Structural details of the C-terminal phosphorylation and palmitoylation/glutathionylation sites.

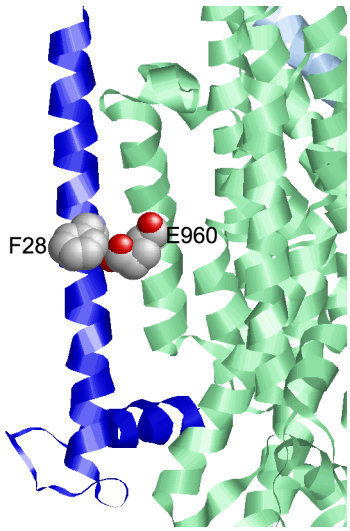

Figure 5: Detail of the interaction between PLM-Phe28 and NKA-α1-Glu960 shown to be important in FRET experiments.

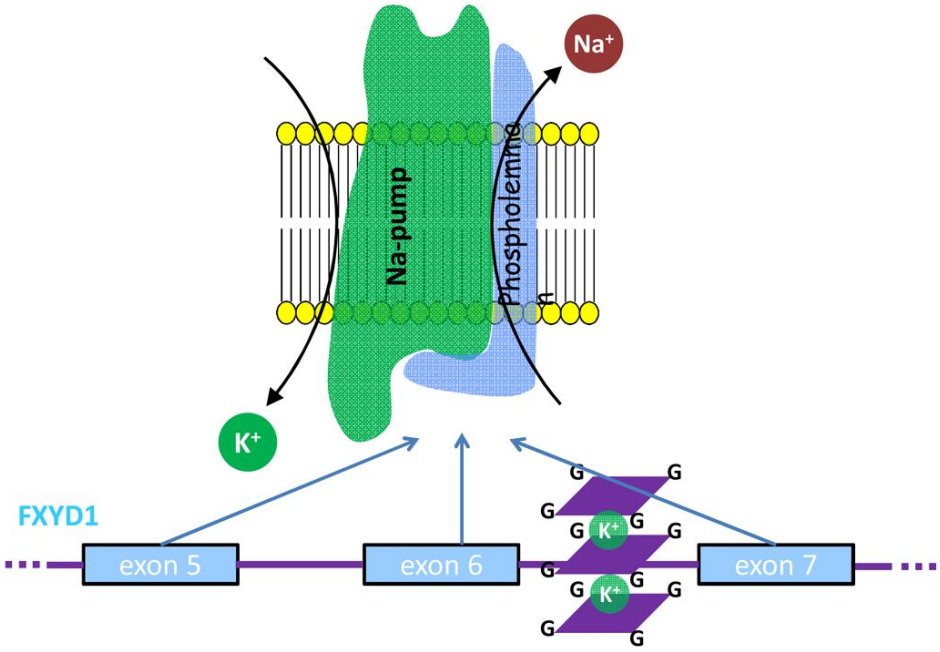

Figure 6: Cartoon of G-quadruplex formation in FXYD1 pre-mRNA between exon 6 and exon 7 that may affect expression of the PLM protein product.