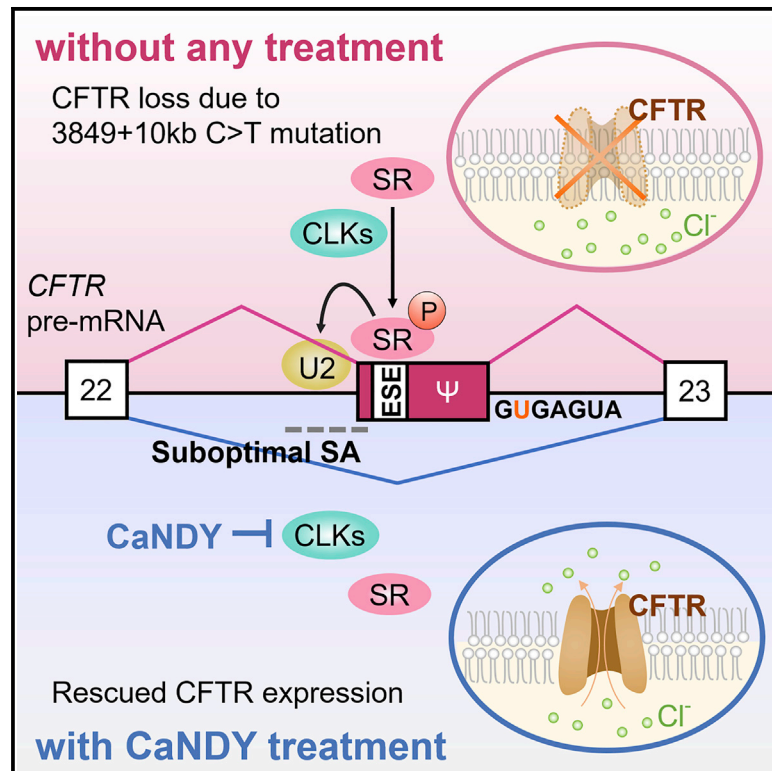


Cell Chemical Biology

Mechanism-Based Personalized Medicine for Cystic Fibrosis by Suppressing Pseudo Exon Inclusion

Graphical Abstract



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In Brief

Shibata et al. propose a mechanism-oriented precision medicine treatment for cystic fibrosis that ameliorates aberrant splicing caused by 3,849 + 10 kb C>T mutation of CFTR. They reveal that CFTR pseudo exon recognition depends on function of CLK and SRSF. Administration of the CLK inhibitor CaNDY restored normal splicing of CFTR and rescued CFTR channel activity.

Highlights

- Mechanism of 3,849 + 10 kb C>T-induced *CFTR* pseudo exon inclusion is unveiled
- SRSFs activated by CLK are required for the *CFTR* pseudo exon recognition
- CLK inhibitor CaNDY was identified as a potent suppressor of *CFTR* pseudo exon
- CaNDY treatment rescues defective CFTR channel activity due to 3,849 + 10 kb C>T

Article

Mechanism-Based Personalized Medicine for Cystic Fibrosis by Suppressing Pseudo Exon Inclusion

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SUMMARY

Cystic fibrosis (CF) is caused by mutations in the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) gene that compromise its chloride channel activity. Here, we present a therapeutic strategy to ameliorate RNA splicing deficiency of *CFTR* with a small molecule. The 3,849 + 10 kb C>T is the most common splicing mutation in CF, creating a pseudo exon with premature stop codon. We reveal that the 3,849 + 10 kb C>T-induced *CFTR* pseudo exon is regulated by phosphorylation of serine/arginine-rich splicing factors, and their functional inhibition by a CDC-like kinase inhibitor restores normal splicing of *CFTR*. Subsequent screening of our focused chemical library identified CaNDY as a rectifier of the aberrant splicing. CaNDY treatment restored normal splicing of *CFTR* with the 3,849 + 10 kb C>T in CF patient cells and functional *CFTR* protein expression in the CF model cells. Our findings open the door for mechanism-based personalized medicine for pseudo-exon-type genetic diseases.

INTRODUCTION

Deep-intronic splicing mutations often cause the inclusion of a pseudo exon, which in turn produces a premature stop codon leading to genetic inactivation. The number of disease-associated pseudo-exonic mutations has dramatically increased because of recent advances in whole-genome sequencing and transcriptome analysis (Dufner-Almeida et al., 2019; Boisson et al., 2019; Gonorazky et al., 2016; Khan et al., 2017; Riant et al., 2014). In contrast, mechanisms involved in pseudo exon recognition remain poorly explored. Understanding the mechanism of a disease-related splicing is crucial for finding an effective therapeutic strategy, as characterization of *SMN2* exon 7 splicing led to the development of nusinersen for spinal muscular atrophy (Hua et al., 2008; Finkel et al., 2017; Mercuri et al., 2018). In our hands, as previously demonstrated, splice-targeting therapeutics were achieved using small-molecule compounds for genetic diseases such as Duchenne muscular dystrophy, NEMO deficiency syndrome, and familial dysautonomia (Nishida et al., 2011; Boisson et al., 2019; Yoshida et al., 2015), by targeting splicing regulators. Therefore, unveiling molecular mechanisms of pseudo exon recognition will provide a therapeutic strategy for pseudo-exonic diseases. We here examine such a concept, using the *cystic fibrosis transmembrane conductance regulator* (*CFTR*) pseudo exon caused by the 3,849 + 10 kb C>T deep-intronic mutation of cystic fibrosis (CF) (Highsmith et al., 1994) as a representative case.

CF is an autosomal-recessive monogenic disease, caused by mutations in the *CFTR* gene that result in functional and/or quantitative defects of the *CFTR* protein (Rowe et al., 2005; Cutting, 2015). More than 2,000 pathogenic *CFTR* mutations have been identified to date and grouped into six classes (class I–VI) by their functional consequences (Elborn, 2016). *CFTR* is an anion channel, essential for regulating transepithelial secretion of chloride and bicarbonate to control water transport and fluid homeostasis (Rowe et al., 2005). As compromised *CFTR* function results in malfunctioning mucosal surfaces in multiple exocrine organs, such as lung, pancreas, and intestine, the estimated median length of patient life is limited to 40 years (Elborn, 2016), with pulmonary infection and respiratory failure being major causes of mortality (Awatade et al., 2018). *CFTR* modulators have recently been clinically introduced for the treatment of CF with class II, III, and IV mutations (Virant-Young et al., 2015). However, treatment for CF patients with class V mutations, which cause splicing defects of *CFTR* mRNA, remain to be established.

The 3,849 + 10 kb C>T deep-intronic mutation is the most common splicing mutation, found in more than 60% of CF patients with class V *CFTR* mutations (Highsmith et al., 1994; Friedman et al., 1999). It creates an 84-nucleotide pseudo exon within *CFTR* intron 22, by providing the essential GU dinucleotide of the splice donor (Highsmith et al., 1994). Despite its importance in CF pathogenesis, the mechanism of pseudo exon recognition remains unclear, and there is no practical strategy to reverse the genetic inactivation of *CFTR* created by pseudo exon

inclusion. In this study, we attempted to unveil the molecular mechanism behind the recognition of the *CFTR* pseudo exon for the development of a therapeutic way to release the 3,849 + 10 kb C>T-driven inactivation of *CFTR* with a small-molecule compound.

RESULTS

Recognition of the *CFTR* Pseudo Exon Requires Serine/Arginine-Rich Splicing Factor-Dependent Splicing Enhancer

For efficient and quantitative evaluation of switched alternative splicing of the *CFTR* pseudo exon, we first established a splicing reporter system for the wild-type IVS22 and the 3,849 + 10 kb C>T mutation of *CFTR* using SPREADD (*CFTR* WT reporter and *CFTR* C>T reporter, respectively, in Figure 1A) to recapture the pathogenic splicing (Boisson et al., 2019; Yoshida et al., 2015). In this system, the normal splicing form (skipping of the pseudo exon) leads to expression of a GFP-fusion protein, whereas the pathogenic splicing form (inclusion of the pseudo exon) results in production of an RFP fusion protein (STAR Methods for further detail). Consistent with previous observations in patient cells (Highsmith et al., 1994), inclusion of the pseudo exon represented by the RFP signal was observed exclusively in the presence of the 3,849 + 10 kb C>T mutation, in contrast to a dominant GFP signal without it (Figures 1B and 1C). Consistency of this *CFTR* SPREADD reporter was also confirmed by RT-PCR. The larger band corresponding to the pseudo exon inclusion dominantly appears for the 3,849 + 10 kb C>T reporter (Figure 1D), indicating that the color change of this reporter reflects pseudo exon inclusion and that splicing regulatory sites required for the splicing switch are located in the inserted region.

We then investigated the molecular mechanism of pseudo exon creation in *CFTR* using this splicing reporter system. Surveying the strength of splice sites surrounding the pseudo-exonic sites, we noticed that the splice acceptor (SA) of the pseudo exon is suboptimal for spliceosomal recognition, with a relatively low 63% C/U content in the polypyrimidine tract and the presence of a less common uridine at the +1 position of the SA (Figure S1A) (Sibley et al., 2016), whereas its splice donor with the 3,849 + 10 kb C>T shows optimal base-pairing with U1snRNP (Figure S1B). These facts prompted us to ask why the weak pseudo-exonic SA is preferentially selected over the strong exon 23 SA. We hypothesized that spliceosomal recognition of the pseudo-exonic weak SA is promoted by a splicing enhancer in the vicinity of the pseudo-exonic SA. We examined associations of the pseudo exon RNA sequence with serine/arginine-rich splicing factors (SRSFs), which are known as alternative splicing regulators to promote spliceosomal recognition of suboptimal splice sites, mainly through an exonic splicing enhancer (ESE) (Fu and Ares, 2014; Cartegni et al., 2002). RNA pull-down assay revealed the association of SRSF1, SRSF4, and SRSF6 with the pseudo-exonic sequence downstream of the SA (RNA #2 in Figures 1E and 1F). The specificity of this interaction was subsequently confirmed by introducing point mutations to disrupt the affinity to SRSFs (RNA #2M in Figures 1F and 1G). The functional importance of SRSF binding to the #2 site was further evaluated by introducing point mutations into

the reporter vector (Figure 1H), and a switched splicing form from the pseudo exon inclusion to skipping was confirmed (Figure 1I), which was accompanied by conversion from an RFP-dominant to a GFP-dominant state on the reporter (Figures 1J and 1K). These data indicate that the downstream SRSF-dependent ESE is required for recognizing the pseudo exon SA.

Inhibition of CDC-like Kinase Induces Skipping of the *CFTR* Pseudo Exon

The above observations suggest a therapeutic opportunity for CF with the class V mutation by targeting the SRSF function. As the splice-promoting activity of SRSF proteins depends on phosphorylation of their RS domain through CDC-like kinases (CLKs) and/or SR protein kinases (SRPKs) (Fu and Ares, 2014; Ghosh and Adams, 2011), we examined the effects of the CLK inhibitor TG003 (Boisson et al., 2019; Nishida et al., 2011; Muraki et al., 2004) and the SRPK inhibitor SRPIN340 (Fukuhara et al., 2006), which were previously developed in our laboratory, on *CFTR* pseudo exon splicing. In the C>T splicing reporter, TG003 treatment suppressed the pseudo exon inclusion (Figures 2A and 2B), whereas SRPIN340 did not (Figures 2A and 2C), indicating that CLKs play a major role in the context of the *CFTR* pseudo exon. We then asked if the suboptimal nature of pseudo-exonic SA provides dependency on CLK/SRSF and responsiveness to TG003. For this purpose, we replaced the weak SA of the pseudo exon with the optimal SA of exon 23 (C>T-SA in Figure 2D, and SA sequences for Figure S1). As expected, C>T-SA was no more dependent on CLK/SRSF (C>T-SA in Figures 2E and 2F), and neither the #2M mutation (C>T(#2M)-SA in Figures 2E and 2F) nor TG003 treatment promoted pseudo exon skipping. These data support the idea that CLK/SRSF dependency is required for responsiveness to CLK inhibition by TG003.

CaNDY Was Identified as a Highly Potent Rectifier of the Aberrant Splicing

Given the *CFTR* pseudo-exon-suppressing activity of TG003, we subsequently sought for a more potent compound in a focused chemical library of 747 in-house kinase inhibitors using the *CFTR* C>T reporter, and identified CaNDY as the top hit (Figures 3A and 3B). CaNDY is a small-molecule compound we previously reported for a dual inhibition of CLK and a dual-specificity protein kinase (DYRK) (Sonamoto et al., 2015). The EC₅₀ value of the *CFTR* pseudo exon suppression by CaNDY was 20.4 times lower than that of TG003 (CaNDY EC₅₀ = 1.2 μM, TG003 EC₅₀ = 24.5 μM) (Figure 3C). This dose-dependent effect of CaNDY was also confirmed by RT-PCR for *CFTR* expressed from a C>T minigene (Figure 3D). Moreover, as the clinical severity in patients with the 3,849 + 10 kb C>T is primarily associated with impaired *CFTR* function in airway epithelial cells (Highsmith et al., 1994; Chiba-Falek et al., 1998), we also tested the effect of CaNDY on the human airway epithelial cell-derived cell line Calu-3 (Figure 3E). We observed the suppression of the pseudo exon inclusion in *CFTR* expressed from C>T reporter in Calu-3 cells following CaNDY treatment, with a rate comparable to that for HEK293 cells. RT-PCR for endogenous *CFTR* revealed no change in the exon 22^23 splicing of *CFTR* without 3,849 + 10 kb C>T in Calu-3 cells (Figure 3E), whereas endogenous *CFTR* was undetectable in HEK293 cells (Figure 3D). Further, we examined the effect of CaNDY on endogenous *CFTR* in the B

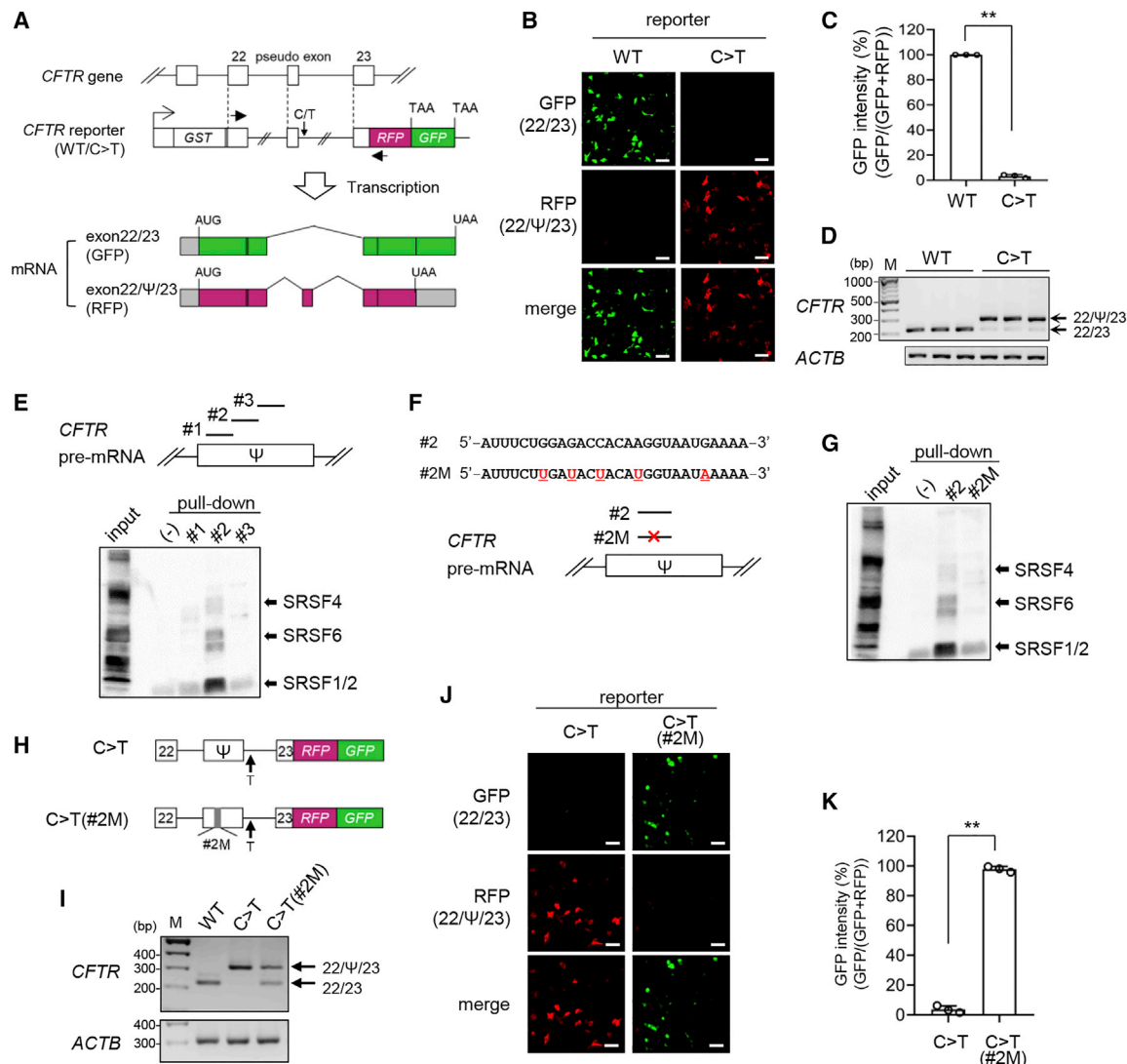


Figure 1. Recognition of the 3,849 + 10 kb C>T-induced *CFTR* Pseudo Exon Requires an SRSF-Dependent ESE

(A–C) (A) Diagram of the *CFTR* splicing reporter. The *CFTR* minigene of exon 22–23 with a wild-type (WT reporter) or the 3,849 + 10 kb C>T-harboring (C>T reporter) IVS22 with a partial truncation (see STAR Methods for details) is fused with the amino-terminal GST and carboxyl-terminal RFP and GFP. Filled arrow, primer for RT-PCR. (B and C) Representative microscopic images of HEK293 cells transfected with the WT or C>T reporter for 24 h (B), and quantification of GFP intensity (%) from the total GFP and RFP signal (C).

(D) RT-PCR for HEK293 cells transfected with the WT or C>T reporter vector for 24 h. *CFTR* splicing was detected by primer set oSS25 and oSS27, with the amplicon of 311 bp for the pseudo-exon-inclusion form (22/Ψ/23) and 228 bp for the skipping form (22/23). *ACTB* was detected by oAM13 and oAM14, serving as a loading control.

(E–G) Western blotting of phosphorylated SR proteins pulled down with biotin-conjugated RNA oligonucleotides (#1, #2, or #3 (E), shown in the diagram on the top, or #2 or #2M (G), their sequences shown in (F)) in HEK293 cell lysate. –, pull-down product without bait RNA. Mutated ribonucleotides are shown in red.

(H) Diagram showing the C>T and C>T(#2M) reporters. The C>T(#2M) reporter harbors point mutations of #2M shown in (F).

(I) RT-PCR for HEK293 cells transfected with the WT, C>T, or C>T(#2M) reporter vector for 24 h. *CFTR* splicing was detected with the primer set oSS25 and oSS27. *ACTB* was detected by oAM13 and oAM14, serving as a loading control.

(J and K) Representative microscopic images (J), and quantification analysis of percentage GFP intensity (K), for HEK293 cells transfected with the C>T or C>T(#2M) reporter for 24 h.

Scale bars, 50 μm in (B and J). 22/Ψ/23 and 22/23 stand for the pseudo exon inclusion and skipping products, respectively, in (B, D, and J). Each open circle represents the mean GFP intensity (%) of five random fields, and the mean ± SD from three independent experiments is shown in (C and K). **p < 0.01 (C and K).

lymphocytes from a patient homozygous for the 3,849 + 10 kb C>T mutation. As expected, CaNDY treatment dose-dependently restored the normal splicing patterns in patient B lymphocytes (Figure 3F). The transcripts of *CFTR* containing the pseudo

exon became visible only when cells were treated with cycloheximide (Figure 3F), indicating that the transcript is degraded by the nonsense-mediated mRNA decay triggered by a premature termination codon produced by pseudo exon inclusion in patient

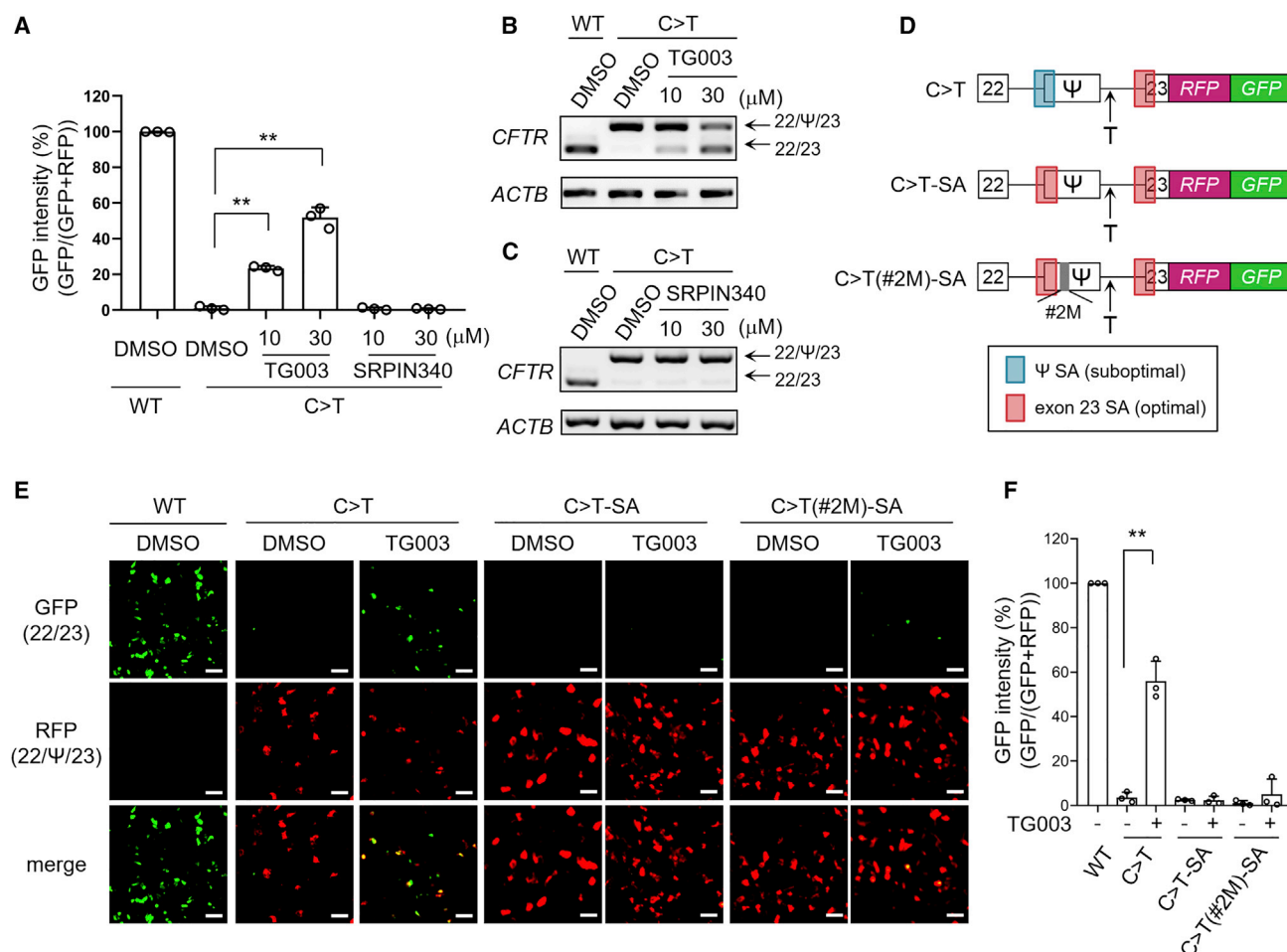


Figure 2. CLK Inhibitor Suppresses Inclusion of the *CFTR* Pseudo Exon in an ESE-Dependent Manner

(A) Percentages of GFP intensity are indicated for HEK293 cells transfected with the WT or C>T reporter for 6 h and treated with the indicated compounds or DMSO (0.1%) for 19 h.

(B and C) RT-PCR for HEK293 cells transfected with the WT or C>T reporter vector for 5 h and treated with the indicated compounds (B, TG003; C, SRPIN340) or DMSO (0.1%) for 19 h. *CFTR* splicing was detected by primers oSS25 and oSS27, and *ACTB* by oAM13 and oAM14.

(D) Diagrams indicating the C>T, C>T-SA, and C>T(#2M)-SA reporters. In the C>T-SA reporter, the pseudo-exonic splice acceptor (SA) is replaced by the conserved SA of exon 23. The C>T(#2M)-SA vector harbors the #2M point mutation in the background of the C>T-SA vector. All three vectors harbor the 3,849 + 10 kb C>T mutation.

(E and F) Representative microscopic images (E) and quantification of percentages of GFP intensity (F) for HEK293 cells transfected with the WT, C>T, C>T-SA, or C>T(#2M)-SA vector for 5 h and treated with TG003 (30 μM) or DMSO (0.1%) for 19 h.

Scale bars, 50 μm in (E). Each open circle represents the mean GFP intensity (%) of five random fields, and the mean ± SD from three independent experiments is shown in (A and F). **p < 0.01. 22/Ψ/23 and 22/23 stand for the pseudo exon inclusion and skipping products, respectively in (B, C, and E).

B lymphocytes. The identities of normal and the pseudo-exon-containing splicing products were confirmed by Sanger sequencing.

Having observed the higher potency of CaNDY compared with TG003 in suppressing the 3,849 + 10 kb C>T-driven pseudo exon, we then examined the underlying mechanism. We first assessed the contribution of the DYRK-inhibiting activity of CaNDY (Sonamoto et al., 2015) to *CFTR* pseudo exon suppression, by knocking down isoforms of *CLK* (*CLKs*) and *DYRK* (*DYRKs*) (Figure 3G and Table S1), and found that *DYRKs* had no or only minor effects on *CFTR* pseudo exon regulation, compared with the major role of *CLKs*. We then evaluated the IC₅₀ value in an *in vitro* kinase assay, and CaNDY showed a slightly higher activity than

TG003 against CLK1 (21.6 nM for CaNDY versus 26.2 nM for TG003) (Figure 3H), and approximately 4-fold higher activity against CLK2 (68.3 nM for CaNDY versus 309 nM for TG003) (Figure 3I). The most drastic difference was evident for CLK3, against which CaNDY shows a 116 nM IC₅₀, whereas TG003 had no inhibition activity up to 3 μM (Figure 3J). We therefore reasoned that the higher activity of CaNDY for pseudo exon suppression compared with TG003 likely resulted from a targeting ability against CLK3. Induced hypophosphorylation of SRSF1/2, SRSF4, and SRSF6 by CLK3 depletion also supports that CLK3 is among the major isoforms for SRSF phosphorylation (Figure 3K). Moreover, the expression level of CLK3 mRNA in human pulmonary tissue is high, according to the Expression Atlas

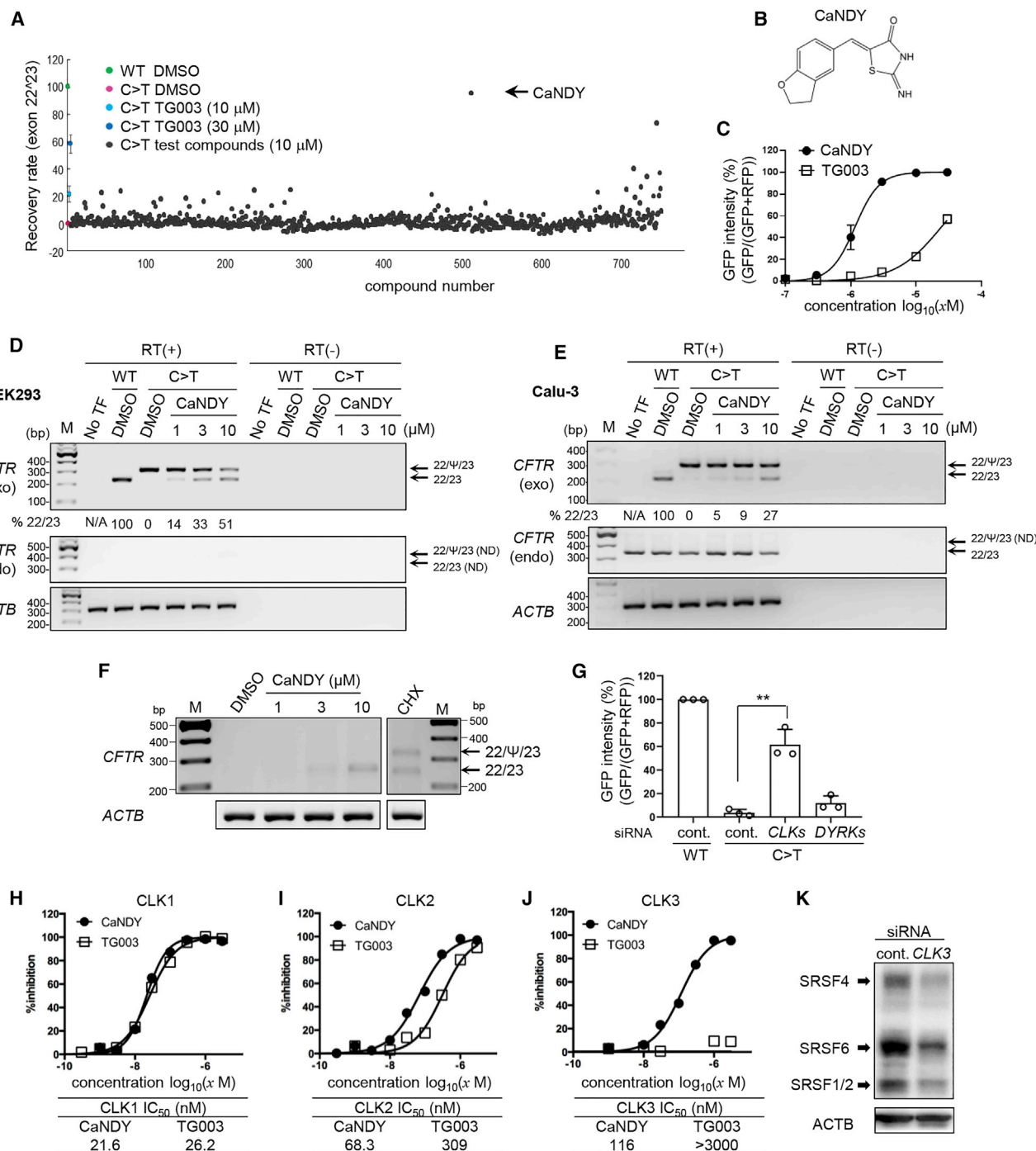


Figure 3. Identification of CaNDY as a Highly Potent Agent in Suppressing the 3,849 + 10 kb C>T-Induced Pseudo Exon of CFTR

(A) Scatterplot for the recovery rates of CFTR exon 22/23 splicing in the focused library screening of CLK inhibitor analogs.

(B) Structure of CaNDY.

(C) Sigmoidal dose-response curve for pseudo-exon-suppression activity of CaNDY and TG003 (0.1, 0.3, 1, 3, 10, or 30 μ M) in HEK293 cells transfected with the WT or C>T reporter and treated with the compounds for 13 h.

(D and E) RT-PCR analysis for HEK293 (D) and Calu-3 cells (E) transfected with the WT or C>T reporter for 5 h and treated with CaNDY (1, 3, or 10 μ M) or DMSO (0.1%) for 19 h. Untransfected cells were also analyzed as a negative control (No TF). Exogenous CFTR was detected by oSS25 and oSS27, and endogenous CFTR was detected by oAM619 and oSS30 (yielding a 430-bp product for pseudo exon inclusion and a 346-bp product for pseudo exon skipping). Rates of normal CFTR splicing (% 22/23) were calculated from the band intensities. ND indicates there was no detectable product after 35 cycles of PCR amplification.

(F) RT-PCR analysis for B lymphocyte GM11860 with CaNDY (1, 3, or 10 μ M), DMSO (0.1%), or cycloheximide (CHX) (100 μ g/mL) for 8 h. Endogenous CFTR was detected with the primers oSS29 and oSS30, yielding a 340-bp product for pseudo exon inclusion and a 256-bp product for pseudo exon skipping. Identities of the CFTR splicing products were confirmed by Sanger sequencing.

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(Papatheodorou et al., 2018). Finally, we examined responsiveness to CaNDY treatment for the C>T-SA and C>T(#2M)-SA reporters. As seen for TG003 (Figures 2D and 2E), we found that C>T-SA and C>T(#2M)-SA were no more responsive to CaNDY (Figure S2), indicating that CLK/SRSF dependency due to the presence of the suboptimal SA is required for responsiveness, which further supports the contention that splice modulation by CaNDY is mediated by its CLK-inhibition activity. We further confirmed that constitutive exons of CFTR without 3,849 + 10 kb C>T mutation in Calu-3 are unaffected by CaNDY treatment (Figure S3A), indicating its selective activity to the pseudo exon. Moreover, neither TG003 nor CaNDY affected exon 22²³ splicing from the CFTR minigene without the mutation (Figure S3B).

CaNDY Rescues Expression of Functional CFTR in the Context of the 3,849 + 10 kb C>T Mutation

CFTR minigenes have been applied for functional evaluation of splice-site and other premature-termination codon variants (Sharma et al., 2014, 2018; Lee et al., 2017). Here, in order to monitor the expression of full-length CFTR in the context of 3,849 + 10 kb C>T mutation, we constructed expression vectors in which a truncated intron 22 with or without the mutation was inserted between exons 22 and 23 of the CFTR coding sequence followed by a HiBiT tag (HiBiT_{WT} and HiBiT_{C>T} in Figure 4A) to emit luminescence upon incubation with LgBiT fragments and substrates. We confirmed that this system mimics pseudo exon splicing by 3,849 + 10 kb C>T mutation (Figure S4), and readily recapitulates restored full-length CFTR protein expression following CaNDY treatment in a dose-dependent manner, with an even level of EGFP cleaved at the 2A site (Figures 4B and 4C) in HEK293 cells.

We next examined the subcellular localization of CaNDY treatment-rescued CFTR with carboxyl-terminally fused AcGFP (CFTR_{WT}-GFP and CFTR_{C>T}-GFP in Figure 4D). When it was transfected into HEK293 cells, we observed the plasma membrane localization of CFTR_{WT}-GFP (CFTR_{WT}-GFP + DMSO in Figure 4E) and poor plasma membranous expression of CFTR_{C>T}-GFP (CFTR_{C>T}-GFP + DMSO in Figure 4E) despite successful vector delivery represented by mKate2 fluorescence. However, plasma membrane localization of CFTR_{C>T}-GFP was restored after 3 μ M CaNDY treatment (CFTR_{C>T}-GFP + CaNDY 3 μ M in Figure 4E). In contrast, VX-809, a CFTR folding modulator approved for a subset of CFTR missense mutations, including F508 deletion, in which trafficking of CFTR to the Golgi apparatus is impaired (Kallen et al., 2018), showed no effect on the 3,849 + 10 kb C>T of CFTR (CFTR_{C>T}-GFP + VX-809 10 μ M in Figure 4E), although restored plasma membrane localization of CFTR_{F508d}-GFP was confirmed with VX-809 (CFTR_{F508d}-GFP + VX-809 10 μ M in Figure 4E), but not CaNDY (CFTR_{F508d}-GFP + CaNDY 3 μ M in Figure 4E). Western blotting of CFTR_{WT}-GFP-expressing cells showed the dominant appearance of CFTR band C (complex-glycosylation form at Golgi apparatus) (white arrowheads

in Figure 4F) over band B (core-glycosylation form at the ER before trafficking to the Golgi) (black arrowheads in Figure 4F), whereas only band B of CFTR was detected in cells transfected with CFTR_{F508d}-GFP, as reported previously (Figure 4F) (Molinski et al., 2017). However, CaNDY treatment rescued the expression of band C from CFTR_{C>T}-GFP, whereas VX-809 could not (Figure 4F, left). In contrast, VX-809 slightly induced the expression of band C from CFTR_{F508d}-GFP, whereas CaNDY did not (Figure 4F, right), consistent with microscopic observations (Figure 4E).

Then, we examined the effect of CaNDY treatment on CFTR channel activity by CFTR-dependent halide influx assay (Merkert et al., 2019). In this assay, CFTR_{WT}-GFP or CFTR_{C>T}-GFP (Figure 4D) was co-transfected with YFP halide sensor vector, and a subsequent I⁻ stimulation induced CFTR-mediated cellular uptake of I⁻ that in turn quenched intracellular YFP fluorescence (Figure 4G). Individual quenching curves were also calculated for the maximal slope (s⁻¹) for a quantitative evaluation (Figure 4H). We obtained a significant reduction in CFTR activity for the CFTR_{C>T}-GFP (magenta line and dots) compared with the CFTR_{WT}-GFP (green line and dots), reflecting the pseudo exon inclusion. Strikingly, 3 μ M CaNDY treatment (blue line and dots) rescued CFTR channel activity from CFTR_{C>T}-GFP at an extent comparable to that of CFTR_{WT}-GFP (Figures 4G and 4H), demonstrating a restoration of functional CFTR expression. Thus, we propose a therapeutic strategy for CF using the 3,849 + 10 kb C>T, in which suppression of SRSF phosphorylation by the CLK inhibitor CaNDY retrieves functional CFTR expression by repressing pseudo exon inclusion (Figure 4I).

DISCUSSION

To compensate for a reduced activity of CFTR, several gene therapy approaches to express CFTR cDNA have been explored with viral or nonviral vectors (Sondhi et al., 2017). Despite promising results in animal models, fully curative effects have not been accomplished because of low expression levels of introduced CFTR (Moss et al., 2007; Alton et al., 2015). Recently a gene editing approach using AsCas12a was also tested to repair mutation of CFTR (Maule et al., 2019), although the application of gene editing for clinical therapeutics still takes time. Recent therapeutic advances were accomplished in CF treatments through the development of CFTR modulators, VX-809, a corrector to promote the ER-to-plasma membrane trafficking of CFTR (Van Goor et al., 2011), and VX-770, a potentiator to enhance impaired channel activity (Van Goor et al., 2011), which improve post-translational action of CFTR caused by missense mutations such as F508del and G551D (Virant-Young et al., 2015; Fajac and Wainwright, 2017) and thus cannot rescue pretranslational defects such as truncation or pseudo-exon-induced inactivation. Although CFTR potentiators are approved for some splice mutations of CFTR, the clinical efficacy is still modest, with an

(G) GFP intensity (%) in HEK293 cells transfected with small interfering RNAs (siRNAs) for CLK isoforms (CLKs for CLK1, CLK2, CLK3, and CLK4), DYRK isoforms (DYRKs for DYRK1A, DYRK1B, DYRK2, and DYRK3), or negative control (cont.) for 72 h and the WT or C>T reporter for 23 h. Each open circle represents the mean GFP intensity (%) of five random fields, and the mean $\pm$ SD from three independent experiments is shown. **p < 0.01.

(H–J) Dose-response curves for kinase inhibitory activity of CLK1 (H), CLK2 (I), and CLK3 (J) in *in vitro* kinase assay.

(K) Western blotting of phosphorylated SRSFs for HEK293 cells transfected with siRNA for CLK3 or the negative control for 96 h. ACTB blotting served as a loading control. ACTB was detected by primers oAM13 and oAM14, serving as loading control in (D, E, and F).

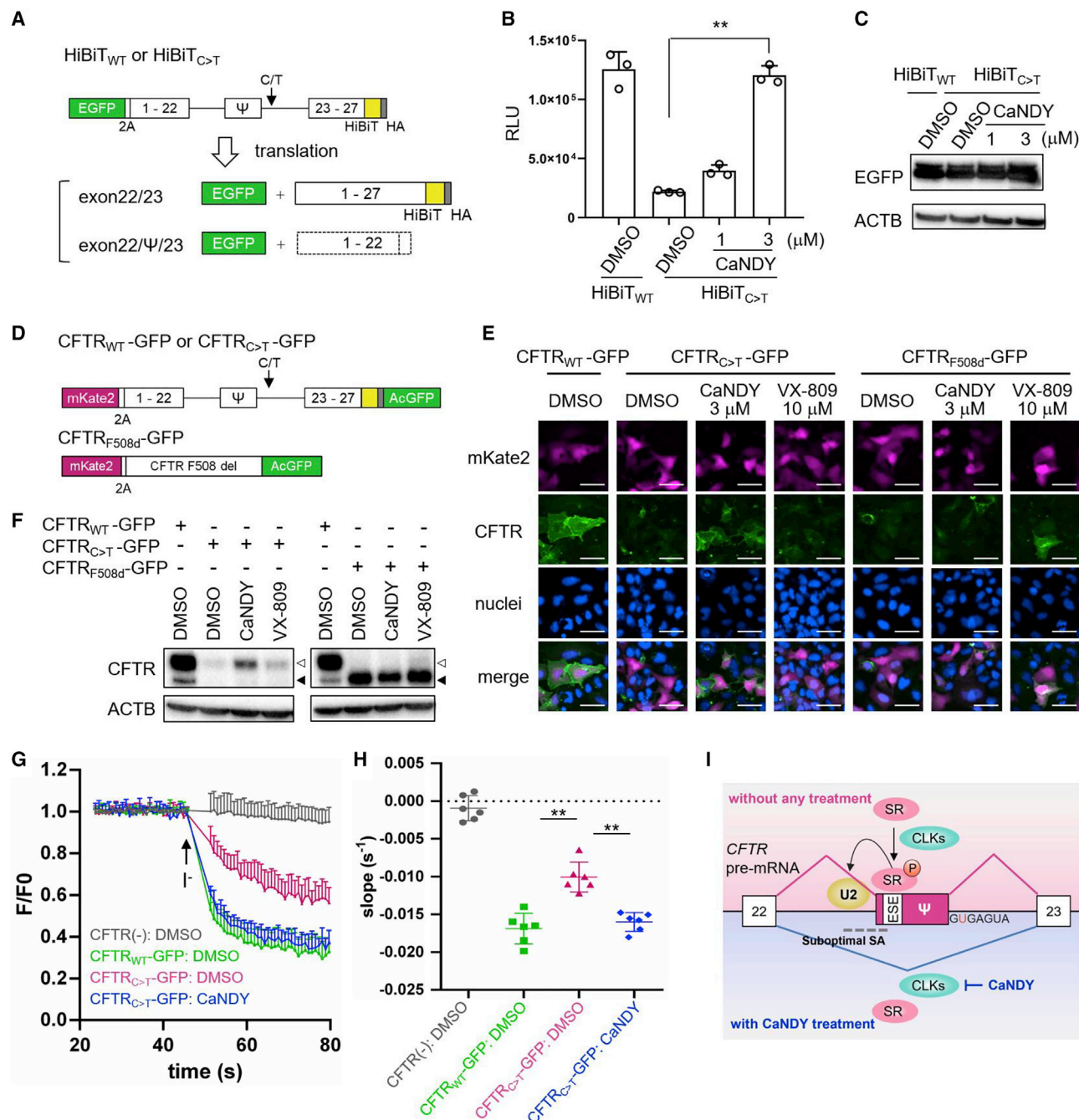


Figure 4. CaNDY, but Not CFTR Modulator VX-809, Rescues Mature CFTR Expression from the 3,849 + 10 kb C>T mutation

(A) Diagram of HiBiT_{WT} and HiBiT_{C>T} expression vectors. Upon translation, cleavage at the 2A site releases amino-terminal EGFP from the full-length or truncated CFTR with or without HiBiT-HA, respectively.

(B and C) HEK293 cells were transfected with HiBiT_{WT} or HiBiT_{C>T} for 5 h and treated with CaNDY (1 or 3 μ M) or DMSO (0.1%) for 48 h. Full-length expression of CFTR was quantified by relative luminescence units (RLU) generated by the C-terminal HiBiT tag using the Nano-Glo HiBiT Lytic Detection System (B), and expression of EGFP was confirmed by western blotting (C). Columns indicate mean $\pm$ SD for independent experiments ($n = 3$, indicated individually by circles).

(D) Structures of CFTR_{WT}-GFP, CFTR_{C>T}-GFP, and CFTR_{F508d}-GFP expression vectors are shown. CFTR_{WT}-GFP and CFTR_{C>T}-GFP vectors harbor a partial IVS22 of wild type or with the 3,849 + 10 kb C>T mutation, respectively, and CFTR_{F508d}-GFP expresses the F508 deletion form of CFTR. In these constructs, mKate2 with the 2A site is fused amino-terminally and AcGFP carboxyl-terminally with the CFTR coding sequence, and inclusion of the pseudo exon results in CFTR truncation without AcGFP translation.

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approximate 3%–10% increase in FEV1 due to a low level of normally spliced CFTR from pre-mRNA with the splice mutations (Rowe et al., 2017; Ramsey et al., 2011; Davies et al., 2018; Keating et al., 2018). In this regard, we expect that a combination of CFTR splice modifiers and previously mentioned CFTR correctors will provide better therapeutic effects for CF cases with 3,849 + 10 kb C>T mutations. In addition, the 3,849 + 10 kb C>T is not completely penetrant, and it is assumed that a partial splice correction may lead to sufficient clinical benefit (Chiba-Falek et al., 1998). Further investigations in patient primary organoids carrying the 3,849 + 10 kb C>T mutation may provide more insight for those clinical benefits of CaNDY.

The regulation of *CFTR* splicing has been extensively studied, and SR-protein-mediated inhibition of exon 9 inclusion (Buratti et al., 2001, 2007) and promotion of exon 12 (Haque et al., 2010; Pagani et al., 2003) have been reported in addition to regulation of the IVS22 pseudo exon shown in this study. The phosphorylation-dependent signaling catalyzed by CLKs and SRPKs is one of the important steps for the regulation of cellular localization and activity of SRSFs (Fu and Ares, 2014). We initially developed TG003 to analyze the role of CLK1 on splicing regulation (Muraki et al., 2004). Recently, CLK inhibitors have attracted extensive attention and have been developed, because the contribution of CLKs to a wide range of diseases, including cancers, has been revealed (Funnell et al., 2017; Fedorov et al., 2011; Iwai et al., 2018). However, CaNDY has unique features in comparison with previous CLK inhibitors: (1) Distinct inhibition profile: most CLK inhibitors, including TG003, show a lower IC₅₀ value for CLK1 than for other CLKs, but CaNDY potently suppresses CLK3 to a level comparable to other CLKs. As CLK3 has been distinguished by a distinct ATP-binding pocket structure compared with other CLK isoforms due to the presence of the 319th alanine (Kallen et al., 2018), such structural differences may reflect the profiles of most CLK inhibitors targeting the ATP-binding pocket. (2) Degradation inducer: CaNDY destabilizes DYRKs and CLKs in living cells by antagonizing interactions between the target kinase and the kinase-specific co-chaperone CDC37 (Sonamoto et al., 2015). These features may contribute to the high potency of CaNDY for suppression of the phosphorylation state of SRSF1/2, SRSF4, and SRSF6, and consequently to the splice-rectifying effect on the 3,849 + 10 kb C>T *CFTR* mRNA.

Genetic inactivation owing to a pseudo-exonic mis-splicing as observed in the case of 3,849 + 10 kb C>T *CFTR* is often found in the context of deep-intronic splicing mutations. The pseudo exons created by an intronic splicing mutation are in principle distinct from constitutive exons for their suboptimal splicing motifs as exemplified for *CFTR* in this study. The accumulation of

more data should indicate how generally pseudo exons are susceptible to CLK inhibitors due to their dependence on SRSFs for their efficient recognition. In fact, we recently reported a recurrent deep-intronic mutation for NEMO deficiency syndrome, and the consequent pseudo exon depends on CLK1/4 and SRSF6 for its recognition (Boisson et al., 2019), and further assessment of the CLK-SRSF pathway for a wide variety of pseudo exons is currently ongoing in our laboratory. Concerning off-target splicing caused by CaNDY, we checked the effect of CaNDY on other *CFTR* exons, but no difference in splicing pattern was observed (Figure S3A).

We further evaluated the preclinical properties of CaNDY to assess its clinical application for CF with the class V mutation, and it showed no genotoxic or cytotoxic effects at a concentration exceeding the EC₅₀, as determined for the CF reporter (Table S2). Moreover, CaNDY administration to rats showed no toxicity even at a concentration 100 times higher than the pharmacological dosage (data not shown). These results might reflect the small ratio of affected splicing by CaNDY. Although we have not counted the number of affected splice events, we previously observed that CLK inhibition mediated by TG003 impacts only ~0.3% of total splicing (Sakuma et al., 2015). There are an increasing number of pseudo-exon-related genetic diseases reported (Vaz-Drago et al., 2017), and recent advances in transcriptome studies led to the discovery of more pseudo-exonic mutations overlooked in past studies (Dufner-Almeida et al., 2019; Boisson et al., 2019; Gonorazky et al., 2016; Khan et al., 2017; Riant et al., 2014; Lee et al., 2017). In this regard, the CLK inhibitors proposed in this study will provide wider applications for numerous pseudo-exonic mutations. We, therefore, hope that our study described here will contribute to the establishment of mechanism-based splicing therapeutics for pseudo-exonic diseases.

SIGNIFICANCE

In this article, we propose a therapeutic strategy for pseudo-exonic genetic diseases with a small-molecule compound, using a class V pseudo-exonic mutation responsible for cystic fibrosis (CF) as a representative case. Pseudo exons are generated by deep-intronic mutations that result in recognition of particular intronic sequences as exons. Whereas a number of pseudo-exonic diseases have been discovered with recent advances in whole-genome sequencing and transcriptome analysis, mechanisms involved in their recognition remain poorly explored. However, it is essential to clarify molecular mechanisms of their

(E and F) Representative images by confocal fluorescence microscopy (E) and western blotting (F) for HEK293 cells transfected with the indicated expression vectors for 5 h and treated with CaNDY (3 μ M), VX-809 (10 μ M), or DMSO (0.1%) for 48 h. Scale bars, 50 μ m in (E). White and black arrowheads indicate CFTR band C (complex-glycosylation form) and band B (core-glycosylation form), respectively, in (F).

(G) Representative traces of YFP fluorescence changes. HEK293 cells were transfected with YFP halide sensor vector and the indicated expression vectors for 5 h and treated with CaNDY (3 μ M) or DMSO (0.1%) for 48 h. Arrows indicate iodide buffer addition. Cells were stimulated with 20 μ M forskolin for 10 min prior to addition of iodide.

(H) Statistical analysis of the maximal slopes of the fluorescence changes. Each dot represents an independent experiment, horizontal lines show the mean value and upper and lower lines show $\pm$ SD for independent experiments (n = 6).

(I) Mechanism of the *CFTR* IVS22 pseudo exon suppression by CaNDY. The 3,849 + 10 kb C>T mutation of the *CFTR* gene creates the pseudo exon splice donor. Recognition of the suboptimal SA of the pseudo exon is promoted by SRSF-dependent ESE activity and exon definition by downstream U1 recognition. Treatment with CaNDY or CLK inhibitors results in suppressed phosphorylation of SRSFs on the ESE, leading to induced skipping of the IVS22 pseudo exon of *CFTR* gene. **p < 0.01 in (B and H). ACTB served as a loading control in (C and F).

recognition for further consideration of therapeutic approaches for pseudo-exonic diseases. CF is a genetic disease caused by mutations in the *cystic fibrosis transmembrane conductance regulator (CFTR)* gene that compromise its chloride channel activity. The 3,849 + 10 kb C>T mutation of *CFTR*, accounting for up to ~2% of patients, is a deep-intronic mutation that creates a pseudo exon. In the present work, we characterized the mechanism of *CFTR* pseudo-exonic recognition and found an essential role for alternative splicing regulator serine/arginine-rich splicing factors, which are activated through phosphorylation by CDC-like kinases (CLKs). In addition, we identified a highly potent CLK inhibitor, CaNDY, in suppressing the pseudo-exonic recognition, and restoration of CFTR channel activity by CaNDY treatment was further demonstrated. This is the first report of a small-molecule-based approach rescuing a pretranslational defect of CFTR, and we believe this approach also stands as a precedent for mechanism-based personalized medicine for pseudo-exon-type genetic diseases.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

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AUTHOR CONTRIBUTIONS

M.A. and M.H. supervised the study. S.S., M.A., and M.H. designed experiments and wrote the paper. S.S. and M.A. performed biological experiments.

DECLARATION OF INTERESTS

S.S. is an employee of Kyorin Pharmaceutical Co., Ltd. M.H. is a founder, shareholder, and member of the scientific advisory board of KinoPharma, Inc., and BTB Drug Development Research Center Co., Ltd. M.H. filed patents PCT/JP2011/003059 for the SPREADD reporter and PCT/JP2011/003655 for TG003. M.H. and M.A. filed patent PCT/JP2018/006070 for CaNDY.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse monoclonal anti-SR proteins Antibody, clone 1H4G7	Thermo Fisher	Cat#33-9400; RRID:AB_87195
Mouse monoclonal anti-beta-Actin (AC-15) antibody	Santa Cruz Biotechnology	Cat# sc-69879; RRID: AB_1119529
Living Colors® A.v. Monoclonal Antibody (JL-8) anti-GFP antibody	Takara Bio	Cat# 632380; RRID: AB_10013427
HRP-conjugated goat polyclonal anti-mouse IgG antibody	Abcam	Cat# Ab5887; RRID:AB_955402
Chemicals, Peptides, and Recombinant Proteins		
VX-809	Chemscene	Cat# CS-0479
TG003	In house	N/A
SRPIN340	In house	N/A
CaNDY	In house	N/A
Focused chemical library of 747 in-house kinase inhibitors	In house	N/A
Critical Commercial Assays		
Nano Glo HiBiT Lytic Detection System	Promega	Cat# N3030
Experimental Models: Cell Lines		
HEK-293 human embryonic kidney cell line	ATCC	CRL-1573
HEK293A cell line	Thermo Fisher scientific	R70507
Calu-3 human lung adenocarcinoma epithelial cell line	ATCC	HTB-55
Human B lymphocyte	Coriell Institute	GM11860
CHL/IU Chinese hamster lung fibroblast	ATCC	CRL-1935
Oligonucleotides		
Refer to Tables S4 and S5		
Recombinant DNA		
pSS3_ WT reporter (CFTR exon 22-23 minigene)	This paper	N/A
pSS4_ C>T reporter (CFTR exon 22-23 minigene with 3849+10kb C>T)	This paper	N/A
pSS7_ C>T(#2M) (C>T reporter with #2M mutation)	This paper	N/A
pSS8_ C>T-SA (C>T reporter with modified pseudo exon SA)	This paper	N/A
pSS9_ C>T(#2M)-SA (C>T reporter with #2M mutation and modified pseudo exon SA)	This paper	N/A
pSS16_ HiBiT _{WT} (CFTR CDS+truncated IVS22 (WT) with N-terminal EGFP-GP2A and C-terminal HiBiT-HA)	This paper	N/A
pSS17_ HiBiT _{C>T} (CFTR CDS+truncated IVS22 (3849+10kb C>T) with N-terminal EGFP-GP2A and C-terminal HiBiT-HA)	This paper	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
pSS21_ CFTR _{WT} -GFP (CFTR CDS+truncated IVS22 (WT) with N-terminal mKate2-GP2A and C-terminal HiBiT-HA-AcGFP)	This paper	N/A
pSS22_ CFTR _{C>T} -GFP (CFTR CDS+truncated IVS22 (3849+10kb C>T) with N-terminal mKate2-GP2A and C-terminal HiBiT-HA-AcGFP)	This paper	N/A
pSS23_ CFTR _{F508d} -GFP (CFTR CDS (ΔF508) with N-terminal mKate2-GP2A and C-terminal AcGFP)	This paper	N/A
CFTR WT_HiBiT	Promega	N/A (Gift from Promega)
CFTR_ΔF508_HiBiT	Promega	N/A (Gift from Promega)
pME-mKate2 no stop vector	Fowler et al., 2016	Addgene plasmid # 80806 (pME-mKate2 no stop)
pcDNA3.1 EYFP H148Q/I152L	Galletta et al., 2001	Addgene plasmid # 25872 (pcDNA3.1 EYFP H148Q/I152L)
Software and Algorithms		
GraphPad Prism Version 8	GraphPad Software, San Diego, CA, USA	www.graphpad.com
Hybrid cell count BZ-H3C software	Keyence, Osaka, Japan	https://www.keyence.com/
Image Lab software	Bio-Rad Laboratories, Inc., Hercules, CA, USA	https://www.bio-rad.com/

RESOURCE AVAILABILITY

Lead Contact

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Materials Availability

Materials used in this study are available upon request under a completed materials transfer agreement.

Data and Code Availability

This study did not generate any additional datasets or code.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Cell Culture

HEK293 (human embryonic kidney-derived cells) (American Type Culture Collection, Manassas, VA, USA) and HEK293A (Thermo Fisher Scientific) were maintained in high glucose Dulbecco's modified Eagle's medium (Nacalai Tesque, Kyoto, Japan) supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA), 100 units/mL penicillin and 100 μg/mL streptomycin (Nacalai Tesque). Calu-3 (human lung adenocarcinoma-derived epithelial cells) (American Type Culture Collection) were maintained in MEM with Earle's Salts and L-glutamine medium (Nacalai Tesque) supplemented with 10% fetal bovine serum (Sigma-Aldrich), 1 mM sodium pyruvate solution (Nacalai Tesque), 100 units/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque). B lymphocyte GM11860 (Coriell Institute, Camden, NJ, USA) were maintained in RPMI1640 (Nacalai Tesque) supplemented with 15% fetal bovine serum (Sigma-Aldrich) and 100 units/mL penicillin, and 100 μg/mL streptomycin (Nacalai Tesque). Chinese hamster lung fibroblasts (CHL/IU) were cultured in Eagle's MEM (Thermo Fisher Scientific, Waltham, MA). Cells were grown at 37°C with 5% CO₂ in humid condition in tissue culture incubator.

METHOD DETAILS

Plasmids

A dual color vector for the splicing reporter of CFTR exon 22 to 23 with and without the 3849+10kb C>T mutation (referred as C>T (pSS4) or WT reporter (pSS3), respectively) was constructed as described previously, using the system of splicing reporter assay for disease genes with dual color (SPREADD) ([Boisson et al., 2019](#); [Yoshida et al., 2015](#); [Kuroyanagi et al., 2010](#)). With this reporter

system, the normal-type splicing (skipping of the pseudo exon) leads to the expression of a GFP-fusion protein. In this case, the RFP cDNA portion is translated with a frame different from the original RFP protein without any stop codon. However, pathogenic-type splicing (inclusion of the pseudo exon) results in the production of an RFP fusion protein and the ORF is discontinuous with GFP cDNA, as we previously reported (Yoshida et al., 2015). Upstream (nt 201-11,705) and downstream (nt 12,390-14,267) sequences of the CFTR pseudo exon were truncated referring to the minigene sequence by Friedman et al. (Friedman et al., 1999). In the SPREADD reporter of CFTR, A>C, A>G, and A>T were introduced at position 58, 67, and 114 of the exon 23, respectively, to avoid a premature stop codon, and the 48th A of the pseudo exon was deleted to induce frameshift for GFP/RFP switching. Similarly, stop codons from frame-shifted RFP were also substituted without affecting fluorescence intensity (Yoshida et al., 2015). The coding sequences from exon 22 to 23 with or without 3849+10kb C>T mutation are shown in Table S3. In the C>T-SA (pSS8) and C>T(#2M)-SA (pSS9), the pseudo exon SA sequence (IVS22+12,078- 12,112; 5'-ATA ATC ACC TTG TGG ATC TAA ATT TCA GTT GAC TT-3', exonic sequence is underlined) was replaced with the exon 23 SA sequence (5'-AAG TGA TCC CAT CAC TTT TAC CTT ATA GGT GGG CC-3', exonic sequence is underlined). CF (#2M) plasmid (pSS7) was prepared by introducing #2M point-mutations into pSS4. CAG promoter and bovine growth hormone polyadenylation signal were used in the plasmids.

For the HiBiT_{WT} (pSS16) and HiBiT_{C>T} (pSS17) vectors, the IVS22 sequence truncated upstream (nt 201- 11,704) and downstream (nt 12,390-14,267) sequences of the *CFTR* pseudo exon were inserted between exon 22 and 23 of the *CFTR* coding sequence (CDS), and subcloned into the pcDNA5 vector (Thermo Fisher Scientific, Waltham, MA, USA) with carboxyl-terminal HiBiT-HA and amino-terminal EGFP-GP2A. CFTR_{F508del}-GFP (pSS23), CFTR_{WT}-GFP (pSS21), and CFTR_{C>T}-GFP (pSS22) vectors were constructed by subcloning F508del-*CFTR* or the *CFTR* CDS with the IVS22 into the pAcGFPN1 plasmid (Clontech Laboratories, Inc. Mountain View, CA, USA) with carboxyl-terminal AcGFP and amino-terminal mKate2-GP2A. A synonymous mutation, ATTGAAAT to ATCGA-GAAC within exon 7 was introduced to rule out potential cryptic promoter. The F508del-*CFTR* expression vector and the HiBiT sequence was provided by Promega Corporation (Madison, WI, USA), and mKate2 was subcloned from pME-mKate2 no stop vector by Philip Washbourne (Addgene #80806) (Fowler et al., 2016). Plasmids used in this study were summarized in Key Resources Table.

Compounds

SRPK inhibitor SRPIN340, and CLK inhibitors TG003 and CaNDY, used in this study were synthesized following steps described in detail in our previous reports (Muraki et al., 2004 for TG003, Fukuhara et al., 2006 for SRPIN340, Sonamoto et al., 2015 for CaNDY). TG003, a mixture of commercially available 5-methoxy-2-methylbenzothiazole (202 mg, 1.12 mmol) and ethyl iodide (2.70 ml, 33.7 mmol) was refluxed for 24.5 h. The precipitate was filtrated, washed with ethyl acetate (20 ml) on a funnel, and dried under reduced pressure to afford 3-ethyl-5-methoxy-2-methylbenzothiazolium iodide (270 mg, 0.805 mmol, 71.9%) as a pale green solid. To a suspension of 3-ethyl-5-methoxy-2-methylbenzothiazolium iodide (502 mg, 1.49 mmol) in acetonitrile (2.0 ml), acetic anhydride (330 μ l, 3.49 mmol) and triethylamine (490 μ l, 3.51 mmol) were successively added at room temperature. After refluxing for 2 h, the mixture was cooled to room temperature and concentrated under reduced pressure. Water (50 ml) was added to the residue, and the mixture was extracted with ethyl acetate (three times with 15 ml). The combined organic extracts were washed with brine (30 ml), dried over Na₂SO₄, filtered, and concentrated under reduced pressure. The residue was purified by silica gel column chromatography (18 g, CH₂Cl₂/ethyl acetate, 4:1) to afford (Z)-1-(3-ethyl-5-methoxy-2, 3-dihydrobenzothiazol-2-ylidene) propan-2-one (TG003) (201 mg, 0.806 mmol, 54.1%) as a pale yellow solid. SRPIN340, piperidine (2.17 ml, 22.0 mmol) was added to a solution of 1-fluoro-2-nitro-4-(trifluoromethyl)benzene (2.09 g, 10.0 mmol) in *N,N*-dimethylformamide (DMF) (5 ml), and the mixture was stirred for 1 h at room temperature. Water was added to this solution, and the product was extracted with ethyl acetate. The combined organic extracts were washed with brine, dried (with Na₂SO₄), filtered, and concentrated under reduced pressure. The crude product was purified by silica-gel column chromatography (*n*-hexane/ethyl acetate = 16:1 $\approx$ 12:1) to give 1-[2-nitro-4-(trifluoromethyl)phenyl]piperidine (9.84 mmol, 98.4%); orange solid. To a solution of 1-[2-nitro-4-(trifluoromethyl)phenyl]piperidine (1.91 g, 6.96 mmol) in methanol (35 ml) was successively added concentrated HCl (7.0 ml, 84 mmol) and anhydrous SnCl₂ (6.63 g, 34.9 mmol) at 0°C, and the mixture was stirred for 18 h at room temperature. Saturated aqueous NaHCO₃ solution was added, and the product was extracted with ethyl acetate. The combined organic extracts were washed with brine, dried (with Na₂SO₄), filtered, and concentrated under reduced pressure. The crude product was purified by silica-gel column chromatography (*n*-hexane/ethyl acetate = 12:1) to give 1-[2-amino-4-(trifluoromethyl)phenyl]piperidine (1.67 g, 6.83 mmol, 98.4%); colorless solid. To a solution of 1-[2-amino-4-(trifluoromethyl)phenyl]piperidine (4.24 g, 17.4 mmol) in CH₂Cl₂ (10 ml) was successively added isonicotinoyl chloride hydrochloride (6.20 g, 34.8 mmol) and triethylamine (7.24 ml, 52.2 mmol) at 0°C, and the mixture was stirred for 0.5 h at room temperature. Water was added to this solution, and the product was extracted with CH₂Cl₂. The combined organic extracts were washed with brine, dried (with Na₂SO₄), filtered, and concentrated under reduced pressure. The crude product was purified by silica-gel column chromatography (*n*-hexane/ethyl acetate = 1:1) to give *N*-[2-(1-piperidinyl)-5-(trifluoromethyl)phenyl]isonicotinamide (SRPIN340) (5.49 g, 15.7 mmol, 90.2%); colorless solid. CaNDY, under an argon atmosphere, to a solution of 2,3-dihydrobenzofuran-5-carbaldehyde (296 mg, 2.00 mmol), pseudothiohydantoin (232 mg, 2.00 mmol), and ammonium acetate (77.0 mg, 0.999 mmol) in acetonitrile (2.0 ml) was added acetic acid (120 μ L, 2.10 mmol) at room temperature and the mixture was stirred with heating at 80°C (bath temperature) for 6 h. After cooling the reaction mixture to room temperature, the precipitate was collected by filtration using a funnel. The collected solid was washed on the funnel with water (3 mL $\times$ 4) and diethyl ether (3 mL $\times$ 2), and then dried under reduced pressure to afford (Z)-5-[[2,3-dihydrobenzofuran-5-yl]methylene]-2-iminothiazolidin-4-one (CaNDY) (492 mg, quantitative) as a pale yellow solid. The compound library for CLK inhibitor analogues was provided by Dr. Takamitsu Hosoya of Tokyo medical and dental university. VX-809 was purchased from Chemscene (Monmouth Junction, NJ, USA). Compounds were dissolved in

dimethylsulfoxide (DMSO) (Sigma-Aldrich) at 50 mM, except 25 mM for CaNDY, and kept at -80°C . Upon compound treatment of cells, each DMSO stock was diluted with DMSO to $\times 1,000$ of test concentration, and 1/1,000 of volume was added to culture media, resulting in 0.1% DMSO concentration in final. For a negative control, DMSO without compounds were added to cell culture to a final concentration of 0.1%.

Genotoxicity Test

Cell viability and small nuclear tests were conducted in Nihon Bioresearch Inc. (Gifu, Japan), using CHL/IU cells. Briefly, tests were examined in two conditions: i) 6 h of compound treatment with S9 fraction-containing media followed by 18 h of recovery in fresh media, and ii) continuous compound treatment for 24 h. 500, 250, 125 $\mu\text{g}/\text{ml}$ were used as test concentrations for CaNDY, and dimethylnitrosamine (DMN) at 500 $\mu\text{g}/\text{ml}$ was used as positive control. Frequency in chromosomal abnormality (numeric and structural) by microscopic analysis and cell number were determined in each condition. More than 5% of frequency in abnormality was considered as positive.

RNA Pull-Down Assay

RNA pull-down assay was conducted as described previously (Boisson et al., 2019; Ajiro et al., 2016). Briefly, the soluble fraction of the HEK293 cell lysate was prepared in lysis buffer (10 mM Tris-HCl (pH7.4), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% SDS, 0.25% sodium deoxycholate, and 10% glycerol, with a cocktail of protease (Nacalai Tesque) and phosphatase (Sigma Aldrich) inhibitors), and incubated with 5'-biotin, 3'-dTdT-attached RNAs (#1 (oSS R1), 5'-biotin-UUU CAG UUG ACU UGU CAU CUU GAU UUC U-dTdT -3'; #2 (oSS R2), 5'-biotin- AUU UCU GGA GAC CAC AAG GUA AUG AAA A-dTdT -3'; #3 (oSS R3), 5'-biotin- UGA AAA AUA UUA CAA GAG UCU UCC AUC-dTdT -3', #2M (oSS R7), 5'-biotin-AUU UCU UGA UAC UAC AUG GUA AUA AAA A-dTdT -3') for 16 h at 4°C , with rotation. RNA-bound fractions were washed three times with TBS, followed by elution with Laemmli buffer. The eluted proteins were analyzed by Western blotting. At least two biological replicates were performed for each assay.

SPREADD Reporter Assay

HEK293 (1.0×10^5 cells/well) or Calu-3 (2.0×10^5 cells/well) in a 12-well plate were transfected with the CFTR reporter plasmid by Lipofectamine 2000 (Thermo Fisher Scientific). Five h after transfection, the cells were treated with the indicated chemical compounds for 13 or 19 h, followed by fixation in 4% paraformaldehyde (Nacalai Tesque) and staining with Hoechst 33342 (Thermo Fisher Scientific). Fluorescence images were obtained with a BX-X710 microscope (Keyence, Osaka, Japan) and quantified using Hybrid cell count BZ-H3C software (Keyence). The cut-off values were set for GFP and RFP fluorescence to remove background noise. GFP intensity (%) was calculated as $\text{GFP intensity (\%)} = \{\text{total GFP intensity} / (\text{total GFP intensity} + \text{total RFP intensity})\} \times 100$. Three biological replications were performed for each assay. For screening the compound library, HEK293 cells (3×10^6 cells in 10-cm dish) were transfected with the WT or C>T reporter plasmid using Lipofectamine 2000 (Thermo Fisher Scientific). Five h after transfection, cells were seeded into a 96-well plate (5.0×10^4 cells/well) and incubated with the library compounds (10 μM). DMSO (0.1%) and TG003 (10 and 30 μM) were included in all plates as a baseline or positive control. DMSO concentration in all wells was kept at 0.1% of the final volume. Thirteen h after incubation, the cells were fixed with 4% paraformaldehyde and stained with Hoechst 33342 (Thermo Fisher Scientific). Fluorescence images were obtained as described above. Recovery rates of exon 22^Δ23 splicing were calculated by the following formula; $\{R(\text{C}>\text{T, cmpd}) - R(\text{C}>\text{T, DMSO})\} / \{R(\text{WT, DMSO}) - R(\text{C}>\text{T, DMSO})\} \times 100$. $R(\text{C}>\text{T, cmpd})$, GFP intensity (%) of the C>T reporter with a compound; $R(\text{C}>\text{T, DMSO})$, GFP intensity (%) of the C>T reporter with DMSO; $R(\text{WT, DMSO})$, GFP intensity (%) of the WT reporter with DMSO. Threshold of the z'-factor was set to > 0.5 for each plate. Compounds that suppressed the number of nuclei by $> 50\%$ compared to the DMSO control were excluded. Compounds shown higher Recovery rate than that of 10 μM of TG003 were confirmed their reproducibility.

Reverse Transcription-Coupled Polymerase Chain Reaction (RT-PCR)

HEK293 (1.0×10^5 cells/well) or Calu-3 (2.0×10^5 cells/well) in a 12-well plate were transfected with the WT or C>T reporter plasmid using Lipofectamine 2000 (Thermo Fisher Scientific). B lymphocyte GM11860 (5.0×10^5 cells/well) in a 12-well plate were seeded. Five h after transfection (HEK293 or Calu-3) or after overnight incubation (B lymphocyte), the cells were treated with the indicated chemical compounds for 19 h (HEK293 or Calu-3) or 8 h (B lymphocyte), followed by total RNA extraction with the RNeasy mini kit (QIAGEN, Hilden, Germany) followed by DNase treatment following the manufacturer's instructions. Reverse transcription was conducted using SuperScript II Reverse Transcriptase (Thermo Fisher Scientific) (HEK293 or Calu-3) or SuperScript IV Reverse Transcriptase (Thermo Fisher Scientific) (B lymphocyte) and oligo (dT)20 primers, followed by PCR with Ex Taq polymerase (Takara Bio, Inc., Shiga, Japan) (HEK293 or Calu-3) or TaqMan Gene Expression Master Mix (Thermo Fisher Scientific) (B lymphocyte). RT-PCR products were separated by electrophoresis. Ethidium bromide-stained images were captured using ChemiDoc (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and analyzed by Image Lab software (Bio-Rad Laboratories, Inc.). The following primers were used for semi-quantitative RT-PCR: 5'-TTC CTT CTC AAT AAG TCC TG-3' (oSS25, forward primer for CFTR exon 22), 5'-CGT CCT CGG AGG AGG CGA-3' (oSS27, reverse primer for RFP), 5'-GTT CAT TGA CAT GCC AAC AG -3' (oSS29, forward primer for CFTR exon 22), 5'-CTT CCC TGA TCC AGT TCT -3' (oSS30, reverse primer for CFTR exon 23), 5'-AGC CAT GAA TAT CAT GAG TAC A -3' (oAM619, forward primer for CFTR exon 21), 5'-CCA ACC GCG AGA AGA TGA CC-3' (oAM13, forward primer for ACTB exon 3), and 5'-AGC TTC TCC TTA ATG TCA CG-3' (oAM14, reverse primer for ACTB exon 4). In addition, primers used for constitutive CFTR splicing detection in Calu-3 in Figure S3A were summarized in Table S4. For real-time RT-PCR, the following

TaqMan gene expression assays by Thermo Fisher Scientific were used for detection: *CLK1* (Hs00964634_m1), *CLK2* (Hs02562748_s1), *CLK3* (Hs00999875_m1), *CLK4* (Hs00252917_m1), *DYRK1A* (Hs00176369_m1), *DYRK1B* (Hs01043777_m1), *DYRK2* (Hs00705109_s1), *DYRK3* (Hs00534092_m1), and *ACTB* (Hs01060665_g1). At least two biological replicates were performed for each assay.

RNA Interference

HEK293 (2.0×10^5 cells/well) in a 6-well plate were transfected with 100 nM of the indicated siRNAs using Lipofectamine RNAiMax (Thermo Fisher Scientific). Thirty one h after transfection, cells were reseeded into a 12-well plate (1.0×10^5 cells/well). Eighteen h after reseeding, cells were used for C>T reporter transfection for fluorescent analysis and RNA extraction for real-time PCR. The following siRNAs by Thermo Fisher Scientific were used in this study: Silencer Select Negative Control siRNA (4390846) as the control siRNA, s3162 for *CLK1*, s3165 for *CLK2*, s3169 for *CLK3*, s32988 for *CLK4*, s4400 for *DYRK1A*, s17490 for *DYRK1B*, s16027 for *DYRK2*, and s16026 for *DYRK3*. Each assay was confirmed reproducibility.

HiBiT and CFTR Subcellular Localization Assay

For the HiBiT assay, HEK293 cells (1.0×10^5 cells in 12-well plate) were transfected with the HiBiT_{WT} or HiBiT_{C>T} reporter plasmid using FuGENE HD Transfection Reagent (Promega). Five h after transfection, cells were incubated with CaNDY (final concentration was 3 μ M, 0.1% DMSO) or 0.1% DMSO control for 24 h, followed by reseeding into a 96-well plate (5.0×10^4 cells/well) and additional 24 h of incubation with CaNDY or 0.1% DMSO. Luminescence was subsequently measured using the Nano Glo HiBiT Lytic Detection System according to the manufacturer's instruction (Promega). Relative luminescence units (RLUs) were normalized to the cell number as determined by the WST-8 cell proliferation assay (Nacalai Tesque). Three biological replications were performed for each assay.

For the CFTR subcellular localization assay, HEK293 cells (1.0×10^5 cells in 12-well plate) were transfected with CFTR_{WT}-GFP, CFTR_{C>T}-GFP, or CFTR_{F508d}-GFP reporter plasmid using FuGENE HD Transfection Reagent (Promega). Five h after transfection, cells were treated with 3 μ M of CaNDY, 10 μ M of VX-809 (Chemscene), or 0.1% DMSO as a control for 19 h, before reseeding to a 96-well plate (5.0×10^4 cells/well). Following an additional 24 h of compound treatment, cells were fixed with 4% paraformaldehyde (Nacalai Tesque) and stained with Hoechst 33342 (Thermo Fisher Scientific). Fluorescence images were obtained with the Opera Phenix (Perkin Elmer, Waltham, MA, USA). Each assay was confirmed reproducibility.

Western Blotting

Total proteins were extracted from the cells with a sample buffer containing a reducing reagent (1 $\times$) (Nacalai Tesque) and protein concentrations were measured using the Pierce 660 nm Protein Assay added Ionic Detergent Compatibility Reagent (Thermo Fisher Scientific). Proteins were separated via 5-20% gradient SDS-PAGE and transferred to a poly-vinylidene fluoride membrane (Merck Millipore, Darmstadt, Germany) by electroblotting; chemiluminescence from the probed antibodies was detected using ChemiDoc (Bio-Rad Laboratories, Inc.). The following antibodies were used for Western blotting: anti-GFP mouse monoclonal antibody (JL-8) (Takara Bio, Inc.), anti-phospho SR protein mouse monoclonal antibody (1H4G7) (Thermo Fisher Scientific), and anti- β -actin mouse monoclonal antibody (Ac-15) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), and HRP-conjugated anti-mouse IgG goat polyclonal antibody as the secondary antibody (Abcam, Cambridge, UK).

In Vitro Kinase Assay

In vitro kinase assay was conducted by Carna Biosciences (Kobe, Japan). Briefly, GST-fused recombinant CLK1 (129-484th amino acids), CLK2 (full-length), and CLK3 (full-length) produced in a baculoviral system, were applied for the *in vitro* kinase assay with an off-chip mobility shift assay using a substrate peptide of DYRKtide-F (1 μ M) in the reaction buffer (20 mM HEPES (pH 7.5), 0.01% Triton X-100, 1 mM DTT, and ATP (10 μ M for CLK1, 150 μ M for CLK2 and 75 μ M for CLK3)). The final DMSO concentration was set to 1% after adding the DMSO solution in each compound. The kinase reaction was conducted for 1 h at room temperature followed by addition of the termination buffer (QuickScout Screening Assist MSA, Carna Biosciences). The entire reaction products were then applied to a LabChip system (Perkin- Elmer, Waltham, MA, USA), and kinase activity was determined by calculation of product rates ($P / (P + S)$) (P, product-derived peak; S, substrate-derived peak).

CFTR Channel Assay

HEK293 cells (1.0×10^5 cells in 12-well plates) were transfected with YFP halide sensor vector by Peter Haggie (Addgene #25872, pcDNA3.1 EYFP H148Q/I152L (Galletta et al., 2001)) and CFTR_{WT}-GFP or CFTR_{C>T}-GFP reporter plasmid using FuGENE HD Transfection Reagent (Promega). Five h after transfection, cells were treated with 3 μ M of CaNDY or 0.1% DMSO as a control for 19 h, before reseeding to a 96-well plate (5.0×10^4 cells/well). Following an additional 24 h of compound treatment, cells were washed three times with HBSS (Nacalai Tesque) and incubated for 10 min with 20 μ M Forskolin (Nacalai Tesque). YFP fluorescence intensity was analyzed for 80 s with 1 read/s in an ARVO X5 Model 2030 (Perkin Elmer). After 45 s, iodide buffer (139.5 mM NaI, 1.7 mM KH₂PO₄, 10.1 mM Na₂HPO₄ and 5 mM D-glucose) was automatically added and the change in YFP fluorescence intensity further monitored. Data were normalized using the formula: $F/F_0 = (\text{fluorescence after iodide buffer addition (F)} / \text{average fluorescence before iodide buffer addition (F}_0\text{)})$. All recorded traces were summarized via calculation of the maximal slope after iodide additions. Each study was performed in triplicate and confirmed reproducibility.

QUANTIFICATION AND STATISTICAL ANALYSIS

Data were analyzed using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

A paired Student's *t*-tests were used to determine the statistical difference between the means of two groups in [Figures 1C, 1K, 2F, 3G, and S2B](#). For [Figures 2A and 4B](#), groups were compared via two-way analysis of variance (ANOVA). Statistical details of individual experiments were indicated in figure legends. *p*-values less than 0.05 were considered statistically significant. The IC₅₀ values of TG003 and CaNDY were determined by fitting to a four-parameter logistic equation in [Figure 3B](#).

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Supplemental Information

**Mechanism-Based Personalized
Medicine for Cystic Fibrosis
by Suppressing Pseudo Exon Inclusion**

Saiko Shibata, Masahiko Ajiro, and Masatoshi Hagiwara

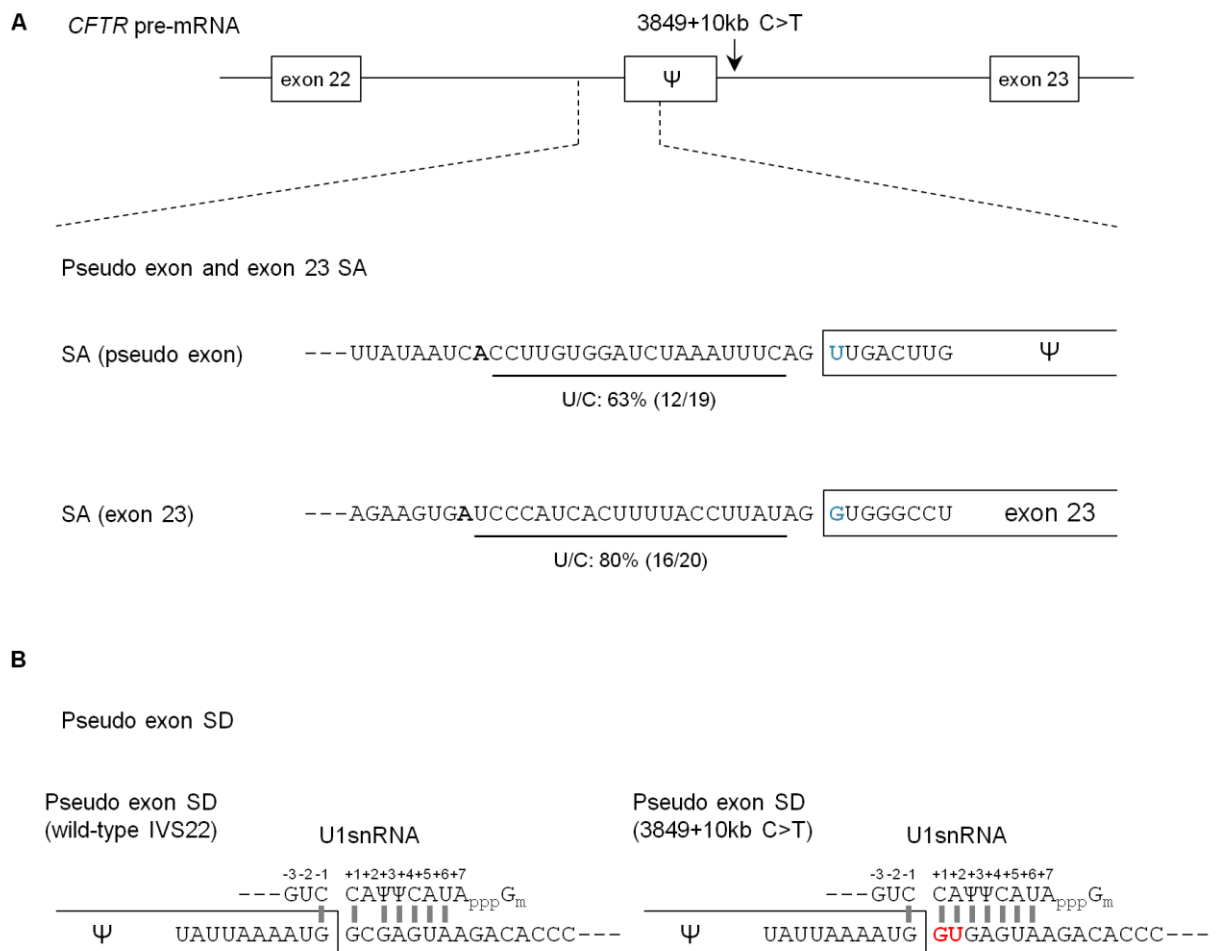


Figure S1. Splice site sequences of the *CFTR* pseudo exon. Related to Figure 1.

(A) The diagram indicates the *CFTR* pre-mRNA spanning exon 22 through exon 23. Splice acceptor (SA) of the *CFTR* pseudo exon (Ψ) and exon 23 are indicated. Branch site adenosine with a close match to yUnAy motif (Gao et al., 2008) are shown in bold capital letter. The splice acceptor site is shown in blue. The %U/C of the polypyrimidine tract is indicated for individual SA. (B) Splice donor (SD) of the *CFTR* pseudo exon (Ψ), with wild type IVS22 and the 3849+10kb C>T splicing mutation are shown with base-comparing to U1snRNA. Essential dinucleotides for SD recognition are shown in red.

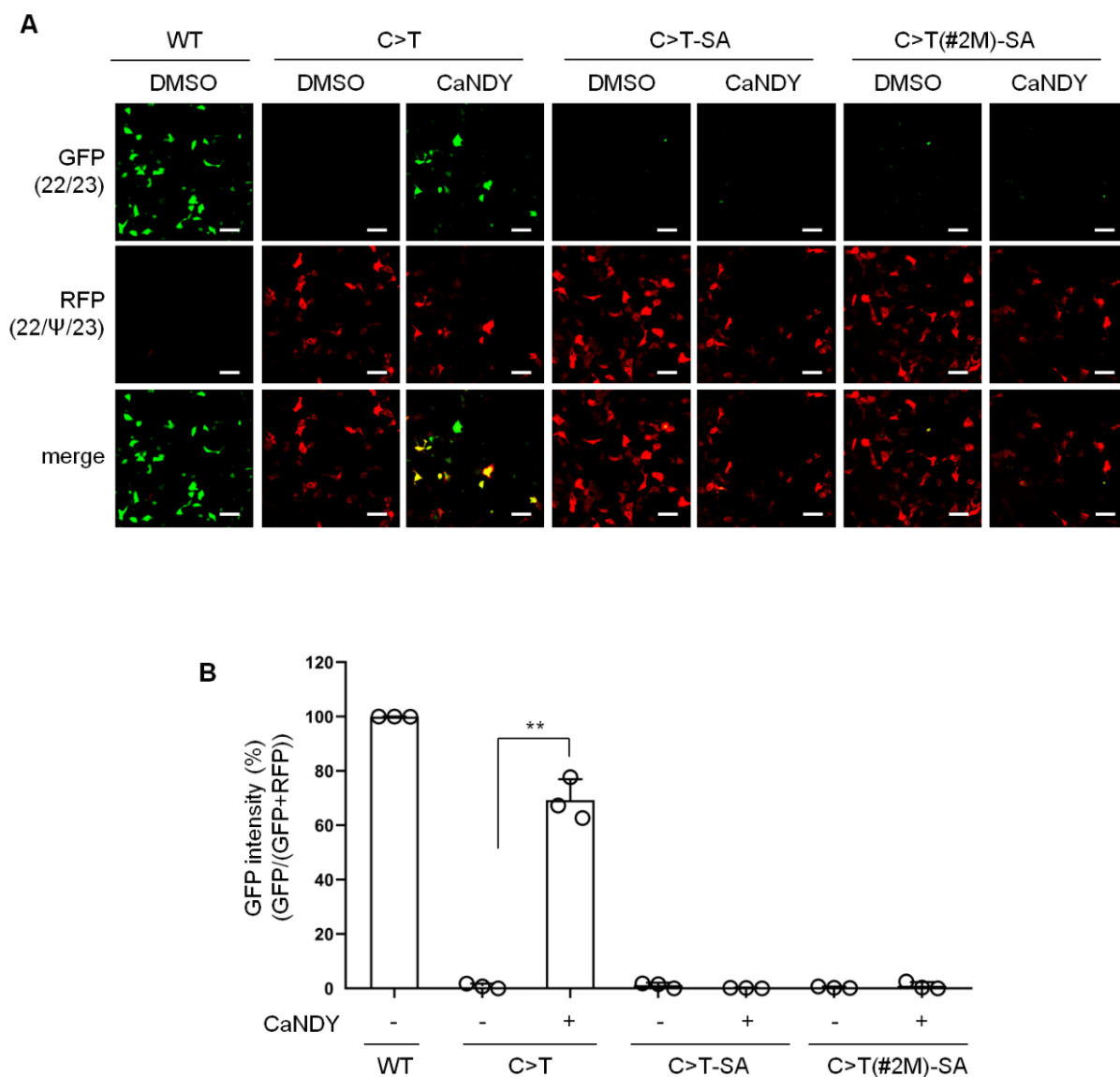


Figure S2. ESE-dependent effect of CaNDY on suppression of the pseudo exon in *CFTR*. Related to Figure 3. (A) and (B) Representative microscopic images (A) and quantification of percentage GFP intensity (B) in HEK293 cells transfected with the WT, C>T, C>T-SA, or C>T(#2M)-SA vector for 5 h followed by treatment with CaNDY (3 μ M) or DMSO (0.1%) for 19 h. Scale bars, 50 μ m in (A). Mean $\pm$ SD of three independent experiments (indicated individually with circles) are shown in (B). **, p-value < 0.01.

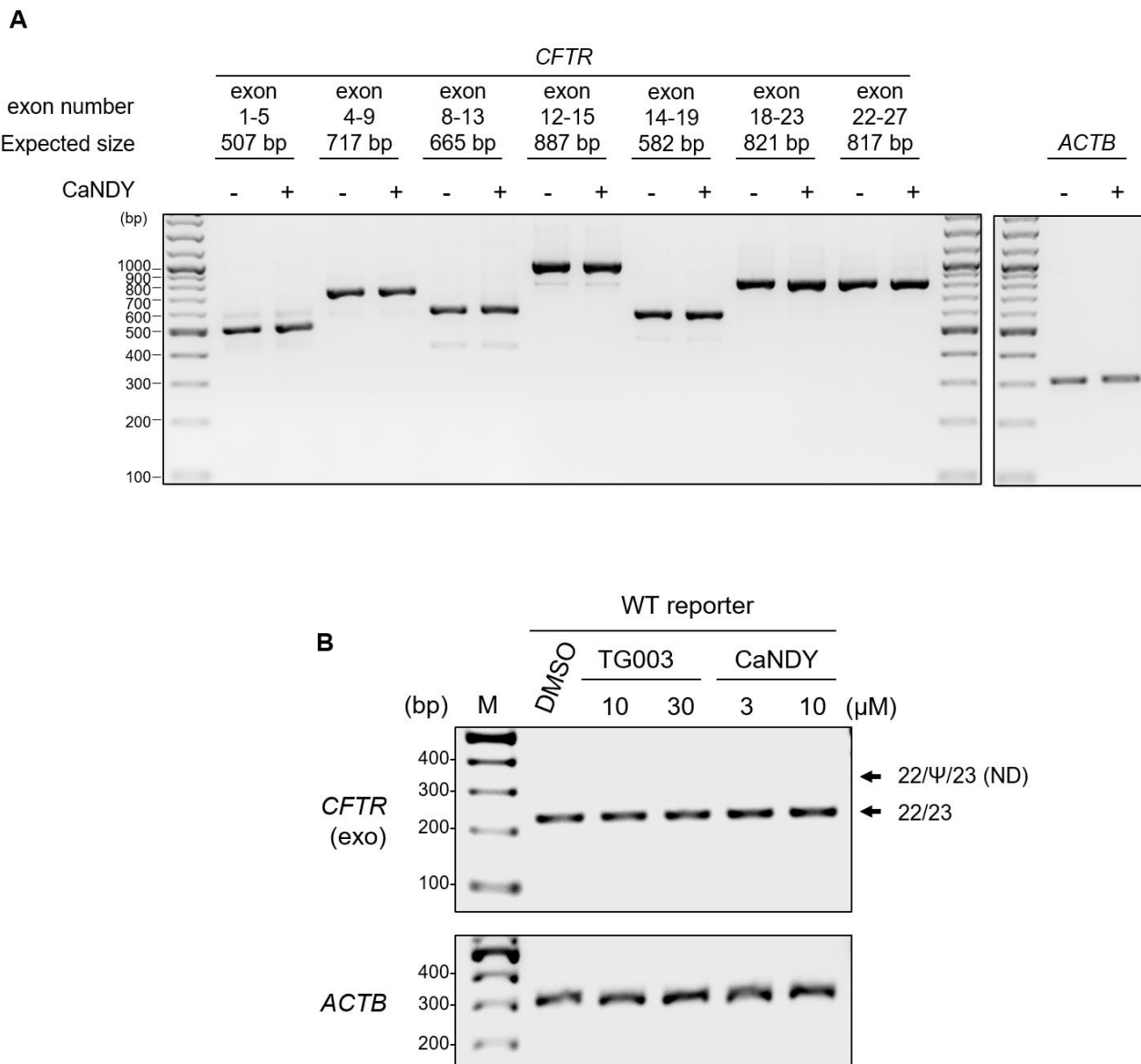


Figure S3. CLK inhibitors have no effect on *CFTR* without splice mutation. Related to Figure 1 and 3. (A) RT-PCR analysis for Calu-3 cells transfected with the C>T reporter for 5 h and treated with CaNDY (3 μM) or DMSO (0.1%) for 19 h. The numbers of exons amplified by specific primer combinations are indicated above the lanes. **(B)** Exogenously expressed *CFTR* was examined for splicing for WT reporter in HEK293 cells, treated with the indicated compounds for 24 h. DMSO (0.1%) served as a mock control. *CFTR* splicing was detected with the primer set oSS25 and oSS27. *ACTB* was detected as a loading control with the primer set oAM13 and oAM14. 22/Ψ/23, the *CFTR* splice product of exon 22, pseudo exon, and exon 23; 22/23, *CFTR* splice product of exon 22 and exon 23. (ND) indicates that RT-PCR products were not detectable.

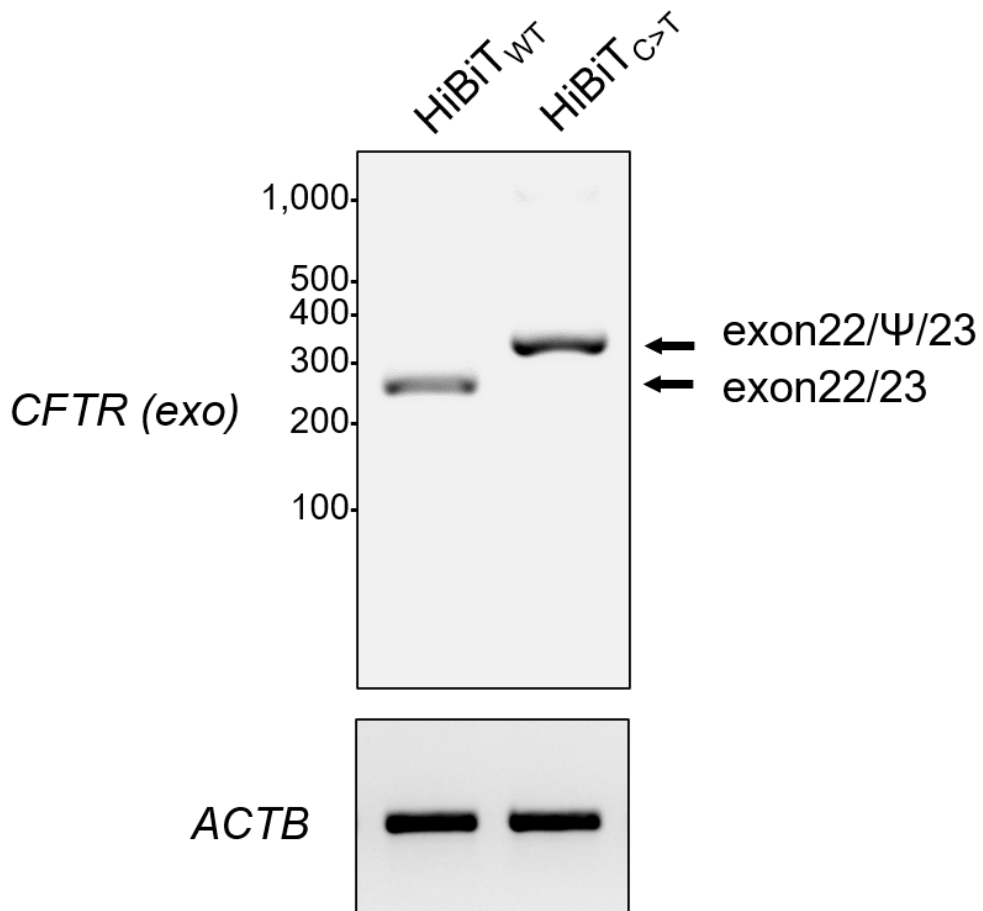


Figure S4. Confirmation of exogenous CFTR splicing expressed from HiBiT vectors. Related to Figure 1, 3, and 4. HEK293A cells, transfected with HiBiTWT or HiBiTC>T for 24 h, were analyzed by RT-PCR for CFTR splicing status. Exon 22^Δ23 was properly processed with HiBiTWT and HiBiTC>T without the previously reported synonymous mutation (Sharma et al., 2018). CFTR exon 22^Δ23 splicing was detected by the primer set oSS29 and oSS30, and ACTB expression was detected by oAM13 and oAM14.

Table S1. Knockdown efficiency of siRNAs used in this study in HEK293 cells. Related to Figure 3.

siRNA	Expression (vs. si-NS transfection)
<i>CLK1</i>	18.8%
<i>CLK2</i>	31.5%
<i>CLK3</i>	20.6%
<i>CLK4</i>	43.4%
<i>DYRK1A</i>	7.2%
<i>DYRK1B</i>	32.5%
<i>DYRK2</i>	23.6%
<i>DYRK3</i>	15.4%

Table S2. Summary of genotoxicity test for CaNDY. Related to Figure 4.

Substance	Conc. (µg/ml)	Exposure- recovery (h)	No. of cells (x10 ⁴ /plate)	Numerical aberration (%)	Structural aberration (%)	Result
DMSO	-	6-18	76.2	1.0	0.3	Negative
CaNDY	125	6-18	75.4	0.3	0.3	Negative
CaNDY	250	6-18	73.1	0.7	0.0	Negative
CaNDY	500	6-18	73.1	0	0.7	Negative
DMN	500	6-18	N/D	0.3	53.7	Positive

Substance	Conc. (µg/ml)	Exposure- recovery (h)	No of cells (x10 ⁴ /plate)	Numerical Aberration (%)	Structural Aberration (%)	Result
DMSO	-	24-0	66.9	0.3	1.0	Negative
CaNDY	125	24-0	63.8	0.0	0.7	Negative
CaNDY	250	24-0	66.1	0.0	0.7	Negative
CaNDY	500	24-0	66.9	1.0	0.7	Negative

Table S3. Coding sequences from exon 22 to 23 of WT or C>T reporters. Related to STAR methods.

WT reporter	
Coding sequence (exon 22/23)	(GST←) GTG CGA TCT GTG AGC CGA GTC TTT AAG TTC ATT GAC ATG CCA ACA GAA GGT AAA CCT ACC AAG TCA ACC AAA CCA TAC AAG AAT GGC CAA CTC TCG AAA GTT ATG ATT ATT GAG AAT TCA CAC GTG AAG AAA GAT GAC ATC TGG CCC TCA GGG GGC CAA ATG ACT GTC AAA GAT CTC ACA GCA AAA TAC ACA GAA GGT GGA AAT GCC ATA TTA GAG AAC ATT TCC TTC TCA ATA AGT CCT GGC CAG AGG GTG GGC CTC TTG GGA AGA ACT GGA TCA GGG AAG AGT ACT TTG TTA TCA GCT TTT TTG CGA CTA CTG GAC ACT GAA GGA GAA ATC CAG ATC GAT GGT GTG TCT TGG GAT TCA ATT ACT TTG CAA CAG TGG AGG AAA GCC TTT GGA GTA (→RFP (not in frame without stop codon)+ GFP)
Amino acid sequence (exon 22/23)	(GST←)VRSVSRVFKFIDMPTEGKPTKSTKPYKNGQLSKVMIENSHVK KDDIWPSGGQMTVKDLTAKYTEGGNAILENISFSISPGQVRVGLLGRGTS GKSTLLSAFLRLLDTEGEIQIDGVSWDSITLQQWRKAFGV (→RFP (not in frame without stop codon)+ GFP)
C>T reporter	
Coding sequence (exon 22/ψ/23)	(GST←) GTG CGA TCT GTG AGC CGA GTC TTT AAG TTC ATT GAC ATG CCA ACA GAA GGT AAA CCT ACC AAG TCA ACC AAA CCA TAC AAG AAT GGC CAA CTC TCG AAA GTT ATG ATT ATT GAG AAT TCA CAC GTG AAG AAA GAT GAC ATC TGG CCC TCA GGG GGC CAA ATG ACT GTC AAA GAT CTC ACA GCA AAA TAC ACA GAA GGT GGA AAT GCC ATA TTA GAG AAC ATT TCC TTC TCA ATA AGT CCT GGC CAG AGG TTG ACT TGT CAT CTT GAT TTC TGG AGA CCA CAA GGT AAT GAA AAA TAT TAC AAG AGT CTT CCA TCT GTT GCA GTA TTA AAA TGG TGG GCC TCT TGG GAA GAA CTG GAT CAG GGA AGA GTA CTT TGT TAT CAG CTT TTT TGC GAC TAC TGG ACA CTG AAG GAG AAA TCC AGA TCG ATG GTG TGT CTT GGG ATT CAA TTA CTT TGC AAC AGT GGA GGA AAG CCT TTG GAG (→RFP)
Amino acid sequence (exon 22/ψ/23)	(GST←)VRSVSRVFKFIDMPTEGKPTKSTKPYKNGQLSKVMIENSHVK KDDIWPSGGQMTVKDLTAKYTEGGNAILENISFSISPGQRLTCHLDFWR PQGNEKYKSLPSVAVLKWWASWEELDQGRVLCYQLFCDYWTLEKESRS MVCLGIQLLCNSGGKPLE(→RFP)

**Table S4. Primers used to investigate splicing of all *CFTR* exons in Calu-3.
Related to STAR methods.**

Target exons	Primer name, orientation, and sequence	
1 to 5	oSS123, forward	5'-ATGCAGAGGTCGCCTCTG-3'
	oSS192, reverse	5'-GCTTGACAGCTTTAAAGT-3'
4 to 9	oSS43, forward	5'-TGGAATGCAGATGAGAATAG-3'
	oSS194, reverse	5'-AAGTTATATTCCAATGTCTTATAT-3'
8 to 13	oSS195, forward	5'-CTGTACAAACATGGTATGAC-3'
	oSS196, reverse	5'-TAGGTATCCAAAAGGAGAGTCTAA-3'
12 to 15	oSS197, forward	5'-CTGAGTGGAGGTCAACGA-3'
	oSS198, reverse	5'-ACTGCTGGTATGCTCTCC-3'
14 to 19	oSS199, forward	5'-AGAAACTGGCTTGAAAATAA-3'
	oSS200, reverse	5'-ACAACTGCTATAGCTCCA-3'
18 to 23	oSS201, forward	5'-GGATGACCTTCTGCCTCTTA-3'
	oSS119, reverse	5'-AAGCTGATAACAAAGTACTCTTCC-3'
22 to 27	oSS16, forward	5'-CCAAATGACTGTCAAAGATCTCACAGCA-3'
	oSS202, reverse	5'-AAGCCTTGTATCTTGCAC-3'

Table S5. List of oligonucleotides used in this study. Related to STAR Methods.

Name	Comments	Sequences
oSS25	forward primer for <i>CFTR</i> exon 22	5'-TTCCTTCTCAATAAGTCCTG-3'
oSS27	reverse primer for <i>RFP</i>	5'-CGTCCTCGGAGGAGGCGA-3'
oSS29	forward primer for <i>CFTR</i> exon 22	5'-GTTTCATTGACATGCCAACAG-3'
oSS30	forward primer for <i>CFTR</i> exon 23	5'-CTTCCCTGATCCAGTTCT-3'
oAM619	forward primer for <i>CFTR</i> exon 21	5'-AGCCATGAATATCATGAGTACA-3'
oAM13	forward primer for <i>ACTB</i> exon 3	5'-CCAACCGCGAGAAGATGACC-3'
oAM14	reverse primer for <i>ACTB</i> exon 4	5'-AGCTTCTCCTTAATGTCACG-3'
oSS R1	RNA #1 for RNA pull-down assay	Biotin-5'-UUUCAGUUGACUUGUCAUCUUGAUUUUCUdTdT-3'
oSS R2	RNA #2 for RNA pull-down assay	Biotin-5'-AUUUCUGGAGACCACAAGGUAAUGAAAAdTdT-3'
oSS R3	RNA #3 for RNA pull-down assay	Biotin-5'-UGAAAAAUUUACAAGAGUCUCCAUCdTdT-3'
oSS R7	RNA #2M for RNA pull-down assay	Biotin-5'-AUUUCUUGAUACUACAUGGUAAUAAAAdTdT-3'