Ivacaftor Reverses Airway Mucus Abnormalities in a Rat Model Harboring a Humanized G551D-CFTR

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ONLINE DATA SUPPLEMENT

Supplemental Methods

Cell Culture

Fischer Rat Thyroid (FRT) cells were stably transfected with either the human *Cftr* cDNA sequence or a hybrid sequence consisting of rat exon1 and intron 1, followed by the human *Cftr* cDNA beginning at exon 2, using the Flp-In system™ (Invitrogen, Waltham, MA, USA) for transfection, as previously described (1). Following selection of stable transfected cell populations, each was clonally purified and tested by Ussing chambers analysis to measure short-circuit current following CFTR agonists, as previously described (2).

Generation of the model

The *hCFTR* G551D mutant rat model was designed and generated by Horizon Discovery (Formerly SAGE Labs, Saint Louis, MO; now part of Envigo, Saint Louis, MO) using Zinc Finger Nuclease (ZFN)-based technology. Briefly, ZFNs targeting to rat *Cftr* exon2 were obtained from Sigma-Aldrich (St. Louis, MO). ZFN mRNA was prepared from each XbaI linearized construct, using T7 RNA polymerase-based *in vitro* transcription method with MessageMax and Poly(A) polymerase tailing kits (Epicentre Biotechnology). ZFN mRNA samples were purified, quantified, and transfected at a 1:1 ratio into rat C6 cells for activity validation by Surveyor mutation assay (Transgenomic SURVEYOR kit) with Cel-1F and Cel-1R primers (Supplemental Figure 3, Tables 1 and 3)(3). The most active ZFN pair targeting CAGCTGGACCACACCAATtttgaGAAAAGGGTACAGAC (lowercases: ZFN cutting site) of the rat *Cftr* exon2 was selected for microinjection. The donor DNA plasmid comprising ~4.4kb human CFTR V470M, G551D cDNA coding sequence (without exon1), SV40 poly A signal sequence, and ~1.0kb homology arms on each side was synthesized, cloned into pUC57 plasmid, and sequence verified by Horizon Discovery (expected protein sequence Supplemental Table 1). The donor DNA plasmid along with the selected pair of *Cftr* ZFN mRNA was microinjected into pronuclei of fertilized one-cell stage eggs isolated from superovulated Sprague-Dawley donor female rats. Following microinjection, 25–30 eggs were transferred into

each pseudopregnant female rat, leading to birth of the founder generation. Tail or toe biopsies from those live births were collected for genomic DNA extraction and analysis to identify founders, as described previously (3). Specifically, genomic DNA extracts were screened for correction integration first by 3 internal PCRs with primer pairs of Cel-1F/2727R, 2678F/4769R and 4752F/Cel-1R, then 5' junction PCR with 5Jxn F1/5Jxn R1 primers and 3'Junction PCR with 3Jxn F2/3Jxn R2 primers. The internal PCR and Junction PCR positive sample was also screened with donor plasmid backbone primers pUC57 F1/pUC57 R1 to determine whether it also has random integration in addition to the targeted integration. All primer sequences are listed in the Tables 1-3. The founder number 52 was positive for 3 internal PCRs, 5' junction and 3' junction PCRs, and negative for plasmid backbone PCR. Therefore, founder 52 was backcrossed to a wildtype Sprague Dawley rat to generate heterozygous F1 rats. All animal generation work was performed in accordance with the approved animal protocols overseen by Horizon Discovery's Institutional Animal Care and Use Committee (IACUC).

Breeding scheme

F1 heterozygous rats were sent to UAB to establish the colony. Heterozygote male and female rats were paired to generate wild-type and hG551D homozygous pups (See Supplemental Figure 2). Litters remained with lactating dams until 21 days of age. Tail snips were taken between 10 - 14 days after birth for collection of genomic DNA, using the Accuris 1 Hour Mammalian Genotyping Kit (Stellar Scientific, Baltimore, MD, USA). 1µL of DNA was mixed with 0.5µL of each primer (forward 5' GGCATCGAAGAGGATTCTGA; reverse 5' GCGCAGTGTTGACAGGTACA, Integrated DNA Technologies, Skokie, Illinois, USA). PCR conditions were 95°C for 5 minutes, followed by 35 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and 68°C for 40 seconds, and a fixed cycle at 68°C for 5 minutes, resulting in an amplification of a 345bp sequence in the middle of the insert for identification. Rats that were homozygous for the insert are here termed hG551D, while rats that host no copies of the insert are hereafter referred to as wild-type (WT).

Husbandry

Derivation of animals was conducted at Horizon Discovery, Inc (microinjection and founder identification/breeding), operated under approved animal protocols overseen by the Horizon Discovery IACUC. Sprague Dawley rats (Ntac:SD) from Taconic Farms (Hudson, New York) were used for microinjection. Animals were bred with housing in standard cages maintained on a 12 h light/dark cycle with *ad libitum* access to food and water. Routine health monitoring of the colony was performed at IDEXX (Columbia, MO) and indicated no evidence of infection with known serious pathogens. At UAB, WT and hG551D rats were maintained in standard housing with *ad libitum* water and a standard rodent diet. To increase body condition, food was supplemented with DietGel 76A or DietGel Boost (Clear H20, Westbrook, ME, USA). To reduce mortality from gastrointestinal obstruction, Go-LYTLEY (Braintree Laboratories, Inc, Braintree, MA, USA) was added to the water at a concentration of 25 - 50%, as previously described (4, 5). For some experiments, $CFTR^{-/-}$ (here termed KO) rats were used as controls, and received the same dietary supplements as the hG551D. Additionally, hG551D and KO were housed with age and sex matched WT rats from weaning until time of experimentation. Rats were maintained on a 12-hour light-dark cycle, in temperatures ranging from 71℉ - 75℉.

Treatment with ivacaftor

Rats received administration of ivacaftor, here referred to as VX-770, which was obtained from Selleckchem (Houston, TX, USA) for some experiments. For the experiments analyzing mucus parameters using μOCT, in Figure 5, the VX-770 was obtained from Vertex Pharmaceuticals (Boston, MA, USA), under a Material Transfer Agreement. Rats for the nasal potential difference study were dosed for 14 days from 21 postnatal days with VX-770 at 30mg/kg/day or 3% methylcellulose vehicle by oral gavage. Rats that were used for mucus studies were dosed beginning at 3 months of age for 14 days with VX-770 at 30mg/kg/day or 3% methylcellulose vehicle control by oral gavage.

Gene Expression

Lung tissue harvested from WT, hG551D, and KO rats was homogenized, followed by RNeasy (Qiagen, Venlo, Netherlands) extraction and purification, following the manufacturer instructions. RNA was converted to cDNA and amplified using the Taqman RNA-to-CT 1-Step Kit (ThermoFisher Scientific, Waltham, MA, USA), according to instructions. Taqman probes for *Cftr* were identified with rat-sequence specificity (Rn01465702_m1), human-sequence specificity (Hs00357011_m1), or double coverage (Rn01455972_m1), using rat *Gapdh* as a housekeeping gene, and normalizing to WT. Amplifications were conducted using a ProFlex PCR System (Applied Biosciences, ThermoFisher Scientific, Waltham, MA, USA). Data were analyzed to present ddCt.

Western Blot

Lung tissue was homogenized in TBS on ice, followed by lysis in RIPA buffer (ThermoScientific, Rockford, IL) with Halt protease inhibitor cocktail (ThermoScientific). Protein was quantitated using the BCA Assay (ThermoScientific), samples were mixed with 4X sample buffer, and incubated at 37℃ for 10 minutes. Equal amounts of protein (15mg) were loaded into each lane of a Novex WedgeWell 6% Tris-Glycine gel (Invitrogen), resolved by SDS-PAGE, and blotted onto nitrocellulose membranes. Membranes were blocked with 1% normal goat serum. Anti-CFTR antibody 596, obtained from the CFTR Antibody Distribution Program at University of North Carolina at Chapel Hill, was incubated overnight at 4°C. Rabbit anti-mouse HRP conjugated secondary was incubated for 1 hour at room temperature. Labeled proteins were detected using SuperSignal West Femto ECL kit (ThermoScientific).

Immunohistochemistry

Trachea from WT, hG551D, and KO rats at 3 weeks of age were excised and fixed with 4% formaldehyde buffered in PBS for at least 4 hours. Tracheae were then moved to 15% sucrose at 4°C for 24 hours, followed by incubation in 30% sucrose at 4°C for 24 hours. Tracheae were trimmed to cross-sectional rings 15mm in width, embedded in OCT media, and flash frozen in liquid nitrogen. Tissues were then cryosectioned at 20°C to 10μm sections and placed on slides. Slides were rehydrated in PBS for 10 minutes at room temperature, followed by an incubation in 1% (w/v) sodium dodecyl sulfate (SDS) in PBS, pH 7.4, for 30 minutes for epitope retrieval. Slides were blocked with normal goat serum in PBS + 0.1% Tween (PBST) for 60 minutes at room temperature. The primary antibody for CFTR was ACL-006 (Alamone Labs, Jerusalem, Israel) at a 1:100 dilution, diluted in blocking buffer and the primary antibody for cilia was α-tubulin (Abcam, Cambridge, MA, USA); this was applied to the slides and incubated at 4°C overnight. Following 5- 5 minute rinses in PBST, an anti-rabbit secondary labeled with Alexa-Fluor 488 (green, Abcam) at 1:1000, or an anti-mouse secondary labeled with Alexa-Fluor 594 (red, Abcam) diluted in blocking buffer was applied to the slides for 60 minutes at room temperature. The secondary step was completed in the dark to preserve the signal. Slides were rinsed 5 times for 5 minutes each in PBST, and Hoescht stain added for 5 minutes. Slides were mounted in Cytoseal 60 (Thermoscientific, Grand Island, NY, USA) and coverslipped. Images were collected using a confocal microscope (Leica SP1 UV Confocal Laser Scanning Microscope).

Tracheal short-circuit current

WT, hG551D, and KO rats between 3 and 6 weeks of age were euthanized and tracheas excised and opened longitudinally, along the dorsal surface. Tracheae were mounted as flat sheets in modified Ussing chambers (area 0.021 cm^2), maintained at 37℃, and bubbled vigorously with 95% O_2:5% CO_2. Short-circuit current (I_{sc}) measurements were performed as previously described(4), under voltage clamp conductions using MC8 equipment and P2300 Ussing chambers (Physiologic Instruments, San Diego, CA). To establish a baseline current,

tissues were equilibrated for 5 minutes in Ringers solution containing 120 mM NaCl, 25 mM $NaHCO_3$, 3.33 mM KH_2PO_4, 0.82 mM K_2HPO_4, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, and 10 mM mannitol. Subsequent bathing solutions were changed at 5 minute intervals, to allow for currents to stabilize; administration of amiloride 100 μM (Sigma-Aldrich, St. Louis, MO, USA) to inhibit the epithelial sodium channel (ENaC); forskolin 10 μM (Sigma-Aldrich); VX-770 at 1 nM, 10 nM, 100 nM, 1 μM , and 10μM; and $CFTR_{Inh}$-172 at 10 μM to block CFTR-dependent current.

Nasal Potential Difference

Rats were anesthetized with ketamine (200 mg/kg), acepromazine (0.6 mg/kg), and xylazine (30 mg/kg) by intraperitoneal injection. Potential difference was measured using AgCl electrode and electronic data capture (AD Instruments, Syndey, Australia) as previously described (4). Nasal cavities were perfused sequentially with 1) Ringer solution containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and 100 μM amiloride (pH 7.3); 2) Cl–free Ringer solution (6 mM Cl_2, pH 7.3) and amiloride; and 3) Cl–free Ringer solution, amiloride, and 20 μM forskolin. Each condition was studied for 5 minutes until a stable signal was achieved. Traces were interpreted in a blinded fashion.

μOCT Image Acquisition and Analysis

Functional microanatomic measurements of *ex vivo* tissue were performed using micro-Optical Coherence Tomography (μOCT), a high-speed, high-resolution microscopic reflectance imaging modality, as previously described (4-7). Trachea were excised and immediately placed on gauze soaked in F12 media, such that the apical surface of the trachea remained media-free, and incubated under physiologic conditions (37^0C, 5% CO_2, 100% humidity; using live-cell imaging incubation systems, Carl Zeiss, Oberkochen, Germany). Trachea were allowed to equilibrate for 30 minutes before imaging. Following baseline image acquisition to ensure the tracheae were viable, tracheae were moved onto F12 media-soaked gauze with 100μM acetylcholine added

and incubated again for 30 minutes, at physiologic conditions, and re-imaged. The acetylcholine images were chosen for analysis to capture the newly secreted mucus to minimize artifact from sacrifice and excision. Mucus transport (MCT) rate was determined using time elapsed and distance traveled of native particulates in the mucus over multiple frames. For each trachea, 5 images, representing 5 regions of interest were acquired at standard distances along the ventral surface with the optical beam scanned along the longitudinal axis. Sites of imaging along the tracheae were chosen to be consistent between tissues and to account for any differences in physiology that might occur based on anatomical distance from the carina. Tissues were imaged with the operator blinded to genotype and treatment, and data were analyzed with reviewers blinded to genotype, treatment, and region of interest.

Particle Tracking Microrheology

Particle-tracking microrheological techniques were used to measure viscosity of mucus on rat tracheae *in situ*. Tracheae were treated with 0.1% benzalkonium chloride (Acros Organics, New Jersey, USA) one hour at 37^0C to stop ciliary beating. Polystyrene (PS) beads were used as previously described (8). Baseline μOCT images were acquired, tracheae were incubated for 30 minutes at 37^0C on media with 100 μM acetylcholine, and then μOCT images were acquired. Images were analyzed using ImageJ and the SpotTracker plugin (http://bigwww.epfl.ch/sage/soft/spottracker/SpotTrackerX2D_.jar). Resulting particle tracks were analyzed with custom Matlab procedures to compute mean squared displacement (MSD) while subtracting spurious bulk motion common to all tracks. Dynamic viscosity was derived from MSD by application of the generalized Stokes-Einstein relation (8).

Statistics

Statistical analysis was performed in GraphPad Prism version 6.0 or later. Inferential statistics (mean, SD, and SE) were computed using ANOVA, unpaired or paired t-test, as appropriate. For multiple comparisons, post-hoc testing was applied only if ANOVA was significant. P values of 0.05 were considered significant. Statistics are presented as means ± SE, except as indicated. Unless otherwise indicated, mean values per animal or tissue explant are shown.

Study Approvals

Procedures involving animals were approved by the IACUC at UAB (IACUC-09479 and IACUC 20532) and Horizon Discovery.

References

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Figure E1

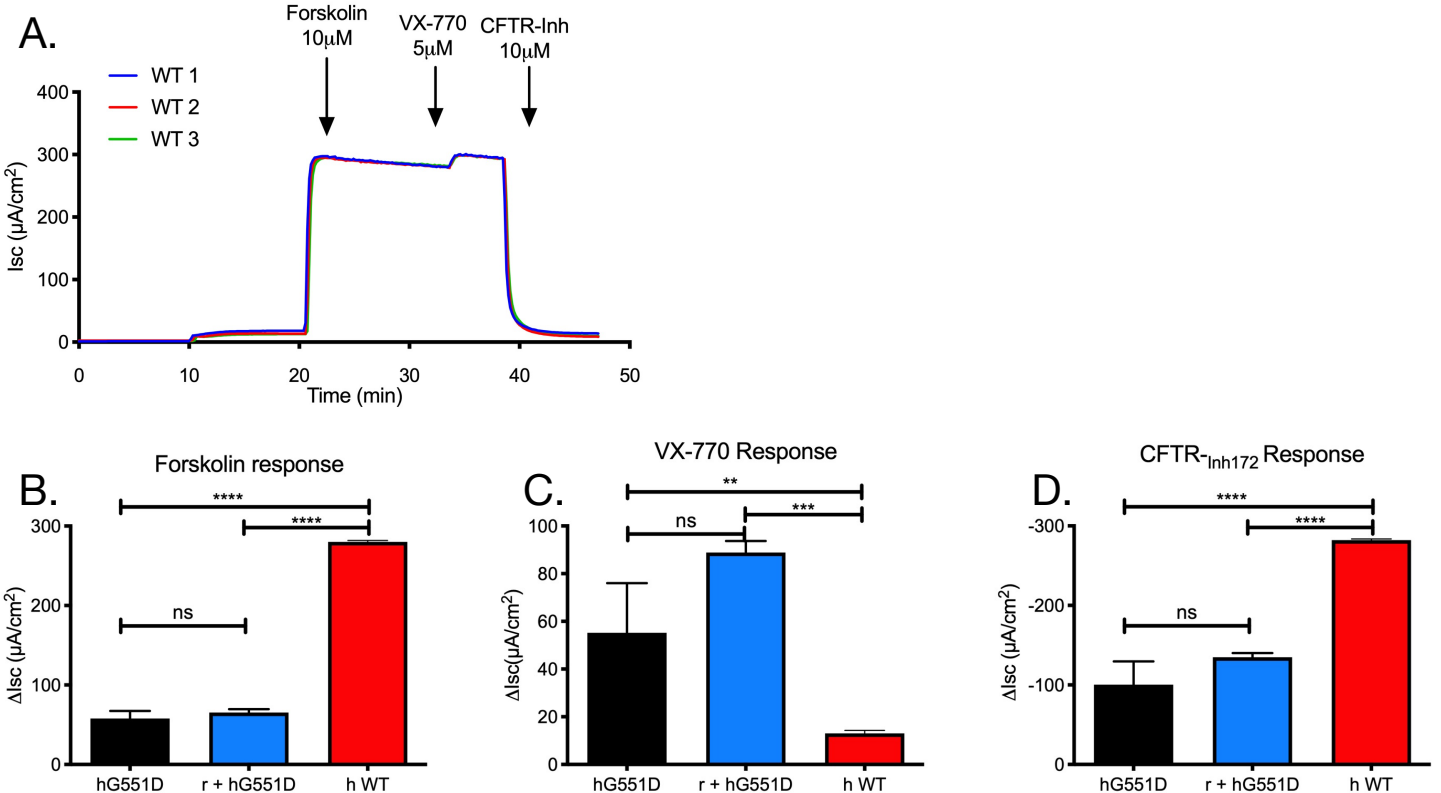


Figure E1. CFTR function in the G551D-CFTR compared to WT-CFTR in FRT cells. A) An FRT cell line hosting the human wild-type (WT) CFTR cDNA was analyzed by short-circuit current in Ussing chambers for comparison to the G551D constructs. B) The change in Isc in response to forskolin administration shows the lack of CFTR activity in the G551D construct compared to WT. C) The change in Isc in response to VX-770 administration shows the specificity of the potentiator to the G551D mutation. CFTR activity in the WT cells are likely maximized by forskolin. D) The Isc response to CFTR-Inh172 inhibitor indicates that the current measured is due to CFTR activity. The addition of VX-770 to the G551D constructs brings CFTR activity to 42% of WT. **P<0.01. ***P<0.001. ****P<0.0001. n = 4 wells/condition.

Figure E2

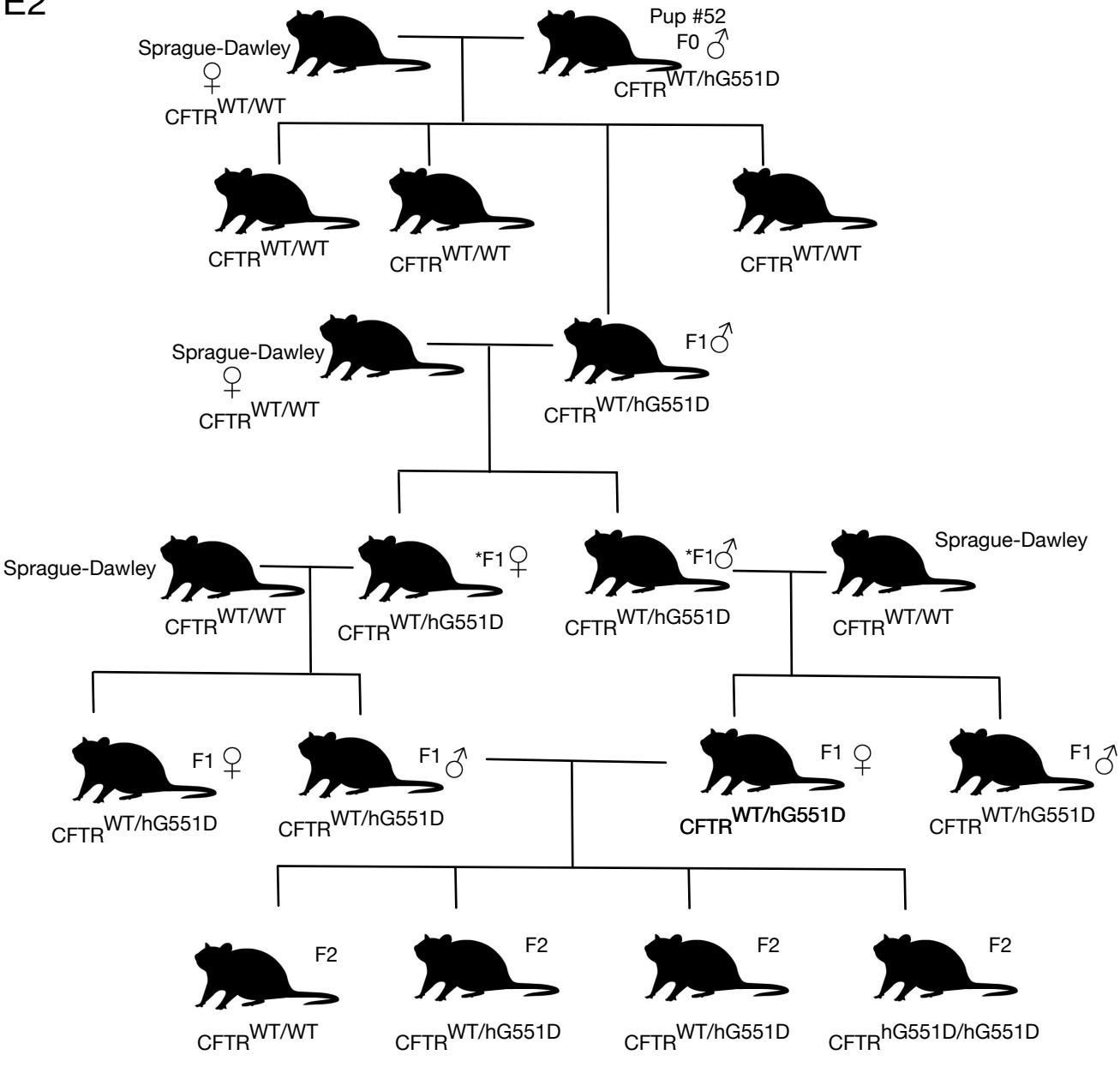
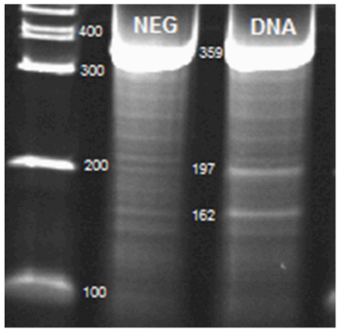


Figure E2. Breeding strategy for hG551D rats. Male founder #52, heterozygous for a targeted insertion of the human *Cftr* cDNA, was bred to a WT Sprague-Dawley female. This litter produced one hG551D heterozygote pup, which was also bred to a WT to produce further heterozygote pups. The resulting generation of heterozygote hG551D, marked with asterisks, was transferred to UAB to establish and expand the colony. The heterozygote progeny from the first litters generated at UAB were bred together to produce F2 experimental animals. This generation produced approximately 25% WT, 50% hG551D heterozygotes, and 25% hG551D homozygous progeny, as expected based on Mendelian predictions.

Figure E3

Surveyor Mutation Detection Assay Results Image



Cel-1 assay resolved on 10% TBE-PAGE

Figure E3. **Surveyor mutation assay.** NEG: Negative control; DNA: ZFN plasmid DNAs in pair. The expected cleavage band sizes are 162bp and 197bp.

Zinc Finger Nuclease Binding/cutting site
CAGCTGGACCACACCAATtttgaGAAAAGGGTACAGAC

Table E1. The Expected hCFTR G551D Protein Sequence

Expected amino acid sequence from the humanized CFGR G551D rat model. The 3 aa residues in green are the only residues that differ from the human CFTR protein.
MQKSPLEKASFISKLFFSWTRPILRKGYRQRLELSDIYQIPSVDSADNLSEKLEREWDREL ASKKNPKLINALRRCFFWRFMFYGIFLYLGEVTKAVQPLLLGRIIASYDPDNKEERSIAIY LGIGLCLLFIVRTLLLHPAIFGLHHIGMQMRIAMFSLIYKKTLKLSSRVLDKISIGQLVSLL SNNLNKFDEGLALAHFVWIAPLQVALLMGLIWELLQASAFCGLGFLIVLALFQAGLGRM MMKYRDQRAGKISERLVITSEMIENIQSVKAYCWEEAMEKMIENLRQTELKLTRKAAY VRYFNSSAFFFSGFFVVFLSVLPYALIKGIILRKIFTTISFCIVLRMAVTRQFPWAVQTWYD SLGAINKIQDFLQKQEYKTLEYNLTTTEVVMENVTAFWEEGFGELFEKAKQNNNNRKT SNGDDSLFFSNFSLLGTPVLKDINFKIERGQLLAVAGSTGAGKTSLLMMIMGELEPSEGK IKHSGRISFCSQFSWIMPGTIKENIIFGVSYDEYRYRSVIKACQLEEDISKFAEKDNIVLGE GGITLSGDQRARISLARAVYKDADLYLLDSPFGYLDVLTEKEIFESCVCKLMANKTRILV TSKMEHLKKADKILILHEGSSYFYGTFSELQNLQPDFSSKLMGCDSFDQFSAERRNSILTE TLHRFSLEGDAPVSWTETKKQSFKQTGEFGEKRKNSILNPINSIRKFSIVQKTPLQMNGIE EDSDEPLERRLSLVPDSEQGEAILPRISVISTGPTLQARRRQSVLNLMTHSVNQGQNIHRK TTASTRKVSLAPQANLTELDIYSRRLSQETGLEISEEINEEDLKECFFDDMESIPAVTTWN TYLRYITVHKSLIFVLIWCLVIFLAEVAASLVVLWLLGNTPLQDKGNSTHSRNNSYAVIIT STSSYYVFYIYVGVADTLLAMGFFRGLPLVHTLITVSKILHHKMLHSVLQAPMSTLNTL KAGGILNRFSKDIAILDDLLPLTIFDFIQLLLIVIGAIAVVAVLQPYIFVATVPVIVAFIMLR AYFLQTSQQLKQLESEGRSPIFTHLVTSLKGLWTLRAFGRQPYFETLFHKALNLHTANW FLYLSTLRWFQMRIEMIFVIFFIAVTFISILTTGEGEGRVGIILTLAMNIMSTLQWAVNSSID VDSLMRSVSRVFKFIDMPTEGKPTKSTKPYKNGQLSKVMIIENSHVKKDDIWPSGGQMT VKDLTAKYTEGGNAILENISFSISPGQRVGLLGRTGSGKSTLLSAFLRLLNTEGEIQIDGVS WDSITLQQWRKAFGVIPQKVFIFSGTFRKNLDPYEQWSDQEIWKVADEVGLRSVIEQFP GKLDFVLVDGGCVLSHGHKQLMCLARSVLSKAKILLLDEPSAHLDPVTYQIIRRTLKQA FADCTVILCEHRIEAMLECQQFLVIEENKVRQYDSIQKLLNERSLFRQAISPSDRVKLFPH RNSSKCKSKPQIAALKEETEEEVQDTRL*