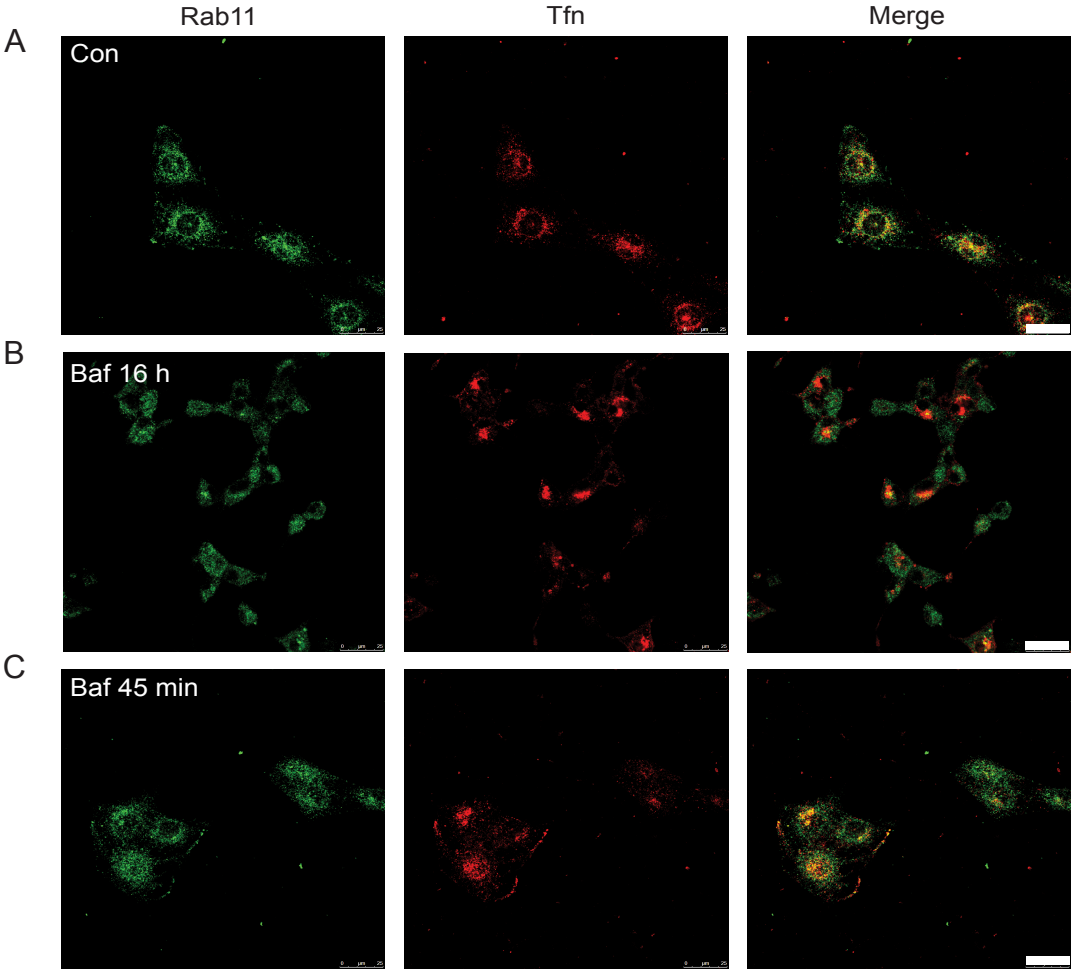
Supplemental Materials

Molecular Biology of the Cell

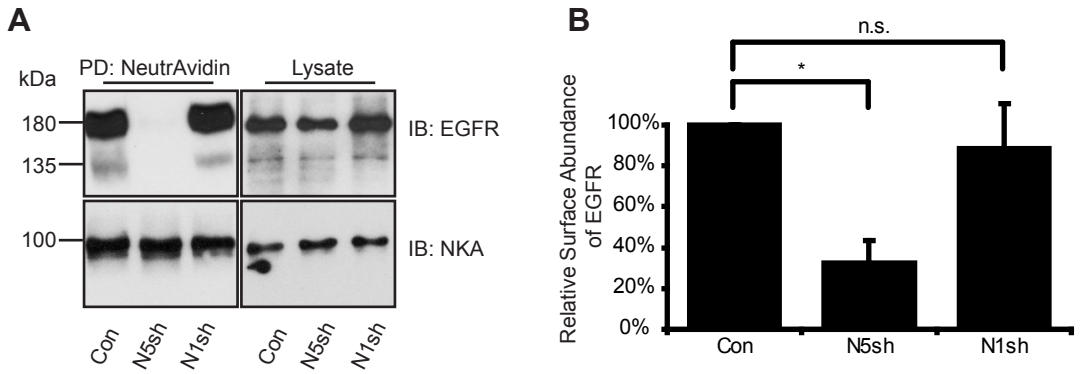
Fan et al.

Supplementary Figure 1



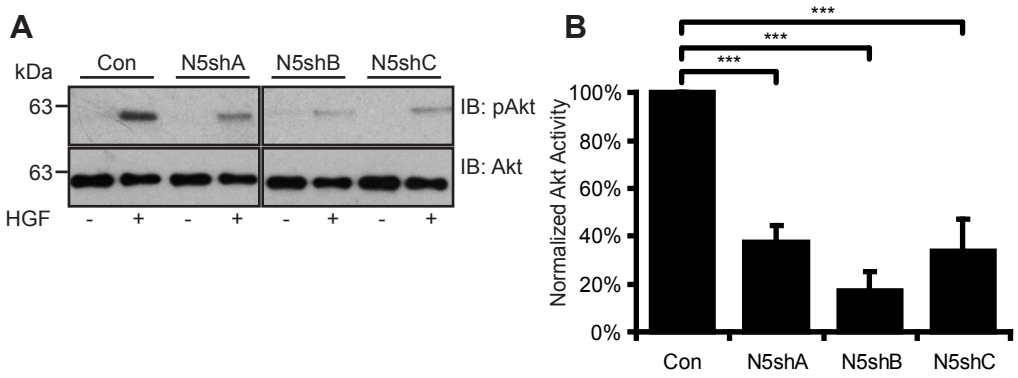
Supplementary Figure 1. Prolonged incubation with Bafilomycin A1 (Baf) causes clustering of transferrin (Tfn). C6 cells were serum starved for 16 h in the absence (A) or presence (B) of 50 nM of Baf, followed by incubation with 30 µg/mL Tfn-Alexa Fluor 568 in the same media at 37 °C for 30 min, and chased for 15 min. When cells were treated with 200 nM of Baf for 45 min. (C), clustering of Tfn was not detected. Cells were fixed and labelled with an anti-Rab11 antibody and Alexa Fluor 488-conjugated secondary antibody as described in Materials and Methods. Bars = 25 µm.

Supplementary Figure 2



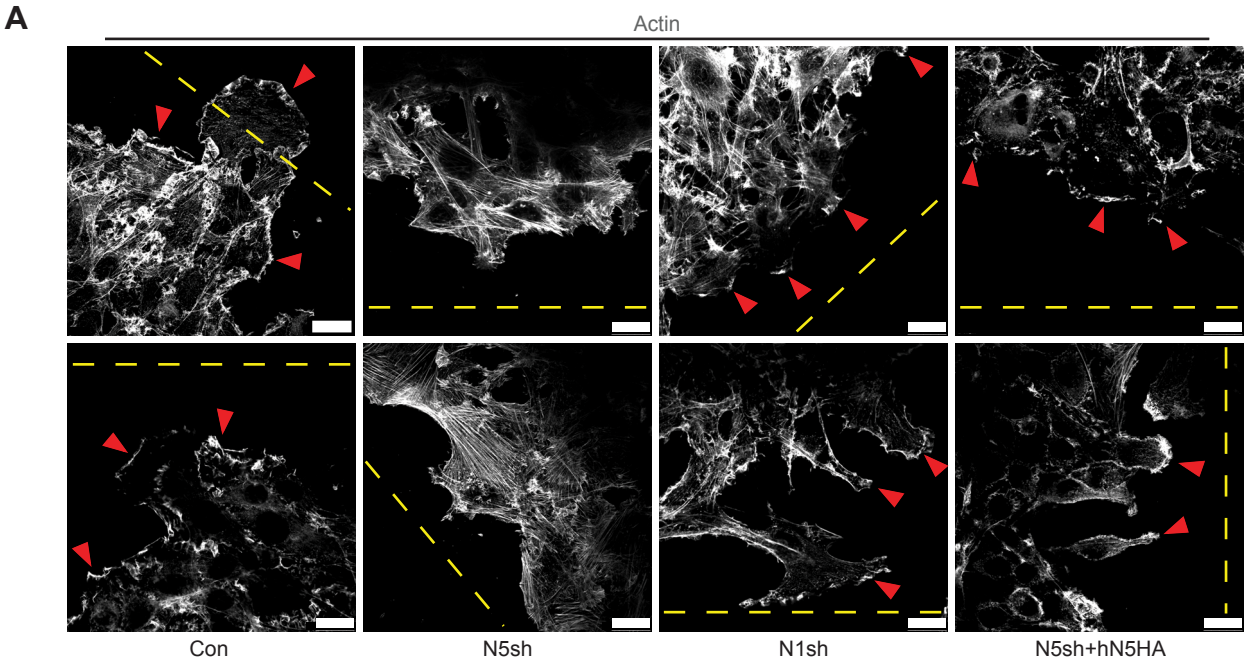
Supplementary Figure 2. Surface expression of EGFR is reduced in NHE5-deficient cells. (A) Control cells (Con), NHE5shA cells (N5sh) and NHE1sh cells (N1sh) were treated with a membrane impermeable biotinylating reagent. Equal amounts of proteins (100 µg) were affinity purified with NeutrAvidin beads and eluted proteins were analyzed by immunoblotting using an anti-EGFR antibody or an anti-Na+/K+ ATPase α-subunit (NKA) antibody. A representative immunoblot was shown. (B) Surface expressions of EGFR were quantified by densitometry and expressed as relative values to the control. The graph presents mean ± SD of three experiments. P values were obtained by Tukey-Kramer test following ANOVA, $F(2,6) = 6.41$ $p = 0.03$. * = $p<0.05$, n.s. = not significant.

Supplementary Figure 3



Supplementary Figure 3. HGF-induced phosphorylation of Akt is diminished in NHE5-knockdown cells. (A) Serum-starved control cells (Con) and N5shRNA cells (N5shA, N5shB and N5shC) were stimulated by 50 ng/mL of HGF for 5 min and Akt-phosphorylation was examined. The same amount of cell lysates were resolved in an SDS-PAGE gel and phosphorylated Akt and total Akt were detected by immunoblotting. Representative immunoblots are shown. (B) Akt-activity was quantified by densitometric analysis of phosphorylated and total Akt. The activity of each cell line was normalized to the level of control cells. The bars represent mean ± SD of three experiments. P-values were obtained by Tukey-Kramer test following ANOVA, $F(3, 8) = 53.48$, $p < 0.0001$. *** = $p < 0.001$.

Supplementary Figure 4



Supplementary Figure 4. Rac1 activation is impaired by NHE5-knockdown.
(A) The actin cytoskeleton was visualized by fluorescently labelled phalloidin during directional migration towards an open space introduced to a confluent monolayer. Images were focused on the bottom layer of leading edge in each image. Yellow dashed lines are imaginary lines paralleled to the wound edge. Red arrow heads point to membrane ruffle structures. NHE5-deficient cells exhibit extensive stress fiber formation at the migrating edge, implicating reduced actin dynamics in this area. This phenotype is partially rescued by re-expression of human NHE5. Bars = 25 μm.
(B) Quantification of positive membrane ruffling in migrating control, N5sh, N1sh, and N5sh+hN5HA cells. Data represent mean ± SD of 8 images per cell line; total number of cells counted are N=224 for control, N=392 for N5sh, N=224 for N1sh, and N=339 for N5sh+hN5HA cells. P-values were obtained by Tukey-Kramer test following ANOVA, $F(3,32) = 23.8$, $p < 0.0001$, using images as the unit of analysis. *** = $p < 0.001$, n.s. = not significant.
(C) Serum-starved control and N5sh cells were stimulated with or without 50 ng/mL of HGF for 5 min. Lysates were subjected to PAK pulldown and immunoblot analysis.
(D) Control cells were incubated in growth media for 24 h with or without 100 μM of Rac1 inhibitor, NSC-23766.
(E) Rac1 associated with membrane fractions was significantly reduced in NHE5-knockdown cells whereas neither association of Secretory Carrier Membrane Protein (SCAMP)1 with the membrane fraction nor association of Receptor for Activated C-Kinase RACK1 with the cytosolic fraction were affected. The same amount (2 μg) of membrane fractions and cytosolic fractions were analyzed by immunoblot.