SUPPORTING ONLINE MATERIAL

MATERIALS AND METHODS

Animals. We previously reported production of *CFTR*+/– male and female pigs (*1*). For this study, they were crossed and the progeny studied. Standard procedures for animal husbandry were used throughout. The Institutional Animal Care and Use Committees of the University of Iowa and the University of Missouri approved all animal experiments. We studied two groups of animals. One group was studied between birth and 12 h and were not fed. The other group was fed colostrum and milk replacer and studied between 24 and 40 h.

Genotyping. Genotyping was performed using genomic DNA isolated from fresh umbilical cord. Tissue was lysed for 15 min at 55 °C in lysis buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-Cl, pH 8.5, 0.5% Nonidet P40, 0.5% Tween, 400 μg/ml Proteinase K) (*2*). Two µl of lysate were used in each PCR (Platinum Taq HF, Invitrogen). PCR conditions: 2 min at 95 ºC, 30 cycles of 95 ºC for 20 sec, 56 ºC for 20 sec, and 68 ºC for 4 min, then 68 ºC for 5 min. Primers Ex10a5F (AGAATTTCATTCTGCTCTCAGT) and GC7R (GAAGACCCTTTACCTTCTTCTA) amplify a 2.0 kb product from wild-type *CFTR* alleles and a 3.7 kb product from gene-targeted *CFTR* alleles. PCR products were electrophoresed on a 1.0% agarose gel. For Southern blotting, 10 μg of genomic DNA was digested with *Bgl*II overnight. Genomic digests were electrophoresed on a 0.7% agarose gel and transferred to a positively charged nylon membrane (Roche) using an alkaline transfer procedure. Blots were pre-hybridized for 15 min at 65 ºC in Rapid-hyb buffer (Amersham). The blot was then hybridized in Rapid-hyb buffer with a ^{32}P-labeled probe specific for a region of *CFTR* that is outside of the targeting vector boundaries. The blot was washed once in 2X SSC/0.1% SDS for 20 min at room temperature followed by two washes in 0.1X SSC/0.1% SDS for 15 min at 65°C. Signal was detected using a phosphorimager. The *CFTR* probe hybridizes at 7.9 kb for wild-type *CFTR* alleles and 9.7 kb for gene-targeted *CFTR* alleles.

Northern blot. Northern blot was performed using total RNA isolated from ileum (RNeasy, Qiagen). Total RNA was electrophoresed on a denaturing gel and transferred to a positively-charged membrane (NorthenMax, Ambion). The membrane was hybridized with ^{32}P-labelled DNA probes corresponding to nucleotides 1-1000 of the porcine *CFTR* cDNA or 1-1002 of porcine *GAPDH* cDNA. Signal was detected using a Fuji FLA7000 phosphorimager. Porcine *CFTR* mRNA was detected at ~6.5kb and *GAPDH* mRNA at ~1.5 kb.

Quantitative RT-PCR. Quantitative RT-PCR using TaqMan chemistry and an ABI 7500 Fast Real-time PCR System was used to measure porcine *CFTR* mRNA. Briefly, total RNA was isolated from ileum (RNeasy, Qiagen). First-strand cDNA was synthesized with oligo-dT primers (SuperScript II, Invitrogen). Sequence-specific primers and probes for porcine *CFTR* and *GAPDH* were designed and ordered using Assays-by-design (Applied Biosystems). For measuring *CFTR* mRNA, primer/probe sets annealing to exon 10 of *CFTR* and *GAPDH* were used in separate reactions (*CFTR*, Forward: TCATGCCGGGCACCATTAAA, Reverse: CGCTTTGATGACACTCCTGTATCTA, Probe FAM-

ACACCAAAGATGATGTTTTC; *GAPDH*, Forward: AAGCTCATTTCCTCGTACGACAAT, Reverse: GGAGGCCATGTGGACCAT, Probe: FAM- TCCACCACCCTGTTGCT). TaqMan Fast Universal PCR Master Mix was used for all reactions. The reaction volume was 20 μl (10 μl of 2x Master Mix without UNG, 1 μl of 20x target primer and probe, 8 μl of Nuclease-free water, and 1 μl of cDNA sample). The reaction plates were covered with optical film and centrifuged briefly. The thermocycler conditions were as follows: 20 sec at 95 ºC, 40 cycles of 95 ºC for 3 sec and 60ºC for 30 sec. All experiments were run in triplicate. Because the efficiencies of *CFTR* and *GAPDH* amplification were not equal, the relative quantification of transcript levels was performed using the standard curve method.

Immunoprecipitation and phosphorylation. We used lysis buffer with 1% NP-40 and centrifugation as previously described (*3*) to prepare soluble protein from cultured airway epithelial cells. Equal amounts (750 μg) of soluble protein were immunoprecipitated with M3A7 antibody (Upstate Technology) and then *in vitro* phosphorylated as described previously (*3*). Immunoprecipitated, phosphorylated proteins were electrophoresed on 6% PAGE, the gels dried and then exposed to phosphorscreen before scanning on Fuji FLA7000 (Fuji Corp.). BHK cells stably expressing wild-type CFTR (a gift from Dr. Gergely Lukacs) were used as controls (7.5 μg) and were treated similarly.

Histopathology. Tissues were collected at necropsy and immediately placed in a bath of 10% neutral buffered formalin and gently agitated for a minimum of 72 hr. Tissues were then processed, paraffin-embedded, sectioned (4-5 μm) and stained with hematoxylin and eosin (H&E) or periodic acid-Schiff (PAS). Histopathological examination was performed by a veterinary pathologist blinded to pig genotype. This allowed for unbiased pathological assessment of tissues ranging from mild/moderate multifocal lesions (e.g. liver parenchyma) to severe lesions with 100% penetrance (e.g. pancreas).

Measurement of nasal Vt. The transepithelial voltage (Vt) across the nasal epithelium was measured using previously described methods (*4, 5*) under propofol anesthesia with the animal spontaneously breathing. The reference electrode was a small Ringer's solution-filled catheter inserted into the leg muscle (25 g needle). The exploring electrode was a 6 french foley catheter filled with Ringer's solution (148 mM NaCl, 2.4 mM KH_2PO_4, 0.4 mM K_2PO_4, 2.25 mM $CaCl_2$, 1.2 mM $MgCl_2$) inserted 4 cm into the nasal cavity, and the nasal mucosa was perfused at a rate of 5 ml/min using Ringer's solution to obtain baseline Vt. Subsequent perfusion solutions included: Ringer's solution containing 100 μM amiloride; Cl^--free Ringer's solution containing gluconate substituted for Cl^- plus amiloride; Cl^--free Ringer's solution containing amiloride plus 10 μM isoproterenol; Cl^--free Ringer's solution containing amiloride, isoproterenol, plus 100 μM ATP; Cl^--free Ringer's solution containing amiloride, isoproterenol, ATP, plus 100 μM GlyH-101. Voltage was measured with a voltmeter connected to a strip chart recorder. Each solution was perfused for 3-5 min. Following completion of the measurements, the epithelium was disrupted by brushing and Vt measured again to determine the zero value of Vt.

Immunocytochemistry. Ileal and tracheal tissues were excised from newborn piglets, immediately placed in ice-cold 30% sucrose, and quick frozen in OCT. Tissues were

cryosectioned at 7 μm thick onto polylysine-coated microscope slides, fixed in 100% MeOH at -20 ˚C for 15 min, permeabilized in 0.2% TX-100 (Thermo Scientific) in PBS, and blocked in Super-Block (Thermo Scientific) with 5% normal goat serum (Jackson ImmunoResearch). Tissue sections were incubated overnight at 4˚C in anti-CFTR antibodies MM13-4, M3A7 (Chemicon), and 24-1 (R&D Systems) and polyclonal antibody to ZO-1 (Zymed) (all at 1:100 dilution), followed by secondary antibodies (goat-anti-mouse A488 and goat anti-rabbit A568 (Molecular Probes) (1:1000 dilution). Sections were mounted with Vectashield containing DAPI (Vector Labs) to visualize nuclei. Images were acquired on an Olympus Fluoview FV1000 confocal microscope with a UPLSAPO 60X oil lens, 1.35 NA. Images were scanned sequentially at 2 μsec/pixel. Images were processed identically using Fluoview FV10-ASW-1.6 confocal software. RGB images were converted to white images for green and red channels and overlayed on a single Z-section of DIC with DAPI for merged image.

Bronchoalveolar lavage (BAL) fluid collection and analysis. Animals were studied between 6 and 12 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). IL-8 levels were measured by antibody capture assay (Thermo Scientific). Standard quantitative microbiologic techniques were used to identify and quantitate the bacteria in BAL.

Statistical Analysis. Data are presented as either mean ± standard error of the mean (SEM) or individual data points. Statistically significant differences between genotype groups were determined using either the Student's t-test or one-way ANOVA and the Student-Newman-Keuls test to determine group differences. Differences were considered statistically significant for $P<0.05$.

FOOTNOTES

Footnote F1.

The penetrance of meconium ileus was greater in newborn *CFTR*–/– piglets (100%) than in newborn humans with CF (~15%). There are several potential explanations for this difference. First, although our pigs were not inbred, they have a more uniform genetic background than humans with CF and hence may show less variability (*6*). Second, patients with two *CFTR* null mutations are very rare, and it is possible that a tiny amount of residual function from CFTR with common CF-associated mutations is sufficient to prevent a greater frequency of meconium ileus in humans. Third, the greater frequency in piglets might be due to anatomical or physiological differences between the species that increase susceptibility to obstruction.

CF mice also develop intestinal abnormalities, although their phenotype differs from the meconium ileus in newborn humans. Some mice die with intestinal disease a few days after birth, and others die around the time of weaning (*7-9*). When the intestine was examined before the onset of weight loss, it could appear similar in *CFTR*+/+ and *CFTR*–/– mice (*10*). In CF mice that developed an obstruction, the intestinal crypts were

dilated, villi were atrophic and sometimes necrotic, and there was increased mucus. Thus, in humans, pigs and mice, the intestine is susceptible to loss of CFTR, although the manifestations can differ.

Footnote F2.
In humans with meconium ileus, surgery is often required to relieve intestinal obstruction. Therefore, three *CFTR*–/– piglets had a laparotomy to remove the obstructing meconium and to place an ileostomy for drainage. We performed an identical procedure in one *CFTR*+/+ piglet. Two *CFTR*–/– and the one wild-type piglet were euthanized during the post-operative period because of technical problems in supportive care. One *CFTR*–/– piglet recovered. We fed him colostrum followed by milk replacer and then standard pig chow. He also received supplemental pancreatic enzymes and fat-soluble vitamins, but not the polyethylene glycol used to treat the intestinal disease in CF mice (*7, 8*). The ileostomy closed spontaneously, and he has grown well for eleven weeks. This is the same pattern observed in humans with CF, who once they recover from meconium ileus develop a pattern of disease like patients without meconium ileus (*11*).

When we discontinued pancreatic enzymes for three days, the *CFTR*–/– piglet's well-formed feces became soft, greasy and yellow like steatorrhea in humans with CF, and then reverted to a normal appearance with reinitiation of pancreatic enzymes. This result is consistent with the pancreatic histopathology and clinical pancreatic insufficiency. After ten weeks, he suddenly stopped eating, failed to pass feces, had an episode of vomiting, and the abdomen became distended. These signs resemble the "distal intestinal obstruction syndrome (DIOS)" observed in humans (*11, 12*). Therefore, as is done in humans, we treated him with oral polyethylene glycol and performed a Gastrografin enema that revealed the obstruction and relieved it. He then resumed eating and normal behavior. These observations are remarkably similar to meconium ileus, pancreatic insufficiency and DIOS in humans with CF.

Although these observations came from a single animal, the meconium ileus had a penetrance of 100%, and therefore the successful treatment with surgery is significant. The pig's subsequent course, which has been identical to what we see in humans, further indicates that this CF pig may prove valuable for those interested in CF. It also suggests that it will be possible for the *CFTR*–/– animals to survive for additional studies focused on CF.

Footnote F3.
Although patients are commonly said to have a "congenital" absence of the vas deferens, the incidence of abnormal vas deferens is low in fetuses and young children, and mucinous obstruction is proposed to cause progressive degeneration (*13-15*).

Footnote F4.
In neonates with CF, the lung parenchyma and airways appear histologically normal at birth. However, the submucosal glands have sometimes been reported to be abnormal in neonates with CF. A report from Oppenheimer (*16*) studied samples from 90 infants and young children with CF and 90 age matched controls. She concluded that there was no

difference in tracheobronchial submucosal glands. Two studies have investigated this issue quantitatively. Chow *et al*. (*17*) examined autopsy material from 21 patients who died within the first 3 weeks of life from meconium ileus. They quantitatively measured submucosal gland size and acinar diameter, and found no difference between CF and non-CF glands. Sturgess and Imrie (*18*) quantitatively examined submucosal glands from patients who had died between birth and 4 months of age. They found no difference between non-CF and CF in multiple measures of submucosal gland size and acinar diameter. However in CF, they reported an increase in the lumen fraction (lumen volume/[acinar cell volume + lumen volume]), an index of dilatation of acini. This increase could be attributed to patients who had lung infection. If only patients who had meconium ileus without obvious pulmonary infection were considered, then there was no difference between the groups. Thus, the evidence suggests a lack of difference between CF and non-CF lungs, including submucosal glands, at the time of birth.

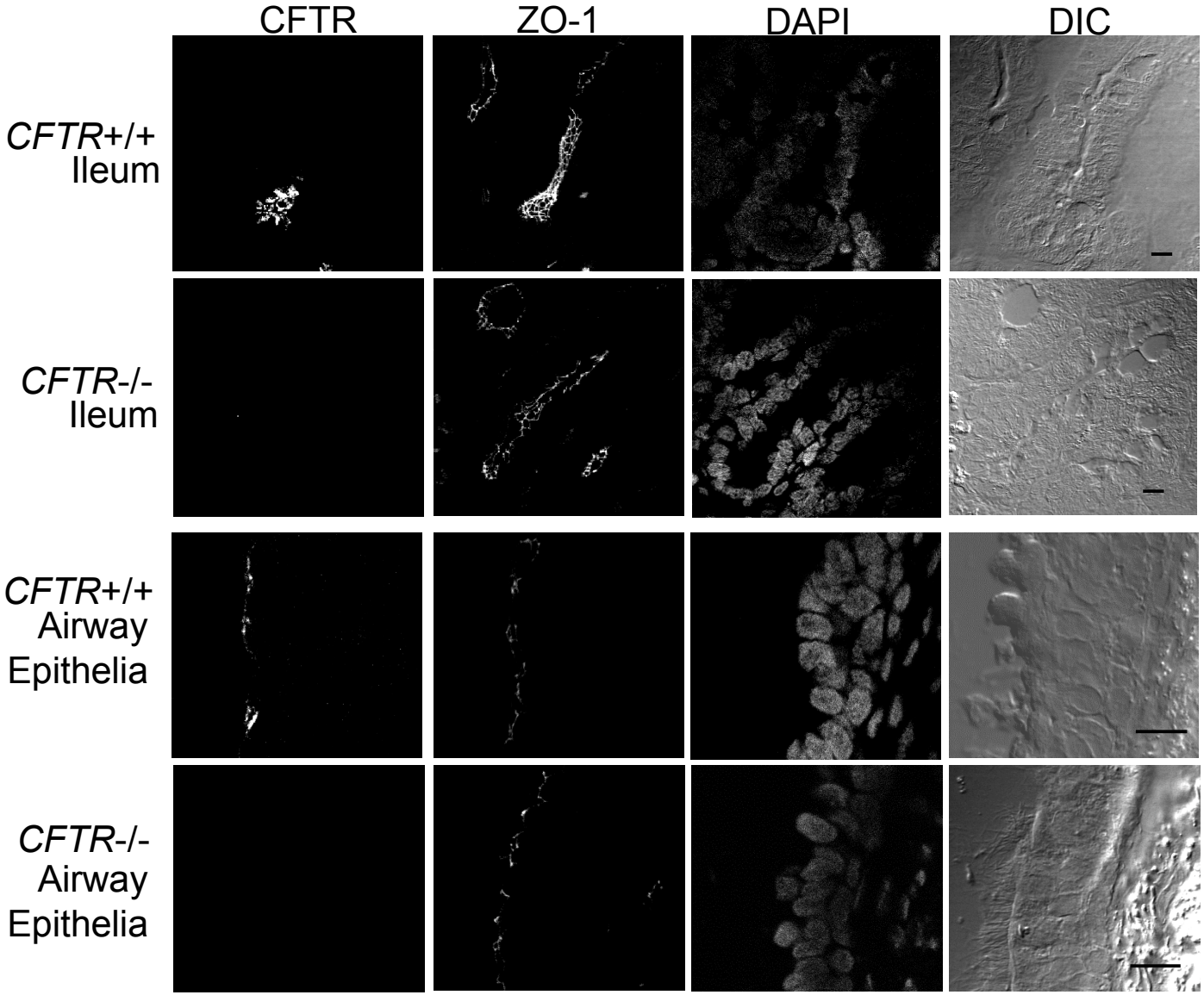

Figure S1. Images show staining for CFTR, ZO-1 and DAPI, plus differential interference contrast. The images in Fig. 1D are a merge of all four of these images. Bars, 10 μm.

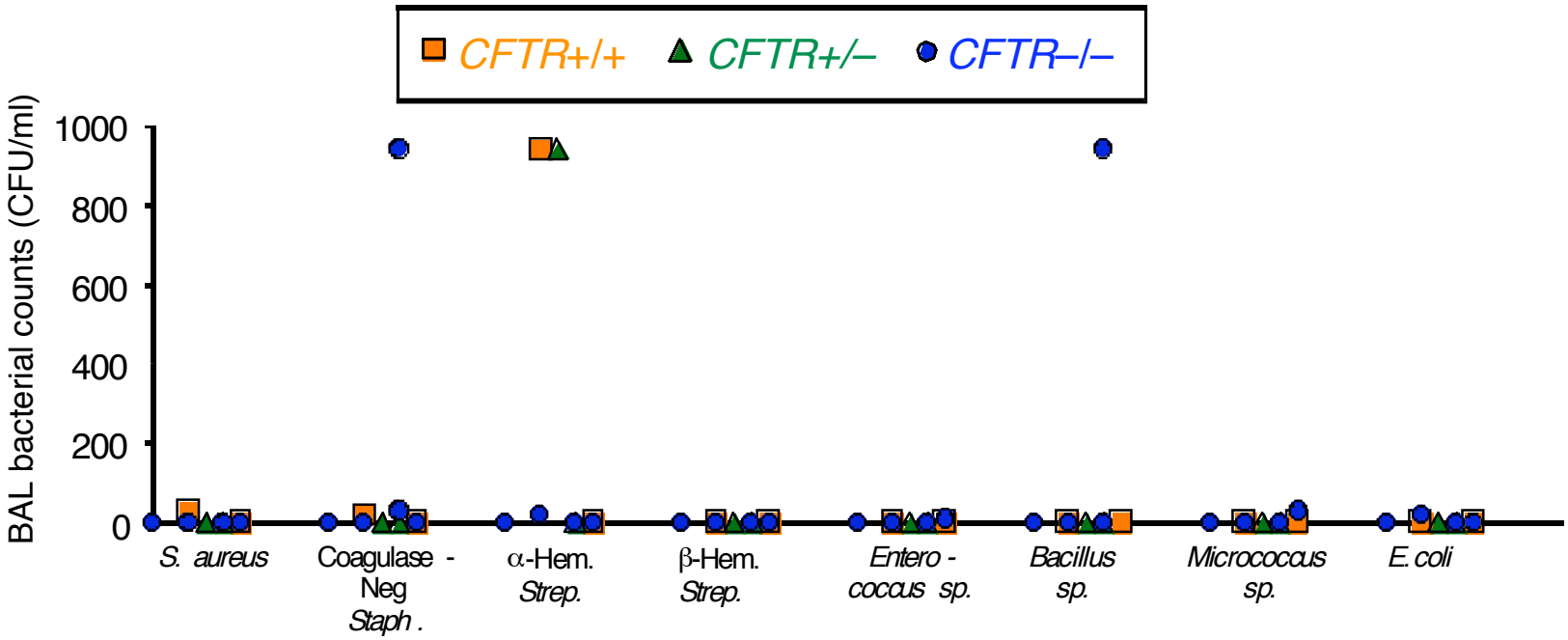

Figure S2. Results from bacterial culture of bronchoalveolar lavage fluid obtained from piglets between 6 and 12 h after birth.

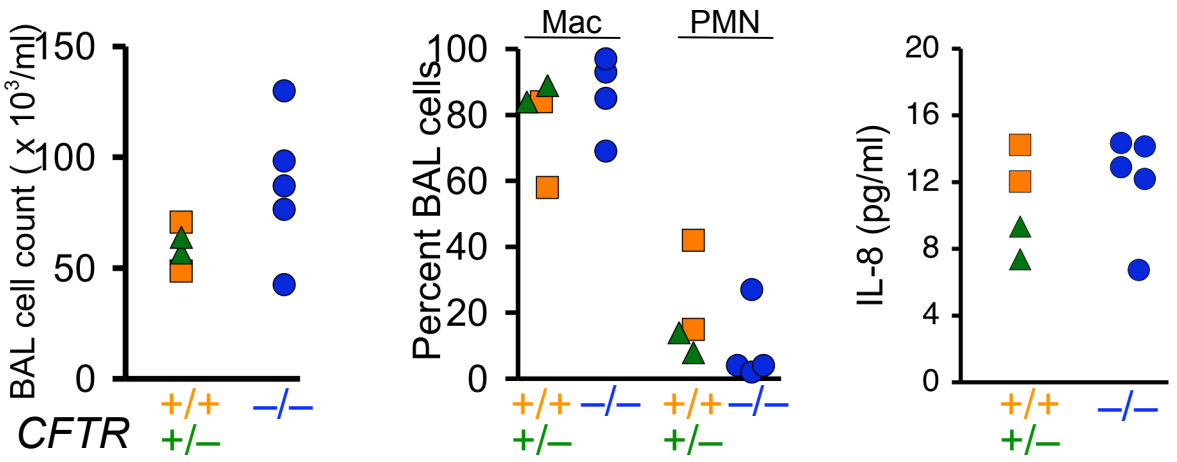

Figure S3. Results from bronchoalveolar lavage (BAL) on unfed piglets <12 hr old. Data are total numbers of cells in the lavage, percentages of macrophages and neutrophils, and levels of IL-8. Data are from 2 *CFTR*+/+, 2 *CFTR*+/–, and 5 *CFTR*–/– piglets. Values were not statistically different, P>0.1.

Table S1. Pathological changes in newborn *CFTR*–/– piglets and human neonates with CF. For references to histopathological changes in humans, see the following references (see Suppl. Refs. (*6, 13-21*).

Organ	Tissue	Lesions in newborn CFTR–/– piglets	Detectable in human neonates with CF
Pancreas	Parenchyma	Reduced amounts of exocrine tissue with increased adipose and connective tissue filling the void	Yes
	Ducts/ductules	PAS+ eosinophilic inspissated material and mucus filling lumen; ductular hyperplasia	Yes
	Acini	Eosinophilic material with rare mucus; atrophic and degenerated acinar cells; reduced zymogen granules	Yes
	Inflammation	None to moderate lymphoid and eosinophilic infiltrate; scattered neutrophils and macrophages in dilated acini and ducts	Yes
Intestine	Duodenum	Brunner glands dilated by inspissated mucus	Yes
	Distal small intestine to proximal colon	Meconium ileus/obstruction	Yes
	Proximal to obstruction	Dilated intestine filled with sticky, viscous meconium	Yes
	Distal to obstruction	Small intestine/colon small, stenotic; variably filled with mucus	Yes
	Complications	*In utero* perforation, chronic peritonitis, atresia	Yes
Liver	Portal regions	Focal biliary cirrhosis; variable ductular hyperplasia, fibrosis, and inflammation	Yes
	Biliary duct/ductules	Eosinophilic material/mucus in lumen; scattered neutrophils	Yes, rare
Gallbladder	Lumen	Small and variably filled with inspissated bile and mucus	Yes
	Cystic duct	Obstructed by mucus and eosinophilic material	Yes
Vas deferens	Duct	None	±
Lung	Parenchyma	None; no inflammation or infection	No
	Airways	None; no inflammation or infection	No

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