Treatment of cystic fibrosis airway cells with CFTR modulators reverses aberrant mucus properties *via* hydration

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CFTR rescue failed to normalise acidic pH, but reduced mucin concentration. Extended rehydration, not pH adjustment, facilitated mucus removal from cell surfaces, suggesting that mucus hydration is the dominant biochemical change in CF airways. https://bit.ly/3iKUzFd

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Abstract

Question Cystic fibrosis (CF) is characterised by the accumulation of viscous adherent mucus in the lungs. While several hypotheses invoke a direct relationship with cystic fibrosis transmembrane conductance regulator (CFTR) dysfunction (*i.e.* acidic airway surface liquid (ASL) pH, low bicarbonate (HCO_3^-) concentration, airway dehydration), the dominant biochemical alteration of CF mucus remains unknown.

Materials/methods We characterised a novel cell line (CFTR-KO Calu3 cells) and the responses of human bronchial epithelial (HBE) cells from subjects with G551D or F508del mutations to ivacaftor and elexacaftor-tezacaftor-ivacaftor. A spectrum of assays such as short-circuit currents, quantitative PCR, ASL pH, Western blotting, light scattering/refractometry (size-exclusion chromatography with inline multi-angle light scattering), scanning electron microscopy, percentage solids and particle tracking were performed to determine the impact of CFTR function on mucus properties.

Results Loss of CFTR function in Calu3 cells resulted in ASL pH acidification and mucus hyperconcentration (dehydration). Modulation of CFTR in CF HBE cells did not affect ASL pH or mucin mRNA expression, but decreased mucus concentration, relaxed mucus network ultrastructure and improved mucus transport. In contrast with modulator-treated cells, a large fraction of airway mucins remained attached to naïve CF cells following short apical washes, as revealed by the use of reducing agents to remove residual mucus from the cell surfaces. Extended hydration, but not buffers alkalised with sodium hydroxide or HCO_3^-, normalised mucus recovery to modulator-treated cell levels.

Conclusion These results indicate that airway dehydration, not acidic pH and/or low [HCO_3^-], is responsible for abnormal mucus properties in CF airways and CFTR modulation predominantly restores normal mucin entanglement.

Introduction

Mutations in the cystic fibrosis transmembrane conductance regulator (CFTR) gene affect mucus-producing organs, notably the lungs and gastrointestinal tract [1]. Defective CFTR protein prevents chloride (Cl^-) and bicarbonate (HCO_3^-) secretion, which alters mucus viscoelastic properties and affects organ function. In the gastrointestinal tract, accumulation of a thick, adherent mucus blocks pancreatic ducts and slows intestinal transit, hindering nutrient absorption and bodily growth [2]. In the lungs, viscous mucus causes obstruction of submucosal gland ducts and small airways, providing a favourable environment for chronic bacterial infection, which is the leading cause of mortality for cystic fibrosis (CF) patients [3, 4].

Mucins, the main polymeric component of mucus, are large, heavily glycosylated proteins that organise in complex multimers to produce the viscoelastic properties of mucus. MUC5AC and MUC5B, the dominant airway mucins, are tightly packed in goblet and secretory cells *via* a low pH/high calcium (Ca^{2+}) environment inside the granules [5]. Upon exocytosis, Ca^{2+} is proposed to be chelated by HCO_3^- in the extracellular milieu, a step considered critical for mucin protein unfolding and the formation of a loose mucus gel [5]. One of the hypotheses proposed for the pathophysiology of CF disease is improper anion (HCO_3^-) secretion, reducing extracellular HCO_3^- concentrations and compromising mucin expansion [6]. Another consequence of low [HCO_3^-] is acidic airway surface liquid (ASL) pH that is proposed to affect electrostatic interactions within the mucus network and plays a role in aberrant mucus properties in CF [7, 8]. In addition to HCO_3^- and pH abnormalities, another prominent hypothesis is that reduced Cl^- secretion leads to airway dehydration, mucin hyperconcentration and mucus stasis [9]. In brief, under this hypothesis, defective CFTR Cl^- transport results in osmotic gradients across the epithelium that favour water hyperabsorption and mucin hyperconcentration [10]. In this dehydrated environment, mucin polymer organisation becomes more entangled with high osmotic pressure, causing the collapse of the periciliary layer and cessation of mucus transport [8, 11–13].

For decades, treatment of CF was reduced to symptom management and infection control with the primary goal of slowing disease progression. In 2012, ivacaftor (VX-770), a CFTR potentiator (a small molecule improving CFTR gating), became the first therapy to address the underlying defect, but benefited only 5% of the CF population who exhibited mild mutations, *e.g.* the G551D gating mutation [14, 15]. Recently, elexacaftor-tezacaftor-ivacaftor (ETI), a triple combination therapy consisting of two corrector compounds (VX-445 and VX-661, small molecules improving CFTR folding) with potentiator VX-770, was shown to efficiently restore function of F508del CFTR, which affects almost 90% of CF patients [16]. Robust clinical benefits from highly effective modulator therapies like ETI demonstrated that CFTR rescue restored airway clearance within days following the initiation of therapy, but the biochemical mode of action of these compounds on pathological CF mucus remains unknown.

To elucidate these biochemical changes, we used wild-type and CFTR-knockout (CFTR-KO) Calu3 cells to demonstrate that lack of CFTR function alone was sufficient to reduce ASL pH and increase the concentrations of both MUC5AC and MUC5B. Using the opposite approach, CFTR function was rescued in CF human bronchial epithelial (HBE) cells with VX-770 and ETI to study the impact of highly effective modulators on ASL pH, mucin concentration, polymeric network organisation and mucociliary transport.

Materials and methods

For full materials and methods see the supplementary materials. Calu3 cells were genetically modified using CRISPR-Cas9 to knock out CFTR [17]. Primary CF HBE cells were obtained from lung transplants from patients heterozygous for G551D or homozygous for F508del mutations, and CFTR function was restored using VX-770 and ETI, respectively [18]. Lack and rescue of CFTR function was confirmed by short-circuit current measurements. Gene expression was assessed using quantitative reverse transcription PCR [19]. Cell washings were analysed using Western blotting to determine the relative changes in MUC5AC and MUC5B content and by light scattering/refractometry (size-exclusion chromatography with inline multi-angle light scattering (SEC-MALS)) for total mucin concentration, as described previously [20, 21]. ASL pH was measured using a microprobe in a temperature/carbon dioxide (CO_2) controlled chamber. Mucociliary transport was measured by video tracking, and mucus biophysical properties were analysed using particle tracking microrheology (PTM), as described previously [12, 22]. Mucus network ultrastructure was analysed *via* scanning electron microscopy (SEM).

Results

Loss of CFTR function affects ASL pH and mucin concentration

CFTR pharmacological inhibition by adding $CFTR_{inh172}$ or GlyH-101 to the apical side of the cells is commonly utilised for bioelectrical measurements. However, this approach is known to hydrate and crosslink the extracellular mucus due to the presence of fluid and/or dimethyl sulfoxide (DMSO), leading to misinterpretation of findings [23]. Hence, the use of a CFTR-KO cell line allowed for direct comparison of cells with functioning and defective CFTR in the absence of pharmacological treatments. We used Calu3 cells, a lung-derived adenocarcinoma cell line normally expressing CFTR and producing MUC5AC and MUC5B, in which the CFTR gene was knocked out using CRISPR-Cas9 to analyse the effects of CFTR loss of function on mucus properties [17].

Measurements of mRNA levels showed a significant knockdown of the CFTR gene in CFTR-KO cells, while MUC5B and MUC5AC gene expression remained unchanged (supplementary figure S1a). Complete knockout of CFTR function was confirmed by absence of forskolin or $CFTR_{inh172}$ responses in transepithelial short-circuit current measurements (figure 1a). One of the characteristics of CF airways is

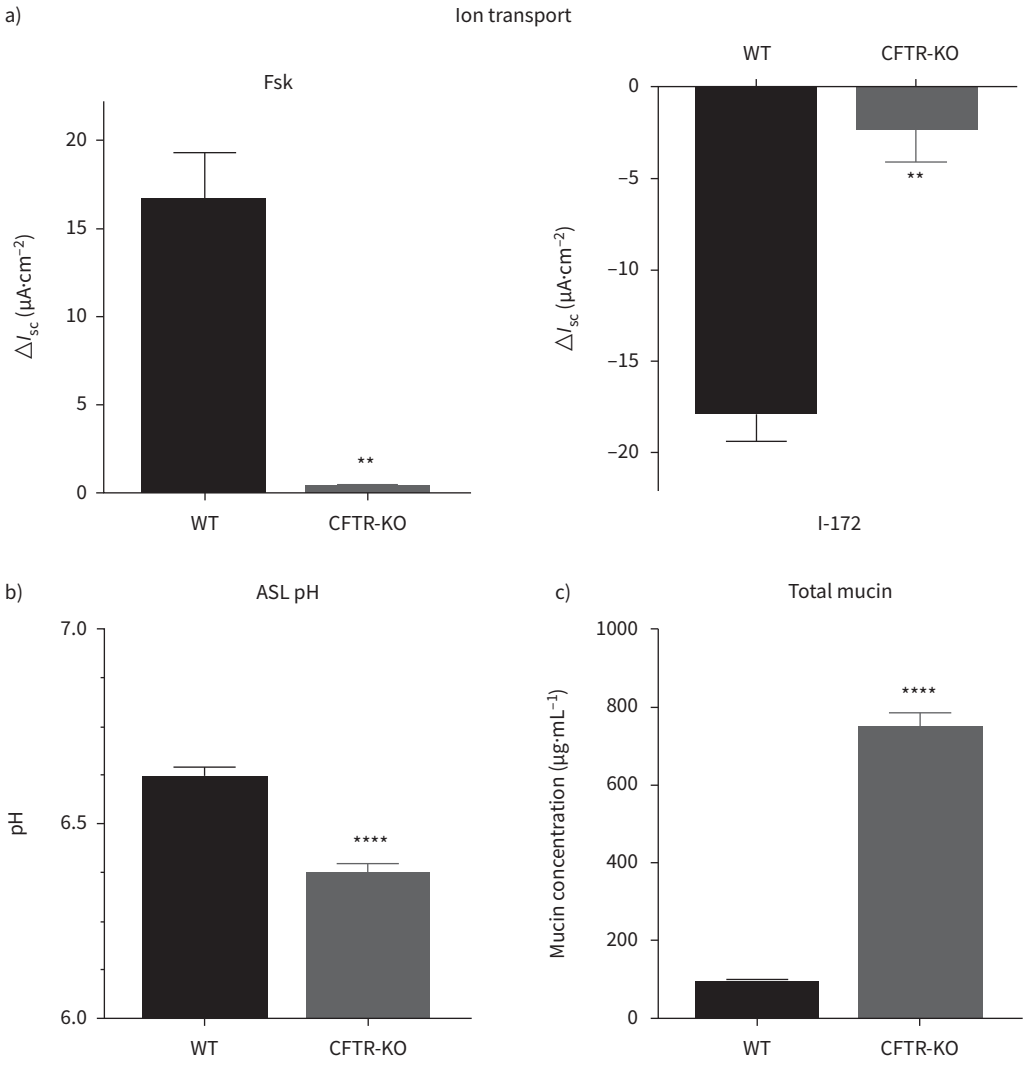

FIGURE 1 Loss of cystic fibrosis transmembrane conductance regulator (CFTR) function affects airway surface liquid (ASL) pH and mucin concentration in CFTR-knockout (KO) Calu3 cells. CFTR gene was knocked out in Calu3 cells *via* CRISPR-Cas9 to generate the CFTR-KO cell line. Wild-type (WT) and CFTR-KO Calu3 cells were analysed *via* different assays. a) Bioelectrical measurements of CFTR-mediated short-circuit current (I_{sc}) for wild-type and CFTR-KO Calu3 cells showing cell response to forskolin (Fsk) and CFTR inhibitor (I-172) (n=3 per group). b) ASL pH of wild-type and CFTR-KO cells measured by microprobe under carbon dioxide and temperature-controlled conditions (n=5 per group). c) Absolute extracellular mucin concentration from wild-type and CFTR-KO apical washings quantified using size-exclusion chromatography with inline multi-angle light scattering. n=4 per group. Error bars indicate SEM. **: p<0.01, ****: p<0.0001 (t-test).

lower ASL pH, which has been shown to impair bacterial killing and affect mucus biophysical properties [7, 22, 24]. An ASL pH measurement assay using a pH microprobe in a temperature- and CO_2-controlled chamber showed that CFTR-KO Calu3 cells exhibited a lower ASL pH than wild-type cells (~0.25 pH unit) and confirmed that CF HBE secretions were significantly more acidic than non-CF controls (~0.2 pH unit) (figure 1b and supplementary figure S2).

To investigate the effect of CFTR function on mucin concentration, 30 min PBS washings from Calu3 cells were analysed using SEC-MALS; CFTR-KO cells showed a 7.7-fold increase in total mucin concentration compared to wild-type cells (figure 1c). In parallel, Western blotting was used to quantify the relative change in MUC5AC and MUC5B content, and CFTR-KO cells showed a 2.4-fold increase in MUC5AC and a four-fold increase in MUC5B signal compared to wild-type cells (figure 2a,b). When observed by SEM, the mucin network ultrastructure was altered in CFTR-KO cultures compared to the

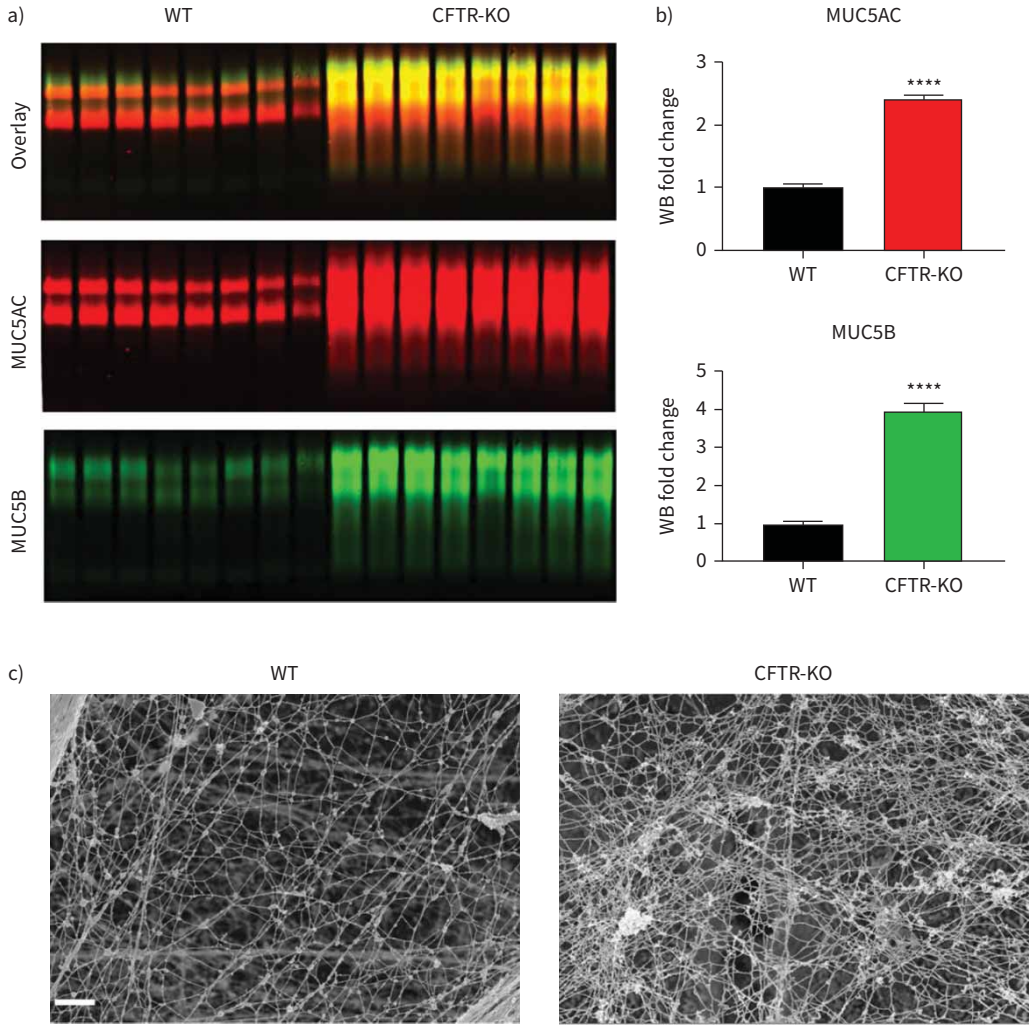

FIGURE 2 Elevated mucin concentration in cystic fibrosis transmembrane conductance regulator (CFTR)-knockout (KO) Calu3 cell supernatant correlates with a tighter mucin network. a) Mucin Western blot of apical washings reveal differences in MUC5AC (red) and MUC5B (green) signals in CFTR-KO compared to wild-type (WT) Calu3 cells. b) Graphs showing average signal intensities of immunoblots of apical washings from CFTR-KO signal intensity normalised to WT for MUC5AC and MUC5B (n=8 per group). c) Representative scanning electron microscope images of a thin layer of extracellular mucus, revealing the network ultrastructure in WT and CFTR-KO Calu3 cultures. Scale bar=1 µm. Error bars indicate SEM. ****: p<0.0001 (t-test).

wild-type meshwork (figure 2c). The CFTR-KO polymeric network appeared more compact and exhibited numerous entanglements. Histological sections revealed the presence of a thicker mucus layer in the CFTR-KO compared to wild-type Calu3 cells, which correlated with a reduced intracellular signal, suggesting intense goblet cell degranulation in response to CFTR dysfunction (supplementary figure S3).

CFTR rescue in G551D HBE cells reverses mucus properties

Ivacaftor (VX-770) has been reported to restore CFTR function in cells with a G551D gating mutation [14]. To assess the effects of CFTR rescue on airway cells *in vitro*, we used primary CF HBE cells heterozygous for the G551D gating mutation to study the effects of the potentiator VX-770 on airway mucus. Bioelectrical measurements confirmed that CFTR function was efficiently restored by VX-770 (figure 3a). Similar to CF Calu3 cells, mucin gene expression was not affected by CFTR rescue (supplementary figure S1b). As shown in supplementary figure S2, ASL pH from CF cultures was slightly more acidic than non-CF cells (6.9 *versus* 7.1). Remarkably, CFTR potentiation did not cause significant alkalisation of the ASL pH (6.89 *versus* 6.92 for DMSO- and VX-770-treated cultures, respectively), but

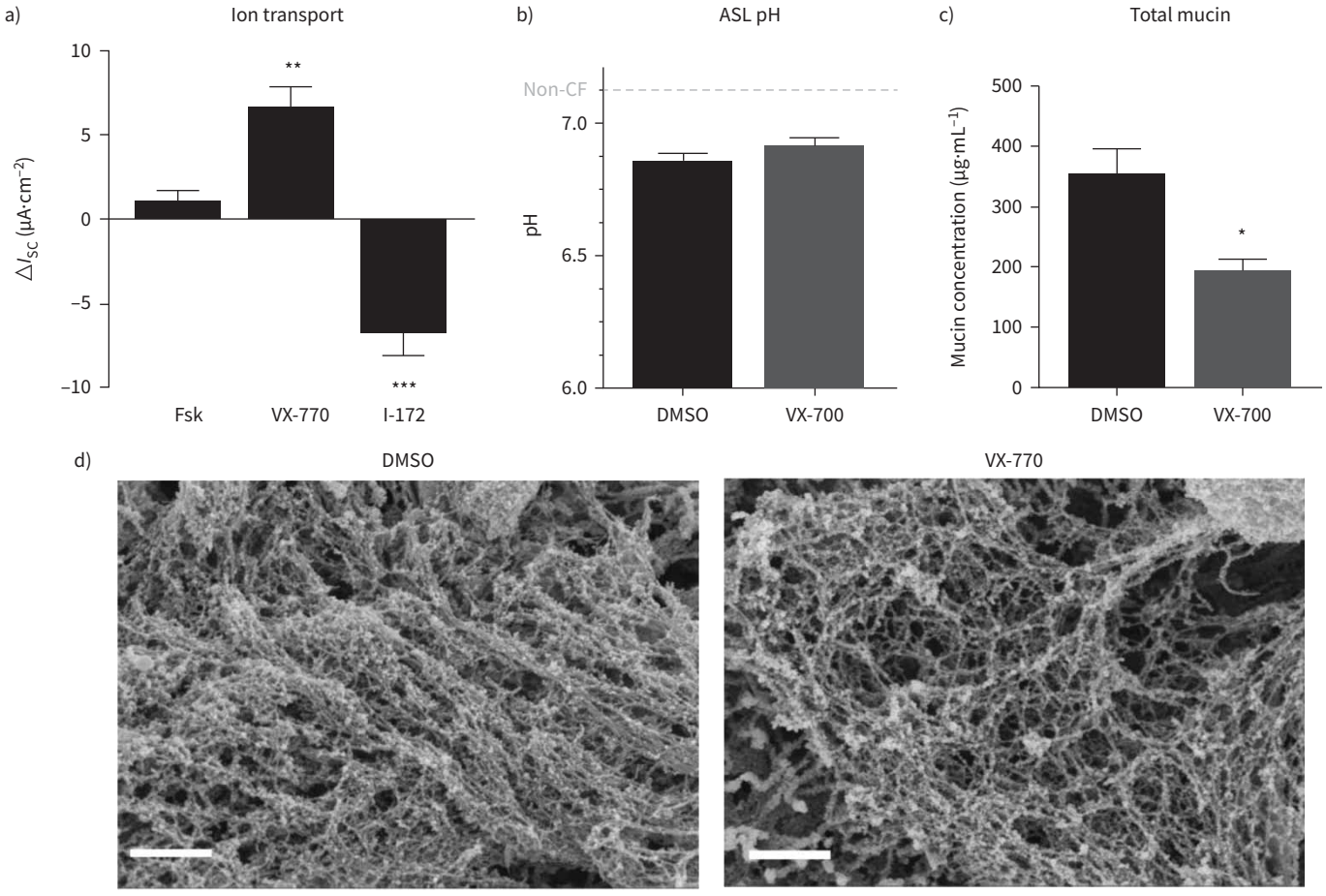

FIGURE 3 Treatment of G551D human bronchial epithelial (HBE) cells with VX-770 reverses aberrant mucus properties. Primary HBE cells with one copy of the G551D mutation were treated with 0.05% dimethyl sulfoxide (DMSO) or 5 μM VX-770 acutely at the time of bioelectrical measurements or daily for 3 days prior to biochemical experiments. a) Bioelectrical measurements of cystic fibrosis transmembrane conductance regulator (CFTR)-mediated short-circuit current (I_{sc}) for G551D HBE cells treated acutely with VX-770 and CFTR inhibitor (I-172) (n=3 inserts per group). b) Airway surface liquid (ASL) pH of DMSO and VX-770-treated cells measured by microprobe under carbon dioxide- and temperature-controlled conditions (n=6 per group). c) Absolute mucin concentration in apical washings as measured *via* size-exclusion chromatography with inline multi-angle light scattering (n=6 per group). d) Representative scanning electron micrographs of the apical mucin network in DMSO- and VX-770-treated G551D cells. Scale bars=1 μm. Error bars indicate SEM. *: p<0.05, **: p<0.01, ***: p<0.001 (t-test).

significantly reduced (~1.8-fold) the absolute concentration of airway mucins in apical washings (figure 3b,c), which correlated with a relaxation of the mucin network (figure 3d). Compared to vehicle-treated cells, the mucus coating VX-770-treated cells displayed a looser appearance with decreased branching and increased mesh size (0.1 *versus* 0.5 μm for the largest pores).

ETI corrects F508del CFTR in vitro *and affects mucus*

Due to limited access to G551D cells and the recent availability of ETI for research, we used F508del HBE cells for further experimentation. In Ussing chamber assays, we confirmed that ETI treatment of F508del HBE cells restored CFTR-mediated short-circuit currents (figure 4a). Consistent with G551D cells, CFTR modulation in F508del cells did not alter ASL pH (6.85 *versus* 6.84 in DMSO- and ETI-treated cultures, respectively), but significantly reduced total mucin concentration in apical washings (figure 4b,c). Mucin mRNA transcript levels remained unchanged following ETI treatment (supplementary figure S1c).

In prior work, it was shown that increases in mucin concentration correlated with increased mucus percentage solids content and reduced mucociliary clearance in CF lungs [11, 25]. Following 3 days of ETI treatment, mucus solids decreased from ~9% to ~3% total solids (or from 8% to 2% organic content) (figure 5a). Mucus stasis was observed in nontreated F508del HBE cells, but following ETI treatment the average mucus velocity increased from 5 to 143 μm·s^{-1} (figure 5b, supplementary videos 1–8). Increased mucus transport correlated with a slight but significant increase in ciliary beat frequency (11.7 *versus*

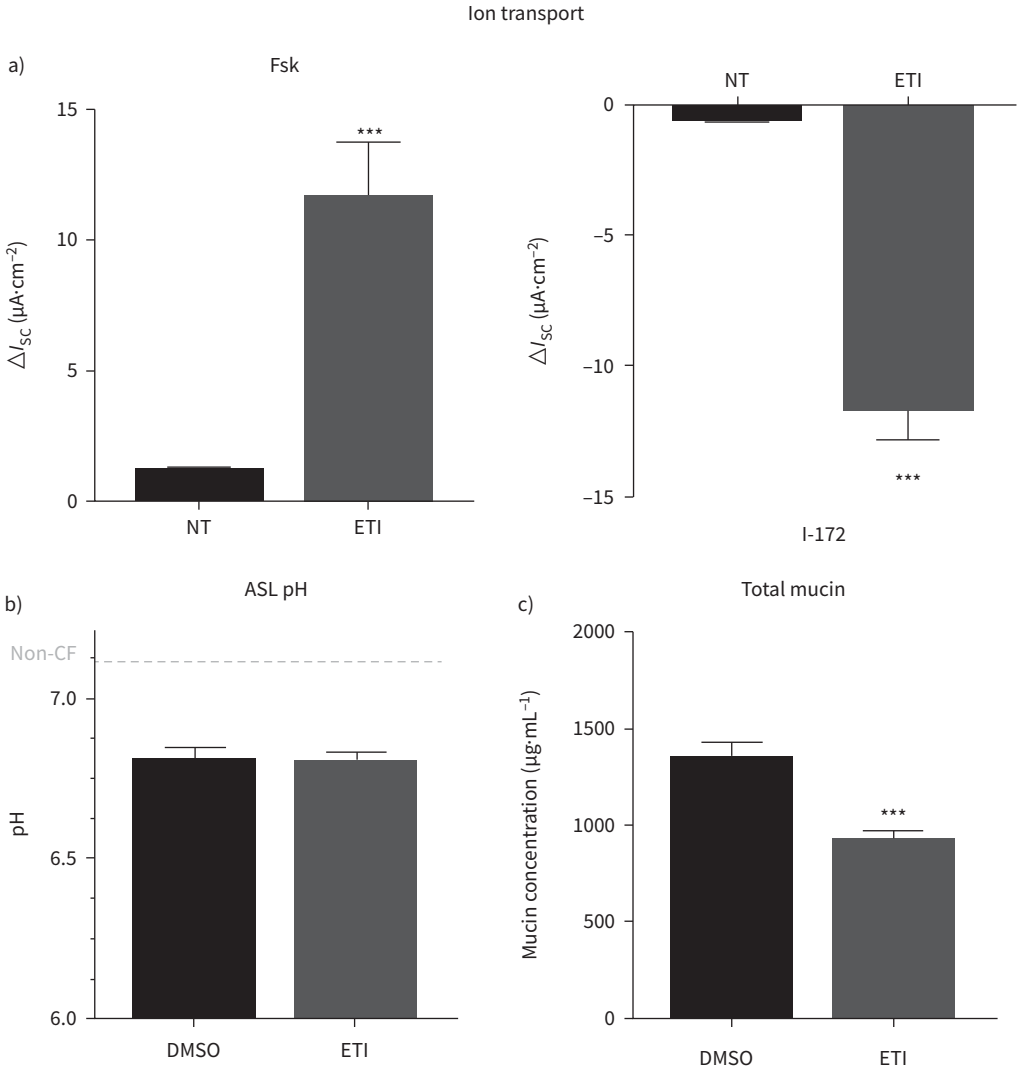

FIGURE 4 Elexacaftor-tezacaftor-ivacaftor (ETI) treatment of F508del cells restores cystic fibrosis transmembrane conductance regulator (CFTR) function and affects mucin concentration, but not airway surface liquid (ASL) pH. Primary human bronchial epithelial (HBE) cells homozygous for F508del were treated for 3 days with 0.06% dimethyl sulfoxide (DMSO) or 3 μM VX-661, 2 μM VX-445 and 1 μM VX-770 (ETI) before biochemical experiments or pre-treated with VX-445 and VX-661 for 2 days then acutely treated with VX-770 at the time of bioelectrical measurements. a) Bioelectrical measurements of CFTR-mediated short-circuit current (I_{sc}) for nontreated (NT) and ETI-treated F508del cells showing cell response to forskolin (Fsk) and CFTR inhibitor (I-172) (n=3 per group). b) ASL pH of DMSO and VX-770-treated cells measured by microprobe under carbon dioxide- and temperature-controlled conditions (n=6 per group). c) Absolute mucin concentration in 30-min apical washings as measured *via* size-exclusion chromatography with inline multi-angle light scattering (n=6 per group). Error bars indicate SEM. **: p<0.01, ***: p<0.001 (t-test).

14.3 Hz in nontreated and ETI groups, respectively) (figure 5c). When examined using SEM, DMSO- and ETI-treated cells revealed striking differences (figure 5d and supplementary figures S4 and S5). In DMSO-treated cells, large sheets of mucus (>200 μm^2) covered the cell surfaces and compressed the cilia. At high magnification, cilia appeared disorganised and flattened, and mucus was seen compressing cilia shafts. In contrast, in ETI-treated cells post-fixative wash, the apical surfaces were nearly devoid of mucus and the cilia were erect and well-organised.

Mucus removal is facilitated by ETI treatment in F508del cells

Apical washings from DMSO- or ETI-treated F508del cells were collected *via* a short (15-min) wash with PBS followed by additional 15-min wash with 1 mM tris(2-carboxyethyl)phosphine (TCEP), a reducing

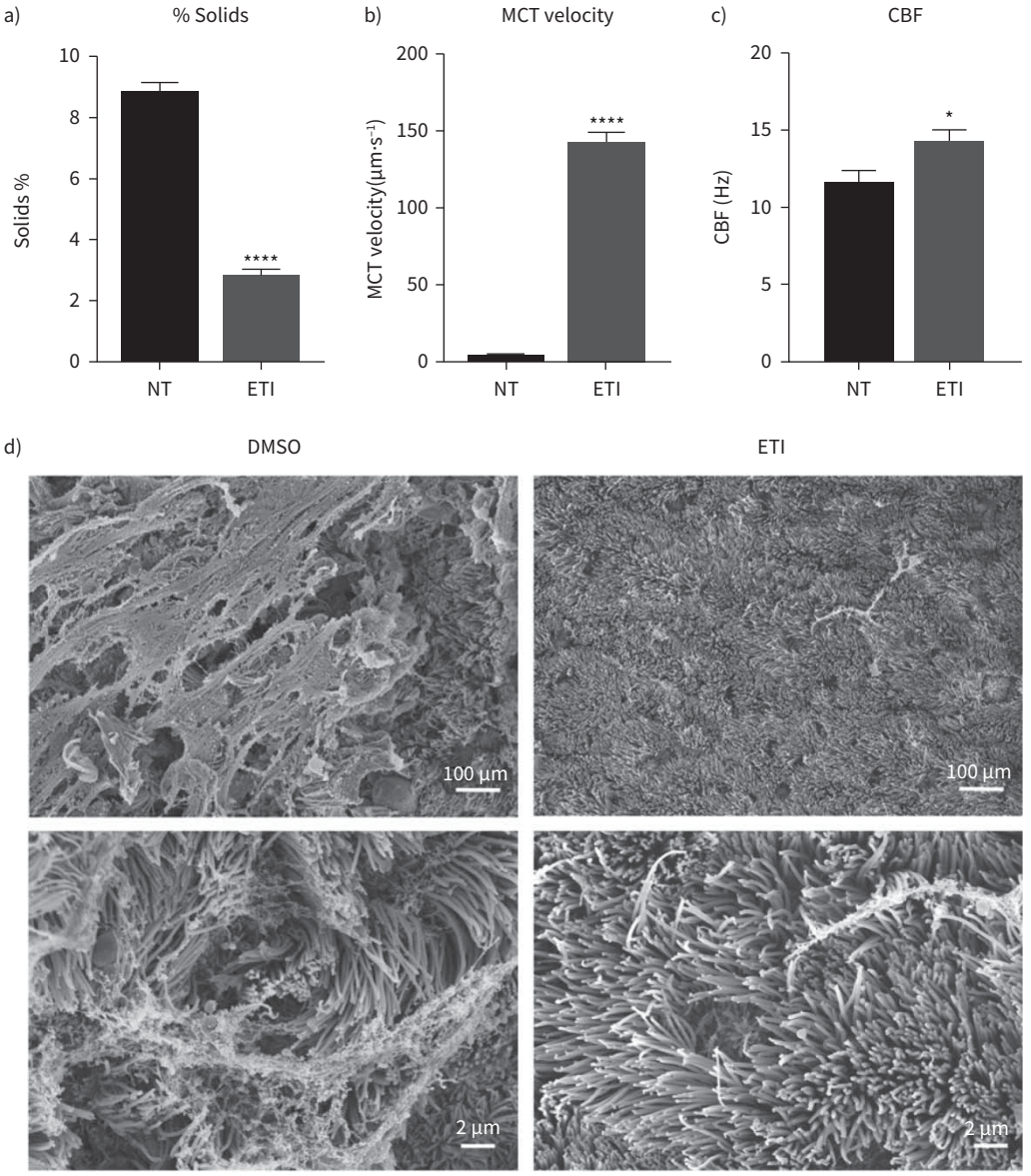

FIGURE 5 Effects of elexacaftor-tezacaftor-ivacaftor (ETI) treatment on mucus percentage solids, mucociliary transport (MCT) velocity, and ciliary beat frequency (CBF) in F508del human bronchial epithelial (HBE) cells. a) Percentage solids of apical mucus was measured as wet/dry ratio before and after 3-day ETI treatment (n=3–6 per group). b) Mucus velocity rates were measured by video tracking before and after a 3-day ETI treatment, n=4 inserts per group. c) CBF was measured by video tracking for non-treated and ETI treated cultures, n=9 inserts per group. d) Representative scanning electron micrographs of dimethyl sulfoxide (DMSO)- or ETI-treated F508del HBE cells showing an apical view of the inserts at low (above) and high (below) magnification power. Scale bars=100 μm and 2 μm. Error bars indicate SEM. *: p<0.05, ****: p<0.0001 (t-test).

agent, to completely remove mucus from the cell surfaces and establish the proportion of residual mucus post-washings [26]. Washings were then subjected to Western blotting (figure 6a). Lane intensity measurements indicated that the short PBS wash removed 33% and 60% more MUC5AC and MUC5B from ETI- *versus* DMSO (vehicle)-treated cells, respectively (figure 6b).

To test whether mucus remained on cell surfaces post-short washing, Western blots were performed on the TCEP washes. Intensity analysis revealed that the majority of the mucus (~62%) remained adhered to the cells in the DMSO group post-PBS wash *versus* ~30% in the ETI group (figure 6b). These results are consistent with the SEM images in figure 5.

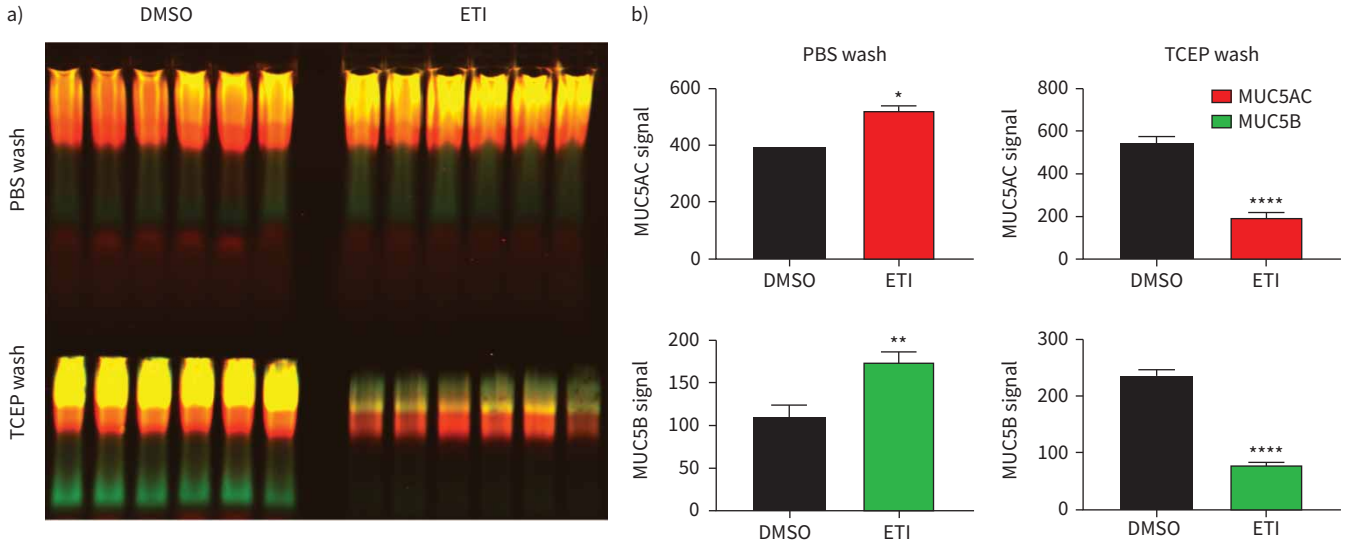

FIGURE 6 Western blot analysis of MUC5AC and MUC5B collection by short washings in dimethyl sulfoxide (DMSO)- and elexacaftor-tezacaftor-ivacaftor (ETI)-treated cells, followed by washings with a reducing agent. Following treatment for 3 days with 0.06% DMSO or 1 µM VX-770, 2 µM VX-445 and 3 µM VX-661 (ETI), the apical surface of F508del human bronchial epithelial (HBE) cells were washed with PBS for 15 min followed by an additional 1 mM tris(2-carboxyethyl)phosphine (TCEP) wash for 15 min. a) Mucin Western blot showing harvested MUC5AC and MUC5B by PBS and additional 1 mM TCEP washings in DMSO- or ETI-treated cells. To stop the reaction, reduced samples were quenched with 1 mM iodoacetamide after 15 min (n=6). b) Graphs showing the intensity analysis of MUC5AC and MUC5B signals for PBS wash and additional TCEP wash. (n=10). Error bars indicate SEM. *: p<0.05, **: p<0.01, ****: p<0.0001 (t-test).

Hydration, but not pH or HCO_3^-, affects mucin recovery in CF cells

To assess the major biochemical change(s) that mediated the efficient wash-induced mucus removal in modulator-treated cells, we washed nontreated F508del HBE cells with different solutions to determine which, if any, solution would result in removal of mucus from cell surfaces similar to ETI-treated cultures. Three of the dominant CF-related hypotheses were tested: acidic airway pH causing new/aberrant mucin interactions; lack of HCO_3^- secretion resulting in mucin compaction; and airway dehydration causing mucin polymer entanglement [1]. To test for the effects of volume, pH and/or [HCO_3^-], PBS wash buffers were delivered neat or alkalised with sodium hydroxide (NaOH) or sodium bicarbonate ($NaHCO_3$) by 0.3 pH units to non-CF levels (figure 1 and supplementary figure S2) and administered for 15 min. To test for mucin disentanglement kinetics, normal PBS and Krebs–Ringer solution (containing 25 mM $NaHCO_3$) were administered for 1 h and 3 h washings, respectively. Mucin Western blot analysis revealed that washing with PBS alone or PBS alkalised with NaOH or $NaHCO_3$ did not normalise mucus removal to ETI levels (figure 7). However, long (1 h) and extended (3 h) washings of CF cultures with PBS alone or buffered with Krebs–Ringer solution normalised both MUC5AC and MUC5B mucin recovery to ETI levels. Extended washes removed 94% of MUC5AC and 83% of MUC5B of the total mucins present on CF cell cultures (supplementary figure S7). Together these results revealed that hydration is sufficient to release mucus in modulator-treated cultures.

To further test whether hydration normalises the biophysical properties of CF mucus, DMSO- and ETI-treated cell washings were analysed by PTM. From the results presented in figure 6, we hypothesised that washes from untreated F508del CF cells harvested only a small fraction of the total mucus, mostly comprised of readily-soluble, non-adherent mucins. In contrast, washes from ETI-treated cultures contained a larger soluble fraction of total mucus (figure 8a,b). PTM measurements of the complex viscosity (η^*) of particles in washes revealed bimodal profiles with low and high viscosity peaks (figure 8c). Using a Gaussian mixture model [27], a hierarchical clustering of bead/particle movements was established for the "thin" (*i.e.* fluid) and "thick" components of mucus. In short-wash DMSO samples, both peaks were in sol-like phases ($G' < G''$), while in ETI samples, the thick peak displayed a gel-like profile ($G' \geq G''$) (figure 8 and supplementary figure S8) [12]. In long-wash samples, the rheological profiles in both control and ETI-treated groups were qualitatively similar and exclusively gel-like, confirming that sustained hydration effected the biophysical properties of control mucus similarly to modulator treatment. In addition, we showed that extended (3 h in Krebs–Ringer buffer) washes showed a similar rheological profile than long (1 h in PBS) washes, revealing two peaks slightly shifted towards the gel-like phase, and that TCEP treatment homogenised and markedly shifted the samples towards a sol-like phase (supplementary figure S9).

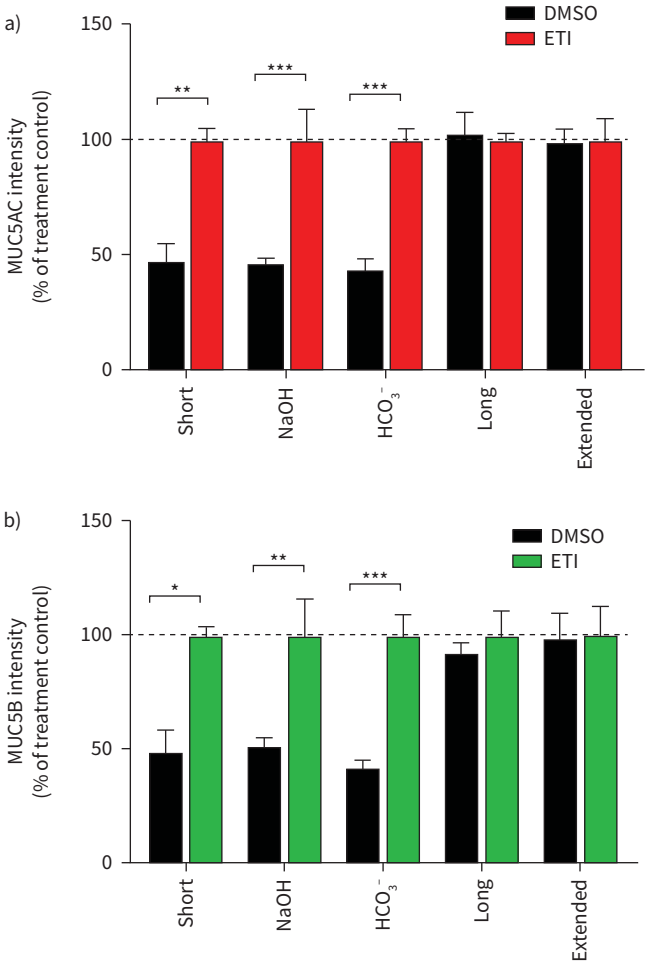

FIGURE 7 Hydration therapy, but not pH correction or bicarbonate ($HCO3^{-}$) addition, normalises mucus to modulator-treated levels in F508del human bronchial epithelial (HBE) cells. Following treatment for 3 days with 0.06% dimethyl sulfoxide (DMSO) or 1 µM VX-770, 2 µM VX-445 and 3 µM VX-661 (elexacaftor-tezacaftor-ivacaftor (ETI)), the apical surface was washed with adjusted buffers. Western blot quantification of a) MUC5AC and b) MUC5B from DMSO- and ETI-treated cultures washed for 15 min with PBS (short), PBS alkalised by 0.3 pH unit with sodium hydroxide (NaOH; OH^{-}) or with sodium bicarbonate ($NaHCO_3$; HCO_3^{-}) or for 1 h with PBS (long) or 4 h with Krebs–Ringer buffer (extended) (n=4–6 per group). Error bars indicate SEM. *: p<0.05, **: p<0.01, ***: p<0.001 (t-test).

Discussion

The recent development of effective modulator therapies has highlighted the role of corrected CFTR in the improvement of airway mucus clearance, but the underlying mechanism by which modulator compounds "reverse" mucus defects have remained unclear. We examined the three prevailing hypotheses (*i.e.* low pH, low [HCO_3^{-}] and dehydration). Our data indicate that dehydration dominates the biochemical changes occurring within the mucin network in response to CFTR malfunction.

Genetically modified cell lines and primary airway cells were used to investigate the effects of CFTR activation/inactivation on mucus in a milieu devoid of bacteria and inflammation. *In vitro* pH measurements on wild-type and CFTR-KO Calu3 cells, as well as non-CF and CF HBE cells, confirmed that in the absence of CFTR function ASL pH decreased by ~0.3 pH units (figure 1 and supplementary figure S2). Previous studies have shown that environmental acidification affects mucus viscoelastic properties, but these changes occurred at 2–3 pH units lower than observed in airway mucus [8, 22]. Small changes (~0.5) in ASL pH, as measured in CF piglet samples, correlated with an increased mucus viscosity, but abnormal mucus clearance persisted in adult animals despite ASL pH normalisation with age [7, 28]. Using G551D and F508del CF HBE cells, we restored CFTR function *via* modulator treatment,

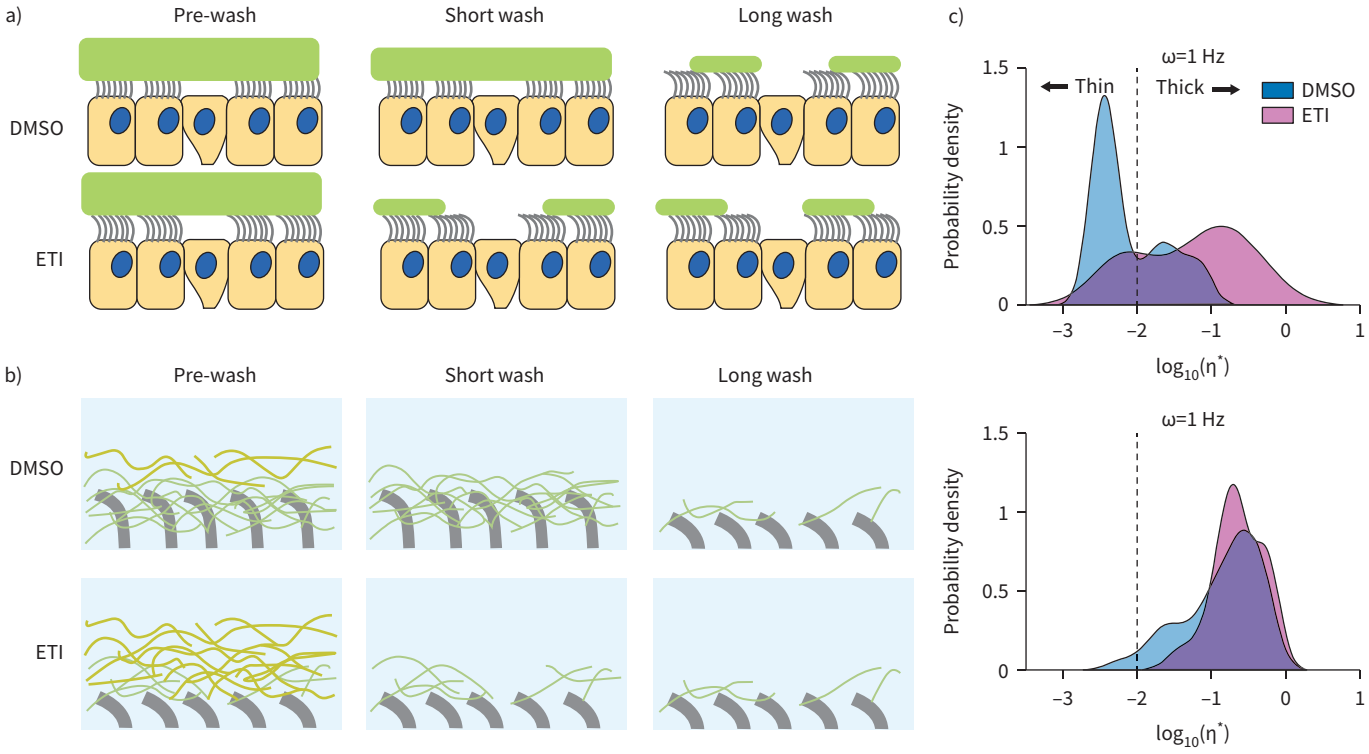

FIGURE 8 Short and long cell washings from dimethyl sulfoxide (DMSO)- and elexacaftor-tezacaftor-ivacaftor (ETI)-treated F508del human bronchial epithelial (HBE) cells analysed by particle tracking microrheology (PTM). Following a 3-day treatment with 0.06% DMSO or 1 µM VX-770, 2 µM VX-445 and 3 µM VX-661 (ETI), F508del CF HBE cells were washed with PBS for 15 min (short) or 1 h (long) before PTM analysis. a) Large-scale schematic view summarising the effects of short and long washes on the mucus layer in DMSO- and ETI-treated cell cultures. b) Small-scale schematic view of the periciliary and mucus layers in DMSO- and ETI-treated cultures following short and long washes. c) Histograms of complex viscosity (η*) analysed *via* PTM, revealing bead probability density of short and long apical washings. Quantitative rheological behaviour of short cell washings were clustered into two distinct modes of viscoelastic behaviour ("thin" and "thick") separated by a dotted line (n=6 inserts per group).

but ASL pH remained unchanged in treated cultures (figures 3 and 4). The absence of pH change may reflect the fact that partial CFTR function does not fully restore HCO_3^- secretion or, more likely, that mucus can buffer small changes in pH as suggested by recent *in vivo* pH measurements in CF infants [29].

In contrast to pH regulation, we consistently showed that mucin concentration was affected by CFTR function. Mucin concentrations were high in association with CFTR dysfunction while CFTR rescue decreased mucin concentration (figures 1, 3 and 4). Of note, neither CFTR dysfunction nor rescue affected mucin gene expression (supplementary figure S1). Western blotting showed that both MUC5AC and MUC5B gel-forming mucin concentrations were affected by CFTR function (figures 2 and 6). Increased mucin concentration may arise from an accelerated release rate of mucin granules, but was not investigated in this study. In the absence of CFTR function, both mucins adhered to the cell surfaces, as evidenced by the compression of the periciliary layer by mucus and the need for a reducing agent to completely remove the mucus (figures 5 and 6).

Increased mucin concentration in CF cultures correlated with a tightening of the mucin network ultrastructure, consistent with hyperconcentrated mucin (figures 1 and 2). Inversely, CFTR rescue produced a relaxation of the mucin network in G551D cells treated with VX-770 (figure 3). Compared to vehicle-treated cells, the mucus network in ETI-treated cells displayed a larger mesh size and was efficiently removed by short PBS washes or fixative submersion (figures 5 and 6, supplementary figures S4, S5 and S6). These physical changes were accompanied with a change in mucus solid contents from 8% to 2% organic solids before and after treatment (figure 5). At >2% organic solids, the osmotic pressure of the mucus layer is greater than the osmotic pressure of the periciliary layer, which causes the collapse of the cilia and abolishes mucus transport [11]. As shown in prior studies [26, 30], we observed mucus stasis in F508del cell cultures before treatment (figure 5, supplementary videos 1–4). Following ETI treatment of

the same cultures, mucus velocity was effectively restored at roughly normal rate (figure 5 and supplementary videos 5–8) [31].

One of the most profound changes produced by modulator treatment was the facilitation of removal of adherent mucus from cell surfaces by short PBS washes. To explore genotype-agnostic therapies aimed at "reversing" abnormal mucus properties in CF, mucus collected by cell washing on ETI-treated cells was the reference standard for successful removal of mucus, and we tested in comparison various washing approaches to remove mucus from the surfaces of vehicle-treated F508del cells (figure 7). We showed that short washes of PBS removed ∼50% of mucus compared to the ETI group. Alkalisation with or without HCO_3^- failed to normalise PBS-recovered mucus to ETI-treated levels. In contrast, sustained 1-h hydration with PBS and extended 3-h washings in Krebs–Ringer solution enabled mucus recovery at the same levels as the ETI-treated group. Hence, prolonged washes with Krebs–Ringer solution containing HCO_3^- had no additional effect compared to 1-h PBS washes.

PTM assays confirmed that ETI and TCEP treatments affected the biophysical properties of mucus (figure 8 and supplementary figures S8 and S9). Short washes on control CF cells showed that the majority of tracked particles signalled a very low viscosity solution, consistent with less mucus recovered in the lavage solution. By contrast, particles tracking in short ETI washes identified more beads in a higher viscosity environment, consistent with a higher mucin concentration. Long washes of both vehicle- and ETI-treated cultures produced bead profiles consistent with higher mucin concentrations that had sufficient time to fully swell. These data indicate that ETI treatment decreased the adherent interactions of mucins with the underlying epithelium, allowing mucins to be rapidly harvested by lavage.

In terms of hydration therapy, our data correlate with the failure of isotonic saline to restore MCC in CF patients and elucidates the limitations of short-acting osmotic therapies due to an absence of sustained hydration [32, 33]. In agreement with previous studies [26], treatment with reducing agent facilitated mucus removal (figure 6) and may provide an additional clinical benefit option for patients who do not qualify for modulator therapy.

Although we showed that hydration significantly alters the mucin network *in vitro*, the presence of bacteria, inflammatory cells, and extracellular DNA *in vivo* may further impact mucus viscoelastic properties, which may affect the efficacy of modulator therapies. Despite their limitations, cellular models provide a controlled environment to study the direct effects of modulator treatment on mucus. Using cell models, we showed that CFTR dysfunction affected mucin concentration and ASL pH. Conversely, CFTR rescue facilitated mucus removal from the cell surfaces, and these biochemical and biophysical changes correlated with a change in mucin concentration but not ASL pH. Assessing novel therapies aimed to reverse abnormal mucus properties complement CFTR modulators, as mucin-based therapies can add to modulator therapy and be universal (*i.e.* independent of genotype and/or inflammation). Indeed, mucin-based therapies may also benefit other muco-obstructive diseases such as COPD and asthma.

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