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In Utero and Postnatal VX-770 administration rescues Multi-Organ Disease in a Ferret Model of Cystic Fibrosis

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Abstract

Cystic fibrosis (CF) is a multi-organ disease caused by mutations in the cystic fibrosis transmembrane conductance regulator (CFTR). In patients with CF, abnormalities initiate in several organs prior to birth. However, the long-term impact of these in utero pathologies on disease pathophysiology is unclear. To address this issue, we generated ferrets harboring a VX-770 (ivacaftor)-responsive *CFTR*^{G551D} mutation. In utero VX-770 administration provided partial protection from developmental pathologies in pancreas, intestine, and male reproductive tract. Homozygous *CFTR*^{G551D/G551D} animals showed the greatest VX-770-mediated protection from these pathologies. Sustained postnatal VX-770 administration led to improved pancreatic exocrine function, glucose tolerance, growth and survival and reduced mucus accumulation and bacterial infections in the lung. VX-770 withdrawal at any age reestablished disease, with the most rapid onset of morbidity occurring when withdrawal was initiated during the first two weeks after birth.

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The results suggest that CFTR is important for establishing organ function early in life. Moreover, this ferret model provides proof of concept for in utero pharmacologic correction of genetic disease and offers opportunities for understanding CF pathogenesis and improving treatment.

Introduction

Cystic fibrosis (CF) is a recessive genetic disease caused by defects in the cystic fibrosis transmembrane conductance regulator (CFTR), an anion channel which moves chloride and bicarbonate across epithelia of many organs (1). Patients with CF have greatly benefited from the discovery of small molecules that increase the amount and/or function of many mutant CFTR forms (2). The two main classes of such drugs are potentiators and correctors, which rescue channel gating or processing defects in CFTR, respectively (2, 3). Alone and in combination, these drugs have shown clinical benefit in an expanding number of CFTR genotypes (2, 4). The best characterized CFTR potentiator in people is VX-770 (ivacaftor), which restores channel gating of CFTR produced by *CFTR*^{G551D} (5), a mutation found in 2-5% of patients with CF, as well as other mutant CFTR forms that produce mutant CFTR protein expression at the cell surface (6, 7). VX-770 has been evaluated in children with CF as young as two years of age (8) and is now approved for use in people with CF with specific *CFTR* mutations who are one year of age and older.

Studies in younger patients are important because early CF manifestations may impact disease progression. For example, the loss of CFTR function impairs proper development of the vas deferens and epididymis such that males with CF are almost always infertile (9). Additionally, gastrointestinal abnormalities and obstruction can occur before and after birth (10), and destruction of the exocrine pancreas occurs in most people with CF within the first two years of life (11). These and other early consequences of CF substantially impact the growth and health of infants with this disease (11). Despite intervention, growth of newborns with CF lags behind healthy controls within the first year of life (12). This can have long-term consequences since impaired growth during the early postnatal period correlates with earlier lung infections in infants with CF (13). Cumulatively, these and other findings suggest that CF disease starts in utero and impacts postnatal health. Thus, it is very important to be able to study these early manifestations of CF in order to understand the pathogenesis of disease and the degree to which CFTR modulators may impact disease progression when administered early in life.

Animal models of CF offer a path to understand how CFTR function affects disease severity and progression in ways that would not be practical in humans. Although large animal CF models such as the pig and the ferret mimic many aspects of early CF disease, mortality associated with gut and pancreatic abnormalities (14, 15, 16) has hindered their utility to study CF pathogenesis. Like infants with CF, CF ferrets are prone to meconium ileus (MI, gut obstruction at birth) and are particularly fragile during the neonatal period (16). However, the incidence of MI in CF ferrets is ~4-5 fold greater than that of infants with CF (10, 16). Despite numerous interventions that prevent gut obstruction and promote growth (elemental diet, stool softeners, pancreatic enzymes, and proton pump inhibitors) and inhibit

lung infection (multiple antibiotics), CF ferrets grow slower than non-CF controls and have a higher rate of neonatal mortality (15–17).

To address this limitation, we reasoned that in utero pharmacological rescue with CFTR modulators could improve survival and also provide the basis for studying disease-relevant processes afforded by precise control of CFTR function. This would facilitate evaluation of the importance of CFTR function during fetal development and the early postnatal period, while also enabling studies of longer-term disease progression. The ferret model was chosen because it manifests many aspects of CF intestinal, pancreatic, and lung disease (15–18) and is modestly sized compared with the pig.

Results

Generating a knock-in ferret harboring the *CFTR*^{G551D} allele

We generated a knock-in ferret harboring the *CFTR*^{G551D} allele and studied the effects of in utero and postnatal administration of the potentiator VX-770 on survival and CF-associated pathologies. To establish the *CFTR*^{G551D} model we used recombinant adeno-associated (rAAV)-mediated homologous recombination in fetal ferret fibroblasts, followed by somatic cell nuclear transfer (Fig. S1A). This generated *CFTR*^{G551D/WT} founders for which a clean homologous recombination event was confirmed by Southern blotting (Fig. S1B), PCR (Fig. S1C) and sequencing (Fig. S1D). A silent codon change at the target locus introduced a BclI restriction site for rapid genotyping (Fig. S1E). These *CFTR*^{G551D/WT} founders were bred to *CFTR*^{KO/WT} and *CFTR*^{WT/WT} ferrets for two generations to produce *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} ferrets. Expression of mRNA produced from the G551D allele in the lungs and intestines of *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} animals was only ~25% and ~50% that produced in *CFTR*^{WT/WT} animals, respectively (Fig. S1F). Neonate lung CFTR protein expression exhibited a similar gene dose-dependent relationship, where *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} expression was ~23% and ~50% of wild type, respectively (Fig. S1G and H).

VX-770 activation of *CFTR*^{G551D} in intestinal organoids, intestine, and pancreatic ductal epithelium

To assess the VX-770 responsiveness of the ferret *CFTR*^{G551D} protein, we performed forskolin-induced swelling assays using intestinal organoids derived from newborn *CFTR*^{WT/WT}, *CFTR*^{KO/KO}, *CFTR*^{G551D/KO}, and *CFTR*^{G551D/G551D} ferrets. Forskolin dose-response experiments demonstrated that VX-770 significantly ($P < 0.001$) increased CFTR-dependent swelling in both *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} organoids as compared to their vehicle (DMSO) control treatment groups (Fig. 1A and Fig. S2A-D). *CFTR*^{WT/WT} organoids gave large swelling responses that were maximal at 0.8 μ M forskolin, while *CFTR*^{KO/KO} organoids failed to swell at any forskolin concentration. In contrast to *CFTR*^{G551D} organoids, there was no difference in swelling between vehicle- and VX-770-treated *CFTR*^{KO/KO} or *CFTR*^{WT/WT} organoids (Fig. 1A and Fig. S2A-D). Consistent with reduced expression from the *CFTR*^{G551D} allele (Fig. S1F), the VX-770-induced swelling response for *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} organoids at 0.8 μ M forskolin was ~30% and 53% that of *CFTR*^{WT/WT} organoids, respectively (Fig. 1B).

Short circuit current (I_{sc}) measurements on freshly excised intestine, from untreated $CFTR^{WT/WT}$ and $CFTR^{G551D/G551D}$ ferrets born from VX-770-treated jills, demonstrated that VX-770 treatment of $CFTR^{G551D/G551D}$ intestine produced a cAMP-inducible chloride current ~28% of that observed in $CFTR^{WT/WT}$ intestine, which was inhibited by the CFTR channel blocker GlyH101 (Fig. 1C and Fig. S2E). Polarized pancreatic ductal epithelium (PDE) generated from $CFTR^{WT/WT}$, $CETAKO/KO$, and $CFTR^{G551D/G551D}$ pancreata demonstrated similar results to that of intestine. $CFTR^{G551D/G551D}$ PDE cultures differentiated in the presence of VX-770 gave cAMP-inducible chloride currents averaging 29% that of $CFTR^{WT/WT}$ PDE cultures (Fig. 1D and Fig. S2F). By contrast, $CFTR^{G551D/G551D}$ PDE cultures differentiated in the presence of vehicle (DMSO) demonstrated no cAMP-inducible or GlyH101-inhibitable chloride currents, similar to $CFTR^{KO/KO}$ PDE (Fig. 1D). Thus, ferret $CFTR^{G551D}$ PDE retains little function to conduct chloride in the absence of VX-770.

Reduction of meconium ileus in $CFTR^{G551D}$ ferrets treated in utero with VX-770

We next determined whether VX-770 could rescue the severe MI phenotype in the CF ferret model. Because most CF ferrets die at birth due to MI (~80%) (16), we chose to administer VX-770 to the pregnant jills during the gestational period of 42 days. We chose E28 as the gestational time to start VX-770 administration because $CFTR$ expression was abundant in both the intestine and pancreas at this time (Fig. S3A-F). This timing would be roughly equivalent to the start of the third trimester in humans. Pharmacokinetic experiments determined the VX-770 dose required to obtain human therapeutic concentrations of VX-770 in fetal tissues of pregnant wild type jills (see Supplementary Materials and Methods). Administration of VX-770 from E28-E42 led to 63% of $CFTR^{G551D/KO}$ kits and 96% of $CFTR^{G551D/G551D}$ kits passing meconium at birth, but had no effect on the incidence of MI in $CFTR^{KO/KO}$ ferrets with 22% of kits passing meconium (Fig. 1E, F). In the absence of in utero VX-770 administration (no treatment or vehicle-treated), the percentage of $CFTR^{G551D/KO}$ (25-28%) and $CFTR^{G551D/G551D}$ (25-29%) kits passing meconium was not significantly different than that observed for untreated- and VX-770-treated $CFTR^{KO/KO}$ ferrets (20-22%) (Fig. 1E, F) (no treatment $P=0.3992$, vehicle $P=0.5398$). Survival of $CFTR^{G551D/ko}$ and $CFTR^{G551D/G551D}$ ferrets in the first month was also significantly enhanced over that of $CFTR^{KO/KO}$ (Fig. 1G) ($G551D/KO$ $P=0.0062$, $G551D/G551D$ $P<0.0001$). Cumulatively, these results demonstrated that the ferret $CFTR^{G551D}$ protein is responsive to VX-770, and when administered in utero, this CFTR potentiator can protect from MI.

In utero VX-770 protection of $CFTR^{G551D/G551D}$ male reproductive tract

We then evaluated whether other in utero developmental abnormalities could be rescued by VX-770. Over 99% of human adult males with CF have congenital bilateral absence of the vas deferens, and some also have abnormalities in the tail and body of the epididymis (9). Vas deferens abnormalities in newborn $CFTR^{KO/KO}$ ferret kits have been reported (16), but the phenotype of the epididymis remains unknown. Here we show that newborn $CFTR^{KO/KO}$ kits lacked not only the vas deferens, but also the epididymis (Fig. 2A, B). These developmental abnormalities were rescued only in $CFTR^{G551D/G551D}$ kits born to VX-770-treated jills, while there was no effect in vehicle-treated jills for $CFTR^{G551D/G551D}$ kits or

vehicle- and VX-770-treated jills for *CFTR*^{G551D/KO} kits (Fig. 2C-F). Histologic examination of the epididymis confirmed only remnant epithelial structures in all genotypes and treatment conditions except *CFTR*^{WT/WT} and VX-770-treated *CFTR*^{G551D/G551D} kits (Fig. 2G-L). Thus, unlike the partial rescue of MI by VX-770 in *CFTR*^{G551D/KO} kits, the development of these male reproductive structures requires greater CFTR expression and function than provided in *CFTR*^{G551D/KO} kits.

Restoration of growth and partial preservation of exocrine pancreas function in VX-770-treated *CFTR*^{G551D} ferrets

To determine the postnatal therapeutic effects of VX-770 administration, we established a dosing regimen based on pharmacokinetic experiments on young kits (see Supplementary Materials and Methods). We then evaluated the impact of VX-770 administration on animal growth and fecal elastase (EL-1) concentration as a measure of pancreatic function during the first several months of life. For *CFTR*^{G551D/G551D} and *CFTR*^{G551D/KO} kits reared on VX-770, growth rates were similar to those of wild type animals and significantly ($P<0.001$) higher than that for *CFTR*^{KO/KO} kits (Fig. 3A). Although the majority of VX-770-treated *CFTR*^{G551D/KO} ferrets exhibited pancreatic insufficiency (PI) within the first month of life, they continued to grow at normal rates even without pancreatic enzyme supplementation while nursing (Fig. 3A-C). Moreover, the majority of VX-770-treated *CFTR*^{G551D/G551D} ferrets maintained pancreatic sufficiency (PS), with EL-1 concentrations in the normal range; only 1 of 8 animals exhibited persistent PI and this occurred during the third months of life (Fig. 3B, C).

The pancreatic histopathology observed at birth in untreated *CFTR*^{G551D/G551D} and *CFTR*^{G551D/KO} kits was similar to that in newborn *CFTR*^{KO/KO} kits and involved luminal dilatation of the exocrine acini (Fig. 3D). This pathology was largely prevented in *CFTR*^{G551D/G551D} kits by in utero treatment with VX-770; the same was true, though to a lesser degree, for *CFTR*^{G551D/KO} kits (Fig. 3D). Pancreatic histologic of the PS *CFTR*^{G551D/G551D} ferrets was similar to that of *CFTR*^{WT/WT} controls, both retaining the acinar cell compartment. By contrast, PI *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} ferrets were characterized by exocrine destruction and fibrosis resembling that observed in *CFTR*^{KO/KO} ferrets (Fig. 3E). Collectively these results revealed that VX-770 improved growth in both *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} ferrets despite large differences in exocrine pancreatic disease.

VX-770-mediated preservation of glycemic stability only in PS *CFTR*^{G551D/G551D} ferrets

We next determined whether VX-770-mediated protection of the exocrine pancreas rescues greater glycemic control in *CFTR*^{G551D} ferrets. Destruction of the exocrine pancreas in *CFTR*^{KO/KO} ferrets leads to spontaneous hyperglycemia and impaired postprandial glucose tolerance following their normal liquid diet at ~1-2 months of age (Fig 4A, B) during a period in which pancreatic inflammation peaks and islet mass is reduced (19). As expected, *CFTR*^{G551D/KO} ferrets with PI and maintained on VX-770 also underwent these glycemic disturbances whereas *CFTR*^{G551D/G551D} ferrets with PS did not (Fig. 4A, B). One *CFTR*^{G551D/KO} animal fluctuated between PS and PI over this period and its postprandial glucose was the lowest for that genotype (Fig. 4B). By contrast, the one *CFTR*^{G551D/G551D}

ferret that later became PI had the highest postprandial glucose of its group (Fig. 4B). We additionally performed formal mixed meal tolerance tests (MMTT) on 3-4 month old animals with a body surface area-normalized caloric intake from solid and liquid food. This analysis confirmed that glucose tolerance was normal in *CFTR*^{G551D/G551D} ferrets that were PS and maintained on VX-770, whereas it was abnormal in *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} with PI despite sustained VX-770 treatment (Fig. 4C-F). Removal of *CFTR*^{G551D/KO} ferrets from VX-770 for a three week period led to no change in glucose tolerance, as indexed by postprandial glucose excursions and area under the curve (AUC) glucose (Fig. 4C, D). These findings are consistent with previous work in the *CFTR* knockout ferret model implicating destruction of the exocrine pancreas and islet remodeling as the primary determinants of impaired glycemic status in CF ferrets (19).

Impact of CFTR function during early postnatal development on growth and survival

After establishing that in utero and early postnatal administration of VX-770 could increase survival and protect exocrine and endocrine pancreatic function, we evaluated the impact of VX-770 treatment cessation postnatally on the health of *CFTR*^{G551D/KO} kits by terminating treatment of *CFTR*^{G551D/KO} kits born to VX-770-treated jills at birth or at 7, 14, or 25 days after birth. In the case of *CFTR*^{G551D/KO} kits, when VX-770 treatment continued only to birth or 7 days of age, growth rates did not differ from those of *CFTR*^{KO/KO} kits (Fig. 5A, B). By contrast, when VX-770 was continued to 14 or 25 days of age, their growth was substantially more rapid than that of *CFTR*^{KO/KO} kits (Fig. 5A, B). Rescue of growth rates to that of wild type animals (Fig. 5A), however, was achieved only when VX-770 treatment was maintained to 25 days. In addition, in *CFTR*^{G551D/KO} kits, increase in survival required VX-770 treatment beyond 7 days of life, and the greatest benefit with respect to these parameters was in the 25-day group (Fig. 5C). The primary reasons for early euthanasia of *CFTR*^{G551D/KO} ferrets after termination of VX-770 administration were rectal prolapse, gut obstruction and lung infection. Analysis of culturable bacteria in the lungs at the time of euthanasia identified bacterial taxa and titers similar to those found in *CFTR*^{KO/KO} ferrets (Fig. 5D) (16, 17). Gross lung pathology showed evidence of atelectasis and air trapping in the lung, and mucus accumulation in the trachea (Fig. 5E-G). Histopathology demonstrated mucus accumulation in the large airways and submucosal glands (Fig. 5H-Q, Fig. S4A-H). Each of these pathologies have been observed in *CFTR*^{KO/KO} ferrets (16, 17). Cumulatively, these findings demonstrate that VX-770 treatment in utero and during the early postnatal period had therapeutic effects in *CFTR*^{G551D/KO} ferrets.

Lung disease following cessation of VX-770 in adult *CFTR*^{G551D/KO} ferrets

To evaluate the rate of lung colonization in healthy adult *CFTR*^{G551D/KO} ferrets, we utilized the cohort that was reared on both VX-770 and multiple antibiotics to ~8 months of age, during which glucose tolerance testing was performed free of potentially confounding effects of lung infection. The triple antibiotic regimen was previously shown to prevent bacterial colonization of the *CFTR*^{KO/KO} lung but not mucoinflammatory disease resulting from airway dehydration (20). Lung infection in adult *CFTR*^{G551D/KO} ferrets was monitored by serial bronchoscopy prior to and following termination of VX-770 and antibiotics (Fig. 6A). The time to detection of culturable bacteria in the lung ranged from 51-134 days following VX-770 removal, and CFU titers increased progressively with time (Fig. 6B).

CFTR^{G55W/KO} ferrets removed from VX-770 eventually contracted terminal lung disease with mucus obstruction in the airways and submucosal glands (Fig. 6C-J), as previously observed in adult *CFTR*^{KO/KO} ferret (17).

Pancreatic and lung disease following cessation of VX-770 in adult *CFTR*^{G551D/G551D} ferrets

The majority of *CFTR*^{G551D/G551D} ferrets were pancreatic sufficient while maintained on VX-770 (Fig. 3B). To assess the effect of VX-770 removal from adult *CFTR*^{G551D/G551D} ferrets, we reduced the VX-770 dose gradually by 2.5 mg/kg per week due to concerns that severe acute onset of pancreatitis might be lethal. In addition to monitoring weight and fecal EL-1, we also assessed plasma pancreatic lipase (measure of pancreatitis). All the animals tested (n=5) developed PI (EL-1 <200 µg/g feces) within 3 weeks of complete VX-770 withdrawal (Fig. S5A). Although plasma concentrations of pancreatic lipase remained relatively constant within each animal (Fig. S5B), weight declined 10-20% from maximum for four of the animals during the three week period after complete VX-770 withdrawal (Fig. S5C). Histopathology of pancreatic sufficient *CFTR*^{G551D/G551D} ferrets removed from VX-770 demonstrated a conversion from normal pancreatic exocrine architecture to a fibrotic pancreas with plugging of the ducts and depletion of acinar cell mass (Fig. 3E, lower right panel). MMTTs on these *CFTR*^{G551D/G551D} prior to and following VX-770 withdrawal demonstrated a diabetic phenotype (blood glucose >200 mg/dL at 120 minutes) in only 1 of 5 ferrets (Animal 4) at 1 month following cessation of VX-770 (Fig. S5D, E). By 3-5 months following cessation of VX-770 postprandial glucose in this animal (Animal 4) normalized, but a third ferret (Animal 3) progressed to a diabetic phenotype (Fig. S5D, E).

Progression of lung disease in these five *CFTR*^{G551D/G551D} ferrets following VX-770 withdrawal was assessed by bronchoscopy and lung histopathology at the time of declining health. The average culturable bacteria (CFU) in the bronchioalveolar lavage fluid (BALF) prior to stopping VX-770 was 73±63 CFU/ml (n=4 ±SEM, range 0-255 CFU/ml, one bronchoscopy could not be obtained). At 1-2 months following complete termination of VX-770, follow-up BALF bacterial titers in these animals averaged 1891±821 (n=5 ±SEM, range 0-4366 CFU/ml). Only one animal did not culture bacteria in the BALF following termination of VX-770 and titers in the others exceeded 1000 CFU/ml (Table S1). Two of these animals (Animal 2 and 3) were euthanized for failure to thrive and lung histopathology in Animal 3 (Fig. 7A-F) and Animal 2 (Fig. 7G-P) showed predominantly distal airway and alveolar disease dominated by neutrophil infiltration. However, the rapid decline of Animal 2 after cessation of VX-770 was accompanied by the mucus occlusion of one lobe (Fig. 7G, H, K-M). Although the timing of terminal disease onset was variable across subjects, VX-770 offered protection from bacterial infections and lung pathologies in *CFTR*^{G551D/G551D} ferrets.

Several *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} ferrets were euthanized while maintained on VX-770 to evaluate whether treatment prevented lung disease. Histopathology of *CFTR*^{G551D/KO} ferrets demonstrated single distal airways in most lobes with intermixed cellular debris and neutrophils (Fig. S6A). In contrast, lung histopathology of *CFTR*^{G551D/G551D} ferrets was normal and submucosal glands lacked mucus accumulation

(Fig. S6G-I, Fig. S4I-P). These findings suggest that *CFTR*^{G551D/G551D} ferrets have sufficient CFTR expression to maintain normal lung clearance and innate immunity while on VX-770.

VX-770 cessation promotes *CFTR*^{G551D/KO} lung inflammation

Mucus accumulation and inflammation are hallmarks of the CF lung (21, 22) and in the *CFTR*^{KO/KO} ferrets, mucoinflammatory lung disease can occur with and without bacterial infection depending on the antibiotic treatment regimen used (17, 20). VX-770 withdrawal in patients with CF carrying the *CFTR*^{G551D} mutation precipitates the deterioration of lung function (23); therefore, we reasoned that termination of VX-770 treatment in *CFTR*^{G551D} ferrets would enhance markers of CF lung disease, such as mucus accumulation and inflammation in the lung. To this end, we performed quantitative proteomics on BALF from three *CFTR*^{G551D/KO} ferrets prior to and one month following cessation of VX-770. Results from this analysis identified 239 proteins, of which 62 were significantly changed ($P < 0.05$) following removal of VX-770 (Fig. 8A and Sheet A in Datafile S1). Sixty-five percent of the differentially regulated proteins were increased in the BALF following VX-770 withdrawal. Proteins of lung epithelial origin that were shown to be significantly ($P < 0.05$) increased in the BALF following VX-770 withdrawal included Mucin 5AC (MUC5AC) and kallikrein 1 (KLK1), a serine protease known to be associated with lung disease (24), whereas significantly ($P < 0.05$) decreased proteins included submucosal gland-secreted Mucin 5B (MUC5B) and lactotransferrin (LTF) and surface airway club cell-secreted Secretoglobulin family 1A member 1 (SCGB1A1) (Fig. 8A). Pathway analysis using ReactomePA also demonstrate significant enrichment (Adjusted P -value, 3.2×10^{-35}) in BALF proteins associated with neutrophil degranulation (R-HSA-6798695) (Fig. 8A, B).

Pathway analysis of the proteomics data using Medical Subject Headings (MeSH) supported a rise in lung disease following VX-770 withdrawal, with enriched disease pathway including *lung disease*, *cystic fibrosis*, *pneumonia*, and *infection*, among others (Fig. 8C and Sheet B in Datafile S1). Similarly, Ingenuity Pathway Analysis (IPA) confirmed enrichment in canonical pathways relating to *acute phase response signaling*, *LXR/RXR*, *complement system*, and *phagosome maturation*, among others (Fig. 8D and Sheet C in Datafile S1). IPA Disease and Functions Pathways also demonstrated enrichment in processes involved in *cellular infiltration of the lung*, *inflammation of the lung*, *leukocyte migration* and an enhancement of pathways (z -score > 2) involved in *quantity of leukocytes*, *quantity of phagocytes* and *quantity of antigen presenting cells* (Fig. 8E and Sheet D in Datafile S1). Each of these analyses support enhanced lung disease and inflammation following withdrawal of VX-770 from *CFTR*^{G551D/KO} ferrets.

Discussion

We have created a *CFTR*^{G551D} ferret model that exhibits multi-organ disease and is responsive to the CFTR potentiator VX-770. We showed that VX-770 can be administered to pregnant ferrets to protect animals from developmental abnormalities caused by in utero CFTR defects, including the prevention neonatal mortality associated with MI. The G551D ferret response to VX-770 is remarkably similar to that of patients with CF (8), including

partial recovery of pancreatic function, slowed lung disease, reduced infections, and weight gain. Furthermore, withdrawal of VX-770 leads to emergence of multi-organ disease similar to that observed in *CFTR*^{KO/KO} ferrets (15–18) and the re-emergence of lung disease seen in patients with CF after withdrawn from VX-770 (23).

Initial characterization of this model revealed similarity to the human condition, therefore providing a path to probe the relationship between CFTR function during development and early life and disease severity and progression later in life. For example, the in utero data suggest that there may be a window during development (E28-P25) when CFTR function is critical for postnatal health and survival of CF ferrets. The similar postnatal growth rates observed for weaning PI *CFTR*^{G551D/KO} and PS *CFTR*^{G551D/G551D} ferrets maintained on VX-770 suggest that factors extrinsic to the pancreas may play a key role in CF health early in life and is consistent with data from patients with CF (8). Additionally, we observed a clear association between abnormal glucose tolerance and exocrine pancreas insufficiency in both *CFTR*^{G551D/G551D} and *CFTR*^{G551D/KO} ferrets, consistent with exocrine pancreatic disease early in life being associated with a higher incidence of CF-related diabetes in humans (25). However, the variable and somewhat less severe postprandial glucose phenotypes in adult *CFTR*^{G551D/G551D} removed from VX-770 were unexpected, with only one of the four surviving animals progressing to a cystic fibrosis related diabetes phenotype at 3-5 months following cessation. This model is expected to prove useful for studying CFTR functions in the pancreas and the mechanisms by which age-dependent pancreatic remodeling events in CF lead to failure of islet function.

These data confirm the in vitro and/or in vivo efficacy of a CFTR modulator on airway and intestinal epithelia, but also provide the first direct evidence that VX-770 increases CFTR function in pancreatic epithelial cells. The hypomorphic nature of the ferret *CFTR*^{G551D} allele likely played a key role in the extent of VX-770-mediated protection of CF ferrets from lung disease, meconium ileus, and exocrine pancreatic dysfunction. The low expression of the knock-in allele is likely due to the presence of a neomycin expression cassette in the adjacent intron of the targeted locus. The LoxP sites flanking the neomycin have not been activated in the ferrets studied here. The current dosing regimen led to ferret VX-770 exposures similar to those associated with maximal efficacy in patients with the *CFTR*^{G551D} mutation (26). Despite the ~50% reduction in mRNA and protein expression from the *CFTR*^{G551D} knock-in allele, VX-770 exposures resulted in partial to complete protection from disease in the various organs studied in *CFTR*^{G551D} ferrets.

Data from humans possessing hypomorphic *CFTR* intronic splice variants suggest that ~8-12% normal *CFTR* transcript is sufficient to prevent disease (27). Based on in vivo concentrations of VX-770 and the in vitro studies of epithelia from the *CFTR*^{G551D} ferrets, the ferret model appears relatively consistent with human data. Future studies titrating VX-770 exposure and varying timing of administration will better elucidate the threshold amount of CFTR activity and treatment window necessary to prevent disease across organs in the ferret model.

This study has some limitations. Despite improved availability of viable *CFTR*^{G551D} ferrets due to VX-770 treatment, the size of cohorts for several of the more labor-intensive

experiments and endpoints were resource limited. With this in mind, longitudinal studies that utilize less invasive translational endpoints, such as nasal potential difference to assess CFTR function and spirometry to quantitatively measure lung disease progression, could provide outcomes closer to those assessed in patients with CF. Regional fluctuations in CF lung colonization over time can also make the use of bronchoscopic BALF collection less reproducible for quantifying bacterial load longitudinally in animals, despite controlling for this by sampling the same lobe; there is limited data in patients with CF to compare our experience. Additionally, we did not investigate the presence of non-bacterial organisms in the lung. Thus, we cannot comment on the contribution of either fungal or viral infections in *CFTR*^{G551D} ferret lung disease progression. Lastly, the in vivo dose-response relationship between VX-770 and *CFTR*^{G551D} function was not fully explored in the present study, thus it remains unknown if optimal efficacy was achieved in all CF animals.

Fundamental questions remain regarding CF pathogenesis that cannot be easily addressed in humans. The current VX-770-responsive *CFTR*^{G551D} model affords a pharmacological switch for selective activation and inactivation of CFTR function and provides a path to address many of these questions. For example, what is the critical window to rescue organ function, and at what point does organ damage become irreversible? Another key question is how well rescue of CFTR function during development and early postnatal periods protects against initiation of lung disease later in life. Furthermore, widespread use of the CF ferret model has previously been limited by the high rate of neonatal mortality and the labor-intensive nature of rearing CF kits, two factors overcome by the G551D ferret model. The implications of this study for human therapy remain to be determined, as examples of in utero treatment of a genetic disease are rare (28). However, the findings from this study emphasize the importance of early interventions in the treatment of CF and offer a model to understand the relation between CFTR function and disease pathophysiology and progression which may inform the development and application of future CF therapies.

Materials and Methods

Study design

The objective of this study was to create a *CFTR*^{G551D} ferret model and to evaluate whether VX-770 could protect animals from disease progression. In vitro endpoints were used to assess the extent to which VX-770 could potentiate *CFTR*^{G551D} and included intestinal organoids, freshly excised intestine, and polarized pancreatic duct epithelium. In vivo endpoints for assessing VX-770 action included: 1) in utero VX-770 treatment and assessment of the extent of protection from developmental abnormalities of the gut, pancreas and male reproductive tract, 2) sustained postnatal VX-770 treatment and assessment of growth, survival, exocrine pancreatic function, glucose intolerance, and lung colonization, and 3) withdrawal of VX-770 during the early postnatal period or in adulthood and analysis of disease progression in the pancreas and lung. The primary data are reported in Table S1.

Generation of *CFTR*^{G551D} ferrets using homologous recombination and somatic cell nuclear transfer

The strategy used to generate the *CFTR*^{G551D} ferret was the same as previously described for the *CFTR*^{KO} ferret (29). Targeting was initiated by infecting primary fetal ferret fibroblasts with recombinant adeno-associated virus (rAAV) harboring the G551D mutant exon 11, neomycin selection cassette, and flanking intronic homology (AAV2.G551D-TG; see Fig. S1). On day 1 following infection, fibroblasts were serially diluted and cultured with G418 (300 µg/ml) for 15 days. G418-resistant clones were screened for homologous recombination PCR (see Fig. S1); clones positive for both the left and right arms of the predicted homologous recombination event were expanded and animals cloned by serial somatic cell nuclear transfer (29). The reconstructed embryos were then transferred into pseudopregnant recipient jills. The genotype of each G551D-targeted ferret founder was confirmed by Southern blotting.

Rearing of ferrets

All animal experimentation was approved by the Institutional Animal Care and Use Committee. VX-770 (20 mg/kg body weight, formulated in elemental liquid diet EleCare, Abbott) was administered orally once a day to pregnant jills, starting on day 28 of gestation (parturition typically occurs on day 42 of gestation). Newborn CF kits were gavaged daily with 5 mg/kg VX-770 formulated in EleCare or EleCare plus the laxative NuLYTELY, Braintree Laboratories. The dose was increased to 10 mg/kg daily at 14 days of age and maintained at that dose thereafter. CF ferrets used for glucose tolerance testing were also maintained on three antibiotics from birth, as previously described (19), to ensure that the lungs would not be infected. These animals were later withdrawn from antibiotics and VX-770 to assess progression of lung disease.

Histopathology

At necropsy, the lung, trachea, pancreata, vas deferens, and epididymis were fixed in 10% neutral buffered formalin, routinely processed, embedded in paraffin, and sectioned at 4 µm. All sections were stained with hematoxylin and eosin (H&E) and select sections for Periodic-Acid Schiff (PAS) for light microscopy. A board-certified veterinary pathologist performed the histopathological examination.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. For all experiments, data are expressed as means ± SEM. The statistical significance of the observed differences was calculated using one- or two-way ANOVA with Bonferroni post-hoc tests. Results were considered significant when $P < 0.05$. Proteomics data was evaluated using Scaffold Q+S ver. 4.7 (Proteome Software) at 0% False Discovery Rate (FDR), and with statistical testing accompanying the Scaffold algorithm. Experiments were not blinded or randomized.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1. Administration of VX-770 in utero enhances *CFTR*^{G551D}-mediated intestinal fluid secretion and protects from development of meconium ileus (MI).

(A) Swelling of intestinal organoids in response to various forskolin doses, for the indicated genotypes, and measured as area under the curve (AUC) in the presence of DMSO or VX-770 (3 μM). Each point represents the AUC for 5-7 independent experiments, each from independent donor animals. Data represent the mean ± SEM. Significant differences were determined by Two-way ANOVA and Bonferroni post-hoc tests, ** $P < 0.01$ and *** $P < 0.001$ for comparison of VX-770 to DMSO within a genotype, and # $P < 0.1$, ## $P < 0.01$, and ### $P < 0.001$ for comparison of VX-770 treatment groups across genotypes. There was no significant difference between any time points when comparing VX-770 to DMSO treatment groups for WT/WT and KO/KO groups. ANOVA P -values for the significance of the VX-770 effect were WT/WT ($P = 0.6471$), KO/KO ($P = 0.9569$), G551D/KO ($P < 0.0001$), and G551D/G551D ($P < 0.0001$). (B) Percent of the WT AUC swelling response for the indicated G551D genotypes and forskolin concentration. The two-way ANOVA P -values for the significance of the genotype effect is given below the legend. The average KO/KO AUC over all time points was subtracted from WT and G551D genotypes prior to making this calculation. (C, D) Short circuit current (I_{sc}) measurements on (C) freshly excised intestine at the level of the jejunum and (D) polarized pancreatic ductal epithelium grown at an air-liquid interface. Shown are the changes in I_{sc} (I_{sc}) in response to sequential addition of amiloride, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), forskolin (FSK) and 3-isobutyl-1-methylxanthine (IBMX), and/or GlyH101 (CFTR inhibitor) as indicated for the given genotypes. Data in represent the mean ± SEM (n in graphs indicates independent

measurements from 3-4 animals per genotype for (C) and independent cultures derived from 3-4 donor animals per genotype in (D). The order of genotypes in (D) from left to right are WT/WT, KO/KO, G551D/G551D + DMSO, and G551D/G551D + VX-770. Significant differences were determined by Two-way ANOVA and Bonferroni post-hoc tests; ns $P>0.05$, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$ for comparison between genotype in (C) and for comparison to KO/KO in (D). (E) Representative images of intestine from neonatal kits of the indicated genotypes and treatment conditions with VX-770 or vehicle administration to jills at E28. Asterisks mark intestinal dilatation and arrows mark the point of intestinal obstruction. (F) Percentage of newborn kits passing meconium at birth. Starting at E28, pregnant jills were treated with VX-770, vehicle only, or left untreated. Numbers in parentheses represent births for each genotype evaluated. Fisher's exact test was used to assess differences between the following groups: 1) Treatment groups across all genotypes: no treatment $P=0.3992$, vehicle $P=0.5398$, and VX-770 $P=1.14\text{e-}6$, 2) Vehicle vs VX-770: KO/KO $P=0.356$, G551D/KO $P=0.003$, G551D/G551D $P=6.16\text{e-}6$, and 3) Genotypes with VX-770 treatment: KO/KO vs G551D/KO $P=9.70\text{e-}4$, KO/KO vs G551D/G551D $P=5.10\text{e-}7$, G551D/KO vs G551D/G551D $P=5.84\text{e-}4$. (G) Survival rates of kits after 1 week of age for the various genotypes. G551D genotypes were born to VX-770-treated jills and maintained on VX-770 after birth, while KO/KO genotypes were not treated with VX-770. Numbers in parentheses represent the number of kits in each group starting at 1 week of age. Log-rank (Mantel-Cox) Test was used to assess significance of differences in survival between genotypes: KO/KO vs G551D/KO ($P=0.0062$), KO/KO vs G551D/G551D ($P<0.0001$), and G551D/KO vs G551D/G551D ($P=0.1186$).

Fig. 2. In utero administration of VX-770 rescues vas deferens and epididymis development only in ferrets homozygous for the G551D allele.

(A-F) Whole mount photomicrographs depicting gross morphology of the testis, vas deferens (Vas), and corpus region of the epididymis in newborn kits of the indicated genotypes and in utero treatment conditions (vehicle or VX-770). Middle row shows enlargements of boxed regions in photomicrographs in top row. Black arrows mark intact vas deferens and corpus of epididymis; red arrows mark regions where the vas deferens and corpus of the epididymis should reside, but is absent. (G-L) Representative H&E-stained sections of the epididymis (corpus) in newborn kits for the above-listed genotypes and treatment conditions. Black arrows mark the intact epididymis; red arrows mark degenerated epithelium or location where the epididymis should reside. The number of animals with in the indicated phenotype evaluated for each genotype and condition were; WT/WT n=6, KO/KO 11=4, G551D/KO vehicle n=3, G551D/KO VX-770 n=5, G551D/G551D vehicle n=3, and G551D/G551D VX-770 n=3.

Fig. 3. Administration of VX-770 to *CFTR*^{G551D} ferrets in utero and postnatally enhances growth and partially preserves pancreatic exocrine function.

In all experiments, treatment with VX-770 commenced at E28 and was sustained through the end of the experiment. (A) Body weight in animals of the indicated genotypes with and without VX-770 treatment. For each genotype, $n=6-11$ animals. Significant differences in weight gain were observed between *CFTR*^{KO/KO} vs. *CFTR*^{G551D/KO} and *CFTR*^{KO/KO} vs. *CFTR*^{G551D/G551D} using Two-way ANOVA and Bonferroni post-hoc tests, $P<0.001$. (B) Fecal elastase (EL-1) concentrations within the first 100 day of life in *CFTR*^{G551D/KO} and *CFTR*^{G551D/G551D} animals maintained on VX-770. The dashed lines correspond to 200 $\mu\text{g EL-1}/\text{gram feces}$, below which is considered to indicate pancreatic insufficiency (PI). Each unique symbol represents an independent animal ($n=9$ *CFTR*^{G551D/KO}; $n=8$

CFTR^{G551D/G551D}). (C) EL-1 concentrations for the indicated genotypes, treatment conditions, and age brackets. One animal in the *CFTR*^{G551D/G551D} group exhibited PI after 2 months of age. For each genotype and age bracket, n=6-20 animals. Significant differences in fecal EL-1 were observed between *CFTR*^{KO/KO} vs. *CFTR*^{G551D/G551D} using Two-way ANOVA and Bonferroni post-hoc tests, ***P*<0.01 and ****P*<0.001. In all graphs, data represent the mean ± SEM (D) Pancreas morphology in newborns of the indicated genotype and treatment, as assessed by hematoxylin and eosin (H&E) staining of sections. Arrows mark exocrine acini that are dilated and filled with eosinophilic secretory material. (E) Pancreas morphology in adult animals (>5 months) of the indicated genotype and treatment, as assessed by H&E staining. PI, pancreatic insufficiency; and PS, pancreatic sufficiency; as judged by fecal EL-1 assay. The animal marked G551D/G551D +VX-770 (PI) is the single pancreatic insufficient *CFTR*^{G551D/G551D} ferret shown in the bottom panel of (B). The animal marked G551D/G551D +VX (PS)→-VX (PI) was an adult pancreatic sufficient *CFTR*^{G551D/G551D} ferret removed from VX-770 for 67 days. Arrows mark islets. Scale bars = 100 μm (D) and 300 μm (E).

Fig. 4. Administration of VX-770 to *CFTR*^{G551D/G551D} ferrets in utero and postnatally improves postprandial glucose tolerance only in animals with pancreatic sufficiency.

(A) Blood glucose at baseline in non-fasted, actively nursing kits of the indicated genotypes at 1-2 months of age. All animals were treated with VX-770 in utero and maintained postnatally. (B) Blood glucose at one hour after gavage feeding an elemental diet for the animals shown in (A). Each data point in (A and B) represents the average of 4-6 measurements per kit on different days. (C) Blood glucose as assessed by mixed meal tolerance test (MMTT) in *CFTR*^{G551D/KO} ferrets at 3-4 months of age. All animals were

treated with VX-770 in utero; for the “On” group treatment was sustained throughout the postnatal period and during testing, but for the “Off” group treatment was terminated three weeks prior to testing. **(D)** Quantitation of blood glucose for animals in (C), by area under the curve (AUC) analysis. **(E)** Blood glucose as assessed by MMTT in 3-4 month old ferrets of the indicated genotypes. All animals were treated with VX-770 both in utero and throughout the postnatal period. For the *CFTR*^{G551D/G551D} group, curves are provided for: the entire cohort (n=7), i.e. including both animals with pancreatic sufficiency (PS, n=6) and pancreatic insufficiency (PI, n=1); and the single *CFTR*^{G551D/G551D} ferret that developed PI. **(F)** Quantitation of blood glucose excursions for animals in (E), by area under the curve (AUC) analysis. Significant differences in **(A, B, F)** were determined by One-way ANOVA and Bonferroni’s post-hoc tests, * $P<0.05$, *** $P<0.001$, ns=non-significant, for comparison to *CFTR*^{KO/KO}, and for **(E)** Two-way ANOVA and Bonferroni post-hoc tests, ** $P<0.01$, *** $P<0.001$ for comparison of the complete *CFTR*^{G551D/G551D} and *CFTR*^{WT/WT} cohorts to *CFTR*^{KO/KO} group. In all graphs, data represent the mean $\pm$ SEM.

Fig. 5. Early postnatal administration of VX-770 to $CFTR^{G551D/KO}$ ferrets is required for dramatic improvements in growth, survival, and slowing of lung disease progression. (A) Growth of $CFTR^{WT/WT}$ and $CFTR^{KO/KO}$ kits during the first three weeks of life. (B) Growth of $CFTR^{G551D/KO}$ kits born to VX-770-treated jills starting at E28, with VX-770 treatment terminated at the indicated age in days (d). (C) Survival curves for $CFTR^{G551D/KO}$ kits receiving VX-770 in utero and postnatally for various lengths of time. Log-rank (Mantel-Cox) Test was used to assess significance of differences in survival between days 0 and 7 vs day 14 ($P=0.0009$; $P=0.0088$, respectively) and between days 0 and 7 vs day 25 ($P=0.0004$; $P=0.0010$, respectively). (D) Quantification of bacterial taxa (left y-axis) and CFU (right y-axis, red dots) in lung homogenates from different $CFTR^{G551D/KO}$ ferrets shown in (C) at the time of euthanasia. Bacterial taxa were identified using matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) fingerprinting. Only the most abundant taxa are listed in the legend. (E-K) Representative lung images of a $CFTR^{G551D/KO}$ ferret removed from VX-770 at 14 days and euthanized at 174

days of age due to poor health, showing gross lung pathology (**E-G**) and H&E stained sections (**H-K**). Boxed region in (**E**) is enlarged in (**F**) and shows air trapping (white arrow) and atelectasis (yellow arrow). (**G**) Thick mucus in the trachea. For histology of all five lobes from this *CFTR*^{G551D/KO} animal see Fig. S4A-H. Boxed region in (**J**) is enlarged in (**K**) and shows mucus accumulation in submucosal glands (SMG). (**L**) Representative SMG image from a wild type ferret for comparison to (**K**). (**M-Q**) H&E stained sections from another *CFTR*^{G551D/KO} ferret removed from VX-770 at 25 days and euthanized at 177 days of age. Lung samples from this animal were not insufflated with fixative at the time of tissue collection. Boxed region in (**N, P**) are enlarged in (**O, Q**), respectively. Histologic images (**H-J, M, N, P**) are all different lobes from each animal with mucus accumulation in the airways marked by asterisks. Growth statistics (**A, B**): Two-way ANOVA and Bonferroni post-hoc tests were used to determine significant differences between groups when comparing the following dataset. For *CFTR*^{WT/WT} (n=11) vs 1) *CFTR*^{KO/KO} (n=11, day 11-21, $P<0.05$), 2) *CFTR*^{G551D/KO} 0d (n=7, day 11-21, $P<0.05$), 3) *CFTR*^{G551D/KO} 7d (n=5, day 15-21, $P<0.05$), 4) *CFTR*^{G551D/KO} 14d (n=8, day 21, $P<0.01$), and 5) *CFTR*^{G551D/KO} 25d (n=6, all days not significant). For *CFTR*^{KO/KO} vs 1) *CFTR*^{G551D/KO} 0d (all days not significant), 2) *CFTR*^{G551D/KO} 7d (all days not significant), 3) *CFTR*^{G551D/KO} 14d (day 12-21, $P<0.05$), and 4) *CFTR*^{G551D/KO} 25d (day 10-21, $P<0.05$).

Fig. 6. Withdrawal of VX-770 from adult *CFTR*^{G551D/KO} ferrets leads to lung disease
(A) Timeline for analysis of *CFTR*^{G551D/KO} ferrets. Bronchioalveolar lavage fluid (BALF) was collected prior to and following withdrawal of VX-770 to follow bacterial colonization of the lung. **(B)** Quantitation of bacterial load (measured as total colony-forming units [CFU] and CFU/ml) in the BALF from *CFTR*^{G551D/KO} ferrets, prior to and following VX-770 withdrawal at 267 days of age. Number of total bronchoscopies are indicated in parentheses. Data represent the mean ± SEM, using the average CFU counts for each animal at each time frame (n=number of animals). One-way ANOVA using Kruskal-Wallis and Dunn's posthoc test was used to assess significant differences in CFU counts between baseline (on VX-770) and the two time points off VX-770, ** $P < 0.01$. **(C-I)** Representative histopathology images of the trachea **(C)** and lung **(D-I)** from an adult *CFTR*^{G551D/KO} ferrets in which VX-770 treatment terminated and the animal was sacrificed 160 day later due to poor health. Separate lung lobes are provided for each low power image. **(E, H, I)** Enlargement of boxed regions in **(D, G, H)**, respectively. **(J)** Representative lung histology from a wild type ferret. Asterisks: basophilic fibrillar material (mucus) and cellular debris. SMG, submucosal gland.

Fig. 7. Withdrawal of VX-770 from adult *CFTR*^{G551D/G551D} ferrets leads to lung disease
CFTR^{G551D/G551D} ferrets were born to E28 VX-770-treated jill and maintained on VX-770 until adulthood, at which time pancreatic function and glucose tolerance testing was performed. VX-770 was then tapered off over a month. (A-F) Lung histopathology from a *CFTR*^{G551D/G551D} ferret (Animal 3) sacrificed 187 days following VX-770 cessation. All low power images represent different lobes. The pathology in this animal was characterized by distal airway disease (A, B, D-F) and pneumonia in some but not all lobes (C). (B, F) Enlargement of boxed regions in (A, E), respectively. Insets in (B, F) are taken at the region

of the black arrows and demonstrate neutrophil and accumulation in the distal airways. (**G-P**) Lung histopathology from an adult *CFTR*^{G551D/G551D} ferret (Animal 2) sacrificed 67 days following VX-770 cessation. (**G**) Breath hold computed tomography image on the day before sacrifice demonstrating an occluded lobe of the lung (asterisk). (**H**) Gross lung pathology showing the atelectatic lobe (asterisk) and abscesses in the distal lung (boxed region with enlargement shown in **I**). (**J**) Bronchoscopy at 47 days following VX-770 cessation demonstrating thick viscous mucus with the indicated CFU titer of bacteria found in BALF. (**K-P**) Lung histopathology from Animal 2 with (**K**) being the atelectatic lobe. (**L, M**) Enlargement of boxed regions in (**K**). All low power images represent different lobes. † Airway mucus. Yellow arrows mark bronchial associated lymphoid tissue expansion.

Fig. 8. Withdrawal of VX-770 from adult *CFTR*^{G551D/KO} ferrets leads to enhanced lung inflammation

Bronchioalveolar lavage fluid (BALF) was collected from three *CFTR*^{G551D/KO} ferrets prior to and one month following withdrawal of VX-770. The BALF proteome was then interrogated in a paired fashion using quantitative proteomics. **(A)** Volcano plot of 239 BALF proteins detected with the Log₂(Fold Change) representing (OFF VX-770)/(ON VX-770) (black circles: not statistically significant or Log₂(Fold Change) was < 1.5 or > -1.5 ; red circles: statistically significant $P < 0.05$ with a Log₂(Fold Change) ≥ 1.5 or ≤ -1.5 ; $N = 3$ paired samples). Proteins (gene names) found in neutrophil granules are marked in blue, while proteins secreted by airway epithelium and submucosal glands are marked in black. **(B)** Volcano plot of 63 BALF proteins enriched $P = 3.2 \times 10^{-35}$ in the ReactomePA neutrophil degranulation pathway. **(C)** List of selected significant disease pathways discovered using Medical Subject Headings (MeSH) analysis. **(D, E)** List of selected significant **(D)** Canonical pathways and **(E)** Diseases or Functions pathways discovered using Ingenuity Pathway Analysis (IPA). The number of proteins found in each pathway is given in brackets and pathways with near significant (> 1.9) or significant (> 2) positive z-scores are highlighted in red.