Supporting Information

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SI Materials and Methods

Recombinant Cell Culture. FRT cells (gift from Michael J. Welsh, University of Iowa College of Medicine, Iowa City, IA) and HEK-293 cells (gift from Neil Bradbury, University of Pittsburgh, Pittsburgh, PA) stably expressing human forms of CFTR or F508del-CFTR were cultured as previously described (1, 2). HEK-293 and NIH 3T3 cells were cultured in HyQ CCM5 (HyClone) with 10% heat-inactivated FBS, whereas FRT cells were cultured in Coons modified Ham F-12 medium, 10% FBS, and 1% penicillin/streptomycin. During compound incubation, the FBS concentration was reduced to 1%. All cells were maintained at 37 °C unless otherwise indicated.

Isolation and Culture of HBE. Whole lungs were provided by the National Disease Research Interchange through an agreement with Cystic Fibrosis Foundation Therapeutics and were obtained from non-CF or CF subjects homozygous for the F508del mutation (F508del-HBE) following autopsy or lung transplantation. After removal, the intact lung was packed in ice-cold PBS solution and processed within 24 h. Non-CF and CF airway epithelia were isolated from bronchial tissue and cultured on 0.4-μm Snapwell culture inserts (catalog no. 3801; Corning) previously coated with NIH 3T3 conditioned media at a density of 5^{-5} cells per insert as previously described (2) with the following modifications: (*i*) Accutase (Innovative Cell Technologies) was used to dissociate the cells, (*ii*) all plastic culture ware and the Snapwell filters (Costar) were precoated with NIH 3T3-conditioned media, and (*iii*) bovine brain extract (kit CC-4133, component CC-4092C; Lonza) was added to the differentiation media. After 4 d, the apical media was removed and the cells were grown at an air:liquid interface for more than 14 d before use. This resulted in a monolayer of differentiated epithelial cells capable of transepithelial ion and fluid transport.

Primary HBE cell cultures during compound incubation were maintained in DMEM/F12, Ultroser G (2.0%; catalog no. 15950–017; Pall), fetal clone II (2%), insulin (2.5 μg/mL), bovine brain extract (0.25%; kit CC-4133, component CC-4092C; Lonza), hydrocortisone (20 nM), triiodothyronine (500 nM), transferrin (2.5 μg/mL: catalog no. 0030124SA; Invitrogen), ethanolamine (250 nM), epinephrine (1.5 μM), phosphoethanolamine (250 nM), and retinoic acid (10 nM).

Ussing Chamber Recordings. All cells were grown on Snapwell cell culture inserts (Costar) and maintained at 37 °C before recording. The cell culture inserts were mounted into an Ussing chamber (VCC MC8; Physiologic Instruments) to record the transepithelial current I_T in the voltage-clamp mode (0 mV). For FRT cells, the basolateral membrane was permeabilized with 270 μg/mL nystatin, and a basolateral-to-apical chloride gradient was established. The basolateral bath solution contained (in mM) 135 NaCl, 1.2 $CaCl_2$, 1.2 $MgCl_2$, 2.4 K_2HPO_4, 0.6 $KHPO_4$, 10 Hepes, and 10 dextrose (titrated to pH 7.4 with NaOH). The apical NaCl was replaced by equimolar sodium gluconate (titrated to pH 7.4 with NaOH). For HBE cells, the I_T was measured in the presence of a basolateral to apical chloride gradient. The basolateral solution contained (in mM) 145 NaCl, 3.3 K_2HPO_4, 0.8 KH_2PO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose, 10 Hepes (adjusted to pH 7.35 with NaOH) and the apical solution contained (in mM) 145 sodium gluconate, 3.3 K_2HPO_4, 0.8 KH_2PO_4, 1.2 $MgCl_2$, 1.2 $CaCl_2$, 10 glucose, 10 Hepes (adjusted to pH 7.35 with NaOH). All recordings were digitally acquired using Acquire and Analyze software (version 2; Physiologic Instruments).

Single-Channel Patch-Clamp Recordings. The single-channel activity of F508del-CFTR and CFTR was measured by using excised inside-out membrane patch recordings as previously described using an Axopatch 200B patch-clamp amplifier (Axon Instruments) (1). The pipette contained (in mM) 150 N-methyl-D-glucamine, 150 aspartic acid, 5 $CaCl_2$, 2 $MgCl_2$, and 10 Hepes (adjusted to pH 7.35 with Tris base). The bath contained (in mM) 150 N-methyl-D-glucamine–Cl, 2 $MgCl_2$, 5 EGTA, 10 NaF, 10 TES, and 14 Tris base (adjusted to pH 7.35 with HCl). After excision, CFTR was activated by adding 1 mM Mg-ATP and 75 nM PKA (Promega). The pipette potential was maintained at 80 mV. The P_o for CFTR and VX-809–corrected and uncorrected F508del-CFTR was estimated based on the number of channels in the patch following VX-770 (1 μM) addition.

CFTR Immunoblot Analysis. FRT, HEK-293, or HBE cells expressing CFTR or F508del-CFTR were incubated for 24 h at 37 °C with or without VX-809 in the assay media. After incubation, cells were harvested in ice-cold D-PBS solution (without calcium and magnesium) and pelleted at 1,000 × *g* at 4 °C. Cell pellets were lysed in 1% Nonidet P-40, 0.5% sodium deoxycholate, 200 mM NaCl, 10 mM Tris, pH 7.8, and 1 mM EDTA plus protease inhibitor mixture (1:250; Roche) for 30 min on ice. Lysates were spun for 10 min at 10,000 × *g* at 4 °C to pellet nuclei and insoluble material. Approximately 12 μg total protein was heated in Laemmli buffer with 5% β-mercaptoethanol at 37 °C for 5 min and loaded onto a 3% to 8% Tris-acetate gel (Invitrogen). The gel was transferred to nitrocellulose and processed for Western blotting by using monoclonal CFTR antibody 769 (gift from John R. Riordan, University of North Carolina, Chapel Hill, NC) or polyclonal to GAPDH (Santa Cruz Biotechnology). Blots were developed by enhanced chemiluminescence. Quantification of the relative amounts of bands C and GAPDH was performed by using NIH ImageJ analysis of scanned films.

CFTR Metabolic Pulse-Chase Analysis. HEK-293 cells expressing CFTR or F508del-CFTR were incubated for 16 h in assay media (HyQ CCM5 with 1% heat-inactivated FBS) with DMSO or compound. For metabolic labeling, cells were starved for 30 min in DMEM without cysteine and methionine with 1% dialyzed FBS in the presence of compound. Cells were then pulsed with [^{35}S] methionine and cysteine EXPRESS35 label (PerkinElmer) for 15 min. Cells were washed and chased in assay media with compound for 0 to 23 h. At each time point, cells were harvested and lysed in RIPA, and CFTR was immunoprecipitated with M3A7 (Millipore). Samples were separated by SDS/PAGE and analyzed by autoradiography. Radioactivity was quantified by PhosphorImager analysis (GE Healthcare).

CFTR Limited Proteolysis Analysis. Twenty-four hours before treatment, HEK-293 cells expressing F508del-CFTR or CFTR were plated to 60% confluence in six T225 flasks. The next day, three flasks were treated with 3 μM VX-809 and three with DMSO. Cells were incubated for 24 h in 5% CO_2 at 37 °C. Each flask was washed once with 10 mL PBS solution and then incubated in 10 mL of Versene (cat. no. 15040; Gibco) for 5 min at room temperature. The cells were dissociated by tapping the flask. Three flasks were combined and the cells were pelleted at 1,500 rpm for 5 min in 4 °C. The cell pellet was suspended in 20 mL of sucrose buffer (250 mM sucrose, 10 mM Hepes, pH 7.2) with protease inhibitor mixture. The cells were lysed by nitrogen cavitation at 300 psi for 5 min. Cell lysates were spun down at

2,900 rpm to remove the nuclei. The supernatant was then spun at 34,000 rpm in an ultracentrifuge for 1 h. The pellet was washed in sucrose buffer to remove protease inhibitors and resuspended in 100 μL. Protein concentration was determined using the BCA method. All microsomes were stored at −70 °C. Stock of proteomics-grade trypsin (cat. no. T6567; Sigma) was made up in trypsin buffer (40 mM Tris, pH 7.4, 2 mM MgCl, 0.1 mM EDTA) and diluted to the following concentrations: 960, 480, 240, 120, 60, 30, and 15 μg/mL. Thirty-five micrograms of protein was resuspended in trypsin buffer to a final volume of 10 μL for each trypsin concentration. Ten microliters of trypsin was added to each tube and incubated for 15 min on ice. The reaction was stopped with 5 μL of 5 mM EDTA and 1 mM PMSF. Ten microliters of 2× Tris-glycine SDS buffer containing 10% β-mercaptoethanol was added to the samples and incubated for 5 min at 37 °C. Samples were run on 4% to 20% Tris-glycine gel and transferred onto nitrocellulose. The membrane was blocked for 1 h in 5% milk with PBS plus 0.1% Tween. Membrane was treated in primary antibody overnight at 4 °C. NBD-1 was probed by using the CFTR antibody 660 and NBD-2 was probed by using the CFTR antibody 596 (provided by John R. Riordan, University of North Carolina, Chapel Hill, NC). Blots were developed by enhanced chemiluminescence and quantified by using NIH ImageJ analysis of scanned films.

Measurement of ASL Height. To measure the ASL height in cultured HBE, the apical surface was first washed two times, followed by addition of 20 μL absorption buffer (89 mM NaCl, 4 mM KCl, 1.2 mM $MgCl_2$, 1.2 mM $CaCl_2$, 1 mM Hepes, 16 mM Na-gluconate, 10 mM glucose) containing 10,000 kDa dextran conjugated to Alexa Fluor 488. The cells were incubated at 37 °C for 2 d before treatment to allow the ASL height to return to baseline levels. All compounds were prepared in differentiation media [DMEM/F12, Ultroser G (2.0%; catalog 15950–017; Pall), fetal clone II (2%), insulin (2.5 μg/mL), bovine brain extract (0.25%; kit CC-4133; component CC-4092C; Lonza), hydrocortisone (20 nM), triiodothyronine (500 nM), transferrin (2.5 μg/mL; catalog no. 0030124SA; Invitrogen), ethanolamine (250 nM), epinephrine (1.5 μM), phosphoethanolamine (250 nM), and retinoic acid (10 nM)] and applied to the basolateral side at desired concentration. After 5 d of treatment, the ASL height was measured by capturing XZ slices of the HBEs by using a Quorum WaveFX spinning-disk confocal system on an inverted Zeiss microscope and ×20 objective. The images were acquired and processed by using Volocity 4.0.

Analysis of Proteasome Activity in Vitro. Jurkat cells expressing 2XUb-β-lactamase reporter were plated in 96-well plates at a density of 1.5×10^6 cells/mL and incubated with the various concentrations of compounds. The cells were treated for 1 h with 100 μg/mL cycloheximide and then loaded with 1 μM CCF2/AM. β-Lactamase activity was measured by quantifying the fluorescence emission from the cells by using a CytoFluor 4000 plate fluorimeter (PerSeptive Biosystems). Background-subtracted emission values at 460 nm and 530 nm were expressed as 460/530 ratio, whereby a high ratio represents high β-lactamase activity.

P-gp and hERG Immunoblot Analysis. HEK-293 cells were transiently transfected with a pcDNA3.1-based vector containing cDNA for the P-gp, G268V-P-gp, or Y490del-P-gp. For hERG immunoblot studies, HEK-293 cells stably expressing hERG or G601S-hERG were used. At 24 h, growth media was replaced for 16 h at 37 °C with or without test compound in assay media. After incubation, cells were harvested in ice-cold D-PBS solution (without calcium and magnesium) plus 1 mM EDTA and pelleted at 1,000 × *g* at 4 °C. Cell pellets were lysed in 1% Nonidet P-40, 0.5% sodium deoxycholate, 200 mM NaCl, 10 mM Tris, pH 7.8, and 1 mM EDTA plus protease inhibitor mixture (Sigma) used at 1:250 for 30 min on ice. Lysates were centrifuged for 10 min at 10,000 × *g* at 4 °C to pellet nuclei and insoluble material. Approximately 5 μg total protein was loaded and separated by SDS/PAGE. The gel was transferred to nitrocellulose and processed for Western blotting by using the P-gp antibody C219 (Abcam) or the 373500 hERG antibody (Merck). Blots were developed by enhanced chemiluminescence. Quantification of the relative amounts of bands B and C was performed by using NIH Image analysis of scanned films.

α1-Antitrypsin Metabolic Pulse-Chase Analysis. HeLa cells were transiently transfected with a pcDNA3.1-based vector containing cDNA for the E342K form of the α1-antitrypsin (AAT) Z mutant (gift from W. E. Balch, Scripps Research Institute, La Jolla, CA). After 24 h, cells were incubated for 16 h in Opti-MEM (Invitrogen) with DMSO or 3 μM VX-809. For metabolic labeling, cells were starved 30 min in DMEM without cysteine and methionine with 1% dialyzed FBS in the presence of compound. Cells were then pulsed with [^{35}S]methionine and cysteine EXPRESS35 label (PerkinElmer) for 15 min. Cells were washed and chased in assay media with compound for 0 to 3 h. At each time point, supernatants were collected and cells were harvested. Cells were lysed in RIPA buffer and media supernatants were adjusted to similar RIPA concentrations. α1-Antitrypsin was immunoprecipitated from both the intracellular (lysate) and extracellular (supernatant) with rabbit polyclonal antibody to AAT (DAKO). Samples were separated by SDS/PAGE and analyzed by autoradiography. Radioactivity was quantified by PhosphorImager analysis (GE Healthcare). Intracellular and extracellular α1-antitrypsin were normalized as the percent intracellular levels at chase time 0.

β-Glucosidase Assay. Primary skin fibroblasts isolated from a 29-y-old man with type I Gaucher disease (GM00372) were obtained from Coriell Cell Repositories and cultured in minimum essential Eagle medium (Lonza) containing 15% unactivated FBS, 100 U/mL of penicillin and streptomycin (Gibco), 1× nonessential amino acids, and 2 mM L-glutamine (Gibco). To monitor β-glucosidase activity assay, primary fibroblast cells isolated from a subject with Gaucher disease were grown to confluence in 24-well plates, after which the growth media was changed to minimum essential Eagle medium with 5% unactivated FBS containing varying concentrations of VX-809. The β-glucosidase chemical chaperone, NN-DNJ (Toronto Research Chemicals), at 10 μM, was used as a positive control for the assay. The cells were allowed to incubate with compound for 3 d, followed by incubation for an additional 24 h with fresh media and compound. The cells were washed with 1 mL of PBS solution followed by the addition of 80 μL of PBS solution and 80 μL of 0.2 M sodium acetate (pH 4.0) to each well. To measure the enzymatic activity of β-glucosidase, 100 μL of a 5-mM solution of 4-methylumbelliferryl β-D-glucoside in 0.2 M sodium acetate was added to each well. The cells were incubated at 37 °C for 1 h and subsequently lysed with 2 mL of 0.2 M glycine, pH 10.8, releasing the substrate and product from the cells. The conversion of 4-methylumbelliferryl β-D-glucoside was measured upon excitation at 330 nm and following the emission at 450 nm with a plate fluorimeter (LJL Biosystems).

Statistical Analyses for in Vitro Studies. Statistical comparisons were made using ANOVA followed by Tukey multiple-comparison test or Student *t* test. We used Prism 5 (GraphPad Software) for our analyses. $P < 0.05$ was determined as significant. All data are presented as mean ± SEM.

Oral Bioavailability of VX-809 in Male Rats. Male rats ($n = 3$ per dose group) were orally administered VX-809 in a vehicle consisting of 0.5% Tween80/0.5% methylcellulose/water at a dose volume of 5 mL/kg. The concentration of VX-809 in plasma

samples was determined with a liquid chromatography/tandem MS method. Pharmacokinetic parameters were calculated by-using WinNonlin Professional Edition software, version 4.0.1. (Pharsight). Oral dosing of 1 mg/kg VX-809 in male Sprague–Dawley rats resulted in a C_{max} of 2.4 ± 1.3 μM with a $t_{1/2}$ of 7.7 ± 0.4 h (mean ± SD; n = 3), indicating that that VX-809 was orally bioavailable and able to reach plasma levels that significantly exceeded in vitro EC_{50} for F508del-CFTR correction.

1. Van Goor F, et al. (2006) Rescue of Delta508-CFTR trafficking and gating in human cystic fibrosis airways primary cultures by small molecules. *Am J Physiol Lung Cell Mol Physiol* 290:L1117–L1130.

2. Weuberger T, Burton B, Clark H, Van Goor F. Use of primary cultures of human bronchial epithelial cells isolated from cystic fibrosis patients for the pre-clinical testing of CFTR modulators (2011). *Methods Mol Biol* 741:39–54.

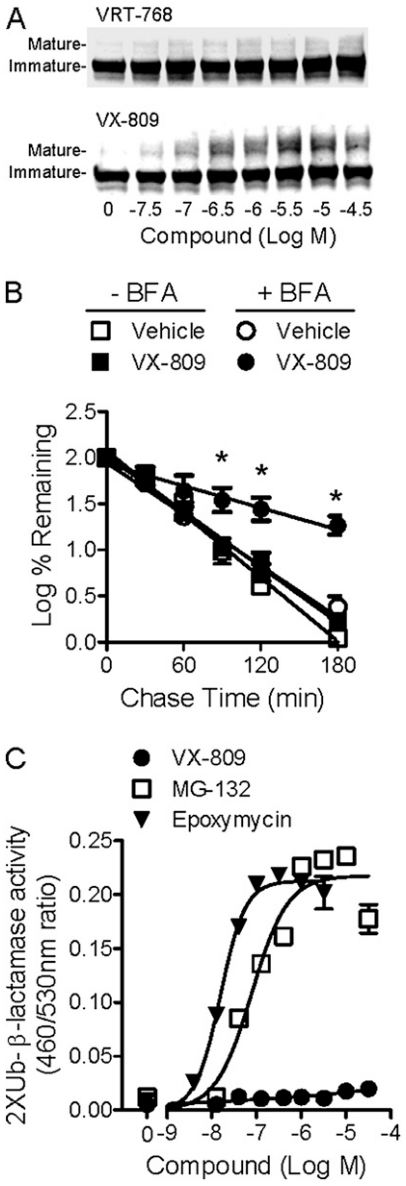

Fig. S1. F508del-CFTR maturation and the decay rate of immature F508del-CFTR following treatment with VX-809. (*A*) Representative Western blot of the glycosylation pattern of F508del-CFTR in FRT cells treated for 48 h with the indicated VRT-768 or VX-809 concentrations. The bands associated with immature and mature CFTR are indicated. (*B*) Decay of the immature F508del-CFTR band (log percentage remaining) during the 180-min chase in vehicle-treated (open symbols) and 3 μM VX-809–treated (filled symbols) cells in the presence (circles) and absence of BFA (squares). The decay rate of immature F508del-CFTR was slower in the presence of BFA than in its absence ($t_{1/2}$ = 78 ± 9 min vs. 26 ± 1 min, respectively; $P < 0.05$; n = 3). The difference in the decay curves with and without BFA in VX-809–treated cells indicated that 28 ± 10% of the immature F508del-CFTR was resistant to degradation in the ER, which was similar to the fraction of F508del-CFTR that exited the ER in the presence of VX-809 (34 ± 4%; n = 3; Fig. 2 *A* and *B*). This suggested that the fraction of VX-809–corrected F508del-CFTR that was more resistant to ER degradation was likely the same fraction exported from the ER in the absence of BFA. (*C*) Effects of VX-809 (●) on cellular proteasome activity. A ubiquitin–β-lactamase fusion protein (2XUb-β-lactamase) was used to assess proteasome activity in Jurkat cells. Cellular β-lactamase activity remaining after 1 h cycloheximide treatment of cells is used as a reporter for proteasome activity. The known protease inhibitors, epoxomycin (▼) and MG-132 (□), are shown as positive controls. Background-subtracted emission values at 460 nm and 530 nm were expressed as a 460 nm/530 nm ratio, whereby a high ratio represents high β-lactamase activity. Each time point represents the mean of four replicates.

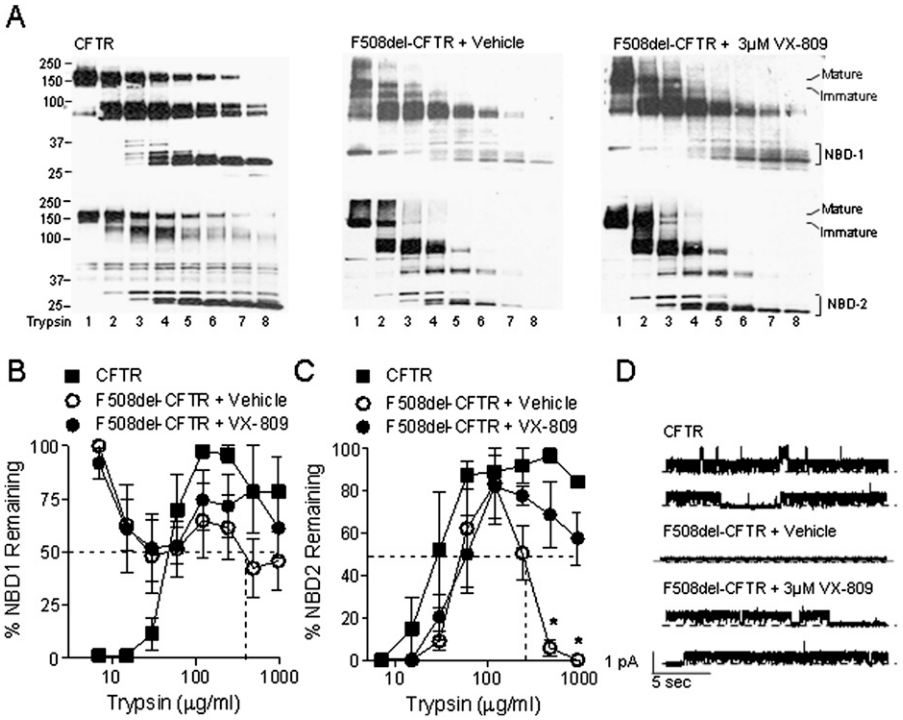

Fig. S2. Effects of VX-809 treatment on the fragment pattern of full-length CFTR, NBD1, and NBD2 from cells expressing F508del-CFTR and F508del-CFTR channel gating. (*A*) Immunoblot of trypsin-digested microsomes from cells expressing CFTR or F508del-CFTR pretreated with vehicle or 3 μM VX-809 for 24 h. The characteristic fragments corresponding to full-length, NBD1, and NBD2 are indicated. (*B*) Quantification of the data described in *A* for CFTR-NBD1 and F508del-NBD1 following treatment with DMSO or VX-809 for 24 h. (*C*) Quantification of the data described in *A* for CFTR-NBD1 and F508del-NBD2 following treatment with DMSO or VX-809 for 24 h. Dotted lines in *B* and *C* indicate the $C_{try50\%}$. (*D*) Representative single-channel recording of CFTR activity in excised membranes from NIH 3T3 cells expressing CFTR or F508del-CFTR. Cells expressing F508del-CFTR were pretreated for 48 h with vehicle or 3 μM VX-809. To activate CFTR, 1 mM ATP and 100 U/mL PKA were added to the bath.

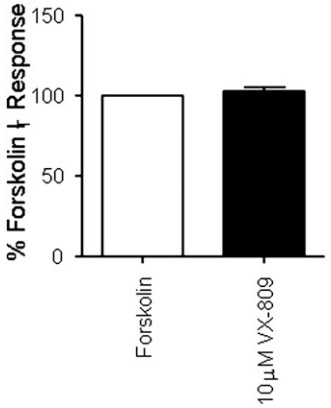

Fig. S3. Acute addition of VX-809 did not increase CFTR function. Forskolin (10 μM)-stimulated I_T before and after acute addition of 10 μM VX-809 in cultured F508del-HBE cells. The lack of an acute effect on chloride forskolin-stimulated chloride secretion suggested that VX-809 is not a CFTR potentiator.

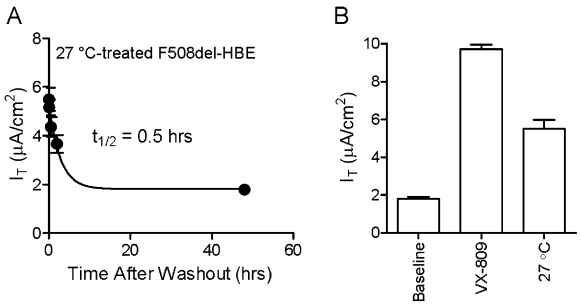

Fig. S4. Cell surface turnover of 27 °C-corrected F508del-CFTR. (*A*) Cultured F508del-HBE were preincubated at 27 °C for 24 h to correct F508del-CFTR. The cultures were subsequently returned to 37 °C and F508del-CFTR activity was monitored 0 to 48 h after compound removal (n = 4 for each time point). (*B*) Chloride (mean ± SEM) transport in F508del-HBE following 24 h incubation at 27 °C or in the presence of DMSO or 3 μM VX-809.

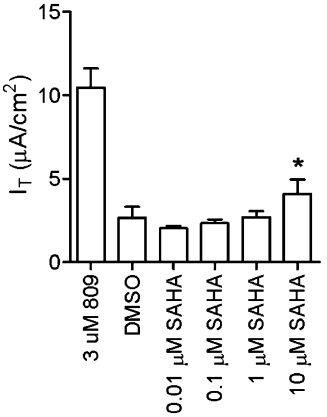

Fig. S5. Effect of SAHA on the forskolin-stimulated I_T in FRT cells expressing F508del-CFTR. Ussing chamber recording of the forskolin-stimulated I_T following 24 h incubation with 3 μM VX-809 or SAHA at the indicated concentrations. Asterisk indicates significant difference compared with controls ($P < 0.05$; ANOVA followed by Tukey multiple-comparison test).

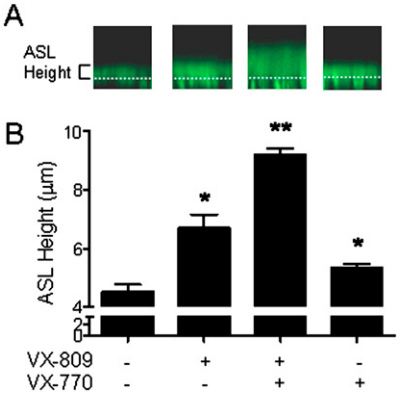

Fig. S6. VX-809 increased the ASL height and CBF in cultured F508del-HBE. (*A*) Confocal image of the vasoactive intestinal peptide-stimulated ASL height after treating the cells for 5 d with vehicle or VX-809 (0.3 or 3.0 μM) in the presence or absence of 3 μM VX-770. (*B*) Quantification of the data in *A* (n = 3; data from two donor lungs).

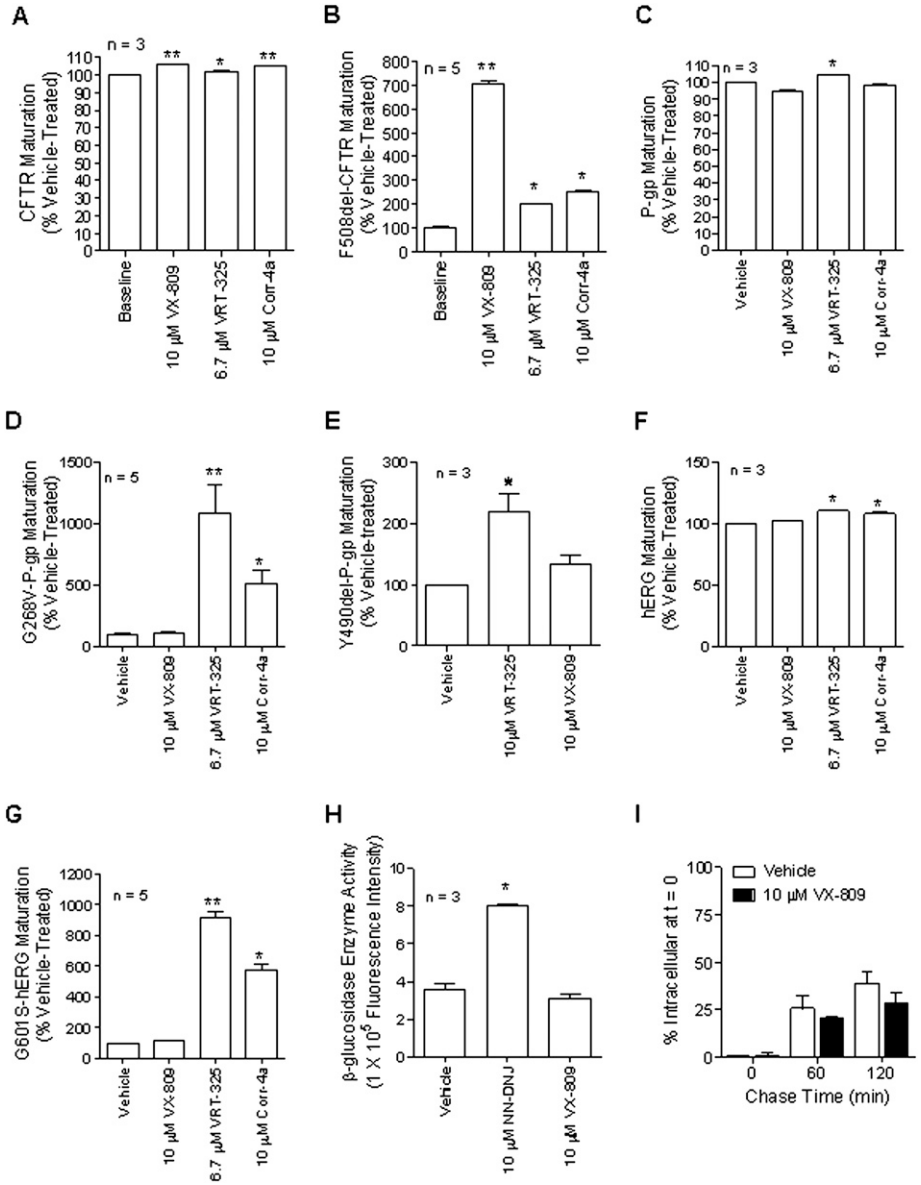

Fig. S7. VX-809 did not increase the maturation of mutant P-gp or hERG. Quantification of glycosylation pattern of G268V-P-gp (*A*) and Y490del-P-gp (*B*) expressed in HEK-293 cells following 48 h pretreatment with vehicle (DMSO), 10 μM VX-809, 10 μM VRT-325, and 10 μM Corr-4a. (*C*) Quantification of the glycosylation pattern of G601S-hERG expressed in HEK-293 cells following 48 h pretreatment with vehicle (DMSO), 10 μM VX-809, 10 μM VRT-325, and 10 μM Corr-4a. Asterisk indicates significant difference vs. controls ($P < 0.05$; paired *t* test).

Table S1. Potency and efficacy of VX-809 alone and combination with VX-770 in F508del-HBE isolated from the bronchi of multiple individuals with CF

			Maximum forskolin-stimulated F508del-CFTR-mediated I_T, μA/cm^2			
Donor lung	n	EC_{50}, log M	Vehicle	3 μM VX-809	3 μM VX-809 + 1 μM VX-770	1 μM VX-770
1	12	−8.1 ± 0.1	3.7 ± 0.6	14.3 ± 1.0	34.1 ± 0.2	10.0 ± 0.8
2	6	−7.3 ± 0.3	1.8 ± 0.2	6.4 ± 0.3	9.9 ± 1.2	3.8 ± 0.5
3	8	−6.8 ± 0.2	1.0 ± 0.1	4.0 ± 0.1	6.6 ± 0.2	1.8 ± 0.1
4	4	−7.1 ± 0.2	1.2 ± 0.1	4.8 ± 0.2	7.2 ± 0.4	2.2 ± 0.2
5	6	−7.1 ± 0.2	2.8 ± 0.1	8.4 ± 0.2	13.0 ± 0.2	5.9 ± 0.3
6	28	−6.9 ± 0.1	1.6 ± 0.2	7.7 ± 0.6	13.9 ± 1	5.0 ± 0.7
7	3	−7.2 ± 0.1	1.4 ± 0.2	8.9 ± 0.6	Not tested	Not tested
Mean ± SEM	NA	−7.2 ± 0.2	1.9 ± 0.4	7.8 ± 1.3	14 ± 3.9	4.8 ± 1.1
Non-CF HBE, %	NA	NA	3.4 ± 0.7	13.9 ± 2.3	25.1 ± 6.9	8.5 ± 2.0