Supplemental Materials for "Chemical Rescue of ΔF508-CFTR mimics Genetic Repair in Cystic Fibrosis Bronchial Epithelial Cells", Singh, O.V. et al.

SM.1.: Both IB3-1 cells (CF genotype ΔF508/W1282X, bronchial epithelial derivation) (16) and genetically repaired-CF (IB3-1 corrected by AAVwtCFTR termed S9) cells were grown on uncoated 75 cm^2 tissue culture flasks (Falcon, Franklin Lakes, NJ) at 37^0C in a 5% CO_2 incubator for 48-72 hrs to reach 80% confluence. The standard growth medium was gentamicin-free LHC-8 (Biosource, Rockville, MD) supplemented with 5% fetal bovine serum (Sigma, St. Louise, MO), 100 $U.mL^{-1}$ of penicillin-streptomycin, 2.5 $\mu g.mL^{-1}$ of Fungizone (Invitrogen) and 80 $\mu g.mL^{-1}$ of tobramycin (Eli Lilly, Indianapolis, IN).

SM.2.: Total CFTR captured protein immune-complex (n = 3) from anti-R domain antisera 169 was separated by first-dimensional isoelectric focusing (IEF) using pre-cast dry 17 cm immobilized pH 4-7 Gradient (IPG) strips on a IPGphor unit (Bio-Rad, Hercules, CA) overlayed with mineral oil, and covered with 17 cm strip holder plate cover. Each IPG strip was re-hydrated for 12 hrs with total capture immune-complex protein in 300 μL of IEF rehydration buffer (7 M Urea; 2 M Thiourea; 4% CHAPS; 0.5% Carrier ampholyte; 40 mM Dithiothreitol (DTT); 0.002% Bromophenol Blue). IEF was carried out using the following conditions: (i) 250 V for 20 min on linear ramp, (ii) 10,000 V for 2 hrs on linear ramp, (iii) 10,000 V at 45,000 V-hr on rapid ramp, (iv) hold at 500 V on rapid ramp until IPG strips were removed from 1st dimension. After isoelectric focusing, the proteins were subjected to reduction and alkylation prior to the

second dimension for the separation by molecular weight. To accomplish this procedure, each strip was subjected to a 2-step equilibration using equilibration buffer I (6 M Urea; 2% SDS; 0.375 M Tris-HCL pH 8.8; 20% Glycerol and 130 mM DTT) followed by equilibration buffer II (6 M Urea; 2% SDS; 0.375 M Tris-HCL pH 8.8; 20% Glycerol and 135 mM Iodoacetamide) for 15 min each just before proceeding for second dimension gel electrophoresis. The second dimensional electrophoresis was performed on 1.0 mm 10% SDS-PAGE gels at 50 V for 30 min followed by 100 W until the blue dye front arrives at the bottom of the gel using an Amersham Pharmacia Iso-DALT electrophoresis unit.

Once the dye reached the end of the gel, the gel cassettes were removed and the gels were place in fixation solution (10% Methanol and 7% acetic acid) for silver staining using the using MS-compatible SilverQuest™ Silver staining kit (Invitrogen). Captured immune-complex protein gels from CF, chemical rescued-CF and genetically repaired-CF were stained in parallel in triplicate under individual set of experiment as manufacturer's instructions on a slowly oscillating rocker table.

SM.3.: Recently introduced technologies such as difference in-gel electrophoresis (DIGE) and a metabolic labeling strategy known as SILAC (stable isotope labeling by amino acids in cell culture) carry potential pitfalls such as problems with labeling strategies, sample complexity, fractionation, relative vs absolute changes and the range of differences. We used 2-DE, a more traditional, and probably still, the most frequently used silver staining method, to estimate protein abundance in cell fractions and in CFTR immunocomplexes. The highly reproducible silver stained gel images were acquired using Molecular Images FX (Bio-Rad, Hercules, CA). Consistently replicate images were

further processed for spot detection, gel alignment and spot quantification by match ratio using Progenesis Nonlinear Dynamics software version 2005. All of the variation in protein spots was contributed by very low abundance proteins near the threshold of detection. Once spot matching was complete, an average gel of all 3 replicate gels was created using the average gel command of the Progenesis Nonlinear Dynamic software. After normalization, using Progenesis background correction, the software was commanded to detect the spot volume of all 3-replicate gels (IB3-1/CF, Chemical rescue, genetically repaired) (Figure S.1a-c). This normalization corrected for loading differences since the normalized intensities are given relative to the median of spot intensities, and it approximates the correction for the non-linear responses at the low and at the high ends of the intensity range. The applications of these corrections in silver stained 2-DE gels are shown in Figures S.1a-c. ThePprogenesis software automatically calculates and reports variation or error of all 2-DE spots represented in a digitally constructed average gel. Figure S.2 shows the individual protein in gel-to-gel coefficient of variation (CV) as a function of spot volume. There is clearly a non-linear relationship between protein spot volume and the coefficient of variation or experimental precision as shown in Figure S.2. The more abundant protein spots show much less gel-to-gel variation (CV) with respect to spot volume while less abundant proteins show much higher gel-to-gel variation. Quantitative variations in proteins are expressed as volumes of spots with reference to a constant spot in each experiment (β- Actin). To compensate for variations in gel staining and differences in protein spot numbers for each gel, normalized spot volumes were used for comparison of protein expression levels. The normalized spot volume was automatically calculated by the software as the single spot volume divided by the total

spot volume and then multiplied by the total spot area. Based on the gel-to-gel variation of less error in high abundant proteins, the differential expression of biological differences in selective proteins (IP-1 – IP-16) in the experimental gel sets of chemical rescued (IB3-1, 4-PBA) and genetically repaired (IB3-1/S9) cells are shown in Figure S.3. Each data point is the mean ±SEM of spot volume relative to average gel of control (IB3-1/CF) cells. The analysis of variance was calculated, the *P* value <0.05 was considered significant.

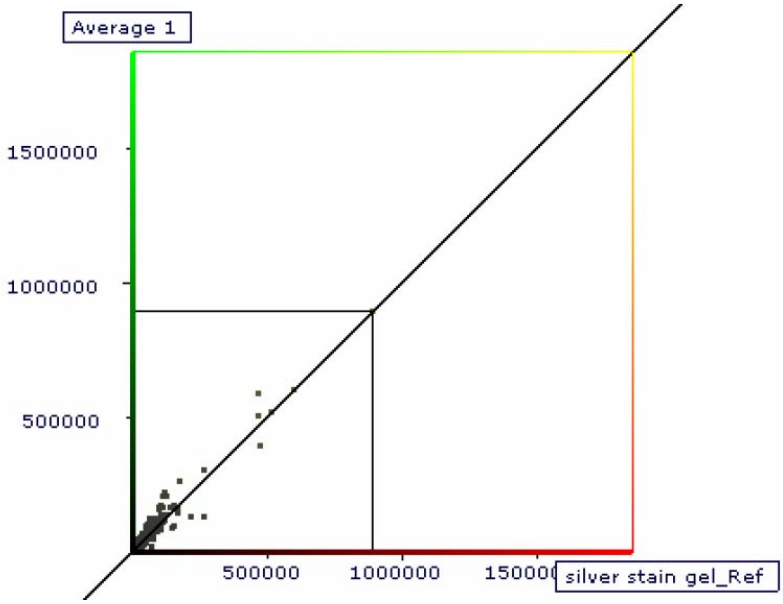

Figure S.1a

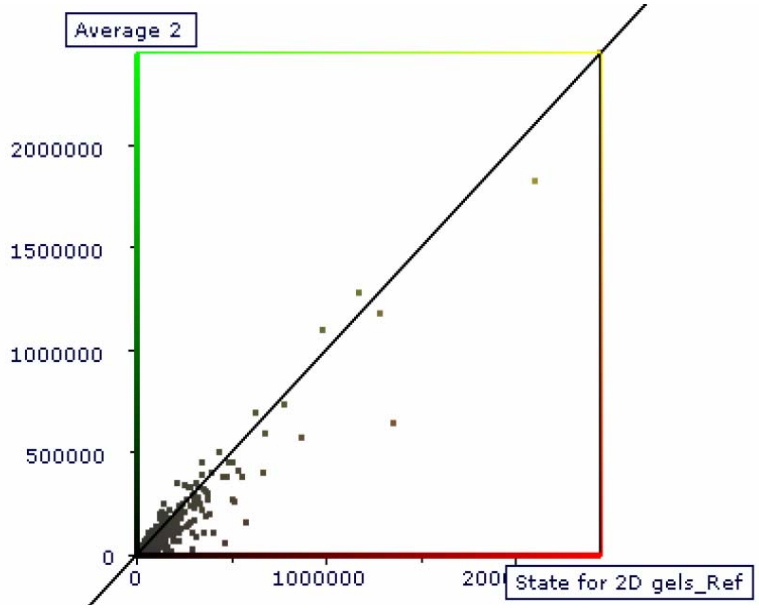

Figure S.1b

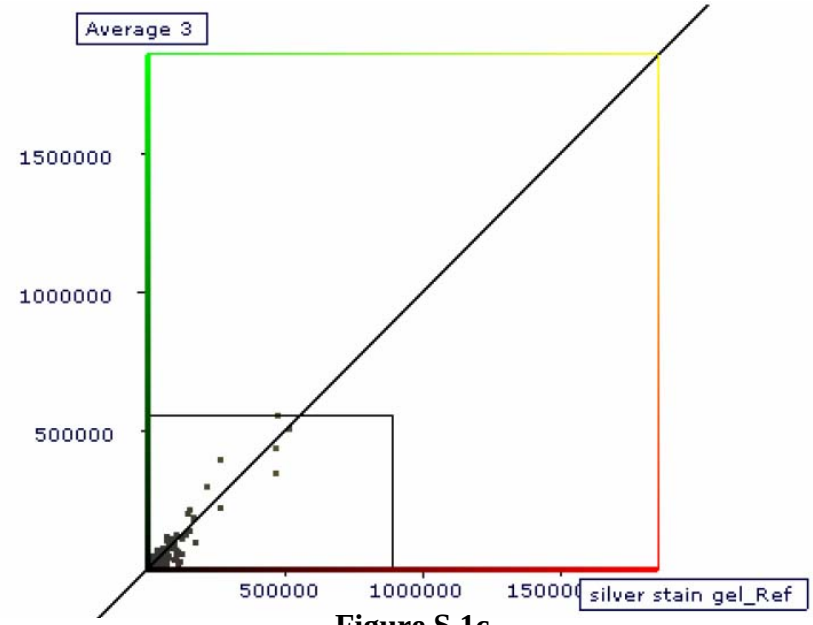

Figure S.1c

Figure S.1a-c. The relative normal distribution of median spot volumes of 3 sets of IB3-1/ CF, chemical rescued (IB3-1, 4-PBA), and genetically repaired (IB3-1/S9) experiments and its approximate corrections with silver stained reference gel of IB3-1/ CF cells.

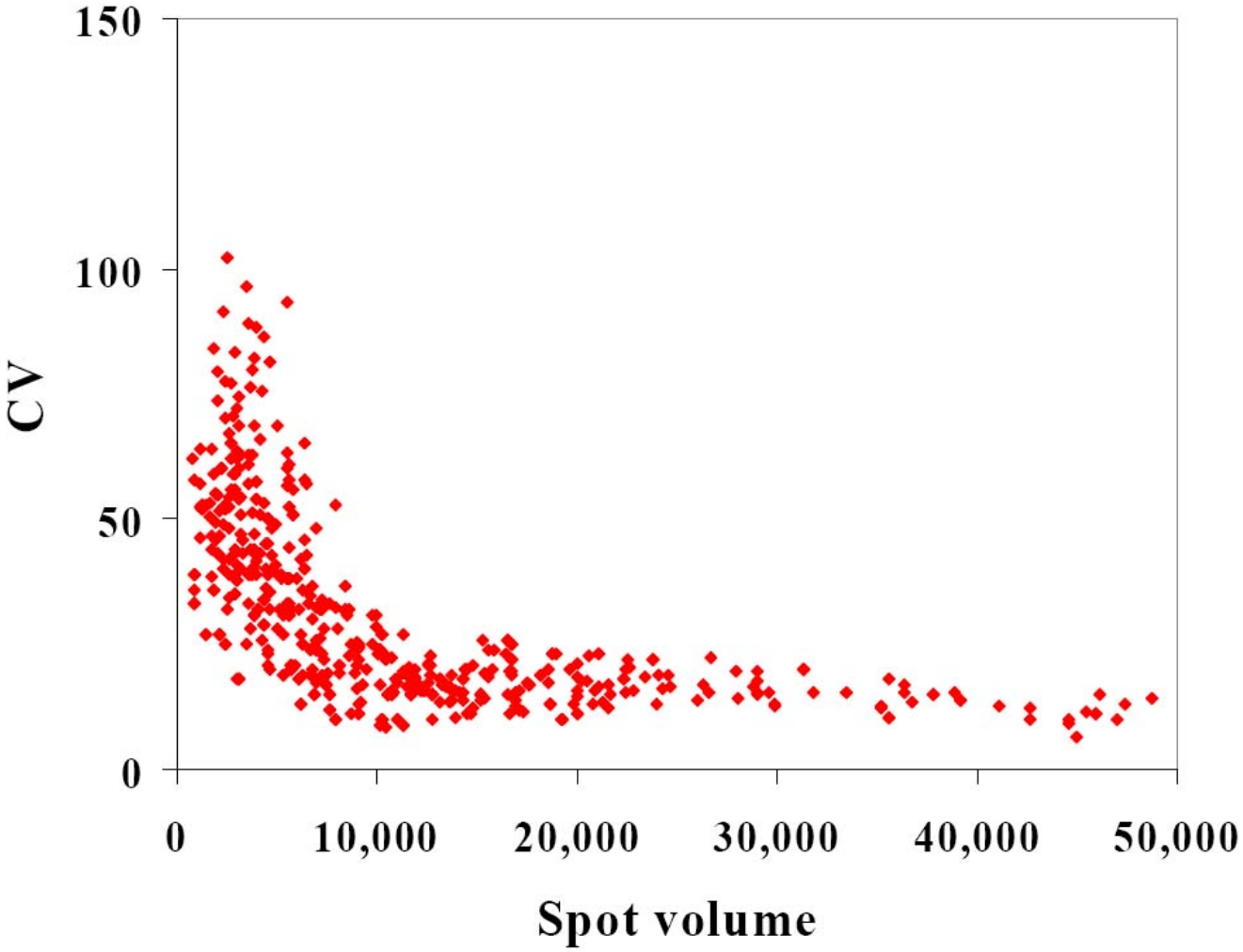

Figure S.2 The coefficient of variation (CV) in the quantitation of silver stained 2-DE gels. The relationship between measured spot volume and the coefficient of variation, data clearly show that low abundant proteins contribute the most error while high abundant proteins are measured with less error.

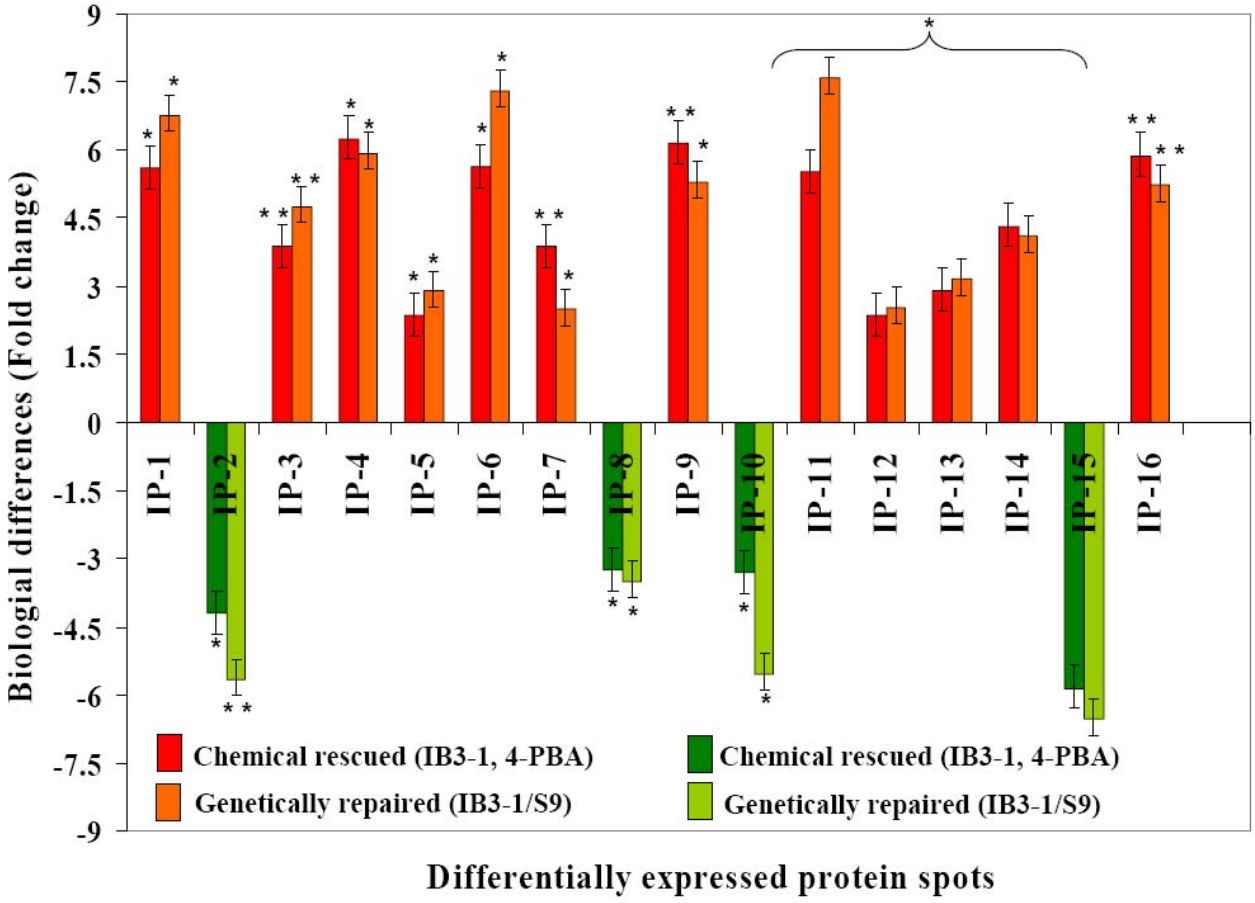

Figure S.3 Differential expression of biological differences in selective protein spots in experimental gel sets of chemical rescued (IB3-1, 4-PBA) and genetically repaired (IB3-1/S9) cells. Each data point is the mean ±SEM of spot volume relative to average gel of control (IB3-1/CF) cells. The analysis of variance was calculated, the *P* value <0.05 was considered significant (*) and most significant (* *).

SM.4. Protein Identification by mass spectrometry:

4.1. Protein in-gel digestion:

The protein gel spots were excised manually with a plastic plunger (The Gel, San Francisco, CA) and digested in parallel with blank spots on the gel as controls (n =3). The protein gel pieces were destained by adding a 1:1 mix of 30 mM potassium ferrocyanide and 100 mM sodium thiosulphate until gel pieces lost the brown color, followed by three washes with 200 μL proteomics grade water (Bio-Rad). Destained gel pieces were washed with 20 mM NH_4HCO_3 and dehydrated with ACN, twice for 10 min each. Finally the dried gel pieces were swelled in 20 μL (10 ng/μL) sequencing-grade modified trypsin (Promega, Madison, WI) in 20 mM NH_4HCO_3, pH 8.5 (Sigma) and incubated on ice for 45 min. Excess trypsin was removed around the gel pieces and replaced with 10 mM NH_4HCO_3, pH 8.5. Protein digestion was continued by incubating gel pieces at 37^{o}C overnight with gentle shaking. Peptides were extracted from gel pieces by adding 200 μL of 0.1% trifluoroacetic acid (TFA) in 60% ACN followed by vigorous shaking for 60 min at 30^{o}C. Supernatant was collected and dried in a speed vac concentrator (Eppendorf) and resulting peptides were solubilized in 5 μL 0.1% TFA.

4.2. Peptide purification for mass spectrometry:

The extracted peptide mixture was desalted with an in-tip reversed phase column (C18 Zip-Tip; Millipore Corp., Bedford, MA). The peptide mixture was eluted from Zip-Tip with 2 μL of matrix solution (10 mg/mL α-cyano-4-hydroxycinnnamic acid (Sigma, St. Louise, MO) in 60% ACN/ 0.1% TFA) and directly spotted onto a matrix assisted laser desorption ionization (MALDI) target plate. Each desalted peptide mixture from triplicate samples were spotted individually on MALDI plate.

4.3. Mass Spectrometry:

Mass analyses was performed using a matrix assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager DE-STR, Applied-Biosystems. Framingham, MA) equipped with a 337 nm nitrogen laser. Samples were analyzed in refelectron mode by TOF at an accelerating potential 20kV. The average of 3 scans (each containing 75 spectra) that passed the accepted criterion of peak intensity were automatically selected and saved using Data Explorer software version 4.0 (Applied Biosystem, Framingham, MA, USA). Mass spectra's were automatically calibrated upon acquisition using two point residual porcine trypsin autolytic fragments (842.51 and 2210.10 [M+H+] ions) and matrix added standard bradykinin and ACTH peaks (757.39 and 2,465.19 [M+H+] ions) obtained from Sigma (St. Louise, MO). Raw spectra were baseline corrected and noise filtered (correlation factor = 7). Spectra were deisotoped and peak automatically collected using "copy peak list" feature of the software. Keratin and trypsin-derived extra peaks were manually removed from monoisotopic standard peak lists. All the submitted masses were accurate to the level of 40 ppm. Processed MALDI-TOF spectra of each identified proteins can be seen in supplementary file.

4.4. Identification of proteins:

Monoisotopic masses of each spectrum in triplicate was searched in the NCBI non-redundant databases (NCBI nr 2005.01.03) using ProFound-peptide Mapping search engine (The Rockefeller University Edition, Version 2005.02.14). Identified proteins were confirmed by searching in MS-Fit search engine using SwissProt.07.05.2006

database. Search was performed from total number of protein entries in the database 203407, which was restricted by experimental molecular weight, p*I* and species search entries. The combined molecular weight (25000 -100000 Da), pI (4.00-7.00) and species (*Homo sapiens*) searches select 4434 entries. The unmatched peptides or miscleavage site were neglected. All mass searches were performed using the short form of Pro-Found-peptide mapping under *Homo sapiens* taxonomic category for only single protein under pre-assumed experimental mass and p*I* range and crosschecked with MS-Fit search engine. The search parameters were allowed complete modification with Iodoacetamide (Cys). Peptides were matched with the theoretical peptide masses of all protein from *Homo sapiens* of the NCBI and SwissProt databases using a tolerance limit of 50 ppm. Other criteria for positive identification of proteins were set as follows: (i) the number of peptides matched averaged more than 10 (minimum =5); (ii) 20-50 ppm or better mass accuracy; (iii) the matched peptides covered at least 25% of the whole protein sequence with a significant Z score (> 90 % probability) and higher Mowse score; (iv) Each identified protein were cross reference to the comparable p*I* and molecular weight (kDa) obtained from experimental image analysis on the 2-D gel. Among 5 maximum reported hits from SwissProt database, a highest Mowse score, at least 25% coverage, with unique peptide and validation through western blots were used to eliminate the redundancy of matched protein to multiple members of a protein family. The measured mass, respective peptide assignments and mass accuracy are provided in supplemental Table 1. Finally, identified proteins were denoted as Immunoprecipitant protein (IP) 1-16 and their validity was cross checked with 1D western blot analysis.

SM.5. 80% confluent CF (IB3-1), chemical rescued-CF (4-PBA treated IB3-1) and genetically repaired-CF (IB3-1/S9) in parallel grown in uncoated 75 cm^2 tissue culture flasks (Falcon, Frankin Lakes, NJ) were washed three times with ice cold phosphate-buffered saline (PBS) (pH 7.4) and incubated at 4^0C for 30 min in 10 ml ice cold PBS containing 10 μL of 1000 x EZ-Link sulfo-NHS-SS-biotin (Pierce Biotechnology Inc., Rockford, IL) stock solution (100 mg/mL, freshly prepared with DMSO) followed by gentle agitation. Reaction was stopped by lysine solution (1 mL, 1 mg/mL). Cells were scraped into ice cold PBS and washed three times by repeated centrifugation at 350 g for 10 min. The final pellet was re-suspended into 1 mL ice-cold hypotonic buffer (10 mM HEPS, pH 7.5, 1.5 mM $MgCl_2$, 10 mM KCL, 1x protease inhibitor cocktail, 1 mM NaF, a and 1 mM Na_3VO_4). In order to improve solublization efficiency of integral plasma membrane proteins, cells were disrupted by sonication (at low power, 1.5) on ice using 2-4 second bursts followed by vortex every 15 min for 5 seconds during 45 min incubation on ice. Cellular lysate was collected by centrifugation at 10 000 g for 2 min at 4^0C. The biotinylated proteins were isolated by incubating total cellular lysate with immobilized NeutrAvidin™ gel slurry (Pierce Biotechnology Inc., Rockford, IL) in QIAshredder™ column (Qiagen Inc. Ca) at 4^0C for 1 h at room temperature with end-over-end mixing rotor device. After washing three times with ice-cold 1 M KCL and of ice-cold 0.1 M Na_2CO_3, pH 11.5 and once with ice-cold hypotonic buffer, the bound proteins with NeutrAvidin gel beads were suspended in 400 μL SDS-PAGE sample buffer (62.5 mM Tris-HCL, pH 6.8, 1% SDS, 10% glycerol, 50 mM DTT). Total plasma membrane protein was extracted by heating the beads for 5 min at 95^0C and centrifugation at 1000 g for 2 min. The supernatant was collected, and the extraction was repeated once.

SM. 6. Cell surface CFTR protein was extracted, using surface biotinylation, 80% confluent cells from all 3 sets (CF, chemically rescued-CF and genetically repaired-CF in parallel) were washed twice with ice-cold PBS containing 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ to inhibit vesicles trafficking. The glycosideic moieties on the cell surface membrane proteins were derivatized with sodium periodate and biotinylated using optimized concentration of EZ-Link biotin-LC-hydrazide (2 mM in 100 mM Sodium acetate, pH 5.5) at 4^0C for 30 min in dark. Cells was scraped into ice cold PBS and extensively washed by repeated centrifugation at 350 g for 10 min. The final pellet was re-suspended into 1 mL ice-cold cell lysis buffer (1% SDS containing 0.2 mM PMSF and 1mM benzamidine). Cells were sonicated briefly and cellular debris were removed by centrifugation at 10 000 g for 10 min at 4^0C. The biontinylated surface protein was bound and eluted as described above for integral plasma membrane protein extraction using immobilized NeutrAvidin™ gel slurry (Pierce Biotechnology Inc., Rockford IL) in QIAshredder columns (Qiagen Inc., USA).

SM. 7. Other primary antibodies used were rabbit polyclonal GRP94 (1:1000), HSP84 (1:1000), GRP58 (1:2000) (abcam, Cambridge, MA) HSC70 (1:1000) (StressGene Biotechnologies, Victoria, BC); mouse monoclonal HSP60 (1:1000), HSP27 (1:1000) (abcam, Cambridge, MA), HSP70 (1:1000) (StressGene Biotechnologies, Victoria, BC); goat polyclonal for GRP78 (1:500), GRP75 (1:500), Actin (1:1000) (Santa Cruz Biotechnology Inc., Santa Cruz, CA), BAG-3 (1:500) (abcam, Cambridge, MA). Secondary antibodies used were donkey anti-rabbit IgG-horseradish peroxidase conjugate

and sheep anti-mouse IgG-horseradish peroxidase conjugate (Amersham, Arlington Heights, IL), and rabbit anti-goat IgG-peroxidase conjugate (Sigma, St. Louise, MO).

SM.8. Pathway studio version 4.0 software was modified from Pathway Assist® software of Ariadne Genomics, Inc. (Rockville, MD) and Pathway Architect from Stratagene (La Jolla, CA) was used for functional connectivity analysis and to characterize the HSP70 system dependent regulation of cytokine expression, respectively. Both software provides comprehensive searches for relationships among genes of interest and generates linkage maps based upon published research. The databases of the software are connected to the extensive lists of known published gene/protein, and extensive literature links in PubMed and Scopus which are based upon automated native languages search strategies.