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S163 is critical for FXYD5 modulation of wound healing in airway epithelial cells

Timothy J. Miller, PhD^{1,2} and Pamela B. Davis, MD, PhD²

¹ Department of Pharmacology, School of Medicine, Case Western Reserve University, Cleveland, Ohio

² Department of Pediatrics, School of Medicine, Case Western Reserve University, Cleveland, Ohio

Abstract

The FXYD family, which contains seven members, are tissue specific regulators of the Na,K-ATPase. Increased expression of FXYD5, a cancer–cell-associated membrane glycoprotein, has been associated with increased cell motility and metastatic potential. To better understand how FXYD5 may modulate cell motility, we analyzed S163, a conserved residue in all FXYD family members located in the C-terminus. Ectopic expression of human FXYD5 S163 mutants in HEK 293 cells showed that negative charge at S163 (S163D) decreased membrane localization, assessed by immunofluorescence. Coimmunoprecipitation studies revealed decreased FXYD5/Na,K-ATPase interaction for S163D compared with wild-type or S163A mutants. Interestingly, FXYD5 overexpression induced expression of vimentin, a marker of epithelial–mesenchymal transition, in murine airway epithelial cells. Because Na,K-ATPase expression is decreased in some forms of cancer and is critical for establishing cell polarity and suppressing cell motility, we analyzed S163 mutants in an epithelial cell scratch-wound model as a measure of cell migration. Wild-type FXYD5 overexpression increased reepithelialization ($p < 0.0001$), which was further increased in S163D mutants ($p < 0.005$). However, S163A mutants inhibited epithelial cell migration compared with wild-type FXYD5 overexpression ($p < 0.0001$). We conclude that negative charge at S163 regulates FXYD5/Na,K-ATPase interaction and that this interaction modulates cell migration across a wound in airway epithelial cells.

The recurrent remodeling of pulmonary epithelium as a result of exposure to environmental stress, viruses, and bacteria requires that airway epithelial cells migrate to wound sites and then polarize in order to maintain epithelial integrity. The requirement to heal lesions in the airway epithelium caused by infection and inflammation might logically result in expression and activation of proteins associated with cell motility and adhesion. While numerous factors are involved in the initiation of the healing process, depolarization of the epithelial

Reprint requests: Timothy J. Miller, PhD, Department of Pediatrics, BRB Rm. 801, Case Western Reserve University, 2109 Adelbert Road, Cleveland, OH 44106. Tel: +216 368 3631; Fax: +216 368 4223; txm20@case.edu.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Data S1: Primers

Data S2: 1BRDU staining of LA4 transfected cells.

Figure S2. Transfection of LA4 airway epithelial cells with FXYD5 does not alter cell proliferation rates. (A) Number of green staining (positive) cells for BRDU incorporation is not significantly different between cells transfected with vector, wt FXYD5 or FXYD5 S163A/D mutations 12 hours post-wounding. The total number of positive cells was counted per field under $\times 10$ objective lens. (B) Vector control BRDU stained LA4 cells under $\times 10$ objective; scale bar = 200 μ m.

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cells along the edge of the wound constitutes an intermediate step in the reorganization of actin characteristically observed during wound healing.¹ This suggests that the activity of ion channels such as the epithelial sodium channel (ENaC) and the Na,K-ATPase may modulate the efficiency of wound repair.

While the primary function of the Na,K-ATPase, located on the basolateral surface of most epithelia, is to exchange three intracellular sodium ions for two extracellular potassium ions, the Na,K-ATPase may also propagate external stimuli within the cell.^{2,3} In particular, signals derived from the β -subunit of the Na,K-ATPase are essential for the development of epithelial cell polarity and suppression of cell motility.^{4–7} The Na,K-ATPase is regulated by members of the FXYD protein family, small type-1 transmembrane proteins characterized by a signature 35-residue domain containing an invariant, extracellular PFXDY sequence.⁸ Currently, the role of FXYD proteins in the regulation of Na,K-ATPase signal transduction and the effect of this association on cell motility and wound repair is unknown.

Recently, members of the FXYD family have been identified as potential markers of tumorigenesis. In particular, increased expression of FXYD5, also known as Dysadherin, has been correlated with increased tumor progression and invasiveness.^{9–11} Knockdown of FXYD5 expression has correlated with decreased cell motility, whereas transfection of FXYD5 into liver cells led to decreased cell–cell adhesion, increased cell motility and diminished expression of E-cadherin.^{10,12} Overexpression of FXYD5 also increased cortical F-actin and membrane filopodia, two prerequisites for wound closure,^{10,12} and implies that FXYD5 may be a critical determinant regulating the role of the Na,K-ATPase in cell adherence and motility. Previous reports have shown that FXYD5 is expressed in the basal layer of squamous epithelia and has been shown to be upregulated in cystic fibrosis airway epithelia.^{8,13} Therefore, we investigated how a conserved serine residue affects FXYD5/Na,K-ATPase association and how this altered cell motility in an in vitro model of airway epithelial cell migration.

MATERIALS AND METHODS

Cell lines

The mouse lung epithelial cell line LA4 and human embryonic kidney (HEK) 293 cells were obtained from the American Type Culture Collection (ATCC, Manassas, VA). LA4 cells (ATCC #CCL-196), originally isolated from a mouse lung adenoma, were grown in Kaighn's modification of F12 medium (F12K, Mediatech Inc., Herndon, VA) and HEK 293 cells were grown Earle's modification of MEM media (Mediatech Inc.). All media were supplemented with 10% heat-inactivated FBS.

Reverse-transcriptase polymerase chain reaction (RT-PCR), cloning and site-directed mutagenesis of FXYD5

FXYD5 cDNA was isolated by RT-PCR using the Superscript II One-Step RT-PCR kit (Invitrogen, Carlsbad, CA) and primers designed from accession numbers NM014164 (human) and NM008761 (mouse), which contained a *Hind*III and *Not*I restriction site on the 5' and 3' end, respectively (see supporting information Data S1). The following RT-PCR conditions were used: reactions were incubated at 50 °C for 30 minutes, followed by 2 minute initial denaturation at 95 °C and 40 cycles of 94 °C, 1 minute, denaturation, 1 minute. Fifty-eight percent primer annealing, 45 seconds of primer extension. RT-PCR products were digested with *Hind*III and *Not*I restriction enzymes and agarose gel purified using the Qiaquick gel purification kit (Qiagen Inc., Valencia, CA). cDNAs were subcloned in pBSK2 vector to create pBhF5k (human) and pBmF5k (murine) and an N-terminus Flag tag inserted into human FXYD5 as previously described.¹³ To create a C-terminal Flag-

tagged FXYD5, pBhF5k was digested with *NotI* and *TfiI*, agarose gel purified and used to ligate an in-frame Flag-tag (see supporting information Data 1). Similarly, a C-terminus Flag-tag was inserted into murine FXYD5 to create pBmF5kFlag. The original and modified versions of FXYD5 were then subcloned into the previously described pKCERegfpSV expression vector¹⁴ using *HindIII/NotI* to create pKCERhF5kFlag, pKCERhF5kQ22Flag and pK CERmF5k. The Quickchange site-directed mutagenesis kit was used to introduce alanine or aspartic acid at serines 163 to create pKCERhF5kS163A, pKCERhF5kQ22FlagS163A, or pKCERhF5kS163D, pKCERhF5kQ22FlagS163D, pKCERmF5kS163D, respectively.

Transfection of LA4 and HEK 293 cells

In order to facilitate detection of FXYD5, which is difficult in untransfected cells, a transfected cell model was used. Cells were plated in 10 cm tissue cultures plates (Co-star Inc., Cambridge, MA) at 75% confluency. After 24 hours, medium was changed to serum-free Optimem (Invitrogen) and cells were transfected with 50 μ L Lipofectamine 2000 (Invitrogen) and 20 μ g plasmid DNA per plate. Lipid-DNA complexes were incubated 4–5 hours on cell monolayers at 37 °C/5% CO₂ after which transfection medium was replaced with complete medium. Cells were then cultured for an additional 24–36 hours and then assayed. Transfection efficiency was generally > 80% in both LA4 and HEK 293 cells, assessed by green fluorescent protein (GFP) immunofluorescence.

Isolation of crude membranes

Crude membranes were prepared as previously described.¹⁵ The pellets were resuspended in 25mM imidazole, 1mM ethylenediaminetetraacetic acid (EDTA), 10mM RbCl, and stored at 4 °C.

Immunoprecipitation and immunoblot analysis

Crude membrane protein (125 μ g) from HEK 293 cells was resuspended in 10mM RbCl and 200 μ M ouabain and solubilized as previously described.¹⁵ Aliquots of solubilized membrane were incubated with 1 μ g of antibody raised against the α_1 subunit of the Na,K-ATPase (Upstate Bio-tech Inc., Charlottesville, VA), 1 μ gM2 Flag antibody or a nonspecific control antibody (NF- κ B, Santa Cruz Biotechnology Inc., Santa Cruz, CA) at 4 °C for 3 hours. Fifty microliters of Protein G agarose beads was added and incubated overnight on a rocking platform at 4 °C. The beads were washed two times with 25mM imidazole, 1mM EDTA, 100mM RbCl, 200 μ M, and 0.2 mg/mL C₁₂E₁₀, resuspended in 50 μ L Laemmli buffer, incubated at 37 °C for 20 minutes and loaded onto 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After semidry transfer to nitrocellulose, proteins were blocked with 1% bovine serum albumin (BSA), the immunoblots cut into sections and incubated with antibodies raised against either mouse anti- α_1 subunit of the Na,K-ATPase (1 μ g/mL), anti-M2 Flag tag (1 mg/mL) (Sigma, St. Louis, MO), anti-vimentin (Santa Cruz), anti-RhoA (26C4, Santa Cruz) or anti-FXYD5 562 antibody¹³ in 1% BSA/Tris-buffered saline for 1 hour at room temperature. The blot was then incubated with species specific secondary antibodies (1: 4,000) (Jackson Laboratory, Bar Harbor, ME) conjugated to HRP and visualized using Pierce's Supersignal Pico West detection kit.

Indirect immunofluorescence

HEK 293 or LA4 cells were cultured in four-well chamber slides (Permanox, Electron Microscopy Sciences, Hatfield, PA) to 80% confluence and transfected with 4.5 μ L Lipofectamine 2000 and 1.5 μ g DNA per well. At 24–36 hours posttransfection, monolayers were washed once with phosphate buffered saline (PBS), fixed with 2% paraformaldehyde/PBS (293) or methanol (LA4) for 10 minutes and washed with PBS twice. Nonspecific

antibody binding was blocked by addition of 4% bovine serum albumin (Invitrogen) for 45 minutes. Primary anti-M2 Flag mouse antibody (2 mg/mL) was diluted in 1% BSA/PBS, incubated on monolayers for 45 minutes and aspirated. Two hundred and ninety-three cells were washed twice with PBS and then incubated in 1% BSA/PBS containing goat antimouse or goat anti-rabbit IgG Alexa-Fluor 568 (red) (1: 250, Invitrogen) for 45 minutes. LA4 cells were stained with anti-Flag antibody coupled to anti-rabbit Alexa-fluor 488 (green) and actin-phalloidin (red). Cells were washed twice, the nuclei counterstained with Hoechst (Invitrogen), mounted with Fluormount-G, and allowed to dry over-night.

Immunofluorescent localization was assessed on a Zeiss 200M Axiovert inverted microscope (Carl Zeiss Microimaging Inc., Thornwood, NY), with a DG4 switchable fluorescent light source (Sutter Instrument Company, Novato, CA) and a 12-bit CoolSnap HQ camera (Roper Scientific, Tucson, AZ) under control of MetaMorph v 6.2 (Molecular Devices, Sunnyvale, CA). Images were obtained with a $\times 10$, $\times 63$, or $\times 100$ numerical aperture 1.3 fluar lens using excitation and emission filter passbands of 260 ± 20 and 645 ± 30 nm, respectively. Typical exposure times for individual frames were 200 ms.

Scratch-wound assay

Mouse LA4 airway epithelial cells were seeded at 80% confluency in 12-well plates (Costar Inc.) and transiently transfected as described above. Transfection efficiency was assessed using GFP fluorescence of parallel-transfected wells (Figure 4A). Twenty-four hours posttransfection, wells were scraped with a 200 μ L yellow pipette tip and the wound was imaged under $\times 10$ objective lens. Each well was wounded three times, and the wound area was measured at six points along each wound to establish baseline values using MetaMorph v 6.2 (Molecular Devices). Sixteen hours later, the wounds were measured again. Data were expressed as the percentage of the gap that had been closed during that period. Graphpad Prism (Graphpad Software Inc.) was used to calculate ANOVA statistics using the Student–Neuman–Kuels regression analysis for pairwise comparisons. Error bars represent standard error of the mean (SEM).

RESULTS

Mutations in Ser163 alter FXYD5 cellular localization

We inserted a Flag-tag into the N- or C-terminus of human or mouse FXYD5 and transiently transfected HEK 293 or LA4 cells (Figure 1). Immunoblot analysis showed expression of all constructs; however, the N-terminal Flag-tagged human FXYD5 appears to be more efficiently translated and detected in the membrane preparations of HEK 293 cells (Figure 1, lane 3) compared with the C-terminal construct (Figure 1, lane 2). Although location of the Flag tag did not affect the apparent molecular weight of either species (human, 35 kDa; mouse, 25 kDa), the C-terminus Flag tag is inefficient for immunofluorescent detection in vivo (data not shown), as others have shown.¹⁵ Interestingly, the appearance of multiple distinct bands in membrane preparations of the N-terminal Q22-Flag construct suggest the formation of detergent resistant multimers, similar to those observed with other FXYD family members.^{16,17} The Q22Flag construct, however, is efficiently detected in the membrane of HEK 293 cells (Figure 2). Similar results were observed in LA4 cells (data not shown). Therefore, N-terminal Q22Flag-tagged human FXYD5 was used to analyze S163 mutations in human cells.

In HEK 293 cells transiently transfected with pKCERhF5kQ22Flag, there is clearly a stronger localization of FXYD5 at homotypic cell borders suggesting that FXYD5 may have a role in cell–cell adhesion (Figure 2C and D). S163 is conserved across all human FXYD family members, including FXYD5, the most divergent FXYD family member. We mutated S163 to alanine or aspartic acid to either remove a potential phosphorylation site or simulate

addition of negative charge, respectively, and transiently transfected HEK 293 cells with N-terminal Flag-tagged FXYD5 vectors (Figure 2). Anti-Flag antibodies show indirect immunofluorescence staining of transfected Flag-tagged wild-type and S163A mutants localized to the cell membrane (Figure 2C–F). However, HEK 293 cells transfected with Q22Flag-tagged FXYD5 containing the S163D mutation show less FXYD5 at the cell membrane and increased intracellular FXYD5 staining (Figure 2G and H). These data suggest that negative charge at S163 regulates FXYD5 membrane insertion and suggest a possible regulatory mechanism for FXYD5/Na,K-ATPase interaction that may lead to altered cell motility.

S163 mutations alter FXYD5/Na,K-ATPase interaction

Members of the FXYD family, including FXYD5, are reported to interact with the Na,K-ATPase. For FXYD5, this offers a mechanism by which FXYD5 can regulate cell motility and adhesion, because the Na,K-ATPase is implicated in these processes. Because S163D mutations alter the cellular localization of FXYD5, we sought to determine if S163 mutations affect FXYD5/Na,K-ATPase interaction by coimmunoprecipitation analysis in HEK 293 cells. Human Q22Flag-FXYD5/Na,K-ATPase complexes were solubilized in the nonionic detergent C₁₂E₁₀, immunoprecipitated with either anti- α_1 Na,K-ATPase or anti-Flag antibodies and identified by Western blot analysis. As previously observed, FXYD5 interacts with the α subunit, although the efficiency of coimmunoprecipitation of the tagged FXYD5 may be less due to the presence of other FXYD proteins in HEK 293 cells (Figure 3A),^{15,18} which can also interact with the α subunit. Serine to alanine mutations did not alter interaction with the Na,K-ATPase. However, replacing S163 with a negatively charged amino acid such as aspartic acid appeared to inhibit the FXYD5/Na,K-ATPase interaction (Figure 3A and B). We observed decreased total S163D protein in crude membrane preparations, consistent with our observations with indirect immunofluorescence (Figure 3B). Therefore, FXYD5 interacts with Na,K-ATPase and negative charge at S163 can disrupt this interaction.

FXYD5 expression induces vimentin expression without altering RhoA or Na,K-ATPase expression in airway epithelial cells

It was recently shown that FXYD5 expression is increased in cystic fibrosis airway epithelia and modulates Na,K-ATPase activity.¹³ We were able to achieve > 80% transfection efficiency in LA4 murine airway epithelial cells, assessed by GFP fluorescence (Figure 4A), and immunofluorescent localization of Flag-tagged FXYD5 showed that FXYD5 localized to the tips of lamellipodia (Figure 4B). Taken together with data demonstrating that FXYD5 expression correlates with metastatic potential, this suggested that FXYD5/Na,K-ATPase association, in addition to modulating ion transport activity, may alter the expression of proteins involved in cell motility via a secondary signaling function. We found that expression of the Na,K-ATPase and RhoA, two proteins involved in cell motility, was unchanged after FXYD5 overexpression. Similar to our results in HEK 293 cells, we found that mutating S163 to aspartic acid decreased localization in membranes prepared from LA4 cells. Interestingly, we determined that increased FXYD5 expression induced the expression of vimentin, which is commonly associated with an epithelial–mesenchymal transition, independent of S163 mutation (Figure 4C).^{19,20}

S163 modulates cell migration in a scratch-wound assay of murine airway epithelial cells

The induction of vimentin expression, independent of S163 mutation, suggested that increased expression of FXYD5 may stimulate cell motility in airway epithelial cells. We tested whether FXYD5 affected epithelial cell migration in a scratch-wound model of cell motility by transfecting LA4 murine airway epithelial cells with wildtype FXYD5, S163 mutant or control vectors (Figure 5A). Overexpression of wild-type FXYD5 clearly

increased reepithelialization of the cell monolayer compared with control (Figure 5B; $p < 0.001$). Nevertheless, S163A mutants inhibited cell migration whereas S163D mutants accelerated it (Figure 5B; $p < 0.05$). Cell migration results were not due to altered cell proliferation rates as measured by BRDU staining (supporting information Data S2). These data support previous findings that increased expression of FXYD5 increases cell motility, and indicate that negative charge (mimicking phosphorylation) at S163 may increase cell motility through altered FXYD5/Na,K-ATPase interaction. Furthermore, our data suggest that increased FXYD5 expression modulates cell motility downstream of vimentin expression.

DISCUSSION

Although a primary function of the Na,K-ATPase is to maintain the monovalent cation balance between the interior and the exterior of the cell, it is also a critical participant in establishing polarity of epithelial cells and in cell motility. Rajasekaran and colleagues^{7,21–24} have shown that the Na,K-ATPase β -subunit is required for mediating E-cadherin-dependent cell–cell adhesion and is able to suppress invasion of carcinoma cells, suggesting that the Na,K-ATPase has a role in cell physiology in addition to its function as an ion pump. FXYD5 has been reported to have an inverse relationship with E-cadherin and its over-expression in cancer cells reduces E-cadherin at the cell surface and promotes metastasis. The mechanism by which this effect is mediated may include regulation of the Na,K-ATPase pump number or activity. In this study, we found that FXYD5 coimmunoprecipitates with the α subunit of the Na,K-ATPase, and in addition, that FLAG-FXYD5 appears in the basolateral membranes of epithelial cells, the location of the Na,K-ATPase.

Such an association may be expected. FXYD5 is a member of a small family of tissue specific regulatory subunits of the Na,K-ATPase. Found primarily in the lung, kidney, lymphocytes, and squamous epithelia, FXYD5 has been shown to interact with the Na,K-ATPase and increase maximal pump activity.¹³ Although quantity of FXYD5 is certainly important, as showed by transfection studies, it may be that its functional state is equally relevant for the overall effect of FXYD5. Functional interactions between the α/β Na,K-ATPase complex and FXYD1 (PLM), FXYD2 (γ), FXYD3 (Mat-8), FXYD4 (CHIF), FXYD5 (RIC), and FXYD7 have been shown.^{25–29} There is good evidence that the FXYD proteins lay within a groove created by the M2, M4, M6, and M9 helices of the α -subunit and that multiple α/β /FXYD contact sites occur.^{30–34} Structural studies have shown for other members of the family, though not specifically for FXYD5, that extracellular segments mediate the effect of FXYD proteins on the apparent ATP affinity, while the transmembrane domain regulates the effects of FXYD on the Na,K-ATPase cation affinities.^{32,35} A recent study confirmed that conserved residues within the transmembrane domain of FXYD5 regulate α/β structural interactions.³⁶ We focused on serine 163, which is conserved across all family members and is predicted to lie just intracellular to the transmembrane domain.⁸ It has been reported that FXYD2 can be phosphorylated by PKC at this conserved serine.³⁰ Mutation of S163 to alanine had no effect on transfected FXYD5 membrane localization when compared with wild-type transfected cells, but FXYD5 S163D transfected cells showed decreased membrane localization of FXYD5-Flag. It has been suggested that phosphorylation of some FXYD proteins, such as FXYD1, FXYD2, FXYD7, and FXYD10, regulates interaction with the Na,K-ATPase and that FXYD phosphorylation maximizes pump activity, much like the interaction of phospholamban with the sarcoplasmic reticulum Ca-ATPase (SERCA).³⁷ It is currently unclear whether phosphorylation of FXYD5 similarly relieves Na,K-ATPase enzyme inhibition. However, we observed that the S163A mutant coimmunoprecipitated with the α -subunit, whereas the S163D mutant displayed reduced association, suggesting that S163 phosphorylation may inhibit FXYD5/ α subunit

interaction. Our studies are in line with previous structural data on FXYP7 that indicate S163 is located along the face of the FXYP5 transmembrane domain that contacts the M2, M4, M6, and M9 pocket within the α subunit. We now propose that, similar to FXYP2, phosphorylation at S163 may induce decoupling of FXYP5 from the α/β complex.

In support of this proposal, we observed that the S163D mutation alters the association of FXYP5 with the Na,K-ATPase, which is required for epithelial polarization and suppression of cell motility. Interestingly, we found that overexpression of FXYP5, independent of S163 mutation, increased vimentin expression in membrane preparations of FXYP5 transfected airway epithelial cells. Inhibition of vimentin expression has been shown to delay wound repair in vimentin knockout mice and others have shown that vimentin expression is induced in cells at the edge of a wound.^{38,39} Our data suggest that FXYP5 may operate downstream of vimentin to regulate wound healing. Thus, we sought to determine if FXYP5 S163 mutations affected cell motility in an airway epithelial cell wound model. As might be predicted from studies with ectopically expressed FXYP5, overexpression of wild-type FXYP5 increased epithelial cell migration in murine airway cells after 16 hours in a scratch-wound model. Interestingly, S163A mutants reduced reepithelialization of the wound, suggesting that S163A mutants, which retain the ability to interact with the Na,K-ATPase in pulldown assay, may be a “dominant negative” mutation able to lock FXYP5 into a conformation able to associate with the Na,K-ATPase in the cell membrane but which prevents downstream signaling necessary for increased motility. Conversely, the S163D mutant, which does not interact strongly with the α subunit and does not clearly localize to the membrane, increased cell migration across the wound, indicating that disruption of FXYP5/Na,K-ATPase interaction may promote signals leading to increased cell motility. Although there is no consensus phosphorylation sequences surrounding S163, and it has not yet been shown to be phosphorylated *in vivo*, we speculate that phosphorylation, or addition of negative charge at S163, disrupts the interaction of FXYP5 and the Na,K-ATPase at the surface of the cell, which promotes cell motility and may facilitate wound closure. Conversely, prevention of such phosphorylation locks the enzyme at the membrane in a state nonfunctional with respect to motility. Previous authors have demonstrated that in two-dimensional models of cell wounding, cell migration is the first step in repairing the epithelial monolayer. Because decreased expression of α/β Na,K-ATPase subunits is also associated with epithelial–mesenchymal transition typically leading to more mobile, metastatic cancer cells, and overexpression of FXYP5 has recently been shown to down-regulate α/β expression oocytes, S163 phosphorylation may be a mechanism for increasing cell motility. In conclusion, we now show that FXYP5 modulates airway epithelial cell motility and we speculate that phosphorylation at S163 may have a profound effect on the ability of FXYP5 to interact with the Na,K-ATPase and regulate wound repair.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Immunoblot of FXYD5-Flag in HEK 293 or LA4 cells. Immunoblot of crude membranes prepared from HEK 293 (10 μ g, lanes 1–3) or LA4 (50 μ g, lanes 4–5) cells transiently transfected with vector (lanes 1,4), pKCERhF5kFlag (lane 2), pKCERhF5kQ22Flag (lane 3), or pKCERmF5kFlag (lane 5) probed with anti-M2 Flag antibody (1 μ g/mL).

Figure 2.

Mutations in Ser163 alter FXDY5 membrane localization in HEK 293 cells. HEK 293 cells transfected with control vector (A, B), pKCERhF5kQ22Flag (C, D), pKCERhF5kQ22Flag-S163A (E, F) or pKCERhF5kQ22FlagS163D (G, H) were stained with anti-M2 Flag antibodies to detect the Flag-tag in the external N-terminus (red; all panels). Nuclei were costained using Hoechst dye (blue; panels A, C, E, G). Representative images show that S163A mutations do not affect FXDY5 membrane localization whereas S163D mutants exhibit decreased surface staining. Images taken with $\times 100$ objective lens; scale bar = 20 μm .

Figure 3.

Ser163 modulates interaction with Na,K-ATPase. Immune complexes were precipitated from crude membrane preparations of HEK 293 cells using antibodies against the α -subunit of the Na,K-ATPase (IP: α 1, 3A), against the M2-Flag epitope (IP:Flag, 3B), or a nonspecific control antibody (NF- κ B, IP:con). Five percent of total crude membrane input used for immunoprecipitation or immunopellet complexes were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The membranes were cut in half to detect α_1 Na,K-ATPase or FXYD5-Flag proteins and probed separately using either anti-Flag antibody directly conjugated to HRP (IB:FXYD5-Flag) or anti- α -subunit Na,K-ATPase antibody (IB: α 1-NKA).

Figure 4.

FXYD5 expression alters vimentin but not RhoA or Na,K-ATPase expression in LA4 airway cell membrane preparations. (A) Representative GFP fluorescent images of murine LA4 airway cells transfected with pKCEREGFP, demonstrating > 80% transfection efficiency. Images taken under $\times 63$ objective lens; scale bar=45 μm . (B) FXYD5-Flag localizes at lamellipodia (*arrows*) compared with negative control (*inset*); images taken under 100 $\times$ objective, scale bar=20 μm . (C) Immunoblot analysis of 25 μg crude membranes prepared from LA4 cells transfected with pKCERmF5k, pKCERmF5kS163A, pKCERmF5kS163D or vector control demonstrating α_1 -Na,K-ATPase and RhoA expression is unchanged, whereas vimentin is increased. Similar to HEK 293 cells, the S163D mutation alters membrane localization. Actin is shown as a loading control.

Figure 5.

FXD5 modulates cell migration in a scratch-wound model of murine airway epithelial cells. (A) Murine LA4 airway epithelial cells transfected with control vector, pKCERmF5k, pKCERmF5kS163A, or pKCERmF5kS163D immediately following or 16 hours postwounding. (B) Cell motility was significantly increased after transfection with wild-type FXD5 ($n = 18$, $*p < 0.001$, compared with control vector). S163A mutations inhibited wound closure ($n = 16$; $**p < 0.05$ vs control), whereas S163D mutations increased wound closure ($n = 18$; $***p < 0.05$ vs wt). Images taken under $\times 10$ objective lens; scale bar = 200 μm .