SUPPLEMENTAL FIGURE LEGENDS

Figure S1.FSK-stimulated Chloride secretion in Caco-2 cells. Basolateral exposure of FSK resulted a rapid elevation of short circuit current (Isc), in Caco-2 monolayers mounted in Ussing Chambers compared to vehicle (DMSO). The arrows indicate the time of addition of each agent. Experiments were repeated at least 3 times and summarized results were expressed in inset. **significant difference at P<0.001 compared to control (paired t-test).

Figure S2. ATP but not Ace increased intracellular calcium in HEK 293 cells. A, Image shown is HEK293 cells stably expressed mcherry-tagged mouse Ano6 grown onto cover glass for measurement of Fura 2 fluorescence. B, Dose and time course for ATP and C, Ace + ATP induced increase in $[Ca^{2+}]i$ in HEK 293 cells, as detected by ratiometric fura 2 fluorescence. Stimulation with ATP activates $[Ca^{2+}]i$ rise. Ace either alone or in combination with the ATP did not augment $[Ca^{2+}]i$ rise. Cells treated with 1μM Ace followed by the addition of 100μM ATP. Ace was not able to increase $[Ca^{2+}]i$ but subsequent addition of ATP evoked $[Ca^{2+}]i$ rise in these cells. Data are representative of three independent experiments. Scale bars: 10μm.

Figure S3. Deletion of KR motifs from N-terminus does not affect surface distribution of Ano6 in HEK 293 cells. A, Western blot analysis (with GFP antibody) for protein expression of wild-type mAno6, KR motifs truncated Ano6, mutant Ano6 transiently expressed in HEK 293 cells. B, Confocal images demonstrate surface distribution of GFP-tagged Ano6, or KR motifs truncated, or mutants of Ano6 transfected within HEK293 cells. Schematics of different step-wise deletions or mutants of mAno 6 channel protein are presented corresponding to their confocal images. N-terminal deletion of KR motifs or mutation in these motifs did not mistarget the mAno6 channel to the surface. Red box represents potential PIP_2 binding sites; the mutants are robustly expressed at the membrane surface. Scale bars: 10μm.

Figure S4. Ano 6 silencing in Caco-2 cells. A, qPCR analysis of Ano6 expression in cells after lenti-shRNA transduction specific for Ano6 genes, which are shown as mRNA levels relative to levels of GAPDH. Values are means ± S.E.. B, Inhibition of Isc in normal and after knockdown of Ano6 expression by lenti shRNA. **significant difference at P<0.001 compared to control (paired t-test); NS, statistically not significant.

Firgure S5: Interaction of RhoA and PIP5K during Ano6 activation: Endogenous RhoA and PIP5K interaction with mAno6-GFP was measured upon stimulation with Ace. RhoA (A) but not PIPK (B) co-immunoprecipitates with mANO6-GFP transiently expressed in HEK 293 cells. mANO6-GFP was immunoprecpitated by anti-GFP antibody followed by detection of Rho A and PIP5K by western blotting using anti-RhoA and anti-PIPK antibody.(Input) 10% of mAno6-GFP

transfected HEK293 cell lysate. Representative GFP blots were presented (centre) to indicate the level of Ano6-GFP precipitation for these experiments.

Figure S1

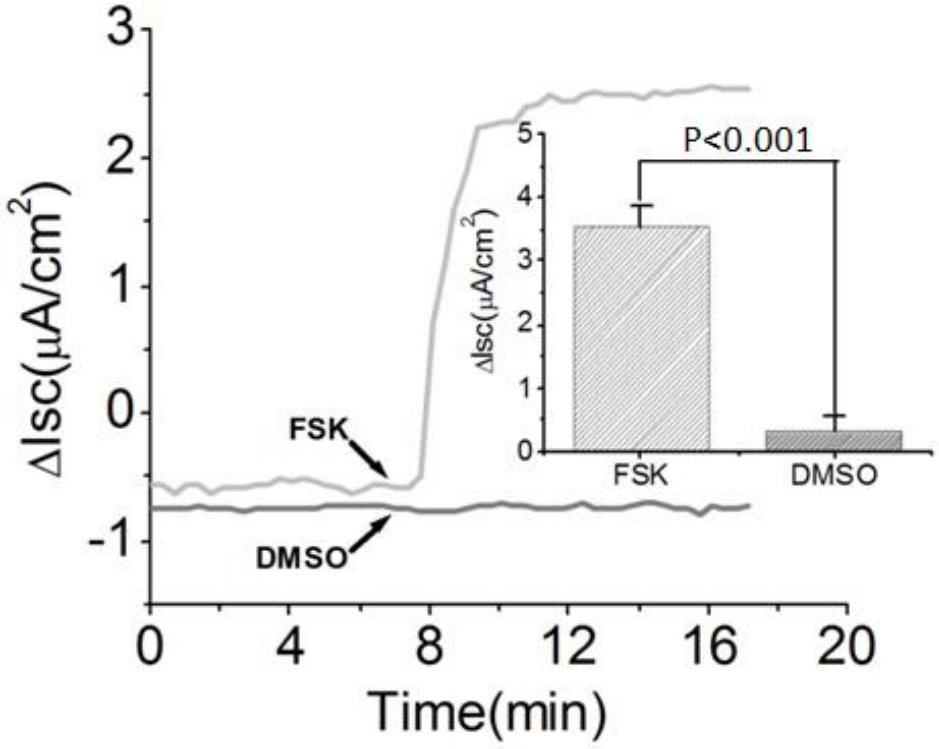

3
2
1
0
-1
ΔIsc(μA/cm²)
FSK
DMSO
0
4
8
12
16
20
Time(min)
P<0.001
5
4
3
2
1
0
ΔIsc(μA/cm²)
FSK
DMSO

Figure S2

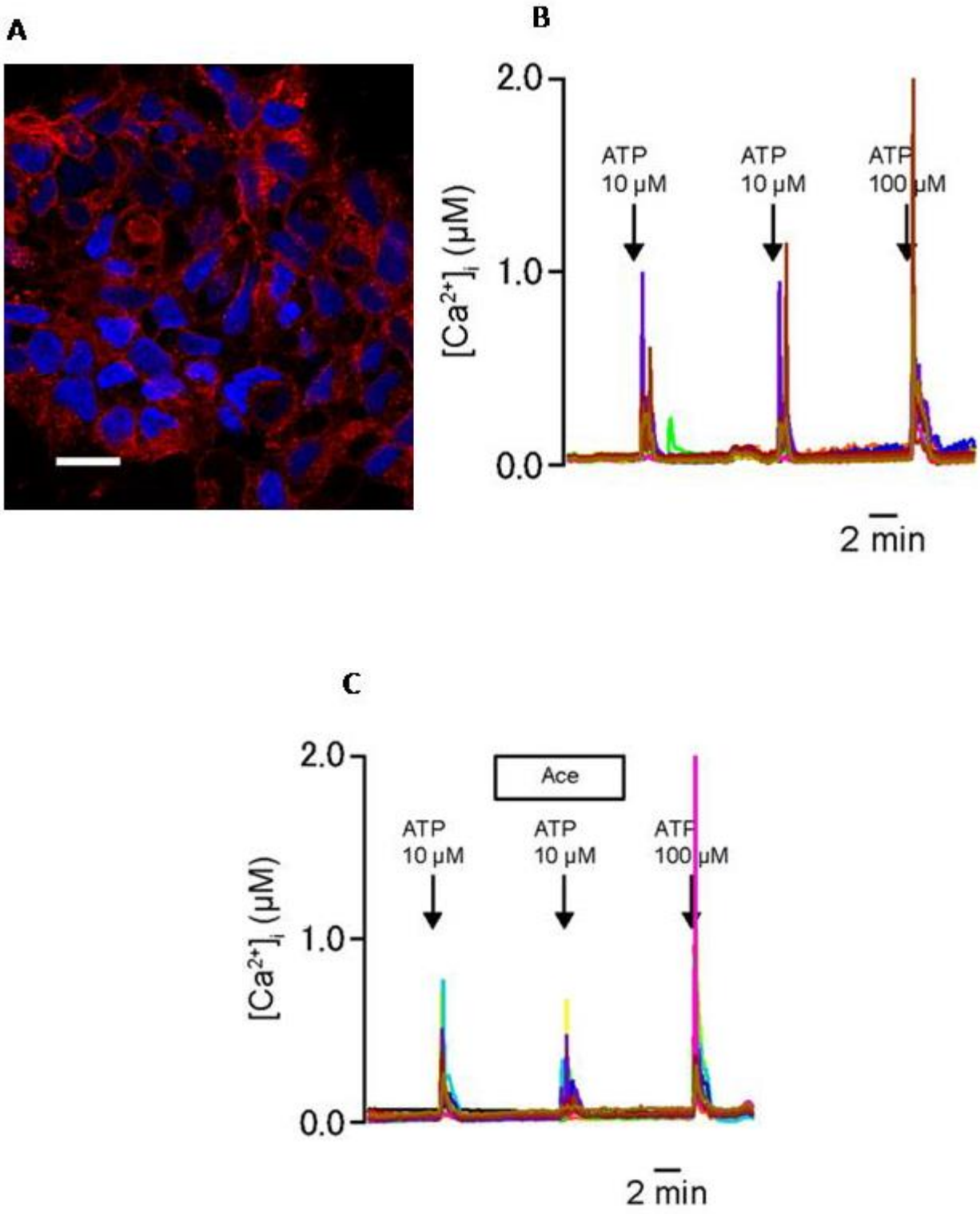
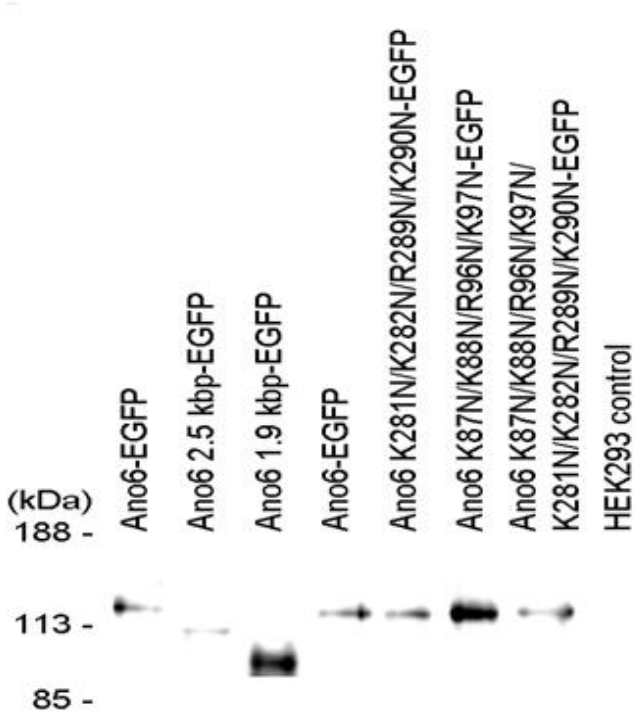

A
B
2.0
1.0
0.0
[Ca2+]i (μM)
ATP
10 μM
ATP
10 μM
ATP
100 μM
2 min
C
2.0
1.0
0.0
[Ca2+]i (μM)
Ace
ATP
10 μM
ATP
10 μM
ATP
100 μM
2 min

Figure S3

A


(kDa)
188 -
113 -
85 -
Ano6-EGFP
Ano6 2.5 kbp-EGFP
Ano6 1.9 kbp-EGFP
Ano6-EGFP
Ano6 K281N/K282N/R289N/K290N-EGFP
Ano6 K87N/K88N/R96N/K97N-EGFP
Ano6 K87N/K88N/R96N/K97N/ K281N/K282N/R289N/K290N-EGFP
HEK293 control

B

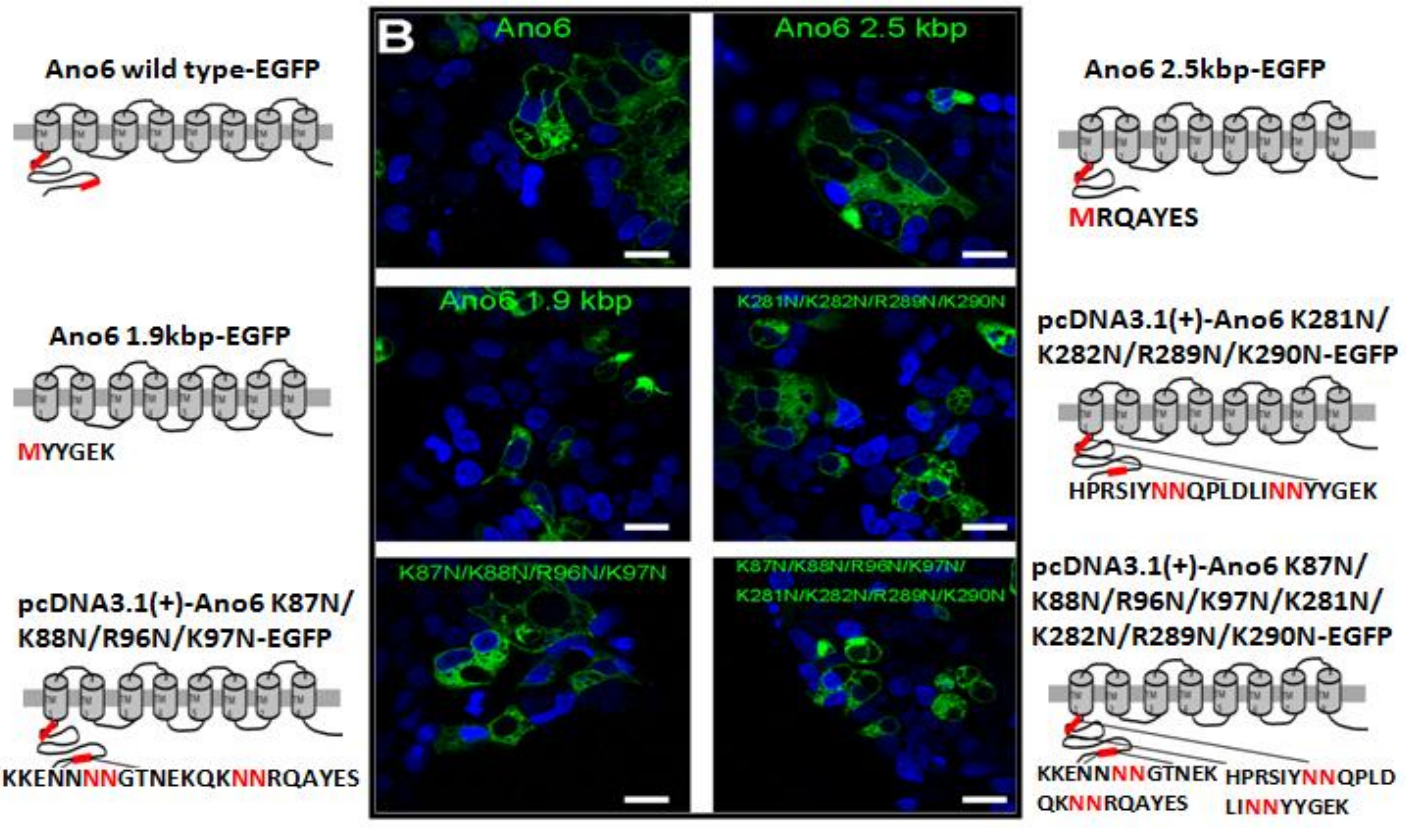

Ano6 wild type-EGFP
Ano6 2.5kbp-EGFP
MRQAYES
Ano6 1.9kbp-EGFP
MYYGEK
pcDNA3.1(+)-Ano6 K281N/ K282N/R289N/K290N-EGFP
HPRSIYNNQPLDLINNYYGEK
pcDNA3.1(+)-Ano6 K87N/ K88N/R96N/K97N-EGFP
KKENNNNGTNEKQKNNRQAYES
pcDNA3.1(+)-Ano6 K87N/ K88N/R96N/K97N/K281N/ K282N/R289N/K290N-EGFP
KKENNNNGTNEK QKNNRQAYES
HPRSIYNNQPLD LINNYYGEK
B
Ano6
Ano6 2.5 kbp
Ano6 1.9 kbp
K281N/K282N/R289N/K290N
K87N/K88N/R96N/K97N
K87N/K88N/R96N/K97N/ K281N/K282N/R289N/K290N

Figure S4

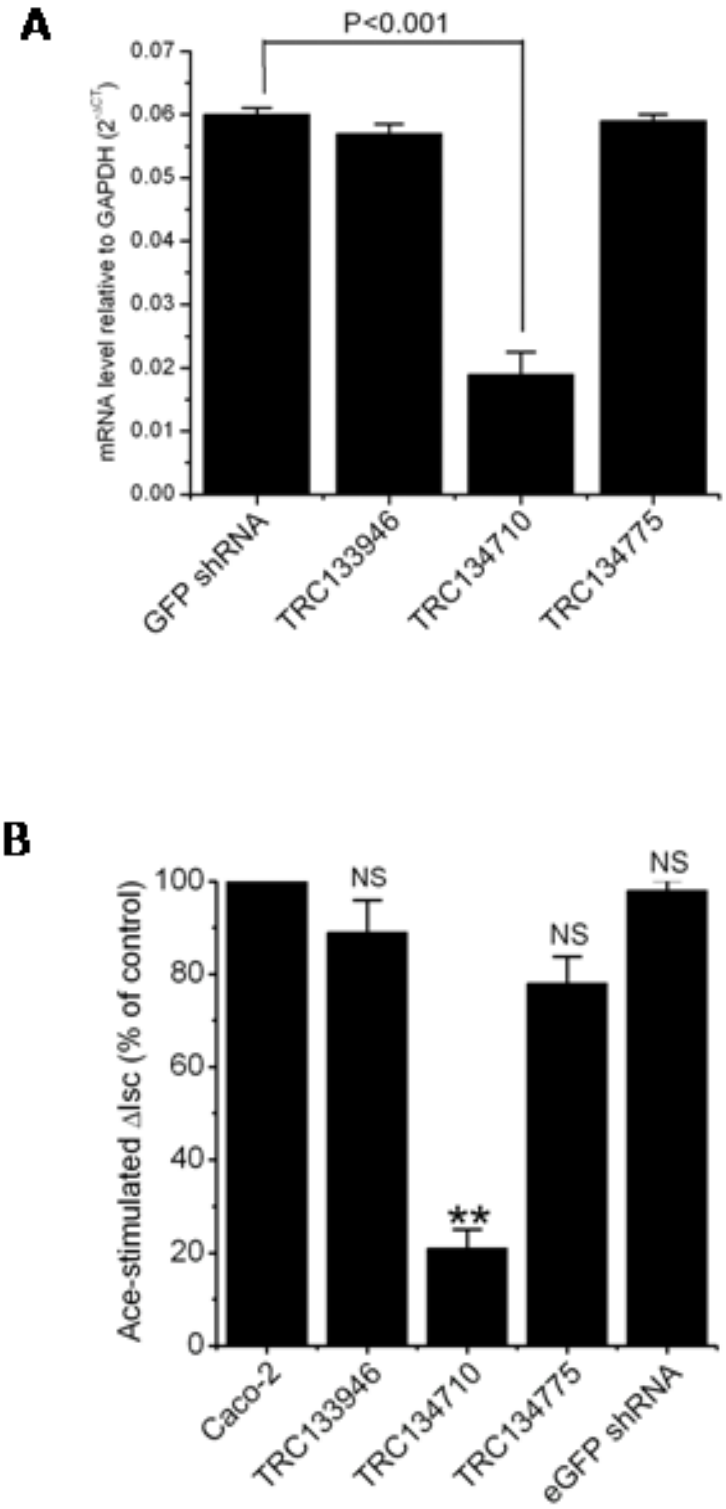
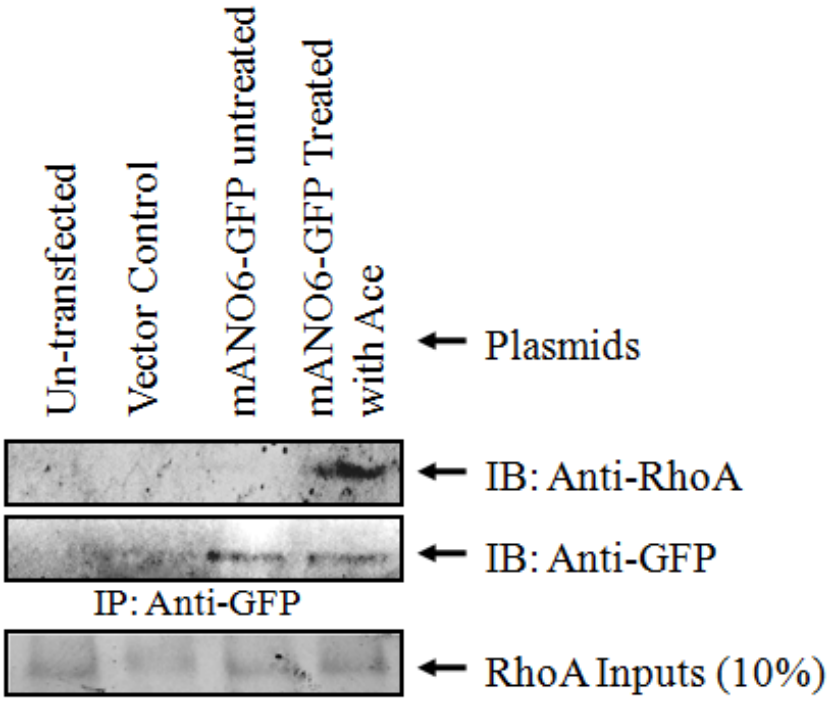
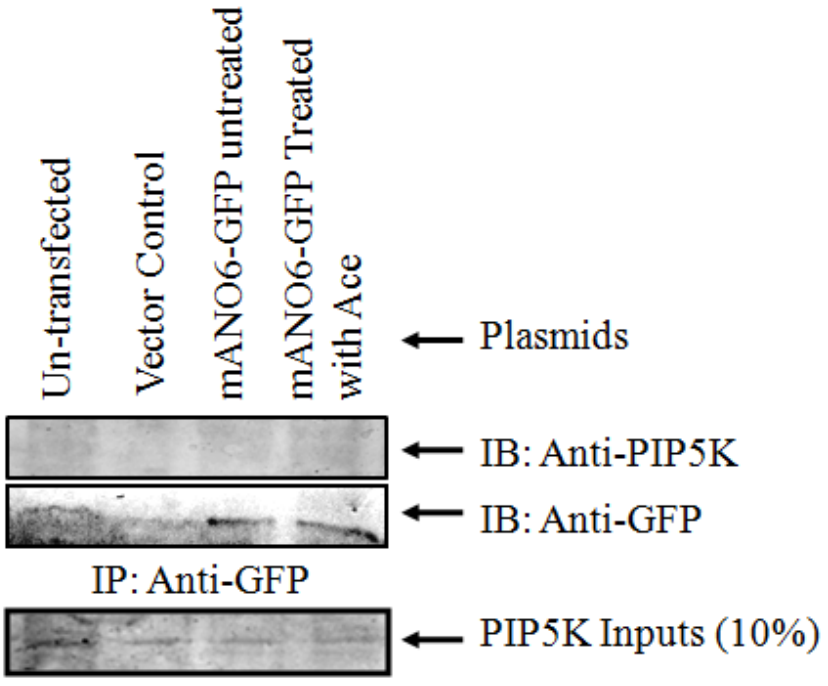

A
P<0.001
mRNA level relative to GAPDH (2^-ΔCT)
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0.00
GFP shRNA
TRC133946
TRC134710
TRC134775
B
Ace-stimulated ΔIsc (% of control)
100
80
60
40
20
0
NS
NS
NS
**
Caco-2
TRC133946
TRC134710
TRC134775
eGFP shRNA

Figure S5

A


Un-transfected
Vector Control
mANO6-GFP untreated
mANO6-GFP Treated with Ace
Plasmids
IB: Anti-RhoA
IB: Anti-GFP
IP: Anti-GFP
RhoA Inputs (10%)

B


Un-transfected
Vector Control
mANO6-GFP untreated
mANO6-GFP Treated with Ace
Plasmids
IB: Anti-PIP5K
IB: Anti-GFP
IP: Anti-GFP
PIP5K Inputs (10%)