SUPPLEMENTAL MATERIAL

MATERIALS AND METHODS

Animals

We previously reported generation of *CFTR*$^{+/\Delta F508}$ and *CFTR*$^{+/-}$ pigs *(1)*. Animals were produced by mating *CFTR*$^{+/\Delta F508}$ male and female pigs or *CFTR*$^{+/-}$ male and female pigs. Newborn CF pigs and littermates were obtained from Exemplar Genetics. The University of Iowa Animal Care and Use Committee approved all animal studies.

For studies of newborn animals, pigs were euthanized 8-15 hr after birth (Euthasol, Virbac). This time minimizes changes from infection, inflammation, feeding, and exposure to environmental influences; it ensures that the animals are all of approximately the same age; and it provides time for genotyping and preparation for experiments. For some animals, within 8-15 h of birth, *CFTR*$^{+/+}$ and *CFTR*$^{\Delta F508/\Delta F508}$ piglets underwent surgical placement of an ileostomy or cecostomy to prevent complications from meconium ileus. In the immediate postoperative period, animals were managed as previously described *(2)*. Thereafter, piglets were fed milk replacer and weaned to a solid diet. Animals received: a) Oral pancreatic enzyme replacement therapy (PancreVed, Vedco) with meals (4000 IU lipase/120 mL milk replacer or ~10,000 IU lipase/kg/day divided between meals). b) Oral fat-soluble vitamins (PancreVed, Vedco). c) Either an oral H2 blocker (1 mg/kg) (famotidine) or oral proton pump inhibitor (1 mg/kg) (omeprazole, Sandoz) once a day. d) Oral polyethylene glycol 3350 (Paddock Laboratories, MinneapPharmaceuticals) with each meal titrated to maintain soft stools.

Case #2 (euthanized on day 62) received oxytetracycline from day 23-27 for a pustular dermatitis and again from day 54-62 for a possible infectious cause of melanotic stools. Case #3 (euthanized on day 87) developed a fever and tachypnea on day 12 and received ceftiofur (day 12-18) and oxytetracycline (day 15-18) for treatment of presumed pulmonary infection with fever and tachypnea. On day 39, the animal then developed a polyarthritis and receive prolonged antibiotic treatment (oxytetracycline day 39-56 and ceftiofur day 43-56).

Production of primary cultures of differentiated airway epithelia

Epithelial cells were isolated from the various tissues by enzymatic digestion, seeded onto permeable filter supports, and grown at the air-liquid interface as previously described *(3)*. Differentiated epithelia were used at least 14 days after seeding.

Histopathological analysis

At necropsy, pigs were examined for gross lesions and the findings were documented. Tissues were fixed in 10% neutral buffered formalin for 48-96 hr. Tissues were then routinely processed, embedded, sectioned (4 μm), and stained with hematoxylin and eosin (HE) for general examination. Additional sections were selectively stained with Masson's trichrome (MT) or amylase pretreated sections with Periodic acid-Schiff (PAS). Morphometric analysis of the pancreas *(4)* and trachea *(5)* were performed as previously described.

Bronchoalveolar lavage (BAL) fluid collection and analysis

Animals were studied between 8 and 15 h after birth. BAL was performed immediately following euthanasia. We instilled 5 ml of normal saline through an intratracheal catheter three

times. The total number of recovered cells was quantified with a hemacytometer and morphologic differentiation of cells was performed on cytospin preparations that were stained with Diff-Quick Stain kit (Baxter). BAL levels of IL-8 were determined on recovered supernatant after centrifugation (1600 x g for 10 min) using a standard sandwich ELISA (R&D Systems). Absolute IL-8 values are not directly comparable to our previous studies because we used different assay protocols.

Microbiologic studies

Standard microbiologic techniques were utilized to identify and quantify bacteria present in bronchoalveolar lavage liquid and lung homogenate samples. Samples were serially diluted and plated onto blood agar (tryptic soy agar with sheep blood; Remel), Colombia colistin-nalidixic acid agar (Remel), Chocolate agar (Remel), Mannitol Salt Agar (Remel), MacConkey agar (Remel), and *Burkholderia cepacia* selective agar (Remel). Organisms were identified with standard microbiological procedures. Some identifications were confirmed by API 20E or API 20NE (bioMérieux), Vitek (bioMérieux) or 16S rRNA gene sequencing (University of Iowa Clinical Microbiology Laboratory and Iowa State University Diagnostic Laboratory).

Northern blot and quantitative RT-PCR

Northern blot was performed using total RNA isolated from duodenum (RNeasy-Lipid Tissue mini-kit, Qiagen). Total RNA was electrophoresed on a denaturing gel and transferred to a positively charged membrane (Roche). The membrane was hybridized with ^{32}P-labelled DNA probes corresponding to nucleotides 1-1000 of the porcine *CFTR* cDNA. Signal was detected using a Fuji FLA7000 phosphorimager. Porcine *CFTR* mRNA was detected at ~6.5kb.

Quantitative RT-PCR used TaqMan chemistry and an ABI 7500 Fast Real-time PCR System to measure porcine *CFTR* mRNA. Briefly, tissue was collected in RNAlater (Ambion) and total RNA was isolated as above. First-strand cDNA was synthesized with random hexamers (SuperScript III, Invitrogen). Sequence-specific primers and probes for porcine *CFTR* and *β-actin* were from ABI. For measuring *CFTR* mRNA, primer/probe sets annealing to exon 10 of *CFTR* (Ss03389420_m1, pCFTR) and *β-actin* (Ss03376160_u1, ACTB) were used in separate reactions. For each tissue, amounts of *CFTR* mRNA were normalized to ß-actin mRNA. These normalized values were then expressed relative to that in wild-type duodenum.

CFTR isolation

For cultured epithelia, cells were scraped from apical surface into lysis buffer (LB) (in mM: 50 Tris-HCl, pH 7.4, 100 NaCl, 0.1 PMSF) and a cocktail of protease inhibitors (7 μg/ml benzamidine-HCl, 1 μg/ml pepstatin A, 2 μg/ml aprotinin, 2 μg/ml leupeptin) plus 1% TX-100, rotated 1 hr at 4 ˚C and centrifuged at 10,000 x g for 5 min to pellet cells. Soluble supernatant was used to isolate CFTR by immunoprecipitation.

Intestines were removed immediately upon necropsy and placed in an ice-cold solution of (mM):150 NaCl, 1 DTT, 0.1 PMSF and the cocktail of protease inhibitors. Intestines were everted onto plastic rods and shaken vigorously at 37 ˚C for 10 min. Intestines on plastic rods were placed in Hanks Buffered Salt Solution (in mM: 5.33 KCl, 0.441 KH_2PO_4, 4.17 $NaHCO_3$, 137.93 NaCl, 0.338 Na_2HPO_4, 5.56 glucose) (Invitrogen) containing 5 mM EDTA and 1 mM EGTA plus the protease inhibitors and shaken at 1800 rpm on VWR VX-250 multi-tube vortexer

at 4 ˚C for 60 min. Intestines were removed from plastic supports and the external muscle layer was removed. Remaining submucosa and epithelium were minced and placed in BD Cell Recovery Solution (BD Biosciences) and shaken at 4 ˚C for 90 min. Dispersed tissue was filtered through gauze and centrifuged at 800 x g for 5 min at 4 ˚C to isolate cells. Individual cells, villi and crypts were present in the cell pellet. Cells were dispersed in (in mM) 12 Tris-HCl, pH 7.4, 300 mannitol, 10 KCl, 0.5 EDTA, 30% glycerol and protease inhibitors as above and frozen at -80 ˚C.

Tracheal cells were isolated by enzymatic digestion of the entire trachea in HBSS (without $CaCl_2$ or $MgCl_2$), 1 EGTA, 20 EDTA, 0.05% collagenase, 1, 500U/50 ml of DNase, and the protease inhibitors with shaking at 4 ˚C for 2 hrs. Cells were filtered, centrifuged and dispersed as above.

Production of recombinant CFTR

293T cells were transfected with pcDNA3 vectors encoding porcine wild-type CFTR or CFTR-ΔF508 as previously described (6). Cells were lysed 48 h after transfection, solubilized in lysis buffer (LB) [50 mM Tris (pH 7.4) 50 mM NaCl, 1% Triton X-100 and proteinase inhibitors (PI), 2 μg/ml aprotinin, 7 μg/ml benzamidine-HCl, 1 μg/ml pepstatin A and 2 μg/ml leupeptin), and centrifuged at 14,000 × *g* for 15 min at 4°C to separate soluble from insoluble pellet. Protein was measured using the BCA assay (Thermo-Fisher). 10-25 mg of protein was used per lane as control for electrophoresis of intestinal and tracheal samples.

Immunoprecipitation and phosphorylation

Protein assays were performed using the BCA assay (Thermo-Fisher). Indicated amounts of intestinal tissue or cells from 1-2 trachea were homogenized in the Tris-mannitol buffer on ice with 20 strokes of the loose-fitting and 15 strokes of the tight-fitting pestle of a Potter-Elvejehm homogenizer. Membrane pellets were isolated by centrifugation at 200,000 x g at 4 ˚C for 30 min. The pellets were solubilized in a commercial detergent mix, Membrane Solution 2 (Profoldin, Ca). Soluble proteins were separated from insoluble pellets by centrifuging at 200,000 x g for 20 min. CFTR was immunoprecipitated from the supernatant of soluble proteins with anti-CFTR antibodies M3A7 and MM13-14 (Upstate Biotechnology) and *in vitro* phosphorylated with ^{32}P-ATP and the catalytic subunit of PKA (Promega) *(6)*. Washed precipitates were electrophoresed on 6% SDS-PAGE. Gels were stained, destained, dried and exposed to phosphoscreens before imaging on a Fuji FLA7000 imager (General Electric).

Immunocytochemistry

Ileal and tracheal tissues were excised from newborn piglets, immediately placed in ice-cold 30% sucrose, and quick-frozen in OCT with liquid N_2. Tissue segments were kept at -80 ˚C. Tissues were cryosectioned into 7 μm sections, fixed in 100% MeOH at -20 ˚C for 10 min, permeabilized in 0.2% TX-100 (Thermo-Fisher) in PBS, and blocked in Super-Block (Thermo-Fisher) with 5% normal goat serum (Jackson ImmunoResearch). Tissue sections were incubated for 2 hrs at 37 ˚C in anti-CFTR antibodies MM13-4, M3A7 (Chemicon), and polyclonal antibody to the tight junction protein ZO-1 (Zymed) (all at 1:100 dilution), followed by secondary antibodies (goat-anti-mouse Alexa-Fluor488 and goat anti-rabbit Alexa-Fluor568; Molecular Probes/Invitrogen) (1:1000 dilution). Sections were mounted with Vectashield (Hard-set)

containing DAPI (Vector Labs) to visualize nuclei. Images were acquired with identical parameters on an Olympus Fluoview FV1000 confocal microscope with a UPLSAPO 60X oil lens. Images were scanned sequentially at 2 μsec/pixel. Post collection enhancements were done identically, except where indicated in the figure legends to amplify CFTR-ΔF508 signal.

Electrophysiological measurements of freshly excised and cultured epithelia

Epithelial tissues were excised from the nasal turbinate and septum, and from trachea through 2nd generation bronchi immediately after animals were euthanized. Tissues and cultured epithelia were studied in modified Ussing chambers. Epithelia were bathed on both surfaces with solution containing (mM): 135 NaCl, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 10 dextrose, 5 HEPES, pH 7.4, at 37 °C and gassed with compressed air. Na-gluconate was substituted for NaCl in low Cl^- bath solutions. Transepithelial voltage (Vt) was maintained at 0 mV to measure short-circuit current (Isc). Transepithelial electrical conductance (Gt) was measured by intermittently clamping Vt to +5 and/or -5 mV.

For studies of HCO_3^- transport, we bathed the epithelia in a solution of (in mM): 118.9 Na-gluconate, 25 $NaHCO_3^-$, 2.4 K_2HPO_4, 0.6 KH_2PO_4, 5 Ca-gluconate, 1 Mg-gluconate and 5 dextrose, bubbled with 5% CO_2/95% air.

A standard protocol was the following. 1) Measurements under basal conditions. 2) 100 μM apical amiloride to inhibit ENaC Na^+ channels. 3) 100 μM apical DIDS (4,4-diisothiocyano stilbene-2,2-disulfonic acid) to inhibit most anion channels other than CFTR. 4) 10 μM forskolin and 100 μM IBMX (3-isobutyl-2-methylxanthine) to increase cellular levels of cAMP

leading to phosphorylation and activation of CFTR. 5) 100 μM apical GlyH-101 to inhibit CFTR. 6) 100 μM bumetanide to inhibit basolateral Na-K-2Cl transporter in cultured epithelia.

To directly measure apical CFTR-mediated Cl^- transport, we pretreated cultured tracheal epithelia apically with amiloride (100 μM) and DIDS (100 μM) in symmetrical bath solutions (139.8 mM Cl^-). We then imposed a large Cl^- concentration gradient across the epithelia by replacing NaCl in the apical solution with Na-gluconate (final Cl^- concentration 4.8 mM) containing the same amount of amiloride and DIDS. To assess apical CFTR Cl^- currents, we permeabilized the basolateral membrane with nystatin (0.36 mg/ml) and activated CFTR by adding 10 μM forskolin and 100 μM IBMX apically. After currents reached a plateau, we added 100 μM GlyH-101 apically to inhibit CFTR.

Statistical analysis

Data are presented as means ± SEM. Differences were considered statistically significant at $P < 0.05$. Statistical analysis of morphometric data was performed with a 1 way ANOVA and Bonferroni post-test. For electrophysiological assays involving three comparisons ($CFTR^{+/+}$, $CFTR^{\Delta F508/\Delta F508}$, and $CFTR^{-/-}$), the variances were often significantly unequal. We therefore used Welch's unpaired t tests *(7)*, and considered differences to be statistically significant if $P < 0.05/3$ (i.e., $P < 0.017$) *(8)*.

SUPPLEMENTAL REFERENCES

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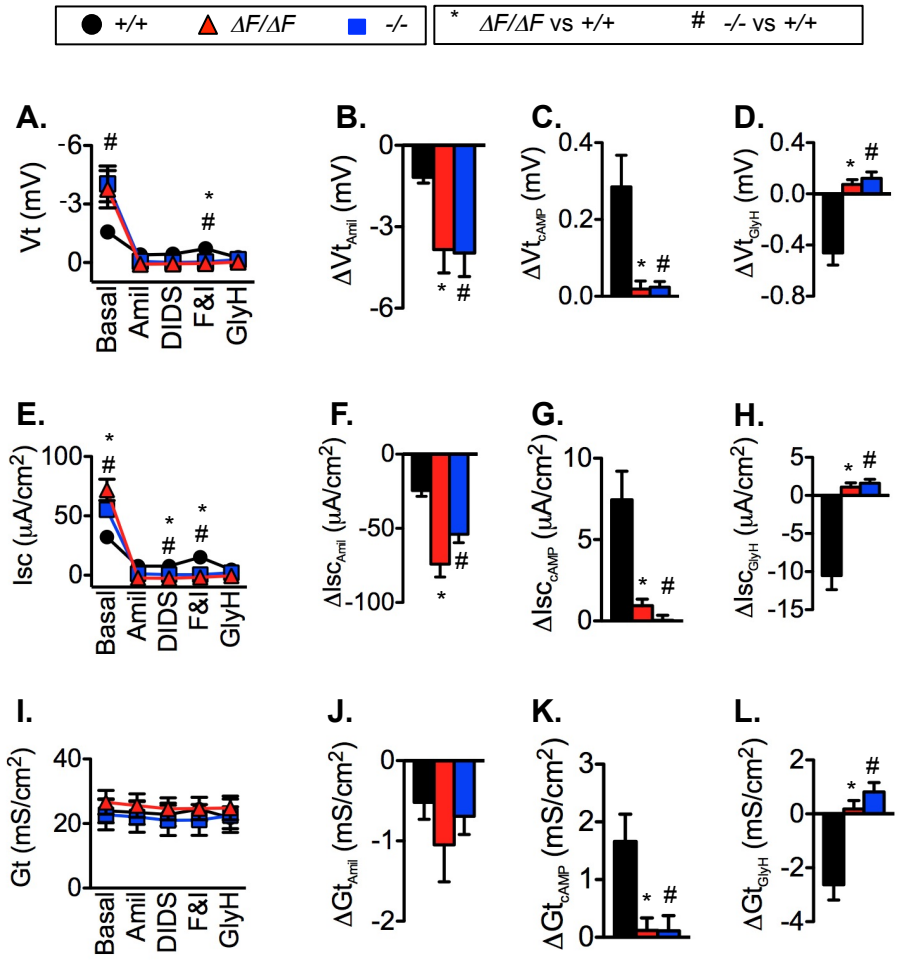

Figure S1. Electrophysiological properties of freshly excised porcine nasal epithelia. Data are from *CFTR*$^{+/+}$ (11 tissues, 8 pigs), *CFTR*$^{\Delta F508/\Delta F508}$ (12 tissues, 8 pigs), and *CFTR*$^{-/-}$ (20 tissues, 10 pigs) epithelia. Data from *CFTR*$^{-/-}$ and most CFTR$^{+/+}$ pigs were previously reported *(9)*.
* indicates *CFTR*$^{\Delta F508/\Delta F508}$ differs from *CFTR*$^{+/+}$, # indicates *CFTR*$^{-/-}$ differs from *CFTR* $^{+/+}$, and † indicates *CFTR*$^{\Delta F508/\Delta F508}$ differs from *CFTR*$^{-/-}$, all at $p<0.017$ by unpaired t test with Welch's correction. **A)** Transepithelial voltage (Vt) and response to sequential apical addition of 100 μM amiloride, 100 μM DIDS, 10 μM forskolin and 100 μM IBMX, and 100 μM GlyH-101. **B)** ΔVt_{amil} indicates change in Vt with addition of amiloride. **C)** ΔVt_{cAMP} indicates change in Vt with addition of forskolin and IBMX. **D)** ΔVt_{GlyH} indicates change in Vt with addition of GlyH-101. **E-H**) Short-circuit current (Isc) measurements that correspond to Vt measurements in panels A-D. **I-L)** Transepithelial conductance (Gt) measurements that correspond to Vt measurements in panels A-D. Changes with DIDS were small and did not differ by genotype.

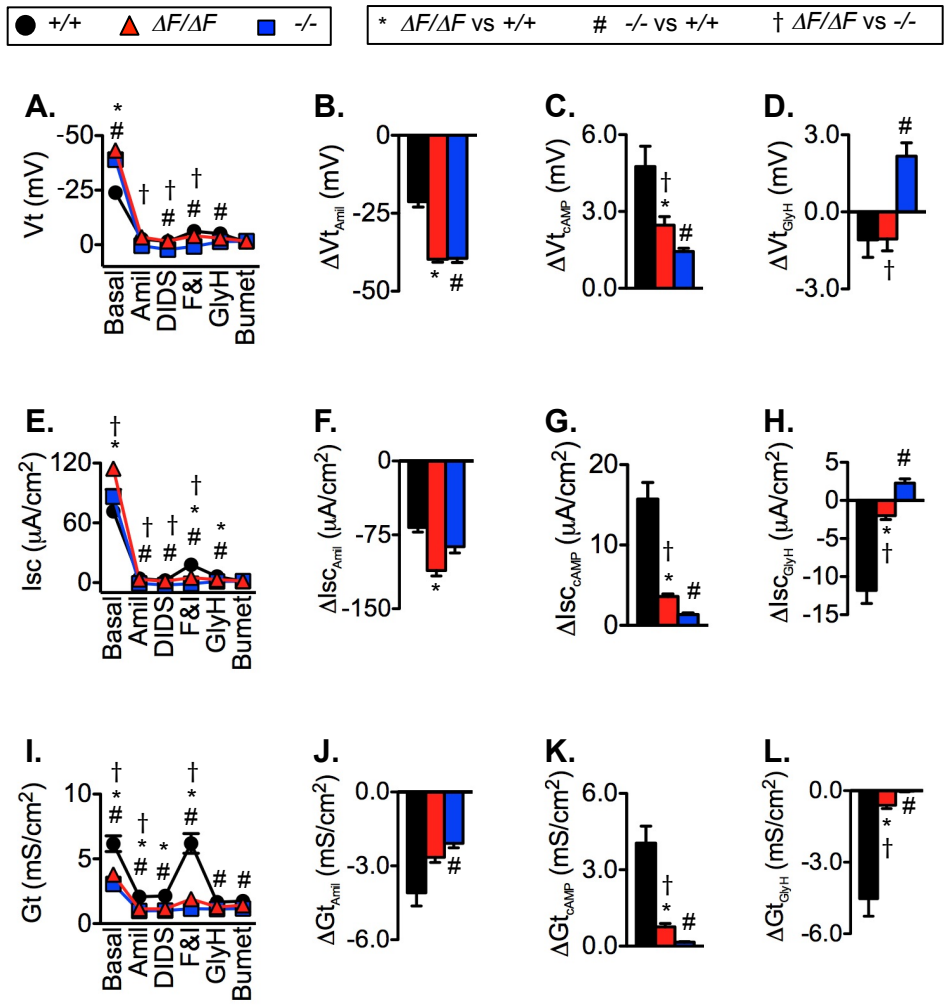

Figure S2. Electrophysiological properties of differentiated primary cultures of porcine nasal epithelia. Data are from *CFTR* $^{+/+}$(18 tissues, 14 pigs), *CFTR*$^{\Delta F508/\Delta F508}$ (31 tissues, 21 pigs), and *CFTR*$^{-/-}$(27 tissues, 21 pigs) epithelia. See legend of Fig. S1 for details

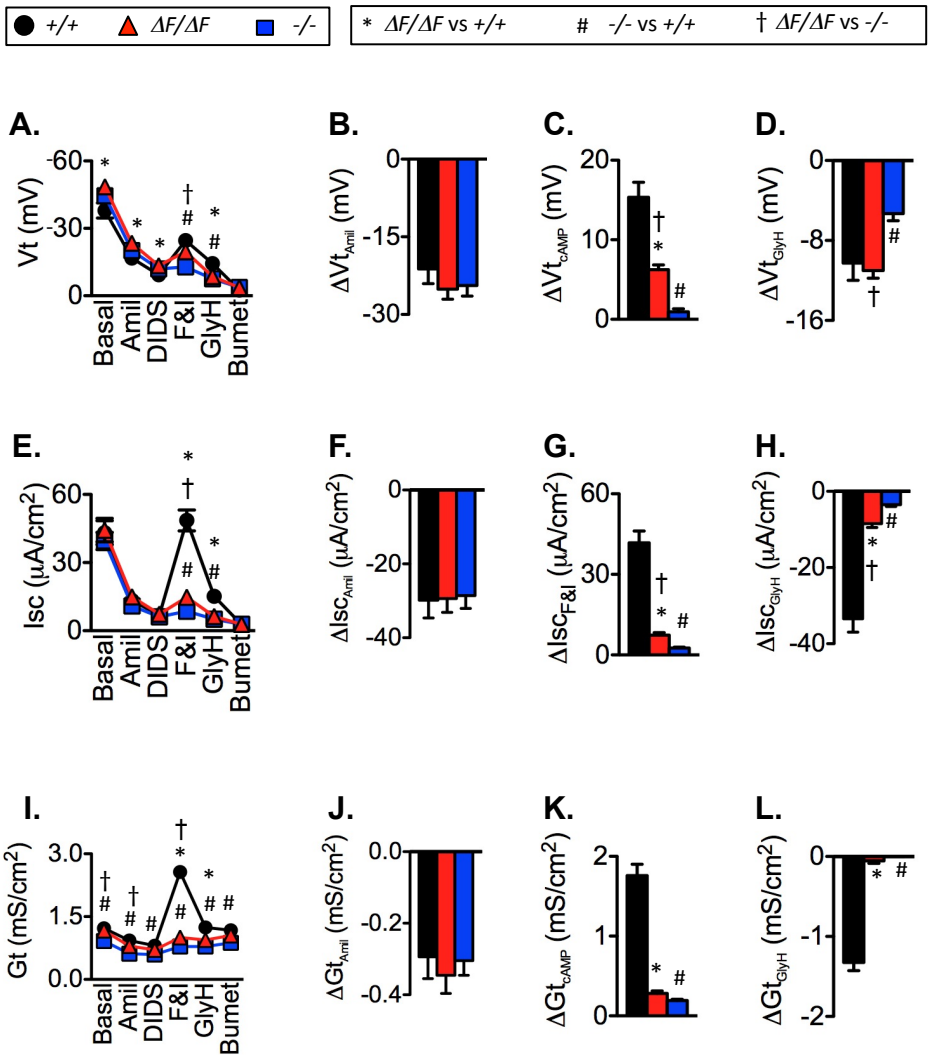

Figure S3. Electrophysiological properties of differentiated primary cultures of porcine tracheal epithelia. Data are from *CFTR*$^{+/+}$(17 tissues, 17 pigs), *CFTR*$^{\Delta F508/\Delta F508}$ (19 tissues, 19 pigs), and *CFTR*$^{-/-}$ (22 tissues, 22 pigs) epithelia. See legend of Fig. S1 for details.