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# Identification of a Carboxyl Terminal Motif Essential for The Targeting of $Na^+$:$HCO_3^-$ Cotransporter NBC1 to The Basolateral Membrane

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Running title: A Basolateral Membrane Targeting Domain in NBC1

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## Abstract

The $Na^+$:$HCO_3^-$ contransporter NBC1 is located exclusively on the basolateral membrane and mediates vectorial transport of bicarbonate in a number of epithelia, including kidney and pancreas. To identify the motifs that direct the targeting of kidney NBC1 to basolateral membrane, wild type and various carboxyl (C)-terminally truncated kidney NBC1 mutants were generated, fused translationally in-frame to GFP, and transiently expressed in kidney epithelial cells. GFP was linked to the N terminus of NBC1 and labeling was examined by confocal microscopy. Full length (1035 a.a.) and mutants with 3 or 20 amino acid deletion from the C-terminal end of NBC1 (lengths 1032 and 1015 a.a., respectively) showed strong and exclusive targeting on the basolateral membrane. However, deletion of 26 amino acid residues from the C-terminal end (length 1010 a.a.) resulted in retargeting of NBC1 to the apical membrane. Expression studies in oocytes demonstrated that the NBC1 mutant with 26 amino acid residues deletion from the C-terminal end is functional. Additionally, deletion of the last 23 amino acids or mutation in the conserved residue F (phenylalanine) at position 1013 on the C-terminal end demonstrated retargeting to the apical membrane. We propose that a carboxyl terminal motif, with the sequence QQPFLS, which spans amino acid residues 1010 to 1015, and specifically the amino acid residue F (position 1013), are essential for the exclusive targeting of NBC1 to the basolateral membrane.

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## Introduction

The $Na^+$:$HCO_3^-$ contransporter NBC1 mediated bicarbonate transport and is located on the basolateral membrane of kidney proximal tubule and a number of other epithelia including pancreatic duct, gastric parietal cells, small intestine, and pulmonary alveolar cells (1-6). NBC1 functions in tandem with apical $Na^+/H^+$ exchanger 3 (NHE3) and is essential for bicarbonate reabsorption in kidney proximal tubule (4, 7, 8). In pancreas, NBC1 is responsible for bicarbonate transport into pancreatic duct for eventual secretion into the pancreatic duct (4, 9, 10). Two well known variants of NBC1 are expressed in various epithelial tissues. These are the kidney (kNBC1) and pancreatic (pNBC1) variants, which differ only in their N-terminal sequence, where amino acids 1 to 41 in kNBC1 has been replaced with 85 amino acids in pNBC1 (3). The carboxyl terminal end of NBC1 is identical in kidney and pancreatic variants (1-3). Kidney NBC1 has a stoichiometry of 3 equivalent of bicarbonate for each sodium whereas pancreatic NBC1 has a stochiometry of 2 equivalents of bicarbonate per each sodium (11,12). This alteration in stochiometry allows for the kidney NBC1 to function in the absorptive mode and for the pancreatic NBC1 to function in the secretory mode (4, 9-12).

In all tissues examined to date NBC1 is found to be located on the basolateral membrane of epithelial cells (4-6) strongly suggesting the presence of specific amino acid motif(s) that direct its targeting to the basolateral membrane. To identify possible motifs that are responsible for the targeting of NBC1 to the basolateral membrane, various C-terminally truncated NBC1 mutants were generated, fused translationally in-frame to GFP and transiently expressed in cultured kidney epithelial cells. The results demonstrate

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that deletion of amino acid residues 1010 to 1015, with the sequence QQPFLS, results in the retargeting of NBC1 preferentially to the apical membrane. Site directed mutagenesis of the conserved amino acid residue F (phenylalanine) at 1013 within this domain recapitulates the retargeting of NBC1 to the basolateral membrane. The sequence QQPFLS is preserved in NBC1 from various species and also in a number of transporters, especially the F (phenylalanine) among the sequence is very conservative in NBC1 protein from species to species and they may play an important role in basolateral targeting of these transport proteins.

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**Methods and Material.**

**Construction of GFP-NBC1 full length and mutants**. The full length and various C-terminally truncated NBC1 mutants were generated by PCR, using the human full length NBC1 DNA (3257bp and 1035 a.a residues) as a template (AF007216). The truncated NBC1 proteins have C-terminal deletions of 3, 20, 26, or 50 amino acid residues and are designated as NBC1 CD3, CD20, CD26, or CD50, respectively. The resulting wild type and various C-terminally truncated NBC1 mutants were amplified and fused translationally in-frame to GFP by cloning into pcDNA3.1/NT-GFP-TOPO vector (Invitrogen, Carlsbad, California).

Full length kNBC1 was amplified using the following primers: t986730: 5' GAATTCGAGATGGCACTGAAAATGTGGAAGGG (sense) and t968120: 5' AGCGGCCGCCTCAGCATGATGTGTGGCGTTCAAGGAATGT (antisense). These primers were used to generate full length NBC1 cDNA from PCI-NEO NBC1 plasmid (1). This resulted in the expression of GFP-NBC1 fusion protein with the GFP fusing to the N-terminus of kNBC1 (GFP-NBC1). Using a similar approach, primer 1 (sense, above) and primer 3 (t80925: 5'GCGGCCGCTCAGTGGCGTTCAAGGAATGTTGGTGA (antisense), were used to generate the NBC1-CD3 cDNA. This resulted in the expression of the GFP-NBC1 fusion protein, which coded the truncated NBC1 (CD3), with deletion of the last 3 amino acid residues on the carboxyl terminal end. For CD20, CD26, and CD50 (deletion of the last 20, 26, and 50 amino acid residues, respectively) the following (antisense) primers were used : CD20 3' primer: 5' GCGGCCGCTCAGCAGCTTAGGAA AGGTTGCTGTTCCATGATGTCCATTGGAAT3'; CD26 3' primer--- 5'

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GCGGCCGCTCAGCAGCTTAGGAAAGGTTGCTATTCCATGATGTCCATTGGAA T3'; and CD50 3' primer--- 5'TCAGTC ACTGTCCAGACTTCCCTTCTT3'. In separate experiments we also generated NBC1 CD67 construct (67 amino acid deletion) using 3' primer : 5' CTATTTCTTGTCCTTTTCTGGAATGAC3'. For the last series of experiments we generated NBC1 CD23 construct (23 amino acid deletion) using 3' primer : 5' GCGGCCGCTCAAGGTTGCTGTTCCATGATGTCCATTGGAAT 3'. The sense primer in all mutants was the same as in full length NBC1 (above).

The diagram shown in Fig. 1 depicts the schematic constructs of human NBC1 truncated mutants.

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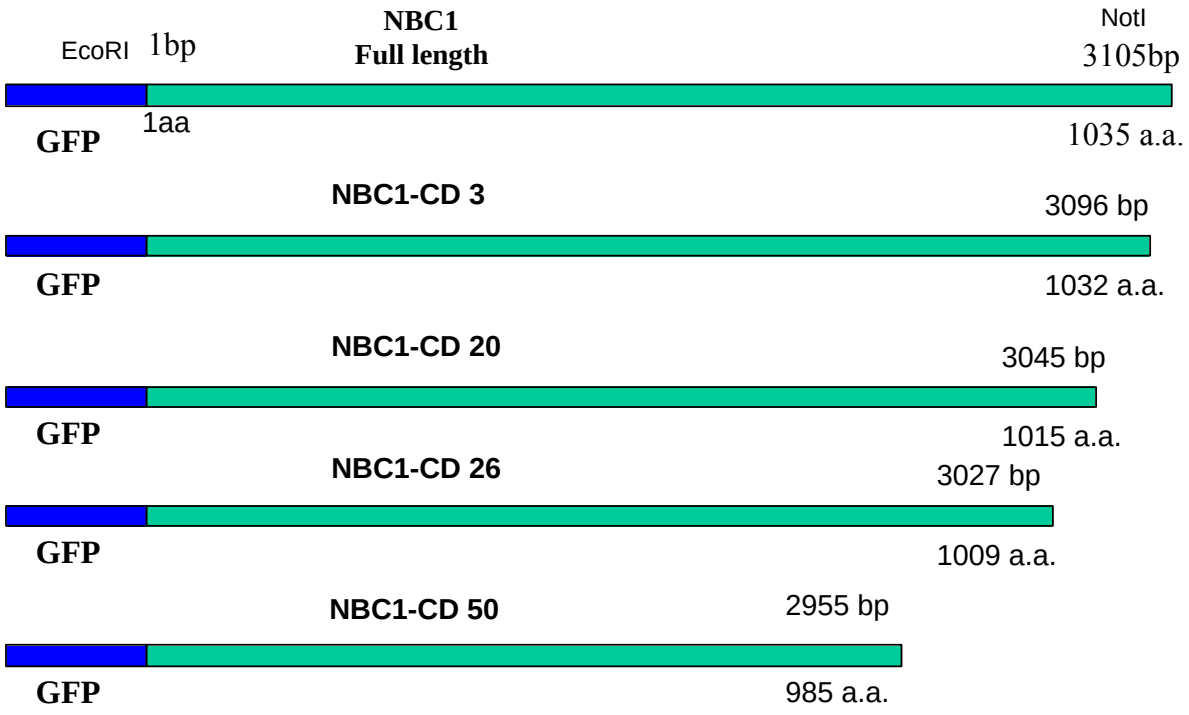
Schematic Diagram Demonstrating Various Human NBC1 Truncated Mutants With Carboxyl Terminal Deletion




CD: Carboxyl Terminal Deletion

**Site directed mutagenesis.** Single mutation of the conserved residue F (phenylalanine) at position 1013 was performed according to established methods and using manufacturer's instruction (QuickChange, Site-Directed Mutagenesis Kit, Strategene, LaJolla, CA). The primers used to mutate F to A at position 1013 were 5' GACATCATGGAACAGCAACCTGCCCTAAGCGATAGC3' and 5' GCTATCGCTTAGGGCAGGTTGCTGTTCGATGATGTC 3'.

**Transient expression of GFP-NBC1 and GFP-NBC1 mutants in MDCK cells.** MDCK cells were transiently transfected with the GFP-NBC1 and GFP-NBC1 mutants and studied 48 hrs later according to established methods (2, 13). Briefly, cells were plated in 24 well plates and transfected at 80% confluence using 1 μg of DNA and 4 μl of Lipofectamine 2000 (Invitrogen, Carlsbad, California). The transfection efficiency was monitored using PCMV-SPORT β-gal plasmid as control. A change in cell color in response to X-gal addition was used as a marker for beta-gal expression. All cells were co-labeled with peanut lectin or phalloidin as markers of apical membrane labeling or cell membrane labeling.

**Con-focal microscopy and immunofluorescence labeling.** MDCK cells were washed 3 times with PBS, fixed 20 min with 3% formaldehyde in PBS and washed 3 more times with PBS. Afterward, cells were permeabilized for 4 min with 0.1% TX-100 in PBS, washed 3 times with PBS and co-stained with PNA-lectin (Molecular Probes, Eurogene, Oregon) or the selective F-actin dye TRITC-phalloidin (Sigma, St. Louis, MO). Afterward cells were washed 3 times with PBS, mounted on glass slides in Slow Fade with DAPI (Molecular probes). Images were taken on a Zeiss LSM510 confocal microscope. Both Z-line and Z-stack images were obtained using the LSM 5 Image

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software (14) in order to analyze the membrane targeting of GFP-NBC1 full length and mutant constructs.

**Functional expression of full length GFP-NBC1 and GFP-NBC1 mutants in oocytes.** Stage IV-V oocytes were isolated as previously described and used for expression studies according to established methods(15). The capped GFP-NBC1 (full length), GFP-NBC1 CD26 (with 26 amino acid residues deletion at the C-terminal end) and GFP only (no NBC insert) cRNAs were generated using mMESSAGE mMACHINE$^{TM}$ T7 Kit (from AMBION INC., AUSTIN, TX) according to manufacturers instruction. Fifty nanoliters cRNA (0.5 µg/µl) was injected with a Drummond 510 microdispenser via a sterile glass pipette with a tip of 20-30 µm. After injection the oocytes were maintained in a solution of the following composition (in mM): 96 NaCl, 2.0 KCl, 1.0 $MgCl_2$, 1.8 $CaCl_2$, 5 HEPES, 2.5 Na pyruvate, 0.5 theophylline, 100U/ml penicillin and 100 µg/ml streptomycin; pH 7.5. Injected oocytes were stored in an incubator at $17^0$C and were used for electrophysiological experiments after 2-4 days. We did not perform intracellular pH recording with BCECF on GFP constructs due to interference of the GFP signal with BCECF recording. However, pHi studies were performed on NBC1 mutants without GFP insert.

***Electrophysiology studies.*** Oocytes were placed on a nylon mesh in a perfusion chamber and continuously perfused (3 ml/min perfusion rate). The perfusion solution had the following composition (in mM): NaCl 96, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8 and Hepes 15 (pH 7.5). After a stabilization period, when the membrane potential (Vm) was constant, the perfusion solution was switched to a $CO_2$/bicarbonate-containing solution of the following composition (in mM): $NaHCO_3$ 30, NaCl 66, KCl 2, $MgCl_2$ 1, $CaCl_2$ 1.8 and

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Hepes 15 (pH 7.5 and gassed with 5% $CO_2$). Experiments were performed at room temperature (22-25$^0$C). Membrane potentials were recorded with conventional microelectrode techniques by glass microelectrodes (resistance 3-5 mΩ) filled with 3M KCl and connected to an Axoclamp 2A amplifier (Axon Instruments, Foster City, CA) (15). The digitized signals were stored and analyzed on a personal computer using Axotape (Axon Instruments, Foster City, CA).

***Statistics.*** Results are given as SEM. Statistical comparisons between the groups were performed by ANOVA. The data were considered significant if $p < 0.05$.

***Materials.*** High-fidelity PCR Amplification Kit, GFP Fusion TOPO TA Expression Kit and Lipofectamine$^{TM}$ 2000 were purchased from Invitrogen (Carlsbad, CA). mMESSAGE mMACHINE$^{TM}$ T7 Kit was purchased from AMBION INC (Austin, TX). EndoFree Plasmid Maxi Kit was purchased from QIAGEN (Valencia, CA). DMEM medium was purchased from Life Technologies. All other chemicals were purchased from Sigma Co. (Saint Louis, MO).

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## Results

In the first series of experiments we examined the expression and targeting of GFP with or without the NBC1 insert in MDCK cells. Figure 1a (left and right panels) shows that transfection of cultured cells with GFP vector alone (no NBC1 insert) results in the accumulation of GFP in the cytoplasm (Z-line images in left panel) with no localization on the membrane (Z. stack images in right panel). However, transfection with GFP-NBC1 full-length cDNA shows exclusive localization of the GFP in the membrane (as visualized by Z-stack, front view) in cells co-labeled with phalloidin (Fig. 1b). The specific membrane labeling (apical vs. basolateral) was examined using the Z-line image (side view) analysis. The results are demonstrated in Fig. 1c. Similar studies were performed with the GFP-NBC1 construct in MDCK cells co-labeled with PNA-lectin dye, a very specific marker of apical membrane labeling in polarized cells (16). The Z-line (side view) images of the results (NBC1-GFP and PNA-lectin double labeling) are depicted in Fig. 1d. As demonstrated, the full length NBC1 is targeted exclusively to the basolateral membrane domain (Fig. 1c and 1d). These results are consistent with published reports on the basolateral localization of NBC1 in epithelial cells (4-6).

In the next series of experiments we examined the expression of GFP-NBC1 mutants with 3 (CD-3) or 20 (CD-20) amino acid deletion from their carboxyl terminal end (Method). Figure 2a is a Z-stack (front view) image analysis of the expression of GFP-NBC1 CD3 co-labeled with phalloidin in MDCK cells, showing GFP-NBC1 CD3 localization on the cell membrane. The Z-line (side view) image analysis of the expression studies doubly labeled with phalloidin or PNA-lectin demonstrate that NBC1-CD3 mutant is targeted to the basolateral membranes (2b and 2c). Figure 3 depicts

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similar results with NBC1 mutant lacking the last 20 amino acids from the C-terminal end. The Z-stack (front view) image analysis of the expression of GFP-NBC1 CD20 co-labeled with phalloidin (3a) is shown in MDCK cells and demonstrates exclusive localization on the cell membrane. The Z-line image analysis (side view) of the expression studies demonstrates that NBC1-CD20 mutant is targeted to the basolateral membrane (3b and 3c).

The next series of experiments examined the expression of the GFP-NBC1 mutant that lacked the last 26 amino acids of NBC1 carboxyl terminal end (CD26). Transfection studies were performed as in the above. The Z-stack analysis (front view) of the expression of GFP-NBC1CD26, co-labeled with phalloidin, shows strong membrane as well as sub-membrane labeling with the GFP (Fig. 4a). The Z-line (side view) image analysis of the results show the surprising and unexpected finding of apical membrane targeting of NBC1 (Fig. 4b and 4c). Experiments were performed with both phalloidin (4b) and PNA-lectin binding (4c). Both methods clearly confirm that deletion of the last 26 amino acids from the C-terminal end causes the mistargeting of NBC1 to the apical membrane (4b and 4c). Some images show residual labeling of the basolateral membrane (4b and 4c) but clearly the majority of the labeling is localized on the apical membrane with little cytoplasmic expression.

To determine whether further truncation of the carboxyl terminal end affects its membrane localization, NBC1 mutants with 50 amino acid residues deletion were generated, coupled to GFP and transiently expressed in MDCK cells. Figure 5a shows transfection of MDCK cells with the NBC1-CD50 mutant and indicates that GFP-NBC1 CD50 localized on the cell membrane as well as in the cytoplasm. As demonstrated in

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Fig. 5 (panels b and c), NBC1 mutant with 50 amino acid residues deletion shows persistence of labeling on the apical membrane. As noted, this mutant also demonstrates significant intracytoplasmic expression in addition to the apical labeling, indicating that progressive trun

cation of the C-terminal end of NBC1-beyond the last 26 amino acids on the C-terminal end may interfere with the targeting of NBC1 to cell membrane.

In the next series of experiments we examined the functional activity of NBC1-GFP CD26 mutant in oocytes by membrane potential measurement (1, 2, 14). For comparison, oocytes injected with GFP-NBC1 full length or GFP only (no NBC insert) cRNA were utilized. We were specifically interested in NBC1-CD26, as this is the mutant with the shortest truncation, which shows mistargeting to the apical membrane (Fig. 4). As demonstrated (Fig. 6, bottom panel, representative tracing), exposure to $CO_2/HCO_3^-$ in oocytes expressing the full length GFP-NBC1 (2-4 days after cRNA injection, 0.5 μg/μl) resulted in an immediate membrane hyperpolarization, which reached a peak within 1 min. The hyperpolarization showed very little decay during the time of exposure to $CO_2/HCO_3^-$ and was reversible upon returning to the $CO_2/HCO_3^-$ -free perfusion solution. This is consistent with published reports and indicates that NBC1 is highly electrogenic (1, 2, 15).

As noted, exposure to $CO_2/HCO_3^-$ in oocytes expressing GFP-NBC1 CD26 mutant (2-4 days after cRNA injection, 0.5 μg/μl) also caused significant membrane hyperpolarization (Fig. 6, middle panel, representative tracing). Control oocytes (GFP only) showed no alteration in membrane potential measurement in response to

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$CO_2/HCO_3^-$ exposure (Fig. 6, top panel, representative tracing). The summary results of the effect of $CO_2/HCO_3^-$ on membrane potential were – 114.6 ± 6.0 mV in full length GFP-NBC1-injected oocytes (n=5), -106.1 ± 5.6 mV in GFP-NBC1 CD26-injected oocytes (n=6) and -62 ± 3.8 mV in GFP alone injected oocytes (n=5). The baseline membrane potentials (before exposure to $CO_2/HCO_3^-$ ) were -49.4 ± 3.2 mV in full length GFP-NBC1-injected oocytes, –53.2 ± 4.1 mV in GFP-NBC1 CD26 –injected oocytes, and –50.9 ± 4.2 mV in GFP-only injected oocytes. As indicated, oocytes injected with either the full length or the mutant NBC1 showed significant hyperpolarization ($p<0.01$ vs. no $CO_2/HCO_3^-$) whereas GFP-only injected oocytes did not show any alteration in membrane potential in response to $CO_2/HCO_3^-$ exposure. The magnitude of hyperpolarization in response to $CO_2/HCO_3^-$ exposure was not different between the full length and the CD26 truncated mutant ($p>0.05$). Taken together, these results demonstrate that the GFP-NBC1 mutant with the deletion of the last 26 carboxyl terminal amino acid residues is functionally active.

In the last series of experiments we examined the membrane targeting of NBC1-CD23 and mutants in which the conserved residue at position 1013 on the C-terminal end was mutated (F1013A) by site directed mutagenesis. As demonstrated in Figure 7 (panel A), deletion of the last 23 amino acid residues causes the retargeting of NBC1 to the apical membrane, indicating that the sequence FLS (residues 1013-1015) is essential for the targeting of NBC1 to the basolateral membrane. To identify the amino acid residue(s) responsible for the exclusive targeting of NBC1 to the basolateral membrane, mutants in which the conserved residue F was mutated (F1013A) were used for transfection in MDCK cells. As demonstrated in Fig. 7 (panel B), the F1013A mutation caused the

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retargeting of NBC1 to the apical membrane.

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## Discussion

The targeting of NBC1 to the basolateral membrane was examined using a series of mutants with progressive truncation of the carboxyl terminal end that were fused in frame to GFP and visualized by confocal microscope. The results demonstrated that the full length NBC1 and mutants with up to 20 amino acid deletion from the carboxyl terminal end are targeted to the basolateral membrane (Figs. 1-3). However, the NBC1 mutant with 26 amino acids deletion from its C-terminal end showed mistargeting to the apical membrane domain, with some labeling observed on the basolateral membrane (Fig. 4). Additional truncations for up to 67 amino acid residues showed persistence of labeling on the apical membrane but also resulted in accumulation of mutant proteins in the cytoplasm (Figs. 5 and personal observation). Membrane potential recording in oocytes demonstrated that the NBC1 mutant with 26 amino acid residues deletion mediates $Na^+$:$HCO_3^-$ cotransport (Fig. 6), indicating that the apically targeted molecule is functionally active. Additionally, truncation of the last 23 amino acids or mutants in which the conserved residue F (phenylalanine) at position 1013 on the C-terminal end was mutated by site directed mutagenesis (F1013A) demonstrated retargeting to the apical membrane. We further find other truncated mutants (upto 67 amino acid deletion) are functional, though at a much smaller rate, when expressed in non-GFP forms and assayed by BCECF (data not shown).

NBC1 is located basolaterally in various epithelia and mediates vectroial transport of bicarbonate. In kidney, NBC1 is located on the basolateral membrane of proximal tubules and mediates the exit of bicarbonate from cell to blood (4-6, 17, 18). In pancreas, NBC1 is located on the basolateral membrane of ducts and mediates the entry of bicarbonate

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from blood to cell (4-6, 19, 20). Studies on NBC1 have demonstrated that the kidney variant (kNBC1) has a stoichiometry of 3 equivalent of bicarbonate per sodium whereas the pancreatic variant (pNBC1) has a stoichiometry of 2 bicarbonate per sodium (11, 12). The difference in the stoichiometry allows for the change in the direction of NBC1 movement from absorptive (i.e. in kidney) to secretory (i.e. in pancreatic duct).

The most salient feature of the current study is the identification of a carboxyl terminal motif and residues that are essential for the targeting of NBC1 to the basolateral membrane. It is noteworthy that in the absence of this motif (CD-23 or CD-26 amino acid deletion) or in mutants in which the conserved residue F (phenylalanine at position 1013) is mutated, NBC1 is predominantly mistargeted to the apical membrane rather than being accumulated in the cytoplasm. This observation raises the possibility that other existing motifs may be responsible for the targeting of NBC1 to the apical membrane. Alternatively it is plausible that the mistargeting of NBC1 to the apical membrane occurs by default (as a result of the deletion of basolateral targeting domain). Further deletion of the carboxyl terminal end beyond 26 amino acids (up to 67 a.a. residues) resulted in more intra-cytoplasmic accumulation of NBC1 (Fig. 5 and personal observations), indicating that the carboxyl terminal end may play important role in membrane targeting of NBC1.

The apically targeted NBC1 is functionally active (Fig. 6), raising the likelihood that possible mutations or deletions in the identified motif (QQPFLS) in human, do not impair its functional activity. However such a mutation or deletion may cause significant impairment in bicarbonate reabsorption due to the absence of an exit pathway for bicarbonate transport in the basolateral membrane. In other words, the mistargeting of NBC1 to the apical membrane may impair the vectroial transport of bicarbonate and lead

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to proximal tubular acidosis. Whether motifs on the amino terminal end of NBC1 play any role in basolateral membrane targeting of NBC1 remains speculative at the present and warrants detailed examination.

Recent studies have identified mutations in the $Cl^-/HCO_3^-$ exchanger AE1, which are associated with distal renal tubular acidosis, a bicarbonate wasting condition. AE1 is normally located on the basolateral membrane of alpha(A) intercalated cells in cortical and outer medullary collecting duct and, in tandem with apical H+-ATPase, is responsible for majority of bicarbonate reabsorption in these nephron segments (21, 22). Bicarbonate wasting in these subjects raised the possibility that AE1 mutants either became functionally inactive or were retained intracellularly. However, studies with AE1 mutants have been less conclusive (23-26) casting doubt on the explanations regarding bicarbonate wasting in humans with AE1 mutations. Very recent studies have deciphered this puzzle (16, 27). In studies in cultured polarized kidney epithelial cells, Dennovald et al and Rungroj et al showed that AE1 mutants tagged with GFP are mistargeted to the apical membrane (16, 27). Given the functionality of AE1 mutants in non epithelial cells, Denovald et al and Rungroj et al concluded that the mistargeting of AE1 to the apical membrane may actually worsen the bicarbonate wasting, as the apically located AE1 secretes bicarbonate into the lumen in exchange for luminal chloride (16, 27). This should lead to renal bicarbonate wasting and worsen distal renal tubular acidosis. It is worth mentioning that other mutations cause the retention of AE1 in the ER or in late endosomes/lysosomes therefore causing distal renal tubular acidosis by reducing bicarbonate exit across the basolateral membrane of the collecting duct (25, 28).

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A Genbank search of other members of NBC/AE superfamily demonstrated that a motif with high homology to the one identified in our studies is also expressed in NBC2, NBC4, NCBE, AE1 and AE2, all known bicarbonate transporters located on the basolateral membrane of epithelia. An analysis of other known chloride/bicarbonate or anion exchangers from SLC26 family which share little homology with NBC/AE superfamily demonstrates that those anion exchangers that are targeted to the basolateral membrane (SLC26A1 and SLC26A7) contain motifs with high homology to the current motif (Genbank NM_022042 for A1, from residue 675 to 678, with a.a. sequence QLFL, with full length of 701 a.a. ; Genbank NM_134266 for A7, from residue 343 to346, with a.a. sequence QEFL, with full length of 663 a.a.). However, SLC26 exchangers that are targeted to the apical membrane (SLC26A3 and SLC26A4) do not express any motif with high homology to the current motif.

In conclusion, NBC1 mutants lacking the last carboxyl terminal 23 or mutant in which the conserved residue F1013 mutated into A1013 show mistargeting to the apical membrane. We propose that a carboxyl terminal motif, with the sequence QQPFLS, which spans amino acid residues 1010 to 1015, and specifically the amino acid residue F(phenylalanine) at position 1013, are essential for the exclusive targeting of NBC1 to the basolateral membrane. Future studies should clarify the underlying mechanism of this targeting rearrangement and attempt to identify the molecules that interact with NBC1 through this motif.

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**Figure legends**

**Fig. 1. Transfection of MDCK cells with GFP vector only (no NBC1 insert) or GFP-NBC1 full length cDNA.**

**1a. Expression of GFP only (no NBC1 insert) . Left panel: Z-line image analysis; Right panel: Z-stack analysis.** The results indicate that transfection with GFP vector alone with no NBC1 insert results in the accumulation of GFP in the cytoplasm and does not reach plasma membrane. Green: NBC1-GFP; red: phalloidin.

**1b. Expression of GFP-NBC1 full length : Z-stack image analysis.** As demonstrated, full length NBC1 is targeted to the plasma membrane. Green: NBC1-GFP; red: phalloidin.

**1c. Expression of GFP-NBC1 full length : Z-line image analysis.** As demonstrated, full length NBC1 is exclusively targeted to the basolateral membrane. Green: NBC1-GFP; red: phalloidin.

**1d. Expression of GFP-NBC1 full length : Z-line image analysis.** Co-labeling with PNA-lectin which exclusively binds to apical membrane demonstrates the localization of full length NBC1 to the basolateral membraneGreen: NBC1-GFP; red: PNA-lectin.

**Fig. 2. Transfection of MDCK cells with GFP-NBC1 CD3 cDNA.**

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**2a. Expression of GFP-NBC1 CD3 : Z-stack image analysis.** As demonstrated, the NBC1 with deletion of the last 3 carboxy terminal amino acids is targeted to the plasma membrane. Green: NBC1-GFP; red: phalloidin.

**2b. Expression of GFP-NBC1 CD3 : Z-line image analysis.** As demonstrated, the NBC1 with deletion of the last 3 carboxy terminal amino acids is exclusively targeted to the basolateral membrane. Green: NBC1-GFP; red: phalloidin.

**2c. Expression of GFP-NBC1 CD3 : Z-line image analysis.** Co-labeling with PNA-lectin which exclusively binds to apical membrane verifies the localization of NBC1-CD3 to the basolateral membrane. Green: NBC1-GFP; red: PNA-lectin.

**Fig. 3. Transfection of MDCK cells with GFP-NBC1 CD20 cDNA.**

**3a. Expression of GFP-NBC1 CD20 : Z-stack image analysis.** As demonstrated, the NBC1 with deletion of the last 20 carboxy terminal amino acids is targeted to the plasma membrane. Green: NBC1-GFP; red: phalloidin.

**3b. Expression of GFP-NBC1 CD20 : Z-line image analysis.** As demonstrated, the NBC1 with deletion of the last 20 carboxy terminal amino acids is exclusively targeted to the basolateral membrane. Green: NBC1-GFP; red: phalloidin.

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**3c. Expression of GFP-NBC1 CD20: Z-line image analysis.** Co-labeling with PNA-lectin which exclusively binds to apical membrane verifies the localization of NBC1-CD20 to the basolateral membrane. Green: NBC1-GFP; red: PNA-lectin.

**Fig. 4. Transfection of MDCK cells with GFP-NBC1 CD26 cDNA.**

**4a. Expression of GFP-NBC1 CD26 : Z-stack image analysis.** As demonstrated, the NBC1 with deletion of the last 26 carboxy terminal amino acids is targeted to the plasma membrane. Green: NBC1-GFP; red: phalloidin.

**4b. Expression of GFP-NBC1 CD26 : Z-line image analysis.** As demonstrated, the NBC1 with deletion of the last 26 carboxy terminal amino acids is predominantly retargeted to the apical membrane with residual labeling on the basolateral membrane. Green: NBC1-GFP; red: phalloidin.

**4c. Expression of GFP-NBC1 CD26: Z-line image analysis.** Co-labeling with PNA-lectin which exclusively binds to apical membrane confirms the apical localization of NBC1-CD26 with residual labeling on the basolateral membrane. Green: NBC1-GFP; red: PNA-lectin.

**Fig. 5. Transfection of MDCK cells with GFP-NBC1 CD50 cDNA.**

**5a. Expression of GFP-NBC1 CD50 : Z-stack image analysis.** As demonstrated, the NBC1 with deletion of the last 50 carboxy terminal amino acids is targeted to the

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plasma membrane with some intracytoplasmic accumulation. Green: NBC1-GFP; red: phalloidin.

**5b. Expression of GFP-NBC1 CD50 : Z-line image analysis.** As demonstrated, the NBC1 with deletion of the last 50 carboxy terminal amino acids is predominantly targeted to the apical membrane with appearance of cytoplasmic localization and residual labeling on the basolateral membrane. Green: NBC1-GFP; red: phalloidin.

**5c. Expression of GFP-NBC1 CD50: Z-line image analysis.** Co-labeling with PNA-lectin which exclusively binds to apical membrane confirms the apical localization of NBC1-CD50. Cytoplasmic localization and residual labeling on the basolateral membrane are observed. Green: NBC1-GFP; red: PNA-lectin.

**Figure 6. Membrane potential recordings in oocytes injected with full length or mutant GFP-NBC1 (CD26) cRNA (representative tracings).** Membrane potential was measured by conventional intracellular microelectrodes in *Xenopus* oocytes 2-4 days after injection with full length GFP-NBC1 or mutant GFP-NBC1 NBC1 (CD26) cRNA. After an equilibrating period, the perfusion solution was switched to a solution containing 10 mM $HCO_3$- and gassed with 1.5% CO2, 98.5%O2 at pH 7.5. A continuous line indicates the time of exposure to CO2/$HCO_3^-$. Exposure of oocytes that were injected with either full length GFP-NBC1 (bottom panel), mutant GFP-NBC1-CD26 (middle panel) or GFP only (no NBC insert) (top panel) to CO2/$HCO_3^-$ resulted in an immediate and sustained hyperpolarization in the full length and mutant NBCs (middle and bottom panel) but not

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the GFP only (top panel). The hyperpolarization was reversible upon removal of $CO2/HCO_3^-$ from the perfusion medium. Exposure of control (water-injected) oocytes to $CO2/HCO_3^-$ also did not alter the membrane potential (data not shown).

**Fig. 7. Transfection of MDCK cells with CD-23 deletion or mutated GFP-NBC1 cDNA.**

**7a. Expression of GFP-NBC1 CD23: Z-line image analysis.** Co-labeling with PNA-lectin which exclusively binds to the apical membrane demonstrates the predominant apical localization of NBC1-CD23. Residual labeling on the basolateral membrane is observed. Green: NBC1-GFP; red: PNA-lectin.

**7b. Expression of mutated GFP-NBC1 cDNA (F1013A) : Z-line image analysis (left and right panels).** As demonstrated, the NBC1 cDNA with mutation of residue 1013 (F1013A) shows predominant retargeting to the apical membrane with residual labeling on the basolateral membrane. Green: NBC1-GFP; left panel: red, phalloidin; right panel: red, PNA-lectin.

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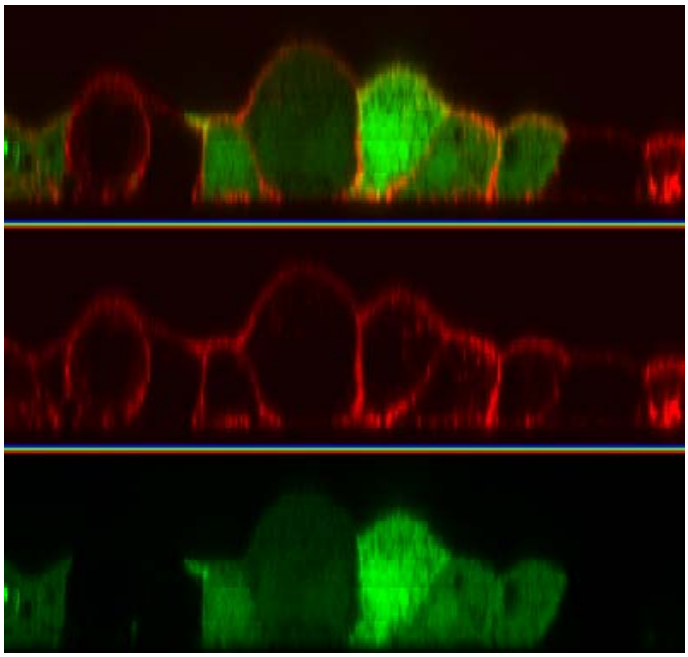
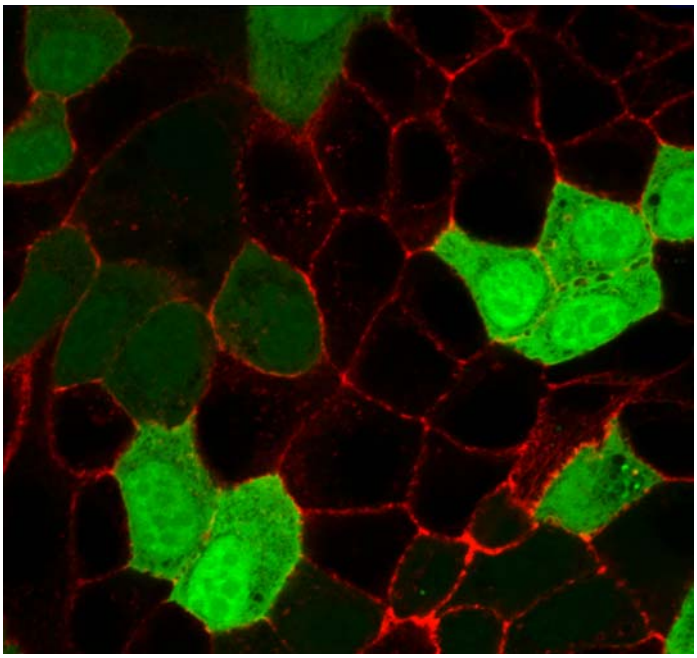
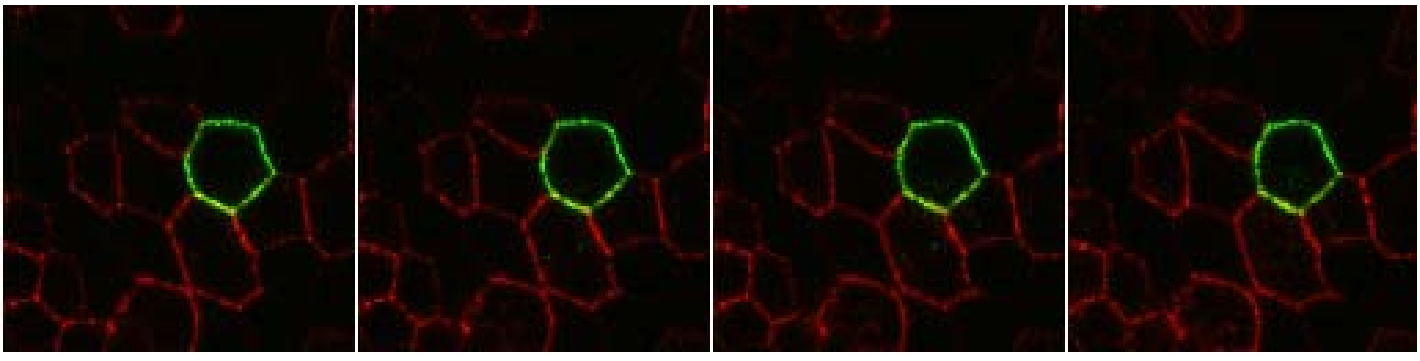
**Fig. 1. Transfection of MDCK cells with GFP only (no NBC1 insert) or GFP-NBC1 full length cDNA.**

**1a. Expression of GFP only (no NBC1 insert) . Left panels: Z-line image analysis of GFP alone (bottom panel), phalloidin (middle panel) and merged image (top panel); Right panel: Z-stack analysis. Green: GFP only; Red:Phalloidin.**





**1b. Expression of GFP-NBC1 full length : Z-stack image analysis. Green: GFP-NBC1 full length; Red:Phalloidin.**



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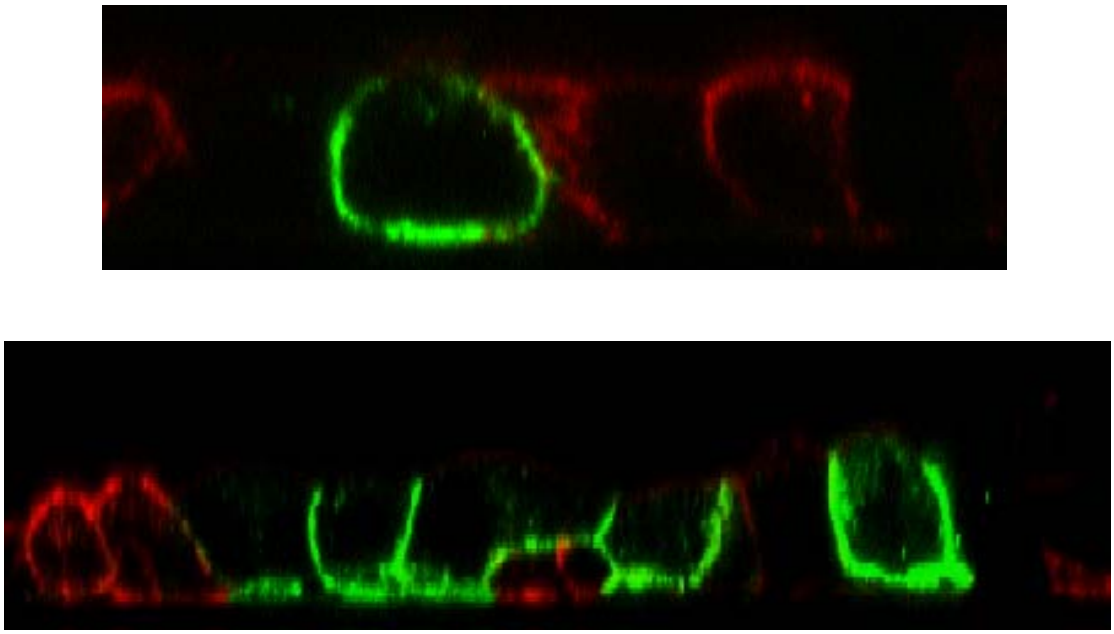
**1c. Expression of GFP-NBC1 full length : Z-line image analysis (top and bottom panel) . Green: GFP-NBC1 full lenght; Red: Phalloidin**



**1d. Expression of GFP-NBC1 full length : Z-line image analysis (top and bottom panels). Green: GFP-NBC1 full length; Red: PNA- lectin.**

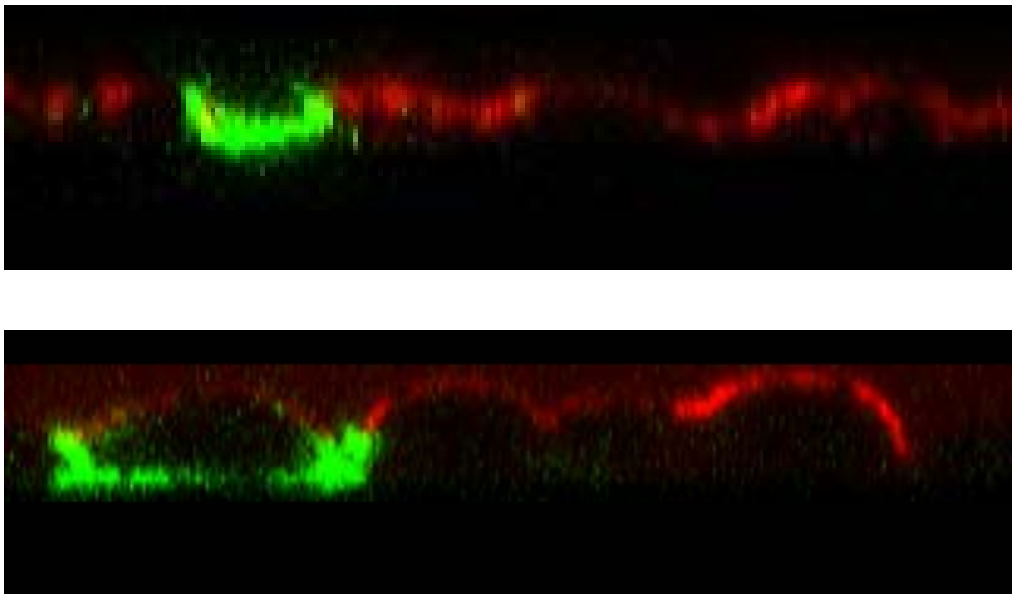


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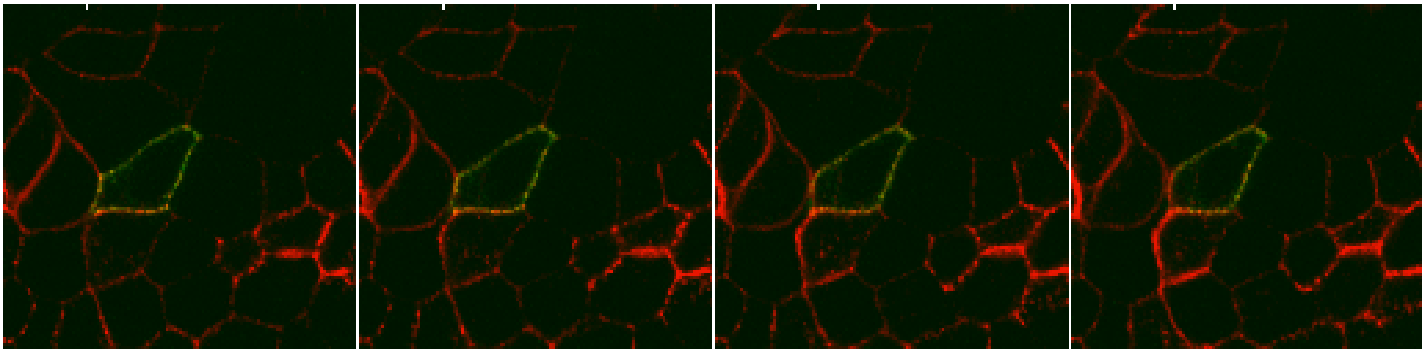
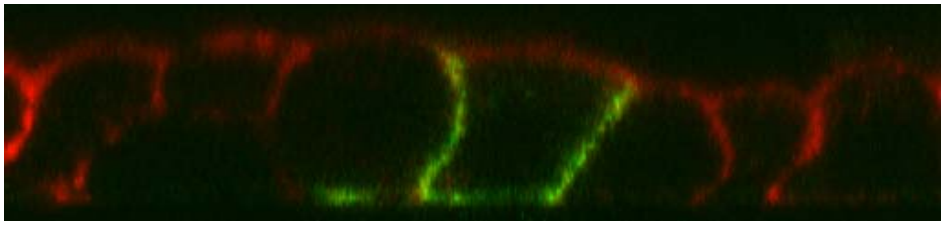
**Fig. 2. Transfection of MDCK cells with GFP-NBC1 CD3 cDNA.**

**2a. Expression of GFP-NBC1 CD3 : Z-stack image analysis. Green: GFP-NBC1 CD3; Red: Phalloidin.**



**2b. Expression of GFP-NBC1 CD3 : Z-line image analysis. Green: GFP-NBC1 CD3; Red: Phalloidin**



**2c. Expression of GFP-NBC1 CD3 : Z-line image analysis. Green: GFP-NBC1 CD3; Red: PNA- lectin**

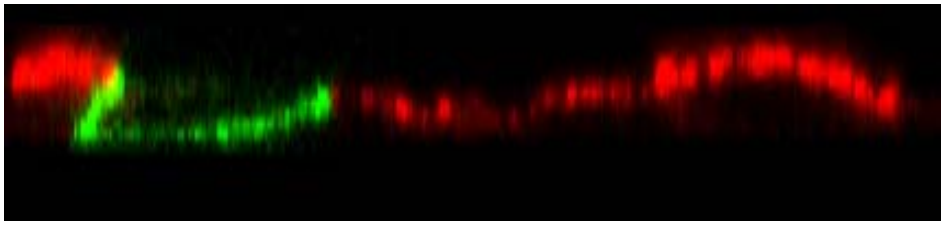


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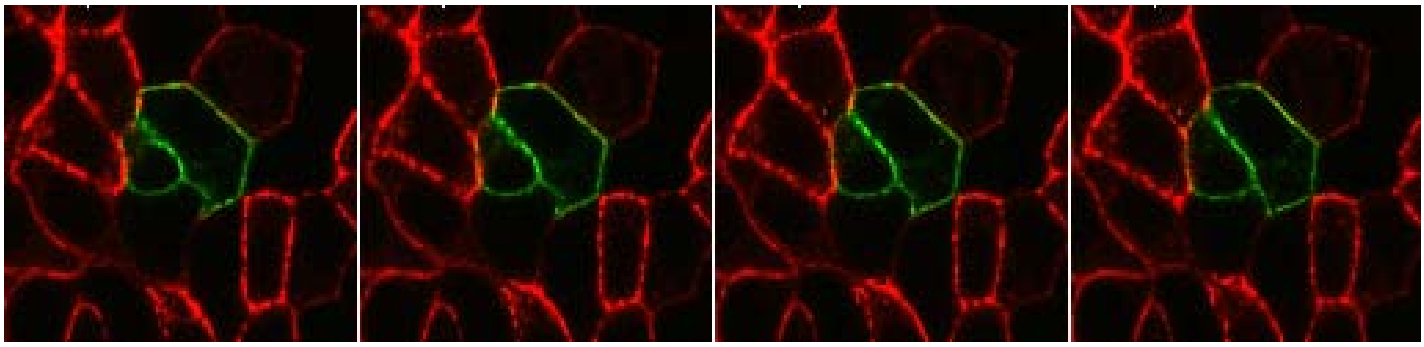
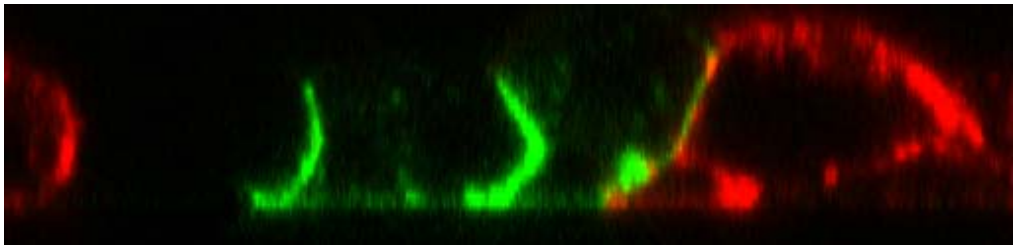
**Fig. 3. Transfection of MDCK cells with GFP-NBC1 CD20 cDNA.**

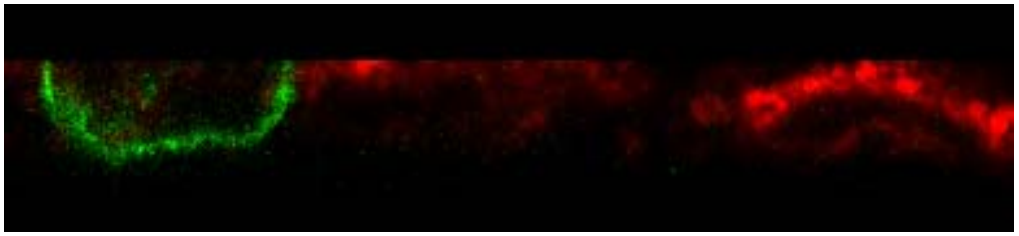
**3a. Expression of GFP-NBC1 CD20 : Z-stack image analysis. Green: GFP-NBC1 CD20; Red: Phalloidin.**



**3b. Expression of GFP-NBC1 CD20 : Z-line image analysis. Green: GFP-NBC1 CD20; Red: Phalloidin**



**3c. Expression of GFP-NBC1 CD20: Z-line image analysis (top and bottom panels). Green: GFP-NBC1-CD20; Red: PNA- lectin.**



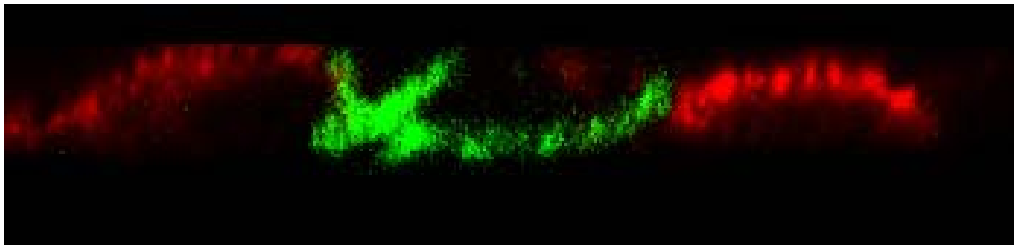


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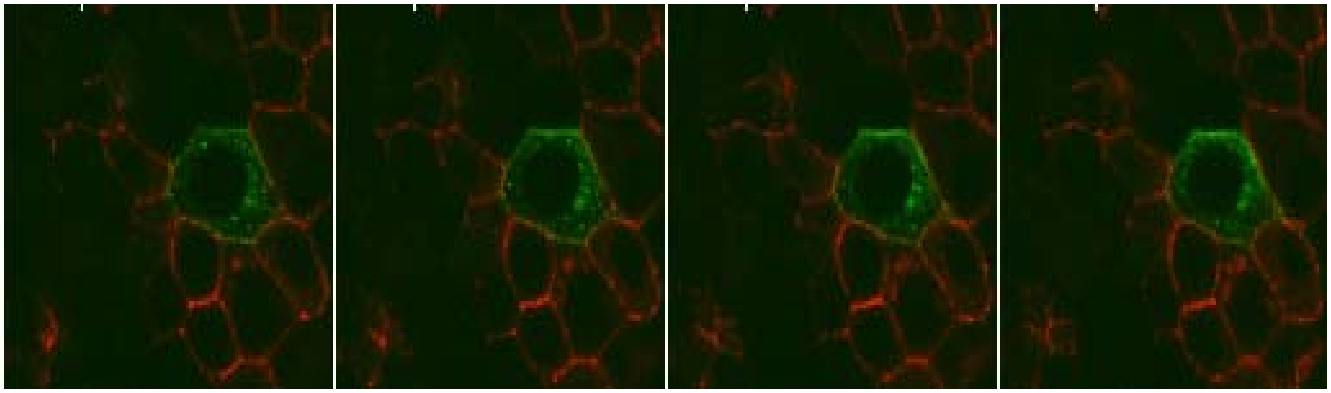
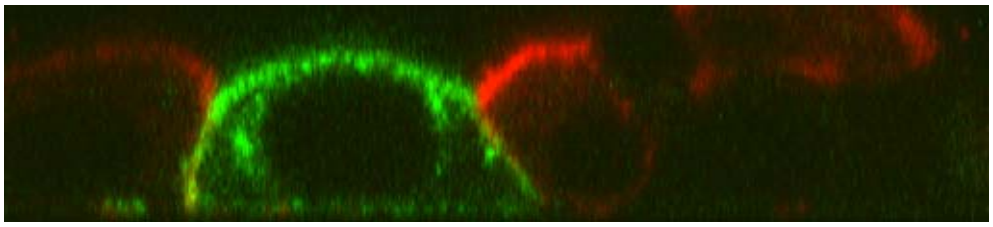
**Fig. 4. Transfection of MDCK cells with GFP-NBC1 CD26 cDNA.**

**4a. Expression of GFP-NBC1 CD26 : Z-stack image analysis. Green: NBC1-GFP CD26; red: phalloidin.**



**4b. Expression of GFP-NBC1 CD26 : Z-line image analysis. Green: NBC1-GFP CD26; red: phalloidin.**



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**4c. Expression of GFP-NBC1 CD26: Z-line image analysis (top and bottom panels). Green: GFP-NBC1-CD26; Red: PNA- lectin.**

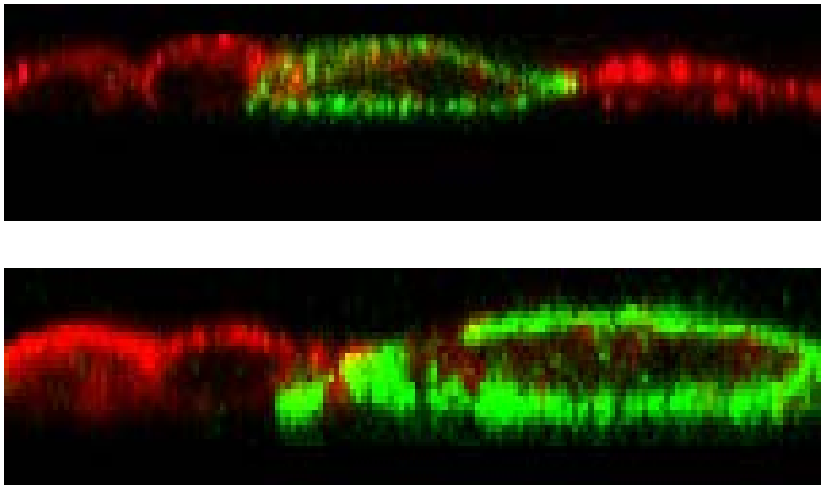

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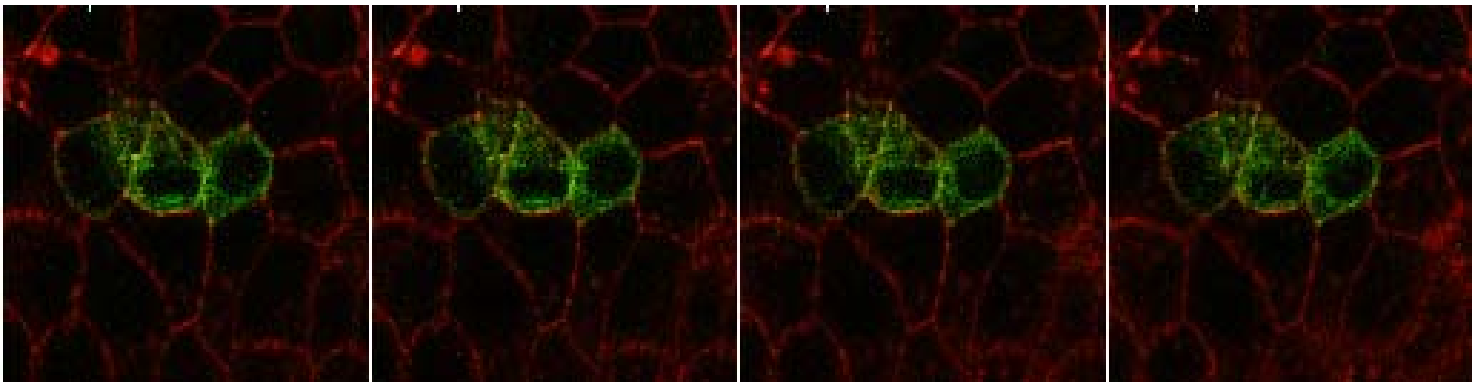
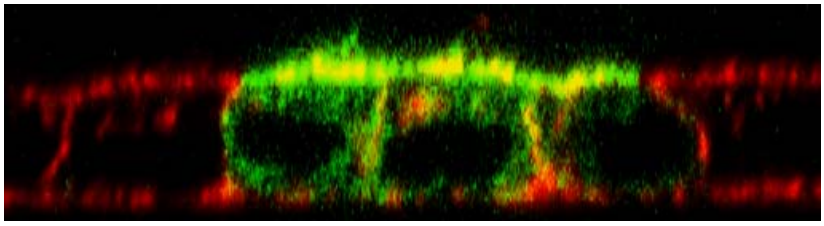
**Fig. 5. Transfection of MDCK cells with GFP-NBC1 CD50 cDNA.**

**5a. Expression of GFP-NBC1 CD50 : Z-stack image analysis. Green: NBC1-GFP CD50; red: phalloidin.**



**5b. Expression of GFP-NBC1 CD50 : Z-line image analysis. Green: NBC1-GFP CD50; red: phalloidin.**



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**5c. Expression of GFP-NBC1 CD50: Z-line image analysis (top and bottom panels).**

**Green: GFP-NBC1-CD50; Red: PNA- lectin.**

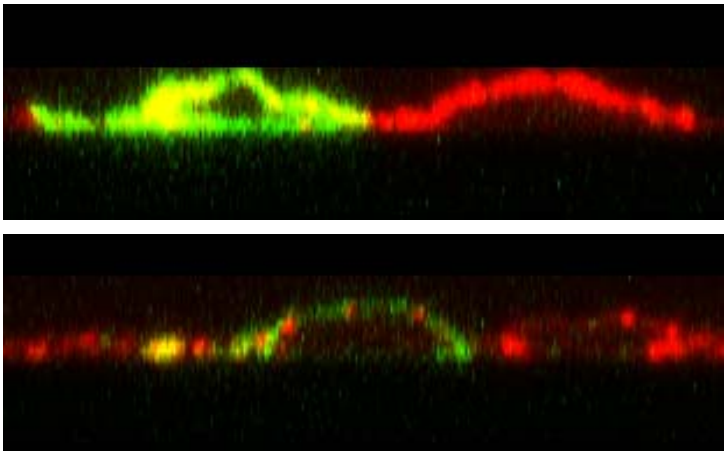

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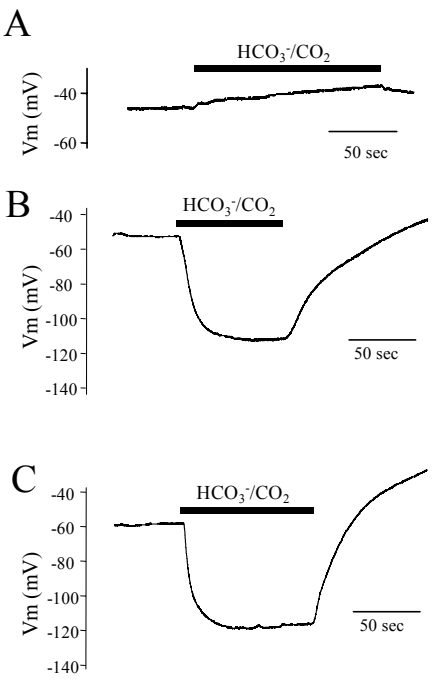
**Fig. 6. Functional expression of GFP-NBC1 (full length) and GFP-NBC1 CD 26 mutant. Representative tracings depicting membrane potentials recordings in oocytes.**




A: GFP only; B: GFP-NBC-CD26; C:GFP-NBC1 full length

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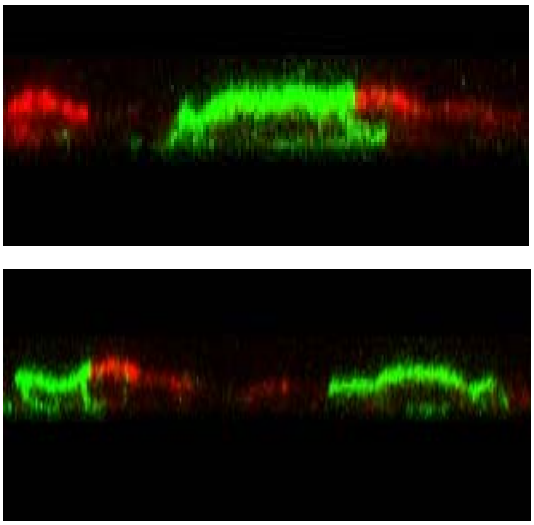
**Fig. 7. Transfection of MDCK cells with CD-23 deletion or mutated GFP-NBC1 cDNA.**

**7a. Expression of GFP-NBC1 CD23: Z-line image analysis (top and bottom panels).** Green: NBC1-GFP; red: PNA-lectin.



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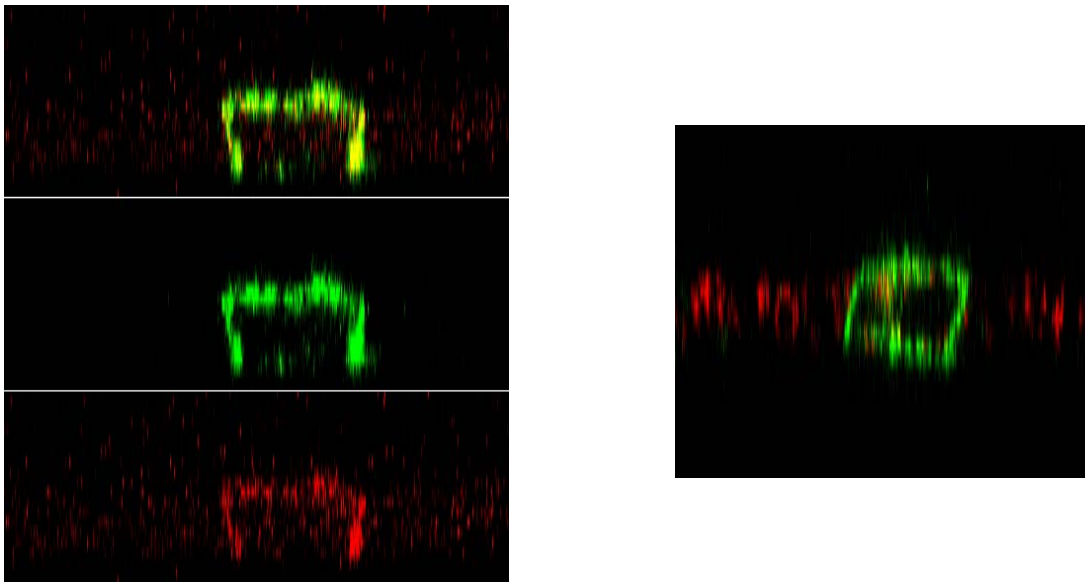
**7b. Expression of mutated GFP-NBC1 cDNA (F1013A) : Z-line image analysis (left and right panels).** Green: NBC1-GFP; red: PNA-lectin.



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**Identification of a carboxyl terminal motif essential for the targeting of Na+: HCO3- cotransporter NBC1 to basolateral membrane**

Hong C. Li, Roger T. Worrell, Jeffrey B. Matthews, Holeh Husseinzadeh, Liza Neumeier, Snezana Petrovic, Laura Conforti and Manoocher Soleimani

*J. Biol. Chem. published online July 25, 2004*

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