Myosin VI mediates the movement of NHE3 down the microvillus
in intestinal epithelial cells

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Summary

The intestinal brush border (BB) Na+/H+ exchanger, NHE3, is acutely regulated through changes in its endocytosis/exocytosis. Myosin VI, a minus-end directed motor, has been implicated in endocytosis at the inter-microvillar (MV) cleft and vesicle remodeling in the terminal web. We asked if myosin VI also regulates NHE3 movement down MV. Basal NHE3 activity and surface amount, determined by BCECF/fluorometry and biotinylation, respectively, were increased in myosin VI knock-down (KD) Caco-2/Bbe cells. Carbachol (CCH) and forskolin (FSK) stimulated NHE3 endocytosis in control but not in myosin VI KD cells. Importantly, immuno-EM results showed NHE3 preferentially localized in the basal half of MV in control but in the distal half of myosin VI KD cells. Dynasore duplicated some aspects of myosin VI KD: it increased basal surface NHE3 activity and prevented FSK-induced NHE3 endocytosis; but NHE3’s distribution along the MV was intermediate in dynasore-treated as compared to either myosin VI KD or untreated cells. We conclude that myosin VI is required for basal and stimulated endocytosis of NHE3 in intestinal cells and suggest that myosin VI also moves NHE3 down MV.

Key words: Na/H exchanger; Na absorption; endocytosis; trafficking; myosin VI; brush border
Introduction
Acute regulation of the activity of the intestinal brush border (BB) Na⁺/H⁺ exchanger, NHE3, during digestion contributes to intestinal water and Na⁺ homeostasis. This regulation occurs primarily by changes in plasma membrane NHE3 expression via modulation in the rates of its endocytosis and exocytosis. The great majority of NHE3 resides in the intestinal MV under basal conditions and its activity is down-regulated early in the post-prandial state by stimulated endocytosis. The steady-state distribution of NHE3 along the MV is not known, nor is the machinery regulating it under basal and stimulated conditions.

In membrane trafficking, motor proteins moving along cytoskeletal tracks play major roles in transporting cargo or vesicles between donor and acceptor compartments. There are three classes of mammalian motors: myosins, which move along actin filaments; and kinesins and dyneins, which use microtubules. Only myosin motors are relevant to membrane protein movements within/on MV, since each MV contains a core of cross-linked actin filaments but no microtubules. The myosin superfamily is made up of ~20 different classes, and the intestinal BB, which comprises the MV, inter-MV region and underlying terminal web, contains at least 14 myosin members (Hodge and Cope, 2000; Berg et al., 2001; McConnell RE et al., 2011). However, myosin VI is the only negatively-directed myosin isoform in this group capable of moving material from the MV to the inter-MV region (Wells AL et al. 1999).

Myosin VI has been suggested to take part in at least three distinct steps of apical endocytosis in polarized epithelial cells (reviewed by Hasson T., 2003). These include movement of cargo from the MV to the clathrin-coated pit area, clathrin-coated vesicle formation, and movement of uncoated vesicles through the actin-Arp2/3 complex-rich terminal web to early endosomes (reviewed by Hasson T., 2003). There is evidence supporting the second and third steps, but evidence for myosin VI-dependent movement of cargo from the MV to inter-MV region remains indirect (Gottlieb et al., 1993; Biemesderfer et al., 2002; Birn et al., 1997; Christensen and Nielsen, 1991; Aschenbrenner et al., 2003; Yang LE et al.,2005; Ameen N et al., 2007; Yang LE et al.,2008; Riquier AD et al.,2009; Riquier AD et al.,2010; Collaco A et al., 2010; Kravtsov DV et al., 2012). In this study, we provide direct evidence that myosin VI is required to move NHE3 along the MV length to the inter-MV region of intestinal epithelial cells and confirm its role in basal and stimulated endocytosis. Our approach was to knock down myosin VI in polarized Caco-2/Bbe
cells and assay the activity, protein abundance, and surface distribution of NHE3 using biochemical and morphological methods.

Results

Myosin VI is present in the MV of intestinal epithelial cells

The expression of myosin VI in the BB of intestinal epithelial cells is well-documented (Hegan et al., 2012, Buss et al., 2001 and Biemesdorfer et al., 2002). We confirmed myosin VI’s presence in BB of Caco-2 cells and mouse ileum and determined the extent to which it overlapped with villin, a BB marker in wt Caco-2/Bbe cells, and aquaporin7, a MV marker in mouse ileum. ~45% of myosin VI overlapped with BB villin in Caco-2/Bbe cells (Fig. 1A a1-a4 and Fig. 1B), illustrating that it is abundant throughout the BB. Myosin VI’s overlap with aquaporin, which is restricted to the MV membrane, was ~9% (Fig. 1A b1-b4 and Fig. 1B). There was no signal in the ileum of myosin VI KO mice (Fig. 1A d) or in the myosin VI KD Caco-2/Bbe cells (Fig. 1A f). These results demonstrate that myosin VI is present throughout the BB (including the MV) in both Caco-2/Bbe cells and mouse small intestine.

Knocking down myosin VI does not dramatically change Caco-2/Bbe cell morphology

To study the regulatory role of myosin VI in NHE3 localization and transport activity, we knocked down the protein in Caco-2/Bbe cells. Myosin VI expression was reduced ~90% by western blot (Fig. 2A), and not detected by immunofluorescence (Fig. 1A f) in myosin VI KD cells. Light microscopic images showed similar morphologies between control and KD cells (not shown), and transmission electron microscopic (TEM) studies confirmed this view (Fig. 2 B, C). It should be noted that individual MV in both control and KD cells were variable in length and surface density, as has been seen in other studies (Marzesco AM et al., 2009; G.Wilson, I.F. et al., 1990). Nonetheless, the MV lengths were comparable (Fig. 2D). The KD cells did not show an apparent lifting of the plasma membrane in the inter-MV region as reported for intestinal cells in vivo (Hegan, et al., 2012; Chen and Donowitz, unpublished observations). Finally, the localizations of tight junction proteins ZO-1 (supplementary material Fig. S1 a,b), occludin (supplementary material Fig. S1 c,d) and claudin-1(supplementary material Fig. S1 e,f) were similar in control and KD cells, as were the trans-epithelial resistances (Fig. 2E).
Knocking down myosin VI increased NHE3 activity and surface NHE3 amount

**NHE3 activity is increased.** To test the effect of knocking down myosin VI on NHE3 function, NHE3 basal and stimulated activities were measured by BCECF/fluorometry. Basal NHE3 activity was increased > 60% after myosin VI KD (Fig. 3A,B). It should be noted that NHE3 activity measured with a ratiometric fluorescent pH sensitive dye is not influenced by changes in cell surface area (Donowitz M et al., 1985). If myosin VI were necessary for NHE3 endocytosis, we would predict that myosin VI KD would greatly reduce the inhibition of NHE3 activity caused by CCH or FSK, both of which stimulate NHE3 endocytosis. The results shown in Fig. 3 confirm this prediction: while CCH and FSK treatment reduced NHE3 activity in control Caco-2/Bbe cells, neither had any effect on NHE3 activity in myosin VI KD cells (Fig. 3 C,D; P<0.05). We then asked whether myosin VI was necessary for the EGF stimulatory effect on NHE3 activity, which we had previously shown was due to an increase in surface NHE3 (Sarker R et al., 2011). Unlike the effects of CCH or FSK, EGF treatment resulted in similar increases in NHE3 activity in control and myosin VI KD cells (Fig. 3E). A summary of the role of myosin VI in NHE3 basal and acutely regulated activity is shown in Table 2. Together, these data indicate that myosin VI is necessary for setting basal NHE3 activity in the MV and for regulating the transporter’s endocytosis after exposure of cells to CCH or FSK. Importantly our results show that myosin VI is not required for exocytosis of NHE3 (Janecki et al., 2000; Li et al., 2001).

**NHE3 surface amount is increased.** We next asked whether myosin VI KD altered the surface expression of NHE3 protein. We compared several parameters of NHE3 expression in control and myosin VI KD cells: total NHE3 expression level by immuno-blot analysis and quantitative IF; and apical membrane NHE3 amounts by cell-surface biotinylation and quantitative immuno-blot analysis. There was a significant increase in total NHE3 expression in KD versus control cells (Fig. 4 A-D). We also measured the extent of overlap between NHE3 and fluorophore-labeled WGA, which was applied to fixed cells at 4°C prior to their permeabilization and staining for NHE3 (Fig. 4C). The extent of overlap between NHE3 and WGA, which represents MV NHE3, was increased in the myosin VI KD cells (Fig. 4E). Finally, we compared the amount of MV NHE3 in control and myosin VI KD cells using surface biotinylation after exposure to CCH or FSK. In control cells, surface NHE3 was significantly reduced after treatment with CCH or FSK (Fig. 5 A,C). However, myosin VI KD cells, which we already showed had increased amounts of NHE3 on the cell surface, showed no subsequent decrease in surface NHE3 upon exposure to
either CCH or FSK (Fig. 5 B,D). Again, these results suggest that myosin VI sets the amount of surface NHE3 expression and activity under basal conditions and conditions that stimulate endocytosis.

Myosin VI regulates the distribution of NHE3 along the MV length

Endocytosis of apical membrane proteins in polarized epithelial cells with BB, such as the intestine, can be separated into three steps: 1) Movement to and accumulation of MV membrane proteins in the inter-MV region; 2) internalization via clathrin-coated vesicles (also non-clathrin dependent processes) that form in the inter-MV region; and 3) movement of vesicles through the actin-rich terminal web to early endosomal compartments (Aschenbrenner et al., 2003; Hasson, 2003). Evidence for myosin VI’s involvement in the second and third steps has been reported by others (Gottlieb et al., 1993; Biemesderfer et al., 2002; Birn et al., 1997; Christensen and Nielsen, 1991; Aschenbrenner et al., 2003; Yang LE et al., 2005; Ameen N et al., 2007; Yang LE et al., 2008; Riquier AD et al., 2009; Riquier AD et al., 2010; Collaco A et al., 2010; Kravtsov DV et al., 2012), and our results described so far clearly support its requirement for both basal and agonist-stimulated endocytosis of NHE3 in Caco-2/Bbe cells. However, NHE3’s localization along the MV in control and myosin VI KD cells has not been determined; consequently, we used immuno-EM to address this question. After para-formaldehyde fixation of filter-grown control and myosin VI KD cells, surface HA-NHE3 was labeled first with anti-HA then gold-labeled secondary antibodies, after which the cells were fixed with glutaraldehyde before being processed for EM.

As shown in Fig. 6, ~85% of the MV NHE3 in control cells was located in the first 600 nm of MV length and the rest >600 nm from the MV base (Fig. 6; Table 3). In contrast, less than 50% of the MV NHE3 in myosin VI KD cells was located in the first 600 nm and >50% were >600 nm from the MV base (Fig. 6; Table 3). This shift in NHE3 distribution along the MV length is significant and suggests that the increase in NHE3 surface activity we consistently detected in myosin VI KD cells (Fig. 3A-E) could be represented by the NHE3 present in the outer MV half (see Discussion).

Finally, we used dynasore to determine if the effect of myosin VI KD on basal NHE3 localization was duplicated by blocking only endocytosis at the level of the inter-MV region. Dynasore is a potent inhibitor of dynamin, a large GTPase that mediates scission of clathrin-coated pits and consequent formation of clathrin-coated vesicles (Macia E et al., 2006). We treated control and myosin VI KD cells with or without 80 µM dynasore for 30 min and then assayed NHE3 activity
using fluorometry. As shown in Fig. 7A, the NHE3 activity was increased by dynasore treatment in control cells, but not in myosin VI KD cells, which already exhibited increased NHE3 activity. Importantly, treatment of myosin VI KD cells with dynasore did not show an additive effect, indicating that the change in NHE3 activity with dynasore treatment and KD of myosin VI were both due to an increase in surface NHE3 (supplementary material Fig. 2A-C). These results are consistent with dynasore and myosin VI acting in the same pathway under basal conditions, but not necessarily at the same steps. In further studies, the effect of dynasore on FSK inhibition of NHE3 was determined. As with myosin VI KD, FSK’s inhibition of NHE3 was blocked by dynasore in control cells (Fig. 8).

Since dynasore and myosin VI KD had similar effects on NHE3 activity, we asked whether they caused similar changes in the NHE3 distribution along MV. Again immuno-EM was used. As shown in Fig. 7B and Table 4, dynasore treatment of control cells resulted in more NHE3 located >600nm from the MV base than that in untreated control cells (~45% compared to <20%, respectively; \( P<0.001 \)). However, untreated myosin VI KD cells still showed more NHE3 in the outer half of MV than did control cells treated with dynasore (Fig. 7, ~63% compared to ~45%, respectively; \( P<0.001 \)). Finally, dynasore treatment of myosin VI KD cells had no further effect on the MV distribution of NHE3 than myosin VI KD alone (Fig. 7B, Table 4). A diagram illustrates the different distributions of NHE3 under the four conditions (Fig. 7C). Since the mechanism of dynasore’s action is thought to involve only the inter-MV region, the difference in NHE3’s MV distributions in myosin VI KD cells versus control cells treated acutely with dynasore suggests that myosin VI KD results in the loss of MV NHE3’s tip-to-base MV movement.

**Effect of myosin VI KD and dynasore on NHE3 BB mobility**

FRAP was performed on NHE3 in the apical domain of Caco-2/Bbe cells to determine if dynasore and myosin VI KD affected NHE3 differently. Compared to relatively immobile NHE3 under basal conditions, NHE3 mobility was greatly increased in the myosin VI KD, consistent with myosin VI’s anchoring a significant amount of MV NHE3. In contrast, NHE3 mobility was slightly but not significantly reduced after dynasore treatment. The combination of dynasore with myosin VI KD increased NHE3 mobility to a level between that of dynasore treatment alone and myosin VI KD. This last result extends our immune-EM results to suggest that myosin VI KD and dynasore treatment differentially affect MV NHE3 mobility.
DISCUSSION

Our study presents the strongest evidence to date that myosin VI moves cargo down the MV length of intestinal cells in addition to its role in subsequent steps of cargo endocytosis. By studying the Na⁺ absorptive Caco-2/Bbe cell, in which myosin VI was knocked down by shRNA, we showed that NHE3 function and localization in the MV are myosin VI-dependent. Basal MV NHE3 was increased by myosin VI KD while the second messenger-induced (FSH and CCH) decrease in MV NHE3 activity and amount was blocked. Since these two agents have been shown to stimulate endocytosis without altering the rates of exocytosis (Gekle M et al., 2002; Lee-Kwon W et al., 2003; Musch MW et al., 2007; Zachos NC et al., 2013), we propose that both basal and stimulated endocytosis require myosin VI. Additionally, since EGF treatment of myosin VI KD cells still led to an increase of MV NHE3 activity, our results further indicate that myosin VI is not involved in the stimulated exocytosis of NHE3 in the Na⁺ absorptive cell. Importantly, our immuno-EM finding that MV NHE3 exhibited different MV distributions in control, myosin VI KD and dynasore-treated cells, convincingly demonstrates that myosin VI is also responsible for moving NHE3 down the MV length. Additionally, while the combination of myosin VI KD and dynasore treatment did not alter the MV distribution of NHE3 protein found in the myosin VI KD cells (our immune-EM results), it significantly reduced the mobile fraction of MV NHE3 compared to that found in the myosin VI KD cells (our FRAP results).

Our results on NHE3 in this study complement and extend those reported for several small intestine apical transporters in the Snell’s Walzer mouse, which lacks myosin VI (Ameen N and Apodaca G., 2007; Hegan PS et al, 2012). For example, CFTR-related Cl⁻ secretion and cell surface expression of CFTR were increased relative to that in wt mice, while its basal and stimulated internalization was reduced (Ameen N and Apodaca G., 2007). Similarly, increased apical expression of NHE3 and NaPi2b in the jejunum of these mice and their reduced trafficking to subapical endosomes were observed (Hegan PS et al, 2012). Our immuno-EM analysis showing that KD of myosin VI in Caco-2/Bbe cells resulted in an increased proportion of NHE3 in the upper half of MV (>80%, Fig. 6,7B) as compared to that in control (40%) or control +dynasore cells (60%), suggests very strongly that myosin VI regulates the movement of NHE3 down the MV.
Myosin 1A is a plus-directed motor that has been convincingly shown to move membrane cargo towards the MV tip, where the barbed ends of the actin filaments attach and actin monomers are added (Tyska MJ and Nambiar R, 2010). Studies of BB transporters in the intestine of myosin 1A-null mice showed that there is virtually complete endocytic removal of transporters such as CFTR into the terminal web region and beyond. In contrast, although there was a significant increase of MV NHE3 in the myosin VI KD cells and Snell’s Walzer mouse intestine (Chen and Donowitz, unpublished), an intracellular pool of NHE3 remained recruitable upon treatment of cells with EGF (see Fig. 3E). This supports previous studies in which suggestions were made that there are multiple intracellular pools of the transporter: an NHE3 (basal) pool that is continually cycling to and from the MV; a reserve pool that requires a signal to move to the MV; and, perhaps, a non-cycling (biogenetic/degradative) pool (Alexander RT et al, 2005; Li X et al, 2001; Murtazina R et al, 2006).

Several outstanding questions remain. How does myosin VI physically associate with MV NHE3, an interaction needed to move NHE3 down the MV? NHE3 itself does not bind myosin VI (Chen, T and Donowitz, MD, unpublished). This is not surprising since many studies have shown that myosin VI binds to its cargos indirectly via adaptors (Tumbarello DA et al, 2013) and the binding motifs in the adaptors vary considerably. For example, the adaptor dab2 uses its C-terminal serine- and proline-rich regions (PTB domain and SYF residues) to bind myosin VI (Morris et al, 2002a; Spudich G et al, 2007), while GIPC uses a PDZ domain (Naccache SN et al, 2006), optineurin uses coiled-coil and ubiquitin-binding domains, T6BP and NDP52 binds to zinc-finger domains, and Tom1 uses an IEXWL amino acid motif (Tumbarello DA et al, 2012; Tumbarello DA et al, 2013; Morriswood B et al, 2007; Sahlender et al, 2005). None of these motifs are present in the cytoplasmic C-terminus of NHE3. Thus, future studies are directed toward identifying the adaptors that mediate the interaction between myosin VI and NHE3, and the mechanisms that regulate NHE3 movements along the MV.

Materials and Methods

Materials

Reagents and antibodies were purchased from the following sources: EZ-Link Sulfo-NHS-SS-biotin (Pierce Chemical, Rockford, IL); restriction endonucleases (New England Biolabs, Ipswich, MA): 2′7′-Bis (2-carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM)
(Invitrogen, Carlsbad, CA); mouse monoclonal anti-hemagglutinin (HA) antibodies (Covance Research Products, Princeton, NJ); mouse monoclonal anti-myosin VI antibodies (Sigma, St. Louis, MO); DNA primers (Operon Biotechnologies, Huntsville, AL); and donkey anti mouse IgG-12 nm gold (Jackson ImmunoResearch Laboratories, Inc, Bar Harbor, Me). Rabbit polyclonal anti-myosin VI antibody was kindly provided by Professor Folma Buss (Cambridge Institute for Medical Research).

Cell culture and adenoviral Infection

The Caco-2/Bbe cell line, originally derived from a human adenocarcinoma, was obtained from M. Mooseker (Yale University, New Haven, CT) and J. Turner (Univ. Chicago, Chicago, IL). Cells were maintained in DMEM + 25 mM NaHCO₃, 0.1 mM nonessential amino acids, 10% fetal bovine serum, 4 mM glutamine, 50 U/ml penicillin, and 50 μg/ml streptomycin, pH 7.4, at 37°C in 5% CO₂. To achieve polarity, cells were cultured on collagen-coated semi-permeable membranes (Transwells, polycarbonate or Anapore) for 12 days after becoming confluent. Cells infected with Ad-HA-NHE3 (Sarker R et al., 2008) were first exposed to 6 mM EGTA in serum-free medium for 2 h at 37°C to increase virus access to the basolateral surface. Appropriate amounts of viral particles (10⁹–10¹⁰ particles/ml) in serum-free medium were added to both chambers of EGTA-treated cells. After 6+ hours, cells were rinsed and incubated further in normal growth medium. Cells used in transport assays were grown to confluence on small pieces of polycarbonate membranes glued to plastic coverslips (0.4 μm pore size, Corning; called “filter slips”). Adenoviral infection of cells on filter slips occurred 12 days after confluence and transport assays were performed 48 hr later.

Lentivirus-shRNA KD of myosin VI

Sequence-verified shRNA lentiviral plasmids in the hairpin-pLKO14-puromycin vector were used for myosin VI gene silencing. Human myosin VI was targeted with three independent shRNAs obtained through the Johns Hopkins HiT center from Open Biosystems (Huntsville, AL) and used to generate lentivirus particles (listed in Table 1). pLKO.1 vector containing shRNA, a packaging vector pCMVdR8.91 containing gag, pol, and rev genes, and envelope vector pCMV-VSV-G were co-transfected into human embryonic kidney 293T cells. Plasmids were prepared using an EndoFree Plasmid Maxi kit (Qigen, Valencia, CA). 2x10⁷ human embryonic kidney 293T cells
were plated on a 10 cm Petri dish and 24 hr later the medium was changed to Opti-MEM serum-free media. Lipofectamine 2000 reagents were used for transfection according to the manufacturer’s protocol (Invitrogen). The transfection conditions were: 10 μg of packaging plasmid + 6 μg of envelope coding plasmid + 10 μg of shRNA coding plasmid solution. Production of lentiviruses was enhanced by replacement of culture media at 16 h post-transfection with 5 ml fresh media containing 10 mM sodium butyrate for an additional 7-8 h (Gasmi M et al., 1999; Sakoda T et al., 1999), after which 5 ml of fresh media was added for 16 h before virus harvesting. Lentivirus supernatants were passed through filters (0.45 μm pore PVDF Durapore, ThermoFisher Scientific, Bedford, MA) and used immediately or stored at -80°C. Negative controls were empty vector and Lenti-shGFP-puromycin (a lentiviral-shRNA construct specific for GFP, which is not endogenously expressed in mammalian cells).

For lentiviral transductions, Caco-2/Bbe cells were plated in 6-well dishes (Transwell) and cultured 24 h to achieve 40-50% confluence. Viral particles were mixed with an equal volume of complete medium, Polybrene added (90 μg/ml, 30 min, 37 °C), and the entire mixture added to the well. Twenty-four hours later, the mixture was replaced with complete media containing 20 μg/ml puromycin. Cells were analyzed after 3-4 passages.

The efficiency of myosin VI KD was assessed by Western blot and immunofluorescence confocal microscopy using specific antibodies against myosin VI. All shRNAs gave similar levels of KD and phenotypes; construct #3 was subsequently used for these studies. Caco-2/Bbe cells transduced with control or myosin VI shRNA were studied 14 days after confluence.

**Measurement of Na+/H+ exchange activity**

Cellular Na+/H+ exchange activity in cells expressing HA-NHE3 was determined fluorometrically using the intracellular pH-sensitive dye BCECF-AM (Molecular Probes, Eugene, OR). Filter-grown cells (polycarbonate) were infected with Ad-HA-NHE3 day 12 after reaching confluence, serum-starved 48 hr later for at least 4 h and then Na+/H+ exchange activity (NHE3) determined as described previously (Janecki AJ et al., 1998; Li X et al., 2004; Murtazina R et al., 2007; Sarker R et al., 2010). HOE-694 (50 μM) was included to inhibit the contributions of NHEs 1, 2 and 8. Cells were loaded for 1h at 37°C with 10uM BCECF-AM in Na+/NH4Cl solution (98 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgSO4, 1 mM NaH2PO4, 25 mM glucose, 20 mM HEPES, and 40 mM NH4Cl, pH 7.4). During dye loading and the NH4Cl prepulse, cells were treated with 25
µM FSK for 15 min, 80 µM dynasore for 30 min or a volume control. For CCH treatment, 25 µM carbachol were added to basal side in TMA+ (~6 min). The filters were mounted in a cuvette, placed in a fluorometer (Photon Technology international, Lawrenceville, NJ), and perfused from both sides with TMA+ medium (130 mM tetramethylammonium (TMA) chloride, 5 mM KCl, 2 mM CaCl₂, 1 mM MgSO₄, 1 mM Na₂HPO₄, 25 mM glucose, 20 mM HEPES, pH7.4) to rapidly acidify the intracellular space. After 2-3 min, the apical TMA+ medium was replaced with Na+ medium containing 130 mM NaCl and no TMA. The basal side continued to be perfused in TMA+. Initial rates of Na+‐dependent intracellular alkalinization (efflux of H⁺, in micromolar per second) were calculated for a given pHi over the first 1 min and expressed as ΔpH/ΔT. Means ± s.e.m were determined from at least three experiments.

**Quantitation of surface NHE3 by biotinylation**

NHS-SS-biotin was used to determine the percentage of total NHE3 on the surface as described previously (Cha et al., 2006). Filter-grown cells were infected with Ad HA-NHE3 12 days post-confluence and studied 48 h later. Cells were serum-starved for 4-6 h, rinsed three times in cold PBS (150 mM NaCl, 20 mM Na₂HPO₄, pH 7.4) then once in borate buffer (154 mM NaCl, 1.0 mM boric acid, 7.2 mM KCl, and 1.8 mM CaCl₂, pH 8.0). 1 mg/ml NHS-SS-biotin in borate buffer was added at 0 and 30 min. Cells were then washed three times with quenching buffer (120 mM NaCl, 20 mM Tris, pH 7.4) to scavenge unreacted biotin, three times in PBS and solubilized with 1 ml N+ buffer (60 mM HEPES, pH 7.4, 150 mM NaCl, 3 mM KCl, 5 mM Na₃EDTA, 3 mM EGTA, and 1% Triton X-100). The cell lysate was centrifuged at 5000 rpm for 10 min and the post-nuclear supernatant was collected, protein concentrations were measured by Bradford assay (Sigma-Aldrich (St. Louis, MO) and adjusted to 1 µg/µl. Of the 1 ml of cell lysate supernatant, 0.9 ml was incubated with Streptavidin-agarose beads (Pierce Chemical, Rockford, IL) for 3 hrs at 4°C. After sedimenting the beads, the supernatant was retained as the intracellular fraction and the avidin-agarose beads were washed five times in N+ buffer (60 mM HEPES, pH 7.4,150 mM NaCl, 3 mM KCl, 5 mM Na₃EDTA, and 3 mM EGTA) with 0.1% Triton to remove nonspecifically bound proteins. The avidin agarose bead-bound proteins, which represent plasma membrane NHE3, were solubilized in 90 µl of loading buffer (5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol, and 1% 2-mercaptoethanol), boiled for 10 min. Two dilutions (30 µl and 60 µl) of total lysate, surface and intracellular proteins from each group were loaded, size-fractionated by SDS-
PAGE (10% gel), and then electrophoretically transferred to nitrocellulose. After blocking with 5% nonfat milk in PBS, the blots were probed with monoclonal anti-HA antibody, rinsed, incubated with anti-mouse IRDye® 488 secondary antibodies (LI-COR) and visualized and signals quantified on an Odyssey Infrared Imaging System (Li-Cor, Lincoln, NE). The signal intensity derived by linear regression was used to obtain a single value for each sample. The percentage of surface NHE3 was calculated [(surface NHE3 signal/total NHE3 signal) × dilution factor of surface and total NHE3 samples] and expressed as percentage of total NHE3.

**Immunocytochemistry, confocal microscopy and image quantitation**

Caco-2/Bbe cells were grown on Anapore filters (25 mm, 0.02 μm, Nunc) then infected at 12 days post-confluence with Ad-HA-NHE3 as described above. After 48 hours, cells were washed with ice-cold PBS and fixed for 30 minutes at 4°C with 3% paraformaldehyde (PFA) in PBS. For dynasore treatment, cells were serum starved for 4hrs and incubated with dynasore (80 µM for 30 min) or equal volume of DMSO and then processed for fixation. Fixed cells were blocked and permeabilized with 1% bovine serum albumin (BSA) and 0.075% saponin in PBS for 1 hour at 4°C. Cells were incubated 1 hour at room temperature with primary antibodies in 1% BSA-PBS, rinsed in 0.1% BSA-PBS (3×5 min), then incubated with secondary antibodies in 1% BSA-PBS for 1 h, and rinsed again in 0.1% BSA- PBS (3×5 min). Cells were rinsed with 0.1% BSA and 0.075% saponin in PBS prior to mounting. Membrane inserts were detached from wells, placed on glass microscope slides, mounted with Fluorogel (Invitrogen), and examined with a Zeiss LSM510 confocal microscope. Images were acquired using a 63× (1.4 NA) water objective or 100× (1.6 NA) oil objective.

For quantitative determination of surface NHE3 using confocal microscopy, cells were grown on filters for 12 days, then infected with Ad-HA-NHE3 and processed for immune-staining 48 hrs later. Cells were fixed in 3% PFA for 30 min at 4°C as described above except that saponin was omitted, and then incubated with wheat germ agglutinin conjugated to Alexa Fluoro 568 (Fl-WGA) to mark the apical surface. After rinsing, the cells were permeabilized with 0.075% saponin and labeled with anti-mouse HA (Alexa Fluoro 488). The total NHE3 fluorescence intensity and percentage of NHE3 on the surface were quantified using Volocity software as described by Zinchuk (2011). Signal intensity was represented as pixels. Overlap was determined with Metamorph co-localization software.
Intact ileum from C57BL/6 wild type and myosin VI-null mice were fixed in 3.5% PFA in PBS at 4°C and paraffin-embedded. Histologic sections (4 µm thick) were mounted onto Superfrost microscope slides (Fisher Scientific Co., Arlington, VA) and heat fixed. Slides were microwaved for antigen recovery in 10 mM sodium citrate buffer, pH 6 (Sigma Chemical Company, St. Louis, MO) at power level setting 9 (Panasonic Model# NN-C980B Conventional Microwave Oven, Secaucus, NJ), for 2-5 min. After cooling for 30 min, sections were washed in PBS and preblocked with 5% normal goat serum (NGS) in PBS for 30 min at room temperature. Sections were then incubated overnight (4°C) with mouse monoclonal Aquaporin7 antibody and rabbit polyclonal myosin VI antibody diluted 1:100 in 5% NGS. Ileal sections were then washed twice in PBS for 10 min and incubated with anti-mouse Alexa-fluor 488 (aquaporin7) and anti-rabbit Alexa-fluor 568 (myosin VI) secondary antibodies, each diluted 1:100 for 1 hour at room temperature. Nuclei were counterstained with Hoechst 33342. Sections were washed twice with PBS and mounted with Gel Mount. Ileal sections were imaged using a Zeiss LSM/510 confocal fluorescence microscope as above. Overlap of myosin VI with the MV marker aquaporin7 was determined with Metamorph software co-localization analysis (See above).

**Electron microscopy (EM) and immuno-EM**

**EM of control and myosin VI KD cells.** Polycarbonate filter-grown cells were processed for conventional EM analysis 14d post-confluencne. After several rinses in PBS, cells were fixed at room temperature for 1 h in 1.6% PFA, 2.5% glutaraldehyde (GA), 3% sucrose and 0.1M Na cacodylate, pH 7.4. Cells were rinsed once in 0.1 M cacodylate plus 3% sucrose, and stored overnight at 4°C. After rinsing in the same solution, cells were incubated 1 h on ice in reduced osmium (0.1 M cacodylate buffer with 0.4% postassium ferrocyanide and 1% OsO4), rinsed quickly at room temperature in ddH2O, incubated for 30 min at room temperature in 2% uranyl acetate in ddH2O, and dehydrated through a graded series of ethanol to 100%. Samples were incubated overnight in 1/1 EPON/ethanol at 4°C, warmed to room temperature, transferred for one h into 100% EPON plus DMP30, then left overnight in fresh EPON plus catalyst with gentle shaking. The next day, fresh EPON+ catalyst was added at two h intervals and incubated in a vacuum chamber for another two h. Filters were cut out of the holders, further cut into small pieces (~2x2 mm), embedded in EPON and cured for 2-3 days at 65°C. Sections were cut on a Riechert Ultracut E with a Diatome Diamond knife, collected on formvar-coated 1x2 mm copper slot grids,
and then stained with uranyl acetate followed by lead citrate. Grids were viewed on a Hitachi 7600 TEM operating at 80 kV and digital images of the apical regions of control and KD cells were captured with an AMT 1K x 1K CCD camera.

**Determination of microvillar lengths.** The lengths of individual MV were measured from images of randomly chosen cells using MetaMorph software and a calibrated ruler set perpendicular to the baseline of the microvilli. At least two microvilli were measured per cell with a total of ~300 MV each from control and KD cells.

**Immuno-EM.** Ultrastructural localization studies were carried out on control and myosin VI KD Caco-2/Bbe cells grown on polycarbonate filters (Transwell, 0.4 μm, Corning Inc.) for 12 days then infected with Ad-HA-NHE3. 48 hr later, cells were serum-starved for 5 h, treated with dynasore (80 μM for 30 min) or equal volume of DMSO and fixed in 2% periodate-lysine-paraformaldehyde for 30 min at 4°C. Fixed cells were brought to RT, rinsed in PBS (2×5 min), quenched in 50 mM NH₄Cl (30 min), blocked in 1% BSA-PBS (30-60 min) and incubated for 1 h with anti-HA monoclonal antibody in blocking buffer. Controls consisted of infected cells fixed as above but incubated in blocking buffer without primary antibody. After one rinse in 0.2% BSA-PBS, cells were incubated for 2-3 h with anti-mouse IgG-12 nm gold in 0.2% BSA-PBS, rinsed twice in PBS, then fixed in GA/PFA, processed and embedded as described above. Oriented blocks were sectioned, so that each section contained ~80-100 cells and each cell was visible from its apical to basolateral surface. Unstained sections were examined at 40,000-50,000× magnification and images of all gold particles associated with MV and the inter-MV region were acquired across each section. ~25% of all cells were labeled with gold in a given section, requiring examination of multiple sections for each condition to accumulate sufficient numbers of gold particles for analysis. Three independent experiments were performed.

**Analysis.** The distribution of gold particles along MV was determined in micrographs using MetaMorph software. The distance of each gold particle from its MV base was recorded (0 was the base), the gold particles located 0-600nm along the MV length and those present >600nm from the MV base were grouped separately and each expressed as a percent of the total gold particles along MV. Comparison was performed with the two-sample Wilcoxon rank-sum (Mann-Whitney) test, as described (Fagerland MW and Sandvik L., 2009).

**Fluorescence recovery after photobleaching (FRAP)**
To quantitate the mobility of NHE3 at the apical domain of polarized Caco-2/Bbe cells, FRAP was used as previously reported (Cha et al., 2004). Cells were cultured on 12 well Transwell plates (#3401, Costar; Transwell Permeable Support, 0.4 mm Polycarbonate Membrane 12mm Insert, 12 well Plate) for 12 days postconfluent. Caco-2/Bbe cells were then infected with Adeno viral HA-NHE3 (Sarker, et al., 2008). Briefly, on the day of 12 days postconfluent, Caco-2/Bbe cells were treated with 6 mM EGTA in Caco-2/Bbe serum free media for 2 hrs before infection. Cells were then infected with Adeno viral HA-NHE3 and incubated for 6+ hours. Then cells were washed with fresh serum free Caco-2/Bbe media and kept overnight before FRAP experiment. To perform FRAP study, surface HA-NHE3 was labeled with monoclonal anti-HA antibody (primary) in phosphate buffered saline (PBS) + 1% BSA solution for 1 hr and labeled with anti-mouse Alexa 488 conjugated antibody in PBS + 1% BSA solution for 1hr. Cells were then washed with PBS three times and kept in serum free D-MEM/F-12 media without phenol red (Life Technology) before FRAP measurement.

FRAP was performed on a stage heated to 37°C of a Zeiss LSM 510/Meta confocal microscope using the 488 nm line of a 400-milliwatt Kr/Ar laser in conjunction with a 100×Zeiss 1.4 NA Plan Apochromat oil immersion objective, with signal collected in the cell apical domain (0.3 μm optical sections starting at the outer limit of the microvillus), and mobile fractions and diffusion coefficients calculated as described previously (Cha et al., 2010). The Zeiss LSM software package allowed autofocusing on the coverslip surface in the reflection mode during the time lapse imaging. Fluorescence was determined within the bleach area (pre-bleach intensity) and then the area was photobleached with full laser power (100% power, 100% transmission). Recovery was followed with low laser power at 9 s intervals, which included up to 30 images, until the intensity had reach a new steady plateau. The mobile fraction was determined by comparing the fluorescence intensity in the bleached region after full recover (F) with the fluorescence intensity before bleaching (Fi) and just after bleaching (Fo). Mobile and immobile fractions were calculated by comparing the intensity ratio in regions of interest (ROI) inside and outside the bleached area just before the bleach and after recovery as described previously (Cha et al., 2010). The postbleach intensities were normalized to correct for maximal loss of fluorescence due to the photobleach. Fluorescence intensity was normalized with prebleach intensity (Fi), which was set to 100 in each experiment.

All data are shown as mean ± s.e.m which were obtained in at least three identical experiments, unless stated otherwise. Statistical comparison was performed by unpaired Student’s t test.
Acknowledgements

This study was supported in part by the National Institute of Health, National Institute of Diabetes and Digestive and Kidney Diseases grants R01-DK26523, R01-DK61765, P01-DK072084, and P30-DK89502 (The Hopkins Digestive Diseases Basic and Translational Research Core Center), and the Hopkins Center for Epithelial Disorders. We acknowledge the generous contribution of anti-myosin VI polyclonal antibody by Folma Buss, Ph.D.

References


**Cha B, Kenworthy A, Murtazina R, Donowitz M.** (2004). The lateral mobility of NHE3 on the apical membrane of renal epithelial OK cells is limited by the PDZ domain proteins NHERF1/2, but is dependent on an intact actin cytoskeleton as determined by FRAP. *J Cell Sci.* 117, 3353-65.


Sarker R., Grønborg M., Cha B., Mohan S., Chen Y., Pandey A., Litchfield D., Mark Donowitz M and Li X. (2008). Casein kinase 2 binds to the C terminus of Na⁺/H⁺ exchanger 3
(NHE3) and stimulates NHE3 basal activity by phosphorylating a separate site in NHE3. *Mol Biol Cell.* 19, 3859-3870.


Figure legends

Fig. 1. A pool of myosin VI localizes on the MV of intestinal epithelial cells. (A) Immunofluorescence/confocal microscopic detection of myosin VI expression and localization in Caco-2/Bbe cells and mouse ileum. In Caco-2/Bbe cells, myosin VI is present throughout the BB, including the MV and in a vesicular pattern in the terminal web and other intracellular compartments (a1- a4). In mouse ileum, myosin VI is present to a smaller extent on the MV and in the terminal web (b1- b4). As a negative control, anti-myosin VI antibody was used to show there is no myosin VI staining in ileum from myosin VI KO mice (d) and myosin VI KD Caco-2/Bbe cells (f); c is WT control and e is empty vector-infected control. (B) Analysis of the overlap of myosin VI and protein markers of MV by Metamorph co-localization software. Please note this software shows presence of two proteins in the same intracellular compartment but does not establish physical association. In Caco-2/Bbe cells, ~46% of myosin VI overlaps with villin, and in mouse ileum ~9% of myosin VI overlaps with aquaporin 7. For both Caco-2/Bbe cells and mouse ileum, results are mean± s.e.m. of three separate experiments with 5-6 images analyzed in each experiment. (n=3, n represents three individual experiments; n=16, n represents total number of images analyzed).

Fig. 2. Efficient KD of myosin VI in Caco-2/Bbe cells did not significantly alter the ultrastructure of their MV. (A) Myosin VI expression in lenti-shRNA virus-infected Caco-2/Bbe cells. Caco-2/Bbe cells were infected with empty vector (lane 1), lentiviral vector encoding shGFP (lanes 2 and 3), shMyoVI#1 (lane 4), shMyoVI#2 (lane 5) and shMyoVI#3 (lane 6) (see Table 1). After 48 h of incubation, the cells were harvested for Western blot analysis. This experiment was repeated 3 times with similar results. (B, C) Transmission EM of MV in Control (B) and Myosin VI KD (C) cells grown on Transwell filters for 14 days. Representative images are shown. No obvious morphological changes were seen in the myosin VI KD cells, such as pulling away of microvillar membrane from the base of MV, as previously reported in myosin VI KO mouse intestine (Ameen N and Apodaca G, 2007) (D) The lengths of MV in myosin VI KD cells were not significantly different from those in control cells. Results are mean±s.e.m. Single experiment using conventional (ie glutaraldehyde) processing; >100 MV analyzed. 6 experiments using immuno-EM processing; >200 MV analyzed. (E) Measurement of transepithelial electrical resistance (TER) in Caco-2/Bbe cells ~14 days post confluence. The value obtained is expressed
in \( \Omega \cdot \text{cm}^2 \). Results are mean±s.e.m. \((n=3)\). There was no significant difference of TER between control cells and myosin VI KD cells.

**Fig. 3.** Knocking down myosin VI increases basal NHE3 activity and prevents reduction of NHE3 activity by CCH and FSK in Caco-2/Bbe cells, but does not affect EGF stimulation of NHE3. (A, B) Myosin VI KD in Caco-2/Bbe cells increased basal NHE3 activity by more than 60%. Basal transport activity was measured in Adenoviral/HA-NHE3 infected Caco-2/Bbe cells. NHE3 activity was measured from the initial rates of Na\(^+\)-dependent intracellular alkalinization (in micromolar per second). A is a single experiment and B is mean±s.e.m of four experiments. (C-E) Knocking down myosin VI in Caco-2/Bbe cells prevents the inhibition of NHE3 activity by FSK (C) \((P<0.05)\), and CCH (D) \((P<0.05)\), but not its stimulation by EGF (E) \((P<0.05)\). Results are mean±s.e.m of 3 independent experiments.

**Fig. 4.** Total and surface NHE3 were increased under basal conditions in myosin VI KD Caco-2/Bbe cells. (A, B) Western blot (WB) detection and quantification of total NHE3 in myosin VI KD cells. Cells were grown in 6 well plates and infected with Adenoviral/HA-NHE3 at day 12. 48 h after infection, cells were processed for WB. Total NHE3 amount was normalized with \( \beta \)-actin. Results are mean±s.e.m of three independent experiments. In myosin VI KD cells, the total NHE3 amount was significantly increased \((P<0.05)\). (C) NHE3 expression in myosin VI KD cells by immunofluorescence/confocal microscopical analysis. Cells were filter grown for 12 days, infected with Adenoviral/HA-NHE3 and two days later processed for immune staining. Cells were not initially permeabilized and were surface labeled with WGA (Alexa Fluro 568 conjugated wheat germ agglutinin) at 4°C; then cells were permeabilized and labeled with anti-HA. (C) Compared to control cells \((a1, a2, a3)\), NHE3 expression was dramatically increased in myosin VI KD cells \((b1, b2, b3)\). (D) Statistical analysis of total NHE3 intensity by Volocity software. Total NHE3 amount was increased \((P<0.05)\) in myosin VI KD cells. Results are mean±s.e.m of 10 images from 3 independent experiments. (E) Quantification of the percentage of NHE3 on the surface by Volocity software. Percentage of surface NHE3 was increased \((P<0.05)\) in myosin VI KD cells. Results are mean±s.e.m of 15 images from 3 independent experiments.
Fig. 5. Surface NHE3 amount was not changed by FSK and CCH in myosin VI KD Caco-2/Bbe cells but was decreased in control cells. Cells were grown in 6 well plates and infected with Adenoviral/HA-NHE3 at day 12. 48 h after infection, cells were serum-starved for 4-5 h then treated with 25 µM CCH for 5 min or 10 µM FSK for 15 min at 37°C before assessing the amount of NHE3 by cell surface biotinylation. Detection and quantitation of total and surface amounts of NHE3 are shown in A and B (single experiments) and C and D (mean±s.e.m of 3 independent experiments). Two dilutions of total, surface and cytosol fractions were loaded and the surface expression of NHE3 was determined as a percent of total NHE3 and normalized to β-actin; the values for the controls were set to 100 in each experiment. As shown in A and C, CCH and FSK-treated control cells expressed ~40% less surface NHE3 than non-treated cells (P<0.05). In myosin VI KD cells, there was no significant change of surface NHE3 expression after treatment with CCH or FSK compared to non-treated cells (B, D).

Fig. 6. More NHE3 distributes on the outer half of MV in myosin VI KD Caco-2/Bbe cells compared to controls. (A, B) HA-NHE3 was detected by immuno-electron microscopy using gold-labeled secondary antibodies. HA-NHE3 was detected on paraformaldehyde-fixed cells by labeling first with mouse anti-HA antibodies followed by anti-mouse IgG-12 nm gold (Black circles). The cells were then fixed with glutaraldehyde. The circles surround gold particles on the MV of the epithelial cells. mv: MV; scale bar: 0.5 µm. (C) A diagram of a microvillus shows how the HA-NHE3 distribution on the MV was quantitated. The whole MV from the baseline to tip (~1200 nm) was divided into 10-12 sections, 100 nm wide each, and the number of gold particles in each section was counted. (D) Histograms show the fraction of MV NHE3 distributed 0-600nm (dark gray) and >600nm (light gray) from MV base in control and myosin VI KD cells from all individual data points of three separate experiments. ~85% of the MV NHE3 in control cells was located in the first 600 nm of MV length and the rest >600nm from the MV base. In contrast, less than 50% of the MV NHE3 in myosin VI KD cells was located in the first 600nm and >50% >600nm from the MV base.

Fig. 7. Dynasore treatment increases MV NHE3 activity and alters NHE3 MV distribution in control but not myosin VI KD cells. (A) Dynasore treatment (80 µM, 30 min) stimulated basal Caco-2/Bbe NHE3 activity by 40% in control but not myosin VI KD cells (P<0.05). Results are...
mean±s.e.m from 3 independent experiments. (B) Dynasore treatment shifts MV NHE3
distribution in control but not myosin VI KD cells. See Fig. 6 legend for details of immuno-EM
analysis. Histograms show the fraction of MV NHE3 distributed 0-600nm (dark gray) and >600nm
(light gray) from MV base for each of the four treatments. Individual data points from three
separate experiments were grouped and analysed (See Table 4 for details). (C) The proposed
distribution patterns of MV NHE3 in Caco-2/Bbe cells +/-myosin VI KD and +/-dynasore are
presented together with a summary of results of NHE3 surface activities and endocytic/exocytic
behaviors in the different conditions. (D) FRAP analysis shows changes in NHE3 mobile fractions
(Mf) in Caco-2/Bbe cells +/-myosin VI KD and +/-dynasore (80 µM, 30 min) treatment. Mf was
increased by ~160% in myosin VI KD versus control cells (P<0.05). Dynasore treatment to control
cells did not significantly change MV NHE3 Mf, but significantly reduced Mf in myosin VI KD
cells (P<0.05). Results are mean±s.e.m from 3 independent experiments.

**Fig. 8. Dynasore inhibited NHE3 endocytosis stimulated by FSK in Caco-2/Bbe cells.** Basal
NHE3 transport activity was measured in Adenoviral/HA-NHE3 infected Caco-2/Bbe cells
with/without dynasore (80 µM, 30 min) or with/without FSK (25 µM, 30 min) pre-incubation.
Dynasore significantly increased NHE3 activity, and FSK decreased NHE3 activity (P<0.05). The
inhibition of NHE3 activity by FSK was blocked by dynasore. Results are mean±s.e.m from 3
independent experiments.

**Supplementary material**

**Fig. S1. The distributions of tight junction proteins are similar in control and myosin VI KD
Caco-2/Bbe cells.** Confocal microscopy/IF was used to examine the tight junction proteins ZO-1
(a, b), occludin (c, d) and claudin-1 (e, f) in both control cells (a, c, e) and myosin VI KD cells (b,
d, f) respectively (3 independent experiments performed). **Methods:** Caco-2/Bbe cells were grown
on Anapore filters (25 mm, 0.02 µm, Nunc) then infected at 12 days post-confluence with Ad-HA-
NHE3 as described in Methods and Materials. After 48 hours, cells were washed with ice-cold
PBS and fixed for 30 minutes at 4°C with 3% paraformaldehyde (PFA) in PBS. Fixed cells were
blocked and permeabilized with 1% bovine serum albumin (BSA) and 0.075% saponin in PBS for
1 hour at 4°C. Cells were incubated 1 hour at room temperature with primary antibodies in 1%
BSA-PBS, rinsed in 0.1% BSA-PBS (3×5 min), then incubated with secondary antibodies in 1%
BSA-PBS for 1 h, and rinsed again in 0.1% BSA-PBS (3×5 min). Cells were rinsed with 0.1% BSA and 0.075% saponin in PBS prior to mounting. Membrane inserts were detached from wells, placed on glass microscope slides, mounted with Fluorogel (Invitrogen), and examined with a Zeiss LSM510 confocal microscope. Images were acquired using a 63× (1.4 NA) water objective or 100× (1.6 NA) oil objective.

**Fig. S2. Changes of surface NHE3 amount by dynasore with or without myosin VI knockdown.** (A,B) Immunofluorescence confocal microscopy view of surface NHE3. Methods: Cells were grown on filters for 12 days, then infected with Ad-HA-NHE3 and processed for immune-staining 48 hrs later. Cells were fixed in 3% PFA for 30 min at 4°C as described above except that saponin was omitted, and then cells were labeled with anti-mouse HA (Alexa Fluoro 488) and wheat germ agglutinin conjugated to Alexa Fluoro 568 (Fl-WGA) to mark the apical surface. The fluorescence intensity were quantified using Volocity software as described by Zinchuk (2011). The overlapped intensity of green (NHE3) and red (WGA) was treated as surface NHE3. Signal intensity was represented as pixels. (C) Measurement of surface NHE3 intensity by Volocity software and statistical analysis of surface NHE3 amount. Compared to control cells, total intensity of surface NHE3 was significantly increased in dynasore treated cells, myosin VI KD cells, and dynasore treated myosin VI KD cells ($P<0.05$). However, there is no significant difference of surface NHE3 between myosin VI KD cells and dynasore treated myosin VI KD cells. These data show that knocking down myosin VI and dynasore treatment both increased surface expression of NHE3 and the effects were not additive.
Table 1: Sequences of myosin VI shRNAs.

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<th>Reverse</th>
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<td>5’-CCGGGCAGTGCTTTAGTTTGAATGACTCG AGTCATTCAAAACTAAGACACTGATTTTTG-3’</td>
<td>5’-ATTCAAAAAGCAGTGCTTTAGTTTGAATG ACTCGAGTCATTCAAAACTAAGACACTGC-3’</td>
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<td>shRNA3</td>
<td>5’-CCGG GCAATCCACAGGCAAGAATAAACTCG AGTTATTCTTGCTGTGGATTTTTG-3’</td>
<td>5’-AATTCAAAAAGCAATCCACAGGCAAGAAT AACTCGAGTTATTCTTGCTGTTGGATTGC-3’</td>
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Table 2. Disruption of Myosin VI in Caco-2/Bbe/HA-NHE3 cells prevents down-regulation of NHE3 activity by CCH and FSK but not its stimulation by EGF.

<table>
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<tr>
<th>Cell line/NHE3 activity</th>
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<th>CCH inhibition</th>
<th>EGF stimulation</th>
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<tr>
<td>Caco-2/Bbe /GFP KD</td>
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<td>50.5 ± 0.03%</td>
<td>70.2 ± 0.02%</td>
<td>37.4 ± 0.01%</td>
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<tr>
<td>Caco-2/Bbe/Myosin VI KD</td>
<td>Increase 60.3 ± 0.01% (p&lt;0.05)</td>
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<td>-</td>
<td>38.1 ± 0.03%</td>
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- , no inhibition or stimulation

- , no inhibition or stimulation
Table 3. More NHE3 distributes on the outer half of MV in myosin VI KD Caco-2/Bbe cells compared to controls.

<table>
<thead>
<tr>
<th>Group</th>
<th>&lt;600nm</th>
<th>≥600nm</th>
<th>Total # of cells with Au</th>
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<tr>
<td>Ctrl</td>
<td>#Au</td>
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<td></td>
<td>355</td>
<td>86.4</td>
<td>56</td>
</tr>
<tr>
<td>KD</td>
<td>#Au</td>
<td>%</td>
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<tr>
<td></td>
<td>111</td>
<td>44.9</td>
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Statistical analysis for each experiment in Fig. 6D was performed with Two-sample Wilcoxon rank-sum (Mann-Whitney) tests. NHE3 was significantly differently distributed in control and myosin VI KD cells ($P<0.0001$). <600 nm and ≥600 nm refer to distance from baseline. Ctrl: empty vector infected Caco-2/Bbe cells. #Au: number of gold particles counted.
Table 4. Dynasore increases MV distribution, an effect not additive with effect of knocking down myosin VI.

Statistical analysis from the pooled results in Fig. 7B was performed with Two-sample Wilcoxon rank-sum (Mann-Whitney) test. NHE3 in dynasore treated cells was distributed significantly further on the outer half of the microvillus compared to untreated control and significantly closer to the microvillus base compared to myosin VI KD \((p<0.0001)\). The combination of myosin VI KD and dynasore exposure had a similar distribution to myosin VI KD. \(<600\, \text{nm}\) and \(\geq 600\, \text{nm}\) refer to distance from baseline. Ctrl: empty vector infected Caco-2/Bbe cells, D: dynasore, KD: myosin VI KD Caco-2/Bbe cells, #Au: number of gold particles counted.

<table>
<thead>
<tr>
<th>Group</th>
<th>&lt;600nm</th>
<th>≥600nm</th>
<th>Total # of cells with Au</th>
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<td>Ctrl+D</td>
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<tr>
<td>KD</td>
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<td></td>
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<td></td>
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P<0.0001
B  Analysis of overlap between myosin VI and BB/MV markers

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<tr>
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<th>Mouse ileum (myosin VI vs Aquaporin7)</th>
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<td>Percentage of overlap (mean±s.e.m)</td>
<td>45.6±0.03%</td>
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Fig. 2.

A. Western blot analysis showing Myosin VI and β-Actin expression in samples 1 to 6.

B. Control sample showing Myosin VI distribution.

C. Myosin VI KD sample showing Myosin VI distribution.

D. Graph showing height of mv (nm) for control and Myosin VI KD samples.

E. Graph showing TER (Ω·cm⁻²) for control and Myosin VI KD samples.

NS indicates no significant difference.
Fig. 3.
Fig. 4.
Fig. 5.

A

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B

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<td>Myosin VI KD</td>
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<td>Ctrl</td>
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</tr>
<tr>
<td>CCH</td>
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<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
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<td>-</td>
<td>+</td>
</tr>
<tr>
<td>FSK</td>
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<td>+</td>
<td>-</td>
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</tbody>
</table>

C

Surface NHE3 amount/Total NHE3/Actin (Normalized with CON)

D

Surface NHE3 amount/Total NHE3/Actin (Normalized with CON)
Fig. 6.

A. Ctrl

B. Myosin VI KD

C.

mv

500 nm

1200 nm Tip

600 nm

0 nm Baseline
D

Fraction

0.86
0.45
0.55

Ctrl
Myosin VI KD

<600
≥600
Fig. 7.

A

\[ \text{NHE3 activity (}\Delta \text{pH/min)} \]

\begin{align*}
\text{Ctrl} & : 0.00 \\
\text{Ctrl+Dyn} & : 0.15 \\
\text{KD} & : 0.20 \\
\text{KD+Dyn} & : 0.25
\end{align*}

B

\begin{align*}
\text{Fraction} & <600 \\
\text{Ctrl} & : 0.19 \\
\text{Ctrl+Dyn} & : 0.55 \\
\text{KD} & : 0.36 \\
\text{KD+Dyn} & : 0.41
\end{align*}

\begin{align*}
\text{Fraction} & \geq600 \\
\text{Ctrl} & : 0.81 \\
\text{Ctrl+Dyn} & : 0.55 \\
\text{KD} & : 0.64 \\
\text{KD+Dyn} & : 0.59
\end{align*}

\( p<0.05 \)

\( \text{NS} \)
C

<table>
<thead>
<tr>
<th></th>
<th>Ctrl</th>
<th>Dynasore</th>
<th>KD</th>
<th>KD+Dynasore</th>
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</thead>
<tbody>
<tr>
<td>Surface NHE3</td>
<td>1X</td>
<td>1.5X</td>
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<td>Endocytosis</td>
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<tr>
<td>Exocytosis</td>
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<tr>
<td>Migration along MV</td>
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<td>-</td>
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</table>
**Figure D**

Bar graph showing NHE3 Mobile Fraction (%) for different conditions: Ctrl, Ctrl+Dyn, KD, KD+Dyn. The graph indicates statistical significance with:

- Ctrl vs. Ctrl+Dyn: p<0.05
- KD vs. KD+Dyn: p<0.05
- Ctrl vs. KD: NS (Not Significant)
Fig. 8.

NHE3 Activity ($\Delta$ ph/min)

- Ctrl
- FSK
- Dyn
- FSK + Dyn

Significance:
- $p < 0.05$
- NS
Fig. S1.

<table>
<thead>
<tr>
<th>Control</th>
<th>Myosin VI KD</th>
</tr>
</thead>
<tbody>
<tr>
<td>a ZO-1</td>
<td>b ZO-1</td>
</tr>
<tr>
<td>c Occludin</td>
<td>d Occludin</td>
</tr>
<tr>
<td>e Claudin-1</td>
<td>f Claudin-1</td>
</tr>
</tbody>
</table>
Fig. S2.

A | Ctrl | Caco-2/Bbe cells
---|------|-------------------
| a1  | a2   | a3               |
| NHE3 | WGA  | Merged           |
| Dynasore | NHE3 | WGA  | Merged |

B | Myosin VI KD Caco-2/Bbe cells
---|-------------------------------
| Ctrl | a1 | a2 | a3 |
| NHE3 | WGA | Merged |
| Dynasore | NHE3 | WGA | Merged |
Intensity of Surface NHE3 (10^9 Pixels)

- Ctrl
- Ctrl+Dyn
- KD
- KD+Dyn

p<0.05 NS