Supplementary Materials and Methods

^{99m}Tc-Mebrofenin Preparation and Scintigraphic Imaging

^{99m}Tc-mebrofenin N-(2,4,6 trimethyl 3 bromophenyl carbamoyl methyl) iminodiacetic acid derivative was prepared with a Cholecis kit (Schering-CisBio International, Saclay, France) containing lyophilized mebrofenin (40 mg) and stannous chloride (0.6 mg). These compounds were mixed with generator-eluted sodium $^{99m}TcO_4^-$ (740 MBq) at room temperature for 10 minutes, to allow the chelation of ^{99m}Tc reduced extemporaneously by $SnCl_2$, with mebrofenin. Scintigraphic imaging was performed with a high-resolution gamma camera (Biospace Mesures, Paris, France) equipped with a position-sensitive photomultiplier tube and a parallel collimator with 1.3-mm holes. Images were recorded with a 128 × 128-pixel and 16-bit matrix, on the 140-keV photopeak of the ^{99m}Tc with a 20% spectral width. Anterior view planar hepatic, gallbladder, and intestinal phase images were acquired at 1-minute intervals for 25 minutes, 5-minute intervals for the next 20 minutes, and 30-minute intervals until 180 minutes.

Bile Acid Analysis by HPLC-Tandem Mass Spectrometry

Chemicals and reagents. Cholic acid, deoxycholic acid, chenodeoxycholic acid, ursodeoxycholic acid, lithocholic acid, hyocholic acid, hyodeoxycholic acid, and corresponding glycine and taurine conjugates were purchased from Sigma-Aldrich; 3-sulfate conjugates were provided by J. Goto (Niigita University of Pharmacy and Applied Life Science, Niigata, Japan); 23-nor-5β-cholanic acid-3α,12β diol, muricholic acid derivatives, and their glycine and taurine conjugates were purchased from Steraloids, Inc (Newport, RI). Acetic acid, ammonium carbonate, and ammonium acetate were from Sigma-Aldrich.

Standard solutions. The 1 mg/mL stock solutions were prepared in methanol and stored at −20°C. The stock solutions were pooled and diluted to obtain mixed calibration solutions of 31.3 μg/mL to 31.3 ng/mL of BA. For tissue extraction, liver specimens (combined with intestine ± gallbladder for BA pool analyses) were cut into small pieces and boiled for 5 minutes in ethanol. Norcholic acid (1 mg/mL stock solution in 50% ethanol; Steraloids, Inc) was added to ethanol as a recovery standard. Extracts were filtered, adjusted to a specific volume, and treated as a biological fluid. Feces collected over a 72-hour period were homogenized and freeze-dried; 2 mL of 0.1 mol/L NaOH was added to 0.1 g dry feces and the mixture was incubated 1 hour at 60°C; 4 mL of distilled water containing 2 μL of norcholic acid (from a 1-mg/mL stock solution in 50% ethanol) was added and the sample was homogenized for 30 seconds with a Polytron homogenizer (KINEMATICA, Inc, Lucern, Switzerland) and centrifuged for 20 minutes at 20,000 *g*. The supernatant was removed and treated as a biological fluid.

Samples of tissue or stool extracts, serum, or water-diluted bile (1/1000, vol/vol) were mixed with 2 μL of internal standard solution (23-nor-5β-cholanoic acid-3α,12β diol; 1 mg/mL). BAs were released from proteins by incubation with 0.5 mol/L ammonium carbonate for 30 minutes at 60°C. Samples were cleaned by centrifugation (4000 *g* for 10 minutes) and solid-phase extraction on reverse-phase Chromabond C_{18} cartridges (100 mg; Macherey-Nagel, Düren, Germany). Solid-phase extraction was performed on a vacuum manifold. The cartridge was rinsed successively with water (20 mL) and hexane (10 mL) to remove neutral lipids, and then again with water (20 mL). BAs were eluted with methanol, dried by evaporation under a nitrogen stream at 50°C, and dissolved in 150 μL methanol. We injected 5 μL of the resulting solution into HPLC-MS/MS.

HPLC-tandem mass spectrometry conditions. Chromatographic separation was performed with an Agilent Technologies 1100 HPLC apparatus fitted with a Restek C18 Pinnacle II analytic column (250 × 3.2 mm, 5 μm; Restek, Lisses, France). Separation was achieved at a flow rate of 0.3–0.5 mL/min. Mobile phase A was 15 mmol/L ammonium acetate, pH 5.3, and solvent B was methanol. HPLC was performed in series with the turbo ion spray source of the mass spectrometer QTRAP 2000 (Applied Biosystems-Sciex, Concord, Ontario, Canada). Electrospray ionization was performed in the negative mode, with nitrogen as the nebulizer gas. Nebulizer, curtain, and heater gases were set at 40, 20, and 40, respectively. The temperature of the evaporation gas was set at 400°C. The ion spray, declustering, and entrance potentials were set at −4500 V, −60 V, and −10 V, respectively. Tandem mass spectrometry detection was performed with a unit resolution in multiple reaction monitoring mode. The dwelling time for each transition was set at 70 ms. The data were acquired with Analyst software, version 1.4.2 (Applied Biosystems-Sciex, Concord, Ontario, Canada). Multiple reaction monitoring was performed by studying the transition reactions from precursor ions to product ions after collision-induced dissociation of the taurine or glycine residue. For unconjugated monohydroxylated, dihydroxylated, and trihydroxylated bile acids, *m/z* 375, 391, and 407, respectively, were selected as precursor and product ions. For the internal standard (23-nor-5β-cholanoic acid-3α,12β diol), *m/z* 377 was selected. For glycine conjugates, *m/z* 432, 448, and 464 representing monohydroxylated, dihydroxylated, and trihydroxylated BAs, respectively, were selected as precursor ions and *m/z* 74 (the fragment ion of the glycine moiety), was selected as the product ion. For taurine conjugates, *m/z* 482, 498, and 514, corre-

Supplementary Table 1. Primers Used for Real-Time Polymerase Chain Reaction

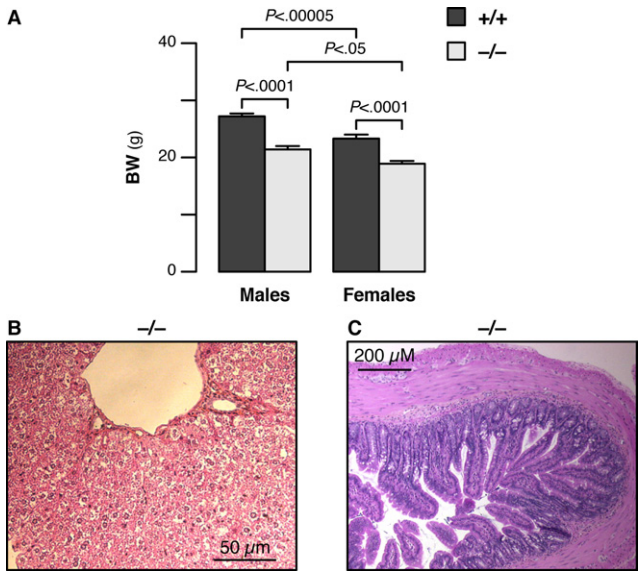
Gene	GenBank accession No	Primer sequences	
Abcb11/Bsep	NM_02102	ATCCTGCTTCTGGACATGGCTA	ATGGGCAACTGAGATGATTGTG
Cck	NM_03116	CGCGATACATCCAGCAGGT	AGCCCATGTAGTCCCGGTC
Cyp7a1	NM_00782	ACTCTCTGAAGCCATGATGCAA	AGCGTTAGATATCCGGCTTCAA
Cyp7b1	NM_00782	TCCGAGAAGTGCAGGAGGAT	TTTCCGGGTCATTGTGTATGAG
Cyp8b1	NM_01001	GAGGAAGCCACCAGGGTCAT	GACGCAGACTCTCCTCCATCA
Fgf15	NM_00800	CGTGTTTCACCGCTCCTTCT	TCTACATCCTCCACCATCCTGA
Fgf receptor 3	NM_00116	CAGGACCCGGCTGACACTT	GCTTGGCAGTACGGTCCTTG
Fgf receptor 4	NM_00801	CATGTCGTCTGCGAGTCAGAG	ACCTTTGGACCCTTTTCTCCC
Ibabp	NM_00837	AGGACGGACAGGACTTCACCT	CCCCATGGTCTGCATTTCAC
Ostα	NM_14593	TACCCTTCTGGCCCTCTGGT	GCACCTGGAACAGAGCAAACT
Ostβ	NM_17893	GATGCGGCTCCTTGGAATTA	CGATTTCTGTTTGCCAGGATG
Shp	NM_01185	TGGCCTCTACCCTCAAGAAACA	ACGGGTCACCTCCAGCAAAAG
Slc10A1/Ntcp	NM_01138	ACCTCCTCCCTGATGCCTTT	GTTGGACGTTTTGGAATCCTG
Slc10A2/Asbt	NM_01138	GTCCCCAAATGCAACTGTCT	CCACATTGCACCCCATAGAAA
Vip	NM_01170	GATGGAAGCCAGAAGCAAGC	CAGAAGGTGGTCCAAAGAGAGG
18S	NR_00327	GAGCGAAAGCATTTGCCAAG	GGCATCGTTTATGGTCGGAA

Bsep, bile salt export pump.

sponding to monohydroxylated, dihydroxylated, and trihydroxylated bile acids, respectively, were selected as precursor ions, and m/z 80 (the SO_3 fragment anion cleaved from taurine) was selected as the product ion. For sulfur conjugates, m/z 97 (the HSO_4 sulfuric anion from the sulfate moiety) was selected as the product ion.

Immunoblot Analysis of BA Transporters

Specimens of gallbladder or ileum from 10 and 2 mice, respectively, that were stored at −80°C, were pooled and homogenized in ice-cold homogenizing buffer containing 10 mmol/L Tris HCl (pH 7.4), 10 mmol/L KCl, 1.5 mmol/L $MgCl_2$, 0.68 mmol/L EDTA and 250 mmol/L sucrose and protease inhibitors (Mini-complete; Roche Diagnostics, Basel, Switzerland), using a Tissue lyser (Qiagen, Hilden, Germany). After tissue fragments and nuclei were pelleted at 800 *g*, membranes were pelleted at 100,000 *g*, and solubilized in 1% Triton X-100. Proteins (5 or 10 μg from ileum and gallbladder, respectively) were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis on 10% polyacrylamide gels. After transfer to nitrocellulose membranes (Bio-Rad, Hercules, CA) in 20% methanol, blots were blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat dried milk, for 1.5 hours at room temperature, and incubated overnight at 4°C with the following primary antibodies: anti-rodent Asbt polyclonal antibody (1/500), anti-mouse Ostα and Ostβ polyclonal antibodies (1/500),[16] and anti-mouse β-actin monoclonal antibody (1/5000; Sigma-Aldrich), followed by peroxidase-conjugated secondary antibodies, for 1 hour at room temperature. Signals were revealed by enhanced chemiluminescence (Thermo Fischer Scientific, Rockford, IL) and quantified using a ChemiGenius bioimaging system with the GeneTools software (Syngene, Frederick, MD).

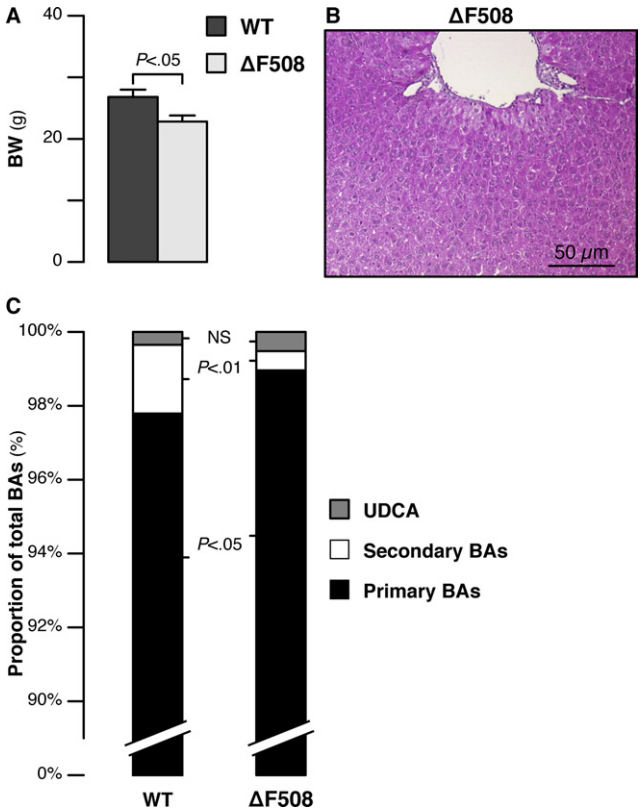


Supplementary Figure 1. Morphologic features in *cftr*$^{-/-}$ mice. The following features were recorded in 3-month-old *cftr*$^{-/-}$ mice and *cftr*$^{+/+}$ control littermates older than 3 months: (*A*) body weight (means ± standard error of the mean of 15 animals), and (*B*) liver and (*C*) ileum histology (normal appearance of H&E-stained tissue sections, representative of 30 *cftr*$^{-/-}$ mice).

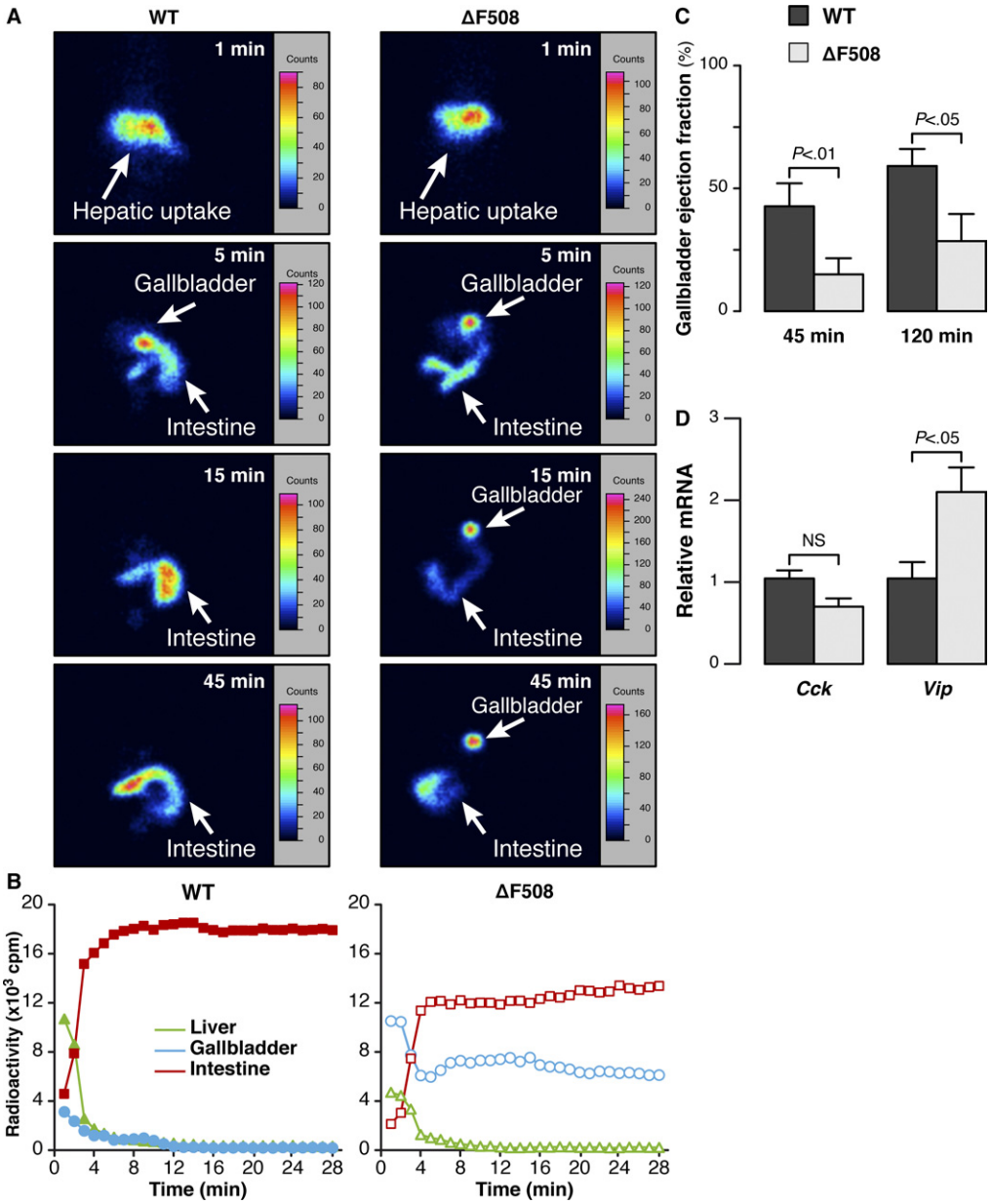
Supplementary Table 2. Liver Weight and Function Tests in *cftr*$^{-/-}$ mice and *cftr*$^{+/+}$ Littermates

	cftr$^{+/+}$	*cftr*$^{-/-}$	*P*
Liver-to–body weight ratio, % (n = 30)	3.86 ± 0.11	3.92 ± 0.12	.94
Bile tests (n = 4)			
Hepatic bile flow, *μL/min/g liver weight*	1.62 ± 0.04	1.56 ± 0.18	.99
Biliary BA output, *μmol/min/g liver weight*	0.07 ± 0.01	0.08 ± 0.05	.99
Serum tests (n = 6)			
BAs, *μmol/L*	12 ± 3.85	12.5 ± 3.96	.79
Cholesterol level, *mmol/L*	2.35 ± 0.16	2.31 ± 0.16	.70
Total bilirubin level, *μmol/L*	1.92 ± 0.31	1.96 ± 0.19	.90
Aspartate aminotransferase level, *IU/L*	60.6 ± 7.3	62.8 ± 6.1	1.00
Alanine aminotransferase level, *IU/L*	14.6 ± 3.7	20.2 ± 2.6	.47
Alkaline phosphatase level, *IU/L*	51.3 ± 5.1	65 ± 5.7	.30

NOTE. Liver-to–body weight ratio, bile measurements, and serum concentrations in the peripheral circulation (ie, vena cava), were determined in *cftr*$^{-/-}$ mice and *cftr*$^{+/+}$ littermates. Values are means ± standard error of the mean.



Supplementary Figure 2. Morphologic features and BA composition in ΔF508 mice. The following parameters were recorded in ΔF508 mice (n = 7) and wild-type (WT) control littermates (n = 7): (*A*) body weight (means ± standard error of the mean), (*B*) liver histology (normal appearance of an H&E-stained tissue section, representative of the 7 ΔF508 mice), and (*C*) BA composition, assessed by HPLC-tandem mass spectrometry analyses. Histograms show the mean proportions of primary BAs (cholic acid, muricholic acid, chenodeoxycholic acid, hyocholic acid), secondary BAs (deoxycholic acid, hyodeoxycholic acid, lithocholic acid), and ursodeoxycholic acid, including their conjugates, in portal blood (total BA concentrations were 118 ± 21.7 μmol/L and 123.39 ± 16.1 μmol/L, in WT and ΔF508 mice, respectively, NS).



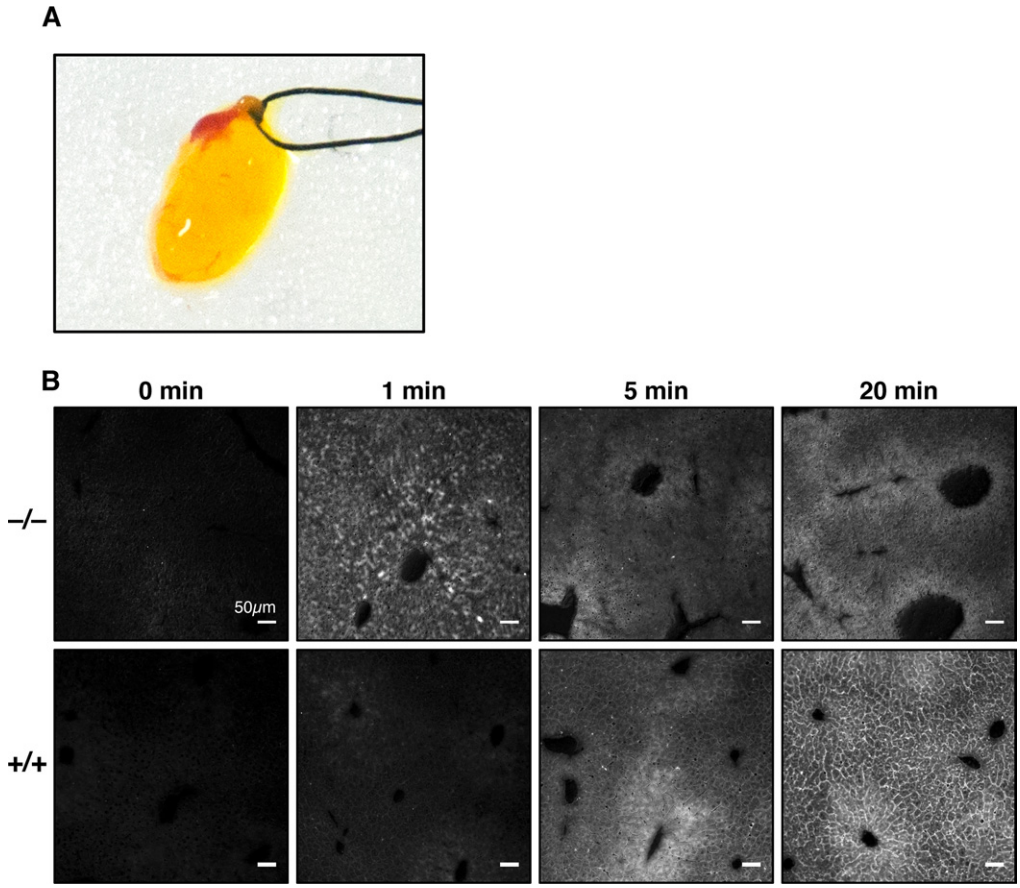
Supplementary Figure 3. Gallbladder motor function in ΔF508 mice. ΔF508 mice (n = 7) and wild-type (WT) control littermates (n = 7) were subjected to (*A–C*) scintigraphic analyses of gallbladder motor function in overnight-fed animals. (*A*) Typical images recorded at the indicated times after intravenous injection of ^{99m}Tc-mebrofenin. (*B*) Representative time-activity curves generated over regions of interest. (*C*) Gallbladder ejection fractions at 45 and 120 minutes (means ± standard error of the mean [SEM]). (*D*) Evaluation of gut-derived signaling to the gallbladder, by reverse-transcription polymerase chain reaction (RT-PCR) analyses of *Vip* and *Cck* gene expression in the duodenum. The mRNA levels are shown relative to the mean value in WT mice (means ± SEM).

Supplementary Table 3. Profiling of Individual Bile Acids in the Liver of *cftr*$^{-/-}$ Mice and *cftr*$^{+/+}$ Littermates

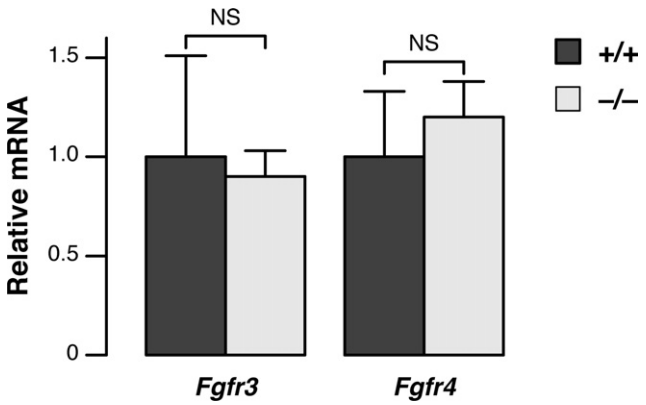
	cftr$^{+/+}$	*cftr*$^{-/-}$	*P*
Total BA concentrations, *nmol/g liver*	26.2 ± 7.6	32.4 ± 3.1	.3
Individual BAs, *%*			
T-CA	44 ± 5.3	49 ± 3.3	.69
T-MCA	20 ± 4.3	28 ± 3.3	.31
T-CDCA	1.34 ± 0.3	0.70 ± 0.14	.056
T-DCA	4.55 ± 1.23	1.95 ± 0.28	.015
T-HDCA	0.37 ± 0.02	0.02 ± 0.006	.008
T-LCA	0.03 ± 0.01	0.02 ± 0.002	.55
T-LCA-3S	0.01 ± 0.005	0.01 ± 0.003	.69
T-UDCA	0.70 ± 0.05	0.84 ± 0.05	.095
T-UDCA-3S	0.06 ± 0.03	0	.054
Total tauro-conjugates	71 ± 5	80.6 ± 2.5	.15
G-CA	0	0.03 ± 0.004	.15
Total glyco-conjugates	0	0.03 + 0.004	.15
MCA	21 ± 3	12.5 ± 1.6	.056
CDCA	0.18 ± 0.05	0.07 ± 0.014	.056
HCA	6.7 ± 2.5	6.2 ± 1.7	.84
Deoxycholic acid	0.23 ± 0.04	0.13 ± 0.03	.056
HDCA	0.5 ± 0.2	0.01 ± 0.005	.008
Ursodeoxycholic acid	0.5 ± 0.1	0.24 ± 0.05	.008
Total unconjugated	29.2 ± 5.1	19.2 ± 2.6	.15

NOTE. Total and individual BA concentrations were determined by HPLC-tandem mass spectrometry analysis in liver tissue from *cftr*$^{-/-}$ mice and *cftr*$^{+/+}$ littermates. Values are means ± standard error of the mean of 5 animals.

CA, cholic acid; CDCA, chenodeoxycholic acid; G, glyco; HCA, hyocholic acid; HDCA, hyodeoxycholic acid; LCA, lithocholic acid; MCA, muricholic acid; T, Tauro; 3S, 3 sulfate.



Supplementary Figure 4. Time-course analysis of the cholecystohepatic shunt. Cholyl-(Ne-NBD)-lysine was injected in the gallbladder of *cftr*$^{-/-}$mice and *cftr*$^{+/+}$ control littermates, after ligation of the cystic duct. At 1, 5, or 20 minutes after injection, the liver was collected and fluorescence was analyzed on frozen tissue sections. (*A*) The absence of cholyl-(Ne-NBD)-lysine leakage from the gallbladder was verified at the end of each experiment. (*B*) Fluorescence appeared sooner (1 minute) in the knockout, and thereafter increased over time in both *cftr*$^{-/-}$ mice and *cftr*$^{+/+}$ mice; basal fluorescence in the liver from noninjected animals is shown in comparison (0 min).



Supplementary Figure 5. Gallbladder expression of *Fgf receptor* in *cftr*$^{-/-}$ mice. *Fgf receptor 3* and *Fgf receptor 4* gene expressions were analyzed by quantitative reverse-transcription polymerase chain reaction in the gallbladder of *cftr*$^{-/-}$ mice and *cftr*$^{+/+}$ control littermates (means ± standard error of the mean of 6 animals).