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COMMENTARY

What FXYDs fix

Michael Habeck and Hanne Poulsen

Cells spend a good part of their energy sustaining ionic gradients over membranes, most prominently the sodium and potassium gradients across the plasma membrane that the Na,K ATPases maintain. These ATP-dependent pumps contain two essential subunits, a catalytic α subunit, and a β subunit required for transport and activity, and the pump may further associate with an additional subunit, a FXYD (pronounced *fixyd*) single span transmembrane protein (Sweadner and Rael, 2000; Clausen et al., 2017). In an earlier issue of the *Journal of General Physiology*, Meyer et al. (2020) explore the consequences of coexpression of several FXYDs on pump function. Endogenous pumps purified from pig kidney and shark rectal glands have been used for crystallographic studies, and they show tripartite complexes comprising an α, a β, and a FXYD (Fig. 1; Morth et al., 2007; Laursen et al., 2013; Kanai et al., 2013). The common motif PFXYD (where X denotes any amino acid) is positioned just on the extracellular side of the membrane, and the TM helix that follows is parallel to TM9 of the α subunit. There are seven FXYD proteins expressed in humans, FXYD1–7, in tissue- and cell-specific manners. Various FXYDs have been studied before, but in the previous work (Geering, 2006), subunits from a range of species have been used and the results reported have sometimes been inconsistent. In their new study, Meyer et al. (2020) have systematically tested the functional effects of five human FXYD proteins on human α1-β1 pumps expressed in *Xenopus laevis* oocytes, thus offering the first comprehensive comparison between the effects of different FXYDs on Na,K ATPases with all-human subunits.

The work of Meyer et al. (2020) reveals that FXYDs affect a number of pump kinetic parameters, including extracellular and intracellular sodium and potassium affinities, turnover, and the voltage dependent E1P-E2P equilibrium. They furthermore address whether FXYDs affect pump trafficking and if the effect of FXYD1 is regulated by PKA.

The most marked differences that Meyer et al. (2020) find are between FXYD2 and FXYD4. Both are strongly expressed in the kidney, but appear in nonoverlapping cell types (Shi et al., 2001). FXYD4 expression is induced by aldosterone, sodium deprivation, and potassium loading; FXYD4 knockout mice respond to high potassium with significantly increased urine production (Aizman et al., 2002). In FXYD2 knockout mice, the FXYD2-less pumps have higher sodium affinity (Jones et al., 2005). Together, these previous results suggest roles in tailoring Na,K ATPase ion affinities for different physiological conditions. The observations reported by Meyer et al. (2020) strongly support this possibility, with FXYD2 elevating potassium affinity and lowering intracellular and extracellular sodium affinity, while FXYD4 has the opposite effects. Thus, these differences in relative affinity for potassium and sodium may provide a physiological mechanism for the organism to conserve either ion preferentially depending on its homeostatic need.

The mechanism by which the FXYDs cause differential affinity of the pump for the monovalent ions remains an open question. Mutations in TM9 of the ATPase α subunit abrogate lowering of potassium affinity by FXYD4 and 7 (Li et al., 2004), and chimeric constructs of FXYD2 and 4 show that their effects on sodium affinity depend on the TM helix (Lindzen et al., 2003). Analogously, the β-subunit TM helix is central for that subunit's modulatory effect (Hilbers et al., 2016). For FXYD1, mutations that affect the interaction of its TM with α TM9 diminish its functional effect on pump activity, although the coimmunoprecipitation suggests that the two subunits remain physically associated (Khafaga et al., 2012). TM interactions of FXYD1, 2, and 4 also stabilize detergent-solubilized pumps (Lindzen et al., 2003), likely because the FXYD facilitates lipid interactions (Mishra et al., 2011). Pump structures reveal several tightly associated lipids, including a cholesterol toward the extracellular side between FXYD and α's TM8–10 (Kanai et al., 2013; Laursen et al., 2013), a phospholipid close to the cholesterol site (phosphatidylserine modeled in HYT4), and a phospholipid toward the cytoplasmic side between FXYD and α's TM2, 6, and 8 (phosphatidylcholine modeled in 3WGU; Fig. 1). These studies suggest that association of the FXYD TM with the α subunit (1) creates hydrophobic binding grooves where specific lipid interactions stabilize the pump, and (2) affect the dynamic restructuring of the α TM helices that enable the catalytic cycle to proceed with binding and release of ions. The TM helix is the best conserved part of the FXYDs, but,

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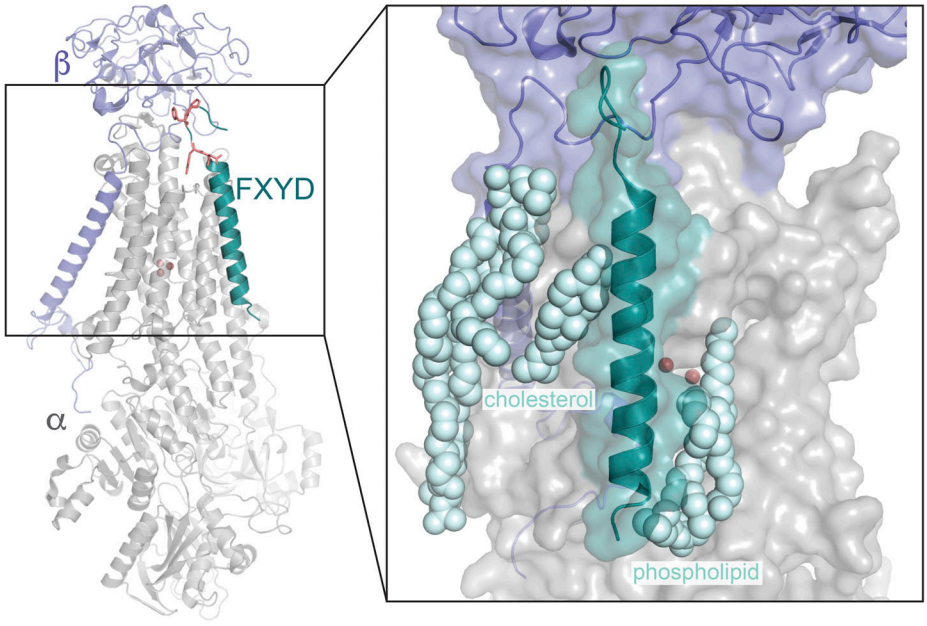


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FXYD1    24 --KEHDPFTYDYQSLQIGGLVIAGILFILGILIVLSRRCRCKFNQQQR-TGEPDEEEGT-FRSSIRRLSTRRR-----
FXYD2    14 --GDVDPFYYDYETVRNGGLIFAGLAFIVGLLILLSRRFRCGGNKKRR-Q--INEDEP--------------------
FXYD3    25 --DKNSPFYYDWHSLQVGGLICAGVLCAMGIIIVMSAKCKCKFGQKSG-H--H-PGET---PPLITPGS-----AQ-S
FXYD4    25 --NKDDPFYYDWKNLQLSGLICGGLLAIAGIAAVLSGKCKCKSSQKQH-S--PVPEKA---IPLITPGS-----ATTC
FXYD5   130 --HEDDPFFYDEHTLRKRGLLVAAVLFITGIIILTSGKCRQLSRLCRN-H--CR------------------------
FXYD6    25 --KEMDPFHYDYQTLRIGGLVFAVVLFSVGILLILSRRCKCSFNQKPR-A--PGDEEAQ-VENLITANATEPQKAEN-
FXYD7    13 --EEPDPFYYDYNTVQTVGMTLATILFLLGILIVISKKVKCRKADSRSES--PTCKSCKSELPSSAPGG-----GGV-
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Figure 1. **Structure and sequence alignment of FXYD.** Top: An overview of the tripartite Na,K ATPase with FXYD (cyan), α (gray), β (blue), and three sodium ions (red) in the middle of the transmembrane domain. The conserved residues of the PFXYD motif are shown as red sticks. The extracellular side is on the top and the intracellular side is on the bottom. In the inset, the transmembrane part of the pump is highlighted, and the pump is turned to position FXYD centrally. Lipid molecules identified in the structure are shown with spheres (pale cyan). PDB accession no. 3WGU. Bottom: An alignment of the transmembrane domains and C termini of seven human FXYDs. The conserved residues of the PFXYD motif are shown on red background. The FXYD2 residues resolved in the crystal structure are cyan. Basic residues in the C termini are purple.

except for two glycines facing TM9, all positions show variation (Fig. 1), and these differences likely explain at least some of the effects that Meyer et al. (2020) report.

What, then, are the functions of the extracellular and intracellular regions of the FXYDs? The PFXYD motif preceding the TM is close to the β subunit, and the rest of the N terminus (16–28 residues except FXYD5, which has a longer N-terminal region) is probably flexible, since the structures do not show defined densities. For FXYD5 and 7, *O*-glycosylation of threonines in the extracellular domain are important for the subunits' trafficking to the membrane (Moshitzky et al., 2012; Tokhtaeva et al., 2016). The intracellular region is also disordered in the structures. The segment just after the TM contains many basic residues (charge of +4–7 for the first 12 residues) as often seen for membrane proteins. The region may be intrinsically disordered or in equilibrium between folded and unfolded states, and it has been proposed to interact with the lipid membrane, possibly in a manner regulated by phosphorylation (Csizmadia et al., 2020). Especially for FXYD1, phosphorylation has been intensely studied. In several muscle tissues, it is the primary substrate of phosphorylation by the PKA and PKC kinases upon stimulation (Palmer et al., 1991), and studies with phosphomimetic FXYD1 mutants show that, instead of its usual inhibitory effect, the phosphorylated version can stimulate pump activity (Mishra et al., 2015). However, the physiological effect of FXYD1 phosphorylation on acute stimulation of pump turnover in muscle remains elusive since FXYD1 knockout mice exhibit normal exercise capacity and no change in muscle contractility or fatigue (Manoharan et al., 2015). In the work reported earlier, Meyer et al. (2020) found that coexpression with FXYD1 reduces the outward current produced, but that oocytes injected with PKA have similar currents regardless of coexpressed FXYD1. As they discussed, different groups have often reached opposing conclusions regarding the effects of FXYD1 phosphorylation, and it remains to be firmly established whether and how the Na,K ATPase ion affinity, maximal capacity, and membrane translocation are regulated by FXYD1 phosphorylation. In most of the other FXYDs, there are also strong candidate positions for phosphorylation; FXYD2, with a very short intracellular C terminus, is the exception. Thus, the role of FXYD phosphorylation in regulating Na,K ATPases remains very much an open question.

The study by Meyer et al. (2020) expands our knowledge of the mechanistic modulatory functions that FXYDs serve when interacting with an α1-β1 Na,K ATPase. In future studies, it will be important to address if the effects that are found also apply when FXYDs associate with other α and β subunits, and what the

physiologically relevant combinations are. The results reported here underscore that the FXYDs can fine-tune the Na,K-ATPase properties to optimize these essential and highly energy-demanding pumps for the specific needs of different tissues and cell types at different times under different conditions. Single-cell RNA sequencing will reveal how the individual subunits are expressed in various cell types to identify likely combinations of α, β, and FXYD isoforms. Furthermore, recent advances in cross-linking mass spectrometry (e.g., Gonzalez-Lozano et al., 2020) may help to identify interactions of low abundant isoform complexes at different developmental stages or pathophysiological conditions.

The roles played by FXYDs in health and disease are still being discovered. Mutations in the *FXYD1* and *6* genes are reported to associate with childhood-onset schizophrenia (Chaumette et al., 2020), and a particular *FXYD2* mutation, G41R, causes dominant hypomagnesemia (Meij et al., 2000; de Baaij et al., 2015). The residue affected is the second of the conserved glycines in the FXYD TM, and Meij et al. (2000) suggested that the mutation hinders the pump from being transported to the plasma membrane. In their model, the lack of Na,K ATPase activity in FXYD2-expressing kidney cells affects sodium and potassium gradients, and thereby the driving force for magnesium. An alternative suggestion has been that FXYD2 can form a cation channel, and that the mutation makes the channel inwardly rectifying (Sha et al., 2008). For several of the FXYDs, it has been described that expression in *Xenopus* oocytes evokes channel-like activity, but the FXYD2 G41R is primarily poorly expressed and trafficked (Sha et al., 2008). That the disease-causing effect of the G41R mutation is due to Na,K ATPase deficiency is supported by a later report that some mutations in *ATP1A1*, in addition to severe problems in the central nervous system, cause renal hypomagnesemia (Schlingmann et al., 2018).

So, what is it that FXYDs fix? It is tempting to speculate that they have evolved to allow an extra level of optimization of the Na,K ATPase's kinetic parameters and its transport and regulation. The pump is essential to virtually all animal cells, but it is also an energetically expensive machine, so rather than relying only on the once-developed scaffold for a catalytic subunit and having to add novel handles to it for fine-tuning, the pump forms complexes with additional subunits, allowing a substantial outcome space of combinations to enable the individual, specialized cell to express Na,K ATPases with the properties best suited for its particular needs.

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