

ARTICLE

G970R-CFTR Mutation (c.2908G>C) Results Predominantly in a Splicing Defect

Meredith C. Fidler^{1,*}, Alexandra Buckley¹, James C. Sullivan¹, Marvin Statia², Sylvia F. Boj², Robert G. J. Vries², Anne Munck³, Mark Higgins¹, Matteo Moretto Zita¹, Paul Negulescu¹, Fredrick van Goor¹ and Kris De Boeck⁴

In previous work, participants with a *G970R* mutation in cystic fibrosis transmembrane conductance regulator (*CFTR*) (c.2908G>C) had numerically lower sweat chloride responses during ivacaftor treatment than participants with other *CFTR* gating mutations. The objective of this substudy was to characterize the molecular defect of the *G970R* mutation *in vitro* and assess the benefit of ivacaftor in participants with this mutation. This substudy assessed sweat chloride, spirometry findings, and nasal potential difference on and off ivacaftor treatment in three participants with a *G970R/F508del* genotype. Intestinal organoids derived from rectal biopsy specimens were used to assess ivacaftor response *ex vivo* and conduct messenger RNA splice and protein analyses. No consistent or meaningful trends were observed between on-treatment and off-treatment clinical assessments. Organoids did not respond to ivacaftor in forskolin-induced swelling assays; no mature CFTR protein was detected in Western blots. Organoid RNA analysis demonstrated that 3 novel splice variants were created by *G970R-CFTR*: exon 17 truncation, exons 13–15 and 17 skipping, and intron 17 retention. Functional and molecular analyses indicated that the c.2908G>C mutation caused a cryptic splicing defect. Organoids lacked an *ex vivo* response with ivacaftor and supported identification of the mechanism underlying the CFTR defect caused by c.2908G>C. Analysis of *CFTR* mutations indicated that cryptic splicing was a rare cause of mutation misclassification in engineered cell lines. This substudy used organoids as an alternative *in vitro* model for mutations, such as cryptic splice mutations that cannot be fully assessed using cDNA expressed in recombinant cell systems.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?

☑ Participants with the *G970R* mutation in the *CFTR* gene have lower sweat chloride responses to ivacaftor treatment than do participants with *G551D* or other non-*G551D-CFTR* gating mutations.

WHAT QUESTION DID THIS STUDY ADDRESS?

☑ This substudy characterized the molecular defect of the *G970R-CFTR* mutation *in vitro* and assessed the benefit of ivacaftor in participants with this mutation.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?

☑ The functional and molecular analyses revealed that the *G970R-CFTR* mutation is not responsive to ivacaftor treatment and identified the mechanism of the *CFTR* defect as a cryptic splicing defect.

HOW MIGHT THIS CHANGE CLINICAL PHARMACOLOGY OR TRANSLATIONAL SCIENCE?

☑ For mutations that cannot be fully assessed using recombinant cell systems, such as cryptic splicing defects, organoid *in vitro* assessments and RNA analyses can be used as alternatives to characterize mutations.

Cystic fibrosis (CF) is an autosomal recessive hereditary disease caused by mutations in the CF transmembrane conductance regulator (*CFTR*) gene that result in decreased quantity and/or function of the CFTR protein.^{1,2} The CFTR protein is an epithelial chloride channel aiding in the regulation of salt and fluid absorption and secretion that is located in the epithelia of multiple organs, including the lungs, pancreas, intestinal tract, liver, and vas deferens.^{3–6} CF is a multisystemic disease with clinical manifestations, including lung function decline, chronic airway infections, pancreatic insufficiency, and malnutrition.⁷

Ivacaftor (Kalydeco^(R); Vertex Pharmaceuticals, Boston, MA) is a CFTR modulator that increases chloride transport by potentiating the channel open probability (P_o (or gating)) of the CFTR protein at the epithelial cell surface.⁸ Currently, ivacaftor is indicated in the United States for people aged ≥ 4 months with CF with ≥ 1 *CFTR* mutation that is responsive to ivacaftor based on clinical and/or *in vitro* assay data⁹; the approved genotypes and ages vary in other regions.^{10–12} In clinical studies of people with CF with eligible genotypes, ivacaftor treatment has been shown to achieve improvements in CFTR function, lung function, risk

¹Vertex Pharmaceuticals Incorporated, Boston, Massachusetts, USA; ²Foundation Hubrecht Organoid Technology, Utrecht, The Netherlands; ³Hôpital Universitaire Robert-Debré, AP-HP, Université Paris Diderot, Paris, France; ⁴University Hospital Gasthuisberg, University of Leuven, Leuven, Belgium. *Correspondence: Meredith C. Fidler (meredith_fidler@vrtx.com)

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of pulmonary exacerbations, pancreatic function, and nutritional status.¹³⁻¹⁷

Among > 340 known CF-causing *CFTR* mutations identified, 10 have historically been classified as class III mutations that result in severe defects in *CFTR* channel gating: *G551D*, *G178R*, *S549N*, *S549R*, *G551S*, *G970R*, *G1244E*, *S1251N*, *S1255P*, and *G1349D*.^{2,18-21} In Fischer rat thyroid (FRT) cells engineered to individually express complementary DNAs (cDNAs) that express *CFTR* proteins coding each of these mutations, chloride transport as determined by electrophysiological studies was < 5% of normal *CFTR*, despite the presence of mature *CFTR* protein at levels similar to those of normal *CFTR*. Ivacaftor increased the channel P_0 of these 10 *CFTR* forms and caused an increase in *CFTR*-mediated chloride transport to levels equivalent to > 10% above baseline. These *in vitro* data supported investigation of the potential clinical benefit with ivacaftor in people with CF with severe gating mutations.⁸ In a clinical study of ivacaftor in participants with CF who had a non-*G551D* severe gating mutation, participants with a *G970R-CFTR* mutation had a numerically lower treatment response than participants with the other gating mutations.¹⁴

Given this result, the molecular defect of the *G970R* mutation was further evaluated as part of the completed open-label extension study KONTINUE.¹⁵ Organoids, a cell culture technology platform,²² enabled generation of an *in vitro* model from each participant that expresses the *CFTR* protein based on the genomic DNA rather than on a cDNA construct. These organoids were used for *in vitro* functional *CFTR* experiments to evaluate the response with ivacaftor and for further mechanistic studies to help clarify the range of functional defects caused by the *G970R* mutation.

METHODS

Clinical substudy design

The *G970R-CFTR* mutation substudy was part of the KONTINUE (VX12-770-112; NCT01707290) open-label study to evaluate the long-term safety and efficacy of ivacaftor in participants with CF who completed the studies KONDUCT (VX11-770-110 (NCT01614457); participants with an *R117H* mutation),¹⁵ KONNECTION (VX12-770-111 (NCT01614470); participants with a non-*G551D* severe gating mutation),¹⁴ and VX12-770-113 (NCT01685801; participants with phenotypic or molecular evidence of residual *CFTR* function).²³ Participants with a *G970R* mutation who had participated in KONNECTION and were enrolled in the ivacaftor treatment arm of KONTINUE had the option to participate in the *G970R* substudy. Participants received treatment with ≥ 12 weeks of ivacaftor 150 mg every 12 hours in KONTINUE before beginning the substudy.

The substudy included 1 on-ivacaftor treatment visit (visit A) and 1 off-ivacaftor treatment visit (visit B), separated by a ≥ 14 -day ivacaftor washout before and after which spirometry, sweat chloride levels, and nasal potential difference (NPD) were assessed. Rectal biopsies were performed at one visit (either visit A or B) for creation of intestinal organoid cell cultures. Ivacaftor treatment resumed after substudy visit B. The substudy was conducted in compliance with institutional review board regulations, International Conference on Harmonisation Good Clinical Practice guidelines, and

the Declaration of Helsinki. All participants provided written informed consent or assent, as appropriate. Substudy visits were conducted at a single site (Universitair Ziekenhuis Gasthuisberg, Leuven, Belgium).

Outcome measures

The sweat chloride test was administered within 1 hour after the start of the NPD assessments. Sweat was collected using a Macroduct device (Wescor, Logan, UT), and samples were evaluated in a blinded fashion by ICON (Farmingdale, NY) using coulometric titration quantification.

Spirometry was performed according to the American Thoracic Society guidelines²⁴ before bronchodilator use and before ivacaftor dosing at visit A. Visit B spirometry was performed before ivacaftor treatment was restarted. NPD measurements were performed according to the Cystic Fibrosis Foundation Therapeutics TDN SOP 528.01 "Standardization of Measurement of Nasal Membrane Transepithelial Potential Difference (NPD)." NPD measurements were conducted 3 hours after ivacaftor dosing on visit A and before ivacaftor treatment was restarted at visit B. Tracings were evaluated in a blinded fashion at the University of Alabama at Birmingham.

Intestinal organoids

Rectal biopsy specimens were collected at either visit A or B and shipped overnight at 4°C to Hubrecht Organoid Technology (Utrecht, The Netherlands). Primary intestinal organoid cell cultures were generated as previously described.²² As control samples to assess ivacaftor response, organoids from the Hubrecht Organoid Technology biorepository that were derived from participants with CF with the following *CFTR* mutations were used: positive controls were two organoid lines with the *S1251N/F508del* genotype (identified as *S1251N/F508del* (A) and *S1251N/F508del* (B)) and one organoid line with the *G551D/F508del* genotype; negative controls were three organoid lines with a stop codon/*F508del* genotype (identified as *W1282X/F508del*, *Y1092X/F508del*, and *R1162X/F508del*). Additionally, an organoid line with the genotype *F508del/F508del* and a *CFTR* wild-type (WT) line were included as controls for Western blot and RNA analysis. All experiments using human tissues for organoid generation described herein were approved by the ethical committee at University Medical Center Utrecht (TcBio 14-008). Informed consent for tissue collection and generation, storage, and use of the organoids was obtained from the participants.

Forskolin-induced swelling assay

Forskolin-induced swelling (FIS) assays were performed on the organoids as previously described.²⁵ A forskolin titration was performed using forskolin 0, 0.008, 0.02, 0.05, 0.12, 0.32, 0.8, 2, and 5 $\mu\text{mol/L}$ with and without ivacaftor 3 $\mu\text{mol/L}$. An ivacaftor titration was performed using ivacaftor 0, 0.01, 0.03, 0.1, 0.3, 1, 3, and 10 $\mu\text{mol/L}$ with forskolin 0.32 $\mu\text{mol/L}$.

Western blot

For *CFTR* protein detection, Western blots were performed as previously described.²⁵

RNA analysis

Bulk RNA sequencing data were generated by Genewiz, USA, from a single biological replicate of each organoid sample (*G970R/F508del* and *F508del/F508del*). Standard bulk extraction from organoid cell pellets was followed with a reverse transcriptase step primed off of the poly-A with oligo-dT to select for messenger RNA (mRNA). Sequencing data were aligned using STAR²⁶ and analyzed using StringTie²⁷ and IRFinder²⁸ to identify novel splice isoforms and intron retention. Abundance estimates for *CFTR* splice variants were made using reads overlapping splice junctions (**Figure S1**).

In addition, RNA was isolated with an RNeasy Mini Kit (Qiagen, Venlo, The Netherlands) at Hubrecht Organoid Technology and shipped to Vertex (San Diego, CA) for processing. RNA samples were incubated for 30 minutes at 37°C in the presence of RNase-Free DNase (Qiagen) to avoid contamination with genomic DNA and repurified using an RNeasy Mini Kit prior to conversion to cDNA using an Omniscript RT Kit (Qiagen). Samples from healthy control (WT), *F508del/F508del* genotype, and *W1282X/F508del* genotype were included as controls. Primer sequences were as follows: forward primer 5'-TCACCAGCACCAGTTCGTAT-3', 62 base pairs downstream of the 5' splice junction of exon 17 (within exon 17), and reverse primer 5'-AAGCCAGCACTGCCATTAGA-3', 69 base pairs downstream of the 5' intron 17 splice site (within intron 17; **Figure 1**). Polymerase chain reaction (PCR) was performed using a ProFlex PCR System (Applied Biosystems, Foster City, CA). Amplification conditions were as follows: initial denaturation at 95°C for 5 minutes, denaturation at 95°C for 30 seconds, annealing at 56°C for 15 seconds, extension at 72°C for 15 seconds, and final extension at 72°C for 5 minutes. To visualize the resulting PCR product, one-tenth of each PCR product was electrophoresed on a 3% agarose gel with ethidium bromide. Images were captured with a GelDoc-It Imaging System (Analytik Jena, Upland, CA).

CFTR sequencing

Organoid samples were provided to Cergentis (Utrecht, The Netherlands) for targeted locus amplification and sequencing of the *CFTR* gene. DNA was analyzed according to methods previously published.²⁹ Biallelic sequencing confirmed the c.1521_1523delCTT (*F508del*) and c.2908G>C

(*G970R*) mutations. No additional exonic mutations, which might indicate the presence of a complex allele, were found.

Statistical analysis

Given the small number of participants in this substudy, no statistical analysis of the clinical data was performed.

RESULTS

Clinical assessment of participants with CF with a *G970R-CFTR* mutation

Three participants with the *G970R* mutation (all with the *G970R/F508del* genotype) enrolled in this *G970R* substudy of KONTINUE (NCT01707290). This *G970R* substudy was conducted after the week 12 visit for all participants. The participants, all of whom were men, were 10, 25, and 39 years of age at baseline in the prior study. Sweat chloride levels, percent predicted forced expiratory volume in 1 second (ppFEV₁), and NPD results for individual participants are summarized in **Table 1**.

From visit A (on ivacaftor treatment) to visit B (off ivacaftor treatment), two participants had a decrease in sweat chloride levels (−7.5 and −11 mmol/L), and one participant had an increase in sweat chloride (+3 mmol/L). Two participants had a decrease in ppFEV₁ (−1.2 and −2.3 percentage points), and one participant had an increase in ppFEV₁ (0.5 percentage points; **Table 1**). The change in total chloride conductance as assessed by NPD varied between −3.7 and +2.2 mV (**Table 1**). No consistent or meaningful trends were observed between the on-treatment and off-treatment values across assessments.

Characterization and functional assessment of intestinal organoids

Intestinal organoid cultures were generated from the three participants enrolled in the substudy.²² *In vitro* organoid studies were conducted to evaluate the *in vitro* phenotype of cells taken from these three participants. In the FIS assay, activation of CFTR by forskolin led to accumulation of fluid in the organoid lumen—a fully CFTR-dependent process that can be quantified.²⁵ In the positive control organoids (heterozygous for *F508del* and a gating mutation known to respond to ivacaftor (*S1251N/F508del* and *G551D/F508del*)), concentration-dependent FIS was enhanced in the presence of ivacaftor 3 μmol/L (**Figure S2**).

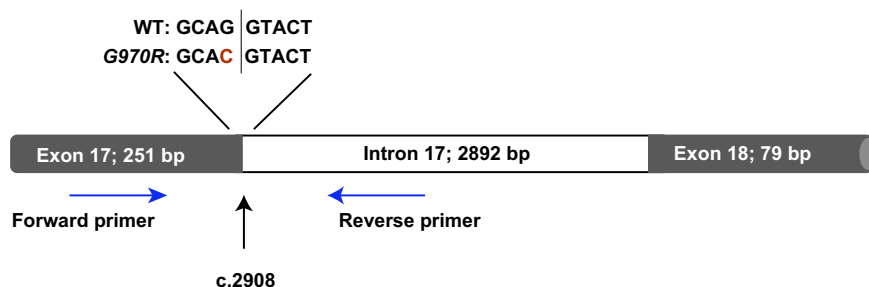


Figure 1 Schematic indicating primer binding locations. The primer set indicated by the blue arrows amplified a 278-bp product, and a product would only be generated from messenger RNA that retained the canonical exon 17 donor site. The position of the variant encoding the *G970R* mutation (c.2908) is indicated in red text. bp, base pair; WT, wild type.

Table 1 Substudy sweat chloride, ppFEV₁, and NPD results

Participant	Time point	Sweat chloride		ppFEV ₁		NPD: ΔTCC (zero chloride + isoproterenol)	
		Value, mmol/L	Absolute change from visit A to B, mmol/L	Value, % predicted	Absolute change from visit A to B, percentage points	Value, mV	Absolute change from visit A to B, mV
001	On treatment	109.0	3	69.4	-2.3	-3.0	-0.4
	Off treatment	112.0		67.1		-3.4	
002	On treatment	122.0	-11	53.7	-1.3	-1.1	2.2
	Off treatment	111.0		52.4		1.1	
003	On treatment	121.0	-7.5	58.8	0.5	-3.9	-3.7
	Off treatment	113.5		59.3		-7.6	

ΔTCC, change in total chloride conductance; NPD, nasal potential difference; ppFEV₁, percent predicted forced expiratory volume in 1 second.

No substantial swelling was observed in the organoids derived from participants with *G970R-CFTR* mutations or from the negative controls (Figure 2 and Figure S3). Thus, swelling observed in organoids from three participants carrying the *G970R* mutation was comparable to that observed in organoids from people with mutations for which no CFTR protein was produced.

Western blots were performed to determine whether mature CFTR protein was present in the *G970R/F508del* organoids (Figure 3a). The positive controls (healthy control and *G551D/F508del* genotype) showed abundant mature protein (C band) and less abundant immature protein (B band). The *G970R/F508del* organoids had a small amount of immature protein (B band) but no apparent mature protein (C band), similar to the *F508del/F508del* organoid.

RNA sequencing studies showed that 3 novel splice variants are created by *G970R-CFTR*: exon 17 truncation, as reported by Amato et al.³⁰; exons 13–15 and 17 skipping; and intron 17 retention (Figure 3b–d and Figure S1a). Exon 17 truncation and exon 17 skipping appear to be the predominant splice variants, together representing 44% of total CFTR transcripts in *G970R/F508del* organoids (Figure S1c). The presence of the retained canonical 3' exon-intron boundary of exon 17 was, however, further confirmed through the analysis of mRNA derived from these *G970R/F508del* organoids (Figure 3e). Normal CFTR splicing at this junction was observed in organoids with *F508del/F508del-CFTR*, WT *CFTR*, and *W1282X/F508del-CFTR*. Together, these data showed that, in organoids, the *G970R* genotype was associated with a splicing defect that resulted in little to no mature CFTR at the cell surface and limited response to ivacaftor.

DISCUSSION

More than 340 CF-causing *CFTR* mutations have been identified and have been classified into 6 categories or classes based on a reduction in either the amount or function of CFTR protein.^{31–33} The *G970R* protein is encoded by a single-nucleotide polymorphism of the last nucleotide in exon 17 (c.2908G>C)—first identified in a screen of Belgian participants with CF.^{34,35} It is a rare CF-causing mutation, with only 11 people with the mutation identified worldwide at the time of this writing.³²

The *G970R-CFTR* mutation has traditionally been considered a mutation that results in a class III gating defect. This classification was attributed to the protein-coding mutation based on conceptual translation and on *in vitro* studies in recombinant cell systems using a *CFTR* cDNA containing the *G970R* mutation.^{19,20,36} Seibert et al.¹⁹ and Caputo et al.³⁶ showed that *G970R-CFTR* cDNA stably expressed in Chinese hamster ovary and COS-7 cells, respectively, produced fully mature, glycosylated CFTR protein that reached the cell surface but was incapable of forskolin-induced iodide transport, and the protein had a lower mean P₀. In a study that analyzed *G970R-CFTR* by using cDNA-expressing FRT cells, Yu et al.⁸ confirmed the production of mature CFTR protein and the reduced P₀ and demonstrated responsiveness to ivacaftor that was comparable to the responsiveness of other *CFTR* mutations that result in a gating defect. In this study, a > 10-fold increase in CFTR-mediated

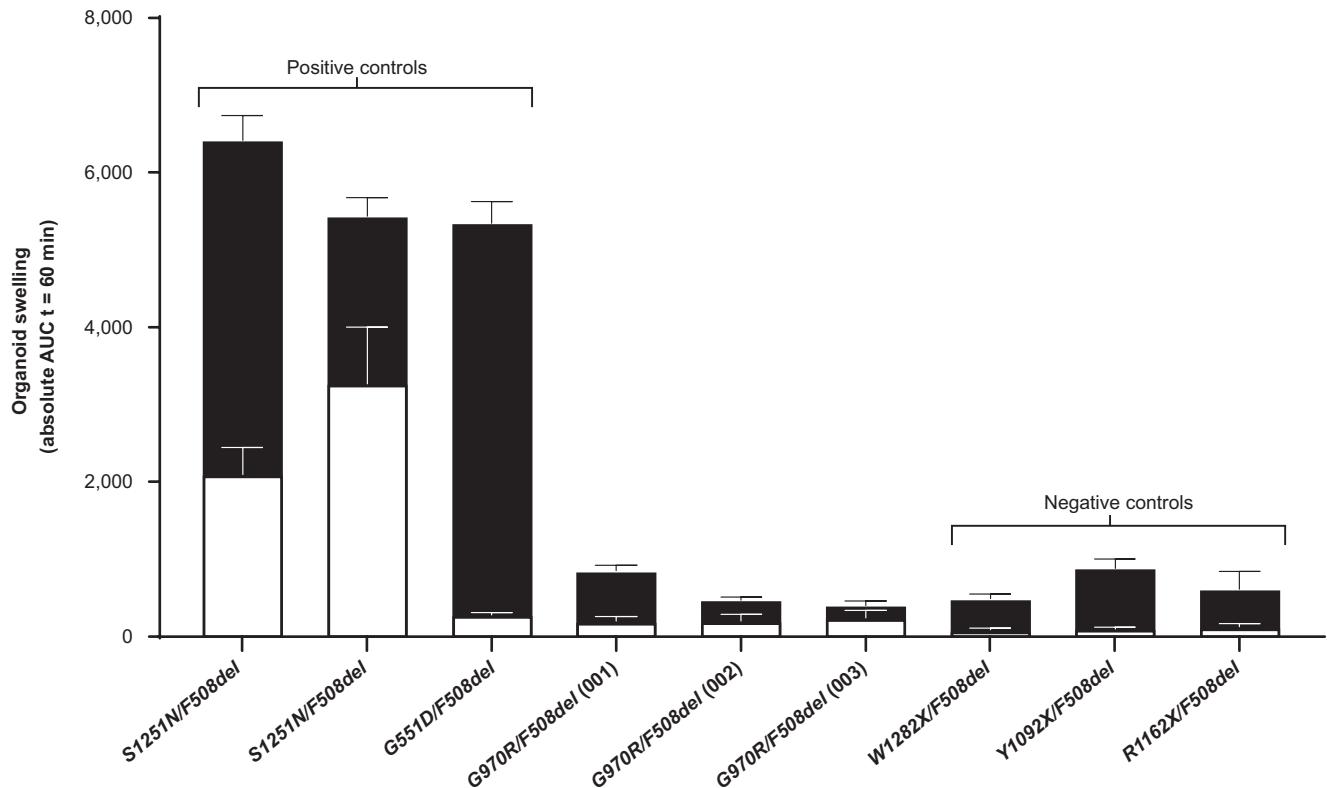


Figure 2 Swelling of rectal organoids at 5 $\mu\text{mol/L}$ forskolin (mean AUC \pm SD at $t = 60$ minutes) with (solid bars) and without (hollow bars) 3 $\mu\text{mol/L}$ ivacaftor. AUC, area under the curve; t, time.

chloride transport was seen in these cells in response to the addition of ivacaftor.⁸

The *in vitro* demonstration of a severe gating defect was consistent with the severe clinical phenotype of participants with CF with a *G970R-CFTR* mutation who exhibit high sweat chloride levels (average of 101 mmol/L) and pancreatic insufficiency (91% of individuals).³² Clinical benefit in 9 of the 10 *CFTR* mutations that result in a gating defect was established in multiple phase II and phase III ivacaftor clinical studies.^{13,14,16,37} However, participants with the *G970R-CFTR* mutation had a lower sweat chloride response during treatment with ivacaftor than did participants with CF with other gating mutations. In this substudy in three participants with CF with the *G970R/F508del* genotype, no consistent or meaningful trends were observed between the on-ivacaftor and off-ivacaftor treatment values for NPD, sweat chloride, or ppFEV₁, thus calling into question the molecular phenotype of the *G970R* mutation.

Cuppens *et al.*³⁴ previously noted that the location of the *G970R* mutation may affect mRNA splicing; however, the recombinant cell systems initially used to evaluate the mutation were based on expression of a mutant *CFTR* cDNA and presumed normal splicing in the native context.^{8,19,36} Primary cell systems, such as human bronchial epithelial cells, provide a physiologically relevant cell system that captures the full biological impact of *CFTR* mutations on mRNA splicing, protein processing and trafficking, and chloride channel function; however, these cells are often not available for rare mutations, including *G970R*. The relatively recent development of conditions for the long-term propagation of human

intestinal epithelial cell cultures, or organoids, derived from rectal biopsy specimens provides an alternative source of primary cells for the study of rare *CFTR* mutations and allows for assessment of mRNA splicing from the native genomic context.²² Organoids can be readily generated from any person with CF.³⁸ Because organoids can be expanded long term, they can be used for mechanistic studies *in vitro*.

In an FIS assay, intestinal organoid cultures from three participants with a *G970R/F508del* genotype showed only minimal response to stimulation in the presence of ivacaftor, similar to negative control organoids derived from participants with *F508del* on one allele and a mutation resulting in the production of no protein on the second allele. Western blot analysis demonstrated the lack of mature CFTR protein production. The *G970R* mutation is caused by a G>C mutation in the last position of *CFTR* exon 17.^{35,39} On the basis of results of studies of experimentally confirmed splice junctions, the last nucleotide of an exon is usually G and is rarely C ($\approx 0.7\%$ of exons),³⁹ suggesting that the c.2908G>C mutation could affect splicing efficiency.

RNA sequencing indicated that the *G970R-CFTR* mRNA creates 3 novel splice variants: exon 17 truncation, as reported by Amato *et al.*³⁰; exons 13–15 and 17 skipping; and intron 17 retention. The presence of 3 different splice variants was unexpected and emphasizes the importance of unbiased approaches when characterizing splice variants. These data are also compatible with the previously reported gating defect seen with a fully translated *G970R-CFTR* protein expressed from cDNA in engineered cell lines and

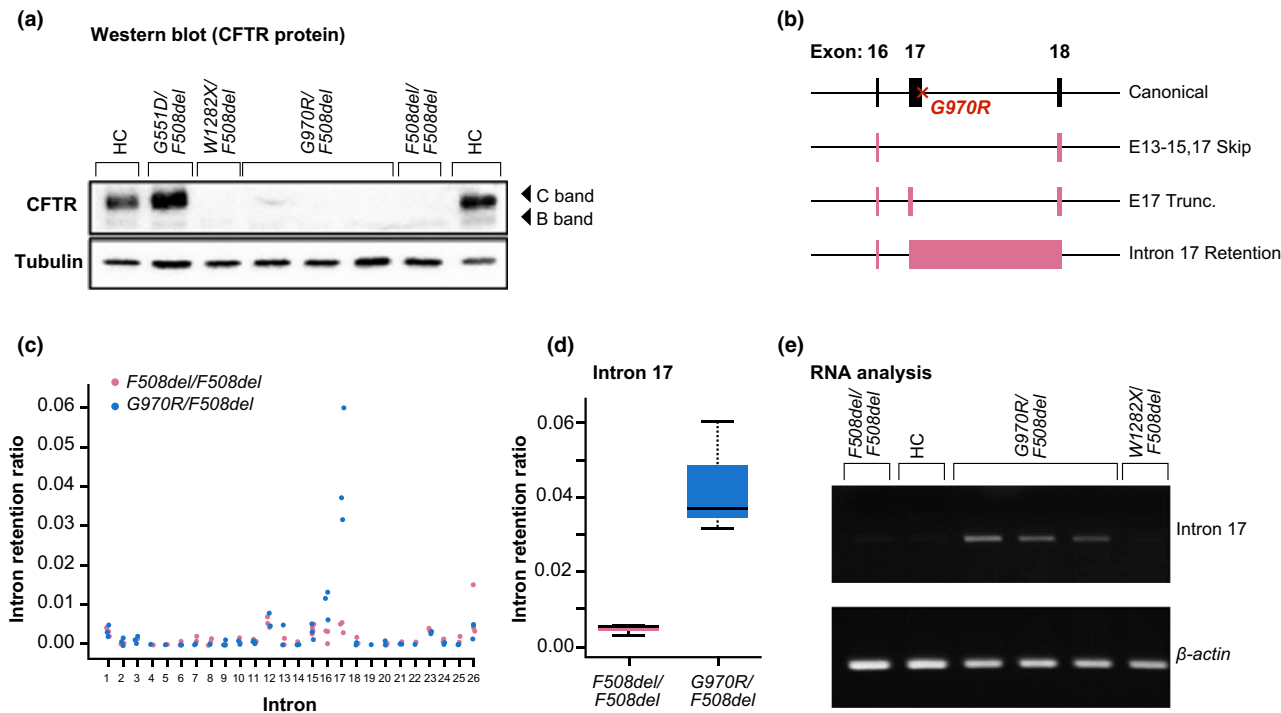


Figure 3 Protein and RNA expression of *G970R-CFTR*. (a) Western blot showing expression of cystic fibrosis transmembrane conductance regulator (CFTR) protein (C band, mature protein; B band, immature protein) in participant-derived intestinal organoids with wild-type (WT) *CFTR* (HC) and *G551D/F508del-CFTR*, *W1282X/F508del-CFTR*, *G970R/F508del-CFTR*, and *F508del/F508del-CFTR* mutations—with tubulin as a loading control. (b) Gene diagram representing *CFTR* splice variants detected in *G970R/F508del* organoids: exons 13–15 and 17 skipping (E13–15,17 Skip), exon 17 truncation (E17 Trunc.), and intron 17 retention. Intron retention ratio calculated with IRFinder for all canonical introns in *CFTR* (c) and for intron 17 (d). (e) Reverse-transcriptase polymerase chain reaction (PCR) of messenger RNA from organoids with *F508del/F508del-CFTR* mutations, WT *CFTR* (healthy controls (HCs)), *G970R/F508del-CFTR* mutations, and *W1282X/F508del-CFTR* mutations—with β -actin as a loading control; shows retention of the 3' splice junction of exon 17 (labeled intron 17).

indicate that any correctly spliced mRNA transcribed from the c.2908G>C-encoding *CFTR* allele would have the gating defect caused by the *G970R* amino acid change. With the methods used here, we can only estimate, not definitively quantify, the amount of correctly spliced c.2908G>C mRNA.

A limitation of *in vitro* model systems using recombinant cell lines and cDNA constructs is that they will not capture cryptic splicing events. Our work shows that primary organoids, like human bronchial epithelial cells, are a physiologically relevant cell system in which to study the effects of *CFTR* mutations on mRNA splicing, protein processing and trafficking, and chloride channel function. Primary cell systems and traditional recombinant cell systems, such as FRT cells, play an important and complementary role in CF research.

The US Food and Drug Administration expanded the approved use of ivacaftor and tezacaftor/ivacaftor (Symdeko[®]; Vertex) for an additional 28 and 21 mutations, respectively, based on the data from *in vitro* studies performed in the FRT cell model. This novel approach to drug approval was based on a robust and validated laboratory assay model and comprehensive understanding of the molecular defects caused by *CFTR* mutations. In addition, verification of data integrity by the regulatory agency—as well as the previously established correlation between *in vitro* cell system responses in the FRT model at Vertex with clinical benefit in large,

randomized trials and well-established safety profiles—contributed to this approach.⁴⁰

The results of this substudy highlight the challenge of using recombinant cell models expressing cDNA constructs to identify *CFTR* mutations that may cause aberrant splicing of *CFTR*. Of the > 2,000 *CFTR* mutations identified, there are rare examples of exonic cryptic splice variants that can result in no or reduced amounts of CFTR protein in native cells.^{41,42} The molecular consequence of these *CFTR* variants may be misclassified when expressed in recombinant cell models using cDNA.⁴² Of the 1,423 unannotated *CFTR* mutations evaluated *in silico*, 33 exonic missense and 6 exonic synonymous mutations were identified as potential splice variants.⁴² Only 6 mutations identified are present in the CFTR2.org database (*R560K*, *R560T*, *G970R*, *G970D*, *I1234V*, and *R1239R*).³² For the *G970R* mutation, their analysis suggested that the G>C change in the last position of the canonical 5' splice donor site of exon 17 weakens the likelihood that this position will be recognized as a splice donor site.⁴² Here, we experimentally validated their prediction and recommend that the *G970R* mutation be reclassified primarily as a splice mutation. Similarly, Lee *et al.*⁴² predict that the c.2909G>A change (*G970D*) on the first nucleotide of exon 18 will weaken the canonical acceptor. However,

experimental data by Amato *et al.*³⁰ demonstrated correct splicing between exon 17 and exon 18 for this mutation using primary, patient-derived tissue. Thus, whereas computational tools can help identify potential cryptic splice mutations, experimental validation using patient tissues is critical for confirmation of splicing defects.

Functional and molecular analyses using participant-derived organoids indicated that the c.2908G>C mutation causes a splicing defect and should no longer be categorized as a CFTR gating mutation. Although there is a robust correlation between the FRT system and data from clinical trials using CFTR modulators, G970R appears to be an exception based on its previously undocumented role as a splice mutation. This substudy used organoids as a complementary *in vitro* model to characterize cryptic splice mutations that cannot be assessed fully in recombinant cell systems expressing cDNA constructs.

Supporting Information. Supplementary information accompanies this paper on the *Clinical and Translational Science* website (www.cts-journal.com).

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Conflict of Interest. M.C.F., A.B., M.H., M.M.Z., P.N., and F.v.G. are employees of Vertex Pharmaceuticals Incorporated and may own stock or options in the company. J.C.S. is a former employee of Vertex and may own stock or options in the company. M.S., S.F.B., and R.G.J.V. are employees of Hubrecht Organoid Technology and report grants from Vertex during the conduct of the study and grants from companies involved in cystic fibrosis research and drug development outside the submitted work, for which Hubrecht Organoid Technology performs contract research organizational work in the area of cystic fibrosis drug development. A.M. reports personal fees from Vertex outside the submitted work. K.D.B. reports support from Boehringer, Protalix, Raptor, Teva, NovaBiotics, Eloxx, Vertex, and Galapagos. All authors received nonfinancial support (assistance with manuscript preparation) from ArticulateScience LLC.

Data Availability Statement. Vertex is committed to advancing medical science and improving the health of people with cystic fibrosis. This includes the responsible sharing of clinical trial data with qualified researchers. Proposals for the use of these data will be reviewed by a scientific board. Approvals are at the discretion of Vertex and will be dependent on the nature of the request, the merit of the research proposed, and the intended use of the data. Please contact CTDS@vrtx.com if you would like to submit a proposal or need more information.

Author Contributions. M.C.F. wrote the manuscript. M.C.F., A.B., J.C.S., M.S., S.F.B., R.G.J.V., A.M., M.H., M.M.Z., P.N., F.v.G., and K.D.B. designed the research. S.F.B. and R.G.J.V. performed the research.

M.C.F., A.B., J.C.S., M.S., S.F.B., R.G.J.V., A.M., M.H., M.M.Z., P.N., F.v.G., and K.D.B. analyzed the data.

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