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Original Article

# Correcting CFTR mRNA splicing defects with the plant cytokine kinetin and its analogues

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ABSTRACT

*Background:* Cystic Fibrosis (CF) results from *CFTR* gene mutations, including splicing defects such as the polymorphic TGnTm repeat, which disrupts exon-10 inclusion and contributes to CF monosymptomatic forms. While recent advances in CF treatment have led to targeted therapies for specific CFTR defects, most splicing variants remain without an effective treatment. Small molecules, like the plant cytokine kinetin, have shown promise in correcting splicing defects in other genetic diseases, offering potential for personalized CF therapies.
*Methods:* This study evaluated the efficacy of kinetin and its analogue, RECTAS, in correcting *CFTR* exon-10 splicing defects caused by TGnTm repeats. Cell models and patient-derived cells were treated with both compounds to assess their ability to enhance exon-10 inclusion. The impact of these treatments on splicing correction and CFTR protein expression was analyzed using molecular and cellular assays.
*Results:* Both kinetin and RECTAS improved exon-10 inclusion, with RECTAS demonstrating superior efficacy, achieving up to a four-fold increase in patient-derived cells compared to kinetin. Additionally, RECTAS consistently rescued exon-10 splicing across various TG-T alleles and successfully restored CFTR protein expression, highlighting its potential as a more potent therapeutic option.
*Conclusions:* These findings identify RECTAS as an effective modulator of *CFTR* splicing. Rather than stand-alone therapeutics, kinetin and its analogues may act as transcript amplifiers, thereby possibly enhancing the efficacy of existing CFTR modulators. This approach could broaden treatment options for splicing-related *CFTR* variants and other genetic disorders.

## 1. Introduction

The *CFTR* gene is approximately 200-Kb long and is composed of 27 exons that are spliced into a 6.5-Kb mRNA [1]. More than 2000 *CFTR* variants have been identified so far (p.Phe508del being the most frequent); the majority of these alterations are distributed within the coding sequence, and about 12 % are splicing variants [Database: www.genet.sickkids.on.ca]. A frequent polymorphism (TGnTm) located at the end of intron 9 (formerly known as intron 8) is known to influence the splicing efficiency of exon 10 [2]. Alleles bearing less T repeats and more TG repeats are associated with a lower rate of exon-10 inclusion, which can be reduced to the 10 % of the wild type in the most severe cases. The skipping of exon 10 leads to an in-frame transcript that encodes for a misfolded and nonfunctional protein [3,4]. Expression of this protein

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isoform in combination with a CF-causing variant *in trans* can be associated with: i) overt CF (rarely); ii) *CFTR*-related disorders (CFTR-RD), like congenital bilateral absence of the vas deferens (CBAVD); or iii) *CFTR*-related metabolic syndrome/cystic fibrosis screen positive, inconclusive diagnosis (CRMS/CFSPID) [5]. Moreover, the 5T allele is also known to modify the expressivity of the *CFTR* p.Arg117His variant when present *in cis* [6].

Currently, drugs targeting specific classes of molecular defects, such as potentiators and correctors, have been developed to act on cellular processing and channel function, respectively, and are opening a new era of personalized CF treatment [7,8]. With few exceptions [9], such therapies are lacking for patients with splicing defects. These include severe mutations that abolish CFTR production, and milder mutations that reduce proper splicing, leading to variable symptoms inversely related to splicing efficiency [6]. Several strategies have aimed to enhance normal splicing, including antisense oligonucleotides, trans-splicing, and modified U1/U7 snRNAs. While effective in cell models and promising in trials, these approaches face challenges such as delivery, duration, side effects, and toxicity—highlighting the need for improved or alternative therapies [10–13].

Ideally, non-toxic, orally-deliverable compounds, which selectively modify RNA splicing, would represent a better option to correct splicing defects. Recent studies reported the successful use of small molecules modulating splicing in different Mendelian conditions, like familial dysautonomia (FD), neurofibromatosis type 1, and spinal muscular atrophy (SMA) [14–16]. Among effective compounds, kinetin, a plant cytokinin used topically for its antiaging properties, was shown to modulate splicing in several genes [14–17]. Kinetin effectively corrected the most common FD-related splicing defect in IKBKAP exon-20 and, when orally administered to eight homozygous patients, significantly increased wild-type IKBKAP mRNA levels in leukocytes. Finally, kinetin treatment was tolerated and safe even in this medically fragile population [18].

More recently, two different kinetin analogues, RECTAS and BPN-15477, were shown to better promote exon-20 inclusion in the same gene as well as splicing defects in other genes, including the c.2988G>*A* variant in *CFTR* [19,20].

The splicing-modulating activity of small molecules appears to be gene- or even exon-specific [21–23]. Whole-transcriptome analysis of kinetin confirmed its high specificity, affecting few targets, mainly splicing factors [24]. While this is promising for clinical use, predicting efficacy across different splicing defects remains challenging. In this study, we evaluated kinetin and its analogues as potential modulators of *CFTR* splicing dysregulation caused by TGnTm variants.

## 2. Materials and methods

This study was approved by the local Ethical Committee of Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico and was conducted according to the Declaration of Helsinki and to the Italian legislation on sensible data recording. Signed informed consent was obtained from all participants and from parents of subjects younger than 18 years.

### 2.1. Plasmid constructs

To study the effect of splicing modifiers on *CFTR* transcripts, we customized the RG6 plasmid (kindly provided by Prof. T.A. Cooper) [25], to contain *CFTR* exon 10. In particular, a gBlocks® Gene Fragment (IDT), comprising exon 10, a portion of exons 9 and 11, and the 3′ and 5′ portions of *CFTR* introns 9 and 10, was cloned into the RG6 vector by replacing the region containing the introns and exons flanking the chicken troponin exon 5 (sequence provided in Supplementary Methods). We produced variants of this vector with the different TG-T repeats ($TG_{12}T_3$, $TG_{15}T_5$, $TG_{13}T_5$, $TG_{11}T_5$, $TG_{11}T_7$, $TG_{10}T_9$) by site-directed mutagenesis using the QuikChange kit (Agilent Technologies).

The plasmid used to investigate the regulatory *in-cis* element CAA, was a kind gift of Prof. E. Buratti [26]. *CFTR* exon10, with its flanking intronic regions, was cloned into the hybrid α-globin-fibronectin minigene plasmid (modified pBS-KS). The constructs carrying an alternative nucleotide in the CAA motif were obtained by site-directed mutagenesis.

For the protein experiments, we cloned the same gBlocks® Gene Fragment with the $TG_{12}T_5$ repeat used for the RG6 construct in a pcDNA3.1 plasmid containing the full *CFTR* coding sequence (kindly provided by Prof. F. Pagani) [27].

### 2.2. Cell cultures and transfection experiments

Caco-2 and HeLa cells were cultured in RPMI-1640 and DMEM, respectively (Merck-Millipore). Both culture media were supplemented with 10 % FBS, L-glutamine (2 mM), and antibiotics (100U/ml penicillin and 100μg/ml streptomycin; Merck-Millipore). The immortalized Cystic Fibrosis airway epithelial cell line, CuFi-1, was cultured as monolayer in flasks precoated with collagen (collagen IV from human placenta; Merck-Millipore) in bronchial epithelial growth medium (BEGM; Lonza). Cells were grown at 37 °C in a humidified atmosphere of 95 % air and 5 % $CO_2$, according to standard procedures.

For transfection experiments, HeLa cells were used to avoid the confounding effect of endogenous *CFTR* transcript. We used Polyplus jetPRIME reagent (EuroClone) for RNA experiments, while lipofectamine 3000 (Thermo Fisher Scientific) for protein analysis.

Nasal brushing samples were collected using a soft sterile cytology brush. After nasal lavage with physiological saline, brushing was performed in both nostrils by gentle circular movements. Freshly-isolated nasal epithelial cells were immersed in washing solution (PBS, DTT 2 mM, EDTA 10 mM) at 37 °C for 1 h on shaking, and then centrifuged at 2300 g for 20 min. Isolated cells were maintained in Bronchial Epithelial Cell Growth Medium supplemented with 10 % FBS and antibiotics.

Cell lines and primary cultures were treated with compounds at a 2–200 μM final concentration for 8–96 h.

### 2.3. Fluorescent-competitive RT-PCR, digital RT-PCR, and semi-quantitative real-time RT-PCR

Total RNA was extracted from cells using the Eurozol reagent (EuroClone) according to manufacturer's instructions. Five hundred ng of total RNA was reverse-transcribed (RT) using the Superscript-III Reverse Transcriptase (Thermo Fisher Scientific) and Random hexamers (Promega).

To measure the relative amount of *CFTR* splicing variants, fluorescent-competitive RT-PCRs were performed using a 6-FAM-labeled primer (Fig. 1A, B). Fragments were separated by capillary electrophoresis and peak areas measured by the GeneMapper software (Thermo Fisher Scientific). The percentage of each isoform was measured by calculating the ratio of the relevant peak over the sum of all the fluorescence peak areas (set as 100 %).

Digital PCR was performed to absolute quantify *CFTR* transcripts in primary cells using the QuantStudio™ 3D Digital PCR Master Mix (Thermo Fisher Scientific) and a custom TaqMan® assay spanning exons 10–11 (Fig. 1C); the Hydroxymethylbilane Synthase (*HMBS*) mRNA was co-amplified for normalization. Reactions were run on the QuantStudio™ 3D Digital PCR System (Thermo Fisher Scientific) using standard 40-cycle conditions. Data were analyzed with the QuantStudio™ 3D AnalysisSuite software according to the manufacturer's instructions. All measurements showed <5 % standard error, confirming assay precision.

Semi-quantitative real-time RT-PCRs were carried out using the SYBR Premix Ex Taq II (TAKARA) on a LightCycler480 (Roche).

### 2.4. Short-read RNA sequencing

Total RNA was extracted from untreated or treated CuFi-1 cells (three replicates per condition) using Eurozol (EuroClone). Libraries were prepared from 500 ng of total RNA with the TruSeq® Stranded Total RNA kit (Illumina) and sequenced (150 bp paired-end) on a NextSeq500 system. Reads were aligned to the human reference genome (hg19) using STAR v2.7.1a, and gene counts were generated with htseq-count v0.11.2. Differential expression between treated and control samples was analyzed using DESeq2. Alternative splicing events were identified with rMATS v3.2.5, considering five major splicing types (Skipped Exon, SE; Mutually Exclusive Exons, MXE; Alternative 5′ Splice site, A5SS; Alternative 3′ Splice site, A3SS; Retained Intron, RI) and selecting events with false discovery rate (FDR)<0.05 and ≥10 % inclusion difference. RNA-seq data are publicly available in the Zenodo repository (DOI: 10.5281/zenodo.14620982). A detailed description of the RNA-seq analysis workflow is provided in the Supplementary Methods.

### 2.5. Western blot experiments

HeLa cells were lysed in RIPA buffer (150 mM NaCl, 1 % Triton X-100, 0.5 % deoxycholic acid, 0.1 % SDS, 20 mM Tris–HCl, pH 7.4), supplemented with protease inhibitor cocktail (Roche). BCA protein assay (Thermo Fisher Scientific) was used to quantitate protein levels before SDS-PAGE. Western blots were performed using anti-CFTR antibody MM13–4 (Merck-Millipore) and ECL substrate (Thermo Fisher Scientific). Blots were imaged with ChemiDoc (Bio-Rad) and quantified using ImageJ (https://imagej.net/).

### 2.6. Long-read RNA sequencing

Caco-2 RNA samples from at least 3 experiments were pooled and poly-A transcripts were selected using the Magnetic mRNA Isolation Kit (NEB). To evaluate the *CFTR* splicing profile of different body districts, commercial RNA from 4 tissues (colon, lung, pancreas, and vas deferens) were used (Thermo Fisher Scientific/BioChain Institute).

Briefly, full-length *CFTR* cDNA libraries were generated by PCR amplification using the LongAmp® Taq DNA Polymerase (NEB) and specific primes designed on exon 1 and 27 of *CFTR* (additional information in Supplementary Methods). Library preparation and PacBio sequencing were performed at the Uppsala Genome Center (Science for Life Laboratory). Raw sequencing data were mapped to the reference genome using GMAP. Full-length reads containing both terminal exons were selected using an R script and transcript isoforms were classified by exon-intron structure [28].

## 3. Results

### 3.1. Kinetin and RECTAS increase CFTR exon-10 inclusion in a human cell line and primary cultures from CF patients

To analyze the effect of kinetin and RECTAS on *CFTR* exon-10 splicing, we used the Caco-2 cell line ($TG_{10}T_7/TG_{11}T_7$), which physiologically expresses high level of *CFTR* transcripts, and presents a significant amount of exon-10 skipping (approximately 40 %, Fig. 1B). We treated cells with different concentrations of the two drugs; after 24 h of treatment, we measured the level of *CFTR* exon-10 splicing by fluorescent-competitive RT-PCRs. These experiments showed that RECTAS is effective at low doses reaching 70 % of inclusion at 5 μM ($p = 0.0098$) and 72 % at 10 μM ($p = 0.0034$), whereas kinetin significantly increases the level of wild-type transcripts only starting from 25 μM (72 %, $p = 0.027$) (Fig. 1B). At the maximum concentration of 200 μM used for kinetin, RECTAS could not be tested, since it showed toxic effects.

To further investigate the efficacy of RECTAS compared to kinetin, we performed a time-course experiment, using the most effective concentration of the two drugs (kinetin: 200 μM; RECTAS: 100 μ M). We observed for both molecules a significant increase in exon-10 inclusion between 4 and 48 h (69–76 %, $p < 0.05$) (Fig. 1B).

To evaluate that both drugs could modulate the in-frame exon-10 splicing in a more disease-related model, we established primary cultures from nasal brushing of 3 CF patients carrying the $TG_{12}T_5$ allele and, *in trans,* a mutation (splicing or nonsense) subject to the nonsense-mediated mRNA decay (NMD) pathway, thus not contributing to our analysis [29–31]. These primary cultures were treated with kinetin, RECTAS, or the vehicle. Digital RT-PCR confirmed that both treatments are effective with RECTAS outperformed kinetin, increasing exon-10 inclusion up to 4-fold (Fig. 1C).

### 3.2. Kinetin/RECTAS effect on splicing depends on the TGnTm genotype

To study the effect of kinetin and RECTAS in increasing the physiological splicing levels determined by different TGnTm alleles, we produced 6 different minigene constructs, each containing a different combination of TG and T repeats (Fig. 2A). We first evaluated by appropriate RT-PCR assays if the produced plasmids could recapitulate *in vitro* the modulation of exon-10 splicing driven by the different selected repeats. As expected, the rare variant $TG_{12}T_3$ showed extremely low levels of wild-type transcripts (9 %). The 3 variants carrying the $T_5$ repeat displayed an increase in the wild-type mRNA transcript inversely proportional to the number of TG ($TG_{15}$: 12 %; $TG_{13}$: 24 %; $TG_{11}$: 49 %). Moreover, the $TG_{11}T_7$ and $TG_{10}T_9$ alleles showed an extremely high level of exon 10 inclusion ( 92 % and 99 %, respectively; Fig. 2B).

Then, we tested the effect of kinetin and RECTAS in rescuing the correct exon-10 splicing (Fig. 2B). Both molecules drastically improved the proper functioning of the acceptor splice site, and RECTAS confirmed to be the most effective compound. In particular, RECTAS increased exon-10 inclusion of 2-fold in the case of the two most severe alleles ($TG_{12}T_3$, $p = 0.0080$; $TG_{15}T_5$, $p < 0.001$). Instead, the other two $T_5$ alleles ($TG_{13}T_5$ and $TG_{11}T_5$) were associated with a lower fold change ($TG_{13}T_5$: kinetin 1.3, $p = 0.033$ and RECTAS 1.71, $p = 0.0086$; $TG_{11}T_5$: kinetin 1.38, $p < 0.0001$ and RECTAS 1.45, $p = 0.013$), which is however notable considering the basal level of correctly spliced transcripts. Finally, the $TG_{11}T_7$ and $TG_{10}T_9$ variants did not show any increase in the wild-type percentage, being their basal level of exon-10 inclusion close to 100 % (Fig. 2B).

### 3.3. Molecular determinants of kinetin activity on CFTR exon-10 splicing

To explore the mechanisms underlying kinetin action, we tested whether the previously described [32] CAA element at the 3′ end of *CFTR* exon 10 contributes to its responsiveness. Site-directed mutagenesis revealed that altering this motif did not affect kinetin-induced exon 10 inclusion, indicating that the CAA element is not required for drug sensitivity (Supplementary Figure 1). In addition, kinetin treatment significantly reduced *TDP43* transcript levels in Caco-2 cells, suggesting that modulation of this splicing regulator may contribute to the observed effects (Supplementary Figure 1). Detailed results are reported in the Supplementary Materials.

### 3.4. Kinetin demonstrates superior splicing selectivity than RECTAS

To comprehensively evaluate the potential impact of kinetin and RECTAS on the transcriptome, we used bronchial epithelium CuFi-1 cells, as they represent a well-characterized cellular model of CF [33]. CuFi-1 cells derive from a CF patient homozygous for the common p. F508del mutation, which is associated *in cis* with the $TG_{10}T_9$ repeat [2], allele characterized by a quasi-total exon-10 inclusion in the *CFTR* transcript (Fig. 2).

Total RNA was extracted from CuFi-1 cells treated either with 200 μM kinetin, 100 μM RECTAS, or with DMSO (vehicle) for 24 h, and analyzed by transcriptome profiling. Differentially expressed genes were

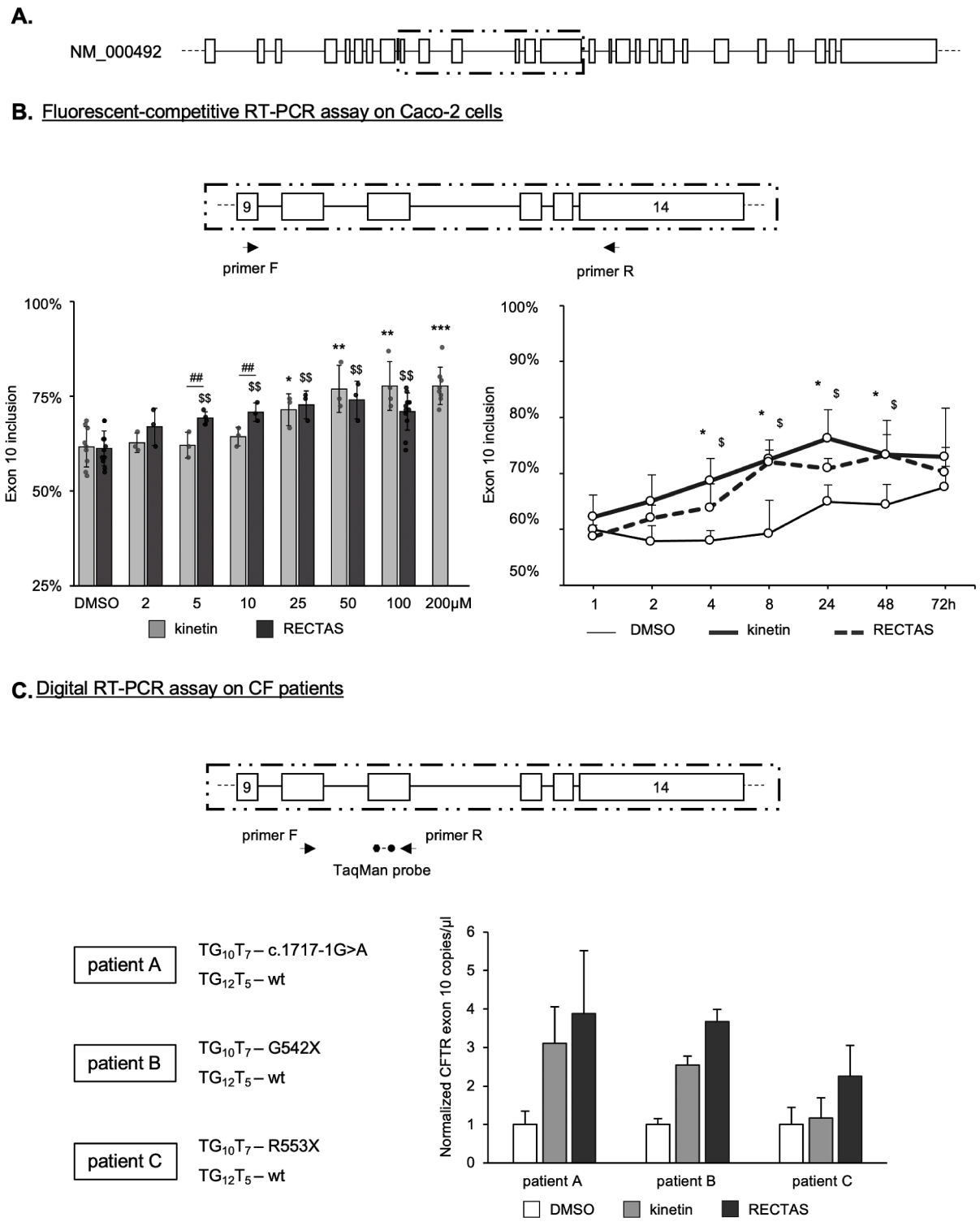



**Fig 1.** Kinetin and RECTAS increase *CFTR* exon-10 inclusion in CaCo-2 cells and in primary cultures from CF patients.
(A) Schematic representation of the *CFTR* genomic region: introns are represented by lines, whereas exons (approximately drawn to scale) are shown as boxes. Dotted rectangle evidence the region of interest.
(B) The primers used in fluorescent-competitive RT-PCR experiments (mapping in exons 9 and 14) are drawn. Kinetin and RECTAS were tested on Caco-2 cells at different doses (from 2 up to 200 μM) for 24 h (Left), or at the maximum doses (200 and 100 μM for Kinetin and RECTAS, respectively) at different time points (Right). In both graphs, the percentage of transcripts with exon-10 inclusion (i.e., wild-type splicing) is reported. Bars/points represent means +SD of at least three independent experiments. In the left graph, dots representing individual experiments are shown. Significance levels of *t*-tests are shown. kinetin vs. DMSO: *=$p < 0.05$, **=$p < 0.005$, ***=$p < 0.001$. RECTAS vs. DMSO: $=$p < 0.05$, $$=$p < 0.005$. kinetin vs. RECTAS: ##=$p < 0.005$.
(C) The primers and the probe used in Taqman digital RT-PCR assays (mapping in exons 10 and 11) are drawn. Cell cultures from nasal brushing of three patients carrying one $TG_{12}T_5$ allele (the complete genotype is reported) were established. Kinetin (100 μM) and RECTAS (50 μM) effect on exon-10 inclusion was evaluated by digital PCR after 24 h treatment. *CFTR* exon 10 copies/μl were normalized co-amplifying *HMBS* transcripts. Treatment with DMSO (vehicle for both kinetin and RECTAS) was used as reference.

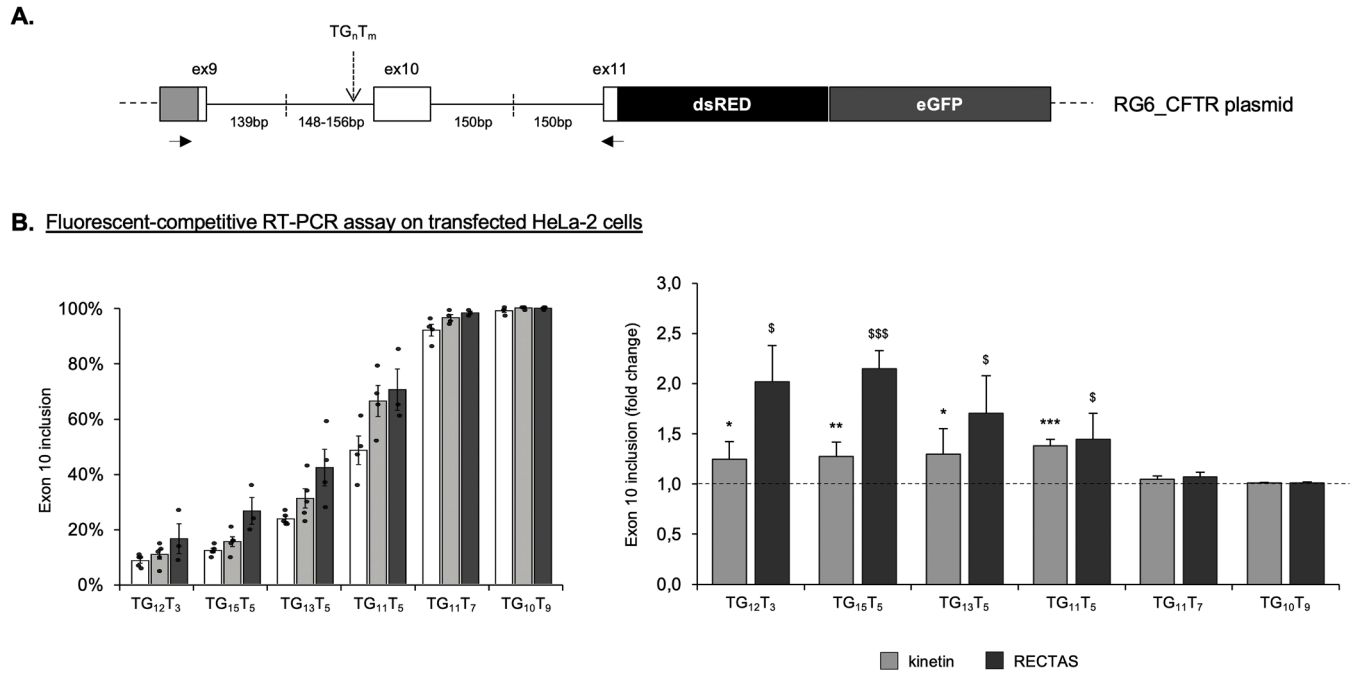



**Fig 2.** Genotype-dependent *CFTR* exon-10 splicing modulation.
(A) Schematic representation of the plasmid used for overexpression experiments: *CTFR* exon 10, a portion of exons 9 and 11, and the regions flanking the splicing sites of introns 9 and 10 (about 150 bp each) were cloned in the RG6 vector (sequence provided in Supplementary Methods). Six different clones, each containing a different combination of TG and T repeats ($TG_{12}T_3$, $TG_{15}T_5$, $TG_{13}T_5$, $TG_{11}T_5$, $TG_{11}T_7$, $TG_{10}T_9$) were produced and transfected in HeLa cells.
(B, Left) Measurements of exon-10 splicing inclusion driven by different TGnTm repeats with or without treatments were performed by fluorescent-competitive RT-PCR: bars represent the mean percentage (±SD) of exon-10 inclusion of at least three independent experiments. Dots representing individual experiments are shown. (Right) Bars indicate the fold change mean values +SD of at least three independent experiments. Significance levels of *t*-tests are shown. kinetin vs. DMSO: *=$p <$ 0.05, **=$p < 0.005$, ***=$p < 0.001$. RECTAS vs. DMSO: $=$p < 0.05$, $$$=$p < 0.001$.

conservatively defined as those showing a FDR <0.05 and an absolute fold change of at least ≥2-fold change (log2 ≥1 or ≤−1). Kinetin treatment altered the transcription of 96 genes (0.5 % of 20,258 expressed genes), whereas RECTAS modified the levels of 347 transcripts (1.7 %). Of these, 44 are modulated by both treatments (Fig. 3A).

Alternative-splicing analysis revealed only 354 (out of 49,874 detected splicing events) and 1141 (out of 57,834) high-confidence alternative-splicing events significantly modulated by kinetin and RECTAS, respectively (FDR <0.05). In both cases, exon-skipping represented the most frequent type of events (64.4 % and 59.8 % for kinetin and RECTAS, respectively; Fig. 3B). As expected, among differentially alternative-splicing events, *CFTR* exon-10 splicing was not evidenced, in line with the CuFi-1 $TG_{10}T_9$ genotype.

### 3.5. Long-read sequencing reveals Kinetin and RECTAS enhance CFTR full-length transcript production by splicing correction

To characterize both basal mis-splicing patterns and the impact of kinetin and RECTAS on full-length *CFTR* transcripts, we employed the PacBio long-read sequencing technology on RNA extracted from Caco-2 cells treated or not with the two compounds. In basal conditions, *CFTR* produces only about 50 % of full-length transcripts (Fig. 4), the remaining 50 % of mRNAs being characterized either by in-frame (the most frequent being exon-10 or exons 12-to-15 skipping) or out-of-frame (the most frequent being exons 13-to-15 skipping) events. Upon treatment, our analysis revealed that both kinetin and RECTAS promote a shift towards wild-type splicing, acting both on in-frame and out-of-frame events across the *CFTR* mRNA (Fig. 4). Noteworthy, kinetin at 25 μM and RECTAS at 100 μM effectively increased the abundance of full-length *CFTR* transcripts (to around 70 %) by acting on multiple alternative splicing events.

### 3.6. Kinetin and RECTAS rescue CFTR protein levels

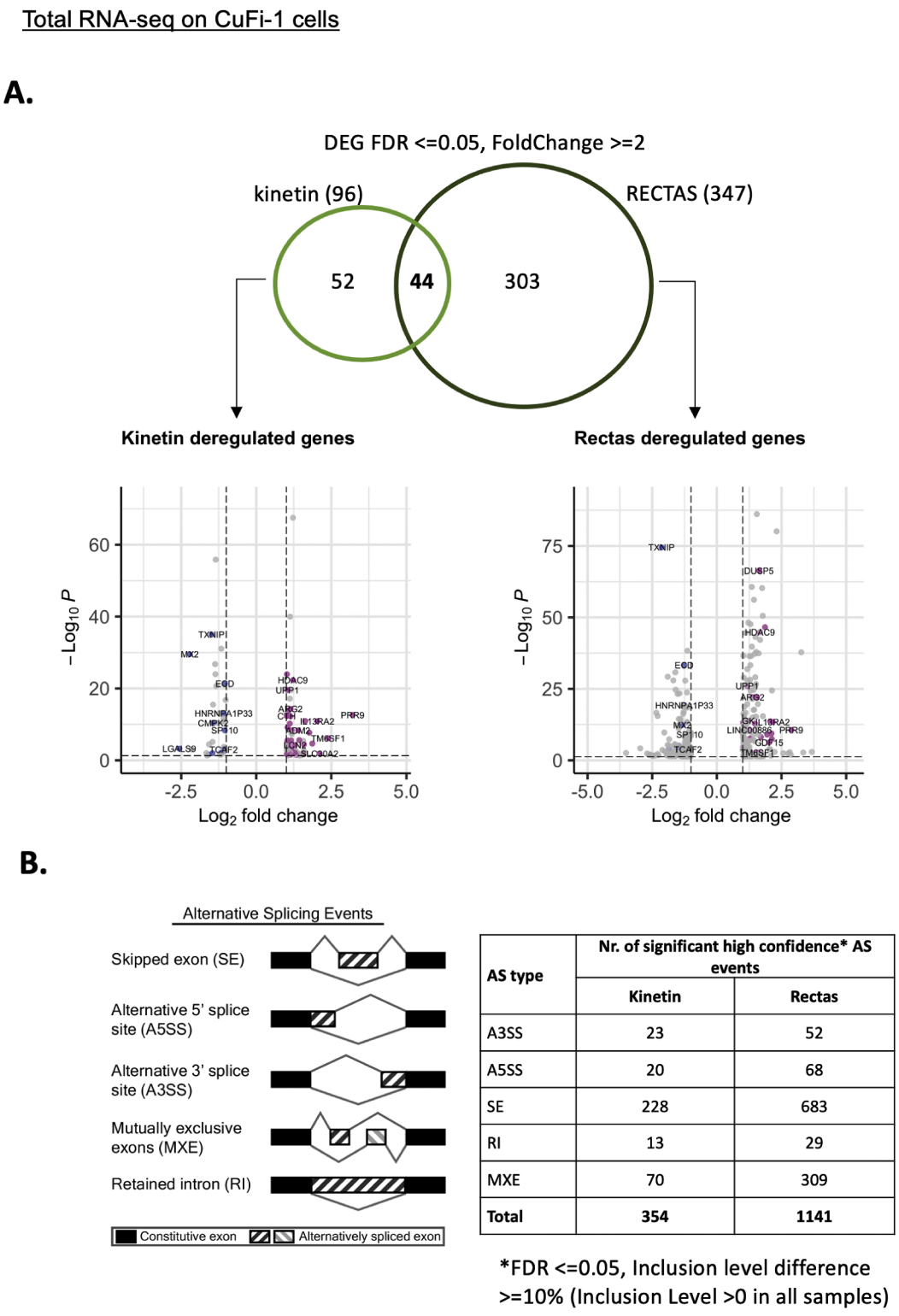
In order to evaluate if the splicing correction had an impact also at the protein level, we performed Western blot experiments on HeLa cells transfected with both full-length *CFTR* coding sequence and its modified version $TG_{12}T_5$—*CFTR* (Fig. 5A), which was competent for exon 10 splicing. The $T_5$ allele was chosen as it is the most commonly associated with CF-related conditions. Transfected cells were treated with kinetin or RECTAS and CFTR was detected using a monoclonal antibody that specifically recognizes CFTR N-terminal [34]. Both treatments significantly increased CFTR protein levels (1.8- and 4-fold, respectively) in cell transfected with the $TG_{12}T_5$ variant. As expected, no change was observed in cells transfected with full-length *CFTR* (Fig. 5B, C).

### 3.7. RECTAS outperforms other kinetin analogues in restoring CFTR exon-10 splicing

Kinetin and RECTAS differ solely by a chlorine atom, prompting us to explore the effects of eight additional kinetin analogues on *CFTR* exon-10 splicing. We selected them based on the assumption that similar structure likely yields similar biological activity. Nonetheless, even at the lowest dose tested, RECTAS remained the most effective compound (Supplementary Results and Supplementary Figure 3).

## 4. Discussion

To date, it is widely perceived that besides DNA analysis, it is essential to explore the mRNA sphere to highlight new *CFTR* defects, otherwise undetectable using conventional mutational screening, thus allowing the identification of variants that quantitatively or qualitatively alter the coding transcript. The minimum level of wild-type *CFTR* mRNA sufficient to prevent clinical manifestations is variable among




| AS type | Nr. of significant high confidence* AS events | |
|---|---|---|
| | Kinetin | Rectas |
| A3SS | 23 | 52 |
| A5SS | 20 | 68 |
| SE | 228 | 683 |
| RI | 13 | 29 |
| MXE | 70 | 309 |
| **Total** | **354** | **1141** |

*FDR <=0.05, Inclusion level difference >=10% (Inclusion Level >0 in all samples)

**Fig. 3.** Kinetin/RECTAS off-target effects on CuFi-1 cell transcriptome.
CuFi-1 cells were treated for 24 h either with kinetin (200 μM) or RECTAS (100 μM).
(A) Top: Venn diagrams show the number of differentially expressed genes after either treatment, as assessed by RNA-sequencing. Bottom: Volcano plots showing differentially expressed genes after treatment with kinetin ($N = 96$) or RECTAS ($N = 347$) in CuFi-1 cells. The x axis represents Log2 fold change of gene expression after treatment, and the y axis represents the p-value (log10 transformed, Log10P). The horizontal dashed line indicates the FDR =0.05. The two vertical dashed lines indicate a 2-fold change. The magenta and blue dots represent genes with significant changes upon both treatments (FDR <0.05, and Log2 fold change $\geq 1$ (magenta) or $\leq -1$ (blue), respectively).
(B) Summary table of significantly altered alternative splicing events (at FDR $\leq 0.05$) identified by rMATS. The complete list of the significantly altered alternative splicing events is included in Supplementary Table 1.

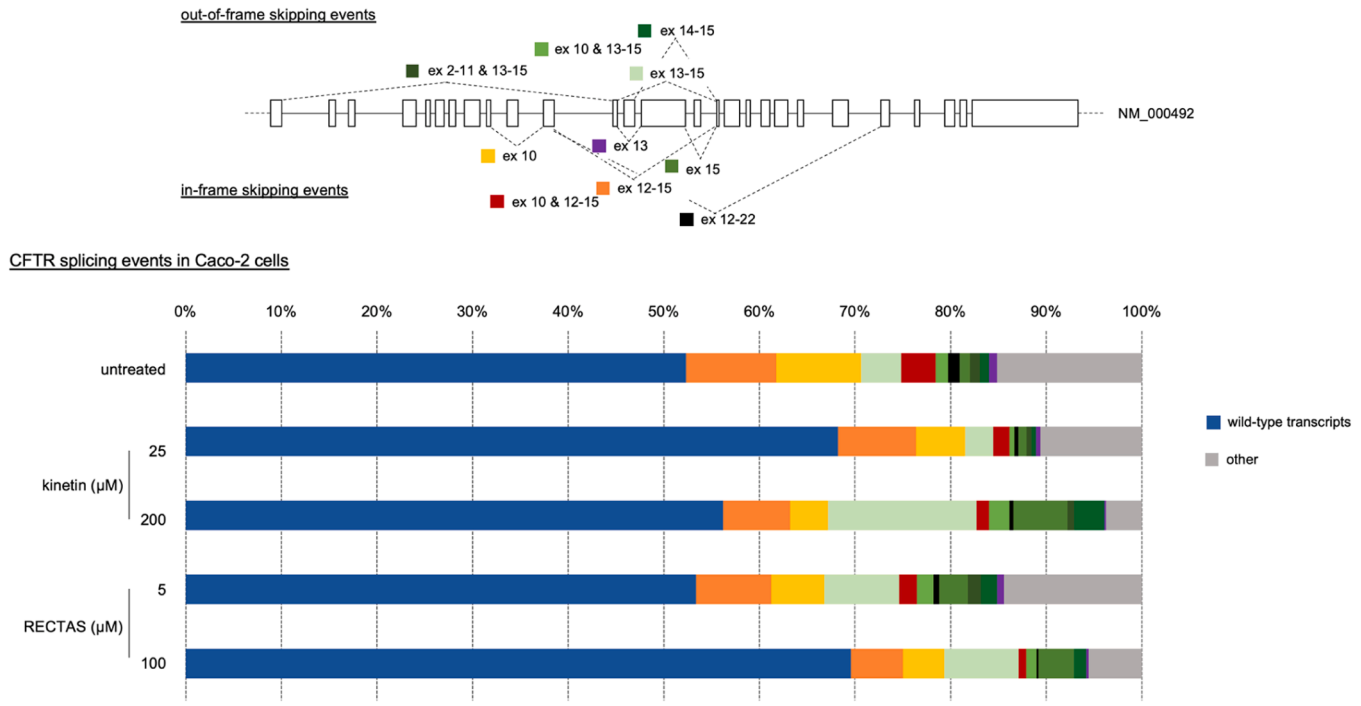



**Fig 4.** Kinetin/RECTAS effects on the *CFTR* splicing using long-read sequencing.
(Top) Schematic representation of the *CTFR* gene with the main alternative splicing events reconstructed by PacBio long-read sequencing, both in-frame and out-of-frame.
(Bottom) Kinetin (25–200 μM) and RECTAS (5–100 μM) effects after 24 h treatment on the levels of the splicing isoforms identified in Caco-2 cells.

different individuals and among different organs involved in CF: for example, vas deferens seem to be the most susceptible tissue to alternative splicing with a consequent reduction of the full-length transcript level [35] (Fig. 6). In particular, while literature evidence shows that as little as 3–5 % of wild-type *CFTR* transcript in the lung is sufficient to declassify CF symptomatology to a mild pulmonary disease [36,37], the functioning protein threshold required for male fertility has not been confirmed yet. Our long-read sequencing analyses revealed variable levels of full-length *CFTR* transcript across tissues, ranging from 22.2 % in vas deferens to 88.2 % in pancreas (Fig.6), supporting the concept that tissue-specific splicing regulation may underlie the phenotypic spectrum of CF-related disorders.

Because even small increases in *CFTR* expression can ameliorate disease severity, CF represents an ideal model for testing small molecules that modulate splicing efficiency. In this study, we investigated the efficacy of the plant cytokinin kinetin and its analogue, RECTAS, in correcting *CFTR* exon-10 splicing defects caused by the presence of the well-studied polymorphic TGnTm repeat. Importantly, the $TG_{12}T_5$ and $TG_{13}T_5$ variants are not classical CF-causing mutations but are instead associated with CFTR-RDs due to partial exon 10 skipping and reduced *CFTR* expression. Although these variants do not typically cause overt CF, their clinical impact should not be underestimated. The $T_5$ allele is relatively common, with an estimated population frequency ranging from 3 % to 5 %, depending on ethnic background, and its penetrance is further modulated by the length of the adjacent TG repeat [38,39]. Individuals carrying these alleles may therefore present with mild or tissue-specific manifestations, underscoring the importance of therapeutic strategies, such as kinetin and RECTAS, that can amplify *CFTR* expression even in the presence of partial splicing defects.

Our preliminary results on primary cultures from nasal brushing of CF patients suggested the efficacy of both treatments also in the correction of defects due to the $T_5$ allele (the most severe one), with RECTAS showing up to a 4-fold increase in exon-10 inclusion. However, the small sample size of patient-derived cells limits the generalizability of these findings: future studies in larger cohorts will be essential to validate these results and to ensure their clinical relevance.

As previously observed in the IKBKAP-familial dysautonomia mis-splicing defect correction [19], also in our experiments RECTAS exhibited superior efficacy compared to kinetin, particularly at lower doses. However, RECTAS also exhibited a broader off-target activity at the transcriptome level, consistent with its more pronounced splicing modulation profile (Fig. 3) [18,40]. Conversely, kinetin displayed a more selective action, in line with previous reports [41]. These observations emphasize that, while both compounds can effectively increase wild-type transcript levels, their global impact on RNA processing must be carefully considered before any therapeutic application.

Based on our long-read sequencing data, both molecules appear to influence multiple endogenous *CFTR* splicing events, suggesting that these compounds may act as broad transcript amplifiers rather than selective splicing correctors (Fig. 4). By enhancing the overall production of correctly spliced *CFTR* mRNA, they could increase the pool of transcripts available for translation into functional protein. This property makes them particularly attractive as combinatorial agents, to be used alongside *CFTR* modulators such as ivacaftor or compounds improving protein folding and trafficking. Such an approach could potentiate the therapeutic benefit, especially in individuals carrying mild or partially penetrant *CFTR* variants where residual protein function can be leveraged.

Finally, while our results confirm the ability of kinetin and RECTAS to enhance *CFTR* exon 10 inclusion and protein expression (Fig. 5), their off-target profiles highlight the need for improved analogues with greater specificity. Overall, these findings position kinetin-like compounds as promising molecules also to boost endogenous CFTR expression and complement current modulator therapies, potentially benefiting individuals with CFTR-RDs caused by splicing inefficiency.

## Data availability statement

Short-read Illumina RNAseq data (raw and processed) and raw long-read PacBio RNAseq data were deposited in Zenodo with doi: 10.5281/

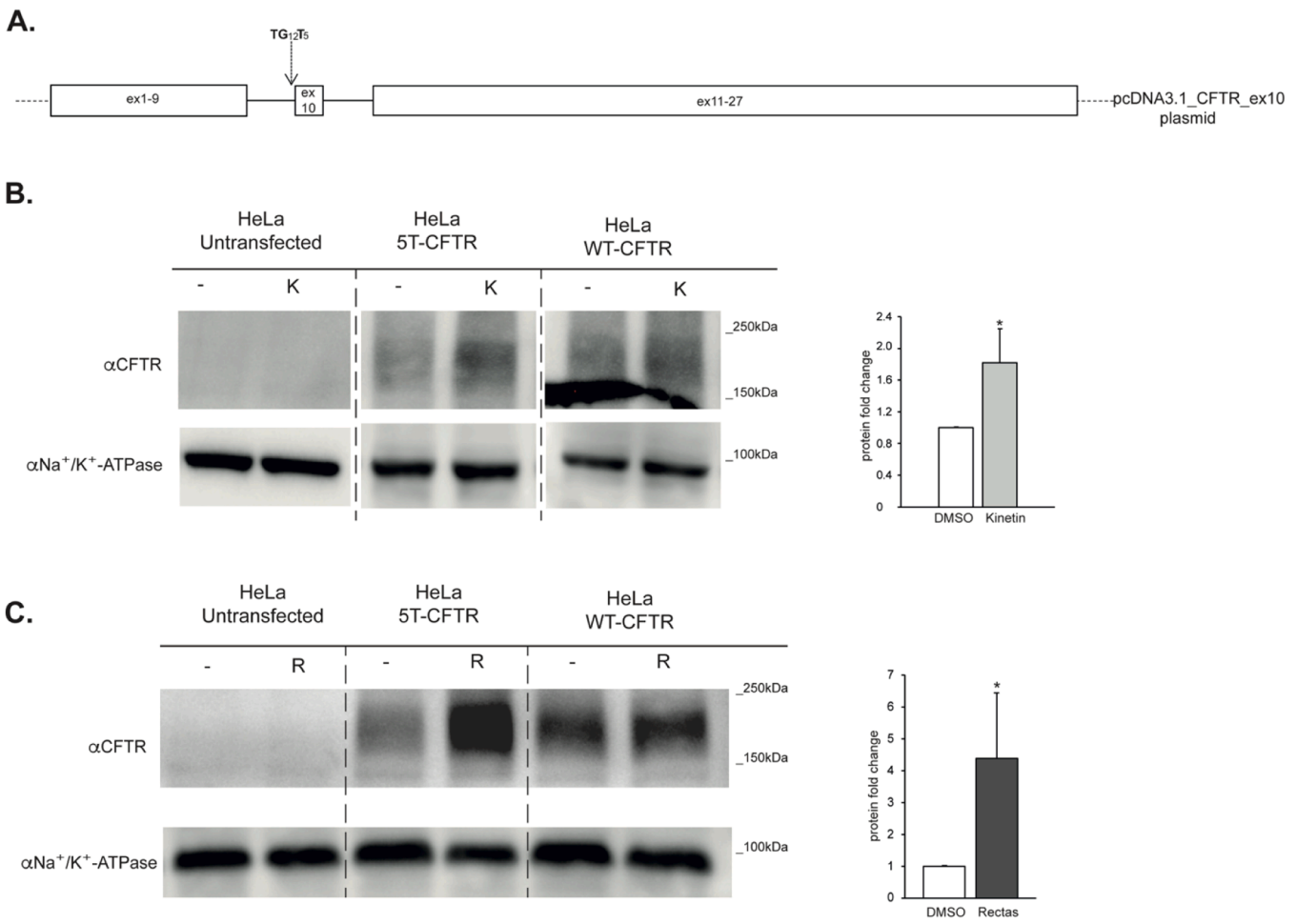



**Fig 5.** Rescue of CFTR protein levels.
(A) Schematic representation of pcDNA3.1 plasmid containing the full coding sequence of *CFTR* including exon 10 and portions of intron 9 and 10 with the $TG_{12}T_5$ repeats.
HeLa cells were transfected with the wild-type and $TG_{12}T_5$ version of the same plasmid and treated either with kinetin (B) or RECTAS (C) for 72 h. (Left) A representative Western blot of three independent experiments is shown. 150 μg of the total proteins were loaded onto a 7 % polyacrylamide maxi-gel; primary antibodies used were: monoclonal antibody to CFTR (diluted 1:1000), and polyclonal antibody to Na+/*K*+-ATPase (diluted 1:1000). (Right) Average CFTR protein levels measured in HeLa cells transfected with the $TG_{12}T_5$ CFTR plasmid, setting the untreated as 1. Na+/*K*+-ATPase was used as internal reference. The results were analyzed by unpaired *t*-test. *: $p < 0.05$.

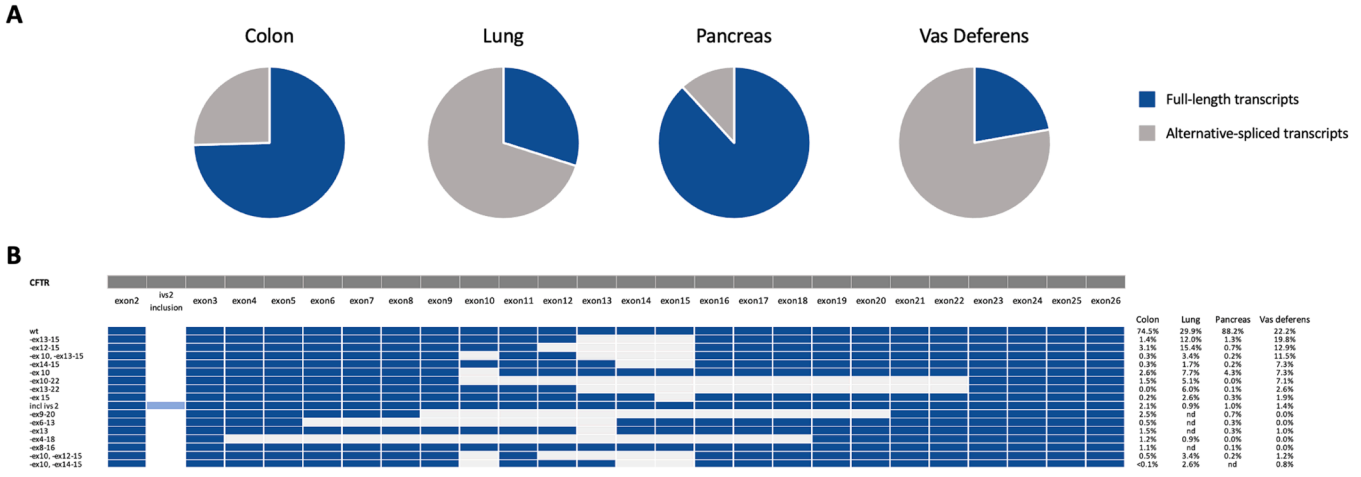



**Fig 6.** *CFTR* splicing profile in different tissues.
(A) Relative proportions of full-length and alternative-spliced *CFTR* transcripts in tissue relevant for CF using the PacBio long-read sequencing technology.
(B) Schematic representation of the splicing pattern of the main *CFTR* splicing isoforms reconstructed by long-read sequencing with the relative amount in the different tissue analyzed. nd: not detected.

zenodo.14620982 and are publicly accessible.

## Declaration of competing interest

All the authors declare no conflicting interest

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jcf.2025.11.006.

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