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A helper-dependent adenoviral vector rescues CFTR to wild type functional levels in CF epithelial cells harbouring class I mutations

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Abstract:

Cystic Fibrosis (CF) is a genetic disorder affecting multiple organs, including the pancreas, hepatobiliary system and reproductive organs however lung disease is responsible for the majority of morbidity and mortality. Management of CF involves CFTR modulator agents including corrector drugs to augment cellular trafficking of mutant CFTR as well as potentiators that open defective CFTR channels. These therapies are poised to help most individuals with CF, with the notable exception of individuals with class I mutations where full length CFTR protein is not produced. For these mutations, gene replacement has been suggested as a potential solution.

In this work, we used a helper dependent adenoviral vector (HD-CFTR) to express CFTR in nasal epithelial cell cultures derived from CF subjects with class I CFTR mutations. CFTR function was significantly restored in CF cells by HD-CFTR and reached healthy control functional levels as detected by Ussing chamber and membrane potential (FLIPR) assay. A dose response relationship was observed between the amount of vector used and subsequent functional outcomes; small amounts of HD-CFTR were sufficient to correct CFTR function. At higher doses, HD-CFTR did not increase CFTR function in healthy control cells above baseline values. This latter observation allowed us to use this vector to benchmark *in vitro* efficacy testing of CFTR-modulator drugs. In summary, we demonstrate the potential for HD-CFTR to inform *in vitro* testing and to restore CFTR function to healthy control levels in airway cells with class I or CFTR nonsense mutations.

Introduction:

Cystic fibrosis (CF) is a progressive, life-threatening, autosomal recessive disease that is caused by mutations in the cystic fibrosis transmembrane conductance regulator *(CFTR)* gene [1, 2]. Although CF involves multiple organs, lung disease is the main cause of morbidity and mortality in CF patients [3]. Dysfunctional CFTR leads to aberrant ion and fluid homeostasis at epithelial surfaces. In the lung, this results in the depletion of airway surface liquid, mucociliary dysfunction, increased bacterial colonization, inflammation, bronchiectasis and fibrosis.

Traditional pulmonary treatments for CF have targeted the consequences of CFTR dysfunction and include inhaled antibiotics, anti-inflammatory drugs, agents to enhance mucociliary clearance and nutritional therapies. In the last decade, CFTR modulator drugs have been introduced to target the basic CFTR defect. The first clinically approved modulator was the CFTR potentiator ivacaftor (IVA), which enhances CFTR channel-open probability [4]. The next clinically approved modulators were the first generation CFTR correctors, including, lumacaftor (LUM) and tezacaftor (TEZ), which improve intracellular trafficking of the p.Phe508del CFTR protein, increasing the amount of mature CFTR protein on the cell surface [5]. In combination with IVA, both LUM and TEZ have shown clinical benefits in patients homozygous for p.Phe508del CFTR [6-9]. Finally, the next generation of correctors, (e.g. elexacaftor, ELE), which have a different structure and mechanism of action compared to the first-generation correctors have recently been approved by the FDA. Triple combination regimens (including ELE/TEZ/IVA = trikafta) have shown efficacy in patients with at least one allele of p.Phe508del CFTR [10-14].

Unfortunately, all available combination therapies require the presence of some CFTR protein in the cell and thus are not predicted to improve CFTR function in the minority of CF patients with mutations where no protein or truncated protein is produced. Recent data suggests some function may be rescuable with the addition of nonsense mediated decay inhibition in some class I mutations, but these approaches are not clinically available and may lead to expression of other truncated genes products increasing off target effects [15]. Gene therapy offers the potential to specifically restore CFTR function to cells with class 1 CFTR mutations [16].

While gene therapy has been a therapeutic goal since the discovery of CFTR, there are a number of challenges related to gene therapy for CF lung disease. These include overcoming the physical and immunological barriers that resist introduction of exogenous DNA to the epithelial cell. As a consequence, no clinical trials have shown sustained therapeutic gene expression [17]. However, there has been significant progress in the development of gene therapy vectors and delivery methods in recent years [16]. Using the epithelium-specific gene expression cassette developed by us and helper-dependent adenoviral (HD-Ad) vectors [18], we have demonstrated efficient reporter and CFTR gene delivery to the airways of mice [19] and pigs [20]. HD-Ad vectors are adenovirus-based vectors in which all viral coding sequences are deleted [21]; thus these vectors are less immunogenic and can carry DNA constructs of up to 37 kb [22]. We have also demonstrated that HD-Ad vectors can be used to deliver genes to mouse and pig airway basal cells [23] which are considered the stem and progenitor cells of airway epithelia [24]. Gene delivery to pig airways can be carried out by aerosolization or instillation with an AeroProbe catheter [25] inserted into the working port of a bronchoscope.

We have shown that HD-Ad vectors can be used to deliver *CFTR* gene to CF primary nasal epithelial cells [23, 26]. However, it is also not clear how much CFTR expression is needed in order to restore a relevant amount of function. Here, we test the therapeutic potential of an HD-Ad vector expressing the human *CFTR* gene (HD-CFTR) to restore CFTR function in CF nasal epithelial cells carrying class I or nonsense mutations. We demonstrate that HD-CFTR can restore CFTR function in CF cells to a healthy control range. Further, the vector did not enhance CFTR function in healthy control cells allowing this vector to be used to benchmark *in vitro* responses.

Methods:

CF patient nasal epithelial cells, cell culture and vector transduction

All subjects (or guardians) provided written informed consent and were recruited under a Research Ethics Board approved study (REB# 1000044783). Primary nasal cell cultures were obtained through the CF Canada-SickKids Program for Individualized CF Therapy (CFIT). Nasal cells from five different CFTR mutation combinations were included in this study. The protein name is given for the mutations or the cDNA name when no protein name is available. Specifically we studied, p.Gly542X/ p.Asn1303Lys (n=1), p.Phe508del/ p.Trp1282X (n=2), c.489+1G>T/c.489+1G>T (n=2), p.Trp1282X/ p.Trp1282X (n=3), and p.Phe508del/ p.Phe508del (n=3). Non-CF individuals were volunteers from our institute (n=3) who did consent to participate in this research and undergo nasal brushing to donate epithelial cells.

Nasal epithelial cells were brushed from the inferior turbinate and cultured as previously described [26-31]. Following two to three passages, cells were switched to air liquid culture for

another 2 weeks. For vector delivery, cells were first treated with 6 mM EGTA for 40 min and then HD-CFTR or HD-GFP was added to the apical surface at varying MOI (infectious vector particles per cell). Ussing experiments and western blots were performed 3 days following transduction. For mixing experiments, cells were counted manually.

Human bronchial epithelial cells (HBE) were used as a source of protein controls for western blot studies. CF-HBE (CFF-16HBEge CFTR F508del V470, i.e. p.Phe508del) were obtained from Cystic Fibrosis Foundation Therapeutics (Lexington, MA) and Wild-type HBE (WT-HBE = 16HBE14o) were obtained from Drs. D Gruenert and B Illek (UCSF).

Helper dependent Adenovirus (HD) vector production

HD-GFP vector (expressing green fluorescent protein) was constructed with a cytomegalovirus (CMV) promoter while HD-CFTR used a K18 (keratin 18) promoter. The K18 promoter was chosen to drive CFTR gene expression in differentiated epithelial cells. The CMV promoter was chosen to more broadly allow GFP expression in all cells. These vectors were produced as previously described [26, 29, 32, 33]. Briefly, HD-Ad vectors were amplified by serial passage in 116 cells with NG163 helper virus and were purified by 2 rounds of CsCl density gradient centrifugation. Vector particle numbers were calculated by absorbance at 260 nm [34].

GFP detection with microscope and Flow cytometry

GFP was detected in live cells with a fluorescence microscope (Leica DM IRB) 3 days after transduction with HD-GFP. For flow cytometric analysis, ALI cell cultures were trypsinized and suspended in PBS as a single cell suspension. Cells were fixed with 2% PFA for 10 min and

washed. Cells were analysed on a Becton Dickinson LSR II CFI (SickKids Flow Cytometry Facility).

Western Blot and Fluorescence immune staining

Nasal cells cultures were lysed in modified radioimmunoprecipitation assay (RIPA) buffer (50mM Tris-HCl, 150 mM NaCl, 1mM EDTA, pH 7.4, 0.2% SDS and 0.1% Triton X-100) containing a protease inhibitor cocktail (Roche, Mannheim, Germany) for 10 min. Proteins were analyzed by SDS-PAGE on 6% Tris-Glycine gels (Life Technologies). Protein was transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA) and incubated in 5% milk to block non-specific background; CFTR bands were detected with anti-CFTR 596 monoclonal antibody at 1:500 dilution (Cystic Fibrosis Foundation Therapeutics), followed by secondary antibody (HRP conjugated anti-mouse IgG, Pierce Biotechnology, Waltham, Massachusetts, USA). The chemiluminescent reaction was captured by Li-Cor Odyssey Fc (LI-COR Biosciences, Lincoln, NE) and analyzed by Image Studio Lite.

For fluorescence immune staining, culture membranes were embedded in O.C.T. and cryosections were incubated in 4% paraformaldehyde (PFA) solution. Immunofluorescent labeling was performed with anti-CFTR monoclonal antibody (24-1, R&D Systems) and CF555 goat anti-mouse secondary antibodies (Biotium, MT-MCD [26]). Microscope images were taken under an inverted microscope using Nikon digital camera and Quorum spinning disk confocal system with an Olympus IX81 inverted microscope. Images were processed by Velocity 6.3 software.

Ussing chamber

Primary nasal epithelial cells were grown on transwells and studied in a non-perfused Ussing chamber (Physiologic Instruments, San Diego, CA). Cells expressing mutant CFTR were transduced as described above. Where indicated, cells were treated with either 0.1% DMSO or the CFTR modulator 3µM VX-809, 48h before the experiments at 37°C. The buffer solution (126 mM NaCl, 24 mM $NaHCO_3$, 2.13 mM K_2HPO_4, 0.38 mM KH_2PO_4, 1 mM $MgSO_4$, 1 mM $CaCl_2$ and 10 mM glucose) was maintained at pH 7.4 and 37^{o}C and continuously gassed with a 5% CO_2 / 95% O_2 mixture. The transepithelial potential (Vte) was recorded in open-circuit mode and the baseline resistance (Rte) was measured following repeated, brief short-circuit current pulses (1 µA every 30 sec). The results are presented as equivalent transepithelial current (Ieq), which was calculated using Ohm's law. CFTR function was determined after inhibition of the epithelial sodium channel (ENaC) with amiloride (30µM, Spectrum Chemical, Gardena, CA) and following cAMP activation with forskolin (10 µM, Sigma-Aldrich, US) and when indicated 1 µM VX-770. CFTR activity was confirmed as Ieq difference following CFTR inhibition with $CFTRInh_{-172}$ (10 µM, EMD Millipore Corp. US) [30, 35].

Membrane potential assay

Nasal cells were grown on 24 transwell plates and 30 min before the experiment, HBSS solution (Multicell) was added to the basal side and the blue membrane potential dye dissolved in chloride-free buffer (150 mM NMDG-gluconate, 3 mM potassium gluconate, 10 mM HEPES, pH 7.30, 300 mOsm) was added to the apical side. Transwells were placed in a fluorescence plate reader (SpectraMax i3, Molecular Device) at 37°C (excitation: 530 nm, emission: 560 nm) [31]. After reading baseline fluorescence, CFTR was stimulated with 10µM forskolin (Sigma).

The assay was terminated with 10μM $CFTRinh_{172}$ (Cystic Fibrosis Foundation Therapeutics). Changes in membrane potential were normalized to the point before addition of agonist and the DMSO control response.

Statistical analysis

GraphPad Prism 7.0 soft- ware (San Diego, CA) was used for all statistical analysis. Student's t -tests, One-way/two-way ANOVA were conducted as appropriate, and p -values < 0.05 were considered significant. Data with multiple comparisons were assessed using Tukey's multiple-comparison test with $\alpha = 0.05$.

Results:

Deficient CFTR function in CF cell cultures is restored with the introduction of a small proportion of healthy control cells.

Nasal cells from CF patients bearing nonsense mutations showed no CFTR protein on Western blot and no CFTR function in Ussing chamber studies. Treatment with VX-809/VX-770, which is designed to augment p.Phe508del-CFTR function, had no effect on CFTR function or protein quantity in these cells (**Figure 1**).

An alternate approach to overcome the basic CFTR defect, particularly for class I or nonsense mutations, is gene replacement therapy. We first sought to determine how much correction (i.e. what proportion of cells) would be needed to see a change in CFTR function in our airway cell model. To test this, we performed experiments in which we mixed CF cells with an increasing number of non-CF cells and measured CFTR protein and CFTR function. Assessment of protein

abundance showed an increase in core-glycosylated (mature) CFTR protein with increasing proportion of non-CF cells (**Figure 2A**). This increase in protein abundance was paralleled by an increase in CFTR function (**Figure 2B&C**). While there was a trend seen in forskolin induced current, the values were not statistically different. However, for CFTR172inh sensitive current, 3% (1/32) of non-CF cells resulted in 32% of the CFTRinh172-sensitive CFTR function seen in a culture composed of 100% non-CF cells (healthy control); 6.25% (1/16) of non-CF cells resulted in a level of CFTRinh172-sensitive CFTR function that was not significantly different from that seen healthy control cells. This suggests that a small number of functional cells are required in a population of cells to impart physiologically relevant CFTR current.

HD-CFTR results in significant functional CFTR that plateaus with higher HD-Ad vector doses

Next, we wanted to evaluate the effectiveness of the HD-CFTR in correcting nasal cells bearing CFTR class I mutations. Transduction of p.Trp1282X/ p.Trp1282X nasal cells with incrementally higher MOI (infectious vector particles per cell) of HD-CFTR led to increased levels of CFTR protein expression (**Figure 3A & B)**. As higher MOI of HD-CFTR were used, CFTR function reached a plateau at an MOI of 12.5 to 25 and did not increase further in Ussing chamber analyses (**Figure 3C & D**). The CFTRinh172-sensitive current was greater than the forskolin induced current suggesting that there was CFTR activity present at baseline in these cells.

To estimate the level of transduction efficiency of our vector, we transduced CF nasal cells with a reporter HD-Ad vector expressing GFP at dose of 50 MOI and at day 14 of ALI culture,

approximately 40-80% of cells were GFP positive (**Figure 4**). We used this dose (50 MOI) to transduce nasal cells bearing class I CFTR mutations since this dose of vector resulted in CFTR function at the plateau range in transduced CF cells (**Figure 3C**). Western blot analysis demonstrated a substantial increase in abundance of mature CFTR protein (band C) which was comparable to the abundance seen in nasal cells from healthy controls (**Figure 5A**). Further the ratio of mature to immature CFTR protein, an indicator of CFTR processing, was similar between HD-CFTR treated CF cells and healthy control cells (**Figure 5B).** Immunofluorescence staining of CFTR in HD-CFTR transduced cells confirmed restoration of CFTR protein to the apical membrane of cells (**Figure 5C**).

HD-CFTR transduction increased CFTR function to levels comparable to (but not above) healthy controls

Since a small proportion of non-CF cells in our mixed cell experiments was sufficient to confer healthy control CFTR functional levels of the entire cell culture, we wanted to evaluate whether HD-CFTR could induce CFTR function exceeding levels seen in healthy control cells. Therefore, we performed transduction experiments in both CF and non-CF primary nasal cells with a vector dose of 50 MOI. With this MOI of HD-CFTR, the CFTR function in transduced CF cells measured by Ussing chamber, was in the range of values seen in healthy control cells (**Figure 6 and supplementary figure 1**) and transduction did not enhance CFTR channel activity in healthy control cells.

HD-CFTR as a means to benchmark *in vitro* CFTR activity

Given that HD-CFTR mediated functional changes did not exceed control values, we explored the possibility of using HD-CFTR to create an upper functional limit or benchmark achievable *in vitro* CFTR rescue on an individual patient basis. For these experiments, we studied cells from subjects homozygous for p.Phe508del -CFTR. HD-CFTR enhanced CFTR protein expression in these cells (**Figure 7A, B**). CFTR corrector VX-809 also impacted CFTR protein expression (**Figure 7C**). Consistent with this enhanced protein expression, both HD-CFTR and VX-809 enhanced CFTR function when compared to vehicle (DMSO or HD-GFP) treated cells **(Figure 7D, E, F & Supp Figure 2&3).** Taking the HD-CFTR values as a surrogate for a target healthy control value (i.e. 100%), VX-809/VX-770 treatment increased CFTR function to 57% of the forskolin inducible current and 31% of the CFTR inhibitor 172 sensitive current that was seen in the HD-CFTR corrected cells.

Discussion:

In this study, we demonstrated the ability for an HD-CFTR to correct CFTR function in airway cells bearing class I CFTR mutations, including nonsense and splicing mutations. We found that only a small portion of wild-type CFTR expressing nasal cells are required to confer healthy control range CFTR activity. Importantly, HD-CFTR treatment did not lead to overexpression of CFTR function, but increasing HD-CFTR doses reached a plateau effect in respect to measurable CFTR function in CF cells and no increase in CFTR function in healthy control cells. This work has some important ramifications.

First, this work suggests that HD-CFTR can result in clinically meaningful correction of CFTR function in subjects with class I CFTR mutations. Our experiments were performed in patient

derived nasal epithelial cell cultures. *In vitro* outcomes in nasal cells and clinical outcomes (lung function) seen with small molecule therapy have been correlated [36]. There are a number of factors that contribute to variability in both nasal cell outcomes and lung function measures, and thus demonstrating an *in vitro* response in nasal cells does not guarantee a clinical response in the individual who donated the nasal cells for study. However, the link between *in vitro* nasal cell outcomes (current changes with drug) and lung function changes with the same drug at the individual and population level speak to the clinical relevance of this model system and in part have led the FDA to justify label extension of ivacaftor to CFTR mutations demonstrating positive *in vitro* findings [37].

Our study also provides some sense about the transduction efficiency required for CFTR functional correction. We noted significant improvement in CFTR-induced transepithelial currents *in vitro* with less than 6% of cells expressing CFTR. One limitation of this work is that we did not analyze the ratio non-CF to CF cells after 2 weeks of ALI culture. It is possible that CF or non-CF cells have a relative growth advantage and this may lead to altered cell ratios different from the initial seeding ratios. However, the idea that few cells expressing CFTR are required to generate a physiologic CFTR current is consistent with recent findings suggesting that the ionocyte, a rare cell in the airway, is responsible for most of the CFTR activity seen in the airway [38]. While the overall epithelial current may be corrected with patchy CFTR expression, it is not clear that other CFTR related epithelial functions such as mucociliary clearance will also improve. This is the subject of future investigations [39]. Our model also allows for testing the longevity of gene expression since nasal epithelial cultures maintained in laboratory conditions can survive for more than a year [40].

In our model, tight junctions were transiently disrupted with EGTA to allow the adenoviral vector to access more receptors (found on the basolateral surface of epithelial cells). *In vivo*, we have also used 0.01% of lysophosphatidylcholine to enhance transgene transduction efficiency [23]. While disruption of the epithelial barrier may increase the risk of adverse effects (such as infiltration of micro-organisms), our large animal (pig) models tolerated this procedure well and, in the future, strategies could be employed to mitigate these risks (e.g. coadministration of antibiotics).

While replicating many aspects of the human airway, our epithelial cell model does not include an adaptive immune response or innate immune cells (neutrophils, macrophages). This is an important consideration as the lung immune response may impact vector uptake [16, 41]. This is a consideration for any viral vector. Recently, non-viral, nanoparticle based delivery methods have shown efficacy *in vitro* and in mouse models for generating CFTR transgene expression [42]. This is noteworthy as non-viral vectors have traditionally been much less efficient when compared to viral vectors however they offer the advantages of reduced pathogenicity, low cost and ease of production [43]. Although we did not incorporate an animal model in this work, we have previously examined *in vivo* models to test HD-Ad delivery. With transient immunosuppressant administration, we have shown that host inflammation and immune reactions can be significantly reduced and long term transgene expression and vector redelivery can be achieved in mouse lungs [19]. In our pig model, a bronchoscope guided vector delivery method [23] resulted in a transduction efficiencies of up to 20% in the large and small airways of the pig lung. In our future work, vector delivery with nebulization [17, 44, 45] can be used to assess the CFTR expression and function in the lung. Future preclinical testing of this vector in animal models, such as pigs, will be useful.

Second, this work addresses a concern that CFTR gene therapy may inadvertently result in excessive CFTR function [46]. CFTR gene expression is tightly regulated temporally and spatially [47, 48]. Previous work has highlighted that over-expression of CFTR may impact cell proliferation and differentiation and importantly, may have impacts on non-epithelial cells as well [49-52]. We previously demonstrated that CFTR transduction of basal cells does not negatively impact their differentiation [29]. Here we show that exogenous CFTR expression introduced by our vector, did not enhance CFTR function in non-CF cells. In cells bearing nonsense CFTR mutations, there was a non-linear relationship between transduced CFTR protein levels and CFTR function; thus, a small amount of CFTR protein led to a large functional correction that plateaued as protein levels increased. This suggests that the exogenous CFTR delivered by this vector is regulated and would not be predicted to result in excessive CFTR activity *in vivo*. Given the role of CFTR in regulating airway surface liquid volume and pH, this is an important consideration. One limitation with our methodology is that we used a single dose of forskolin to stimulate CFTR. Ideally, testing a broad range of forskolin doses would have allowed for better discrimination of functional responses relative to protein amount as has been done previously [53]. However, we were concerned with maximal responses and in that regard, using a saturating concentration of forskolin does provide us with an estimate of the maximal currents in transduced cells. Previous studies have used a variety of promoters upstream of the CFTR transgene including the SV40 promoter, the Rous sarcoma virus long terminal repeat promoter (Av1Cf2) or the Ad5 major late promoter (Av1Cf1) [49-52]. It is unclear if the K18 promoter in our vector is in part responsible for the plateauing functional response seen. The

transduced CFTR may also be regulated at the protein function (gating) level, though this remains to be proven.

However, current gene therapy strategies have not reached an efficiency level where excess transgene function is a primary concern and experiments remains focused on trying to enhance suboptimal gene expression. Thus, a pertinent outcome of our work is that, this HD-CFTR vector could be used *in vitro* to benchmark CFTR functional responses. While CFTR modulators have shown tremendous success and promise, a challenge for modulator therapies is to quantify CFTR functional responses to various modulation strategies *in vitro* to help inform the potential for an *in vivo* response [36, 53, 54]. Measuring a fold change in current exaggerates changes in cells with low baseline activity. The absolute current seen will vary with cell and culture conditions. Heterogeneity also exists in CFTR function of healthy control tissue challenging the use of these cells as a target for functional responses. Some of the variability in nasal cell CFTR function is understood and is related to the media used, the age of the cells (population doublings) and the amount of CFTR protein expressed. However, some of the variability is not well explained. Our observation that exogenous CFTR did not enhance CFTR function in healthy control cells or above control values in CF cells, led us to explore the use of HD-CFTR as tool to benchmark *in vitro* CFTR function. The idea of restoring a functional CFTR gene to benchmark *in vitro* function is not new. Though benchmarking was not the primary goal, Crane *et al* CRISPR edited CF pluripotent stem cells, differentiated these cells to an anterior foregut phenotype and then measured CFTR function [55]. Incubation with VX-809/770 in unedited p.Phe508del cells restored a proportion of the function seen in edited cells. Our approach offers technical and financial advantages and allows differentiated nasal cells to be

studied. Thus, this vector may be used for CF care by providing a means to objectively compare different strategies for a given patient over time.

In summary, we have tested the effects of a helper-dependent adenoviral vector expressing CFTR in patient derived nasal epithelial cell cultures. We demonstrate that this vector can result in restoration of CFTR function to a clinically relevant level, that this function appears to be regulated and that these vectors may be used to provide an *in vitro* benchmark for testing drug responses.

Figure legends:

Figure 1. Nasal cells from patients homozygous for W1282X lack functional CFTR

(A) Representative Western blot of CFTR protein expression in nasal epithelial cell cultures with p.Trp1282X-CFTR. Positive control: WT-CFTR HBE cell line; Ladders: protein marker ladder; Negative control: KO-CFTR HBE cells; DMSO: vehicle control; VX-809: cells treated with CFTR modulator 3μM VX-809 for 48 hours. CNX: calnexin (as a loading control. **(B)** Representative tracings from Ussing chamber experiment. First tracing shows cells treated with DMSO control, second tracing shows cells treated with 3 μM VX-809 for 48 hours and acutely with VX-770. Amil denotes Amiloride; Fsk denotes forskolin; CFTRinh denotes CFTR inhibitor 172. **(C)** Bar graph shows the forskolin stimulated transepithelial current in nasal cells from p.Trp1282X/ p.Trp1282X donors, treated *in vitro* with 3 μM VX-809 for 48 hours and acutely with VX-770 or DMSO vehicle control. n=5; ns = no significant difference by student paired t-test.

Figure 2. CFTR protein and function in cultures of mixed CF and non-CF cells.

(A) CFTR protein detected by Western Blot. Healthy control and p.Trp1282X/ p.Trp1282X CFTR cells were mixed in the ratio as indicated and cultured on ALI for 2 weeks. n=3. CF cells from the same donor and healthy control (non-CF) cells from 3 different individuals were used in the experiments. Total cells seeded on insert were held constant; fraction indicates the proportion of healthy control cells. CNX: calnexin, as a sample loading control. Bar graphs show summary of CFTRInh172-induced current changes measured in Ussing chamber all in presence of/after amiloride after addition of **(B)** forskolin and **(C)** subsequent ΔIeq after addition of CFTR

inhibitor 172. Statistical test was done using one-way ANOVA. p value is to compare with healthy control cells; "ns" means no significant difference.

Figure 3. Dose response of HD-CFTR vector to CFTR protein expression and function. **(A)** Representative Western-blot of CFTR protein expression three days after HD-CFTR transduction of cells from a p.Trp1282X/ p.Trp1282X donor at indicated MOI (6.25 to 100). CNX: calnexin as a loading control. **(B**) Relationship of CFTR protein expression level and HD-CFTR vector dose (MOI). Experiments were repeated 3 times, each with nonsense mutation CF cells from a different patient, n=3. **(C)** Relationship of CFTR function and the dose of HD-CFTR vector. ΔIeq of the transduced cells was measured in an Ussing chamber following amiloride and then forskolin (first panel) and then CFTR inhibitor 172 (second panel). n=3. **(D)** Original traces of Ussing chamber experiments measuring the transepithelial potential difference. Increasing MOI increased the forskolin-induced transepithelial current as well as the $CFTR_{Inh\text{-}172}$ –sensitive currents.

Figure 4. Transduction efficiency of HD-GFP in differentiated primary epithelial cells. **(A)** Representative images are shown. GFP expression from HD-GFP vector in class I CF cells on the ALI culture transwell was detected directly with a fluorescence microscope. Three days after transduction, the control cells (no-vector, upper panels) and HD-GFP vector transduced cells (lower panels) were imaged with bright field (left panel) and green channel (middle panel) at apical view. The right panels show membrane cryotron sections stained with DAPI to display nuclei. Signals were detected directly with green and blue channels. **(B)** Quantification of GFP positive cells with flow cytometry. Both control (no vector) and HD-GFP transduced cells after 3

days of transduction were trypsinized and single cells were prepared from transwells for flow cytometer after fixing with 2% PFA. Data were analyzed with Flowjo. GFP positive gating was based on comparison with fluorescence of no vector treated cells. Left panel shows side scatter versus GFP fluorescence dot plot in no vector cells; middle panel shows side scatter versus GFP fluorescence dot plot in HD-GFP vector treated cells. GFP positive cells were calculated from collected total cells (10,000 cells) and presented as percentage (right panel), n=6.

Figure 5. CFTR protein expression in CF and non-CF cells.

(A) Representative Western blot analysis of CFTR protein expression in CF or healthy control cells treated with no-vector, HD-CFTR or HD-GFP at 50 MOI. The cells from healthy control and CF donors were cultured in air liquid interface for 2 weeks. Western blotting was performed three days post transduction of HD-vectors. Different mutations are indicated on top of the panel. C/(C+B): CFTR protein band C/ CFTR protein band C + band B. CNX: calnexin (as loading control). **(B**) Quantification of CFTR protein expression in cells 3 days post HD-vector transduction in p.Gly542X/ p.Asn1303Lys (n=1) and p.Phe508del/ p.Trp1282X, (n=2) n=3; c.489+1G>T/c.489+1G>T, n=2 p.Trp1282X/ p.Trp1282X, n=3; healthy control, n=3. **(C)** CFTR protein expression pattern from HD-CFTR in differentiated CF primary cells. CFTR protein was detected by immunofluorescence staining with anti-CFTR antibody on ALI cultured p.Phe508del/ p.Trp1282X CF cells treated with no-vector (left) and HD-CFTR (middle left), HD-CFTR treated cells stained without primary antibody (middle right), HBE CFTR knockout cells on submerged culture stained with anti-CFTR antibody as a negative control (right). red: CFTR; blue: DAPI

Figure 6. HD-CFTR vector leads to CFTR *in vitro* function in the healthy control range

CFTR function was determined by Ussing chamber 3 days post transduction. All measurements were performed in presence of amiloride. **(A**) shows the forskolin-induced transepithelial current (ΔIeq FSK) and **(B)** the $CFTR_{Inh\text{-}172}$ –sensitive currents (CFTRinh) following treatment with HD-CFTR or HD-GFP at 50 MOI. No vector cells were also included as controls. ns: no significant difference between healthy control and each of 3 groups of CF cells transduced with HD-CFTR vector. p.Phe508del/ p.Trp1282X group, n=2; c.489+1G>T/c.489+1G>T, n=2; p.Trp1282X/ p.Trp1282X mutation group, n=3; Healthy control group, n=3. Statistical test was done using one-way ANOVA.

Figure 7. Levels of CFTR protein and function in cells homozygous for p.Phe508del treated with HD-CFTR or CFTR modulators.

Cells studied from donor homozygous for p.Phe508del CFTR. (**A**) Representative Western blot and quantification of CFTR protein in ALI cultured cells treated with no vector, HD-CFTR or HD-GFP at 50 MOI 3 days post. **(B)** CFTR protein was quantified with Image Studio Lite. n=3. **(C)** Representative Western blot of cells treated for 48 hours with vehicle (DMSO) or 3 μM VX-809. **(D&E)** CFTR function was determined by Ussing chamber 3 days post transduction. All measurements were performed in presence of/after amiloride. First panel shows ΔIeq after forskolin and second panel shows ΔIeq after subsequent addition of CFTR inhibitor 172. CFTR function was enhanced to 57% of the forskolin inducible current (D) and 31% of the CFTR inhibitor 172 sensitive current (E) with VX-809 when compared to HD-CFTR **(F)** Membrane potential assay was performed to forskolin and CFTRInh172 responses three days post transduction of HD-vectors.

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Figure 1

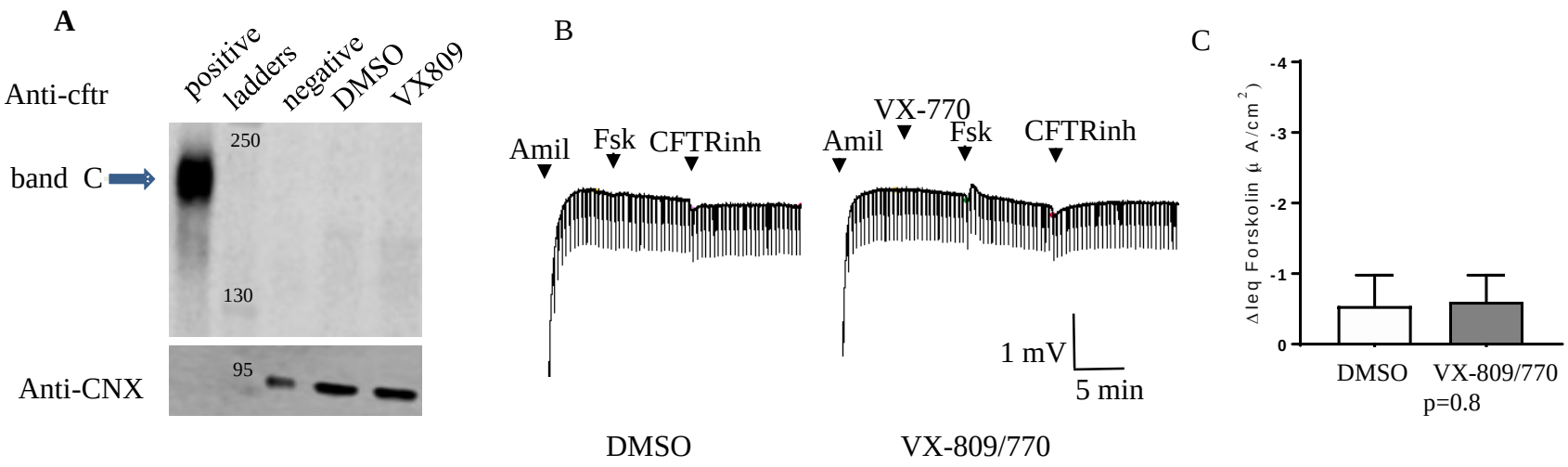
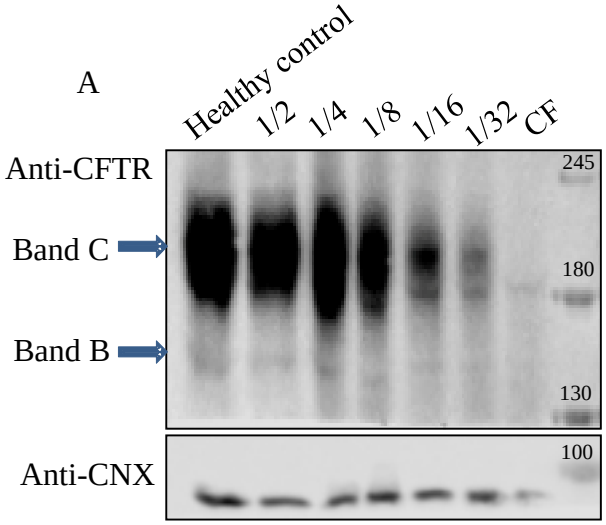
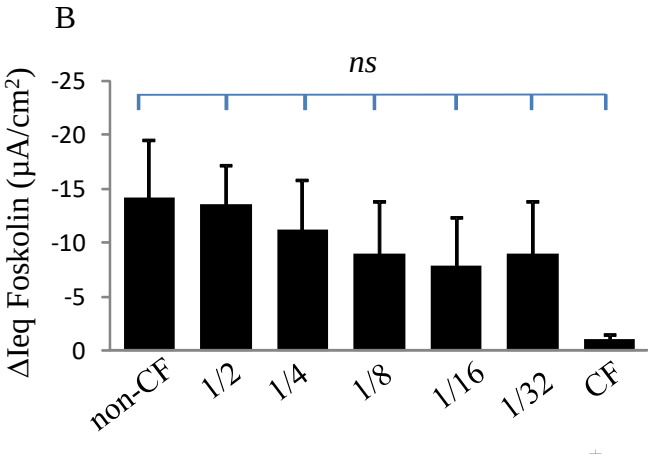

A
positive ladders
negative
DMSO
VX809
Anti-cftr
band C
250
130
Anti-CNX
95
B
Amil
Fsk
CFTRinh
VX-770
Amil
Fsk
CFTRinh
1 mV
5 min
DMSO
VX-809/770
C
Δ Ieq Forskolin (μ A/cm²)
-4
-3
-2
-1
0
DMSO
VX-809/770
p=0.8

Figure 2


A
Healthy control
1/2
1/4
1/8
1/16
1/32
CF
Anti-CFTR
Band C
Band B
245
180
130
Anti-CNX
100


B
ns
ΔIeq Foskolin (μA/cm²)
-25
-20
-15
-10
-5
0
non-CF
1/2
1/4
1/8
1/16
1/32
CF

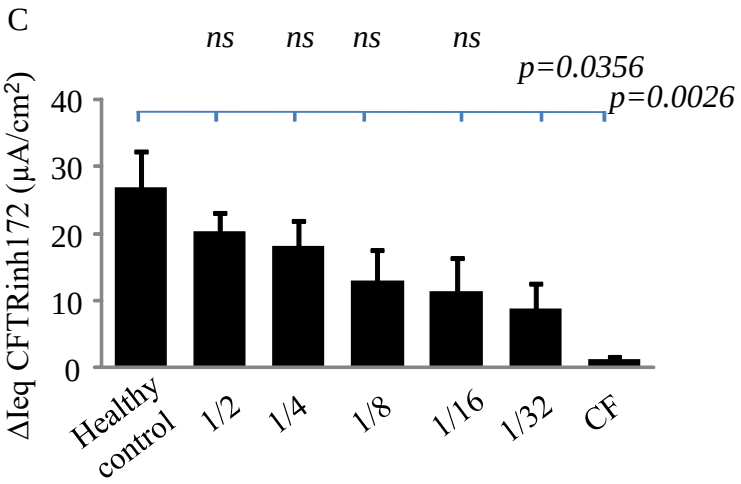

C
ns
ns
ns
ns
p=0.0356
p=0.0026
ΔIeq CFTRinh172 (μA/cm²)
40
30
20
10
0
Healthy control
1/2
1/4
1/8
1/16
1/32
CF

Figure 3

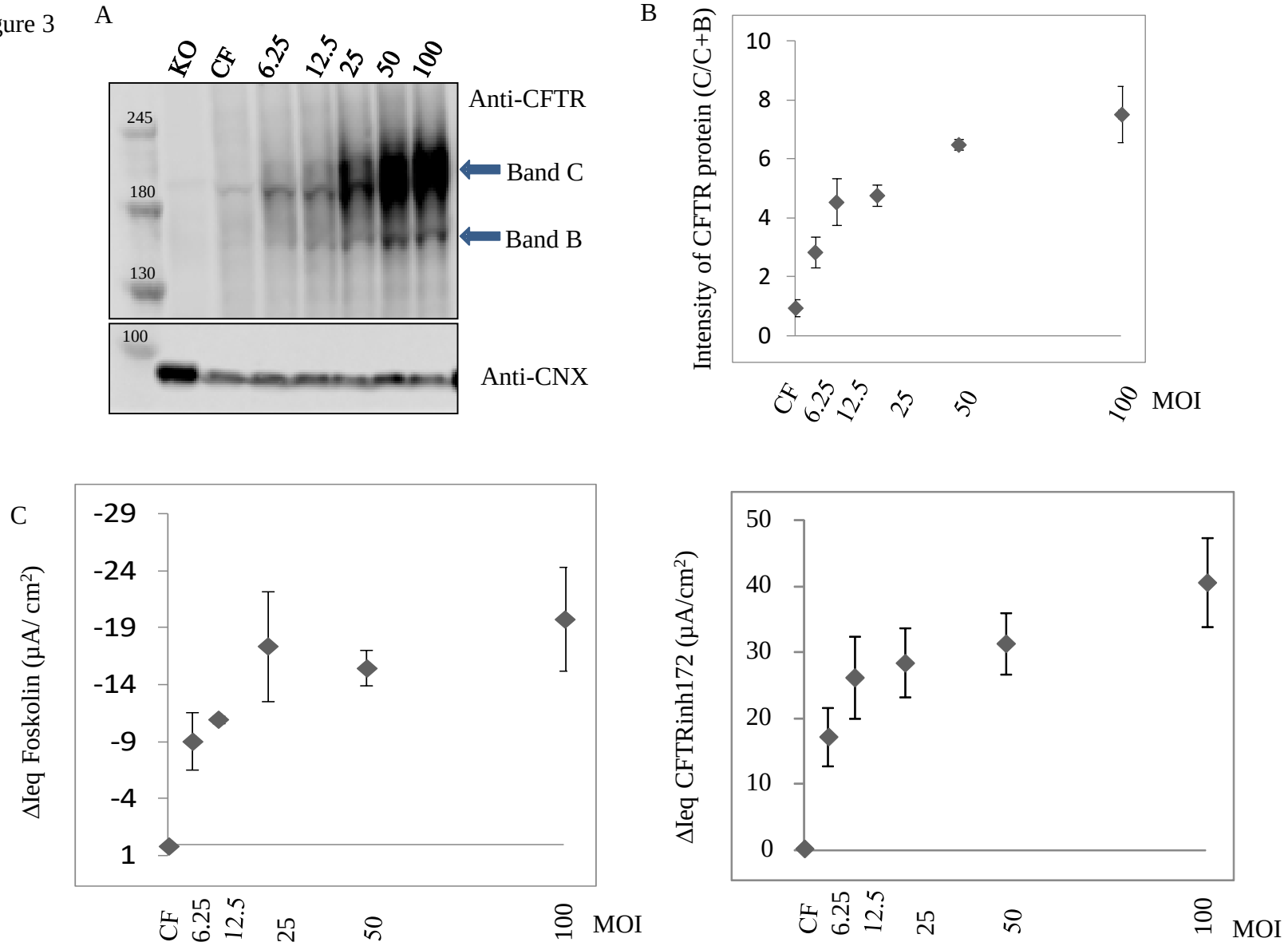
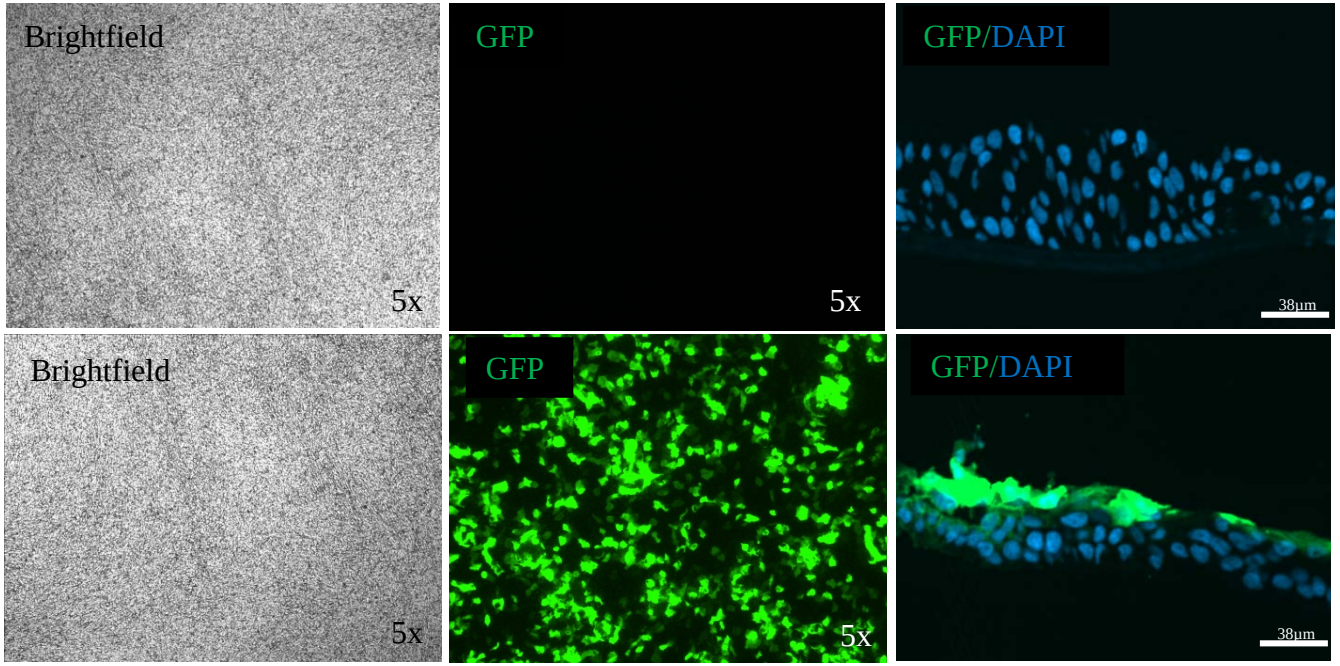

A
KO
CF
6.25
12.5
25
50
100
245
180
130
Anti-CFTR
Band C
Band B
100
Anti-CNX
B
Intensity of CFTR protein (C/C+B)
10
8
6
4
2
0
CF
6.25
12.5
25
50
100
MOI
C
ΔIeq Foskolin (µA/ cm²)
-29
-24
-19
-14
-9
-4
1
CF
6.25
12.5
25
50
100
MOI
ΔIeq CFTRinh172 (µA/cm²)
50
40
30
20
10
0
CF
6.25
12.5
25
50
100
MOI

Figure 4

A


Brightfield
GFP
GFP/DAPI
5x
5x
38µm
No vector
Brightfield
GFP
GFP/DAPI
5x
5x
38µm
HD-GFP

B

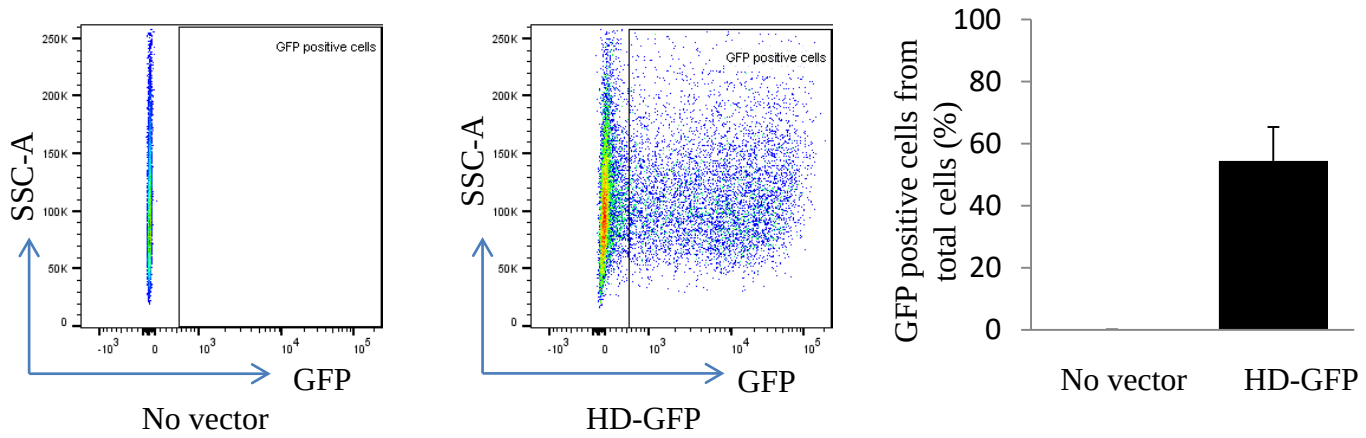
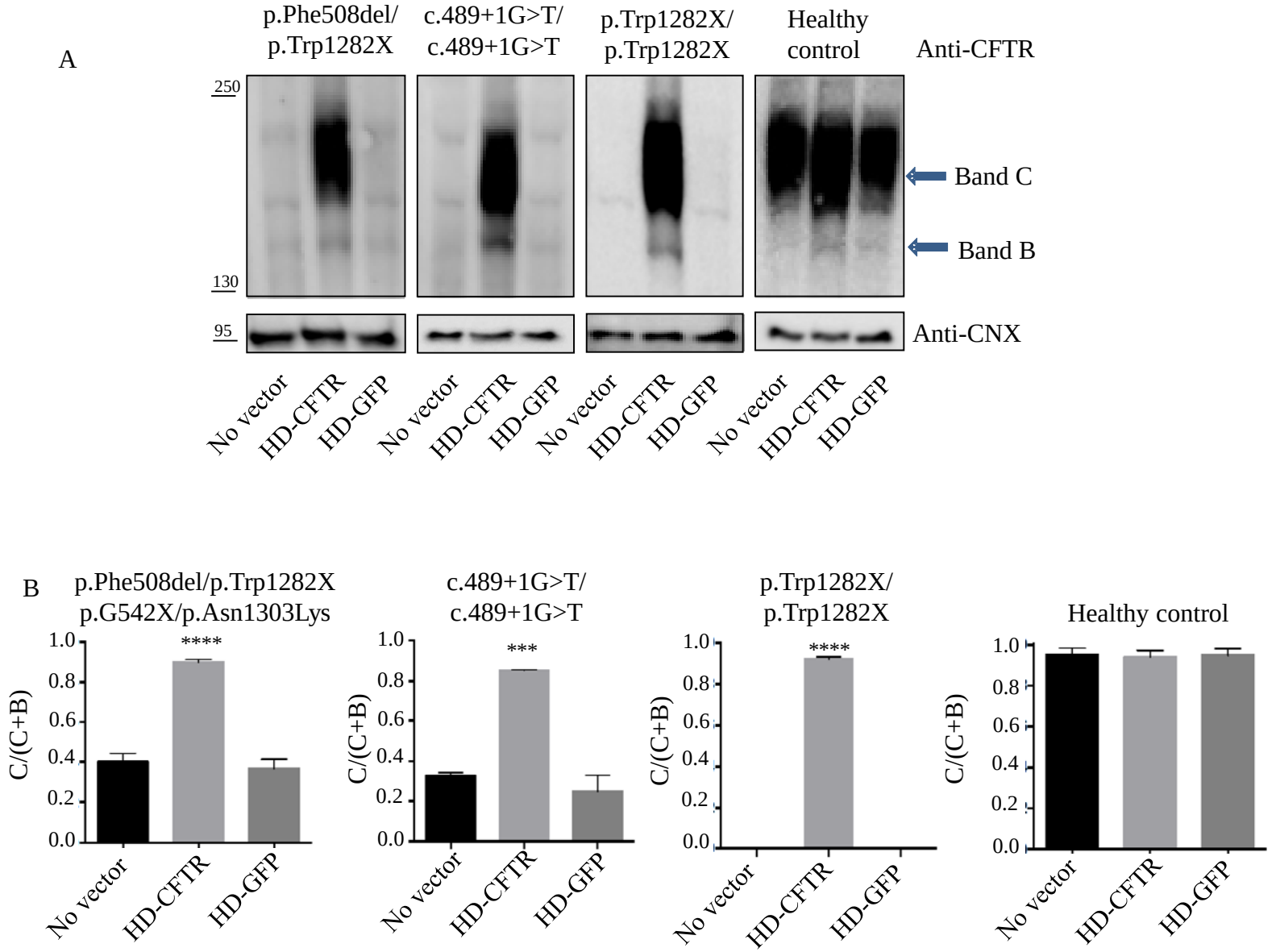
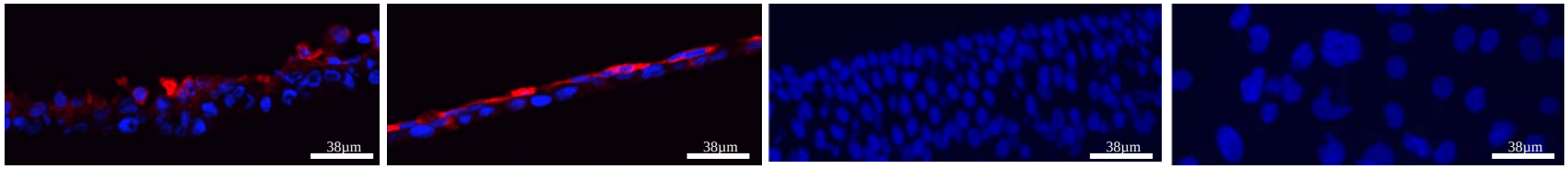

GFP positive cells
SSC-A
GFP
No vector
GFP positive cells
SSC-A
GFP
HD-GFP
GFP positive cells from total cells (%)
100
80
60
40
20
0
No vector
HD-GFP

Figure 5


A
p.Phe508del/ p.Trp1282X
c.489+1G>T/ c.489+1G>T
p.Trp1282X/ p.Trp1282X
Healthy control
Anti-CFTR
250
130
95
Band C
Band B
Anti-CNX
No vector
HD-CFTR
HD-GFP
B
p.Phe508del/p.Trp1282X
p.G542X/p.Asn1303Lys
c.489+1G>T/ c.489+1G>T
p.Trp1282X/ p.Trp1282X
Healthy control
C/(C+B)
1.0
0.8
0.6
0.4
0.2
0.0
****
***
****
No vector
HD-CFTR
HD-GFP

C



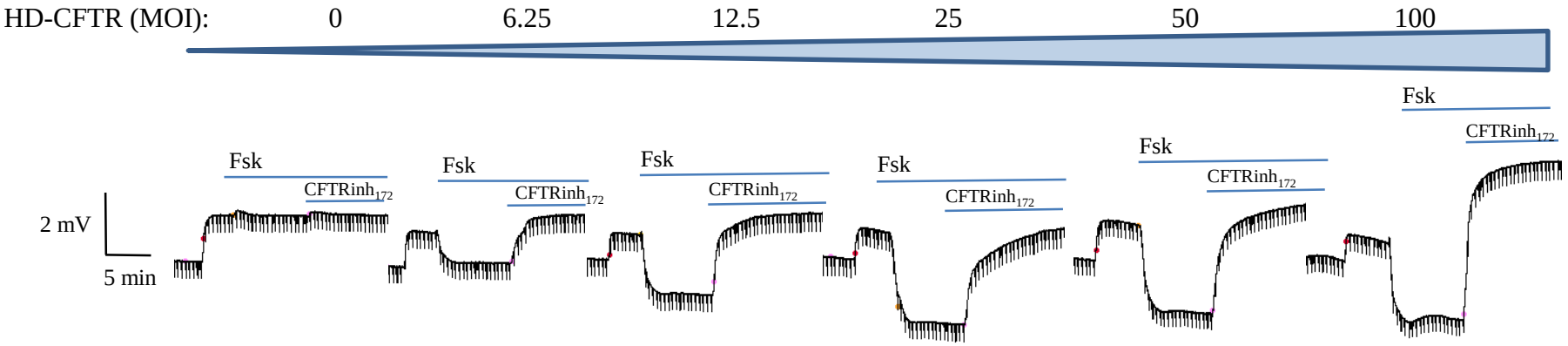
No vector

HD-CFTR

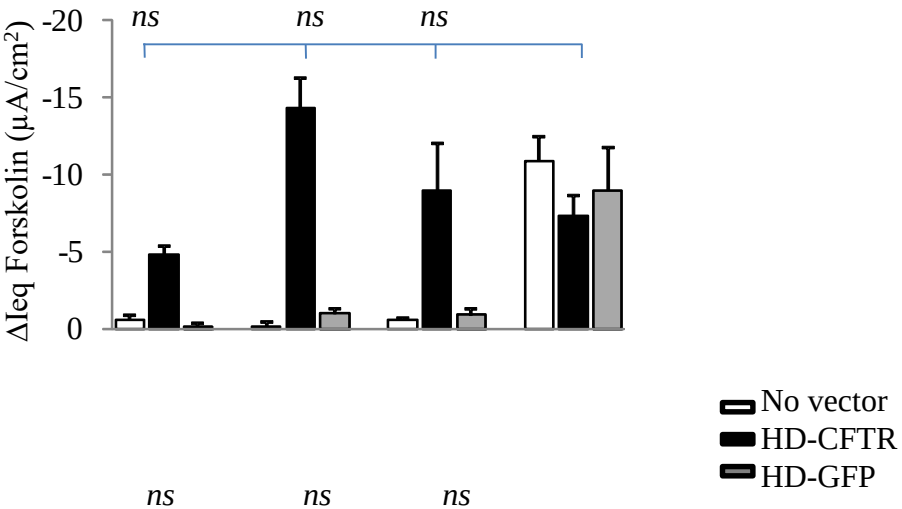
HD-CFTR, and secondary Ab only

CF knockout cells, stained with anti-CFTR Ab

D



A


ns
ns
ns
-20
-15
-10
-5
0
ΔIeq Forskolin (μA/cm²)
No vector
HD-CFTR
HD-GFP

B

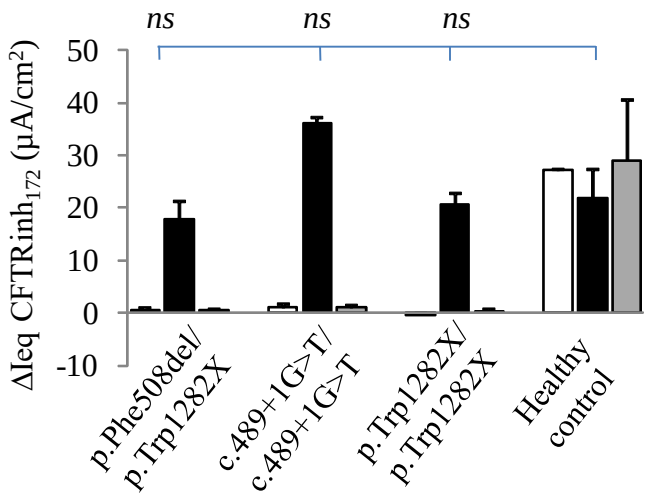

ns
ns
ns
50
40
30
20
10
0
-10
ΔIeq CFTRinh172 (μA/cm²)
p.Phe508del/ p.Trp1282X
c.489+1G>T/ c.489+1G>T
p.Trp1282X/ p.Trp1282X
Healthy control

Figure 6

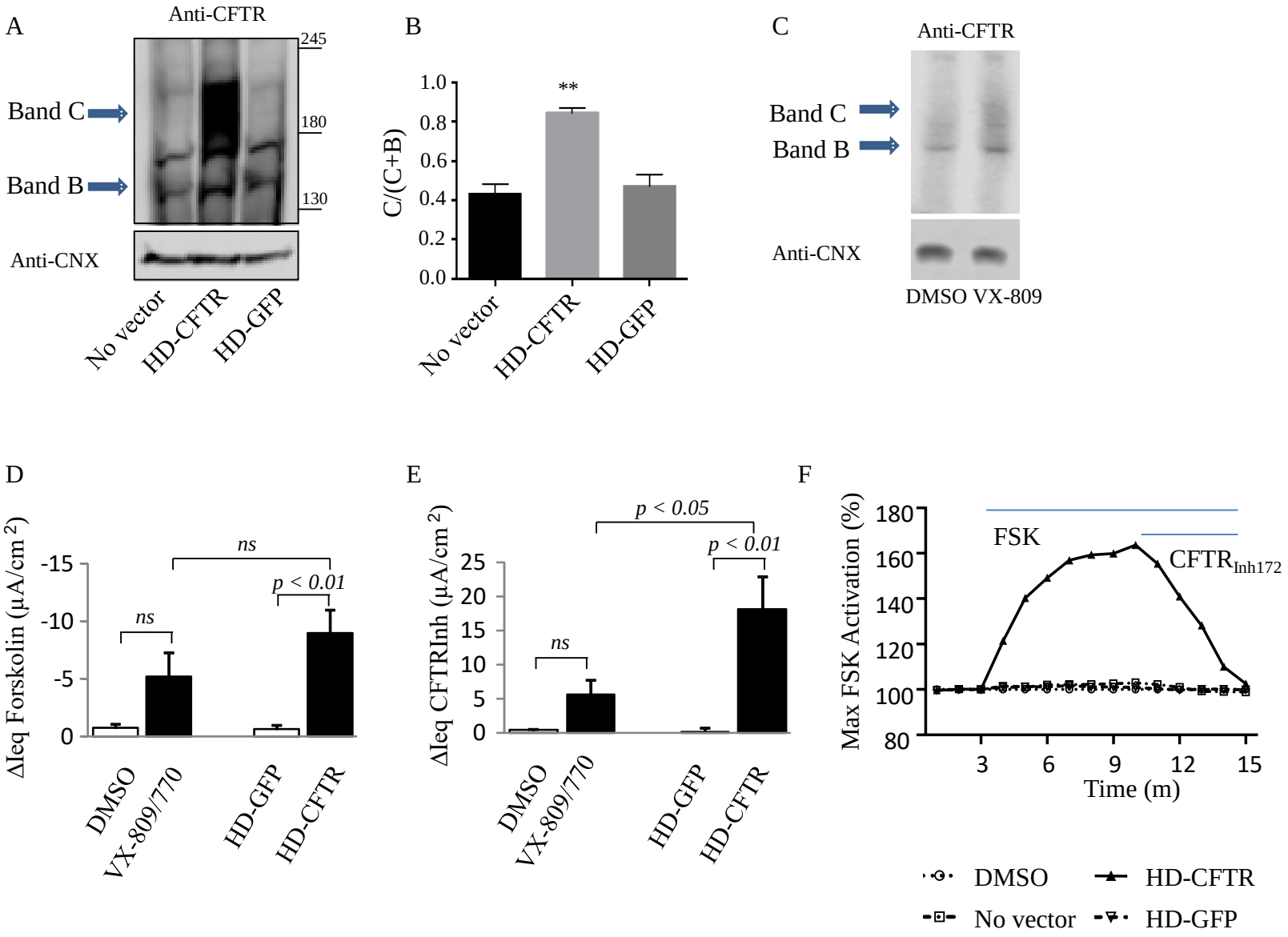
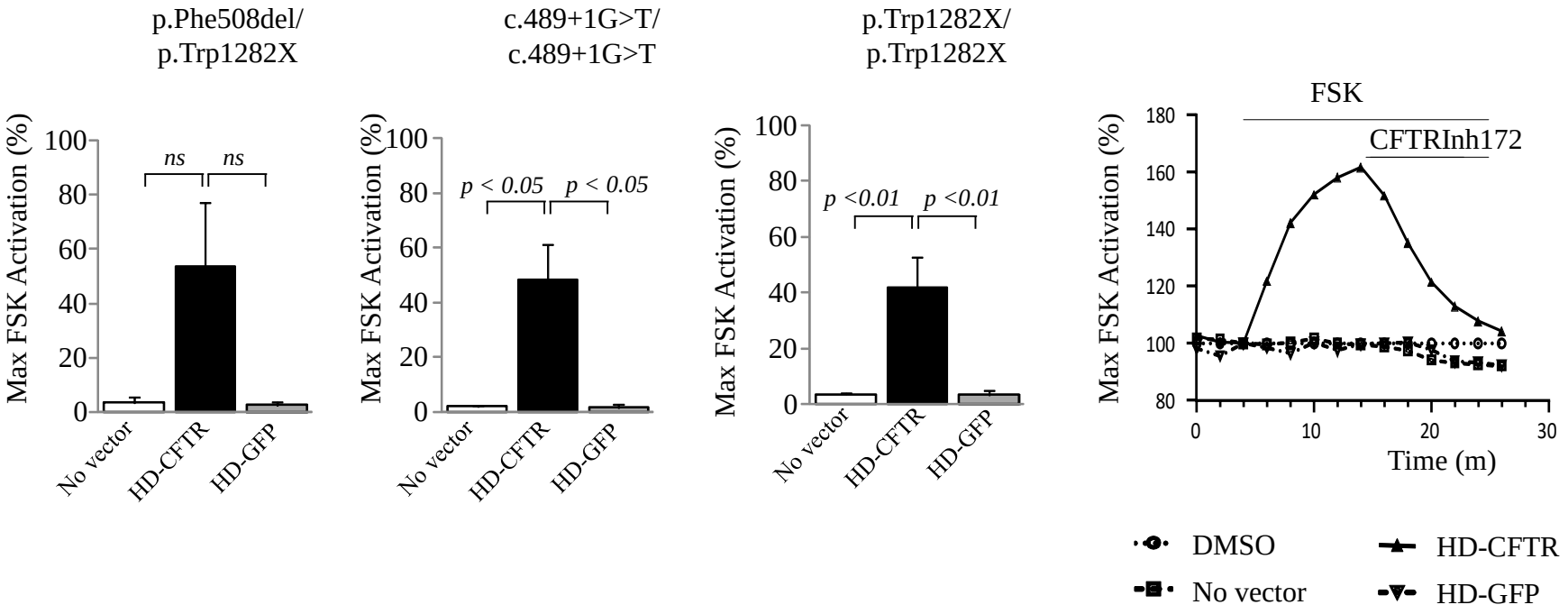

A
Anti-CFTR
245
180
130
Band C
Band B
Anti-CNX
No vector
HD-CFTR
HD-GFP
B
C/(C+B)
1.0
0.8
0.6
0.4
0.2
0.0
**
No vector
HD-CFTR
HD-GFP
C
Anti-CFTR
Band C
Band B
Anti-CNX
DMSO VX-809
D
ΔIeq Forskolin (μA/cm 2)
-15
-10
-5
0
ns
p < 0.01
ns
DMSO
VX-809/770
HD-GFP
HD-CFTR
E
ΔIeq CFTRInh (μA/cm 2)
25
20
15
10
5
0
p < 0.05
p < 0.01
ns
DMSO
VX-809/770
HD-GFP
HD-CFTR
F
Max FSK Activation (%)
180
160
140
120
100
80
FSK
CFTRInh172
3
6
9
12
15
Time (m)
DMSO
HD-CFTR
No vector
HD-GFP

Figure 7

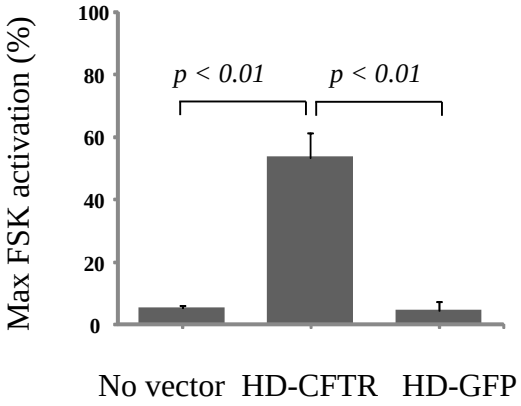
Supplementary Figure 1. CFTR function analyzed in CF primary nasal epithelial cells by membrane potential assay. CF cells, including p.Phe508del/ p.Trp1282X group, n=2; c.489+1G>T/c.489+1G>T group, n=2; p.Trp1282X/ p.Trp1282X mutation group n=3, were cultured on ALI for 2 weeks and treated with no-vector, HD-CFTR or HD-GFP. Membrane potential assay was performed three days post transduction of HD-vectors.

Supplementary Figure 2. CFTR function analyzed in p.Phe508del/ p.Phe508del primary nasal epithelial cells by membrane potential assay. The CF cells were treated with no-vector, HD-CFTR or HD-GFP after 2 weeks ALI culture. n=3

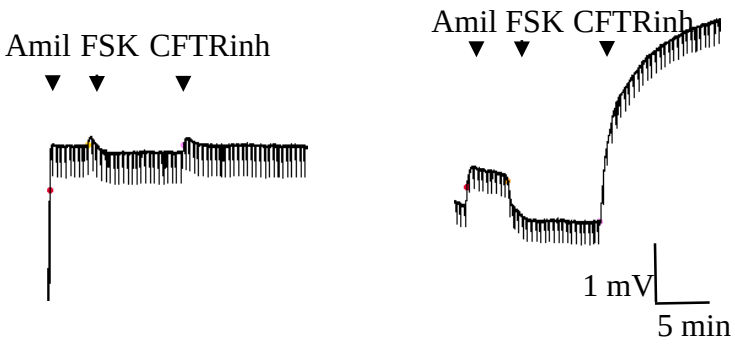
Supplementary Figure 3. Ussing trace of p.Phe508del/ p.Phe508del CF cells corrected with HD-CFTR. The p.Phe508del/ p.Phe508del primary nasal epithelial cells were cultured 2 weeks in ALI and treated with HD-GFP (left panel) or HD-CFTR(right panel). Ussing Chamber experiment was performed after 3 day HD-vector transduction.



Supplementary Figure 1



Supplementary Figure 2



Supplementary Figure 3