

REVIEW

FXYP proteins and sodium pump regulatory mechanisms

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The sodium/potassium-ATPase (NKA) is the enzyme that establishes gradients of sodium and potassium across the plasma membrane. NKA activity is tightly regulated for different physiological contexts through interactions with single-span transmembrane peptides, the FXYP proteins. This diverse family of regulators has in common a domain containing a Phe-X-Tyr-Asp (FXYP) motif, two conserved glycines, and one serine residue. In humans, there are seven tissue-specific FXYP proteins that differentially modulate NKA kinetics as appropriate for each system, providing dynamic responsiveness to changing physiological conditions. Our understanding of how FXYP proteins contribute to homeostasis has benefitted from recent advances described in this review: biochemical and biophysical studies have provided insight into regulatory mechanisms, genetic models have uncovered remarkable complexity of FXYP function in integrated physiological systems, new posttranslational modifications have been identified, high-resolution structural studies have revealed new details of the regulatory interaction with NKA, and new clinical correlations have been uncovered. In this review, we address the structural determinants of diverse FXYP functions and the special roles of FXYPs in various physiological systems. We also discuss the possible roles of FXYPs in protein trafficking and regulation of non-NKA targets.

Introduction

The Na⁺/K⁺-ATPase (NKA), or “sodium pump,” is the ion-motive ATP-dependent transporter that establishes the sodium and potassium gradients that serve as the foundation of myriad transport and cell signaling processes (Skou, 1957). With each enzymatic cycle, this transporter harnesses the energy of a single ATP to pump three sodium ions out of the cell and two potassium ions into the cell. A considerable fraction of the cell’s ATP budget is allocated to this critical process. In tissues that require intensive Na transport (brain and kidney), NKA accounts for 60–70% of ATP consumption. Even in tissues with competing energy-intensive functions (such as contracting muscle), NKA activity still accounts for at least 10% of ATP (Pirkmajer and Chibalin, 2016). While the basic mechanisms of sodium/potassium transport are common to all cells, NKA activity is exquisitely tuned to meet the particular needs of diverse tissues within the body over a wide range of physiological conditions. For example, NKA is adapted to power the secondary transport requirements of the digestive or renal systems. Elsewhere, NKA is optimized to support the unique electrophysiological demands of excitable cells in the nervous system or muscle, where action potentials may increase ion flux by 100-fold above basal function (Albers and Siegel, 1999). In the heart,

sodium/potassium transport must be responsive to autonomic control, dynamically adapting to changes in heart rate between rest and exercise. Even within a single physiological system, NKA may be adapted to very different conditions, such as the wide range of ion concentrations along the nephron of the kidney. Specialization even occurs at a subcellular level, with different transport requirements of cardiac muscle cell sarcolemmal and transverse tubule (T-tubule) membranes or neuronal axon and soma.

Some of the necessary NKA functional tuning is accomplished by differential expression of four genes encoding the major subunit of NKA and three genes encoding the auxiliary subunit. Different isozymes form from tissue-specific combinations of these subunits (Blanco, 2005; Clausen et al., 2017) yielding pumps with different kinetic properties (Stanley et al., 2015; Han et al., 2009; Despa and Bers, 2007; DiFranco et al., 2015; Crambert et al., 2000). NKA transport properties and signaling are directly modulated by the availability of substrates and posttranslational modifications of the αβ subunits such as phosphorylation, palmitoylation, and glutathionylation (Shattock et al., 2015; Figtree et al., 2009; Howie et al., 2013; Pirkmajer and Chibalin, 2016). The NKA also interacts with a variety of regulatory partners (Cui and Xie, 2017). Of particular

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interest is a family of NKA-regulating membrane proteins called FXYP (Phe-X-Tyr-Asp) proteins, previously reviewed by Käthi Geering in 2006 (Geering, 2006).

The goal of the present review is to provide a reference that consolidates key papers relating to each of the FXYP protein family members, compare and contrast the structure and function of these FXYPs, and discuss unanswered questions and unresolved controversies in the field. As the review is centered on human FXYP protein regulation of NKA, it provides limited analysis of other aspects of NKA structure-function mechanisms. The scope of this review also does not include the very interesting and extensive literature in FXYP protein function in marine organisms and other nonhuman species. These topics are discussed in more detail in other resources (Pirkmajer et al., 2017; Yang et al., 2013; Mahmoud et al., 2000; Tipsmark, 2008). While this review is necessarily an incomplete map of this large and growing field, it is our hope that it will serve as a starting point for investigators interested in grappling with the many exciting problems that remain unsolved.

Structural features of FXYP proteins and regulatory complexes

All mammalian FXYP proteins are type I membrane proteins with a single transmembrane (TM) α -helix flanked by extracellular N-terminal and intracellular C-terminal domains. A handful of conserved residues presumed to be important for NKA binding and regulation are concentrated in the middle of the primary sequence (Fig. 1 A). The conserved PFXYP motif that gives the name to this family is located in the extracellular N-terminal domain. Two glycines in the TM domain and a juxtamembrane serine are also conserved across family members. The latter is most frequently followed by a pattern of basic residues and cysteines in the more variable cytoplasmic domain. At least one cysteine is always present three and/or five residues after the conserved serine, suggesting they are critical for NKA regulation, as discussed below.

NMR has revealed the secondary/tertiary structure of several important FXYP proteins and provided insight into structural dynamics. Fig. 1 (B–D) shows structures of human phospholemman (PLM; FXYP1; Franzin et al., 2007; Fig. 1 B), human γ subunit (FXYP2; Gong et al., 2015; Fig. 2 C), and rat corticoid steroid hormone-induced factor (CHIF; FXYP4; Franzin et al., 2007; Fig. 1 D) and highlights key structural features of FXYP proteins. Some FXYP proteins contain unique sites that can be posttranslationally modified, tuning FXYP regulatory potency. The chief example of this dynamic functional responsiveness is PLM, which bears a cytoplasmic domain containing several functionally significant sites for modification (C40, C42, Ser63, Ser68, and Thr69, see Fig. 1, A and B). Ser63 and Ser68 are particular to PLM but conserved across species (Pavlovic et al., 2013a; Wypijewski et al., 2013; Pirkmajer et al., 2017; Pirkmajer and Chibalin, 2019). The diverse posttranslational modifications of FXYP proteins may serve to fine-tune NKA function according to tissue-specific needs.

The regulatory complex of NKA with FXYP proteins has long been compared with that of an analogous P-type ATPase, the SERCA Ca^{2+} pump, for which a wealth of structural information

is available. An early assumption was that the FXYP protein would bind to the M2/M6/M9 cleft (Fig. 2 B) in a manner reminiscent of PLB binding to the Ca-ATPase. Consistent with this hypothesis, Lindzen et al. (2006) showed cross-linking of FXYP1, FXYP2, and FXYP4 to NKA α helix M2.

However, x-ray crystal structures revealed that the γ subunit (FXYP2) was bound to the outside of NKA TM helix 9 (M9; Morth et al., 2007; Nyblom et al., 2013; Laursen et al., 2013; Kanai et al., 2013; Shinoda et al., 2009; Fig. 2, A and B), and the same position was observed for FXYP10 in the shark NKA structures (Shinoda et al., 2009). Mutagenesis studies seem to confirm the interaction with M9, mediated by residues Gly20, Ala24, Gly25, Phe28, Gly31, and Val35 of PLM (FXYP1; Li et al., 2004; Lindzen et al., 2006). It is not obvious how FXYP binding to M9 is allosterically transmitted across two helices to alter the apparent ion-binding affinities at the ion-binding sites 15 Å away. This may be due to FXYPs altering the interaction of M9 with M8, which is directly involved in forming Na^+ -binding site III (Mishra et al., 2015), but it may also involve stabilization of one of the two major NKA conformations, as discussed below.

Béguin et al. (2001) showed that the N-terminal FXYP domain is required for a stable interaction between γ subunit and CHIF with the NKA, while Lindzen et al. (2003) demonstrated that the stability of detergent-solubilized NKA-CHIF and NKA- γ subunit complexes is determined by three TM residues that may be involved in hydrophobic interactions (Lindzen et al., 2003). In keeping with the FXYP TM helix being an important determinant of FXYP-NKA association, F28 from PLM (conserved in most FXYPs except Mat-8 and CHIF) has been shown essential for PLM and FXYP7 association with NKA (Khafaga et al., 2012; Crambert et al., 2005). These results are congruent with our study of PLM, where mutations of TM residues Leu27 and Ile32 to alanine increased PLM affinity for the NKA but had no effect on function (Himes et al., 2016), suggesting that increased binding between FXYP proteins and the NKA may be associated with nonkinetic effects on NKA, such as increasing FXYP protein and NKA complex stabilization or NKA trafficking. Lindzen et al. found that Cys49 in CHIF, as well as the respective Phe36 and Phe48 to cysteine mutations of the γ subunit and PLM, cross-links with Cys140 in M2 of the NKA α subunit (Lindzen et al., 2006). Note that this study used rat FXYPs. Due to different numbering conventions, Cys49 corresponds to Ala30 in human CHIF, Phe36 corresponds to Phe38 of human γ subunit, and Phe48 corresponds to Phe28 in human PLM (Fig. 1). This cross-linking is somewhat surprising, since the FXYP binding site lies on the outside of M9, which is far from M2 in the crystal structures (Fig. 2 B). This may imply movement of the FXYP and M2 during the conformational changes of the reaction cycle (Young and Artigas, 2021). In addition, the binding interface may be more loosely defined than has been previously appreciated, as discussed in the Unresolved controversies, unanswered questions, and future directions section.

The roles of the extracellular, TM and cytoplasmic domains of FXYP proteins in modulating NKA binding and function have been investigated with chimera studies. Coimmunoprecipitation experiments suggested γ subunit associates more strongly with the NKA compared with CHIF, but exchanging the TM domains

A

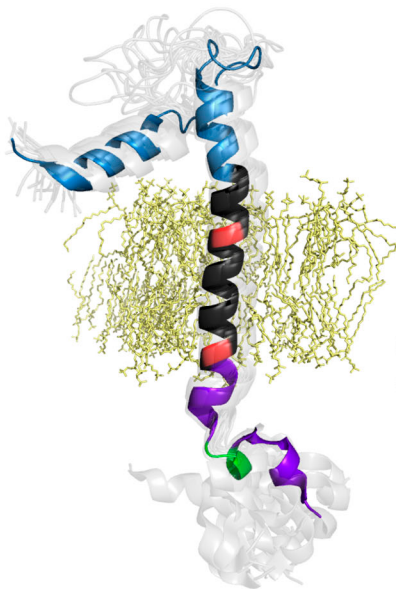
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FXYP1 -----
FXYP2 -----
FXYP3 -----
FXYP4 -----
FXYP5 SPSGRICLLTIVGLILPTRGQTLKDTTSSSSADSTIMDIQVTPRAPDAVYTELQPTSPPTWPADETPQPQTQQLQEGTDGPLVTDPEHKSTK
FXYP6 -----
FXYP7 -----

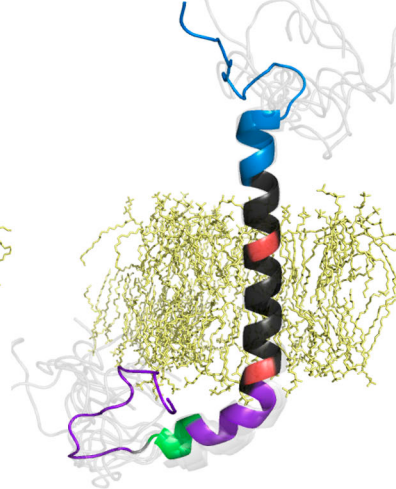
--MASLGHILVFCV-----GLLT-MAKAESPKEHDPFTYDYQSLQIGGLVIAGILFILGILIVLSRRRCCKFNQQQTGEPEDEE-EGTFRSSIRRLSTRRR-----
FXYP2 -----MT-----GLSM-DGGGSPKGDVDPFYDYETVRNGLIFAGLAFIVGLLILLRRFRCCGNNKRRQINDEDEP-----
FXYP3 -----MQKVTGLGLL-----VFLAGFPVLDA-NDLEDKNSPFYDWHSLQVGLICAGVLCAMGIIIVMSAKCKCKFGQKSG-HHPGET-PPL-----ITPGSAQS-----
FXYP4 -----MERVTLLALL-----L-LAGLTALEANDPFANKDDPFYDWNKQLSLGICGGLLAIAAGIAAVLSGKCKCKSSQKQHSVPPEKA-IPL-----ITPGSATTC-----
FXYP5 AAHPTDDTTTLSEKSPSTDVQTDPPQ-TLKPSGFHEDDPFYDEHTLRKGLLVAAVLFTIGIIILTSGKCRQLSRCLRNRCR-----
FXYP6 -----MELVLVFLC-----SLAPMVL-ASAAEKEKEMDPFYDYQTLRIGLVFAVVLFSVGILLILSRRCCKSFNQKPRAPGDEEA-QVE--NLITANATEPQKAEN
FXYP7 -----MATPT-QTPKAPEEDPFYDYNTVQTVGMLTATILFLLGILIVISKVKCKRKAADSRSESPCKSCKSELPSSAPGGGGV-----

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B



C



D

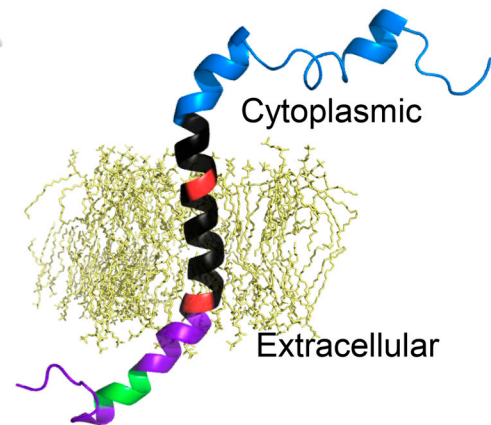


Figure 1. FXYP protein primary and tertiary structures. (A) Amino acid sequence alignment of human FXYP proteins. Signal sequences are orange, the extracellular domain is purple with the FXYP motif highlighted in green, the TM domain is black with two red conserved glycine residues, and the cytoplasmic domain is blue. The sequence numbering starts with the first purple residue in the extracellular domain. (B–D) NMR structures of human PLM (B), human γ subunit (C), and rat CHIF (D) proteins. When available, structures show overlay of all possible configurations obtained from NMR analysis. Conserved residues are highlighted in the structure. The FXYP motif is green and two glycine residues are red. FXYP5's longer N terminus is shown in the top part of the sequence alignment. Structures are made with the PLM, γ subunit, and CHIF structures (PDB accession nos. 2OJ1, 2MKV, and 2JP3, respectively) with approximate position of the lipid bilayer according to Geering (2006).

of the γ subunit and CHIF reversed the relative affinities (Lindzen et al., 2003). In contrast, switching the extracellular and cytoplasmic domains did not alter the apparent affinity for NKA, suggesting that it is primarily the TM domain that stabilizes the NKA–FXYP complex. This interpretation is supported by the observation that mutation of CHIF TM residues Met55 and Ala56 to the respective γ subunit residues swapped the NKA binding affinities ($K_{0.5}$) of CHIF and γ (interestingly, without a functional effect; Lindzen et al., 2003). Likewise, exchanging the TM domains of the dysadherin (FXYP5) and CHIF switched their apparent affinities for NKA. Mutation of FXYP5 residues at the equivalent positions of CHIF residues 55 and 56 to Met and Ala respectively reversed the affinities of CHIF and FXYP5 (Lubarski et al., 2007). Scanning alanine mutagenesis of the PLM TM domain showed that most substitutions actually increased the apparent affinity of the NKA interaction (Himes et al., 2016). This is

suggestive of adaptation of PLM for submaximal NKA $K_{0.5}$. This concept is discussed more below in the Unresolved controversies, unanswered questions, and future directions section.

Compared with the TM domains, the extramembranous domains are larger and have much less conserved sequences between the FXYPs. This has attracted attention to the extra- and intracellular domains as possible determinants of the differential function. Specifically, the FXYP domain interacts with both the α and β subunits (Fig. 2 D), and mutation of FXYP residues nearly abolished NKA binding to both CHIF and the γ subunit in oocytes (Béguin et al., 2001). Positive residues on the cytoplasmic side were also important for γ subunit binding (Béguin et al., 2001). Mutation of these residues on CHIF did not alter NKA binding but did alter CHIF's regulation of NKA. A chimera consisting of the PLM TM domain and CHIF extra- and intracellular domains increased PLM affinity for NKA (Lifshitz et al.,

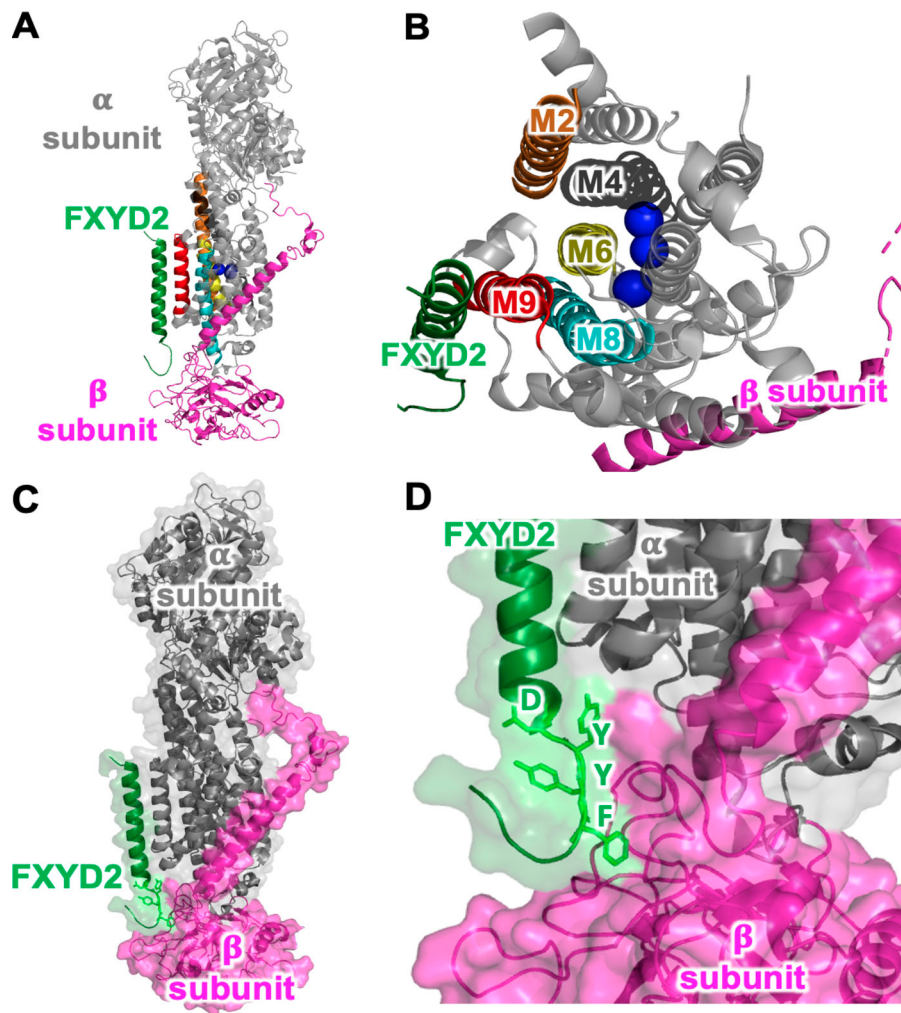


Figure 2. Quaternary structure of NKA-FXYD complex and their interaction. (A) Lateral view of the NKA crystal structure in the E1 conformation positioning FXYD2 (γ -subunit) in the structure. (B) View of the NKA TM domain from the extracellular side. Essential subunits α (gray) and β (pink) are associated with FXYD2 (green), which interacts with the α subunit M9 (red). Three bound Na^+ ions are blue, M9 is red, M8 is cyan, and M2 is orange. (C) Surface representation of α (gray), β (pink), and FXYD2 (green) complex. (D) Enlarged contacts between NKA subunits and FXYD2 regulator. Structures are made with PyMOL using the NKA structure (PDB accession no. 3WGU).

2006), so some stabilization of the complex may be provided by the extramembranous regions.

While the regulatory importance of the NKA-FXYD regulatory complex is well established, there is another FXYD complex of unknown functional significance, the FXYD homo-oligomeric complex. Nondenaturing gel electrophoresis suggested PLM may form tetramers (Beevers and Kukol, 2006; Wong et al., 2008; Beevers and Kukol, 2007) that may be analogous to multimers formed by SERCA regulators (Glaves et al., 2019; Singh et al., 2019). PLM tetramers have been observed in live cells using photon counting histogram analysis and fluorescence resonance energy transfer (FRET; Song et al., 2011; Himes et al., 2016). Oligomerization of PLM may increase with phosphorylation, a mechanism that could support relief of NKA inhibition (Song et al., 2011). Another study showed that PLM phosphorylated at Ser63 increased coimmunoprecipitation with unphosphorylated PLM in heart muscle from mice, while coimmunoprecipitation with NKA decreased (Wypijewski et al., 2013). The authors also showed that dephosphorylation of PLM with PP2A decreased total phosphorylated PLM at Ser63 but had no impact on the amount of phosphorylated PLM bound to NKA or NKA current, as quantified by patch clamp. These results suggest that there are two distinct pools of PLM that do not

exchange. The significance of these apparently independent PLM pools is unknown.

FXYD1 (PLM)

Of all of the FXYD proteins, PLM (FXYD1) has been the most intensively studied. Palmer et al (1991) identified PLM as the most prevalent target for PKA phosphorylation in purified cardiac sarcolemmal vesicles. Since then, PLM has been found in skeletal muscle, smooth muscle, cerebellum, choroid plexus, and liver (Feschenko et al., 2003; Pavlovic et al., 2013a). Interest in PLM as a regulator of NKA function grew when protein sequencing showed this 15-kD protein was a γ subunit orthologue (Palmer et al., 1991). Crambert et al. showed that PLM coimmunoprecipitated with the NKA in native heart and skeletal muscle and provided the first suggestion of PLM's function. Electrophysiology (Crambert et al., 2002; Meyer et al., 2020) and Na-sensitive dye (Despa et al., 2005; Khafaga et al., 2012) measurements revealed PLM decreases the apparent affinity of NKA for intracellular sodium.

NKA-PLM structure-function mechanisms

PLM is also of particular interest because several posttranslational modifications modulate its regulatory function.

Sympathetic stimulation activates signaling pathways that target PLM for phosphorylation at three C-terminal residues, Ser63, Ser68, and Thr69 (Fig. 1B), by PKA and PKC (Fuller et al., 2009; Walaas et al., 1994), relieving inhibition of NKA (Bossuyt et al., 2006; Mishra et al., 2015). β -Adrenergic stimulation of PKA and PKC decreased FRET between PLM and NKA (Bossuyt et al., 2009; Despa et al., 2005). Stopped-flow kinetics experiments showed that PLM-binding slowed the enzymatic transition from E_2 to E_1 . Complementary computational simulations suggested that the cytoplasmic portion of PLM interacts with a negatively charged cleft between the N and P domains in the NKA in the E_2 conformation (Mishra et al., 2015), interfering with domain interactions and motions. Mishra et al. proposed that upon PLM phosphorylation, the change in the charge of the cytoplasmic domain promotes exit from this cleft and a translocation of the cytoplasmic domain to the membrane interface, relieving inhibition of NKA. This hypothesis is compatible with another FRET study that showed a small increase in the distance between a donor fused to the N terminus of the NKA α subunit (in the A domain) and an acceptor fused to the C terminus of PLM (in the cytoplasmic domain; Song et al., 2011). That study also showed a small increase in PLM oligomerization and an apparent reciprocal decrease in the affinity of PLM for NKA. Thus, relief of inhibition after phosphorylation may be due to a combination of decreased PLM-NKA binding and a structural change of those PLM-NKA regulatory complexes that persist after PLM phosphorylation.

PLM and dynamic regulation of NKA

The functional importance of PLM phosphorylation is supported by ex vivo experiments in which genetic ablation of PLM abolished the PKA- or PKC-mediated regulation of NKA. Quantification of Na^+ efflux with a fluorescent indicator showed NKA activity increased after β -adrenergic stimulation in WT mice but did not change in PLM knockout mice (Despa et al., 2005). Others have shown similar results in mice with unphosphorylatable PLM, in which the PLM phosphorylation sites have been mutated to alanines (Shattock et al., 2015). One study showed using NMR that mice with unphosphorylatable PLM had increased intracellular Na^+ compared with WT mice (Eykyun et al., 2015). These results suggest that PLM phosphorylation by β -adrenergic stimulation may protect against Na^+ overload. This is corroborated by in vivo studies that demonstrate mice with nonphosphorylatable PLM have worse systolic and diastolic dysfunction after aortic constriction compared with WT mice (Boguslavskyi et al., 2014). Other studies have shown PKC increases the activity ($V_{\max}$) of the NKA $\alpha 2$ isoform, but not NKA $\alpha 1$ (Bossuyt et al., 2009; Bibert et al., 2008). This specific stimulation of NKA $\alpha 2$ activity is noteworthy, since the density of NKA $\alpha 2$ is approximately five times higher in the T-tubules, where the sodium-calcium exchanger (NCX1) is located (Berry et al., 2007; Despa and Bers, 2007). Thus, the relief of PLM inhibition of NKA may be most pronounced in those subcellular regions where Na^+ transport is most closely coupled to NCX1-dependent Ca^{2+} extrusion. While such studies in intact cellular preparations have generally supported phosphorylation-dependent relief of NKA inhibition, it has been challenging to

recapitulate this in well-defined in vitro systems. Many in vitro preparations have failed to show a stimulatory effect of PKA (Main et al., 1997; Ishizuka and Berlin, 1993; White et al., 2010). This may be due to the loss of some unknown regulatory species during the preparation (Hilgemann, 2020).

PLM in protein trafficking

PLM phosphorylation by PKC at Ser69 (analogous to Thr69 residue in humans) has also been shown to increase PLM trafficking to the plasma membrane (Lansbery et al., 2006), and we have recently presented evidence that PKA increases the plasma membrane density of human NKA heterologously expressed with human PLM in *Xenopus laevis* oocytes (Meyer et al., 2020). In these scenarios, enhanced transport activity may be important when cardiac function increases during sympathetic stimulation. For example, during exercise, circulating catecholamines and sympathetic innervation of the heart activate adrenergic PKA/PKC-dependent signaling pathways, increasing heart rate and trans-sarcolemmal Na^+ and Ca^{2+} flux. This creates the potential for Na^+ and Ca^{2+} overload and cardiac arrhythmias (Figtree et al., 2009). Relief of NKA inhibition through increased plasma membrane insertion of phosphorylated PLM and NKA may serve as safety mechanisms, limiting $\text{Na}^+/\text{Ca}^{2+}$ overload during stress.

PLM modulation of NKA kinetics

Though there is a wide consensus that interaction with unphosphorylated PLM decreases the apparent Na^+ affinity of NKA and PLM phosphorylation increases the apparent Na^+ affinity of NKA (Crambert et al., 2002; Bibert et al., 2008; Despa et al., 2005; Han et al., 2006; Meyer et al., 2020), the effect of PLM and PLM phosphorylation on $V_{\max}$ are not fully established. Two studies showed that injection of synthesized unphosphorylated PLM cytoplasmic peptide (residues 54–72; Pavlović et al., 2007) or overexpression of human unphosphorylated PLM protein (Mishra et al., 2015) decreases the $V_{\max}$ of NKA. Injection of a synthesized phosphorylated PLM cytoplasmic peptide or overexpression of human phosphorylated PLM protein increased $V_{\max}$. Two other studies looked at the effects of PLM on $V_{\max}$ under more physiological conditions. These studies showed that stimulation of ventricular myocytes with isoproterenol increased PLM phosphorylation without changing the $V_{\max}$ of NKA, suggesting the effect of PLM is to reduce Na^+ affinity without impairing pump maximal turnover rate (Despa et al., 2005; Han et al., 2006). This property may serve as a safety measure against Na^+ overload by allowing NKA to overcome inhibition by PLM and operate at peak capacity when intracellular Na^+ levels rise. PLM phosphorylation also protects against Ca^{2+} overload via the nitric oxide synthase (NOS) pathway. Increased intracellular Ca^{2+} has been shown to activate NOS, increasing NO. NO increased the apparent Na^+ affinity of NKA and NKA current in WT mice, but not mice with unphosphorylatable PLM (Pavlovic et al., 2013b).

Additional posttranslational modifications

In addition to being phosphorylated, PLM is also glutathionylated. Bibert et al. (2011) found that peroxynitrate, an oxidizing

agent, increased the amount of glutathionylated PLM in rabbit ventricular myocytes loaded with biotin-tagged glutathione. To determine which cysteines were glutathionylated, the authors coexpressed NKA with WT PLM, Cys40Ala PLM, Cys42Ala PLM, or Cys40Ala/Cys42Ala PLM mutants in *Xenopus* oocytes. Exposure to peroxynitrate induced glutathionylation in the Cys40Ala mutant, but not in the Cys42Ala PLM or Cys40Ala/Cys42Ala PLM mutants. These results suggest that PLM is glutathionylated at Cys42 in response to peroxynitrate. The authors also measured NKA current using voltage clamp and found that exposure to peroxynitrate decreased NKA current in the C42A and Cys40Ala/Cys42Ala mutants, but not the Cys40Ala single mutant, concluding that glutathionylation at Cys42 increases NKA activity (Table 1).

Cys40 and Cys42 of PLM are conserved among the FXYP proteins (Fig. 1A); these key residues are also important sites for functional regulation by palmitoylation. Howie et al. demonstrated that Cys40 is the primary palmitoylation site using PE-Gylation assay in HEK cells expressing PLM-YFP C40A or PLM-YFP Cys42Ala (Howie et al., 2014). Cells expressing PLM Cys42Ala, but not PLM Cys40Ala, exhibited decreased NKA current, suggesting that PLM palmitoylation at Cys40, but not Cys42, inhibits the NKA. PLM palmitoylation was increased after phosphorylation of PLM by PKA and PKC. PLM phosphorylation may induce PLM palmitoylation by interacting with DHHC5, a potent inducer of palmitoylation of proteins in the plasma membrane. DHHC5 coimmunoprecipitated with phosphorylated PLM in ventricular myocytes. Inhibition of palmitoylation by 2-bromopalmitate increased NKA activity in HEK-293 cells (Tulloch et al., 2011). This suggests that palmitoylation makes PLM more inhibitory, which seems to be in contradiction to the role of PLM phosphorylation in relieving inhibition of NKA. This paradox may arise from the complex effects of phosphorylation/palmitoylation in modulating protein expression, degradation, and trafficking. In HEK cells, the half-life of the Cys40Ser/Cys42Ser mutant was much shorter than that of WT PLM (1.7 h and 4 h, respectively), suggesting palmitoylation slows the rate of PLM degradation (Tulloch et al., 2011).

In cardiac myocytes, ischemia-reperfusion increased PLM palmitoylation and induced massive endocytosis. This response was blunted in PLM knockout mice (Hilgemann et al., 2013). Interestingly, the rate of endocytosis was increased by overexpression of PLM and PKC phosphorylation of exogenous PLM. These data suggest that PLM palmitoylation and endocytosis may have a role in the pathological response to cardiac ischemia. This mechanism may be the basis for the long-observed decrease in NKA after ischemia-reperfusion (Beller et al., 1976) and consequent cardiac dysfunction.

FXYP2 (γ subunit)

FXYP2, also known as the γ subunit, was initially considered to be the third subunit of the NKA complex. In contrast to PLM, the γ subunit is expressed as two splice variants in the kidneys and the pancreas (Arystarkhova et al., 2002a; Arystarkhova et al., 2002b; Arystarkhova et al., 1999; Arystarkhova, 2016). Kuster et al. (2000) used tryptic peptide mapping and mass spectrometry to show that the two distinct γ subunit variants,

termed the γ a subunit and γ b subunit, differ in six residues at their N termini, with γ a having two extra N-terminal residues. Both γ -subunit variants are primarily expressed in the thick ascending limb (Arystarkhova et al., 2002b) and induce identical reduction of the NKA apparent affinity for sodium (Béguin et al., 2001; Arystarkhova et al., 1999; Therien et al., 1999; Béguin et al., 1997; Meyer et al., 2020). The γ a and γ b subunits do seem to have differential posttranslational modifications that have different effects on NKA kinetics (Arystarkhova et al., 2002a). However, the physiological significance of having two γ -subunit isoforms remains a mystery.

The high expression of the γ subunit in the kidney suggests it plays a role in renal physiology. The NKA is located on the basolateral membrane of the renal epithelial cells. Na^+ from the lumen of the nephron enters these cells through apical Na^+ transporters. The majority of sodium reabsorption occurs in the proximal convoluted tubule or thick ascending loop of Henle. The long-accepted concept is that at baseline, FXYP expression conforms to physiology in the kidney. The γ subunit and CHIF fine-tune Na affinity for nephron segments: affinity is low in the proximal tubule, connecting tubule, and medullary ascending limb (the γ subunit); intermediate in the cortical ascending limb (no major FXYP detected); and high in the collecting duct (CHIF; see Table 1 for effects on Na affinity). Surprisingly, however, genetic ablation of the γ subunit yielded no differences in renal function or significant changes in urine Na^+ , K^+ , Mg^{2+} , and creatinine (Arystarkhova et al., 2014; Jones et al., 2005). It is probable that compensatory mechanisms are responsible for the lack of renal effects. Instead, γ -subunit-knockout mice showed decreased blood glucose levels, increased β cell mass, and improved glucose tolerance compared with WT mice, suggesting that γ subunit may have a functional role in the pancreas (Arystarkhova et al., 2013). Studies have shown that the γ subunit is highly expressed in human pancreatic islet cells (Flamez et al., 2010) and that the majority of the γ subunit is internalized (Arystarkhova et al., 2013). However, the function of the γ subunit in these cells remains unknown.

The γ subunit may mediate adaptation to various stressors, such as hypertonicity (Wetzel et al., 2004). Exposing NRK-52E cells to a hyperosmotic medium (with sodium chloride, sucrose, or urea) induced expression of the γ a variant. In addition, hypertonic stimulation induced a decrease in both sodium affinity and V_{max} of the NKA (Table 1). This effect was reversed by γ -subunit small interfering RNA (siRNA) knockout. These results suggest that under hypertonic conditions, cells may increase γ -subunit expression, resulting in decreased NKA activity. The authors also found similar results in response to oxidative stress and heavy metals, suggesting the γ subunit may participate in a general adaptive response to stressors. Interestingly, induction of the γ a variant also resulted in decreased expression of PLM, the primary FXYP in NRK-52E cells (Arystarkhova et al., 2007). This result suggests that certain stimuli may induce expression of FXYP proteins different than those expressed in the tissues from which the cells originate.

A naturally occurring Gly41Arg mutation of the γ subunit is responsible for isolated dominant hypomagnesemia (Meij et al., 2000; Mayan et al., 2018). One of the key driving forces for

Table 1. Summary of FXYD protein characteristics

FXYD protein	Other name	Expression pattern	Effects on NKA	Posttranslational modifications and effects on NKA	Physiological/pathological role
FXYD1	PLM	Expresses in multiple tissue types	↓Sodium affinity (Crambert et al., 2002; Despa et al., 2005; Bossuyt et al., 2009; Meyer et al., 2020)	Phosphorylation at Ser63, Ser68, and Ser69 ↑NKA activity (Fuller et al., 2009; Despa et al., 2005), glutathionylation at Cys42 ↑NKA activity (Bibert et al., 2011), palmitoylation at Cys40 and Cys42 ↓NKA activity (Tulloch et al., 2011; Hilgemann et al., 2013)	PLM is required for the sympathetic stimulation of the NKA in cardiomyocytes (Despa et al., 2005)
FXYD2	γ Subunit	Restricted to kidney and pancreas	↓Sodium affinity (Béguin et al., 1997; Arystarkhova et al., 1999, 2002a; Therien et al., 1999; Meyer et al., 2020)	Unknown	Unknown
FXYD3	Mammary tumor (Mat-8)	Restricted to tissues rich in smooth muscle, skin, and salivary gland	Mixed results (Crambert et al., 2005; Bibert et al., 2006; Wujak et al., 2016)	Glutathionylation at Cys40 and Cys42 ↑NKA activity (Bibert et al., 2006)	Unknown
FXYD4	CHIF	Restricted to kidney, inducible in colon with corticosteroid	↑Sodium affinity and V_{max} (Béguin et al., 2001; Meyer et al., 2020)	Unknown	CHIF increases sodium reabsorption in the colon and kidneys during sodium deprivation (Aizman et al., 2002)
FXYD5	Related ion channel, dysadherin	Expressed in multiple tissue types and almost completely absent in brain tissue	↑Sodium affinity and V_{max} , ↓potassium affinity (Lubarski et al., 2007; Mathias et al., 2008)	Palmitoylation and glycosylation	Dysadherin increases cancer invasiveness (Ino et al., 2002)
FXYD6	Phosphohippolin	Expressed in multiple tissue types	↓Sodium affinity (Delprat et al., 2007b; Meyer et al., 2020)	Unknown	Unknown
FXYD7	N/A	Brain	↓Potassium affinity, ↑sodium affinity (Béguin et al., 2002; Meyer et al., 2020)	Glycosylation at Thr5 and Thr9 ↑FXYD7 stabilization and translocation to the plasma membrane (Moshitzky et al., 2012)	Unknown

Expression patterns were obtained from the GTEx Portal. N/A, not applicable.

transepithelial Mg^{2+} transport in the distal convoluted tubule and thick ascending limb is the sodium gradient set up by NKA (de Baaij et al., 2015). It has been suggested that the Gly41Arg γ subunit interacts with the NKA to disrupt the sodium gradient, leading to isolated dominant hypomagnesemia (Mayan et al., 2018). Because the γ subunit interacts with NKA at the distal convoluted tubule (Arystarkhova et al., 2002b), the Gly41Arg mutation of the γ subunit may be expected to alter its effect on the NKA. In a study by Pu et al., Gly41Arg- γ was shown to have increased Na^+ affinity for NKA compared with WT (Pu et al., 2002). However, increased Na^+ affinity for NKA would decrease the amount of intracellular Na^+ and increase the driving force for transcellular Mg^{2+} transport. Thus, Gly41Arg γ subunit may mediate hypomagnesemia by a different mechanism. Several studies have shown that there is decreased Gly41Arg γ subunit

trafficking to the plasma membrane compared with WT γ subunit (Pu et al., 2002; Sha et al., 2008; Meij et al., 2000). This might be explained by the arginine residue in the middle of a hydrophobic membrane span (Meij et al., 2003). However, the role of Gly41Arg γ -subunit misrouting in hypomagnesemia induction is unclear.

FXYD 3 (Mat-8)

Mat-8 is an 8-kD TM protein expressed in breast tumors, the uterus, stomach, and colon (Morrison et al., 1995). It has two isoforms: a short isoform and a long isoform that has an extra 26-amino acid insertion after the TM domain (Bibert et al., 2006). Both isoforms associate with the NKA. However, the functional role of Mat-8 in regulating NKA activity is still unsettled. Two-electrode voltage-clamp experiments in *Xenopus*

oocytes showed that the short Mat-8 isoform decreased Na^+ affinity of NKA, while the long isoform increased Na^+ affinity of NKA (Li et al., 2005; Bibert et al., 2006). In contrast, Wujak et al. (2016) measured NKA activity in lung epithelial cells using a Ussing chamber system and found NKA activity was not affected by overexpressing Mat-8 (Table 1).

Mat-8 has also been suggested to increase NKA activity during oxidative stress by relieving glutathionylation of the NKA $\beta 1$ subunit (Bibert et al., 2011). Although Mat-8 is not native to cardiac tissue, Bibert et al. exposed rat ventricular myocytes to purified Mat-8 and peroxynitrite, which resulted in increased Mat-8 glutathionylation and decreased $\beta 1$ subunit glutathionylation. Mutating two potential sites for Mat-8 glutathionylation, Cys40 and Cys42, reversed the effects of Mat-8 on $\beta 1$ subunit glutathionylation in response to peroxynitrite. These results suggest that during oxidative stress, glutathionylation of Mat-8 activates the NKA by reducing glutathionylation of the β subunit, which may promote cell survival. Indeed, Mat-8 is overexpressed in some cancer cells, while reducing Mat-8 (short isoform) using siRNA (Grzmil et al., 2004) impairs growth of prostate cancer cells. Thus, Mat-8 may be worth exploring as a target for future therapeutic applications.

FXD4 (CHIF)

FXD4 (Fig. 1 D) was discovered as a protein orthologue of PLM and the γ subunit that was induced by corticosteroid hormone (Attali et al., 1995), hence the name corticosteroid hormone induced factor (CHIF). CHIF is highly expressed in the distal colon and principal cells of kidneys (Capurro et al., 1996; Pihakaski-Maunsbach et al., 2006). Béguin et al. showed that CHIF coimmunoprecipitated with NKA. Interestingly, CHIF increased the apparent affinity of the transporter for Na^+ and increased NKA activity in *Xenopus* oocytes (Béguin et al., 2001; Meyer et al., 2020), which contrasts with the actions of PLM and the γ subunit (Table 1). The major phenotype of CHIF knockout mice is increased Na^+ wasting in the urine under the stress of Na^+ deprivation, but not under normal conditions (Aizman et al., 2002). Because 95% of Na^+ is reabsorbed before the collecting duct, luminal Na^+ concentration in the collecting duct is low. During Na^+ deprivation, CHIF-bound pumps have the high-affinity needed for reabsorption of a more dilute Na^+ in the collecting duct. Thus, CHIF absence in knockout animals results in Na^+ wasting and increased urine volume under these conditions. CHIF has also been shown to protect mice treated with furosemide, a diuretic that inhibits the Na-K-2Cl cotransporter (NKCC) in the thick ascending limb of the loop of Henley. CHIF-knockout mice treated with furosemide had a much lower survival rate than WT mice (Aizman et al., 2002). These results suggested that CHIF may play a protective role in diseases associated with Na^+ wasting, such as chronic kidney disease, or during treatment with diuretics.

A similar physiological role of CHIF has been described in the colon (Goldschmidt et al., 2004). In CHIF-knockout mice, amiloride-sensitive sodium reabsorption was reduced by 42% in the distal colon under control conditions and by 58% under a Na^+ -restriction diet. The decreased basal reabsorption under control conditions differs from the results shown in the kidneys,

where the CHIF-knockout phenotype was only revealed by Na^+ deprivation stress. This can be explained by the low sodium concentration in the distal colon. Knockout of CHIF would decrease the Na^+ affinity of NKA, leading to increased Na^+ wasting.

FXD5 (dysadherin)

FXD5 is known as dysadherin because of its role in modulating cell-cell adhesion. Though dysadherin's function has been explored primarily in this context, the protein is also expressed in normal lung, kidneys, intestines, and spleen (Lubarski et al., 2005). In kidneys, dysadherin is expressed in the connecting tubule, the collecting tubule, and the intercalated cells of the collecting duct (Lubarski et al., 2005). Like CHIF, dysadherin may increase Na^+ absorption by enhancing the activity of NKA, which is expressed at very low levels in the cortical collecting duct. Dysadherin coimmunoprecipitated with NKA in *Xenopus* oocytes, and expression of dysadherin increased NKA pump current (without changing NKA expression; Lubarski et al., 2007), suggesting an increase in V_{max} (Table 1). In support of dysadherin being an NKA potentiator, experiments by Miller and Davis measuring NKA-specific $^{86}\text{Rb}^+$ uptake revealed dysadherin increased the $K_{0.5}$ for K^+ and decreased the $K_{0.5}$ for Na^+ in canine kidney cells (Mathias et al., 2008). Thus, like CHIF, dysadherin may increase NKA activity by increasing the apparent affinity for Na^+ and increasing the maximal turnover rate. This may support Na^+ reabsorption in the cortical collecting ducts, where the last 3% of Na^+ is reabsorbed.

Dysadherin mRNA expression was also increased in the nasal epithelia from cystic fibrosis patients (Miller and Davis, 2008a), though the role of dysadherin in cystic fibrosis pathology awaits elucidation. The protein has a well-established association with cancer (Ino et al., 2002), and a role in determining cancer invasiveness was suggested by its up-regulation in several cancer cell lines (Lubarski-Gotliv et al., 2016). Transfection of dysadherin into liver cancer cells decreased E-cadherin expression, and dysadherin injection into the spleens of immunodeficient mice increased the number of metastatic nodules (Ino et al., 2002). NKA is required for mediating E-cadherin-dependent cell-to-cell adhesion and cell motility (Rajasekaran et al., 2001; Rajasekaran et al., 2003). Carbohydrate moieties on the NKA β subunit mediate interactions with β subunits on neighboring cells, and inhibiting N-glycosylation of the β subunits weakens this interaction (Vagin et al., 2006; Vagin et al., 2007). It was suggested that dysadherin decreases cell-to-cell adhesion by reducing glycosylation of the NKA $\beta 1$ subunit (Lubarski et al., 2011). To study this hypothesis, Lubarski et al. (2011) recorded the time-dependent increase in transepithelial resistance in rodent kidney collecting duct cells. They found that dysadherin reduced glycosylation of the NKA $\beta 1$ subunit, which was associated with decreased formation of cell-to-cell contacts and transepithelial resistance.

In a more recent study, FXD5 was shown to mediate substitution of $\beta 1$ isoforms to $\beta 3$ isoforms (Lubarski-Gotliv et al., 2017). The authors found that in mammary gland tumor cells, $\beta 3$ isoforms were replaced by $\beta 1$ isoforms after FXD5 silencing. Since annexin A2 binds to $\beta 1$ to inhibit cellular motility, the authors hypothesized that FXD5 affects metastasis by

influencing interaction of the β subunit with annexin A2. When secreted to the cell membrane, annexin A2 binds to plasminogen and tissue plasminogen activator, leading to the conversion of plasminogen to plasmin. Plasmin is then able to activate matrix metalloproteinase 2/9 (MMP-2/9), which degrades the extracellular matrix, allowing for metastatic progression. The authors found that silencing FXYD5 decreased annexin A2 and MMP-2/9 secretion, as measured by Western blotting and gelatin zymography, respectively. Thus, the authors proposed that FXYD5-mediated substitution of $\beta 1$ to $\beta 3$ increases annexin A2 secretion, which in turn promotes MMP-9 activation, ECM degradation, and metastatic spread.

In addition, regulation of cell motility may be governed by dysadherin posttranslational modifications, including phosphorylation at Ser163, a site that is conserved among all FXYD proteins (Fig. 1 A). Phosphorylation may decrease dysadherin's association with NKA and activate signaling pathways that promote cell motility (Miller and Davis, 2008b). As part of this process, phosphorylated dysadherin may change its localization from the plasma membrane to the cell interior (Miller and Davis, 2008b). In an airway epithelial cell scratch wound-healing model, phosphomimetic dysadherin increased reepithelialization of the cell monolayer, suggesting that phosphorylation of Ser163 increases cell motility.

FXYD6

Yamaguchi et al. described and cloned FXYD6 (Yamaguchi et al., 2001). It was originally named phosphohippolin because it was discovered in the hippocampus. However, it is also highly expressed in different regions of the CNS, cerebellum, hypothalamus, and cerebral cortex (Kadowaki et al., 2004). FXYD6 associates with both NKA $\alpha 1\beta 1$ and $\alpha 1\beta 2$ complexes in *Xenopus* oocytes (Delprat et al., 2007a). FXYD6 association with $\alpha 1\beta 1$ resulted in a 30% reduction in apparent affinity for intracellular Na^+ with no change in the apparent affinity for extracellular K^+ (Delprat et al., 2007b; Meyer et al., 2020; Table 1), concomitantly accelerating the NKA turnover rate by speeding up Na^+ deocclusion by ~50%. In addition to modulating NKA kinetics, our recent study showed FXYD6 drastically reduced plasma membrane functional expression of $\alpha 1\beta 1$ in *Xenopus* oocytes (Meyer et al., 2020), suggesting this protein may also function as a regulator of NKA localization to the plasma membrane. This is reminiscent of studies that showed that PLM phosphorylation (Bibert et al., 2008; Meyer et al., 2020; Moorman et al., 1992) and palmitoylation (Hilgemann et al., 2013) modulate NKA trafficking. The recent observations contribute to the emerging picture that FXYD proteins have important roles beyond simple regulation of NKA's affinity for transported ions.

In the brain, FXYD6 is exclusively found in neurons and is thought to participate in neuronal excitability. Immunofluorescence microscopy revealed plasma membrane colocalization of FXYD6 with the $\alpha 1$ isoform of NKA in primary auditory neurons and in differentiated PC12 cells (Delprat et al., 2007a; Delprat et al., 2007b). However, the distribution in non-differentiated PC12 cells appears to be mostly perinuclear (Delprat et al., 2007b). Interestingly, there is a special spatiotemporal distribution of FXYD6 in the brain (Kadowaki et al.,

2004). FXYD6 expression in the hippocampus, cerebellum, and forebrain increases during the first 3 wk of life, peaking during week 3, after which expression gradually decreases. FXYD6 appears to participate in the development of multiple brain structures. Dynamic expression of FXYD6 is also observed in the inner ear (Delprat et al., 2007a) and cerebellum, where it localizes to the parallel fibers and cell membranes of granule cells and the axons and dendrites of basket cells (Kadowaki et al., 2004). The increase in FXYD6 expression in the cerebellum between the second and third postnatal weeks coincides with granule cell migration and axon elongation in mice suggesting FXYD6 may be involved in the development in these cells in specific cerebellar lobules (Saito et al., 2001). Shindo et al. showed FXYD6 is also expressed in type II taste cells (Shindo et al., 2011). They proposed that the reduction in $\alpha 1\beta 1$ pump sodium affinity produced by FXYD6 is necessary for extrusion of sodium following an increase in intracellular sodium and responsible for the slower inactivation of sodium currents seen in type II taste cells relative to type III (Shindo et al., 2011). Interestingly, FXYD6 expression does not correlate with $\alpha 1\beta 1$ expression in all structures where it presents, suggesting FXYD6 may have additional interacting partners.

More recent reports suggest that FXYD6 may regulate synaptic transmission. A proteomics study of synaptosomes from GABAergic neurons found that FXYD6 was enriched in plasma membrane fractions and synaptosomes containing the vesicular Na^+ -dependent transporter VGLUT1 (Biesemann et al., 2014). FXYD6 was detected in all brain regions and immunostaining showed dendritic, axonal, somatic, and presynaptic plasma membrane localization (Biesemann et al., 2014). FXYD6 (and FXYD7) showed a high degree of colocalization with synaptic protein PSD95 (Shiina et al., 2010).

FXYD6 may be important for several pathologies. Polymorphisms in the FXYD6 gene have been linked to schizophrenia (Choudhury et al., 2007). However, other studies have not detected such an association (Iwata et al., 2010; Jiao et al., 2011). Like FXYD5, FXYD6 is highly expressed in cholangiocarcinoma (Chen et al., 2014), osteosarcoma, and hepatocellular carcinoma (Gao et al., 2014; Yang et al., 2014). Additional studies may reveal whether FXYD6 plays a direct role in cell growth and migration or is simply a result of abnormal protein expression in these cells. In either case, it may prove a clinically useful biomarker.

FXYD7

FXYD7 has been exclusively found in the brain, with mRNA and protein being detectable in the neurons (and to a lesser extent in glia) of multiple regions, including the hippocampus, brain stem, hypothalamus, cerebrum, and cerebellum (Béguin et al., 2002). Béguin et al. (2002) found that anti-FXYD7 antibodies coimmunoprecipitated with the NKA $\alpha 1$ isoform but not with the $\alpha 2$ or $\alpha 3$ isoforms. However, when coexpressed in *Xenopus* oocytes, FXYD7 was found to associate with $\alpha 1\beta 1$, $\alpha 2\beta 1$, and $\alpha 3\beta 1$ pumps. These coexpression experiments demonstrate interactions with $\alpha 2$ and $\alpha 3$ can occur. However, whether FXYD7 regulates these isoforms in vivo is less clear.

Despite the uncertainty about the role of FXYD7 in brain function, studies in *Xenopus* oocytes have provided insight into

its regulation of NKA kinetics. Electrophysiological recordings showed FXYP7 reduced the apparent affinity for external K^+ , an effect that depended on the presence of external Na^+ and voltage (Béguin et al., 2002; Meyer et al., 2020; Table 1). FXYP7 also increased the apparent affinity for intracellular Na^+ (Meyer et al., 2020). Béguin et al. proposed the FXYP7-dependent reduction in NKA K^+ affinity serves as a mechanism to aid in the reuptake of extracellular K^+ in neurons and astrocytes. Following neuronal activity, the concentration of extracellular K^+ is significantly increased and must be restored in order to maintain neuronal function, because high extracellular K^+ would depolarize the membrane, leading to neuronal hyperexcitability and seizures (Dichter et al., 1972; Traynelis and Dingledine, 1988; Takahashi et al., 1981). A subpopulation of $\alpha 1\beta 1$ pumps with an increased $K_{0.5}$ for K^+ resulting from association with FXYP7 would serve as a reserve pool of transporters. This subpopulation would be relatively inactive at resting K^+ but marshalled for K^+ reuptake into neurons and glia when external K^+ levels increase after intense neuronal activity. A similar role has been proposed for PLM and FXYP6 in cardiac tissue and auditory neurons, respectively (Crambert et al., 2002; Delprat et al., 2007b).

FXYP7 is unique among the FXYP protein family because it is able to translocate to the plasma membrane of *Xenopus* oocytes independent of its association with NKA (Moshitzky et al., 2012), suggesting FXYP7 may have additional roles in addition to $\alpha 1\beta 1$ regulation. Western blot analysis of oocytes expressing FXYP7 yields three species with different mobility (14, 18, and 19 kD) due to posttranslational O-glycosylation of at least two out of three N-terminal threonine residues (Thr3, Thr5, and Thr9; Crambert et al., 2004; Moshitzky et al., 2012). Interestingly, only the 18-kD species was detected in rat brain, which corresponds with the most prominent FXYP7 species detected in oocytes (Béguin et al., 2002). Evidence suggests O-glycosylation also plays a role in the FXYP7 stability and export from the endoplasmic reticulum and Golgi apparatus (Crambert et al., 2004). Mutation of the three N-terminal threonines greatly reduced FXYP7 expression at the plasma membrane of H1299 cells. Similarly, truncation of the first 15 residues in the N terminus significantly reduced the amount of $\alpha 1$ subunit coimmunoprecipitated with FXYP7, demonstrating the importance of the N terminus in association with NKA (Crambert et al., 2004; Moshitzky et al., 2012). Additional structural determinants within the C terminus appear to be involved in FXYP7 processing and surface expression. Valine residue (Val80) acts as an endoplasmic reticulum export signal and promotes O-glycosylation and protein stability (Crambert et al., 2004). Deletion of the Val80 residue reduces the rate and degree of FXYP7 O-glycosylation and resulted in decreased FXYP7 expression at the plasma membrane in *Xenopus* oocytes (Crambert et al., 2004). The presence of an endoplasmic reticulum export signal may serve as a mechanism to modify functional FXYP7 expression based on cellular demands.

Structural determinants of FXYP protein regulation of NKA kinetics

A recent study by our laboratory used electrophysiological techniques to perform a detailed comparison of the kinetic

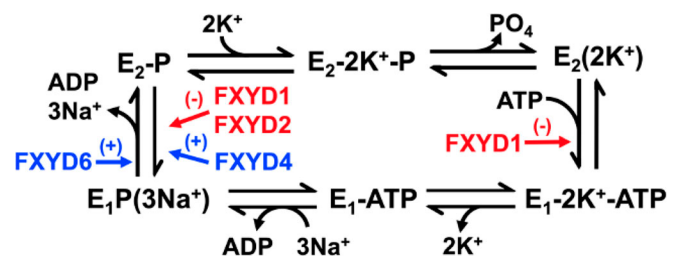


Figure 3. **A simplified Post-Albers diagram showing the enzymatic transitions of the NKA ion transport cycle.** Effects of FXYPs on forward (clockwise) or reverse steps are highlighted, with stimulatory effects that increase the rate of a transition shown in blue and inhibitory effects shown in red.

effects of five FXYP proteins on human NKAs (Meyer et al., 2020). Our experiments included evaluation of the transient currents, which inform of the conformational change between $E_1P(3Na^+)$ and E_2P (Moreno et al., 2020), as well as measurement of the apparent affinity for intracellular Na^+ using patch clamp. Transient current measurements demonstrated that PLM, the γ subunit, and FXYP6 poise the equilibrium of the $E_1P(3Na^+)$ to E_2P+Na^+ transition toward Na^+ -free E_2P (Fig. 3), therefore reducing apparent affinity for external Na^+ by up to twofold. In contrast, CHIF and FXYP7 favor occupancy of $E_1P(3Na^+)$, therefore increasing affinity for external Na^+ by up to twofold. Although our experiments cannot rule out direct effects on apparent affinity, the changes in conformational equilibrium we observed are sufficient to explain the changes in apparent affinity for intracellular Na^+ observed with each FXYP (Table 1). Different FXYP proteins influence different partial reactions. In particular, PLM and the γ subunit slow down the voltage-dependent transition to $E_1P(3Na^+)$ without altering the transition to E_2P , while FXYP6 accelerates the transition to E_2P without altering the transition to $E_1P(3Na^+)$, thereby increasing the NKA overall turnover rate (Fig. 3). On the other hand, while CHIF accelerates the transition to $E_1P(3Na^+)$ (Fig. 3), it remains unclear which actual reaction rates are affected by FXYP7. Thus, these diverse kinetic effects of FXYP proteins may involve distinct structural determinants in each of them. However, given that some of these effects are small in absolute terms (less than twofold changes in apparent affinity for any ion on any side of the membrane), identification of the actual determinant of each kinetic effect may be prone to large influences that depend on the chosen experimental system (e.g., whole-cell systems versus isolated patch membranes or detergent-solubilized protein).

As discussed, several reports have addressed the effect of point mutations, deletions and domain swaps (chimeras) on different FXYP subunits in an attempt to understand both the determinants of the binding interaction and the functional efficacy of FXYP proteins. Consistent with the reasoning laid out in the previous paragraph, a common structural determinant for the effects of all FXYP proteins remains to be found, and even the structural correlative of kinetic effects for each FXYP protein are far less clear than the determinants of interaction (compare structural determinants of FXYP-NKA interaction). Béguin et al. (2001) reported that cationic amino acids in the

C-terminal domain are responsible for CHIF's increased apparent affinity for Na⁺. However, Lindzen et al. (2003) show that the residues responsible for the opposite effects of CHIF and the γ on NKA's apparent affinity for Na⁺ are located in the TM domain. Crambert et al. (2005) show that four residues in the TM of FXYP7 are responsible for the altered apparent affinity for ions, some affecting only affinity for K⁺ and others altering affinity for both Na⁺ and K⁺. Future studies could revisit these problems using NKA and FXYP proteins originated from a single species while evaluating the functional effect on whole-cell, isolated membrane, and detergent-solubilized protein.

Unresolved controversies, unanswered questions, and new directions

While our understanding of FXYP protein regulation of sodium/potassium transport has made great advances over the last two decades, there are still key questions that are likely to be the subject of intensive research in the coming years. Below, we have categorized some of the most intriguing controversies and unsolved problems.

Effects of FXYP proteins on non-NKA targets

While interest in the regulatory function of PLM has focused primarily on its well-established modulation of NKA activity, other candidates have been proposed as PLM targets, including the L-type Ca²⁺ channel (Zhang et al., 2015) and NCX1 (Zhang et al., 2006; Wang et al., 2014). L-type Ca²⁺-current amplitudes were larger in cardiac myocytes isolated from PLM-knockout animals, while expression of the PLM TM domain in PLM-knockout mice restored L-type Ca²⁺ current amplitudes to normal. PLM and the NCX1 were found to coimmunoprecipitate and colocalize (Zhang et al., 2006; Wang et al., 2014), although FRET between PLM and NCX1 tagged with fluorescent proteins could not be detected (Bossuyt et al., 2006). It has been hypothesized that phosphorylated Ser68 PLM may directly inhibit NCX1 based on the observation that overexpressing the phosphomimetic PLM mutant Ser68Glu in PLM-knockout myocytes resulted in decreased NCX1 current without affecting NKA current (Zhang et al., 2006). Compared with WT mice, transgenic mice expressing the phosphomimetic Ser68Glu PLM mutant tolerated cardiac ischemia-reperfusion better, showing less severe contractile dysfunction (Wang et al., 2014). The investigators proposed direct inhibition of NCX1 by phosphorylated S68 PLM decreases Ca²⁺ extrusion, preserves intracellular Ca²⁺ levels, and ameliorates contractile dysfunction. However, an alternative interpretation of those observations is that phosphorylated PLM relieves NKA inhibition, reducing intracellular Na⁺, which itself can improve outcomes after ischemia-reperfusion (Hasenfuss and Maier, 2008). Overall, the concepts of PLM regulation of NCX1 and the L-type Ca²⁺ channel have been controversial; NKA regulation is still regarded as the most relevant FXYP function.

Structural biology

One exciting area of future investigation will be additional determinations of novel structures of FXYPs (and other similar peptides) and analysis of the structure and dynamics of the complexes these peptides form with target ATPases. This high-

priority area will benefit from the ongoing revolution in membrane protein structural biology that has been powered by technical advances in cryo-EM and computational modeling (Rui et al., 2016). In particular, high-resolution cryo-EM could provide insight into differences between the regulatory complexes of NKA-activating and inhibiting FXYPs. Single-particle analysis could uncover conformational heterogeneity (Nakane et al., 2018), perhaps revealing how FXYPs stabilize with a particular poise of the pump (Meyer et al., 2020). Such high-resolution structural information, complemented with structural constraints from other techniques, is the foundation for computational studies (Sánchez-Rodríguez et al., 2015; Rui et al., 2016). These have become increasingly useful as growing processing power enables longer simulations of larger systems. The timescale of molecular dynamics now overlaps the time regimen of interesting structural transitions (Ragumova et al., 2020), and analytical approaches provide exploration of the energy landscape that governs key conformational changes (Das et al., 2017). In addition, we anticipate that new membrane protein-protein docking methods (Alford et al., 2015) will be useful for quantifying the energetics of binding of FXYPs to different $\alpha\beta$ complexes, generating testable hypotheses about novel modes of tissue-specific interactions. We previously used this approach to reveal multiple modes of interaction for the PLB-SERCA complex, observing alternative binding sites and identifying several favorable orientations of the peptide at each interface (Alford et al., 2020).

Such observations are in keeping with the emerging theme of broad specificity in recognition of peptide regulators by transport ATPases. Many micropeptides differentially regulate SERCA (Singh et al., 2019; Anderson et al., 2016; Makarewich et al., 2018), and even synthetic peptides with random hydrophobic sequences bind and regulate the Ca transporter (Afara et al., 2008). Likewise, NKA has a promiscuous binding site appropriate for recognizing a broad diversity of FXYP protein partners. Taken together, these previous computational and physical studies point to the possibility that P-type ATPases do not interact with regulatory partners in a stringent "lock-and-key" recognition mechanism but instead form a polymorphic "fuzzy complex" characterized by dynamic disorder (motions) and static disorder (heterogeneity). A loosely defined regulatory interaction could account for the differing conclusions derived from cross-linking experiments (Lindzen et al., 2006) versus x-ray crystal structures of NKA bound to FXYPs (Morth et al., 2007; Nyblom et al., 2013; Laursen et al., 2013; Kanai et al., 2013; Shinoda et al., 2009; Ogawa et al., 2009).

Such ambivalence and structural heterogeneity will create challenges for the search for determinants of inhibition or stimulation. Indeed, few aspects of NKA functional regulation have been attributed to discrete structural elements. Future studies that attempt to discover unifying principles of FXYP protein regulation of NKA may benefit from strategies developed for investigation of intrinsically disordered proteins (Appadurai et al., 2019).

Phosphorylation and other posttranslational modifications

We have known that PLM is phosphorylated by PKA and PKC for more than 30 yrs, and it would seem at first to be a tractable

problem to elucidate the functional mechanism by which PLM phosphorylation modulates NKA regulation. However, our recent article highlighted some of the challenges faced in trying to demonstrate unequivocally that PKA phosphorylation directly increases function of $\alpha\beta$ PLM by modulating its kinetic parameters (Meyer et al., 2020). A key problem is the difficulty to demonstrate a direct increase in NKA current or ATPase activity after PLM phosphorylation with protein kinases. We observed an increase in apparent affinity for Na^+ without a direct increase in current after addition of PKA under conditions of subsaturating $[\text{Na}]$. It is possible that some unknown intracellular essential factor (Hilgemann 2020) is missing from cell-free systems such as excised membrane patches or membrane preparations. In addition, dilution may contribute to loss of multimolecular complexes and scaffolding by changing the balance of association and dissociation. Finally, such preparations create uncertainties related to phosphorylation/dephosphorylation rates, perhaps contributing to discrepancies between in vitro and in vivo studies.

These uncertainties are inherent to kinase regulation studies and are very hard to manage. The magnitude of the direct effect of FXYDs on the measured kinetic parameters (such as apparent affinity) is small, even though the physiological effects of such small kinetic changes can be drastic (Meyer et al., 2020). An additional complication is that in oocytes, dephosphorylated PLM reduces the amount of NKA present at the plasma membrane by up to 50%, and this effect is relieved by injection of PKA. If similar trafficking regulation occurs in more complete physiological systems like ventricular myocytes, then an increase in current may reflect a change in apparent affinity or an increase in the number of pumps at the plasma membrane.

Finally, the interdependence of phosphorylation with other posttranslational modifications such as palmitoylation and glutathionylation (which have their own kinetic and trafficking effects) adds to the list of unknowns. How these influences work in concert in an integrated system is still not well understood.

FXYDs in physiology

As investigators have moved beyond well-defined in vitro preparations to physiological models, additional nuances of FXYD protein function have been uncovered. For example, a PLM-knockout mouse phenotype of more dilute urine was attributable not to modulation of NKA activity, as one might expect, but rather to increased trafficking of AQP2 to the apical membrane (Arystarkhova et al., 2017). Similarly, PLM has also been proposed to play a role in glucose transport in fat cells. In these cells, inhibiting PLM phosphorylation decreased GLUT4 trafficking to the plasma membrane (Walaas et al., 1999). We anticipate that FXYD protein regulation of trafficking non-NKA targets will be an important area of future investigation. The γ subunit has also been implicated in glucose metabolism, as γ ablation decreased blood glucose levels and improved glucose tolerance (Arystarkhova et al., 2013). This possible role for FXYDs in pancreatic function is an important area that requires further exploration. Such studies entail the same drawbacks of other genetic manipulations, namely the possibility of compensatory mechanisms that obfuscate the native physiological

function of the FXYD in question. Indeed, γ -subunit-knockout mice show no difference in kidney function, despite showing effects on NKA regulation in the nephron. This is not surprising, since the kidney is highly adaptive and may undergo compensatory changes, such as up-regulation of other FXYDs (FXYD switching; Arystarkhova et al., 2007). Further study on these the compensatory changes will be required to better understand the physiological role of γ subunit in the kidneys. Physiological experiments will be essential for understanding the integrative function of FXYD proteins. We will be particularly interested to see future developments in elucidating the physiological roles of FXYD6 and FXYD7 in neurophysiology.

Kinetic effects of FXYDs

The field has benefitted from extensive characterization of the effects of FXYDs on NKA ion affinity and V_{max} , but these are high-level kinetic parameters that describe overall enzyme function. Much is still unknown about the mechanistic basis for these changes. How does a particular FXYD alter the rate of a particular enzymatic step in the Post-Albers cycle (Fig. 3)? One possibility is that different FXYDs stabilize particular NKA conformations (Meyer et al., 2020), making preceding or subsequent steps more (or less) energetically favorable. FXYDs could alter the free energy of an intermediate state and accelerate or inhibit structural transitions through that state. Single-molecule fluorescence measurements could reveal how different FXYDs alter the dwell time and order of conformations in the catalytic sequence (Dyla et al., 2017). Insight into such mechanisms may also be gleaned from future computational studies that explore the energetics of structure changes (Fernández-de Gortari and Espinoza-Fonseca, 2018), particularly if these studies can provide comparative analysis of different FXYDs with opposing functions. Preferential stabilization of NKA conformations may also manifest as physically measurable parameters such as enhanced $K_{0.5}$ of FXYDs to different enzymatic states, or “melting temperature” measurements of regulatory complexes. In addition, electrophysiological measurements provide evidence that a fraction of the NKA population may divert into an “inactivated” pathway under conditions of low intracellular sodium (Lu et al., 2016). Inactivated states could reflect long-lived K^+ occluded states in the absence of Na^+ , and recent patch-clamp experiments suggest that these states may be altered by FXYDs (Meyer et al., 2020). Future studies that combine electrophysiology and quantification of FXYD-NKA binding energetics may reveal whether diversion to the putative inactivated pathway is mediated by NKA structural stabilization by inhibitory FXYDs such as PLM.

Translation and clinical significance

With so many fundamental questions still unanswered, motivation is high for mechanistic structure-function studies. In addition, efforts are ongoing to improve translation of mechanistic knowledge into clinical insights. There is fast-growing literature showing noteworthy correlations of FXYD protein changes with various disease states, but many of these are descriptive studies. The remaining challenge is to clarify the functional significance of these changes. At a minimum, FXYD

proteins may serve as useful biomarkers, but is the change in expression and the resulting change in NKA function mechanistically connected to the pathology? Moreover, one may speculate that the broad receptivity of the NKA regulatory binding sites may create the potential for misregulation of transport by “poison peptides.” Many disease states are characterized by increased proteolysis, including heart disease (Inserte et al., 2006; Inserte et al., 2005; Singh and Dhalla, 2010; Singh et al., 2004; French et al., 2006; Roczakowsky et al., 2020; Düsterhöft et al., 2017). Helical TM segments are ubiquitous elements of membrane protein structure. Might TM fragments inappropriately bind to the low-specificity site on NKA and modulate transport function? We predict that recent advances in mass spectrometry and other analytical techniques will power future discovery of novel NKA-binding species and reveal how this regulatory interactome changes in health and disease. In addition, quantitative comparison of the affinity of different FXYPs and FXYP-like peptides for NKA will improve our understanding of how they compete for NKA binding as the relative concentrations of FXYP species changes in various diseased tissues.

Finally, it is our hope that our growing foundation of mechanistic knowledge can serve efforts in rational design of NKA-targeting therapies. These may include small-molecule drug candidates that mimic FXYP regulation of NKA ion affinity and kinetics. NKA-inhibiting cardiac glycosides such as digitalis have been used for hundreds of years for the treatment of heart failure (Withering, 1785), but these compounds have a very narrow therapeutic window, severely limiting their clinical utility (Vamos et al., 2019). Glycosides enhance calcium handling by simple inhibition of the NKA V_{max} . This inhibition impairs maximal Na/K transport rates and risks sodium and calcium overload, leading to potentially lethal arrhythmias. Because of the high and growing prevalence of heart failure, improved NKA-dependent inotropic drugs are urgently needed. One may anticipate development of new small molecules that can exploit endogenous NKA regulatory mechanisms, namely, modulating specific catalytic intermediates and changing Na^+ and K^+ affinity without impairing turnover during conditions of Na overload.

In addition, the small size and physiological specialization of FXYPs make them attractive candidates for gene therapy strategies. While clinical trials based on ion transporter gene delivery have yielded disappointing results so far (Greenberg et al., 2016), we consider it likely that the small FXYP peptides will be a more tractable payload for viral vectors. Indeed, correction of altered PLM expression with delivery of lentiviral construct showed promise for improving Rett syndrome in a mouse model (Deng et al., 2007). Another high-priority target pathology is heart failure. One would expect an inotropic effect (enhancing cardiac contraction strength) for inhibitory FXYPs, as we have previously observed for WT FXYP and a super-inhibitory mutant (Himes et al., 2016). Conversely, NKA-activating species will likely be negatively inotropic, which may also be therapeutically useful for some conditions. While increased NKA activity is negatively inotropic in healthy hearts, NKA stimulation improved contractility in a large animal model of heart failure with reduced ejection fraction (Bundgaard et al., 2010). Moreover, NKA stimulation may be appropriate for

treatment of cardiac hypertrophy, which is associated with decreased function/expression of NKA (Verdonck et al., 2003a; Pogwizd et al., 2003; Verdonck et al., 2003b). Furthermore, we envision possible applications of FXYP gene delivery or expression modulation for the treatment of neurological disorders such as Parkinson’s disease, Alzheimer’s disease, schizophrenia, and other pathological conditions.

Summary

This is an exciting time for the field as new functional mechanisms and clinical correlations are uncovered. These discoveries generate hope for future therapeutic approaches that may build on the broad foundational knowledge of FXYP protein regulatory mechanisms. We anticipate that the next few years will see a rapid growth in our understanding of this diverse family of regulatory micropeptides as new investigators bring advanced methods to bear on the many tantalizing questions that remain unanswered.

Acknowledgments

Christopher J. Lingle served as editor.

We thank Sean Cleary and Dr. Qi Qian for helpful suggestions and Dr. Petra Cechova for providing lipid membrane representations.

This work was supported by the National Institutes of Health (awards HL092321 and HL143816 to S.L. Robia and award NS116433 to P. Artigas) and the National Science Foundation (award MCB 2003251 to P. Artigas).

The authors declare no competing financial interests.

Author contributions: J.Q. Yap and S.L. Robia contributed to the conception the manuscript. J. Seflova created the figures. J.Q. Yap, J. Seflova, R. Sweazey, P. Artigas, and S.L. Robia contributed to writing the manuscript, approved the final version, and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Submitted: 27 June 2020

Accepted: 3 February 2021

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