frontiers | Frontiers in Genetics

TYPE Mini Review
PUBLISHED 24 April 2023
DOI 10.3389/fgene.2023.1166529

OPEN ACCESS

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SPECIALTY SECTION
This article was submitted to RNA, a section of the journal Frontiers in Genetics

RECEIVED 15 February 2023
ACCEPTED 27 March 2023
PUBLISHED 24 April 2023

CITATION
Jackson JJ, Mao Y, White TR Jr., Foye C and Oliver KE (2023), Features of CFTR mRNA and implications for therapeutics development. *Front. Genet.* 14:1166529. doi: 10.3389/fgene.2023.1166529

Features of CFTR mRNA and implications for therapeutics development

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Cystic fibrosis (CF) is an autosomal recessive disease impacting ~100,000 people worldwide. This lethal disorder is caused by mutation of the *CF transmembrane conductance regulator (CFTR)* gene, which encodes an ATP-binding cassette-class C protein. More than 2,100 variants have been identified throughout the length of *CFTR*. These defects confer differing levels of severity in mRNA and/or protein synthesis, folding, gating, and turnover. Drug discovery efforts have resulted in recent development of modulator therapies that improve clinical outcomes for people living with CF. However, a significant portion of the CF population has demonstrated either no response and/or adverse reactions to small molecules. Additional therapeutic options are needed to restore underlying genetic defects for all patients, particularly individuals carrying rare or refractory *CFTR* variants. Concerted focus has been placed on rescuing variants that encode truncated CFTR protein, which also harbor abnormalities in mRNA synthesis and stability. The current mini-review provides an overview of CFTR mRNA features known to elicit functional consequences on final protein conformation and function, including considerations for RNA-directed therapies under investigation. Alternative exon usage in the 5′-untranslated region, polypyrimidine tracts, and other sequence elements that influence splicing are discussed. Additionally, we describe mechanisms of CFTR mRNA decay and post-transcriptional regulation mediated through interactions with the 3′-untranslated region (e.g. poly-uracil sequences, microRNAs). Contributions of synonymous single nucleotide polymorphisms to CFTR transcript utilization are also examined. Comprehensive understanding of CFTR RNA biology will be imperative for optimizing future therapeutic endeavors intended to address presently untreatable forms of CF.

KEYWORDS

CFTR, mRNA stability, synonymous single nucleotide polymorphism, suppressor tRNA, miRNA, peptide nucleic acid (PNA), antisense oligonucleotide, readthrough compound

Introduction

Cystic fibrosis (CF) is a monogenic disorder caused by loss-of-function of the CF transmembrane conductance regulator (CFTR), a chloride and bicarbonate channel expressed in respiratory, gastrointestinal, and exocrine tissues (among others) (Collins, 1992; Marson, 2018). With more than 2,100 disease-associated variants reported in *CFTR* (Sosnay et al., 2013; The Clinical and Functional TRanslation of CFTR CFTR2, 2021; Cystic Fibrosis Mutation Database, 2022), these variants are categorized by molecular attributes

such as little-to-no transcript and/or polypeptide production, decreased protein maturation, or impaired channel gating (Oliver et al., 2017; Ensinck and Carlon, 2022) (Figure 1). Recent advancements in CF therapeutics have led to clinical approval of small molecules ("modulators") primarily designed to rescue defects in CFTR folding or trafficking, otherwise termed "processing" variants. While at the forefront of personalized medicine, significant portions of the global CF population do not benefit from these modulators based on *CFTR* genotype (McGarry and McColley, 2021). Thus, there remains a critical need for efficacious treatment strategies to address presently off-label variants. Increasing evidence places CFTR mRNA quality and utilization as a key contributor to patient heterogeneity and pharmacologic responsiveness (Clarke et al., 2021; Rauscher et al., 2021; Bengtson et al., 2022). In this brief review, we summarize ways in which mRNA structure, stability, and abundance robustly influence CFTR functional expression. We also highlight new CF treatment paradigms targeted at the level of RNA.

Features within the CFTR 5′-untranslated region (UTR)

Intron excision and exon ligation are essential steps in pre-mRNA processing known as splicing (Ben-Dov et al., 2008). Through this mechanism, it has been well established that different patterns of intron removal can lead to diverse mRNA sequences and protein products originating from a single gene. A contributor to variable CFTR splice forms encompasses alternative exon usage within the 5′-UTR (Figure 2). In human CFTR, upstream 5′-exons generated from alternative transcriptional start sites demonstrate direct splicing to exon-2, effectively skipping the exon-1 (Chou et al., 1991; Yoshimura et al., 1991; Koh et al., 1993; Mouchel et al., 2003; Davies et al., 2004). Resulting transcripts exhibit Gibbs free energies reflective of stable RNA secondary structures. Such features would induce prolonged translational pausing and ribosomal dissociation (Wen et al., 2008), thereby downregulating gene expression. Moreover, loss of exon-1 would remove diphenylalanine motifs crucial for transport of CFTR from the Golgi apparatus to the plasma membrane (Mouchel et al., 2003).

Alternative 5′-exons may also introduce a series of upstream open reading frames (uORFs) (Davies et al., 2004; Calvo et al., 2009; Barbosa et al., 2013). These elements are proximal to consensus nucleotides surrounding the initiation codon, i.e. Kozak sequence, which influence the rate of AUG recognition by ribosomes. In the 5′-UTR of native CFTR transcripts, the presence of an uORF and RNA secondary structure reduce translation initiation and mRNA stability (Lukowski et al., 2015). The Kozak context of the uORF is suboptimal with

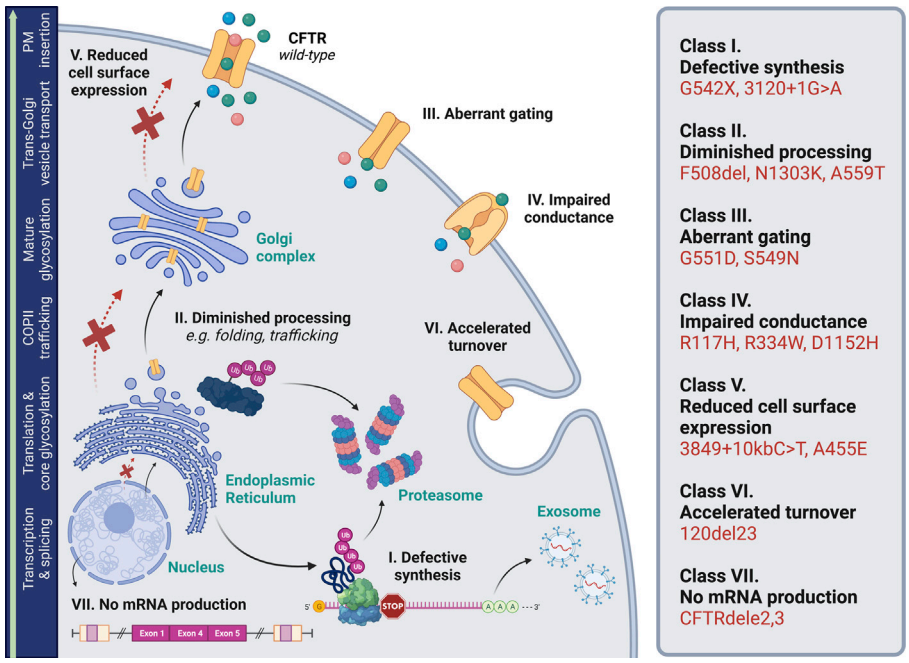

FIGURE 1
Biosynthetic pathway of CFTR. This ATP-binding cassette class-C transporter facilitates efflux of chloride (green molecule), bicarbonate (pink molecule), and water (blue molecule) across the plasma membrane (PM). Biogenesis encompasses transcription and splicing in the nucleus; translation, folding, and core glycosylation at the endoplasmic reticulum; transport to and through the Golgi, including further glycosylation and other post-translational modifications; and vesicular delivery to the cell surface. *CFTR* variants disrupt one or more steps in this pathway, and thus, are categorized into seven sub-classes based on molecular features (De Boeck and Amaral, 2016; Cottrill et al., 2020). These include defects in: (I) protein synthesis (e.g., nonsense codons, splice variants); (II) processing (e.g., folding abnormalities); (III) channel gating; (IV) ion conductance through a malformed pore; (V) expression at the cell surface (defects in trafficking are denoted with a red "X"); (VI) plasma membrane stability and turnover; and (VII) mRNA production. Abbreviations: coat protein complex II (COPII); ubiquitin (Ub); premature termination codon ("stop"). Image was generated using BioRender.

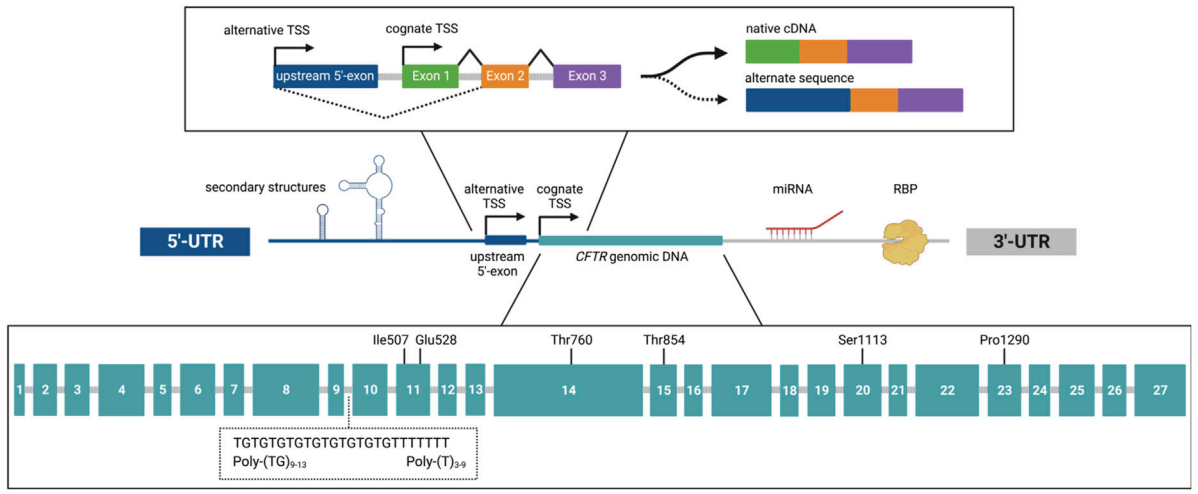

FIGURE 2
Major contributors to CFTR mRNA structure, abundance, and utilization. In the 5′-untranslated region (UTR), RNA secondary structures inhibit translation initiation and mRNA stability. Upstream 5′-exons introduced by an alternative transcriptional start site (TSS) demonstrate skipping of exon-1 *via* direct splicing to exon-2 (upper panel). The 3′-UTR facilitates enhanced or reduced CFTR mRNA expression through interaction of RNA-binding proteins (RBPs) with cytosine repeats, poly-uracil tracts, or AUUUA motifs. Engagement of miRNAs with the 3′-UTR also diminishes CFTR transcript abundance. Within the gene body, synonymous single nucleotide polymorphisms at residues Ile507, Glu528, Thr760, Thr854, Ser1113, and Pro1290 confer alterations to CFTR mRNA secondary structure, splice forms, and translation efficiency (lower panel). In addition, extended repeats of thymidine (9T) and/or thymidine-guanine (13TG) in intron 9 induce alternative splicing and skipping of exon-10. Image was generated using BioRender.

zero out of two critical nucleotides encoded, providing an infrequently recognized start codon. In addition, an RNA hairpin inhibits scanning ribosomes from potentially initiating (or re-initiating) translation at downstream AUG codons. This RNA secondary structure contributes to ribosomal stalling and dissociation as well, thereby diminishing mRNA half-life.

CFTR sequence elements that influence splicing

Polypyrimidine sequences located near splice branch/acceptor sites impact RNA primary sequence and secondary structure. For example, thymidine-guanine (TG) repeats and polythymidine (poly-T) tract immediately adjacent to exon-10 (Figure 2) have been shown to influence alternative splicing (Chu et al., 1993; Pagani et al., 2000; Hefferon et al., 2004). An inverse relationship exists between the length of the polypyrimidine segment and subsequent mRNA abundance–e.g. an extended TG repeat sequence and poly-T tract confer enhanced exon-10 skipping, reduced CFTR transcript expression, and diminished channel activity (Chu et al., 1993; Groman et al., 2004). Formation of RNA secondary structures by dinucleotide repeats can mask splicing signals in CFTR and/or generate features that stimulate (or interfere with) protein-protein, RNA-protein, or RNA-RNA interactions (Niksic et al., 1999; Pagani et al., 2000; Buratti et al., 2001). Findings have shown higher splicing efficiency of exon-10 correlates with shorter TG repeats, which undergo self-base-pairing to form RNA hairpins with low stem loop thermostability (Hefferon et al., 2004)—suggesting that the transient presence of these structures improves pre-mRNA processing.

Splicing is also influenced by the presence of pseudoexons, which are intronic sequences that exhibit characteristics of exons (e.g., 5′- and 3′-splice sites) but are absent in mature transcripts (Grellscheid and Smith, 2006). Existence of pseudoexons in mRNA is associated with disease pathology in CF and other disorders (Dhir and Buratti, 2010; Vaz-Drago et al., 2017; Petersen et al., 2022). Earlier investigations have established that pseudoexon inclusion occurs from single nucleotide variants within *CFTR* that introduce new 5′-splice sites (Chillón et al., 1995; Buratti et al., 2007). In addition to engagement of inhibitory RNA-binding proteins and/or absence of binding sites for enhancive splicing factors, RNA secondary structures have been suggested to mediate splicing efficiency in this setting (Buratti et al., 2007). Evidence indicates that unused 5′-donor sites are common in native *CFTR* introns, although they may be positioned within RNA hairpins and therefore inaccessible (Petersen et al., 2022). Experimental manipulation of the RNA stem loops can expose the normally unemployed 5′-splice sequences, leading to inclusion of pseudoexons (Dhir and Buratti, 2010). These aspects of intron processing are important considerations for gene editing-based therapeutic strategies under development for CF.

Attributes of the CFTR 3′-UTR

The 3′-UTR consists of non-coding mRNA between the cognate stop codon and poly-adenine tail. This sequence is best known for

mediating mRNA localization, nuclear export, half-life, and translation initiation (Mignone et al., 2002; Matoulkova et al., 2012; Mayr, 2019). Alternative splicing or polyadenylation sites may produce altered 3′-UTR sequences that also affect the 3′-coding region (if the stop codon is involved) and/or regulatory features (Mayr, 2019). In the context of CFTR, adenine (A)- and uracil (U)-rich sequences have been established as strong contributors to mRNA stability (Mignone et al., 2002). CFTR possesses three AUUUA motifs, in addition to many U- and cytosine (C)-repeats (Zielenski et al., 1991). The AUUUA and poly-U tracts deplete mRNA half-life through formation of destabilizing ribonucleoprotein complexes, whereas the C-repeats putatively enhance mRNA stability through interactions with poly-C-binding proteins (Baudouin-Legros et al., 2005).

Negative post-transcriptional regulation of CFTR also occurs in cell type-specific manner through 3′-UTR interactions with microRNAs (miRNAs) (Figure 2). These molecules are single-stranded, non-coding RNAs approximately 19–25 nucleotides in length. Several miRNAs have been observed to directly bind the CFTR 3′-UTR, suppress protein production, and contribute to manifestations of CF lung disease (Megiorni et al., 2011; Ramachandran et al., 2013; Viart et al., 2015). Depletion of CFTR abundance mediated by miRNAs is further exacerbated by exposure to bacterial infection or proinflammatory cytokines, which augment expression of miRNAs that correspondingly inhibit CFTR transepithelial ion transport (Ramachandran et al., 2013).

An emerging area of CF therapeutics development involves anti-miRNA approaches. For example, miRNA-binding blocker oligonucleotides (MBBOs) are single-stranded RNA sequences engineered to mask miRNA binding sites. Recently described MBBOs employ locked nucleic acids, which are high-affinity RNA analogues that possess a ribose ring connected by a methylene bridge between the 2′-oxygen and 4′-carbon atoms (Viart et al., 2015). This modification "locks" the ribose ring in an ideal conformation for base pairing and enhances thermal stability of the MBBO-RNA hybrid. MBBOs designed to impair miRNA binding to the CFTR 3′-UTR have been shown to enhance mRNA abundance, protein maturation, and channel activity (Viart et al., 2015).

Additional miRNA-targeting technologies include peptide nucleic acids (PNAs), which are DNA analogues with a pseudo-peptide backbone composed of N-(2-aminoethyl) glycine units (Gambari et al., 2020). PNAs bind with strong affinity to complementary sequences of DNA or RNA, although uptake is limited in eukaryotic cells. The latest scientific reports indicate that PNA intercalation through the plasma membrane can be increased by loading the PNAs onto lipid-polymer nanoparticles (McNeer et al., 2015; Comegna et al., 2021; Piotrowski-Daspit et al., 2022) or linking poly-arginine tails to the PNAs (Papi et al., 2022). To date, numerous PNAs have been shown to inhibit target miRNA binding sites and augment CFTR mRNA and protein expression (McNeer et al., 2015; Zarrilli et al., 2017; Sultan et al., 2020; Fabbri et al., 2021; Piotrowski-Daspit et al., 2022). Off-target effects are an important consideration of anti-miRNA based interventions, as single miRNA species may recognize complementary sequences across multiple genes. Therefore, technologies such as PNAs or MBBOs must be carefully optimized for sequence context to mask miRNA target(s) within a specific gene of interest.

Contributions of synonymous single nucleotide polymorphisms (SNPs)

Synonymous (or "silent") SNPs are prevalent variations found throughout the human genome, in which a single nucleotide identity is changed without altering the encoded amino acid. While synonymous SNPs have been traditionally viewed as benign during protein biogenesis, emerging evidence indicates these polymorphisms impact polypeptide folding and sensitivity to small molecule therapies in a variety of disease states (KomarGenetics, 2007; Sauna et al., 2007; Hunt et al., 2009). Advancements in the CF field have further elucidated the impact of silent SNPs on CFTR splicing, mRNA utilization, protein maturation, and channel function.

The most commonly reported *CFTR* variant, c.1521_1523delCTT (*p*.Phe508del), is caused by deletion of cytosine in the third position of isoleucine-507 (ATC), as well as the first two thymidines present in phenylalanine-508 (TTT) (Figure 2). As a result, Phe508 is lost, and a synonymous SNP is introduced into Ile507 (ATC>ATT). Additional consequences include the appearance of two enlarged, single-stranded loops in CFTR transcripts (Bartoszewski et al., 2010). These secondary structures are predicted to cause mRNA misfolding, decreased ribosome velocity, and diminished co-translational assembly of *p*.Phe508del protein. Newer evidence indicates both ATC and ATT are decoded by similar low-abundance tRNAs (Polte et al., 2019), suggesting the proposed reduction in local ribosome speed would be independent of tRNA availability.

When the mutated Ile507-ATT codon is reverted back to ATC, the silent substitution yields native-like mRNA structure, augments *p*.Phe508del transcript levels, and amplifies protein expression of both the immature ("band B") and mature ("band C″) glycoforms (Bartoszewski et al., 2010; Lazrak et al., 2013). This synonymous SNP would also confer a silent codon change in Phe508 (TTT>TTC), which has been documented in humans (rs1400451895) (Karczewski et al., 2020). *In vitro*, Ile507-ATC renders *p*.Phe508del more sensitive to correction by clinically approved CFTR modulators (Bali et al., 2016), indicating that synonymous SNPs may influence pharmacologic responsiveness.

Recent investigations demonstrate that additional silent SNPs induce alterations to CFTR mRNA expression, protein topology, and transepithelial ion transport (Figure 2). For example, the following three synonymous SNPs in *CFTR* exhibit significantly decreased transcript abundance: c.2280G>A (*p*.Thr760=), c.3339T>C (*p*.Ser1113=), c.3870A>G (*p*.Pro1290=) (Kirchner et al., 2017). The synonymous SNP, c.1584G>A (*p*.Glu528=), elicits retention of intron-11 and skipping of exon-11, albeit at a low frequency (Bampi et al., 2020). A prevalent silent SNP, c.2562T>G (*p*.Thr854=), reduces local ribosome speed through a mechanism dependent on tRNA abundance. This codon substitution diminishes CFTR protein stability and channel conductance (Kirchner et al., 2017). Moreover, c.2562T>G has been shown to epistatically modulate functional expression of certain rare CFTR variants. When c.2562T>G is present *in cis*, this synonymous SNP produces subtle structural rearrangements that counteract destabilizing effects of CFTR missense alleles such as *p*.Gly551Asp, *p*.Asp579Gly, and *p*.Asp614Gly (Rauscher et al., 2021). Together, findings underscore the impact that silent SNPs

contribute to CFTR mRNA utilization and protein folding trajectory. Such effects are likely to influence the spectrum of disease symptoms, and may help predict therapeutic response in precision theratyping studies.

Quality control of CFTR transcripts

Eukaryotic cells perform co-translational mRNA surveillance as a means to sustain expression of properly synthesized transcripts, as well as degrade aberrant mRNAs and recycle associated ribosomes. Three mRNA decay pathways have been extensively described: non-stop decay (NSD), no-go decay (NGD), and nonsense-mediated decay (NMD) (Shoemaker and Green, 2012; Simms et al., 2017; Embree et al., 2022). The NSD complex facilitates RNA cleavage by detaching ribosomes that become jammed at the 3′-end of transcripts lacking a native termination codon. NGD factors recognize and detach ribosomes that become stalled on transcripts bearing nucleotide modifications, secondary structures, and/or other features that interfere with efficient ribosomal decoding. NMD machinery monitors ribosomes undergoing translation termination and eliminates mRNAs harboring premature stop codons (i.e. "nonsense" alleles).

To our knowledge, NSD and NGD remain unexplored in the context of CFTR processing. These pathways still bear relevance, however, as both NSD and NGD are triggered by stalled ribosomes. CFTR is predicted to harbor ribosomal queues at stall sites as a result of elevated initiation rates (Matsumura et al., 2011; Park and Subramaniam, 2019), which may be alleviated by suppressing certain components of translation machinery. The NGD complex binds to the 60S P stalk protein, uL11 (RPL12), to facilitate ribosomal subunit disassembly (Becker et al., 2011). Approximately 50% reduction in uL11 expression attenuates translation initiation and elongation (Oliver et al., 2019). Findings also indicate uL11 depletion enhances protein maturation/stability and transepithelial ion transport exhibited by *p*.Phe508del and other CFTR missense variants (Veit et al., 2016; Oliver et al., 2019). Interestingly, siRNA-mediated knockdown of eukaryotic initiation factor 3a also improves *p*.Phe508del protein trafficking and function (Hutt et al., 2018). Tuning translation initiation by partially silencing uL11 or eukaryotic initiation factor 3a could mitigate NSD- and/or NGD-stimulated downregulation of CFTR expression conferred by stalled ribosomes.

Conversely, NMD has been experimentally verified to influence transcript abundance of numerous refractory CFTR variants (Oren et al., 2017; Sharma et al., 2018; Clarke et al., 2021). NMD is initiated by recognition of features such as long 3′-UTRs, uORFs, and/or exon-exon junction complexes downstream of stop codons [reviewed in (Yi et al., 2021)]. In humans, terminating ribosomes are distinguished by conserved up-frameshift (UPF) proteins: UPF1, UPF2, and UPF3B. UPF1 engages the mRNA and ribosome, after which UPF2 binds and triggers ATPase and helicase activity of UPF1. UPF3B stimulates UPF2-mediated activation of UPF1 and links UPF2 to exon-exon junction complexes. Transcripts become targeted for degradation in response to UPF1 phosphorylation by the serine/threonine protein kinase complex, SMG1-SMG8-SMG9. Phosphorylated UPF1 activates endonucleolytic cleavage of mRNA by SMG6, followed by deadenylation, decapping, and exonucleolytic cleavage by SMG5, SMG7, and Xrn1, respectively. Molecular agents designed to target branch-specific NMD factors are actively being pursued in preclinical testing, as a means to address defects in CFTR mRNA synthesis and stability (Table 1).

RNA-directed therapeutic strategies

Multiple subtypes of *CFTR* variants–such as frameshifts, splicing defects, and nonsense codons–exhibit susceptibility to premature mRNA decay and protein degradation. These variants are largely insensitive to (and ineligible for) clinically approved CFTR modulators, thus representing a significant unmet medical need. Recent emphasis has been placed on development of gene transfer, gene editing, and mRNA therapies (reviewed in (Da Silva Sanchez et al., 2020; Egan, 2021; Harrison, 2022; Sui et al., 2022)). Here, we briefly summarize small molecule and short nucleotide-based approaches that directly interact with translation machinery to circumvent cellular disposal of aberrant CFTR transcripts and associated polypeptides (Table 1).

Compounds that stabilize CFTR mRNA, and therefore augment both immature and mature CFTR protein, are termed "amplifiers" (Molinski et al., 2017). Certain small molecules in this class increase CFTR transcript abundance and translation efficiency through enhanced association with polysomes and poly (rC)-binding protein 1 (PCBP1) (Giuliano et al., 2018; Dukovski et al., 2020). These amplifiers require a PCBP1 consensus element within the CFTR ORF to elicit effects on translation. Due to a distinct mechanism of action, many amplifiers demonstrate additivity or synergy with clinically approved CFTR modulators (Molinski et al., 2017; Smith et al., 2021; Bengtson et al., 2022). When combined with the highly effective modulator therapy, elexacaftor-tezacaftor-ivacaftor, the amplifier PTI-428 (nesolicaftor) augments mRNA half-life and protein function of the *p*.Phe508del CFTR variant (Bengtson et al., 2022). Moreover, PTI-428 overcomes the negative effects of miRNA expression on CFTR mRNA abundance, and reduces cytokine production in the setting of acute inflammation (Bengtson et al., 2022).

Robust attention has also been devoted towards engineering small molecules that restore full-length protein expression of CFTR nonsense variants. Translation is arrested when a ribosome encounters any of the three termination codons (UAA, UAG, UGA), which are recognized by eukaryotic release factor 1 (eRF1). Following GTP hydrolysis by eRF3a, the nascent peptide is released and ribosomal subunits are disassembled (Hellen, 2018). Compounds that specifically deplete eRF1 or eRF3a enhance full-length protein production and channel activity of CFTR premature termination codons (Sharma et al., 2021; Lee et al., 2022). Additional small molecules have been designed to reduce ribosome fidelity through a variety of mechanisms and confer read-through of CFTR nonsense alleles (Du et al., 2008; Crawford et al., 2021), although none of these pharmacologic agents have been clinically approved (Table 1).

Suppressor tRNAs have emerged as a promising new method for overcoming CFTR nonsense alleles. For example, anticodon engineered transfer RNAs (ACE-tRNAs) possess altered anticodons that pair to termination codons (Porter et al., 2021). ACE-tRNAs are stabilized by

TABLE 1 Therapeutic strategies presently employed for CF. Clinically approved agents are emphasized, as well as interventions undergoing clinical trials or advanced preclinical testing. Abbreviations: miRNA-binding blocker oligonucleotide (MBBO); peptide nucleic acid (PNA); antisense oligonucleotide (ASO); upstream open reading frame (uORF); translation suppression element (TSE); premature termination codon (PTC); nonsense-mediated decay (NMD); anticodon edited (ACE) transfer RNA (tRNA); exonic splicing enhancer (ESE); lipid nanoparticle (LNP); adeno-associated virus (AAV).

Therapeutic strategy		Sponsor	Intervention(s)	Clinical stage	References and/or clinicaltrials.gov identifiers
Gating and/or conductance variants					
Small molecules	Potentiator	Vertex Pharmaceuticals	Ivacaftor (VX-770)	Approved	KALYDECO (2020)
	Potentiator	Vertex Pharmaceuticals	Deuterated-Ivacaftor (VX-561)	Phase II	NCT03911713
Processing, trafficking, and/or expression variants					
Small molecules	1 Corrector +1 Potentiator	Vertex Pharmaceuticals	Lumacaftor (VX-809) + Ivacaftor	Approved	ORKAMBI (2019)
	1 Corrector +1 Potentiator	Vertex Pharmaceuticals	Tezacaftor (VX-661) + Ivacaftor	Approved	SYMDEKO (2020)
	2 Correctors +1 Potentiator	Vertex Pharmaceuticals	Elexacaftor (VX-445) + Tezacaftor + Ivacaftor	Approved	TRIKAFTA (2021)
	2 Correctors +1 Potentiator	Vertex Pharmaceuticals	VX-121 + Tezacaftor + Deuterated-Ivacaftor	Phase III	NCT05422222
					NCT05444257
					NCT05033080
					NCT05076149
	2 Correctors +1 Potentiator	Abbvie	ABBV-2222 + ABBV-3067 + ABBV-576	Phase II	NCT03969888
	Amplifier	Proteostasis Therapeutics	Nesolicaftor (PTI-428)	Reached Phase II trials before testing ended due to merger with Yumanity Therapeutics	NCT03591094
					NCT03500263 (PR Newswire, 2020)
MBBOs	miRNA blocker	Academic investigators	MBBO-1, MBBO-2, MBBO-3, MBBO-4	Preclinical	Viart et al. (2015)
PNAs	miRNA blocker	Academic investigators	ɣPNA/DNA nanoparticles; PNA-a145, PNA-a101	Preclinical	McNeer et al. (2015), Zarrilli et al. (2017), Sultan et al. (2020), Fabbri et al. (2021), Papi et al. (2022), Piotrowski-Daspit et al. (2022)
ASOs	Steric blockers of 5′-UTR uORF or TSE stem loop	Ionis Pharmaceuticals	uORF-ASO, TSE-ASO	Preclinical	Sasaki et al. (2019)
Nonsense variants					
Small molecules	PTC read-through	Eloxx Pharmaceuticals	Exaluren (ELX-02)	Phase II	NCT04135495
					NCT04126473
	PTC read-through	PTC Therapeutics	Ataluren (PTC-124)	Reached Phase III and "N-of-1" Phase IV trials before testing ended due to failure of meeting clinical endpoints	NCT03256968
					NCT03256799
					NCT00803205
					NCT01140451
					NCT02139306
	PTC read-through	Southern Research Institute	SRI-41315	Preclinical	Ng et al. (2021), Sharma et al. (2021)
	NMD inhibition	Cystic Fibrosis Foundation	SMG1i	Preclinical	Gopalsamy et al. (2012), Laselva et al. (2020), Venturini et al. (2021), de Poel et al. (2022)
	NMD inhibition	Academic investigators	CC-90009	Preclinical	Baradaran-Heravi et al. (2021), Nishiguchi et al. (2021), Lee et al. (2022)

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TABLE 1 (*Continued*) Therapeutic strategies presently employed for CF. Clinically approved agents are emphasized, as well as interventions undergoing clinical trials or advanced preclinical testing. Abbreviations: miRNA-binding blocker oligonucleotide (MBBO); peptide nucleic acid (PNA); antisense oligonucleotide (ASO); upstream open reading frame (uORF); translation suppression element (TSE); premature termination codon (PTC); nonsense-mediated decay (NMD); anticodon edited (ACE) transfer RNA (tRNA); exonic splicing enhancer (ESE); lipid nanoparticle (LNP); adeno-associated virus (AAV).

Therapeutic strategy		Sponsor	Intervention(s)	Clinical stage	References and/or clinicaltrials.gov identifiers
	NMD inhibition	Academic investigators	SJ6986	Preclinical	Baradaran-Heravi et al. (2021), Nishiguchi et al. (2021), Lee et al. (2022)
ASOs	NMD inhibition	Ionis Pharmaceuticals	SMG1-ASO, SMG6-ASO	Preclinical	Keenan et al. (2019), Sanderlin et al. (2022)
tRNA suppressors	PTC read-through	Academic investigators	ACE-tRNAs	Preclinical	Lueck et al. (2019), Porter et al. (2021), Ko et al. (2022)
Splice variants					
ASOs	Splice-switching	SpliSense	SPL84	Phase I/II	Oren et al. (2021), Oren et al. (2022), PR Newswire (2022)
	Splice-switching	Academic investigators	ASO-23A, ASO-23B; ESE-1, ESE-2, ESE-3	Preclinical	Michaels et al. (2020), Kim et al. (2022), Michaels et al. (2022)
Variant-agnostic approaches					
LNP vehicle	mRNA delivery	Arcturus Therapeutics	LUNAR® platform (ARCT-032)	Phase I	NCT05712538
	mRNA delivery	Vertex Pharmaceuticals	VX-522	Phase I	NCT05668741
	mRNA delivery	ReCode Therapeutics	SORT-LNP™ platform	Preclinical	BusinessWire (2023)
AAV vector	Gene delivery	4D Molecular Therapeutics	4D-710	Phase I	NCT05248230

post-transcriptional modifications and charged by endogenous aminoacyl-tRNA synthetases. Together, these alterations allow ACE-tRNAs to read-through CFTR premature stops, effectively rescuing channel function of certain CFTR nonsense variants to levels predictive of clinical benefit (Lueck et al., 2019; Ko et al., 2022). Additional species of suppressor tRNAs have been engineered with pseudouridine (Ψ) modifications in the anticodon stem loop, which confer stop codon read-through *via* incorporation of near-cognate amino acids (Albers et al., 2021). Although to date, this approach has only proven successful in bacteria.

Antisense oligonucleotides (ASOs) are presently under investigation as a therapeutic strategy for NMD inhibition and regulation of alternative splicing (Table 1). ASOs are short (~15–30 nucleotides), single-stranded DNA, RNA, or DNA:RNA analogs that bind and functionally alter complementary RNA sequences. Chemical modifications to ASOs can either: (1) sterically impair RNA-binding proteins, nucleic acids, or other factors involved in RNA utilization (Khorkova and Wahlestedt, 2017); or (2) recruit RNase-H to facilitate degradation of the RNA target (Crooke, 2017). ASOs employing steric hindrance have been clinically approved to treat diseases apart from CF, and have been well-tolerated in thousands of human subjects using a number of administration routes (Mahfouz et al., 2020; Singh et al., 2020; Akoumianakis et al., 2021). These ASOs do not require a delivery vehicle, thereby reducing risk of innate or adaptive immune responses in patients receiving repeat doses.

In the setting of CF, preclinical investigations indicate ASO-dependent silencing of SMG1 or SMG6 significantly increases transcript expression, protein trafficking, and transepithelial ion transport of CFTR nonsense variants (Keenan et al., 2019; Sanderlin et al., 2022). ASOs that sterically block uORFs in the 5′-UTR have also been shown to augment transcription and function of CFTR (Sasaki et al., 2019). Other ASOs have been developed as splice-switching oligonucleotides that overcome cryptic splice site activation. For example, the *CFTR* variant c.3718–2477C>T (3849+10kbC>T) generates a new 5′-splice site that causes incorporation of a cryptic exon bearing a nonsense codon (Highsmith et al., 1994). ASO-mediated inhibition of the alternative splice site prevents inclusion of the cryptic exon and restores function to 3849+10kbC>T (Michaels et al., 2020; Oren et al., 2021). This approach is presently undergoing clinical testing (PR Newswire, 2022).

Additional splice-switching ASOs facilitate exon removal. ASO-dependent skipping of exon-23 rescues functional expression of CFTR premature termination codons such as *p*.Trp1282Ter (Kim et al., 2022; Michaels et al., 2022; Oren et al., 2022). These ASOs preserve the CFTR reading frame while conferring enhanced mRNA stabilization and channel activity. Because C-terminal residues important for post-translational processing and channel gating would be retained, this strategy is being pursued for splicing and nonsense variants that occur within exon-23 and other in-frame *CFTR* exons (e.g. exon-9) (Martinovich et al., 2022). Minimizing

exposure of NMD suppression and/or splice-switching to localized area(s) of delivery remains a prominent consideration.

Discussion

Advancements in CFTR modulator treatments have revolutionized approaches to personalized medicine for people living with CF. However, effective therapeutic strategies are still needed to address underlying genetic defects for patients with presently untreatable forms of the disease. Sequence context plays an important role during CFTR processing, although contributing mechanisms remain poorly understood. Nucleotide identity, mRNA secondary structures, and additional features surrounding the variant–including proximity to the 5′- or 3′-end of *CFTR*–are likely to influence capacity for functional restoration. Tuning ribosome fidelity, translational velocity, and/or mRNA surveillance are viable methods for improving mutant CFTR biosynthesis. Central to the success of such approaches is CFTR mRNA quality. Future studies will be required to elucidate the most ideal sequence and/or modifications necessary for optimal transcript abundance, stability, and utilization.

Author contributions

JJ, YM, CF, and KO performed primary literature searches. JJ, YM, and CF drafted the initial manuscript. JJ, YM, and TW revised the manuscript. KO conceptualized the work, critically reviewed scientific content, and revised the manuscript.

Funding

This work was supported by the National Institutes of Health (R00HL151965), U.S. National Cystic Fibrosis Foundation (OLIVER22A0-KB), and the Atlanta Pediatric Research Alliance (00109545).

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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