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.

Fig. S1.

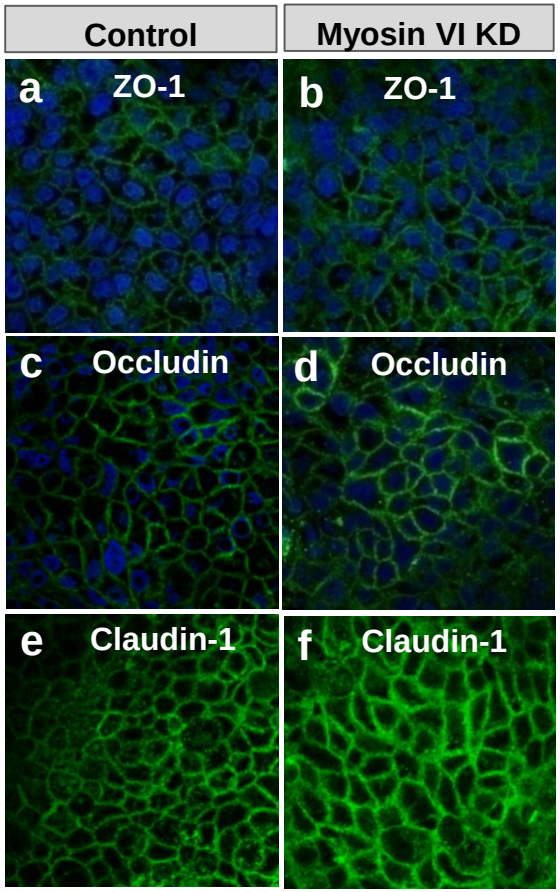
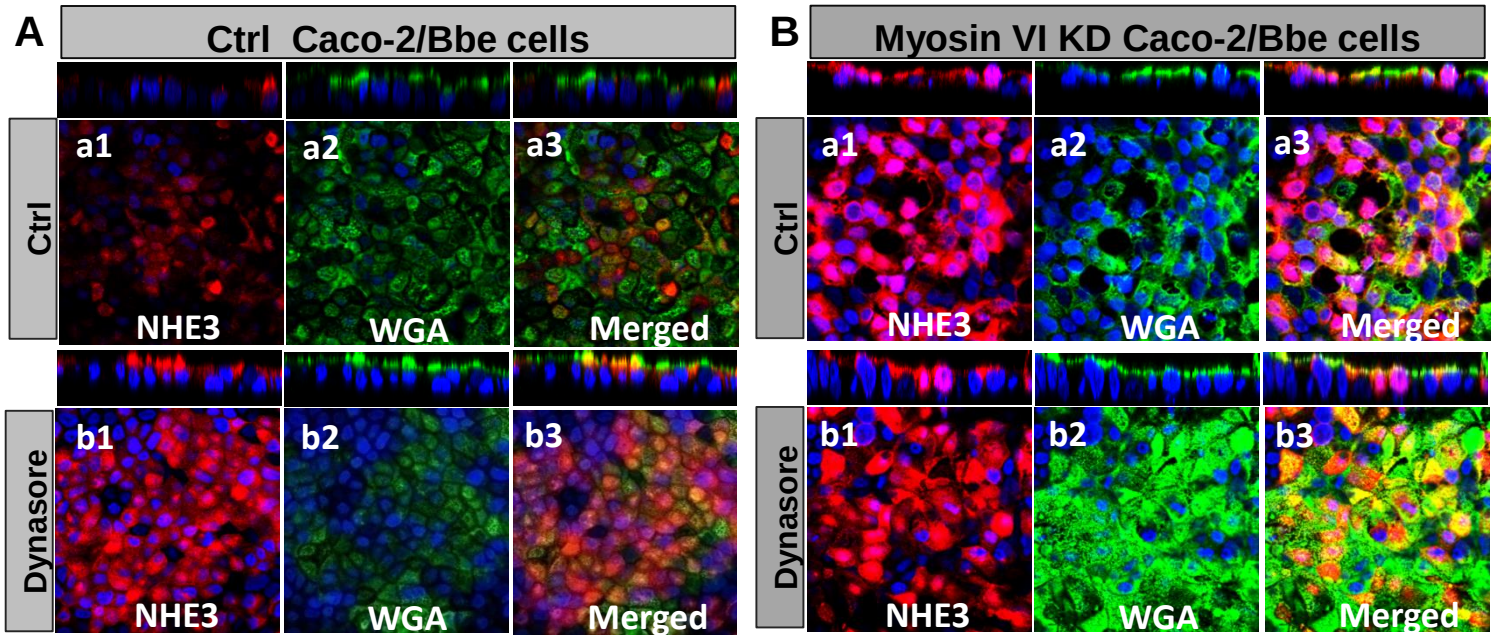

Control
Myosin VI KD
a
ZO-1
b
ZO-1
c
Occludin
d
Occludin
e
Claudin-1
f
Claudin-1

Fig. S2.


A
Ctrl Caco-2/Bbe cells
B
Myosin VI KD Caco-2/Bbe cells
Ctrl
Dynasore
a1
a2
a3
b1
b2
b3
NHE3
WGA
Merged

C

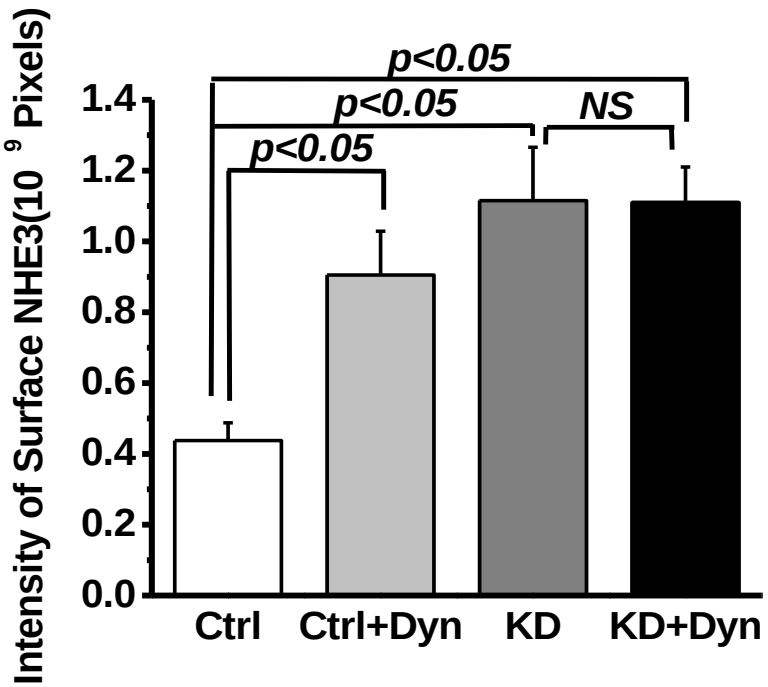

Intensity of Surface NHE3(10 9 Pixels)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
p<0.05
p<0.05
p<0.05
NS
Ctrl
Ctrl+Dyn
KD
KD+Dyn

Table S1: Sequences of myosin VI shRNAs.

	Forward	**Reverse**
shRNA1	5’-CCGGGCAGTGTCTTAGTTTGAATGACTCG AGTCATTCAAACTAAGACACTGATTTTTG-3’	5’-ATTCAAAAAGCAGTGTCTTAGTTTGAATG ACTCGAGTCATTCAAACTAAGACACTGC-3’
shRNA2	5’-GCTCATTCTGCTTCTGTAATGCTCGAG CATTACAGAAGCAGAATGAGCTTTTTG-3’	5’-AATTCAAAAA GCTCATTCTGCTTCTGTAAT GCTCGAGCATTACAGAAGCAGAATGAGC-3’
shRNA3	5’-CCGG GCAATCCACAGGCAAGAATAACTCG AGTTATTCTTGCCTGTGGATTGCTTTTTG-3’	5’-AATTCAAAAAGCAATCCACAGGCAAGAAT AACTCGAGTTATTCTTGCCTGTGGATTGC-3’