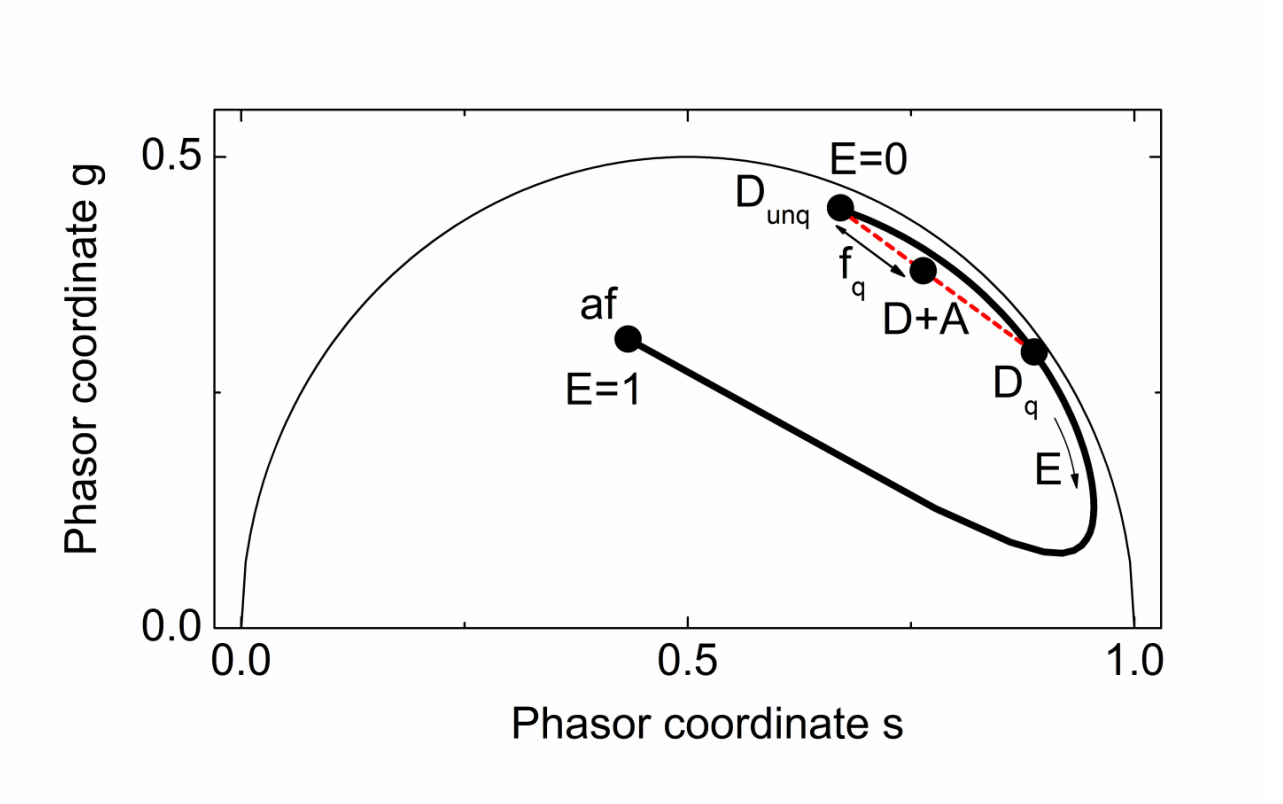
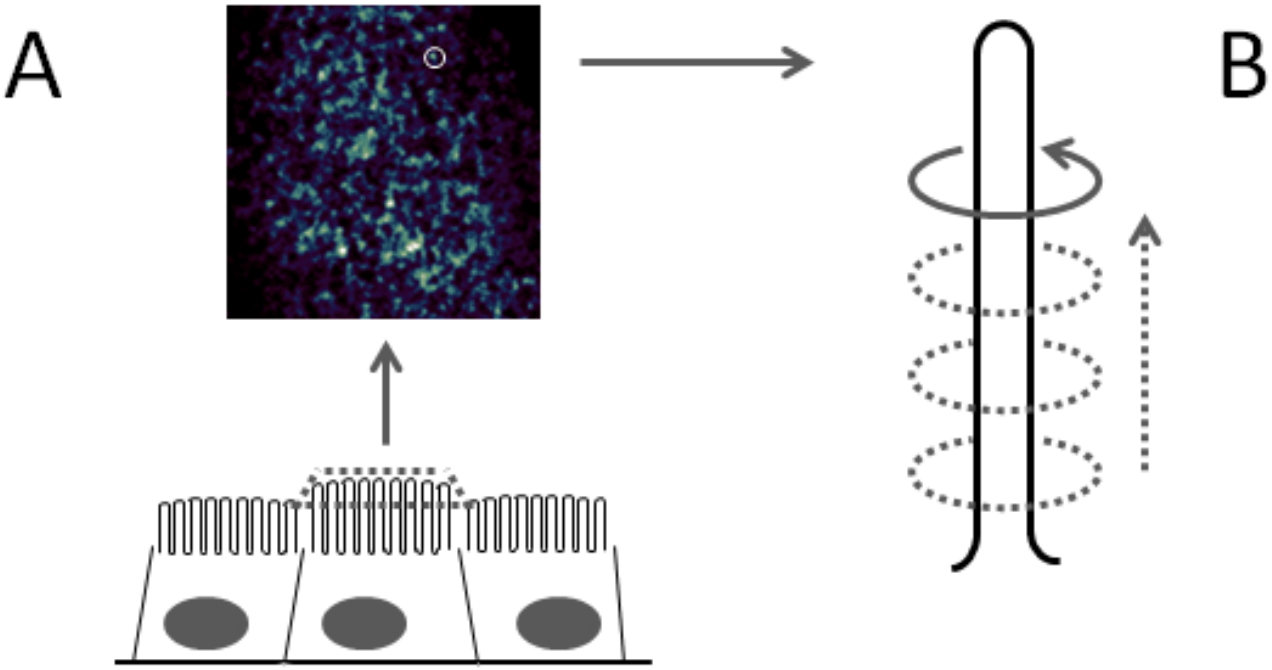
Supplementary figure 1



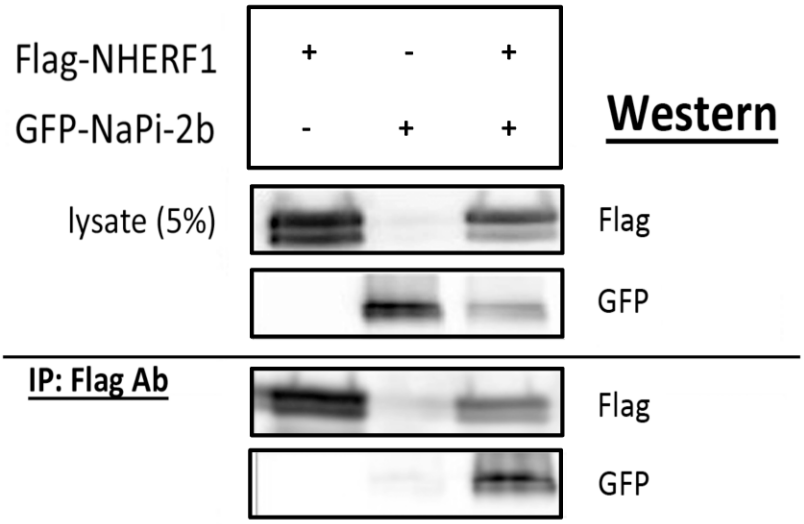
Supplementary Figure 1. Representative diagram of phasor approach and calculations of FRET efficiency and fraction of interacting donors. Frequency-domain lifetime measurements at each of the pixels of an image resulted in a value of the modulation and phase that were used to determine the radial and angular coordinates in the phasor plot. The phasor associated to each image was determined as the average phasor of the pixels corresponding to the cell apical membrane and the phasor of the autofluorescence (af) was determined by imaging non-transfected cells. For each experiment, the unquenched donor (D_{unq}) and the af phasor have been determined and a trajectory between them has been calculated (solid line). This trajectory represent the FRET efficiency (E) varying from E=0 at the D_{unq} position to E=1 at the af phasor position. Any phasor along this trajectory corresponds to pure species of donor quenched (D_q) with FRET efficiency E. However, in a given experiment each pixel may contain a mixture of quenched and unquenched donors with relative fractions f_q and f_{unq} respectively. In order to quantify FRET results we analyzed the shift of the phasor of the donor in presence of the acceptor with respect to the donor only and we can trace a segment (dashed line) from the only donor (D_{unq}) to the donor + acceptor (D+A) phasors that will also intersect with the efficiency trajectory. This intersection point is used to calculate the FRET efficiency (E) since this point represents the maximum FRET detectable if all the donors were ideally paired with an acceptor (D_q). Moreover, we can also calculate the fraction of donors undergoing FRET (f_q) from the distance of the phasor D+A from D_{unq} divided by the length of the entire segment.

Supplementary figure 2



Supplementary Figure 2. Schematic representation of the Modulation Tracking (MT) imaging method. A) First a raster scan is performed with the confocal microscope to localize the microvilli at the apical membrane. One isolated microvillus is selected and the scanner is pointed on its coordinates. **B)** The selected microvillus is tracked by scanning the laser spot in circular orbits around the microvilli. A single particle tracking algorithm based on the calculation of the Fast Fourier Transform (FFT) of the intensity along the orbit is used to keep the microvillus always at the center of the scanned orbit. The position of the microvillus is tracked while scanning at different sections along its length to obtain a 3D image of the microvillar surface. The orbit period is set to 8ms and the position of the center of the scanning orbit is updated every 64ms, a time resolution sufficient to follow the relatively slow movements of the microvillus. The final 3D reconstruction of the microvilli surface is painted in color scale according to the fluorescence intensity recorded in one channel (Figure 4).

Supplementary Figure 3



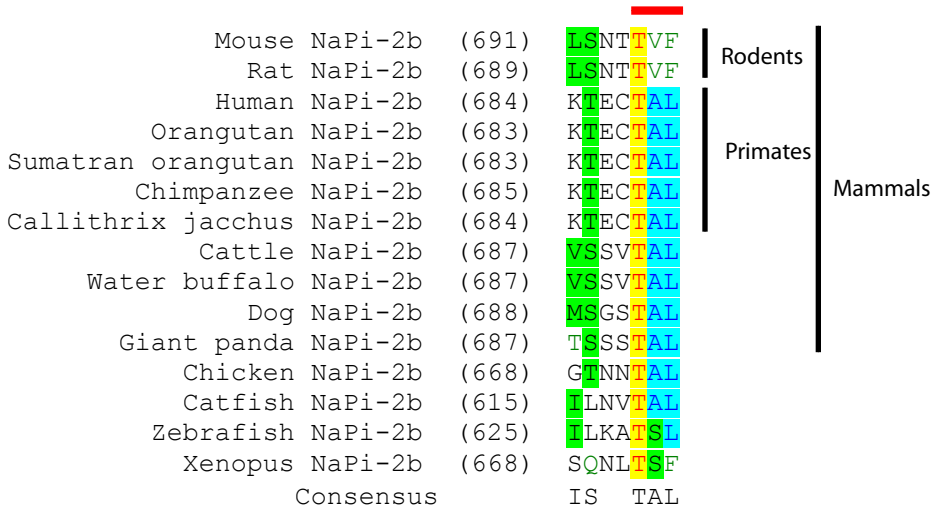
Supplementary Figure 3. GFP-NaPi-2b co-precipitates with Flag-NHERF1. HEK cells were transfected with Flag-NHERF1, GFP-NaPi-2b or both constructs. In the upper panel, Western blotting of the total lysates demonstrates the fusion proteins are appropriately expressed. In the lower panel, following Flag co-precipitation, Western blotting showed GFP-NaPi-2b was co-precipitated when cells were co-transfected with Flag-NHERF1.

Supplementary figure 4

A

Class I	-X- S/T -X- ϕ
Class II	-X- ϕ -X- ϕ
Class III	-X- D/E -X- ϕ

ϕ- hydrophobic residue
X- any residue



Mouse- *Mus musculus*, NP_035532.2; Rat- *Rattus norvegicus*, NP_445832.1; Human- *Homo sapiens*, AAI46667.1; Orangutan- *Pongo pygmaeus*, Q5REV9; Sumatran orangutan- *Pongo abelii*, NP_001124770; Chimpanzee- *Pan troglodytes*, XP_003310312; *Callithrix jacchus*, XP_002745982; Cattle- *Bos Taurus*, Q27960; Water buffalo- *Bubalus bubalis*, ADW66550; Dog, *Canis lupus familiaris*, XP_545968; Giant panda- *Ailuropoda melanoleuca*, XP_002924589; Chicken- *Gallus gallus*, NP_989805; Catfish- *Pelteobagrus fulvidraco*- ADM18964; Zebrafish- *Danio rerio*, NP_571699; Xenopus- *Xenopus laevis*, NP_989302.

B

Species	CFTR	NHE3	NaPi-2a	NaPi-2b	NaPi-2c
Human	-D-T-R-L	-S-T-H-M	-A-T-R-L	-C-T-A-L	-S-Q-Q-L
Mouse	-E-T-R-L	-S-T-H-M	-A-T-R-L	-T-T-V-F	-S-Q-Q-L
Rat	-E-T-R-L	-S-T-H-M	-A-T-R-L	-T-T-V-F	-S-Q-Q-L

Supplementary Figure 4. Evolutionary conservation of NaPi-2b putative PDZ-binding site at its C-terminus. A) Comparison of NaPi-2b protein sequences from different species were performed by ClustalW alignment analysis. The alignment of the last 7 amino acids on the C-terminal tail of 15 different species is shown. All the sequences analyzed fitted on the class I PDZ-binding motif consensus. Sequence accession numbers have been included below the table. **B)** The 4 last amino acids of the C-terminal tails of different epithelial transporters are shown in the table. Most of these transporters present also a class I PDZ-binding motif including CFTR, NHE3, NaPi-2a and NaPi-2b. The exception is NaPi-2c transporter that contains a sequence that is not perfectly matching with any of the described consensus motifs.