Figure S1. ΔF508 Forms a Cavity at the NBD1/ICL4 Interface in CFTR, Related to Figure 1. (A) View of ICL4 NBD1 interface in the model of WT CFTR as described in Figure 1B. (B) Murine ΔF508 NBD1 (pdb 3SI7) docked on the CFTR model as described in Figure 1B. Comparison of the surface between the WT and ΔF508 reveals changes local to F508 forming a pocket and perturbation of the ICL4 interaction surface (Figures 1B and S1A).

Figure S2. Independent Coupling Matrices for Each of the Four Methods, Related to Figure 2. Four heat maps of coupling values for each statistical method are shown without normalization as described in Figure 2A. (A) Upper left matrix is from Statistical Coupling Analysis (SCA), (B) upper right is from Explicit Likelihood Subset of Covariation (ELSC), (C) lower left is OMES, and (D) lower right is from McBASC.

Figure S3. Positions in NBD1 Coupled to F508, Related to Table 1. (A) The structure of murine NBD1 (pdb 1R0W, grey ribbon) is shown with the side chains of fourteen 508-coupled positions from Table 1 indicated in green and two positions, D529 and S573, indicated in yellow and magenta, respectively. The four NBD1 previously identified second-site suppressor positions (I539, G550, R553, and R555) are indicated in orange. (*Top*) Canonical view of NBD1 looking from the perspective of the membrane. (*Bottom*) A 90° rotation about the x-axis. (B) Plot of coupling scores to the 508 position from SCA *versus* the frequency of the most common amino acid for every position in NBD1 (389-673). The sixteen representative 508-coupled positions (green, yellow, and magenta circles) account for 60% of the top 20 scoring positions in SCA. Positions in NBD1,

which are dominated by a single amino acid, frequency > 0.75, have low coupling scores, right most side of plot. The abrupt drop in coupling scores for these positions is not evident when a similar plot is produced using traditional conservation scores. The four second-site suppressor positions were not identified through the coupling analysis, since these positions have a single amino acid frequency > 0.75 and are thus above the drop off. (C) Plot of coupling scores to the 508 position from ELSC versus the frequency of the most common amino acid in NBD1. (D) Plot of coupling scores to the 508 position from OMES versus the frequency of the most common amino acid in NBD1. (E) Plot of coupling scores to the 508 position from McBASC versus the frequency of the most common amino acid in NBD1.

Figure S4. Structural Context of D529F and S573E Suppressor Mutations, Related to Figure 3. The mutations D529F and S573E increase the folding efficiency of NBD1 by different mechanisms (Figure 3). (A) View of the helical subdomain of human NBD1 (pdb 1XMI), F494-A566, from the perspective of the NBD1/NBD2 domain interface. Left panel, D529 is shown in yellow, F508 in green, R555, one of the previously identified second-site suppressor mutations is shown in orange sticks, and Q525 is shown in grey sticks. R555 makes a salt bridge with D529 as well as a hydrogen bond with the carbonyl backbone of Q525. *Right*, structure of human NBD1 with second-site suppressors (pdb 2BBO). Identical view as left panel but contains the R555K suppressor mutation. (B) *Left*, the S573 position, shown in magenta, is typically an Asp or Glu residue, serving as the catalytic base, in the vast majority of the Walker B motifs in the ABC transporter superfamily. This is a canonical view of the α/β core of human NBD1

(pdb 1XMI) from the perspective of the membrane. The adenosine head group of ATP is shown in grey and the three phosphates of ATP are shown in orange. Mg^{2+} is represented as a green sphere while F508 is shown in a green stick representation. Right panel, T_m of human WT and S573E as a function of ATP concentration. For every ATP concentration assessed (40 μM – 100 mM), the T_m is higher for S573E.

Figure S5. Comparison of Various Methods for Quantification of Maturation of CFTR, Related to Figure 4. (A) Representative western blot (mAb 596) of WT, ΔF508, and sixteen 508-coupled positions as described in Figure 4A. Band C and Band B from three independent experiments were quantified by densitometry and plotted using common methods for comparisons of mature CFTR. (B) Plot of normalized ratios Band C/Band B red filled circles and linear fit, and of Band C/Total (Band B + Band C) blue filled circles and fit. (C) Total CFTR as measured by ELISA linearly correlates with relative quantity of Band C, as do total CFTR as measured by densitometry and mature CFTR protein as measured by cell surface biotinylation. (D) Statistical parameters for the five methods. W496V CFTR has the lowest quantity of Band C, and is significantly different from ΔF508 CFTR, p-value < 0.05, for three of the five methods; Band C/Band B, Band C/Total, and ELISA.

Table S1. Crystallographic and Refinement Data for the Murine ΔF508 Structure, Related to Figure 5 and 6.

Parameter	Value
Space Group	$P42_12$
Unit cell dimensions (Å) a	170.5
b	170.5
c	109.4
α, β, γ (°)	90, 90, 90
Resolution (Å)	44.9-2.25 (2.28-2.25)*
Completeness (%)	100 (100)
Multiplicity	11.2 (11.3)
Unique reflections	76,676 (2,535)
R_{merge} †	0.074 (0.858)
I/σ_I	34.4 (2.7)
Wilson B ($Å^2$)	45.7
Refinement Resolution (Å)	49.8-2.25
No. non-solvent atoms	8,505
No. solvent atoms	291
Cutoff F_o/σ_{Fo}	0
Avg. *B*-factors	52.8

non-solvent ($Å^2$)	
solvent ($Å^2$)	45.3
***R*-values** R_{work}	0.181
R_{free}	0.227
Ramachandran statistics[§] outliers (%)	0.0
most favored region (%)	96.9
r.m.s. deviations Bonds (Å)	0.007
Angles (°)	1.1

*Values in parentheses are for the highest resolution shell.

† $R_{sym} = \sum_h \sum_i \left| I_{h,i} - \langle I_h \rangle \right| / \sum_h \sum_i I_{h,i}$ where the outer sum (h) is over the unique reflections and the inner sum (i) is over the set of independent observations of each unique reflection.

[§]From MolProbity (Davis et al., 2007).

Table S2. Impact of Suppressor Mutations on ΔF508 NBD1 Folding, Maturation, and Function, Related to Figure 5 and 6.

		Relative Yield NBD1 (β-gal.) (n)	Relative Yield CFTR (ELISA) (n)	Relative Conductance (10 μM Forskolin + 100 μM IBMX) (n=3)	Relative Conductance (10 μM Forskolin + 100 μM IBMX + 20 μM Inh-172) (n=3)	Current Density (pA/pF) (10 μM Forskolin + 100 μM IBMX) (n)	Current Density (pA/pF) (10 μM Forskolin + 100 μM IBMX + 10 μM Inh-172) (n)
WT	WT	1.00 ± .03 (18)	1.00 ± .03 (6)	1.00 ± 0.05	0.18 ± 0.13	82 ± 2 (5)	1.1 ± 0.1 (5)
	ΔF508	0.43 ±.03 (18)	0.11± .01 (6)	0.17 ± 0.03	0.22 ± 0.03	1.7 ± 0.1 (5)	0.4 ± 0.2 (4)
	R1070W	N/A	0.22 ± .02 (3)	0.47 ± 0.03	0.21 ± 0.10	ND	ND
ΔF508	I539T	0.79 ± .03 (9)	0.14 ± 0.01 (3)	0.21 ± 0.08	0.29 ± 0.10	0.38 ± 0.03 (6)	0.23 ± 0.05 (6)
	G550E	0.78 ± .03 (9)	0.17 ± 0.02 (3)	ND	ND	ND	ND
	R553M	0.59 ± .06 (9)	0.11 ± 0.01 (3)	ND	ND	ND	ND
	R555K	1.03 ± .06 (9)	0.18 ± 0.01 (3)	0.25 ± 0.05	0.22 ± 0.03	18 ± 6 (6)	0.46 ± 0.05 (6)
	G550E-R553M-R555K (3M)	0.67 ± .0 (9)	0.16 ± 0.01 (3)	ND	ND	ND	ND
	R1070W	N/A	0.17 ± 0.03 (3)	0.48 ± 0.07	0.21 ± 0.10	27 ± 9 (5)	1.1 ± 0.2 (5)
ΔF508- R1070W	I539T	N/A	0.76 ± 0.03 (6)	1.71 ± 0.23	0.35 ± 0.10	88 ± 8 (6)	1.2 ± 0.1 (6)
	G550E	N/A	0.35 ± 0.03 (6)	ND	ND	ND	ND
	R553M	N/A	0.20 ± 0.02 (6)	ND	ND	ND	ND
	R555K	N/A	0.62 ± 0.04 (6)	1.90 ± 0.42	0.31 ± 0.06	76 ± 5 (6)	0.8 ± 0.2 (6)
	G550E-R553M-R555K (3M)	N/A	0.84 ± 0.06 (6)	ND	ND	ND	ND

ND – not determined

EXTENDED EXPERIMENTAL PROCEDURES

ΔF508 NBD1 Structure Determination

The crystals had the symmetry of space group P4212, and they diffracted X-rays to a dmin value of 2.25 Å. The diffraction data were indexed, integrated, and scaled using HKL2000 (Otwinowski and Minor, 1997). Statistics regarding the data set are found in Table S1. Negative intensities were corrected using the TRUNCATE procedure (French and Wilson, 1978). The crystal appeared to be isomorphous with that used to determine the structure of wild type murine NBD1 (Lewis et al., 2004) (pdb accession code 1R0W). The 1R0W model, stripped of waters and heterogens, was therefore used as the starting model for the current structure. To eliminate statistical bias and allow for a random assignment of reflections to the "free" set for the calculation of Rfree, the starting coordinates were "shaken" such that they adopted new positions, resulting in a coordinate rms deviation between the starting and shaken coordinates of 0.5 Å. The shaken coordinates were then subjected to the rigid-body, simulated annealing, individual ADP, and TLS refinement protocols available in PHENIX (Adams et al., 2010). Coot (Emsley and Cowtan, 2004) was used to manipulate the model between rounds of refinement. The statistics from the final model are found in Table S1.

Homology Model of ΔF508 CFTR

The crystal structures of murine WT (pdb 1R0W) and ΔF508 (pdb 3SI7) NBD1 were docked onto the Sav1866 (2HYD) structure as described in the main text using Pymol (Schrodinger, 2010). Contact distances were calculated from the atomic coordinates of the homology models using Perl scripts.

Analyses of Evolved Sequences

The coupling matrices for each of the four independent methods SCA (Lockless and Ranganathan, 1999), ELSC (Dekker et al., 2004), McBASC (Fodor and Aldrich, 2004; McLachlan, 1971), and OMES (Fodor and Aldrich, 2004; Olmea and Valencia, 1997) were calculated as described in the main text.

ATP Dependence on Stability of NBD1

Human WT and S573E NBD1 proteins were expressed and purified as described in the main text. Additionally, each T_m for the two proteins was determined as a function of ATP concentration from 40 μM to 100 mM.

Quantification Methods of Mature CFTR

Plasmids that express each of the sixteen 508-coupled mutants were used to transfect HeLa TetOn cells (Clonetech). The sandwich ELISA was performed as follows: 100 λ of capture anti-body, A596 (provided by J. Riordan, UNC) was bound to Nunc Immobilizer plates (Nalgene Nunc Intl.) at a dilution of 1:1000 in binding buffer (100 mM Phosphate buffer, pH 8.0). After 24 hours, the cells were lysed using CRIPA buffer, a modified RIPA, (20 mM Tris pH 7.5, 150 mM NaCL, 0.5% CHAPS w/v, 0.5% DOCA, 0.1% SDS, and Complete Mini Protease Inhibitor tablet (Roche Applied Science) for one hour at 4°C. The antibody bound plates were washed three times with TBST and loaded with 100 λ of lysate clarified by centrifugation at 16,000 rcf and normalized to total protein. After an overnight incubation, the plates were washed three times with TBST and incubated for two hours with 100 λ of detection primary (3G11, W. Balch, Scripps) at a 1:1000 dilution

in blocking buffer (3% BSA in PBS and sodium azide), washed three times with PBST, and then incubated for two hours with 100 λ of diluted (1:2000 in blocking buffer) HRP conjugated α-rat secondary (The Jackson Laboratory). The plates were then washed three times with PBST and 100 λ of HRP colorogenic *o*-Phenylenediamine (OPD) substrate was added (Sigma-Aldrich Corp.). After 30 minutes, the reaction was stopped by adding 100 λ of 2.5 M Sulfuric Acid and absorbance read at 490 nm on either a Spectramax M5 or a Gemini plate reader (Molecular Devices Inc.). Western blot analysis of samples was performed as previously described (Thibodeau et al., 2005; Thibodeau et al., 2010) using the A596 antibody (provided by J. Riordan, UNC) at 1:5000 dilution.

Cell surface biotinylation experiments were performed using EZ-Link Sulfo-NHS-SS-Biotin and NeutrAvidin Agarose Resin as recommended by the manufacturer (Thermo Scientific). The samples were then analyzed by western blot analysis as previously described. Densitometry measurements from western blots of Band B and Band C from three independent experiments of whole cell lysate samples or samples derived from cell surface biotinylation experiments were performed using ImageJ (NIH). Ratio of Band C/Band B and Band C/Total were calculated, normalized to the respective WT CFTR ratio for each transfection set, and plotted against relative Band C using Prism (Prism Software Corp.). Band C/Band B was fit to a straight line and Band C/Total was fit to the equation $y=x/(x+C)$, where C is a constant.

ELISA measurements, Total CFTR (Band B + Band C), cell surface biotinylation (CSB) were plotted against the relative intensity of Band C from WT CFTR and all fit to

a linear equation. Comparison of the five quantification methods (ELISA, Band C, Total CFTR, Band B, and Band C/Total) was performed using a Student's T-test (two tail, heteroscedastic) between three independent experiments. The p-values for each of the methods and mutants were calculated against ΔF508 using the Student's T-test.

CFTR-dependent Transepithelial Conductance in FRT Monolayers

FRT cells were transfected with the plasmids used for the CFTR maturation studies with Lipofectamine LTX with Plus reagent (Invitrogen) following the manufacturer protocol optimized with 1μg of CFTR plasmid, 1.2 λ of Plus reagent, 1.5 λ of Lipofectamine LTX, and 300,000 cells in 600 λ. 200 λ of the transfection mix per well was plated in a 24 well plate HTS transwell plate (Costar) with 750 λ of Coon's F-12 (Biochrom AG) growth media in the outer well. 72 hours post-transfection, transepithelial conductance (G_t) measurements were performed using an EVOM^2 Epithelial Voltohmmeter with a STX100 electrode (World Precision Instruments) at 37 °C. G_t was measured 10 minutes after treatment with final concentration of 10 μM Forskolin + 100 μM IBMX (Sigma) and 5 minutes after 20 μM Inh.-172 (CFFT), respectively.

CFTR-dependent Whole –Cell Current in Hela Cells

HeLa TetOn (Clontech) cells were transfected with the plasmids used for the CFTR maturation experiments. 1 μg of CFTR plasmid and 100 ng of PCMV GFP were co-transfected using 2 λ of Lipofectamine 2000 (Invitrogen) in a 6-well format. 24 hours post-transfection, the whole-cell configuration of the patch-clamp technique was used to measure the Cl^- current. The pipette solution contained (mM) 145 $NMDG^+$-Cl^-, 1 $MgCl_2$, 2 EGTA, 5 ATP, and 10 HEPES (pH 7.3 with Tris). The bath solution was (mM) 145

$NMDG^{+}$-Cl^{-}, 1 $MgCl_2$, 1 Ca Cl_2, 10 HEPES and 10 Glucose (pH 7.4 with Tris). The current was recorded with an Axopatch 200B patch-clamp amplifier and digitized at 2 kHz. The membrane conductance was probed by stepping the membrane potential from a holding potential of 0 mV to membrane potentials −40 and +40 mV steps for 200 ms. Whole cell current responses were measured in response to 10 μM forskolin plus 100 μM IBMX and 10 μM Inhibitor-172 (Inh.-172). Pipettes had resistances between 3 and 5 MΩ when filled with pipette solution and seal resistance exceeded 8 GΩ. Current recording and analysis was performed with pClamp 9.2 software and analyzed with Origin 8 software.

SUPPLEMENTAL REFERENCES

Adams, P.D., Afonine, P.V., Bunkóczi, G., Chen, V.B., Davis, I.W., Echols, N., Headd, J.J., Hung, W., Kapral, G.J., Grosse-Kunstleve, R.W.*, et al.* (2010). PHENIX: a comprehensive Python-based system for macromolecular structure determination. Acta Crystallographica *D66*, 213-221.

Davis, I.W., Leaver-Fay, A., Chen, V.B., Block, J.N., Kapral, G.J., Wang, X., Murray, L.W., Arendall, W.B., Snoeyink, J., Richardson, J.S.*, et al.* (2007). MolProbity: all-atom contacts and structure validation for proteins and nucleic acids. Nucleic Acids Res *35*, W375-W383.

Dekker, J.P., Fodor, A., Aldrich, R.W., and Yellen, G. (2004). A perturbation-based method for calculating explicit likelihood of evolutionary co-variance in multiple sequence alignments. Bioinformatics *20*, 1565-1572.

Emsley, P., and Cowtan, K. (2004). Coot: Model-building tools for molecular graphics. Acta Crystallographica Section D: Biological Crystallography *60*, 2126-2132.

Fodor, A.A., and Aldrich, R.W. (2004). Influence of conservation on calculations of amino acid covariance in multiple sequence alignments. Proteins *56*, 211-221.

French, S., and Wilson, K. (1978). On the treatment of negative intensity observations. Acta Crystallographica Section A, Crystal Physics, Diffraction, Theoretical and General Crystallography *34*, 517-525.

Lewis, H.A., Buchanan, S.G., Burley, S.K., Conners, K., Dickey, M., Dorwart, M., Fowler, R., Gao, X., Guggino, W.B., Hendrickson, W.A.*, et al.* (2004). Structure of nucleotide-binding domain 1of the cystic fibrosis transmembrane conductance regulator. EMBO Journal *23*, 282-293.

Lockless, S.W., and Ranganathan, R. (1999). Evolutionarily conserved pathways of energetic connectivity in protein families. Science *286*, 295-299.

McLachlan, A.D. (1971). Tests for comparing related amino-acid sequences. Cytochrome c and cytochrome c 551. Journal of molecular biology *61*, 409-424.

Olmea, O., and Valencia, A. (1997). Improving contact predictions by the combination of correlated mutations and other sources of sequence information. Folding & design *2*, S25-32.

Otwinowski, Z., and Minor, W. (1997). Processing of X-ray diffraction data collected in oscillation mode. Methods in Enzymology *276*, 307-326.

Schrodinger, LLC (2010). The PyMOL Molecular Graphics System, Version 1.3r1.

Thibodeau, P.H., Brautigam, C.A., Machius, M., and Thomas, P.J. (2005). Side chain and backbone contributions of Phe508 to CFTR folding. Nat Struct Mol Biol *12*, 10-16.

Thibodeau, P.H., Richardson, J.M., 3rd, Wang, W., Millen, L., Watson, J., Mendoza, J.L., Du, K., Fischman, S., Senderowitz, H., Lukacs, G.L.*, et al.* (2010). The cystic fibrosis-causing mutation deltaF508 affects multiple steps in cystic fibrosis transmembrane conductance regulator biogenesis. J Biol Chem *285*, 35825-35835.