SUPPLEMENTARY MATERIALS

Materials and methods

Molecular Biology. To generate GST fusion constructs of SLC26A6, in each case cDNA sequences were amplified by PCR with primers containing BamHI restriction sites to facilitate cloning of the PCR product into pGEX-5X-3 (Pharmacia Biotech). PKC phosphorylation site mutants, SLC26A6-S553A and SLC26A6-S582A, as well as the carbonic anhydrase binding site mutant SLC26A6-ΔCAB, were constructed using a mega-primer mutagenesis strategy, described previously (Alvarez et al., 2001). First-round PCR forward primer for all mutants was 5'-gccacaggcatctcctat-3'. First round PCR mutagenic reverse primers were 5'-gagcagtttcttcttctgggcgat-3' and 5'-ggggcggcagcctgttt-3' for SLC26A6-S553A and SLC26A6-S582A, and 5'-gaagttgacattcacaccaca-3' for SLC26A6-ΔCAB, respectively. The CAB motif was mutated to 546NVNF549. Products from the first PCR round were used as forward mega-primers for the second round of amplification, with 5'-cgtagaatcgagaccgaggag-3' as reverse primer. Second-round products were subcloned back into hSLC26A6 cDNA using *Bsu36*I and *EcoRV* at 5' and 3 ends, respectively.

CAII Binding Assays. The ability of CAII to bind the C-terminal tail of human SLC26A6 was investigated using a microtiter assay, described previously (Vince and Reithmeier, 2000; Vince and Reithmeier, 1998). Briefly, 200 ng of purified human CAII (Sigma-Aldrich) was chemically coupled to wells of a 96-well plate using 1-cyclohexy-3-(2-morpholinoethyl) carbodiimide metho-p toluene sulphate (Sigma-Aldrich). Wells were then washed with PBS (140 mM NaCl, 3 mM KCl, 6.5 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.5) and blocked with 2% (w/v) BSA in PBS. After washing with antibody buffer (100 mM NaCl, 5 mM EDTA, 0.25%(w/v) Gelatin, 0.05%

(w/v) Triton X-100, 50 mM Tris, pH 7.5) plates were incubated with increasing concentrations (0-200 nM) of purified GST fusion proteins or GST alone.

Measurement of SLC26A6 chloride/bicarbonate exchange activity. HEK293 cells, grown on 7.5 x 11 mm glass poly-L-lysine-coated coverslips (Erie Scientific Co, USA), were transfected or co-transfected with cDNA encoding SLC26A6, or SLC26A6 and V143Y CAII mutant, or SLC26A6 and AT_{1a}-AngII receptors. Two days post-transfection, coverslips were rinsed in serum-free DMEM and incubated in serum-free DMEM containing 2 μM BCECF-AM (Molecular Probes), 37 ºC, 20 min. Coverslips were mounted in a fluorescence cuvette and perfused at 3.5 ml/min alternately with Ringer's buffer (5 mM glucose, 5 mM potassium gluconate, 1 mM calcium gluconate, 1 mM $MgSO_4$, 2.5 mM NaH_2PO_4, 25 mM $NaHCO_3$, 10 mM Hepes, pH 7.40) containing either 140 mM NaCl (Cl^- containing) or 140 mM sodium gluconate (Cl^--free). Both buffers were continuously bubbled with air/5% CO_2. Fluorescence changes were monitored in a Photon Technologies International RCR fluorometer at excitation wavelengths 440 and 502 nm and emission wavelength 528 nm. To study the effect of AngII, PMA or ACTZ on anion exchange activity, two consecutive Cl^- removal-re-addition protocols were carried out on the same coverslip, the first in the absence and the second in the presence of the specified compound. After the first Cl^- removal-re-addition pulse, the cells were then exposed either to AngII (1 μM, Sigma), PMA (150 nM, Sigma) or ACTZ (150 μM, Sigma) for a further 10 min. The initial rates of $\Delta pH_i/\Delta t$ obtained during the first and the second treatment were then compared. After each experiment, fluorescence data were converted to pH_i by calibration using the nigericin/high potassium method (Thomas et al., 1979). The initial rate of change of pH_i determined during the removal and re-addition of Cl^- was determined by linear

least squares fit. All transport data have been corrected for background activity of HEK293 cells transfected with pcDNA3.1 vector alone. The intrinsic buffer capacity (β_i) was negligible at pH_i values above 7.10 (Sterling and Casey, 1999), so that β_{CO2} = βtotal (β_{tot}), where β_{CO2}=2.3 x [HCO_3^-]. Total flux of proton equivalents was calculated as: J_{H+}= βtotal x ΔpH_i (Roos and Boron, 1981).

Confocal microscopy. Cells grown on 22x22 mm poly-L-lysine-coated coverslips were transiently transfected as described above. Cells were washed in PBS, and fixed for 20 min in 3.5% (w/v) paraformaldehyde in PBS containing 1 mM $CaCl_2$, 1 mM Mg Cl_2. After three washes with PBSCM, the cells were incubated for 5 min in PBS, containing 0.1% (vol/vol) Triton X-100. Slides were blocked for 25 min with PBSG 0.2% (w/v) gelatin in PBS and with a 1:1000 dilution of goat anti-CAII antibody (Santa Cruz, CA), and rabbit polyclonal anti-SLC26A6 antibody (the anti-N-terminal antibody, previously reported (Lohi et al., 2003)) (1:1000 dilution) for 1h in a humidified chamber at room temperature. After three washes with PBS containing 0.2% gelatin, coverslips were incubated for 1h in a dark humidified chamber with a 1:1000 dilutions of Alexa Fluor 594-conjugated chicken anti-goat IgG, and with Alexa Fluor 488-conjugated chicken anti-rabbit IgG. Coverslips were mounted in Prolong Antifade Solution (Molecular Probes, Eugene, OR, U.S.A.) and imaged with a Zeiss LSM 510 laser scanning confocal microscope (Germany), mounted on an Axiovert 100M controller with A x63 (NA1.4) lens. Images of HEK293 cells transiently transfected with SLC26A6 and α_{1a} adrenergic receptor cDNAs were taken using the same conditions of illumination and exposure. Images were quantified by MetaMorph® Software, with saturation at 50% intensity. MetaMorph®

compared the images (SLC26A6 and either CAII or α_{1a} adrenergic receptor signal), pixel-by-pixel, and determined the percent overlap of the fluorescent signals.

Protein Expression and cell surface processing assays. SLC26A6 WT and mutant proteins were expressed by transient transfection of HEK293 cells (Alvarez et al., 2004), using the calcium phosphate method (Alvarez et al., 2001). Cells were grown at 37ºC in an air/CO_2 (19:1) environment in DMEM medium, supplemented with 5% (v/v) fetal bovine serum and 5% (v/v) calf serum. Assays to assess the degree of cell surface processing and biotinylation of SLC26A6 proteins were performed as described previously (Fujinaga et al., 2003). The effect PMA on SLC26A6 cell surface expression was performed as described before (Fujinaga et al., 2003), in HEK293 cells transfected with SLC26A6 cDNA. After 48h, cells were serum starved for 1h, and incubated with 200 nM of αPMA (control), or 200 nM of PMA, for 1h.

Supplementary Figures Legends

Figure 1. Cell-surface expression of SLC26A6-WT and mutants. A, HEK-293 cells, transfected with SLC26A6-WT, SLC26A6-ΔCAB, SLC26A6-S553A, or SLC26A6-S582A cDNA, were incubated with the membrane-impermeant, lysine-directed compound, EZ-Link™ Sulpho-NHS-SS-Biotin. Cells were solubilized, and proteins were incubated with Streptavidin resin. Biotinylated proteins associated with the resin were eluted with SDS-PAGE sample buffer. Protein samples (total (T), and unbound (U) fractions) were electrophoresed on polyacrylamide gels and transblotted to PVDF membrane. Bound fraction was not shown because it is not possible to fully elute bound protein from streptavidin resin, because of the tight biotin/streptavidin interaction (Quilty et al., 2002). Blots were developed using anti-SLC26A6 antibody, incubated with ECL® reagent and imaged with a Kodak Image station. B, Fraction of WT SLC26A6 and mutant SLC26A6 associated with the plasma membrane was quantified by densitometry of the immunoblotted proteins and calculated as ((total-unbound)/total) x 100% (n=3). C, Effect of PMA on SLC26A6 cell surface expression. Experiment was performed as in B, but SLC26A6-expressing cells were treated with PMA (grey bar) or αPMA (black bar) (n=3).

Figure 2. PMA treatment results in phosphorylation of SLC26A6. HEK293 cells were either untransfected or transfected with SLC26A6 cDNA. Cells were treated with either αPMA or PMA, as indicated. Proteins were immunoprecipitated (*IP*) with a polyclonal anti-SLC26A6 antibody, resolved by SDS-PAGE (8% gel), and immunoblotted with a monoclonal anti-phosphoserine antibody. The *arrowhead* indicates the position of the phosphorylated form of SLC26A6.

Figure 3. Expression of SLC26A6 variants. *S*amples (20 μg protein) of HEK293 cells used in transport assays (panel A) were assayed on immunoblots for the presence of SLC26A6 (upper) and β-actin (lower). Samples were from cells transfected with the respective cDNAs: SLC26A6-WT (lane 1), SLC26A6-ΔCAB (lane 2), SLC26A6-S553A (lane 3), and SLC26A6-S582A (lane 4). SLC26A6 and b-actin expression were quantified by densitometry. The average amount of SLC26A6 in each sample was measured as the amount of SLC26A6/amount of b-actin, which is shown relative to WT SLC26A6 at the bottom.

Figure 4. Interaction between SLC26A6 variants and CAII. HEK293 cells transfected with an SLC26A6 variant were plated on glass slides. Cells were incubated for one hour with 200 nM of either PMA, or the biologically inactive α-PMA isomer. Transfected cells were stained with rabbit anti-SLC26A6 antibody, followed by Alexa Fluor 488-conjugated chicken anti-rabbit IgG secondary antibody (SLC26A6 N-terminus antibody, green) or with goat anti-CAII antibody, followed by Alexa Fluor 594-conjugated chicken anti-goat IgG (CAII, red). Co-localization of CAII and SLC26A6 is yellow (merge). Images were collected with a Zeiss LSM 510 laser scanning confocal microscope. Scale bar= 10 μm. A, Transfected cells as indicated and treated with α-PMA. B, S582A-transfected cells were treated with either α-PMA or PMA, as indicated.

Supplementary References

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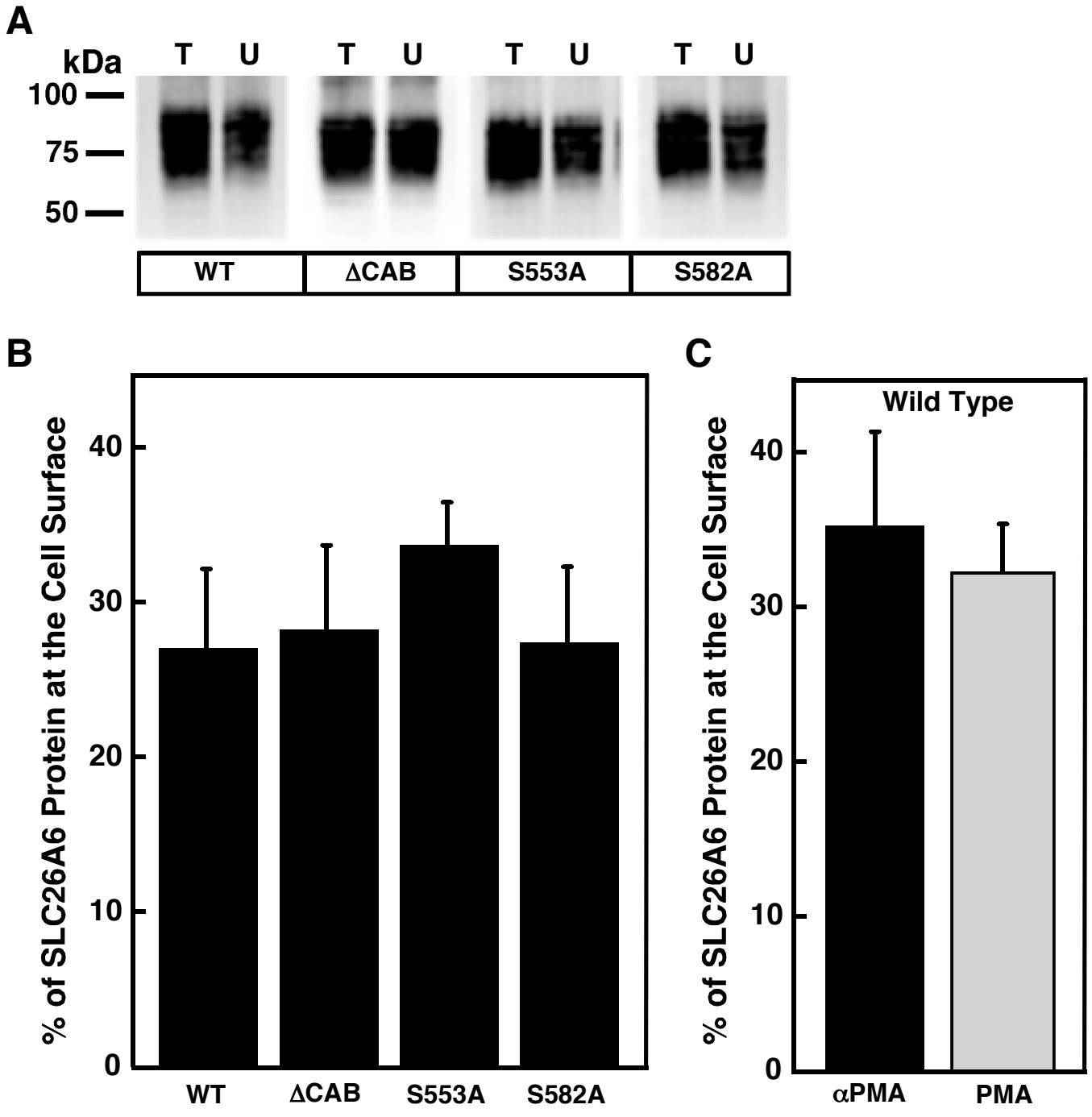
A
kDa
T U T U T U T U
100
75
50
WT
ΔCAB
S553A
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B
% of SLC26A6 Protein at the Cell Surface
40
30
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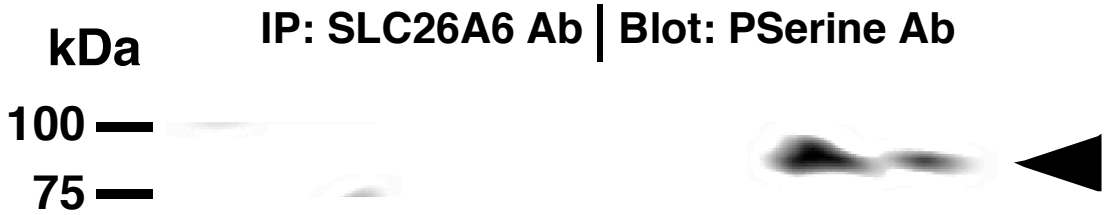
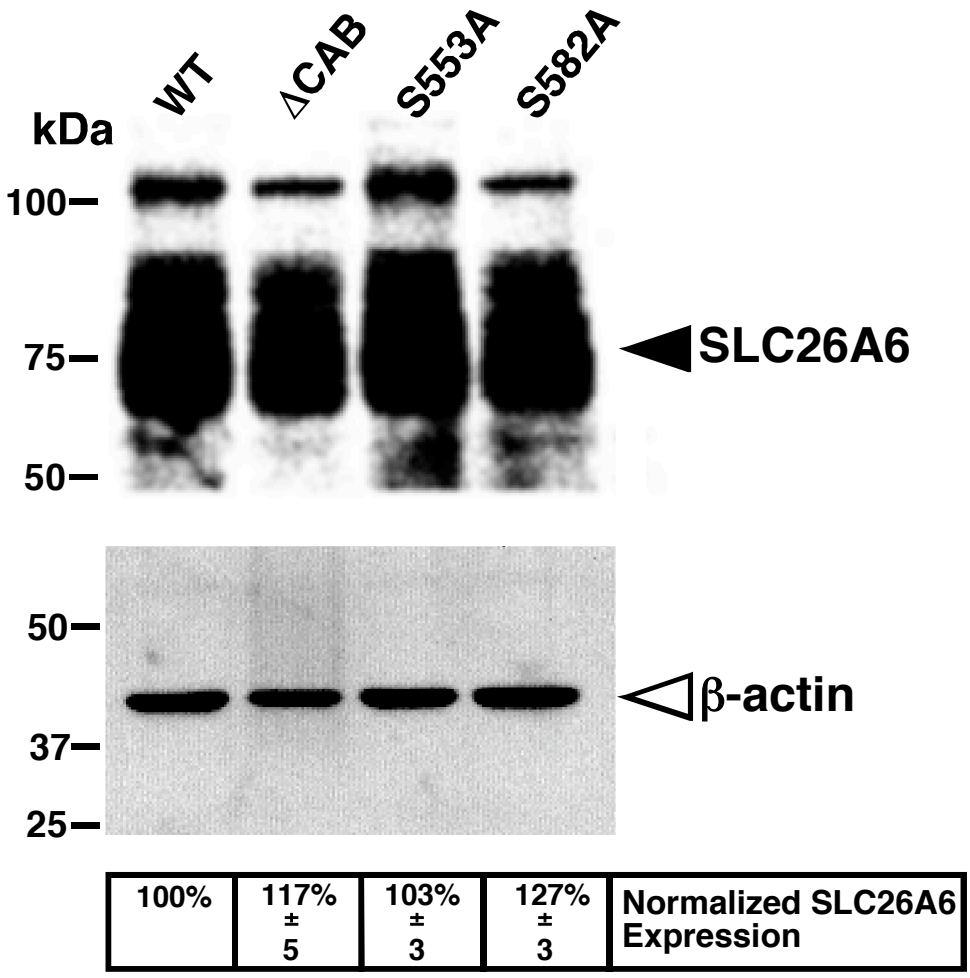
10
0
WT
ΔCAB
S553A
S582A
C
Wild Type
% of SLC26A6 Protein at the Cell Surface
40
30
20
10
0
αPMA
PMA

Suppl. Fig. 1



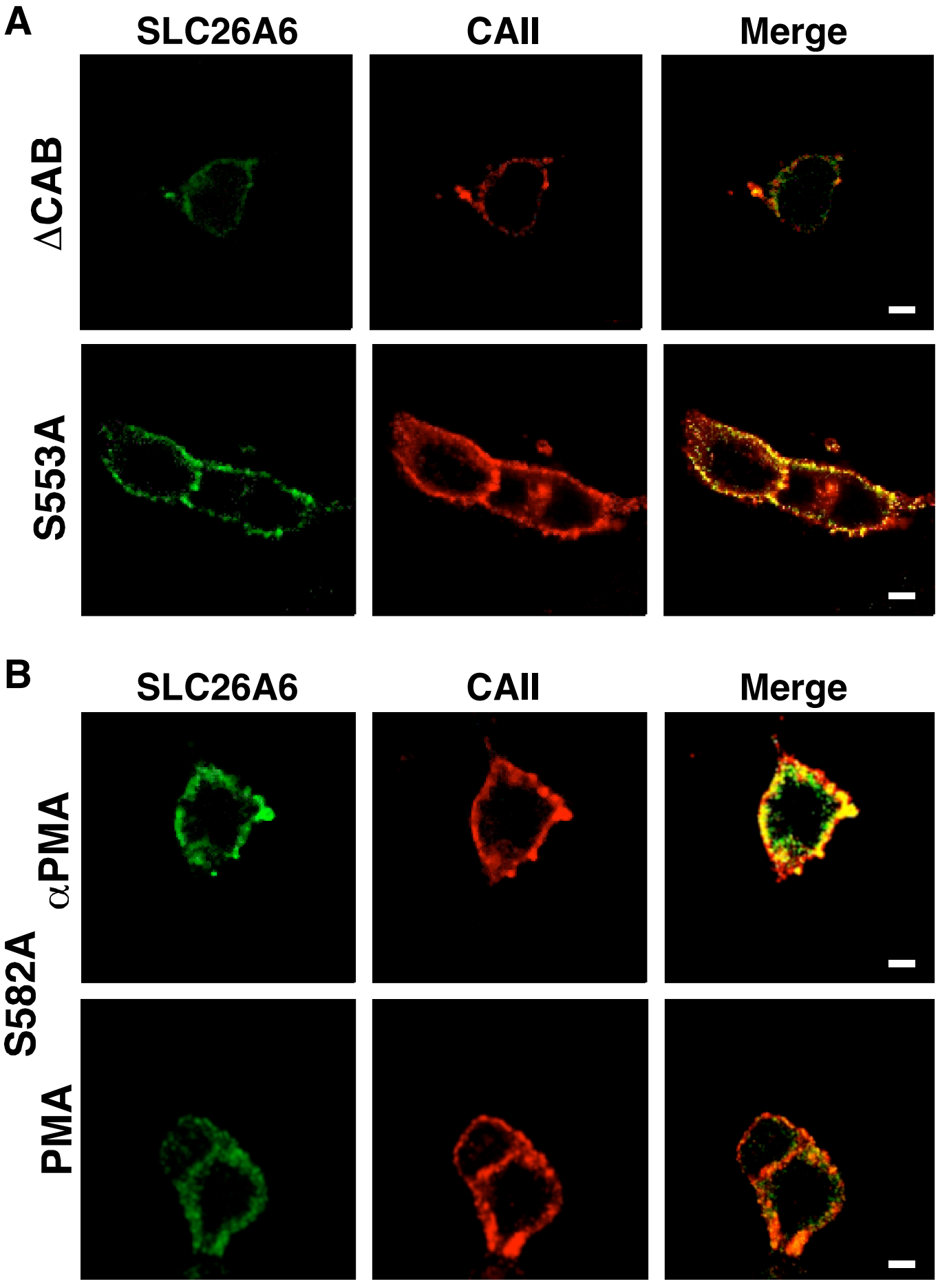
-	-	+	+	SLC26A6
+	-	+	-	200 nM αPMA
-	+	-	+	200 nM PMA

Suppl. Fig. 2



100%	117% ± 5	103% ± 3	127% ± 3	Normalized SLC26A6 Expression

Suppl. Fig. 3


A
SLC26A6
CAII
Merge
ΔCAB
S553A
B
SLC26A6
CAII
Merge
αPMA
S582A
PMA

Suppl. Fig. 4