Supplementary Materials and Methods

Abcam: HSP90 antibody (ab13492), Everolimus (ab142151)

BioSciences: Pierce protease inhibitor tablets (A329663), RIPA lysis and extraction buffer (89900), HALT phosphatase inhibitor cocktail (78420), BSA protein assay kit (23235), Spectra TM multicolour broad range protein ladder (26634), siRNA s100A12 (4392420)

Cell Signalling Technology: pAKT antibody (9271T), Anti-mouse IgG, HRP-linked antibody (7076S), Anti-rabbit IgG, HRP-linked antibody (7074S)

Cayman *Chemical:* VX-809 (22196), VX-661 (21480), VX-770 (15145)

Cell Signalling**:** Annexin V (8555), Flotillin-1 (D2V7J), CD9 (D8O1A), total Akt, pAkt ser473 (9271 S), LC3AB antibody (4108)

LifeLine Cell Technology: Bronchial Life epithelial basal medium with epithelial airway medium complete kit (LM-0007)

LifeSpan BioSciences: CD9 antibody (LS-C675301)

Merck: CD63 antibody (CBL553)

Promega: Sequencing Grade Modified Trypsin (V5111)

Santa Cruz: goat anti-mouse HRP (sc-2005), rabbit anti-mouse IgG-HRP (sc-358914), VCAM antibody E10 (sc-13160), EPCAM antibody (sc-21792), Beta-actin antibody C4 (sc-47778)

Selleck Chemicals: MK-2206 (S1078), VX-770 (S1144), VX-809 (S1565)

Sigma Aldrich: AKT-VIII (A6730), TruePage Precast gels (PCG2010-10EA), Minimal Essential Medium Eagle (M2279-500ML), RPMI-1640 Medium supplemented with L-glutamine (31095-029), SuperSignal West-Femto maximum sensitivity substrate (34095), Whatman cellulose chromatography papers (WHA3030675), Collagen from human placenta (C7521-10MG), Corning transwell collagen coated membrane inserts (8586985)

Thermo fisher scientific: Minimum essential medium with L-glutamine (31095-029), goat anti-mouse Alexa Fluor® 594 (A-11005), anti-rabbit Alexa Fluor® 488 (A 11034), CD47 antibody (MA5-11895)

Cell Lines: Cystic Fibrosis Bronchial Epithelial cells CFBE41o- F508del ($CFTR^{(F508/F508)}$), HBE41o-WT ($CFTR^{(WT/WT)}$), isogenic CFTR null cells NuLi1 ($CFTR^{(WT/WT)}$), CuFi5 ($CFTR^{(F508/F508)}$) and CuFi4 ($CFTR^{(F508/G551D)}$) were obtained from Prof's E Sorscher/J Clancy (University of Alabama, Birmingham).

Compound	Primary Target	Source	Reference
Everolimus	Binds to FKBP12 and complex prevents TOR phosphorylation. Rapamycin analog	Abcam	"Everolimus" Clinical cancer research : an official journal of the American Association for Cancer Research vol. 16,5 (2010): 1368-72
MK-2206	Allosteric Akt inhibitor by stopping autophosphorylation of Akt at T308 and Ser473.	Selleck Chemicals	Hirai *et al* (2010) MK-2206, an Allosteric Akt Inhibitor, Enhances Antitumor Efficacy by Standard Chemotherapeutic Agents or Molecular Targeted Drugs In vitro and In vivo. Mol Cancer Ther July 2010 9; 1965
AKT-VIII	Allosteric Akt inhibitor by binding PH domain.	Sigma Aldrich	Wu *et al* (2010) Crystal Structure of Human AKT1 with an Allosteric Inhibitor Reveals a New Mode of Kinase Inhibition. PLoS One. 2010; 5(9): e12913.
Lumacaftor (VX-809)	Corrector of the misfolded CFTR protein, the root cause of the F508del mutation.	Cayman Chemical	Zhang *et al* (2016) Lumacaftor/Ivacaftor combination for CF patients homozygous for Phe508del-CFTR. Drugs today, vol. 52,4 2016: 229-37
Tezacaftor (VX-661)	Corrector of the misfolded CFTR protein, the root cause of the F508del mutation.	Cayman Chemical	Schmidt *et al* "Cystic fibrosis transmembrane conductance regulator modulators in cystic fibrosis: current perspectives" Clinical pharmacology : advances and applications vol. 8 127-140. 21 Sep. 2016, doi:10.2147/CPAA.S100759
Ivacaftor (VX-770)	Ivacaftor (VX-770) is a selective potentiator of CFTR, targets G551D CFTR and F508del-CFTR	Cayman Chemical	Condren *et al* "Ivacaftor: a novel gene-based therapeutic approach for cystic fibrosis" journal of pediatric pharmacology and therapeutics : JPPT : the official journal of PPAG vol. 18,1 (2013): 8-13

Supplementary Methods

Cell culture

HBE41o- WT and CFBE41o- F508del cells were cultured in Minimum Essential Medium (MEM, Sigma-Aldrich) supplemented with 10% FCS (Fetal Calf Serum), 2 mM glutamine and 1% penicillin/streptomycin. NuLi1, CuFi4 and CuFi5 cells were cultured in BronchiaLife epithelial basal medium with epithelial airway medium complete kit (LifeLine Cell Technology). All cells were grown at 37 °C in 5% CO_2. All inhibitors (list of inhibitors and concentrations used described in supplemental materials) were incubated with cells for 24h. DMSO concentration never exceeded 0.1%.

SiRNA Transfections

CFBE41o- cells were transfected with 5nM siRNA using 4uL Lipofectamine (Invitrogen, Grand Island, NY) as described previously[10]. Briefly, lipid-siRNA complexes were prepared in serum-free media and added to cell suspension in culture medium with 10% FCS. After 24h, medium was replaced. The target siRNAs were to S100A12 (assay ID s12433, Ambion).

Collection and processing of bronchoalveolar lavage fluid (BALF)

All bronchoscopies were performed under general anaesthetic via a laryngeal mask, using a Wolf flexible bronchoscope with a 2.5 mm outer diameter and a 1.2 mm instrument channel, (Wolf 7.73250.172, Richard Wolf Medical Instruments Corp., Vernon Hills, Illinois) and a Pentax flexible bronchoscope with 2.8 mm outer diameter and a 1.2 mm instrument channel, (FB8V HOYA Corporation, Tokyo, Japan). The bronchoscope was inserted through the laryngeal mask and suctioning was avoided until the bronchoscope had passed the carina, thus minimizing upper airway contamination. The bronchoscope was gently wedged in the right middle lobe and 1 ml/kg (to a max of 20 mL) of sterile 0.9% sodium chloride was instilled and returned using low-pressure suction into a suction trap. The bronchoscope remained in position and a second lavage was performed. The process was repeated in the lingula. The samples from right middle lobe and lingula were pooled. BALF was transported from theatre on ice to the processing laboratory. The sample is gently inverted to mix. The BALF was centrifuged at 3000rpm for 10 minutes. BALF supernatant was aliquoted, labelled and stored at -80°C.

Extracellular vesicle fraction isolation from BALF

Extracellular vesicle (EV) fractions were isolated by differential ultracentrifugation. Briefly, BALF aliquots (1-2mL) were centrifuged at 120,000g for 2 h at 4 °C (XL-70 ultracentrifuge, Beckman-Coulter, Villpinte, France) to pellet the EVs. The pellet was then re-suspended in either PBS (for nanoparticle tracking experiments), RIPA (immunoblotting) or had undergone tryptic digestion (Mass Spectrometry). Samples were stored at −80 °C. All experiments were normalized at the time of EV isolation, therefore, no additional normalization of data was performed. All data is normalized within each sample type.

Extracellular vesicle fraction isolation from cell lines

EV fractions were isolated by differential ultracentrifugation. All experiments were done in biological triplicate. Briefly, cells were grown in T75 cell culture flasks. Cells were seeded at 1 x10^6cells/ T75 cell culture flask approximately and allowed to adhere for 24 hours. The following day, media was replaced with serum-free media for CFBE41o- and HBE41o- cells and fresh primary cell media for NuLi1, CuFi4 and CuFi5 cells, 10mL/T75 flask. Cells were grown for 48 hrs. Supernatants from 5x T75 cell culture flasks were used for each biological replicate, for each cell line this was: 25 x10^6 cells approximately. Cellular supernatants were then centrifuged at 3000g for 30 min to remove cell debris. Next, the supernatant was centrifuged at 10,000g for 30 min at 4 °C. The resulting supernatant had undergone ultracentrifugation at 120,000g for 2 h at 4 °C (XL-70 ultracentifuge, Beckman-Coulter, Villpinte, France) to pellet the EVs. The pellet was then re-suspended in either PBS (for nanoparticle

tracking experiments), RIPA (immunoblotting) or had undergone tryptic digestion (Mass Spectrometry). Samples stored at −80 °C.

Protein extraction and immunoblotting

EV and cell extracts were prepared in RIPA lysis buffer, mammalian protease inhibitor cocktail, pH 7.4, phosphatase inhibitor cocktail). Protein concentration was determined by the BCA assay (Thermo Scientific, Rockford, IL). Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis and transferred onto 0.2mm polyvinylidene fluoride (PVDF) membranes as previously described[10]. Membranes were blocked (0.05% Tween 20 and 5% non-fat dry milk or 3% BSA) prior to incubation with primary antibodies. Horseradish-peroxidase-conjugated secondary antibodies were visualized using SuperSignal West Pico or Femto reagents (Thermo Scientific, Rockford, IL). Images were obtained using Vilber Fusion FX imager (Vilber, France).

Nanoparticle Tracking Analysis (NTA) of Biological Samples

Particle size distribution in cellular supernatants and BAL samples was determined by NTA using a NanoSight NS300 system (Malvern Technologies, Malvern, UK) configured with a 488 nm laser and a high sensitivity scientific CMOS camera. Samples were diluted (cellular supernatants 1:5–1:10, BAL 1:500) in particle-free PBS (Gibco, Waltham, MA, USA) to an acceptable concentration, according to the manufacturers recommendations. Samples were analyzed under constant flow conditions (flow rate = 50). Ten successive videos were captured for each sample. Data were analyzed using NTA 3.1.54 software.

Transmission Electron Microscopy

For negative stain microscopy, 10µL 2% uranyl acetate alternative (gadolinium triacetate) (Ted Pella Inc, CA, USA) was aliquoted onto Parafilm (Beemis Company Inc, WI, USA), 5 µL of EV enriched sample was added to the negative stain and covered for 10 minutes incubation/drying. A Formvar/silicon monoxide 200 mesh copper grid (Ted Pella Inc, CA, USA) was placed on the stain/samples and incubated for 1 minute. Excess liquid was removed by blotting. The grid was briefly placed on 10 µL of 2% uranyl acetate alternative (gadolinium triacetate) (Ted Pella Inc, CA, USA).

Images were acquired using a H7560 transmission electron microscope (Hitachi High-Technologies Corporation Europe, UK) at 100 kV. To find a magnification that shows as many EVs as possible on a single image with sufficient detail to distinguish EV morphological features, we manually evaluated images at magnifications of 60,000 and 200,000 X magnification. At least 9 images were acquired per sample. Images were analyzed using Image J (https://imagej.nih.gov/ij/).

Neutrophil (PNC) Isolation

Blood was collected in lithium heparin blood tubes on the day of experiment. Blood was diluted 1:1 with Hank's Balanced Salt Solution (HBSS; Mg^{2+} and Ca^{2+} ion free) and carefully layered over Lymphoprep solution (Stemcell Technology), centrifuged at 600 rcf for 15 min at room temperature (RT) with no brake or acceleration. After centrifugation, four distinct layers are observed, with the pellet of sedimented red blood cells (RBC) with the granulocytes the layer of interest. The RBC pellet was then immediately suspended in 5 ml of 1X HBSS buffer for further processing with 3% dextran (Sigma Aldrich) with the RBC sedimentation allowed to take place for 20 min, at room temperature in the dark. After sedimentation, neutrophil rich supernatant in the upper layer is collected and centrifuged for 5 min at 400 rcf at room temperature. RBC lysis was performed to obtain a pure granulocyte population using RBC lysis buffer (Roche). Enriched granulocyte/neutrophil cells were then re-suspended in RPMI (FBS free) media, counted using a haemocytometer and yield determined by Leishman staining/CD66b positivity using flow cytometry.

Neutrophil (PNC) Migration

Migration assays were performed using 12 mm Costar Transwell -COL collagen-coated 3.0 µM pore PTFE membrane inserts (Sigma Aldrich). Neutrophils were seeded (5×10^5 cell/ml) on the upper chamber. The lower chamber was filled with 500 µl serum free non conditioned RPMI medium (negative control) and or supplemented with HBE41o- EVs, CFBE41o- EVs (10 µg) or fMLP (100 nM) for 3 h. After 3 h, the number of migrated cells was counted under a microscope. Data was counted and analysed using ImageJ.

Neutrophil Transmigration

Neutrophil transmigration through CFBE41o- cells was performed as follows. In brief, CFBE41o- cells (1×10^5) were cultured on 6.5-mm Costar Transwells with 3-µm-pore-size polycarbonate membrane inserts (Sigma Aldrich), for 48 h. Human neutrophils were obtained as described. Neutrophils (1×10^6 cells/well) in RPMI were placed in the upper chamber, and cells were allowed to migrate to the lower chamber containing media alone, fMLP (100 nM) or HBE41o- /CFBE41o- EVs (10 µg) as chemo attractants. After 3h, neutrophils found in the lower chamber were measured in triplicate by microscope.

Neutrophil Staining and Flow Cytometry

Neutrophils were seeded $1x10^5$/ well. 10 ug of EVs (± PKH26 dye) were incubated for 30 mins at 37^oC. Neutrophils ± EVs were centrifuged at 300g for 5 mins at 4C. The pellet was re-suspended in 1X HBSS (100uL, Ca/Mg^{2+} free). Appropriate volume of staining buffer (100uL 1% FBS-HBSS) was added to the suspension. Cells were stained using 10 µl CD66b-APC for 30 mins on ice and protected from light. Following incubation, cells were washed with 1-2 ml of staining buffer. Following the final decanting, stained cells were re-suspended in an appropriate amount of staining buffer for flow analysis.

Flow cytometry was carried out on a BD LSR Fortessa cell analyser and analysed using FlowJo software (TreeStar).

Myeloperoxidase Assay (MPO)

Myeloperoxidase (MPO) release was measured from isolated neutrophils post chemotaxis as per manufacturing instructions (Sigma-Aldrich). Neutrophil release of MPO upon incubation of EVs (10 µg) from HBE41o- WT, CFBE41o- F508del was quantified by a myeloperoxidase (MPO) assay (Sigma-Aldrich). Samples were diluted 1/50 and assayed against TNB standards for colorimetric detection as per manufacturers protocol. All samples were assayed in duplicate (sample blank and sample containing MPO substrate) in order to calculate the change in measurement between each sample blank and its corresponding sample. Only values that were within linear range of the TNB standard curve were used to determine MPO activity.

Mass Spectrometry

Patient BALF, HBE41o- WT and CFBE41o- F508del EV samples were dissolved in 8 M urea, 100 mM Tris pH 8.5. Proteins were reduced with DTT and alkylated with IAA. Protein digestion was performed by overnight digestion with trypsin sequencing grade (Promega) resuspended in diluted TFA and stored at 4 °C until MS analysis, where 10-20 µg of protein were ran on a Thermo Scientific Q Exactive mass spectrometer operated in positive ion mode and connected to a Dionex Ultimate 3000 (RSLCnano) chromatography system. All data was acquired while operating in automatic data dependent switching mode. A high-resolution (70,000) MS scan (300–1600 *m*/*z*) was performed to select the 12 most intense ions prior to MS/MS analysis using high-energy collision dissociation (HCD). Proteins were identified and quantified by MaxLFQ (REF; Accurate Proteome-wide Label-free Quantification by Delayed Normalization and Maximal Peptide Ratio Extraction), Termed MaxLFQ by

searching with the MaxQuant version 1.5 against a human reference proteome database [15]. Modifications included C carbamlylation (fixed) and M oxidation (variable).

Protein /Pathway Analysis

Proteins identified by mass spectrometry were overlaid onto a global molecular network developed from information contained in the Ingenuity database (http://www.ingenuity.com) and Reactome (https://reactome.org). Venn diagrams and pie charts illustrating subcellular enrichments of identified proteins was performed using the functional enrichment analysis tool Funrich (http://www.funrich.org/)

Statistical analysis

All quantified data are presented as the mean ± SEM for at least three independent experiments. For each experiment, statistical tests are indicated in the results section. Student paired *t-test* and unpaired *t-test* analysis was conducted using Prism 6 (GraphPad Prism, San Diego, CA, USA). Unpaired *t-tests* were used for analyzing two different variables (i.e. WT, F508del and the 4 different CF patient groups). One way ANOVA was used for statistical analysis involving three or more groups. Bonferroni multiple comparisons *post-hoc* test was used to compare multiple groups to one group.