Modulation of cell polarization by the Na⁺-K⁺-ATPase-associated protein FXYD5 (dysadherin)

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Lubarski I, Asher C, Garty H. Modulation of cell polarization by the Na⁺-K⁺-ATPase-associated protein FXYD5 (dysadherin). Am J Physiol Cell Physiol 306: C1080–C1088, 2014. First published April 9, 2014; doi:10.1152/ajpcell.00042.2014.—FXYD5 (dysadherin or also called a related to ion channel, RIC) is a transmembrane auxiliary subunit of the Na⁺-K⁺-ATPase shown to increase its maximal velocity (V_{max}). FXYD5 has also been identified as a cancer-associated protein whose expression in tumor-derived cell lines impairs cytoskeletal organization and increases cell motility. Previously, we have demonstrated that the expression of FXYD5 in M1 cells derived from mouse kidney collecting duct impairs the formation of tight and adherence junctions. The current study aimed to further explore effects of FXYD5 at a single cell level. It was found that in M1, as well as three other cell lines, FXYD5 inhibits transformation of adhered single cells from the initial radial shape to a flattened, elongated shape in the first stage of monolayer formation. This is also correlated to less ordered actin cables and fewer focal points. Structure-function analysis has demonstrated that the transmembrane domain of FXYD5, and not its unique extracellular segment, mediates the inhibition of change in cell shape. This domain has been shown before to be involved in the association of FXYD5 with the Na⁺-K⁺-ATPase, which leads to the increase in V_{max}. Furthermore, specific transmembrane point mutations in FXYD5 that either increase or decrease its effect on cell elongation had a corresponding effect on the coimmunoprecipitation of FXYD5 with α Na⁺-K⁺-ATPase. These findings lend support to the possibility that FXYD5 affects cell polarization through its transmembrane domain interaction with the Na⁺-K⁺-ATPase. Yet interaction of FXYD5 with other proteins cannot be excluded.

FXYD5: dysadherin; Na⁺-K⁺-ATPase; cell adhesion; anterior-posterior polarity

FXYD is a group of single-span transmembrane proteins named after its invariant extracellular motif Phe-Xxx-Tyr-Asp (32). They all have been shown to interact with the Na⁺-K⁺-ATPase (the Na⁺ pump) and alter its kinetic properties (9, 11). Thus it is generally accepted that the seven mammalian members of the FXYD group are tissue-specific auxiliary subunits or regulators of the Na⁺-K⁺-ATPase whose role is to adjust its kinetic properties to the specific needs of a particular tissue or physiological state without affecting it elsewhere.

A structurally and functionally unique family member is FXYD5 (also termed dysadherin or related to ion channel, RIC). As other family members, FXYD5 affects the Na⁺-K⁺-ATPase kinetics and increases its maximal velocity (V_{max}) by at least twofold (17–19). However, several studies also reported observations that cannot be readily explained by modulation of the Na⁺-K⁺-ATPase kinetics. FXYD5 has been identified as a cancer-associated protein whose expression inhibits E-cadherin and promotes metastasis (12). Clinical studies have demonstrated correlations between the abundance of FXYD5 and the progression of malignancies and survival chances of patients with various human cancers (21). Silencing FXYD5 was found to decrease individual and collective cell motility, while overexpressing it had the opposite effect (15, 25, 28). In addition, the expression of FXYD5 was associated with changes in cytoskeletal organization, altered cell shape, and impairment of tight and adherence junctions (15, 16, 20, 25, 28).

FXYD5 also differs structurally from other FXYD proteins in that its extracellular domain is much longer (32). This domain is poorly conserved but is characterized by a high abundance of S, P, and T residues suggesting O-glycosylation. FXYD5 was indeed found to be O-glycosylated (17, 34). This O-glycosylation has also been suggested to account for a large discrepancy between the protein size detected by certain anti-FXYD5 antibodies (50–55 kDa) and its calculated mass (<20 kDa) (12, 25, 28, 34). Other studies using different antibodies and epitope-tagged constructs detected a much smaller protein of ~25 kDa that better correlates with its calculated mass (16–18, 20).

The current study characterizes effects of FXYD5 on the morphological changes that follow cell adhesion and in particular the transformation from the initial radial to elongated shape with anterior-posterior polarity. This has been shown in a number of cell lines in which FXYD5 abundance was manipulated by either transfection or knockdown. In all cell lines FXYD5 was found to reduce the rate at which morphological changes of cells take place that are characterized by cell flattening, elongation, and polarization. Structure-function analysis has demonstrated that the transmembrane domain of FXYD5, and not its unique extracellular segment, is responsible for this effect. This domain is also known to be involved in its high-affinity association with the Na⁺-K⁺-ATPase and in its ability to increase the V_{max} of the Na⁺-K⁺-ATPase. Mutating two transmembrane residues that either increase or decrease the FXYD5-Na⁺-K⁺-ATPase association had a comparable effect on cell elongation.

MATERIALS AND METHODS

Expression and silencing of FXYD5 in cultured cells. The mouse kidney collecting duct cell line M1 (30) was purchased from ATCC. Cells were cultured in a 1:1 mixture of DMEM and F12 media (Biological Industries, Beit Haemek, Israel) supplemented with 5% fetal calf serum, 5 μM dexamethasone, and antibiotics (penicillin, streptomycin). Cells were transfected with pIREs-EGFP vector ex...

1 In this and subsequent cancer-related studies this protein is termed dysadherin. We prefer however the alternative name FXYD5, which highlights its identity as a member of a family of Na⁺-K⁺-ATPase modulators.
pressing mouse FXYD5 in which amino acids 103–106 were replaced by a hemagglutinin A (HA) epitope. Transfection was done using jetPEI reagent (PolyPlus Transfection) according to the manufacturer’s instructions. Stably transfected clones were isolated by FACS and assayed for expression of FXYD5 by Western blot using an anti-HA antibody. FXYD4, endogenously expressed in M1 cells, was stably silenced using MISSION short hairpin RNA (shRNA) plasmid DNA (clone ID NM_033648.1, Sigma-Aldrich). Positive clones were selected using puromycin and verified by Western blot with anti-FXYD4 antibody. Human embryonic kidney cell line HEK293 was cultured in a DMEM medium supplemented with 10% fetal calf serum and antibiotics (penicillin, streptomycin). M1 and HEK293 cells were either transiently or stably transfected with the above mouse FXYD5 cDNA, mutated constructs, or different FXYD5/FXYD4 chimera described in Ref. 17.

H1299 cells (human non-small cell lung carcinoma) were cultured in RPMI 1640 (Biological Industries) supplemented with 10% FCS, 1 mM sodium pyruvate, and penicillin-streptomycin. Panc-1 cells (human pancreatic carcinoma) were cultured in DMEM high glucose medium (Biological Industries) supplemented with 10% FCS plus penicillin-streptomycin. Silencing of endogenous FXYD5 in Panc-1 and H1299 cells was done using the pSUPER RNAi system (OligoEngine) according to manufacturer’s instructions and verified by real-time PCR.

For coimmunoprecipitation, cells expressing HA-tagged FXYD5 were lysed in buffer containing C12E10 found to preserve FXYD-Na+–K+–ATPase interactions (10). Soluble material was incubated overnight at 4°C with anti-HA antibody attached to agarose beads (HA Tag IP/Co-IP Kit, Pierce), washed six times with Tris-buffered saline + 0.05% Tween-20, and then eluted with SDS sample buffer.

Cell imaging and image acquisition. For monitoring shape changes of single cells, cells were plated at low density in either uncoated eight-well chamber plates (µ-slide ibidi, Martinsried, Germany) or similar plates coated with either fibronectin or laminin (Sigma-Aldrich). Time-lapse images of predefined areas of 661.83 µm2 of single cells, cells were plated at low density in either uncoated filter bottom cups. Measurements were repeated with and without 10 µM amiloride, and short circuit current (Isc) was calculated as the ratio between voltage and resistance.

Statistics. Statistical significance was determined using an unpaired t-test.

Antibodies used. Anti-FXYD1, FXYD2, and FXYD4 were described previously (5, 14, 27). A monoclonal antibody recognizing the NH2 terminal of the α1 subunit of Na+-K+-ATPase (6H) was kindly provided by Dr. M. J. Caplan, Yale University School of Medicine. A mouse monoclonal anti-HA antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and monoclonal anti-paxillin was from BD Transduction Laboratories (Lexington, KY). A rabbit polyclonal antibody against the COOH-terminal region (R52-V80) of FXYD7 was a gift from Prof. Kathi Geering, University of Lausanne, Switzerland.

RESULTS

FXYD5 is normally expressed in the kidney collecting duct and participates in the modulation of Na+ transport and blood pressure (18, 31). Previously, we have demonstrated that expressing FXYD5 in the mouse collecting duct-derived cell line M1 impairs the formation of tight and adherence junctions as manifested by a large increase of the paracellular permeability of confluent cell monolayers (16). In addition, redistribution of the tight and adherence junction markers ZO-1 and β-catenin was observed. In light of previous studies reporting effects of FXYD5 on single cell morphology and cytoskeletal organiz-
tion (12, 15, 20, 25, 28), we aimed to study also the effect of FXYD5 on single cell behavior.

As shown before, FXYD5 transfected into M1 cells interacts with the Na\(^{+}\)-K\(^{+}\)-ATPase and can be coimmunoprecipitated with the pump’s α and β subunits (16). These cells natively express FXYD4, another FXYD protein expressed in the kidney collecting duct. Stable transfection of cells with FXYD5 resulted in suppression of FXYD4 (Fig. 1A). Such interchange-

![Fig. 2. Effect of FXYD5 on adhered cell shape. WT and FXYD5-transfected M1 cells were plated. The same field of 661 µm × 661 µm was imaged shortly after plating and 20 h later, using DeltaVision microscopy.](image)

![Fig. 3. Effects of FXYD5 on cell shape and cytoskeletal organization. A: WT (circles), FXYD5-transfected (squares) and FXYD4-silenced (triangles) M1 cells were plated. Fields of 100–150 cells were imaged at 30-min intervals over 20 h, and the rate of cell elongation was quantified by determining the percentage of cells in the field that maintain a radial shape as a function of time. Data points are means ± SE of 4 fields for each cell type. B: brightfield and fluorescence images of M1 cells stained for actin (green) and paxillin (red). C: quantification of the number of focal points visualized in paxillin-stained WT and FXYD5-transfected M1 cells. Values are means ± SE of 12 randomly selected cells. *P < 0.0001.](image)
ability of different FXYD proteins has been shown before (1) and may reflect competition for interaction with αβ Na\(^+-\)K\(^+-\)ATPase and degradation of the noninteracting FXYD protein. To discriminate between effects resulting from the expression of FXYD5 and those caused by the suppression of FXYD4, we have also prepared M1 cell clones in which FXYD4 was knocked down by stable transfection with appropriate shRNA (Fig. 1B). Silencing FXYD4 did not result in induction of FXYD1, 2 or 7 and none of the three FXYD proteins was detected in the FXYD4-silenced cells nor in the wild-type cultures (Fig. 1C). Since the anti-FXYD4 antibody cross-reacts with FXYD3 as well, (6), this FXYD protein is not expressed either. Also, no detectable levels of the neuronal FXYD6 (7) were noted by RT-PCR of wild-type or FXYD5-transfected cells. Thus the silencing of FXYD4 in M1 cells is not compensated by the induction of any other mammalian FXYD protein.

Figure 2 depicts an experiment in which wild-type and stably FXYD5-transfected M1 cells were plated, and representative fields were imaged 1 and 20 h after plating. Time-lapse images taken at 30-min intervals are provided as supplementary material (supplemental movie S1; Supplemental Material is available online at the Journal website). Both Fig. 2 and the supplementary images clearly show substantial effect of FXYD5 on the rate of cell shape changes. Initially, all cells have a radial shape typical to loosely attached cells. Over time they flatten, form lamellipodia, and acquire anterior-posterior polarity. However, the nontransfected cells appear to undergo this process at a much higher rate than the FXYD5-expressing ones, and at long times many more cells were polarized in the nontransfected culture. To quantify the above phenomenon the percentage of radial cells was determined as a function of time, and the data from several fields and independent experiments were averaged (Fig. 3A). The effect of FXYD5 was found to be highly significant, and the number of radial cells after a 20-h incubation was threefold higher in the FXYD5-expressing culture. This effect is due to the expression of FXYD5 rather than the suppression of FXYD4 since the FXYD4-silenced culture was indistinguishable from the wild-type FXYD4-expressing cells (Fig. 3A). Effects were noted also in the single cell cytoskeletal organization. Staining for paxillin revealed fewer focal points as well as less ordered actin stress cables in the FXYD5-transfected cells (Fig. 3B). Quantifying the number of focal points per cell demonstrated that the expression of FXYD5 lowers the number of paxillin focal points by more than threefold (Fig. 3C).

The effect of FXYD5 on the rate of cell flattening and elongation appeared as a general one and could be observed in three additional cell lines tested. Two of these H1299 and Panc-1 derived from different human tumors and express FXYD5 endogenously. Silencing FXYD5 in these cells evoked a large increase of the rate at which morphological changes of loosely attached to flattened cells take place (Fig. 4). The third epithelial line tested was HEK293, which like M1 lacks FXYD5. Transient transfection of these cells with FXYD5 lowers the rate of adhesion-driven shape change, like that seen for M1 (Fig. 4). Thus the above phenomenon is independent of cell line (normal epithelia vs. tumor-derived cells) or procedure (transient vs. stable transfection or silencing). It also appeared to be independent of the extracellular matrix, and it is observed in fibronectin- and laminin-coated as well as uncoated plates (Fig. 5).

Possible mechanisms mediating the above phenomenon are considered in DISCUSSION. However, one possibility tested was that this effect is secondary to the FXYD5-induced increase of the Na\(^+-\)K\(^+-\)ATPase activity (18, 19). Such a possibility was assessed by monitoring cell shape
change in the presence of the Na$^+$-K$^+$-ATPase inhibitor ouabain. At physiological extracellular concentrations of Na$^+$ and K$^+$, ouabain inhibits the human α1β1 isoform of the Na$^+$-K$^+$-ATPase with a $K_D$ of 10–30 nM (8, 13). Thus incubating FXYD5-transfected HEK293 cells with 50 nM ouabain should inhibit more than 50% of the pumps, fully reversing an FXYD5-dependent increase in the pump’s $V_{\text{max}}$.

Figure 6 summarizes experiments in which the rate of change in cell shape was monitored in the presence and absence of ouabain. A: time course of the decrease in percentage of radial cells. Values are means ± SE of 5 independent transfections (each averaging 4–5 fields). B: short-circuit current ($I_{\text{sc}}$) was calculated from the transepithelial resistance and voltage measurements in a confluent monolayer of M1 cells cultivated on permeable supports before and after the addition of 10 μM amiloride. Values are means ± SE of 3 cultures. C: cell shape changes were monitored in M1 cells in the presence and absence of 10 μM amiloride. Values are means ± SE of 4 fields each containing 50–150 cells.

Fig. 5. Effects of FXYD5 in different cell supports. M1 cells that do or do not express FXYD5 were plated either on noncoated supports or on supports coated with either laminin or fibronectin. Fields of 40–100 cells were imaged at 30-min intervals as above. Values are means ± SE of 8 fields.

Fig. 6. Possible effect of Na$^+$-K$^+$-ATPase activity on cell shape change. WT and FXYD5-transfected HEK293 cells were plated and imaged over 20 h. To some of the plates either 10 nM or 50 nM ouabain was added. A: time course of the decrease in percentage of radial cells. Values are means ± SE of data from 5 fields in a single experiment. B: percentage of radial cells at time = 10 h. Values are means ± SE of 5 independent transfections (each averaging 4–5 fields).
cell shape change was monitored in the presence and absence of either 10 or 50 nM ouabain. One key observation was that ouabain slowed down cell elongation, irrespective of the expression of FXYD5, and for long incubation periods even reversed it (Fig. 6A). To minimalize this effect, response to ouabain was evaluated at a relatively short incubation period of 10 h (Fig. 6B). Under these conditions no significant effect of ouabain on cell elongation was noted.

An alternative way to inhibit the Na\(^+\)-K\(^+\)-ATPase activity is by lowering intracellular Na\(^+\). In M1 cells this can be done by blocking the epithelial Na\(^+\) channel (ENaC) using amiloride, which largely reduces apical Na\(^+\) entry. The resulting decrease in the steady-state pumping rate can be measured in confluent cell monolayers as a change in the \(I_{\text{sc}}\) (30). As shown in Fig. 6C, blocking ENaC by 10 \(\mu\)M amiloride decreased \(I_{\text{sc}}\) from 25.4 \(\pm\) 1 to 1.3 \(\pm\) 0.2 \(\mu\)A/cm\(^2\). This >15-fold decrease in the steady-state Na\(^+\)-K\(^+\)-ATPase activity is not associated with toxic effects to the cells and thus enables comparison of cell polarization at very different Na\(^+\)-K\(^+\)-ATPase activities. As shown in Fig. 6D the application of amiloride had at most a minor effect on the rate of cell polarization in FXYD5-transfected cells and no significant effect in the wild-type culture. Amiloride and ouabain will both inhibit pump activity but should have opposite effects on cell Na\(^+\) and the membrane electrical potential. The fact that ouabain does not reverse the effect of amiloride, which largely reduces apical Na\(^+\) entry, argues against the involvement of changes in pump activity, membrane potential, or cell Na\(^+\) in the above response.

Next, we have studied the structural domain(s) of FXYD5 mediating the above response. Since the rate of transformation from radial to elongated form is not affected by silencing FXYD4 (Fig. 3A), we have addressed this issue by transfecting cells with several FXYD5/FXYD4 chimera in which the extracellular, transmembrane and intracellular domains were exchanged between the two proteins. The nomenclature used to define different chimera is the following: each chimera is described by a three-letter symbol corresponding to the origins of its extracellular, transmembrane, and intracellular domains. These are either C or R corresponding to the traditional names of FXYD4 and FXYD5 (CHIF and RIC, respectively). Accordingly, CRR corresponds to a chimera whose extracellular domain is that of FXYD4 and the transmembrane and intracellular domains from FXYD5 and vice versa for RCC (Fig. 7A).

Since the unique feature of FXYD5 is its atypically long extracellular domain, we first examined the effects of exchanging the ecto domains of the two proteins. Surprisingly, this manipulation had no effect on the rate at which cells acquired anterior-posterior polarity (Fig. 7B); i.e., the chimera CRR reduced the rate of cell elongation similar to FXYD5, while RCC had no effect and was similar to wild-type or FXYD4-silenced cells. Similar results were obtained by monitoring the transepithelial resistance (TER) of confluent monolayers and further established that FXYD5 does not mediate its effect by its unique extracellular domain (Fig. 7C). Namely, the chimera CRC largely decreased TER like FXYD5, whereas the chimera RCC did not permeabilize tight junctions and, if anything, increased in TER relative to wild-type and FXYD4-silenced cells.

FXYD5 structure-function relationships were further characterized by examining the effects of all six chimera formed by exchanging domains between FXYD5 and FXYD4. Because of the difficulty to successfully transfect M1 cells, this study was carried out using transiently transfected HEK293 cells that do not express endogenous FXYD5. Results summarized in Fig. 8 confirm and extend the above findings in M1 cells. In particular, it was demonstrated that inhibition of cell shape change is determined by the transmembrane domain of FXYD5; i.e., the chimeric protein CRC in which only the transmembrane domain originates from FXYD5 behaves like FXYD5 (Fig. 8A). In agreement, the protein CRC, in which the transmembrane segment of FXYD5 was replaced by the corresponding

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**Fig. 7.** Domains involved in the effect of FXYD5 in M1 cells. A: illustration of the membrane topology of FXYD4 (hatched), FXYD5 (filled), and two chimera formed by swapping the extracellular domains of the two proteins. B: cell shape change in M1 cells expressing FXYD4 (WT), FXYD5, FXYD4 shRNA (shFXYD4), and the two FXYD4/FXYD5 chimera illustrated in A. In each case the percentage of remaining radial cells was determined 20 h after plating. Values are means \(\pm\) SE of 3 experiments with the total number of cells counted in 4–6 fields is given in brackets. *\(P < 0.001\) compared with WT cells. C: transepithelial \(I_{\text{sc}}\) was measured in the above cell clones grown till confluency on permeable supports as described under MATERIALS AND METHODS. Values are means \(\pm\) SE of 4 experiments. *\(P < 0.0005\) compared with WT cells.
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FXYD4 sequence, had no effect on the rate of cell elongation. Exchanging the intracellular or extracellular domains of the two proteins had no effect as well. Figure 8B compares the expression levels of four of the tested constructs that express an extracellular HA epitope. RCR, which does not inhibit cell polarization, is expressed at a similar level as FXYD5, whereas RRC and RCC were expressed at the same level even though only RRC inhibits cell polarization. Thus lack of inhibition of cell polarization by RCR and RCC are not likely to reflect different levels of expression of the transfected proteins.

Structure-function relationships were further characterized by mutating specific FXYD5 transmembrane residues to the corresponding FXYD4 residues. In particular, two point mutations were found to affect cell polarization. These were A150G, which inhibited the effect of FXYD5 on cell elongation, and R145G, which enhanced it (Fig. 9, left). Coimmunoprecipitation of α with FXYD5 using HA-tagged FXYD5 constructs showed similar effects of these mutations on association of the two proteins (Fig. 9, right). Namely, the mutation A150G immunoprecipitated less α than FXYD5 and the α-to-FXYD5 ratio in the immunopellet was 21% of that obtained for nonmutated FXYD5. The mutation R145G on the other hand, increased association, and the immunopellet α-to-R145G ratio was 60% higher than that of the nonmutated FXYD5. These findings demonstrate a correlation between the ability of FXYD5 to inhibit cell elongation and flattening and to immunoprecipitate α and therefore support the notion that the effect of FXYD5 on cell polarization is mediated by its interaction with the Na+/K+-ATPase.

DISCUSSION

FXYD5 was reported to be a cancer-associated protein involved in the modulation of cell motility (12, 15, 28). Other studies have demonstrated that, as with all members of the FXYD family, FXYD5 interacts with the Na+/K+-ATPase and modulates its kinetic properties (17–19). Yet unclear are the possible additional effects of this interaction, and whether changes in cell adhesion and motility are secondary to the interaction between FXYD5 and the Na+/K+-ATPase or are a Na+/K+-ATPase independent function of this FXYD protein.

One manifestation of the cancer-related properties of FXYD5 are changes in cell morphology and cytoskeletal organization (12, 15, 28). In the current study this is also reflected as the reduced rate of transformation from initial radial shape to an elongated one with anterior-posterior polarity. We have demonstrated this effect in four different cell lines. Two were derived from normal epithelia that were stably or transiently transfected by FXYD5. The other two were derived from human tumors that endogenously expressed FXYD5 and were silenced by stable transfection with appropriate shRNA. The fact that a similar inhibition of cell shape changes by FXYD5 was apparent in all four cell lines indicates the generality of this phenomenon.

Next, we have analyzed the structure-function relationships of FXYD5 in slowing shape change. Since FXYD4, a topologically similar and homologous protein, does not affect cell polarization, we addressed this issue by analyzing effects of various FXYD4/FXYD5 chimera as well as point mutation of FXYD5 residues to the corresponding FXYD4 residues. Surprisingly, it was found that the unique extracellular domain of FXYD5 does not mediate the effect on cell flattening and elongation. It could be replaced by that of FXYD4 without affecting the above behavior nor the impairment of tight junctions. On the other hand, the transmembrane domain was found to be essential for inhibiting shape changes of the adhered cells. Replacing it with the corresponding FXYD4 domain fully blocked the response to FXYD5, whereas inserting FXYD5 transmembrane domain in FXYD4 mimicked the reduced elongation rate typical to FXYD5. Further specific transmembrane mutations have identified two point mutations that affect both the anterior-posterior polarity and association with the Na+/K+-ATPase in the same way. One is A150G, which decreased both parameters, and the other R145G, which enhanced them. A high resolution crystal structure of the shark rectal gland, Na+/K+-ATPase and its associated FXYD protein (FXYD10) has been reported by Shinoda et al. (29). This structure predicts that A150 (A27 in FXYD10) is located in the vicinity of A963 A966 and F967 of Na+/K+-ATPase independent function of this FXYD protein.
a major effect on the position of FXYD5 within the plasma membrane.

The similarity between the domain and residues participating in the high-affinity interactions of FXYD5 with the Na\(^{+}\)-K\(^{+}\)-ATPase and its effect on cell shape change lend support to the notion that FXYD5 mediates this effect through its association with the Na\(^{+}\)-K\(^{+}\)-ATPase. Current data do not exclude a role of the extracellular and/or intracellular segments of FXYD5. However, if such role(s) exist, they are not unique to FXYD5 and can be mimicked by the similar domains in FXYD4.

Several mechanisms by which an interaction between FXYD5 and the Na\(^{+}\)-K\(^{+}\)-ATPase can affect cell shape may be considered. One possibility is that this response is secondary to the previously reported increased pumping rate (17–19). Such an increase will lower intracellular Na\(^{+}\) activity, increase membrane potential, alkalize the cytoplasm (by elevating H\(^{+}\) extrusion through the Na\(^{+}\)/H\(^{+}\) exchanger), and decrease cell Ca\(^{2+}\) (due to increased Na\(^{+}\)/Ca\(^{2+}\) exchange). Any of these may in principle give rise to a change in cytoskeletal organization (4, 26). We assessed this possibility by examining effects of amiloride and ouabain. While both treatments inhibit Na\(^{+}\) pumping, their effects on the Na\(^{+}\) gradient and membrane potential are opposite. Partial inhibition of the Na\(^{+}\)-K\(^{+}\)-ATPase with ouabain will dissipate the Na\(^{+}\) gradient and lower the membrane potential. On the other hand, passive Na\(^{+}\) inflow by amiloride will lower cell Na\(^{+}\) and elevate the membrane potential. The fact that the two treatments neither mimicked nor abolished the effect of FXYD5 argues against the possibility that the above effects are mediated by a change in Na\(^{+}\)-K\(^{+}\)-ATPase pumping rate and the subsequent changes in intracellular ion activities or membrane potential.

Several other functions of the Na\(^{+}\)-K\(^{+}\)-ATPase, which do not involve translocation of ions across the cell membrane that may account for the above data, have been reported. Involvement of the Na\(^{+}\)-K\(^{+}\)-ATPase in actin polarization and cell motility has been suggested by Rajasekaran and co-workers (3, 23, 24). The mechanism suggested involves association of phosphatidylinositol 3-kinase to the cytosolic tail of α Na\(^{+}\)-K\(^{+}\)-ATPase and the subsequent activation of Rac1. Independently, β Na\(^{+}\)-K\(^{+}\)-ATPase and its N-glycosylation level were reported to play a role in cell adhesion and cell-cell contact (33, 35, 36). This is further supported by the finding that the three-dimensional structure of the extracellular domain of β resembles a cell adhesion molecule (2). Finally, a number of signaling cascades initiated by the binding of cardiac glycosides to the Na\(^{+}\)-K\(^{+}\)-ATPase have been suggested (22, 37). Thus several different mechanisms may relate the Na\(^{+}\)-K\(^{+}\)-ATPase and its associated proteins to cell polarization. The involvement of any of these in the response to FXYD5 awaits further studies. Finally, it is also possible that despite the above correlations between interaction with Na\(^{+}\)-K\(^{+}\)-ATPase and shape changes, FXYD5 mediates its morphological effects through interaction with other yet unknown proteins.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

Author contributions: I.L. and H.G. conception and design of research; I.L. and C.A. performed experiments; I.L., C.A., and H.G. analyzed data; I.L. and
H.G. interpreted results of experiments; I.L., C.A., and H.G. approved final version of manuscript; H.G. prepared figures; H.G. drafted manuscript; H.G. edited and revised manuscript.

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