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Suppressor tRNAs as personalized therapy for nonsense mutation-associated pathologies

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ABSTRACT

Nonsense mutations – associated with many devastating genetic disorders that currently lack effective treatments – prematurely terminate protein synthesis by converting an amino acid-encoding sense codon into a termination codon. Transfer RNAs (tRNAs), essential players in protein synthesis, have naturally evolved to decode sense codons, while avoiding the three stop codons (UGA, UAG, and UAA) that signal termination of translation. Emerging therapeutic strategies increasingly focus on refactoring natural tRNAs into suppressor tRNAs (sup-tRNAs). These engineered sup-tRNAs recognize nonsense mutation-associated premature stop codons (PTCs), restore translation, and recover protein function. This review summarizes recent advances in the design of sup-tRNAs to decode PTCs and discusses critical milestones in developing sup-tRNAs as a personalized therapeutic approach tailored to individual genetic backgrounds for treating pathologic conditions associated with nonsense mutations.

1. Introduction

Nonsense mutations account for approximately 11 % of all genetic lesions linked to human monogenic disorders and are typically associated with the most devastating loss-of-function pathologies (Mort et al., 2008). Within the coding sequence, nonsense mutations introduce a premature termination codon (PTC), terminating protein biosynthesis and leading to a mostly nonfunctional truncated protein product. Depending on their location, some nonsense mutations trigger mRNA surveillance mechanisms, e.g., nonsense-mediated mRNA decay (NMD), to degrade mRNAs containing PTCs and prevent the accumulation of potentially toxic truncated proteins (Lejeune, 2017; Moon et al., 2017). This dual impact exacerbates the nonsense mutation-mediated loss-of-function effects, underscoring the critical need for therapeutic strategies to effectively address the underlying complex molecular nature of the nonsense mutation-associated pathologies.

For over four decades, the potential of small molecules, also called translational readthrough inducing drugs (TRIDs) (e.g., aminoglycosides, oxadiazole derivatives, nucleoside analogs) to promote readthrough at PTCs and restore synthesis of the full-length functional protein has been explored (reviewed in (Ricci et al., 2025; Spelier et al., 2023; Torices et al., 2025)). More than 30 compounds have been characterized in preclinical studies and many have advanced to clinical trials for various disease indications (summarized in (Ricci et al., 2025; Spelier et al., 2023)). However, their therapeutic efficiency in clinical settings remains unconfirmed, most likely because of low efficacy below a clinical threshold, potential cytotoxicity because of unspecific amino acid incorporation, side effects at natural stop codons (NSCs), and variable specificity depending on the PTC identity and context (Beryozkin et al., 2023; Friesen et al., 2017; Hristodor et al., 2025; Leroy et al., 2023; Mangkalaphiban et al., 2024; Morais et al., 2024; Rowe et al., 2011; Roy et al., 2016; Spelier et al., 2023; Toledano et al., 2024; Trzaska et al., 2020; Tutone et al., 2020; Wangen & Green, 2020). So far, only ataluren (PTC124 or Translarna) (Welch et al., 2007) has been tested in clinical settings and has received conditional approval in Europe to treat patients with Duchenne muscular dystrophy aged two years and older. Despite the initial encouraging results, the prolonged treatment over more than five years failed to confirm the effectiveness of

Abbreviations: aaRS, aminoacyl-tRNA synthetase; AAV, adeno-associated virus; ACE-tRNA, anticodon-edited tRNA; AI, artificial intelligence; ASO, antisense oligonucleotides; BDP1, B-double prime 1; bp, base pairs; BRF1, B-related factor 1; CNS, central nervous system; eEF1A, Eukaryotic Translation Elongation Factor Alpha 1; eRF1, eukaryotic release factor 1; IVT, in vitro transcribed; LNP, lipid nanoparticle; mRNA, messenger RNA; NMD, nonsense-mediated mRNA decay; NSC, natural stop codon; nt, nucleotides; Pol III, polymerase III; PTC, premature termination codon; TF, transcription factor; TRIDs, translational readthrough inducing drugs; tRNA, transfer RNA; sup-tRNAs, suppressor tRNAs; 5'UCE, 5′-upstream control element.

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Translarna. In 2024, EMA discontinued the renewal of its authorization for the European market. Despite the continuous search for new compounds (Leroy et al., 2023; Ricci et al., 2025), the limited clinical success and persistent challenges of pharmacological TRIDs (Spelier et al., 2023), new alternative approaches are urgently needed to address the unmet therapeutic needs of patients with nonsense mutation–associated pathologies. Refactored natural tRNAs into suppressor tRNAs (sup-tRNAs) – a concept inspired by naturally occurring nonsense sup-tRNAs, which have evolved in various organisms to recognize NSCs – have emerged as a versatile and effective strategy for suppressing nonsense mutations (Albers et al., 2023; Ko et al., 2022, 2025; Lueck et al., 2019; Pezzini et al., 2024; Porter et al., 2024; Specht et al., 2025; J. Wang et al., 2022). Recent advancements in RNA delivery technologies, combined with the success and compelling safety profile of mRNA-based vaccines, have accelerated the progress of sup-tRNA research over the past five years (Anastassiadis & Köhrer, 2023; J. Coller & Ignatova, 2024; Dolgin, 2022). Technological and therapeutic breakthroughs in small RNA medicines (e.g., siRNA and antisense oligonucleotide (ASO)-based therapies (Adams et al., 2018; Dweh et al., 2025; Hua et al., 2011; Krey-Grauert et al., 2025; Lauffer et al., 2024; Mercuri et al., 2018) are further driving the developments in the use of sup-tRNAs as innovative gene therapy solutions for monogenic disorders associated with nonsense mutations. By delivering engineered tRNAs that decode PTCs and precisely insert the lost amino acid, sup-tRNAs offer a targeted and potentially effective approach for restoring protein function. However, some challenges remain in establishing sup-tRNAs as a safe and effective

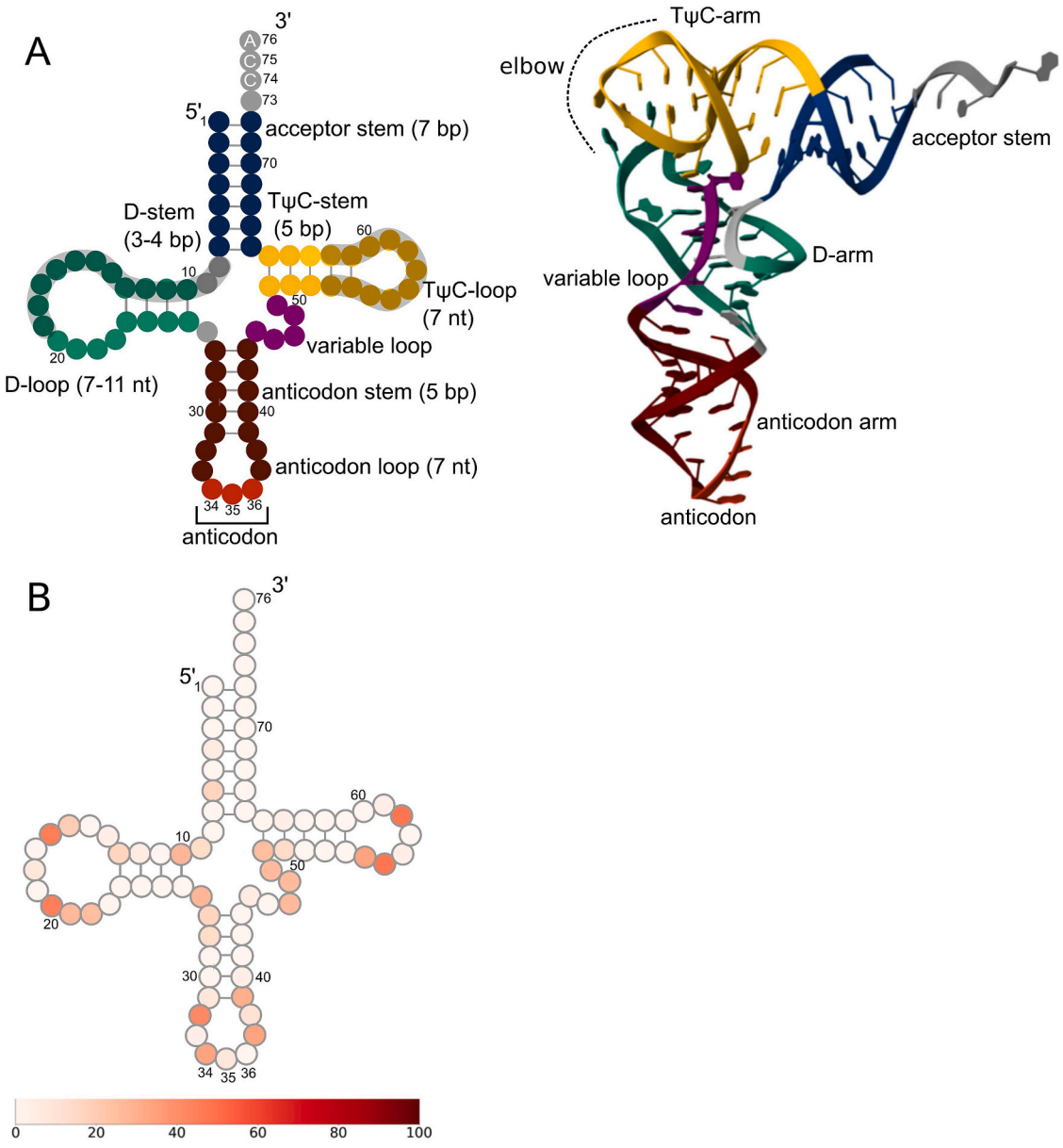

Fig. 1. tRNA structure and modification pattern. (A) tRNA secondary (left) and L-shaped 3D (right) structure (exemplified with the structure of yeast $tRNA^{Phe}$, PDB: 1ehz). tRNA nucleotides establish extensive secondary interactions forming four structured domains: acceptor stem, D-loop and D-stem, anticodon loop and stem, and TψC-loop and TψC-stem, whose lengths are strictly conserved (Kirchner & Ignatova, 2015) (designated as base pairs (bp) for the stems and nucleotides (nt) for the loops). The positions of the A- (8–19 nt) and B-box (52–62 nt), the intrinsic promoter required for transcription, are highlighted with a gray line. The length of the D-loop and variable loop can vary, leading to differences in the total tRNA length (typically between 76 and 90 nt). Despite these length variations, a standardized annotation is used (e.g., the anticodon is always numbered 34–36; the 3'-CCA end is 74–76). Interactions between the D- and TψC-loops stabilize the L-shaped tertiary structure (e.g., the elbow in the right-side structure). (B) Heat map of the modification frequency represented as aggregated frequency at each nt across all nuclear-encoded human tRNAs deposited in the MODOMICS database (Cappannini et al., 2024).

therapy.

Several excellent reviews describing recent developments of delivery vehicles for RNA therapeutics, also suitable for tRNA-therapeutics, including critical milestones for optimizing their safety, have been published (Cullis & Felgner, 2024; Hou et al., 2021; Kontogiannis et al., 2024; Loughrey & Dahlman, 2022; O'Brien Laramy et al., 2025; Suarez-Amaran et al., 2025; Wang, Gao, & Wang, 2024; Wang, Gessler, et al., 2024). Here, we focus on current strategies in sup-tRNA design aimed at enhancing their efficacy, stability, and safety, with a focus on their clinical translation. We compare the two major routes of sup-tRNA delivery (e.g. into cytosol or to the nucleus) and discuss key considerations for sup-tRNA design and use in personalized treatment of patients with nonsense mutations.

2. tRNA repertoires and function

2.1. Canonical function of tRNAs

Transfer RNAs (tRNAs) are pivotal for translation and play a central role in decoding sense codons of mRNAs. tRNAs are loaded with a corresponding amino acid by their cognate aminoacyl-tRNA synthetase (aaRS), enabling sequential insertion of an amino acid into the growing polypeptide chain determined by the sequence of the sense codons of the mRNA (reviewed in (Berg & Brandl, 2021; Kirchner & Ignatova, 2015; Orellana et al., 2022)). tRNAs are conserved in length and structure, e.g., partially double-stranded cloverleaf structure, which further folds into a 3D L-shaped structure (Fig. 1A), to fit the same ribosomal site and ensure similar decoding efficacy of all tRNAs in the cell (Westhof et al., 2022).

The genetic code is degenerate, and the 20 proteinogenic amino acids (except for methionine and tryptophan) are encoded by more than one codon. In humans, 46 different tRNA species (called isoacceptors) serve all 61 sense codons, whereby some isoacceptors decode more than one codon. With one codon, the tRNA establishes canonical base pairs at all three positions. With the other codon, two bases pair canonically, while wobble pairing between the first nucleotide in the tRNA anticodon (position 34, Fig. 1A) and the last nucleotide of the mRNA codon allows non-canonical hydrogen bonding. tRNAs are pervasively post-transcriptionally modified (Fig. 1B), with modifications displaying varying chemical complexity (reviewed in (Pan, 2018; Schultz & Kothe, 2024; Suzuki, 2021; M. Zhang & Lu, 2025)). Modifications in the anticodon loop facilitate decoding, while modifications in the tRNA body mainly modulate stability and interactions with tRNA-interacting proteins. The wobble position (position 34, Fig. 1A) is highly modified, facilitating also non-canonical base pairing with the corresponding nucleotide in the mRNA codon.

The nuclear genomes of higher eukaryotes contain several hundred tRNA genes, a small fraction of which encode tRNAs with identical mature sequences, thus representing identical gene copies (Berg & Brandl, 2021; Ehrlich et al., 2021). The rest of the tRNA genes bear sequence differences in the tRNA body. For example, the human hg38 genome from the GtRNAdb tRNA-database (Chan & Lowe, 2016) contains close to 600 interspersed tRNA genes, which can potentially generate 432 unique tRNA sequences, that is defined as the entire tRNA repertoire (or tRNAome) in one cell or organism. Each tRNA isoacceptor includes multiple tRNAs that share the same anticodon but differ in their body sequences outside the anticodon region; these variants are termed isodecoders. The presence of such a large number of isodecoders in genomes is not entirely clear. The variations of the gene copy number between human individuals, with some tRNA isodecoders completely absent (Iben & Maraia, 2014), question their functional importance. Increasing evidence suggests tissue-specific expression of various isodecoders (Dittmar et al., 2006; Gao et al., 2024; Hughes et al., 2023; Kapur et al., 2024). The sequence variations of the isodecoders could influence their stability, processing, and interactions with other cellular components during translation, thus contributing to the adaptability of the human tRNA repertoire and tissue-specific translational plasticity.

Chloroplast and mitochondrial genomes encode their own tRNAs, which show broader structural heterogeneity, deviating from the conserved structural features of nuclear-encoded cytosolic tRNAs (Fig. 1A). Several excellent reviews describing their biogenesis, structure, and modifications have been published (Chrzanowska-Lightowlers et al., 2017; Suzuki et al., 2011; Tiller & Bock, 2014; Zoschke & Bock, 2018), and thus, these organellar tRNAs are not a focus of this review.

The canonical function of tRNAs is to pair with sense codons during the decoding process at the ribosome. Thereby, cognate tRNAs, whose anticodon matches the sense codon, are selected in the decoding process. tRNAs with a single-nucleotide mismatch to the anticodon, called also near-cognate, are typically rejected by the ribosome in the proofreading step (Noller, 2024; Rodnina et al., 2017). Generally, tRNAs have evolved to avoid the recognition of NSCs (UGA, UAG, and UAA), which are instead recognized by release factors, specifically eRF1 in mammals, resulting in termination of translation and release of the nascent polypeptide from the ribosome (Hellen, 2018).

In rare cases, near-cognate tRNAs can occasionally pair to NSCs, leading to readthrough and extension of the peptide chains. Compared to the NSCs decoding by a near-cognate tRNA, the binding to the release factor is significantly more energetically favorable (Blanchet et al., 2018; Floquet et al., 2012). As a result, the spontaneous readthrough at NSCs is extremely rare, with a frequency lower than 0.1 % (Floquet et al., 2012). This frequency varies among the three different NSCs, with certain near-cognate tRNAs pairing more frequently with specific NSCs, so that at UGA codons, primarily Trp, Arg, and Cys are inserted, whereas Gln, Tyr, and Lys are inserted at UAA and UAG (Roy et al., 2015). In *Saccharomyces cerevisiae* and *Trypanosoma brucei*, the length and the composition of the anticodon stem, in particular the identity of base pair 28:42, determine the stop codon decoding efficiency of near-cognate tRNA (Pavlíková et al., 2024). It remains unclear whether this effect is restricted to species that, compared to humans, have a much lower tRNA set with a different composition.

Post-transcriptional modifications at NSCs (Karijolich & Yu, 2011) or a decrease in the concentration of release factor eRF1 by drug-aided inhibition (Carnes et al., 2003; Gurzeler et al., 2023; Sharma et al., 2021) facilitate readthrough of NSCs by near-cognate tRNAs. Inherited mutations in the *ETF1* gene encoding eRF1 that are linked to pathologies are not known and have only been found in a few acute cancers in a monoallelic state (Dubourg et al., 2002), suggesting strong evolutionary pressure to maintain the functional integrity of this essential protein. Among different posttranslational modifications, pseudouridine (Ψ) at the first position of NSCs is the most potent one in stimulating readthrough by near-cognate tRNAs. At the modified ΨGA, Tyr and Phe are incorporated, and at ΨAA and ΨAG – Ser and Thr (Karijolich & Yu, 2011). However, decoding ΨNN stop codons requires forming two typically forbidden purine-purine base pairs at the second and third position with an unusual Watson-Crick/Hoogsteen geometry (Fernández et al., 2013). Although the readthrough at artificially pseudouridinylated NSCs is relatively high (Adachi & Yu, 2020; Luo et al., 2024; Song et al., 2023), the natural occurrence of NSCs with Ψ at the first nucleotide is extremely rare. It is found in only a handful of human transcripts (Dai et al., 2023), implying stringent evolutionary gatekeeping strictly limits NCS modifications to maintain faithful termination.

2.2. Natural nonsense sup-tRNAs

In some organisms, tRNAs have been identified that decode one of the NSCs, mediating a low level of readthrough and are, thus, termed natural nonsense sup-tRNAs. These natural sup-tRNAs predominantly derive from cellular tRNAs and recognize one of the three NSCs (UGA, UAG, or UAA) as near-cognate or non-cognate tRNAs. The first natural sup-tRNA was identified in 1971 by David Hirsh and named after him (Hirsh, 1971). Replacing G24 with adenosine in the D-stem of $tRNA^{Trp}$ enabled suppression of *E. coli* UGA NSC. The G24A mutation forms an additional internal interaction that stabilizes the tRNA distortion

required for decoding the UGA NSC (Schmeing et al., 2011).

Nucleotide changes in the tRNA body that modulate the conversion of a sense-codon decoding tRNA to sup-tRNA are rare natural cases. Most commonly, changes in the modification pattern in the anticodon or its vicinity enable non-standard base pairing between NSC and tRNA (Beier & Grimm, 2001). In yeast, modifications at or adjacent to the anticodon of tRNAs for Tyr, Gln, Lys, Trp, Cys, and Arg codons reveal that their functions extend beyond sense-codon decoding also to support NSC decoding (Blanchet et al., 2018). Other examples include the nuclear-encoded $tRNA^{Tyr}$ with a GΨA anticodon decoding UAG NSCs, a function that has not been observed for $tRNA^{Tyr}$ with unmodified GUA or QΨA anticodon (Beier, Barciszewska, Krupp, et al., 1984; Beier, Barciszewska, & Sickinger, 1984; Suter et al., 1986). Additionally, yeast sup-tRNAs lacking the i^6A modification at position 37 display a lower level of suppression (Janner et al., 1980; Laten et al., 1978). In the widely used laboratory organisms, *E. coli* or *S. cerevisiae*, some natural tRNAs can acquire mutations in their anticodon following external stress stimuli that allow them to establish a canonical Watson-Crick base-pairing with the NSC (Beier & Grimm, 2001). Broadly, $tRNA^{Sec}$ or $tRNA^{Pyl}$ also belong to the class of natural sup-tRNAs as they incorporate selenocysteine and pyrrolysine at UGA and UAG stop codons, respectively. However, these two tRNAs alone do not effectively recode stop codons and require a coordinated action of specialized elements to facilitate the recognition of the corresponding NSC. For example, $tRNA^{Sec}$ is supported by a distinct mRNA structural element (SECIS) and a dedicated translational factor (SelB), while $tRNA^{Pyl}$ requires a PYLIS sequence (Y. Zhang et al., 2005). Recently, a variant of the natural $tRNA^{Trp}$ has been identified in trypanosomatids, incorporating tryptophan at in-frame UGA codons (Kachale et al., 2023). The anticodon stem of this tRNA variant is shorter by one base pair. Similarly to $tRNA^{Sec}$ and $tRNA^{Pyl}$, this $tRNA^{Trp}$ variant alone is unable to recode an NCS; a concurrent mutation in eRF1 facilitates its suppression function (Kachale et al., 2023).

Notably, compared to natural nonsense sup-tRNAs that recode stop codons a much higher number of frameshift suppressor tRNAs have been evolutionarily selected. Those natural frameshift suppressor tRNAs either retain their original anticodon, while harboring mutations outside the anticodon stem-loop that affect tRNA flexibility and stability, or accommodate an extra nucleotide within the anticodon region, allowing four-nucleotide decoding (Atkins & Björk, 2009; Demo et al., 2021; Fagan et al., 2014; Gaber & Culbertson, 1982; Gamper et al., 2015; Hatfield et al., 1990; Maehigashi et al., 2014; Mendenhall et al., 1987). Mechanistically, restoring shifts in the translational reading frame via frameshift suppressor tRNAs differs from nonsense sup-tRNA-mediated stop-codon readthrough, which requires maintenance of the precise triplet reading frame. As a result, different nucleotide positions within these two classes of natural suppressor tRNAs are subject to distinct evolutionary selection pressure.

3. tRNA biosynthesis cycle – An inspiration for sup-tRNA delivery strategies

3.1. Human tRNA biogenesis cycle

In eukaryotes, transcription of the tRNA genes is mediated by a concerted action of two transcription factors, namely TFIIIC, which recognizes the intragenic A-box and B-box sequences (Fig. 1A), and TFIIIB, which binds to the 5′ upstream regions (Fig. 2). The interaction of TFIIIC with the intrinsic tRNA promoters is mediated by the B-box, which has a much higher affinity and faster binding kinetics than the A-box (Seifert-Dávila et al., 2025). Subsequent interaction of TFIIIC with the A-box (Fig. 2) is necessary to position TFIIIB at a fixed distance

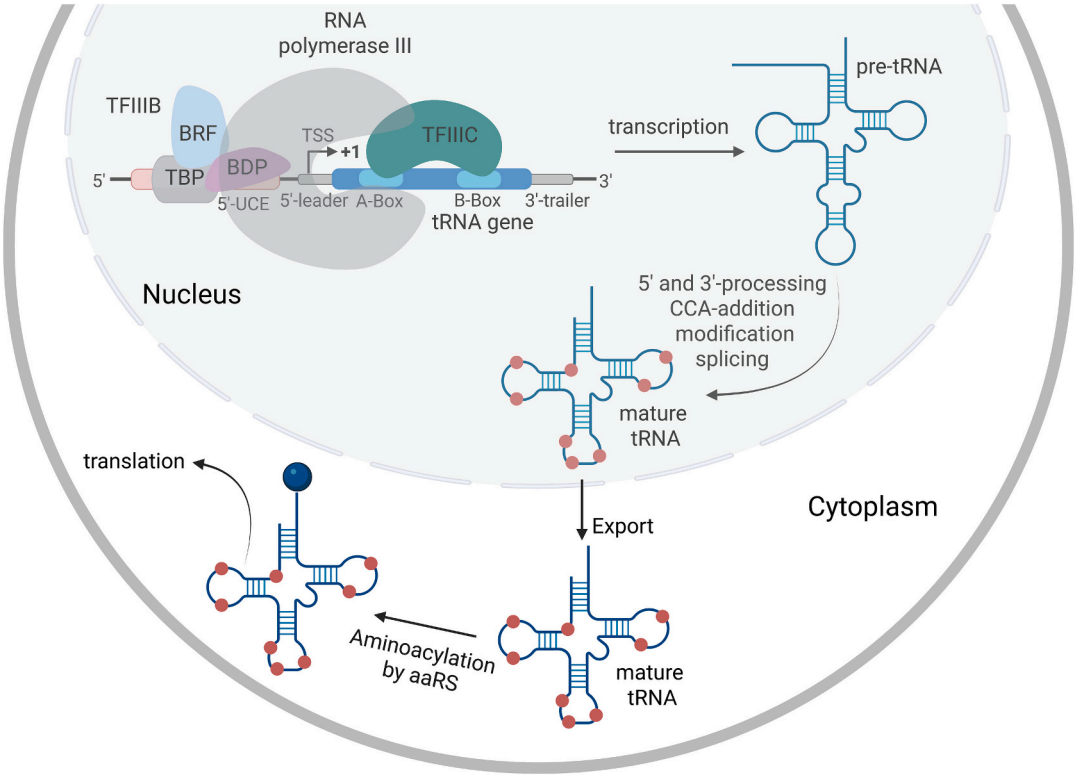

Fig. 2. Biogenesis cycle of nuclear-encoded tRNAs. The transcription factors TFIIIC and TFIIIB (consisting of three subunits – B-double prime 1 (BDP1), B-related factor 1 (BRF1), and TATA-binding protein (TBP)) recruit Pol III to the transcription start site (TSS) in the 5′-upstream control element (5′-UCE). During maturation, tRNAs are processed at their 5′- and 3′-ends; intron-containing tRNAs are spliced; the 3′- CCA-end is added; tRNAs are modified and exported to the cytoplasm, where they are aminoacylated by their cognate aaRS to participate in translation. Red dots represent posttranscriptional modifications; the large blue dot – amino acid group. Created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

upstream of the A-box (Seifert-Davila et al., 2023; Talyzina et al., 2023; Vorländer et al., 2020). The binding of both transcription factors facilitates RNA polymerase III (Pol III) recruitment to initiate tRNA transcription. In particular, the TFIIIB subunit Bdp1 rearranges the Pol III subunits, thereby promoting DNA opening (Abascal-Palacios et al., 2018). While the intragenic A- and B-boxes are highly conserved, the 5′-TFIIIB-binding region is more variable in mammalian tRNA genes and lacks a common sequence signature. Mammalian genomes express multiple splicing variants of the two TFIIIB subunits, BDP1 and BRF1, with tissue-specific expression patterns, that likely exhibit different sequence binding motifs and thus, may modulate the expression of tissue-specific isodecoders (G. Zhang et al., 2011).

Following transcription, the nuclear-encoded tRNAs undergo a complex maturation cycle in the nucleus (Hopper & Huang, 2015), including processing of 5′-leader and 3′-trailer sequences, splicing for some intron-containing tRNAs, 3'-CCA-addition and extensive modification by dedicated enzymes specialized to insert a single modification (Fig. 2). Aberrantly processed pre-tRNAs are eliminated through nuclear surveillance pathways. tRNAs that pass the quality check are exported into the cytosol, the playground of translation (Fig. 2).

Eukaryotic genomes do not encode the 3′-single-stranded CCA ends in their tRNA genes, which are added post-transcriptionally by the CCA-adding enzyme. The 3'-CCA sequence is required for the catalytic function of the cognate aaRS and esterification of the last adenosine with the corresponding amino acid (reviewed in (Gomez & Ibba, 2020; Yakobov et al., 2018)). The 20 aaRS (one for each proteinogenic amino acid) are divided into two major classes, based on distinct structural architecture and functional characteristics. Class I aaRSs are predominantly monomeric, bind the minor groove of the acceptor stem, and aminoacylate the 2′-hydroxyl group of the ribose of A76. In turn, class II aaRSs are commonly dimeric or multimeric, bind the major groove of the tRNA acceptor stem, and aminoacylate the 3′-hydroxyl group of A76. Different parts of the tRNAs (e.g., acceptor stem, anticodon loop and stem, variable loop, Fig. 1A) and/or combinations thereof serve as a unique set of recognition elements for the cognate aaRS (Giegé & Eriani, 2023). Thereby, the anticodon identity or nucleotides thereof serve as a recognition signal for many aaRSs.

Aminoacylated tRNAs (or tRNAs charged with their cognate amino acid) immediately form a ternary complex with GTP and elongation factor eEF1A, which is among the most abundant proteins in the cell. The ternary complex (aminoacyl-tRNA-eEF1A-GTP) reaches the ribosomal A site by diffusion. Through the ternary complex, the ester bond between the amino acid and A76 is protected from hydrolysis in the nearly neutral pH of the cytosol (Peacock et al., 2014).

3.2. Sup-tRNA delivery routes

Considering the tRNA biogenesis cycle (Fig. 2), two possibilities for sup-tRNA delivery emerge: (i) *cytosolic delivery* as a ready-to-use tRNA delivered directly into the cytosol, and (ii) *nuclear delivery* in a form of a plasmid for independent episomal expression (Fig. 3). The delivery routes for sup-tRNAs leverage the same systems currently being developed for mRNA replacement therapies which is facilitated by shared features in their biogenesis (i.e., in the nucleus) and their common function as essential components of the translational machinery (i.e., in the cytoplams of the cell). Both delivery modes for sup-tRNAs have been tested in preclinical settings and establish their efficacy in correcting PTCs associated with nonsense mutations in vivo (i.e., in mouse models) and in in vitro cell systems (e.g. cell culture, patient-derived primary cells) (Albers et al., 2023; J. Wang et al., 2022).

Sup-tRNAs for *cytosolic delivery* are commonly produced by in vitro T7 transcription from a double-stranded DNA template containing the T7 promoter or by chemical synthesis of full-length sup-tRNAs. Usually, transcription with T7 RNA polymerase generates byproducts with shorter length (Lenk et al., 2024) and full-length sup-tRNAs are purified by polyacrylamide gel electrophoresis. The in vitro transcribed (IVT) sup-tRNAs are typically packaged into carriers and delivered into the cytoplasm, where they are aminoacylated by the cognate aaRS and immediately engaged in translation (Fig. 3). Full-length protein production is re-established as early as six hours post administration in vivo (Albers et al., 2023). Over several days, these IVT sup-tRNAs remain active for multiple rounds of aminoacylation and translation, with a

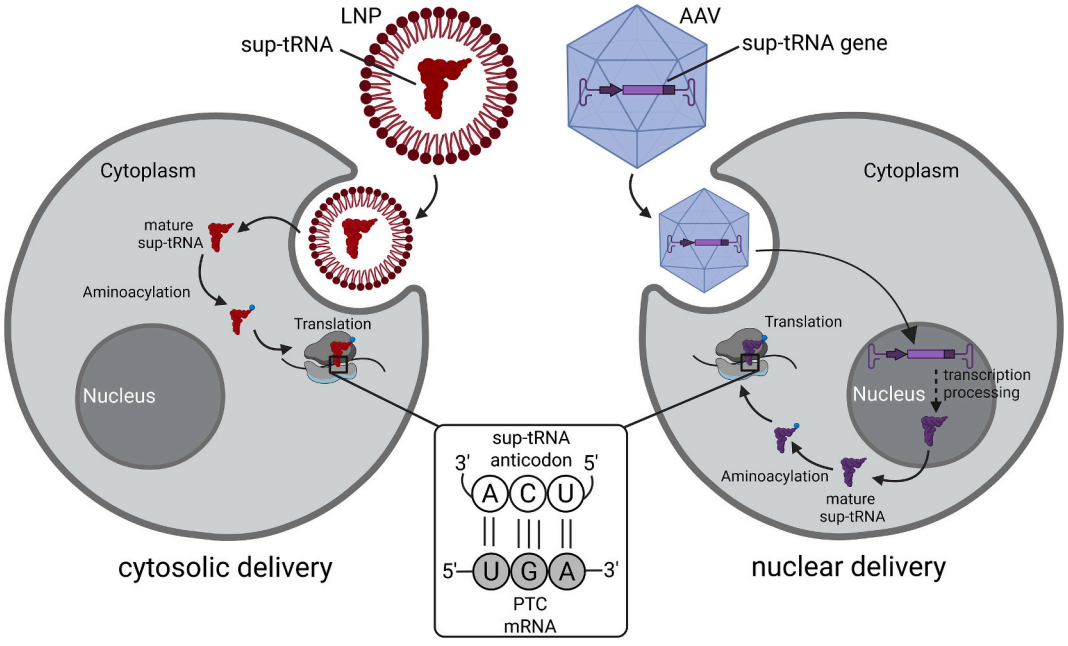

Fig. 3. Delivery routes of sup-tRNAs. Cytosolic delivery (left route). IVT-produced or chemically synthesized full-length sup-tRNA (red) packaged into LNPs is released into the cytoplasm and immediately engaged in translation. Nuclear delivery (right route). A sup-tRNA gene (purple) carried on a small plasmid is packaged into AAV particles and delivered to the nucleus where it enters the endogenous tRNAs biosynthesis pathway. In both cases, the sup-tRNA decodes a target PTC, here exemplarily shown for UGA. Created with BioRender.com. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

lifespan comparable to endogenous tRNAs (Albers et al., 2023). The cytosolic delivery of IVT sup-tRNAs would require a frequent lifelong re-administration. Lipid nanoparticles (LNPs), which have experienced their breakthrough with the mRNA vaccines targeting SARS-CoV-2 virus (Hou et al., 2021; X. Xu & Xia, 2023), are one of the most clinically advanced delivery systems, with multiple current approvals for clinical use. They are typically the system of choice for cytosolic sup-tRNA delivery, because LNPs have limited ability to penetrate the nucleus (Barua & Mitragotri, 2014).

For *nuclear delivery*, the genetic sequence of a sup-tRNA, embedded between a Pol III promoter and terminator element, is cloned into small plasmids (Fig. 3) which engage the host endogenous transcription machinery to produce mature sup-tRNAs following the same expression and processing route as endogenous tRNAs (Fig. 2). This long natural biogenesis process delays the functional onset of sup-tRNA compared to the direct cytosolic administration of IVT sup-tRNA. The nuclear delivery of the sup-tRNAs on small episomally expressed plasmids, which should remain extrachromosomal with no integration into the nuclear genome, provides a durable expression mode that ideally, with a single administration, should persist throughout the lifetime of the cell or organism (Wang, Gao, & Wang, 2024). Typically, recombinant adeno-associated virus (AAV) particles – the gold standard in gene delivery – are preferred vehicles for nuclear delivery. AAVs are a versatile delivery option targeting any tissue, including the CNS by utilizing the innate ability of the viruses to cross the blood-brain barrier (Wang, Gao, & Wang, 2024). AAVs are among the most clinically advanced delivery systems, with worldwide more than 700 AAV-based gene therapy programs in clinical trials (Suarez-Amaran et al., 2025) – developments that will boost the clinical use of sup-tRNAs.

The clinical translation of the sup-tRNAs would greatly benefit from the developments of the delivery toolbox for nucleic acids. Overall, all types of vehicles optimized for the delivery of different RNAs could be utilized for sup-tRNA administrations, except for bioconjugation approaches. In clinical settings, bioconjugation has demonstrated a high safety profile as a delivery system for small RNAs. For example, *N*-acetyl galactosamine covalently linked to an antisense oligonucleotide targeting mRNA transcripts of HBV (Yuen et al., 2022) (Bepirovirsen; clinical trial NCT05330455); siRNA inhibiting the synthesis of angiopoietin-like protein 3 (ANGPTL3) for lowering triacyl glycerides and cholesterol (Watts et al., 2023) (Solbinsiran; clinical trial NCT04644809)) represent two such clinically advanced applications. However, in its current form, bioconjugation remains unsuitable for the delivery of sup-tRNAs. The process requires covalent extension of the RNA entity with different moieties to improve biodistribution to a specific region or cell type (e.g., antibodies), increase receptor-mediated transport (e.g., *N*-acetyl galactosamine), and/or increase lipophilicity (e.g., cholesterol) (Benizri et al., 2019). The intact 5′- and 3′-termini of the sup-tRNAs are essential for their biological activity, thus any persistent covalent conjugation will render them inactive.

3.3. Considerations for choosing cytosolic versus nuclear delivery

The efficacy of the cytosolic deliveries is determined by the half-life of the sup-tRNA, which is comparable to that of the natural tRNAs, e.g., several days to weeks (Albers et al., 2023), and thus, is significantly longer than that of exogenously administered mRNAs (Pateev et al., 2024). To sustain the full-length protein synthesis, repeated dosing is required, with a frequency of re-administration depending on the *in-tissue* stability of the sup-tRNA and the targeted protein. LNP formulations are generally considered safe, including approvals for re-administration. However, repeated dosing has thus far been limited to a small number of cycles, primarily in re-immunization contexts. Multiple re-administrations will require a rigorous evaluation of immunogenicity and general safety. Recent studies have shown some immunostimulatory effects associated with LNPs (reviewed in (Azarnezhad et al., 2020; Chen & Blakney, 2024; Yuan et al., 2024). Each component of the LNP formulations currently approved for clinical use, e.g., ionizable cationic lipid, phospholipid, cholesterol, polyethylene glycol-lipid, likely contributes to the overall immunogenicity. Ongoing efforts are underway to identify alternative polymers with fewer immune-mediated liabilities, which would facilitate the use of LNP carriers for lifelong redosing regimens.

LNPs are suitable for addressing many organs, except the central nervous system (CNS). Recent studies, however, indicate that specific modifications of the LNP components enable delivery to the CNS and efficient crossing of the blood-brain-barrier (Khare et al., 2023; Teixeira et al., 2023; C. Wang et al., 2025), likely to increase the application spectrum of LNPs for nuclear deliveries. LNPs remain a single option for tissues unsuitable for AAV administrations, e.g., pulmonary tissue, because of pre-existing neutralizing antibodies due to prior infections (Chirmule et al., 1999; Xiao et al., 1996). Overall, cytosolic LNP-mediated sup-tRNA administration could be a preferred option for diseases linked to high-metabolizing tissue(s) of onset, where an immediate effect (i.e., rapid restoration of full-length production) would be beneficial. Furthermore, pathologies involving proteins with long half-lives in slow-metabolizing tissues are also attractive targets, as they may allow for extended intervals of re-dosing. Innovative solutions for easy application of LNP-encapsulated drugs (e.g., nebulized preparations for inhalation) (Jiang, Lathwal, et al., 2024; Jiang, Witten, et al., 2024; Meng et al., 2024; Yong et al., 2025) make applications in domestic settings feasible. One significant advantage of cytosolic delivery is that, in the event of person-specific adverse effects, the treatment can be immediately discontinued, and the effect will vanish with the sup-tRNA degradation.

Conceptualized as a single lifelong administration, the AAV-mediated sup-tRNA delivery to the nucleus would require continuous and stable expression of the sup-tRNA from the episomal plasmid. A recent publication reports that the sup-tRNA production remains stable, with a sustained suppression effect observed for at least six months in mice (J. Wang et al., 2022). Although data from animal studies and emerging human trials using AAV vehicles suggest that the expression in whole-gene replacement therapies can be maintained for 4–15 years (Muhuri et al., 2022), extended monitoring of sup-tRNA safety and efficacy, along with testing in various disease models, is necessary before advancing to clinical trials. The durability of therapeutic response (here, constant level of sup-tRNA expression) is key in this strategy, especially since immune responses to AAV vectors prevent re-dosing. In addition, many humans are seropositive, thus precluding a large cohort of the human population from potential treatment with AAV-mediated sup-tRNAs (Earley et al., 2023). Removing AAV-neutralizing antibodies or using immunosuppressive adjuvants to inhibit pre-existing immunity or immune response to AAV vehicles holds promise for re-dosing, should efficacy wane over time (Ertl, 2024). In general, AAV-mediated (single) administrations should be a preferred option for targeting non-dividing cells, as an unequal inheritance and partitioning of the episomal plasmids in the off-spring cells would lead to a gradual loss of the plasmid over successive cell divisions (Kymäläinen et al., 2014), resulting in decreased sup-tRNA expression.

3.4. Safety of sup-tRNAs entities in the sup-tRNA therapeutics

The safety profile of the sup-tRNA therapeutics is collectively determined by both the delivery vehicle and the sup-tRNA entity. Several excellent reviews address the specificity and tropism, safety and toxicity, along with limitations in application of each delivery system (Godbout & Tremblay, 2022; Grossen et al., 2023; Hou et al., 2021; Hudry & Vandenberghe, 2019; Naso et al., 2017; Peters et al., 2021; Wang, Gessler, et al., 2024; Wei et al., 2024; X. Xu & Xia, 2023; T. Zhang et al., 2024), thus those aspects are not covered in this review.

In addition to the classical safety assessments for RNA-based therapies (e.g. general toxicity, biodistribution and tropism, off-target effects in non-targeted tissues and organs), comprehensive evaluations of the

molecular safety and potential risks, including off-target effects at NCSs, adverse effects on host translation and tRNA pools, are necessary to establish sup-tRNAs as safe therapeutics (J. Coller & Ignatova, 2024). Since PTCs share sequence identity with NSCs, a crucial aspect of safety evaluation involves Ribo-seq (also called ribosome profiling (Ingolia et al., 2009), which allows for determining the presence of translating ribosomes downstream of the NSCs with transcript-specific precision. Both in vitro studies in cell culture and in vivo studies in mice, using either LNP-IVT-sup-tRNAs (Albers et al., 2023) or episomal sup-tRNA-AAV delivery (Lueck et al., 2019; J. Wang et al., 2022), demonstrated high specificity of sup-tRNAs for PTCs, with no detectable readthrough at NSCs with the same identity to the PTC or at unrelated NSCs. This specificity is likely driven by evolutionary pressure on sequences upstream of NSC to facilitate faithful termination and limit susceptibility to readthrough (Bharti et al., 2024).

In mice, nuclear AAV-mediated delivery of sup-tRNAs did not significantly alter the endogenous tRNA pool, as assessed by tRNA-seq (J. Wang et al., 2022). In this analysis, tRNAs were grouped by anticodon, so subtle effects on individual isodecoders or isoacceptors might be masked. Similarly, LNP-IVT-sup-tRNA administrations did not perturb endogenous tRNAome (Albers et al., 2023), which is expected given that these tRNAs are delivered as mature molecules and do not engage the host transcriptional machinery. However, for both delivery strategies (Fig. 3), it remains critical to evaluate aminoacylation efficiency to rule out competition for the corresponding aaRS, which could disrupt the aminoacylation balance of endogenous tRNAs.

Histological and serum analysis in mice ten weeks post-administration of AAV-sup-tRNAs showed no abnormalities (J. Wang et al., 2022), indicating a lack of overt toxicity of sup-tRNA therapy. One area that remains underexplored is the immunogenic potential of IVT sup-tRNAs when delivered cytosolically. A recent study using Toll-like receptors (TLR)-transfected HEK293 cells as a system to monitor immunogenicity shows no elevation of IL-8 levels beyond those induced by the transfection reagent alone, suggesting that TLR7 and TLR8 were not activated (Albers et al., 2023). However, these cells represent an artificial system and may not fully recapitulate in vivo responses, particularly under conditions of systemic administration or chronic exposure in durable re-administration regimes, thus, requiring careful evaluation in clinical settings. Furthermore, it remains unclear whether IVT sup-tRNAs, delivered thtough the cytoslic route, are modified in the cell post-delivery, and if so, how these modifications affect the tRNA immunogenicity and therapeutic performance. Thus, further research is needed to evaluate potential innate immune responses triggered at the onset of delivery and throughout the entire life cycle of the IVT sup-tRNAs.

Collectively, although the current results are limited to a small number of sup-tRNAs (Albers et al., 2023; Lueck et al., 2019; J. Wang et al., 2022), those findings are encouraging and suggest that key components of the host translation machinery remain largely unaffected, supporting the high therapeutic potential of sup-tRNAs.

4. Design strategies to enhance the suppression efficacy of sup-tRNAs

More than four decades ago, Kan and colleagues showed the feasibility of sup-tRNA to correct nonsense mutation at the lysine 17 codon in β-globin mRNA linked to β-thalassemia (Temple et al., 1982). They mutated the anticodon of a human $tRNA^{Lys}$ to decode UAG PTC and, using microinjection, introduced this sup-$tRNA^{Lys}$ gene into the nucleus of *Xenopus laevis* oocytes. The sup-$tRNA^{Lys}$ mediated the synthesis of full-length β-globin (Temple et al., 1982). Over the years, multiple efforts across various model systems have been made to develop sup-tRNAs for the treatment of different indications associated with nonsense mutations (Table 1). Despite these efforts, sup-tRNAs have not yet advanced to the clinical stage, likely due to limited efficacy. This highlights the need to develop more efficient sup-tRNAs that exceed the therapeutic threshold, defined as the minimum level of functional protein expression required to achieve meaningful clinical benefit for a given disease.

4.1. Development of sup-tRNAs through engineering of the anticodon

Continuing the critical experiment of Kan and colleagues (Temple et al., 1982), efforts still revolve around engineering the anticodon of various native sense-codon-decoding tRNAs (Table 1). Using high-throughput cell-based assays and nuclear delivery, Christopher Ahern and colleagues systematically exchanged the natural anticodon of more than 200 human tRNA isodecoders from ten tRNA isoacceptor families, naming them ACE-tRNAs (anticodon-edited tRNAs) (Lueck et al., 2019). Thereby, they report that some tRNA isoacceptors when engineered to ACE-tRNAs exhibit a clear preference for a particular PTC identity, e.g. while $tRNA^{Tyr}$ tolerates equally well anticodon changes to pair to both UAA and UGA PTCs, $tRNA^{Trp}$ has higher efficacy at UAG than at UGA PTCs (Lueck et al., 2019). Furthermore, different isoacceptors vary in their tolerance to mutation in the anticodon sequences and conversion to ACE-tRNAs, e.g., $tRNAs^{Ser}$ being more adaptable than $tRNAs^{Gly}$. Even within the same isoacceptor family, individual isodecoders exhibit distinct capacities for accommodating the engineered anticodon (Lueck et al., 2019).

For some aaRSs, the anticodon serves as a critical identity element for aminoacylation with the cognate amino acid (e.g., $tRNA^{Gly}$) (Giegé & Eriani, 2023), that might be the reason for the measured low suppression activity. In addition, structural alterations in the anticodon loop may disrupt the optimal geometry within the ribosomal A site, further compromising the decoding efficiency of sup-tRNA (H. A. Nguyen et al., 2020; Yarus, Cline, Wier, et al., 1986).

Overall, the sup-tRNAs with an engineered anticodon only (or ACE-tRNAs), exhibit low suppression efficacy, which presents a significant challenge and might be insufficient to reach therapeutic thresholds for several nonsense mutation-associated disorders. One strategy to boost the sup-tRNA efficacy involves increasing the administration dosage; nuclear episomal delivery of plasmids with multiple sup-tRNA copies (ranging from 4 to 16) has been used (Blomquist et al., 2023; Ko et al., 2022; Lueck et al., 2019; Pezzini et al., 2024). However, following injection of a plasmid containing ACE-$tRNA^{Arg}$ into the tibialis anterior muscle of mice, the suppression effect was transient, dissipating approximately 20 days post-administration (Lueck et al., 2019), presumably due to suboptimal plasmid design and/or instability. Administration of a plasmid containing two sup-tRNA copies markedly enhanced expression, supporting sustained sup-tRNA expression for six months in mice (J. Wang et al., 2022). Using lower sup-tRNA copies reduces size constraints and is advantageous for packaging, potentially improving delivery efficiency (Bulcha et al., 2021). This suggests that, rather than increasing the number of sup-tRNA copies, efforts should focus on enhancing suppression activity through further optimization of the sequence of individual sup-tRNAs.

4.2. Engineering of the sup-tRNA sequence outside the anticodon to increase efficacy

Different regions of tRNA determine its functional integrity (Fig. 1). The T-stem modulates binding to elongation factor; interactions between the TψC-arm and D-arm stabilize the 3D L-shaped structure (Barraud et al., 2008; Basavappa & Sigler, 1991; Bénas et al., 2000; Jovine et al., 2000; Kim et al., 1974; Moras et al., 1980; Robertus et al., 1974; Shi & Moore, 2000; Westhof et al., 1985; Woo et al., 1980) and the

Table 1
Chronological overview of the studies with sup-tRNAs, including sup-tRNA engineering steps, delivery type and test models.

sup-tRNA(s) and engineering steps	Delivery type	Targeted disease(s), gene(s) and (PTCs)	Experimental design and outcome	Ref.
sup-tRNA^{Tyr}(CUA) -sequence changes: anticodon	Nuclear delivery; transduction with SV40 virions (in vitro)	Virus *SV40*	- cell culture; co-transfection PTC-*Ad2-SV40* and PTC-*TMV* -increase in expression of Ad2^{+}/ND1 and TMV	(Laski et al., 1982)
sup-tRNA^{Lys}(CUA) - sequence changes: anticodon	Nuclear delivery; injection (in vitro)	β -thalassemia *HBB* (K17X)	- microinjection into Xenopus oocytes with patient-derived reticulocyte RNA - increase in β-globin protein levels	(Temple et al., 1982)
sup-tRNA^{Arg}(UCA) - sequence changes: anticodon	Nuclear delivery; transfection with laboratory reagents or with HSV-1 virions (in vitro)	Xeroderma pigmentosum (XP) group A *XPA* (R207X)	- cell culture in patient-derived XP cells - increase in UV survival, but no increase in XPA protein level	(Panchal et al., 1999)
sup-tRNA^{Ser}(UUA) - sequence change: anticodon - plasmid change: tRNA copy number	Nuclear delivery; transfection with laboratory reagents (in vitro); injection in mouse (in vivo)	Chloramphenicol acetyltransferase (model protein) *CAT* (S27X)	- cell culture with PTC-*CAT*, injection into skeletal muscle and hearts of mice expressing PTC-*CAT* gene - increase in CAT activity	(Buvoli et al., 2000)
sup-tRNA^{Gln}(UUA) - sequence change: anticodon	Nuclear delivery; transfection with laboratory reagents (in vitro); injection in mouse (in vivo)	Duchenne muscular dystrophy *DMD* (Q995X)	- cell culture with PTC-*lacZ*; injection into quadriceps muscle of *mdx* mice - increase in protein expression	(Kiselev et al., 2002)
sup-tRNA^{Arg}(UCA) -sequence change: anticodon	Nuclear delivery; transfection with laboratory reagents (in vitro)	Herediatry diffuse gastric cancer *CDH1* (R335X)	- cell culture with PTC-*CDH1* - increase in E-cadherin expression	(Bordeira-Carriço et al., 2014)
ACE-tRNAs (multiple sup-tRNAs) -sequence change: anticodon	Nuclear delivery; transfection with laboratory reagents (in vitro); injection and electroporation in mouse (in vivo)	Cystic fibrosis *CFTR* (G542X, W1282X)	- cell culture with PTC-*NLuc*, electroporation into skeletal muscle of mice expressing PTC-*NLuc*; injection in Xenopus oocytes with PTC-*CFTR* - increase in protein expression and CFTR ion channel activity	(Lueck et al., 2019)
sup-tRNA^{Tyr}(CUA) - sequence change: anticodon - plasmid change: tRNA copy number, promoter	Nuclear delivery; Lentiviral transfection (in vitro); intravenous rAAV-delivery (in vivo)	Mucopolysaccharidosis type I *IDUA* (W402X)	- cell culture of patient-derived fibroblasts with PTC-*IDUA;* injection into mice expressing PTC-*IDUA* - increase in IDUA expression and activity	(J. Wang et al., 2022)
sup-tRNA^{Arg}(UCA), sup-tRNA^{Leu}(UCA), sup-tRNA^{Gly}(UCA), sup-tRNA^{Trp}(UCA) - sequence change: anticodon - plasmid change: tRNA copy number	Nuclear delivery; transfection with laboratory reagents (in vitro) Cytosolic delivery; transfection with laboratory reagents (in vitro)	Cystic fibrosis *CFTR* (G542X, R1162X, W1282X)	- cell culture with PTC-*NLuc and* PTC-*CFTR* - increase in protein expression, CFTR channel activity	(Ko et al., 2022)
sup-tRNA^{Arg}(UCA), sup-tRNA^{Gly}(UCA), sup-tRNA^{Trp}(UCA) -sequence change: anticodon	Nuclear delivery; transfection with laboratory reagents (in vitro)	Virus *HIV-1 gag* (G221X, G226X, G233X, G238X, R229X, R232X)	- cell culture with PTC-*EGFP,* co-transfection PTC-*HIV-1* - increase in protein expression and virus production	(T. Y. Wang et al., 2023)
sup-tRNA^{Arg} (UCA), sup-tRNA^{Ser}(UCA) -sequence change: anticodon; anticodon stem; TψC-stem	Cytosolic delivery; transfection with laboratory reagents (in vitro); intravenous and intratracheal LNP delivery in mouse (in vivo)	Cystic fibrosis *CFTR* (S466X R553X, R1162X)	- cell culture with PTC-*FLuc*; mice expressing PTC-*aLuc;* patient-derived epithelial cells with endogenous PTC-*CFTR*; - increase in protein expression, CFTR channel activity; restoration of ASL - restoration of expression and function of R1162X beyond CF threshold	(Albers et al., 2023)
sup-tRNA^{Arg}(UCA), sup-tRNA^{Leu}(UCA), sup-tRNA^{Gly}(UCA), sup-tRNA^{Trp}(UCA)) -sequence change: anticodon; anticodon loop, TψC-stem; stem GC-content - plasmid change: editing of 3′-trailer and 5′-UCE	Nuclear delivery; transfection with laboratory reagents or scAAV transduction in cell culture (in vitro)	Cystic fibrosis *CFTR* (G542X, R553X, R1162X, W1282X)	- cell culture with PTC-*NLuc*, PTC-*sfGFP* and with luminescent PTC-*CFTR* - increase in protein expression	(Porter et al., 2024)
ACE-tRNA^{Arg}(UCA) - sequence change: anticodon	Nuclear delivery; transfection with laboratory reagents (in vitro)	CDKL5 deficiency disorder *CDKL5* (R59X, R134X, R550X)	- cell culture with fluorescently tagged PTC-*CDKL5* - increase in CDKL5 expression and activity	(Pezzini et al., 2024)
sup-tRNA^{Glu}(CUA) - sequence change: anticodon; anticodon stem and loop, TψC -stem - plasmid change: tRNA copy number, promoter	Nuclear delivery; transfection with laboratory reagents (in vitro)	Cystic fibrosis; *CFTR* (E1418X, E60X) Breast and ovarian cancer *BRCA1* (E1535X, E1836X) *TP53* (E203X, E298X)	- cell culture with reporters (*RLuc*-PTC-*FLuc*; PTC-*GFP*; fluorescently tagged PTC-*CFTR*, PTC-*BRCA1*, PTC-*TP53* - increase of protein expression and CFTR, p53, BRCA1 activity	(Specht et al., 2025)
sup-tRNA^{Arg}(UCA), sup-tRNA^{Leu}(UCA), sup-tRNA^{Gly}(UCA), sup-	Nuclear delivery; AAV transduction in cell culture (in vitro)	Cystic fibrosis *CFTR* (G542X, R553X, R1162X, W1282X)	- cell culture with PTC-*NLuc*, PTC-*CFTR*, patient-derived intestinal cells for organoids; primary enteric monolayers with endogenous	(Ko et al., 2025)

(*continued on next page*)

Table 1 (*continued*)

sup-tRNA(s) and engineering steps	Delivery type	Targeted disease(s), gene(s) and (PTCs)	Experimental design and outcome	Ref.
$tRNA^{Ser}$(UCA) -sequence change: anticodon			PTC-*CFTR* - increase of protein expression and CFTR channel activity	

anticodon-stem-loop is important for accuracy of decoding (H. A. Nguyen et al., 2020; Schrader et al., 2011; Uhlenbeck & Schrader, 2018; Yarus, Cline, Raftery, et al., 1986). Recent studies highlight the potential of leveraging these fundamental principles in the tRNA structure-function relationship to guide rational sequence changes concomitant to anticodon modulation to enhance sup-tRNA efficacy (Albers et al., 2021; Albers et al., 2023; Porter et al., 2024; Specht et al., 2025). For example, sequence changes in the acceptor stem (AC-stem) to increase stability during decoding, along with the TψC-stem to stabilize interactions with the elongation factor, significantly boost the suppression efficacy of sup-$tRNA^{Ser}$ decoding UGA PTC by 5.5-fold, compared to the cognate anticodon-edited variant (Albers et al., 2023). Interestingly, while some tRNA families require a simultaneous sequence tuning in multiple regions (e.g., sup-$tRNA^{Ser}$ in both AC-stem and TψC-stem), others can be optimized by adjustments in just one of those regions (e.g., sup-$tRNA^{Arg}$ in TψC-stem). This strategy led to a successful engineering of efficient sup-$tRNA^{Arg}$, which surpassed the therapeutic threshold for the R1182X mutation in cystic fibrosis (Albers et al., 2023).

At the core of the rational design of the TψC-stem is a biophysical study of bacterial tRNAs by Olke Uhlenbeck and colleagues, that demonstrated that the TψC-stem sequences of different tRNA families have coevolved to align with the physicochemical nature of their cognate amino acid (Schrader et al., 2011; Uhlenbeck & Schrader, 2018). Thermodynamically destabilizing amino acids are compensated by TψC-stems that establish stronger interactions with the bacterial elongation factor (EF-Tu) and vice versa. This compensatory mechanism was first exploited in the design of bacterial sup-tRNAs (Albers et al., 2021), enhancing markedly the suppression efficacy compared to anticodon-edited sup-tRNAs. Implementing changes to mimic the D-loop and D-stem of $tRNA^{Pro}$ and facilitate recruitment of the bacterial translation factor EF-P showed a modest increase in suppression efficacy (Albers et al., 2021). Considering the functional similarity and conserved sites of aminoacyl-tRNAs binding between bacterial EF-Tu and human eEF1A (Andersen et al., 2000), this design principle has also been applied to human sup-tRNAs, yielding a 2- to 4-fold enhancement in suppression activity across multiple sup-tRNA families (Albers et al., 2023). A recent publication from Tao Pan's group profiling the structure and cellular interactions of human chromosomal- and mitochondrial-encoded tRNAs reveals consistency with the prokaryotic tRNA–EF-Tu interaction paradigm (Peña et al., 2025). The energy contributions of the TψC-stem sequence and the amino acid moiety show opposing effects: a positive correlation for the tRNA and a negative correlation for the amino acid (Peña et al., 2025). Together, these effects establish near-uniform binding affinities of elongator tRNAs to the EF1A, underscoring the importance of incorporating this universal biological principle into sup-tRNA design.

Integration of the AC-stem of the natural UGA-decoder $tRNA^{Sec}$ into the TψC-stem-engineered sup-$tRNA^{Ser}$ led to an increase in suppression efficacy, while the same sequence change reduced the suppression activity of the TψC-stem-edited sup-$tRNA^{Arg}$ by 10-fold, implying that tailored strategies are necessary for each tRNA family (Albers et al., 2023). Systematic mutation of nearly every position of each stem of sup-tRNAs to increase their GC content enhanced suppression efficiency for $tRNA^{Leu}$ by 7-fold and $tRNA^{Arg}$ by 2-fold (Porter et al., 2024). Compared to targeted nucleotide changes, high-throughput libraries bear the drawback of altering important recognition elements for the cognate aaRS, leading to no acylation or misacylation, as detected for $tRNA^{Trp}$ mischarged with Arg (Porter et al., 2024). In addition, for sup-tRNAs developed for nuclear delivery, the sequence of the intrinsic A- and B-boxes should be kept unaltered, otherwise the sup-tRNA expression could be compromised (Chang et al., 2002; Newman et al., 1983; Traboni et al., 1984).

4.3. Incorporation of nucleotide modifications into sup-tRNAs for cytosolic administration

To enhance *in-cell* stability and primarily to reduce the immunostimulatory effect of unmodified IVT sup-tRNAs in cytosolic delivery (Cui et al., 2022), sup-tRNAs can be synthesized with modifications. Thereby, a complete substitution of one nucleotide with its modified counterpart, e.g. complete replacement of UTP by ΨTP or m^1ΨTP, in the IVT reaction, as commonly employed for mRNA vaccines (Nance & Meier, 2021), is not feasible. tRNAs are highly structured entities (Fig. 1) and their structure is required for function. Excessive replacement of all uracils with ΨTP or m^1ΨTP interferes with functional folding of RNAs, as evidenced by a drastic reduction in melting temperature (A. D. Biela et al., 2025). Instead, modifications should be introduced in a position-specific manner, either through chemical synthesis of full-length sup-tRNAs or via splint ligation of modified and unmodified tRNA fragments (Y. Xu et al., 2025).

The number of natural modifications per individual human tRNA can vary, with an average of 13 modifications (Pan, 2018). Not all modifications in the parental tRNA would likely be necessary to generate stable, non-immunogenic sup-tRNAs. By leveraging conserved patterns of tRNA modifications (Fig. 1B), it becomes evident that a small subset of modifications might be sufficient (EP21216959.3 (Ignatova & Albers, 2022)). However, given the idiosyncratic nature of tRNA modifications, each sup-tRNA may need a unique combination of modifications. Some tRNA-modifying enzymes are located in the cytosol or partition between both nucleus and cytosol (Kessler et al., 2018), suggesting that unmodified IVT sup-tRNAs may obtain some modifications directly in the cytosol. In addition, the retrograde transport of hypomodified tRNAs to the nucleus (Kramer & Hopper, 2013; Takano et al., 2005) might also be utilized by sup-tRNAs to acquire more modifications. Thus, it remains crucial to determine for each sup-tRNA which modifications are introduced post-delivery by the host cell cell, highlighting the need to selectively introduce only those not installed endogenously. Collectively, this unique array of modifications for each sup-tRNA will likely affect its stability *in cellulo* and efficacy in PTC decoding.

4.4. Tuning episomal sup-tRNA expression for nuclear delivery

In contrast to cytosolic delivery, in which sup-tRNAs are immediately available for translation, the nuclear delivery route employs the host's transcription machinery for sup-tRNA expression. Due to the compact size of sup-tRNA expression cassettes (typically not exceeding 350–400 bp including the flanking sequences necessary for expression), multiple copies can be accommodated within the episomal expression vector without exceeding the ~5 kb packaging limit of AAVs. Depending on the study, between one and sixteen copies of sup-tRNAs have been incorporated into the plasmid-based expression cassette (Buvoli et al., 2000; Lueck et al., 2019; Panchal et al., 1999; J. Wang et al., 2022) (Table 1). However, no consistent trend has emerged regarding the optimal number of copies, as increased copy number does not always correlate with high suppression efficiency (Kiselev et al., 2002; J. Wang et al., 2022).

Commonly strong type 3 Pol III promoters, such as U6 or H1, have been used to achieve high sup-tRNA expression (J. Wang et al., 2022). Natural tRNA promoters, which are type 2 Pol III promoters, have also been exploited for sup-tRNA expression (Porter et al., 2024). Different studies have reported varying outcomes, ranging from pronounced differences to comparable sup-tRNA expression levels between type 2 and type 3 Pol III promoters (Koukuntla et al., 2013; Porter et al., 2024). These differences may arise from variations in the sequences of the internal A- and B-box promoter elements of the tested sup-tRNAs.

Endogenous tRNAs are expressed at varying levels, typically exhibiting two- to ten-fold differences in concentration between isodecoders (Dittmar et al., 2006; Gao et al., 2024; Polte et al., 2019). This natural variability provides a repertoire of promoters that can be exploited to fine-tune the expression of the target sup-tRNA (WO 2020/069194 A1 (J. M. Coller et al., 2020), EP23167017.5 (Ignatova et al., 2023)). tRNA transcription can be further modulated by the 5′-upstream control element (5′-UCE) (Fig. 2, Table 1), which includes the TATA box, transcription start site, and 5′-leader sequence; and by the 3′-trailer determining transcription termination; the 5′-leader and 3′-trailer are removed in the pre-tRNA processing by multiple nucleases. A screening of over 350 natural human 5′-UCE sequences, each 55 bp in length, in human laboratory cell lines (16HBE14o-, HEK293) revealed over 4-fold variation in expression levels, as assessed by suppression efficiency of a reporter construct (Porter et al., 2024). In contrast, 35 bp 3′-trailer sequences have less pronounced effects, with suppression efficiencies varying by approximately 2-fold (Porter et al., 2024).

Combining 5′-UCE and 3′-trailer sequences native to tRNAs enables tighter control of sup-tRNA expression levels, in contrast to U6 or H1 promoters, which typically drive much higher expression levels. It is important to note that sup-tRNA expression levels in disease-relevant tissues – the intended targets for sup-tRNA therapies – may differ substantially from those observed in laboratory cell lines. Therefore, validation of lead sup-tRNA candidates should be conducted in the tissue of disease onset (e.g., patient-derived primary cells, organoids, or multicellular disease models). Crucially, expression levels must be carefully optimized for each sup-tRNA variant to avoid imbalance of the endogenous tRNA pool or saturation of the cognate aaRS activity, that could lead to skewed aminoacylation within a specific endogenous tRNA family.

4.5. Computer-aided sup-tRNA design

tRNAs are complex molecules, with each tRNA region specialized for distinct function and/or stability. During their lifecycle, tRNAs interact with a multitude of partners, e.g., enzymes involved in tRNA processing, modification, degradation, aaRSs, translation initiation and elongation factors, and the ribosome, which rely on recognition of sequence motifs or structural elements. Therefore, the design of sup-tRNAs cannot be entirely exploratory (i.e., involving indiscriminate modification of all nucleotides at all positions), but must be guided by invariant nucleosides and base pairs that determine the intricate structure-function relationship inherent to each tRNA (Albers et al., 2021;Grosjean & Westhof, 2016; Westhof et al., 2022). In addition, the post-transcriptional modifications (Fig. 1B) add another layer of complexity to computer-aided sup-tRNA design. Despite these constraints, a vast combinatorial design space remains available for this relatively small molecule (72–95 nucleotides), offering numerous possibilities for functional optimization.

Considering both functional rules (i.e., aaRS identity elements) and structural constraints (i.e., interactions necessary for establishing cloverleaf conformation and L-shaped tRNA architecture), an earlier study features a semi-de novo, in silico approach to sup-tRNA design (Albers et al., 2021) using the DSS-Opt software (Matthies et al., 2012). A total of 10,000 sequences were computationally generated and ranked based on their predicted folding probability, as calculated by the ViennaRNA package (Lorenz et al., 2011). However, suppression efficacy did not show a linear correlation with folding probability. For example, the second-highest ranked variant exhibited nearly no aminoacylation, highlighting the importance of considering the more complex scheme of aaRS identity features (e.g., stacking interactions within the acceptor stem) (Albers et al., 2021).

Leveraging the fast developments in machine learning and artificial intelligence (AI) approaches, one might envision a computer-aided design of sup-tRNAs – a strategy that one sup-tRNA-focused company, AlltRNA, is currently pursuing. AI-based approaches hold promise for optimizing sup-tRNAs by identifying sequence-function relationships and predicting sequences of efficient sup-tRNA variants. The limited size of the available training datasets is problematic, which may constrain the performance of data-rich models such as deep neural networks. Traditional machine-learning techniques (e.g., random forest algorithm, support vector machines), which demonstrate robustness with smaller data sets and have been effectively employed in RNA-related predictive tasks (Asim et al., 2025; Liu et al., 2025), could be more suitable.

Overall, design strategies for sup-tRNAs should be uniquely tailored to each tRNA isodecoder, taking into account several key aspects of tRNA biology: (i) evolutionarily constraints governing the structure-function relationship within each tRNA family (Grosjean & Westhof, 2016; Westhof et al., 2022); (ii) structural constraints essential for establishing the L-shaped architecture, primarily driven by interactions between nucleotides in the D- and TψC-loops (A. Biela et al., 2023; Du & Wang, 2003); (iii) recognition elements for the cognate aaRS (Giegé & Eriani, 2023); (iv) thermodynamic effects of the amino acid and the compensatory T-stem sequence variations (Schrader et al., 2011; Uhlenbeck & Schrader, 2018); and (v) recognition elements of modifying enzymes to preserve desired modifications (Suzuki, 2021; M. Zhang & Lu, 2025; W. Zhang et al., 2022).

5. Advancing personalized medicine through sup-tRNA therapeutics

The rapid advancement of sequencing technologies continues to reveal new cases of genetic mutations, including those stemming from de novo or somatic nonsense mutations, thus, further expanding the growing body of inherited or non-inherited pathologies attributed to nonsense mutations (Mort et al., 2008). The frequency of nonsense mutations varies across the three PTC identities (UAG (40.4 %), UGA (38.5 %), and UAA (21.1 %), (Mort et al., 2008)) and differs from the usage frequencies of NSCs in humans. Overall, 19 different sense codons encoding in total ten amino acids can be converted into PTCs through nonsense mutations (J. Coller & Ignatova, 2024). Some mutations yield a single PTC identity (e.g., the CGA arginine codon can be mutated to UGA), while others result in all three PTC identities (e.g., Ser codons can be converted to UGA, UAG, or UAA). Theoretically, 19 distinct sup-tRNAs would be needed to suppress all possible pathogenic PTCs. Among these, the most common are the CGA Arg codon mutated to UAG (23.7 % frequency) and the CAG Gln codon mutated to UAG (19.1 %) (J. Coller & Ignatova, 2024).

Utilizing sup-tRNAs to target nonsense mutations necessitates a mutation-tailored strategy, where each sup-tRNA is specifically engineered to match both the identity of PTC and the affected amino acid. This guides the selection of the appropriate native isoacceptor and/or isodecoder to be refactored into a sup-tRNA.

5.1. Nonsense mutation-specific sup-tRNAs or one fits-all approach

A single, highly efficient sup-tRNA targeting a specific PTC can potentially be applied to multiple identical mutations (Anastassiadis & Köhrer, 2023) – either within the same gene or across distinct genes associated with different diseases (Fig. 4). The pathological threshold varies across different disorders (e.g., 10 % for cystic fibrosis (Masvidal et al., 2014; Quon & Rowe, 2016) and hemophilia A (George et al., 2021), 30 % for Duchene muscular dystrophy (Neri et al., 2007); >50 %

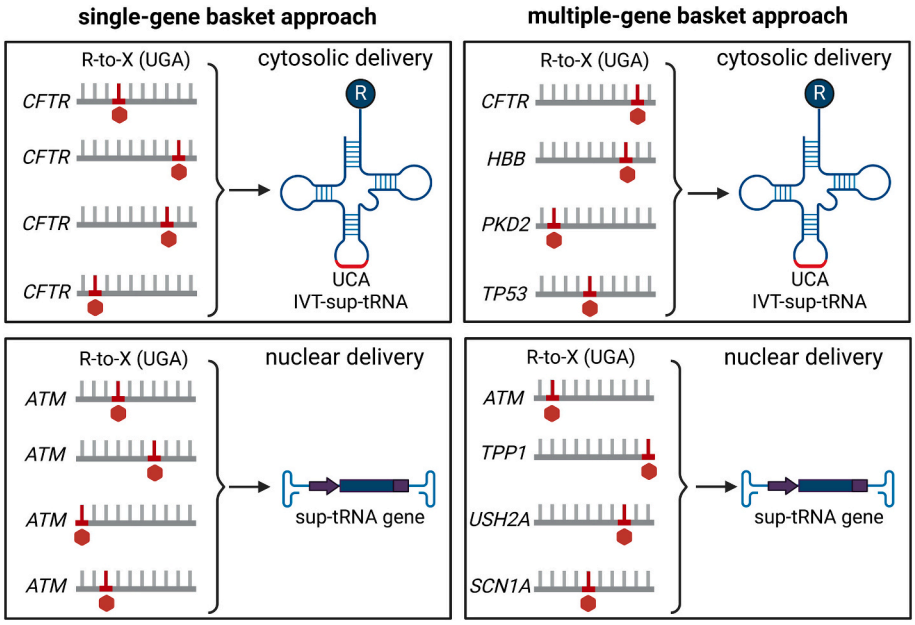

Fig. 4. The basket approach utilizes a single sup-tRNA to treat patients carrying a nonsense mutation with the same identity but at different positions within the same gene (single-gene basket approach) or across pathologies associated with different genes (multi-gene basket approach). The basket approach is applicable for both delivery regimens, cytosolic as IVT sup-tRNAs (upper blocks) and nuclear with sup-tRNA gene expressed episomally (bottom blocks). Abbreviations: *CFTR*, cystic fibrosis transmembrane regulator implicated in cystic fibrosis; *ATM*, ataxia telangiectasia mutated implicated in ataxia telangiectasia; *HBB*, hemoglobin subunit β implicated in β-thalassemia; *PKD2*, polycystin 2 implicated in polycystic kidney disease; *TP53*, tumor protein 53 implicated in cancer; *TPP1*, tripeptidyl peptidase 1 implicated in neuronal ceroid lipofuscinosis; *USH2A*, usherin implicated in usher syndrome; *SCN1A*, sodium voltage-gated channel alpha subunit 1 implicated in dravet syndrome (Table 2). Created with BioRender.com.

for β-thalassemia (Christakopoulos et al., 2023) can yield therapeutic benefit). For a basket trial targeting a single pathological condition (or single-gene basket approach, Fig. 4), sup-tRNA efficacy must meet the disease-specific threshold and be aligned with relevant clinical readouts. In contrast, when aiming to correct a common mutation across multiple pathologies (or multi-gene basket approach, Fig. 4), efficacy should be calibrated to meet the highest therapeutic threshold among the targeted diseases. Ultimately, the goal is to develop sup-tRNAs with maximal suppression efficiency to ensure broad applicability and effectiveness across diverse clinical conditions with varying pathological thresholds. The use of efficient sup-tRNAs for conditions with low therapeutic thresholds offers an additional benefit by enabling dose reduction.

It is important to note that the sup-tRNA efficacy can vary between mutations of the same type, even within one gene, potentially limiting the effectiveness of a basket trail approach. Systematic analysis of the suppression efficacy at various PTCs revealed that translational velocity of the mRNA sequence upstream of the PTC predominantly affects sup-tRNA efficacy (Bharti et al., 2024). In the *CFTR* gene, which is implicated in cystic fibrosis, readthrough efficiency at PTCs of the same identity can vary by up to 10-fold, with some PTCs being nearly uncorrectable (Bharti et al., 2024). The PTCs most refractory to suppression display a characteristic translation profile: the translation velocity of the upstream mRNA region shifts abruptly (i.e., from slow to vast or vice versa), resulting in ribosome collisions that reduce PTC suppression (Bharti et al., 2024). In such cases, mutation-specific solutions may be required, including adjuvant therapies with translational modulators or agents that influence translation dynamics (e.g., alter speed or ribosomal loading to increase the distance between ribosomes and avoid collisions) on the target mRNA.

For isoacceptors, that are refractory to engineering them into sup-tRNAs, e.g., $tRNA^{Gly}$ (Albers et al., 2023), alternative strategies may be required. One such approach involves using non-cognate sup-tRNAs that misincorporate another amino acid at the PTC, thereby mimicking the effect of a missense mutation. Specific proteins, such as dystrophin which is implicated in Duchenne muscular dystrophy, are tolerant to amino acid misincorporations, whereas others are more sensitive to deviations in the amino acid sequence. For some diseases (e.g., cystic fibrosis (Heijerman et al., 2019; Keating et al., 2018; Middleton et al., 2019)), clinically approved small-molecule modulators can restore protein folding and function associated with missense mutations. Combining sup-tRNA-mediated amino acid misincorporation with adjuvant treatment using these modulators may offer a synergistic therapeutic strategy in these cases. By cotsrast, pairing sup-tRNAs with nonsense-mediated decay (NMD) inhibitors is not recommended. Although NMD inhibition elevates mRNA levels, it does not consistently enhance protein production (Albers et al., 2023), and impeding NMD is associated with embryonic lethality and neurodevelopmental dysfunction in mice (Johnson et al., 2019; McIlwain et al., 2010; Medghalchi et al., 2001; L. S. Nguyen et al., 2013; Tarpey et al., 2007). Notably, optimized sup-tRNAs can efficiently antagonize NMD (Albers et al., 2023; J. Wang et al., 2022) by an unknown mechanism, likely involving competition and blocking of NMD factors binding.

Readthrough-inducing antisense oligonucleotides (also named R-ASOs) offer another alternative to enhance the efficacy of suppressor tRNAs (sup-tRNAs) at some refractory PTCs (Susorov et al., 2024). The mechanism of action of R-ASOs is distinct from that of ASOs evaluated in clinical contexts to date (e.g., by RNA interference, RNase H-mediated cleavage, splicing modulation, non-coding RNA inhibition, gene activation, and programmed gene editing), including FDA-approved ASO therapeutics (Krey-Grauert et al., 2025). R-ASOs bind complementarily to mRNA sequences downstream of the PTC, and with maximal efficacy observed when binding initiates around position +8 (Susorov et al., 2024) or + 9 (Kar et al., 2020). This R-ASO binding interferes with translation termination, likely by inhibiting peptide release, thereby promoting readthrough. The efficacy of R-ASO-mediated readthrough is highly dependent on the local sequence context of the PTC and is particularly strong at weak termination signals (e.g., UGA*C*). When combined with sup-$tRNA^{Ser}$ targeting UGA PTCs, the R-ASO substantially enhances readthrough, restoring reporter protein expression to levels approaching that of the wild type (Susorov et al., 2024).

Table 2
Examples of nonsense mutation-associated pathologies with the affected gene(s) (ClinVar database (Landrum et al., 2014)) and tissue of onset, highlighting the possible delivery strategy.

Disease	Affected gene(s)	Possible sup-tRNA delivery route
lung		
Primary ciliary dyskinesia	*TP73, DRC1, DAW1, ZMYND10, DNAH1, CCDC39, TTC14, DNAH5, MCIDAS, CCNO, DNAH8, RSPH9, RSPH4A, RSPH3, DNAAF5, PRKAR1B, DNAH11, CDCA7L, SPAG1, DNAAF11, DNAI1, ODAD2, CFAP300, TTC12, CCDC65, DNAAF2, DNAL1, DNAAF4, HYDIN, DNAAF1, DRC4, GAS8, DNAH9, DNAAF19, DNAI2, CCDC40, ODAD3, ODAD1, DNAAF3, CFAP298, RSPH1, OFD1, RPGR, DNAAF6*	Local LNP-mediated delivery of IVT sup-tRNA (e.g., by inhalation or intratracheal instillation), with redosing
Cystic fibrosis[a]	*CFTR*	
muscles		
Duchenne muscular dystrophy	*DMD*	Systemic (intravenous) or local (intramuscular injections) LNP-mediated delivery of IVT sup-tRNA, with redosing
Ullrich disease	*COL6A2, COL6A3, COL12A1, COL6A1*	
blood		
Hemophilia	*F8, F9*	Systemic (intravenous) LNP-mediated delivery of IVT sup-tRNA, with redosing
β-Thalassemia	*HBB*	
skin		
Xeroderma pigmentosum	*ERCC3, XPC, POLH, XPA, DDB2, ERCC5, ERCC4, ERCC2*	Direct administration into the skin of IVT sup-tRNA embedded in LNPs (with redosing) or single-dose AAV-mediated nuclear delivery of episomally encoded sup-tRNAs
Epidermolysis bullosa	*LAMC2, LAMB3, ITGA6, COL7A1, DSP, DST, PLEC, COL17A1, CD151, EXPH5, KRT5, KRT14, ITGB4, LAMA3*	
ear		
Usher syndrome	*USH2A, CLRN1, ADGRV1, WHRN, PCDH15, CDH23, USH1C, MYO7A, ARSG, USH1G*	Local single-dose AAV-mediated nuclear delivery of episomally encoded sup-tRNAs
Non-syndromic hearing loss	*ESPN, LMX1A, OTOF, ATP2B2, GRXCR1, LHFPL5, MYO6, TMC1, PCDH15, MYO7A, PTPRQ, GJB2, MYO15A, LOXHD1, SYNE4, TMPRSS3, TRIOBP, POU3F4*	
kidney		
Polycystic kidney disease	*DZIP1L, DNAJB11, PKD2, PKHD1, GANAB, ALG8, ALG9, ALG5, PKD1*	Systemic (intravenous) LNP-mediated delivery of IVT sup-tRNA, with redosing
Nephropathic cystinosis[b]	*CTNS*	
Renal tubular acidosis	*ATP6V1B1, SLC4A4, ATP6V0A4, SLC4A1*	
Alport syndrome[c]	*COL4A4, COL4A3, COL4A5*	
liver		
Wilson disease	*ATP7B*	Systemic (intravenous) LNP-mediated delivery of IVT sup-tRNA, with redosing
nervous system		
Neuronal ceroid lipofuscinosis	*PPT1, MFSD8, TPP1, CLN8, CTSD, CTSF, CLN5, CLN6, CLN3, GRN*	Systemic (intravenous) single-dose AAV-mediated nuclear delivery of episomally encoded sup-tRNAs
Dravet syndrome	*SNX27, SCN1A*	
Rett Syndrome	*FOXG1, MECP2*	
Neurodevelopmental disorder with regression, abnormal movements, loss of speech, and seizure (NEDAMMS)	*IRF2BPL*	
Ataxia telangiectasia	*ATM*	
Spinal muscular atrophy	*ASCC1, IGHMBP2, TRIP4, ATP7A*	
Charcot-Marie-Tooth disease	*PLEKHG5, MFN2, LMNA, MPZ, MPV17, DNAJB2, MME, SH3TC2, NEFL, GDAP1, NDRG1, LRSAM1, SURF1, DHTKD1, GBF1, SBF2, BSCL2, IGHMBP2, MTMR2, FGD4, DYNC1H1, SPG11, PMP22 AARS1, PRX, NAGLU, GJB1, PRPS1*	
Parkinson's disease	*PARK7, PINK1, DNAJC6, GBA1, PRKN, VPS13C, SYNJ1, PLA2G6*	
multiple tissues		
Cancers[d]	*APC, ARHGEF12, ATM, BCL11B, BLM, BMPR1A, BRCA1, BRCA2, CARS, CBFA2T3, CDH1, CDH11, CDK6, CDKN2C, CEBPA, CHEK2, CREB1, CREBBP, CYLD, DDX5, EXT1, EXT2, FBXW7, FH, FLT3, FOXP1, GPC3, IDH1, IL2, JAK2, MAP2K4, MDM4, MEN1, MLH1, MSH2, NF1, NF2, NOTCH1, NPM1, NR4A3, NUP98, PALB2, PML, PTEN, RB1, RUNX1, SDHB, SDHD, SMARCA4, SMARCB1, SOCS1, STK11, SUFU, SUZ12, SYK, TCF3, TNFAIP3, TP53, TSC1, TSC2, VHL, WRN, WT1*	Systemic (intravenous) LNP-mediated delivery of IVT sup-tRNA, with redosing; by solid cancers intratumoral administration also possible
Hurler syndrome	*IDUA*	
Alpha-mannosidosis	*MAN2B*	

[a] Multiple tissues, like the intestine, the male reproductive system, are affected; the lung is the major tissue of onset.
[b] Multiple tissues, including skin, are also affected; the kidney is the major tissue of onset.
[c] Multiple tissues, including eyes and ears, are also affected; the kidney is the major tissue of onset.
[d] For cancer, more than 10,000 nonsense mutations are listed in the ClinVar database; here, the most common 63 tumor suppressor genes associated with different cancers (M. Zhang et al., 2021) are shown.

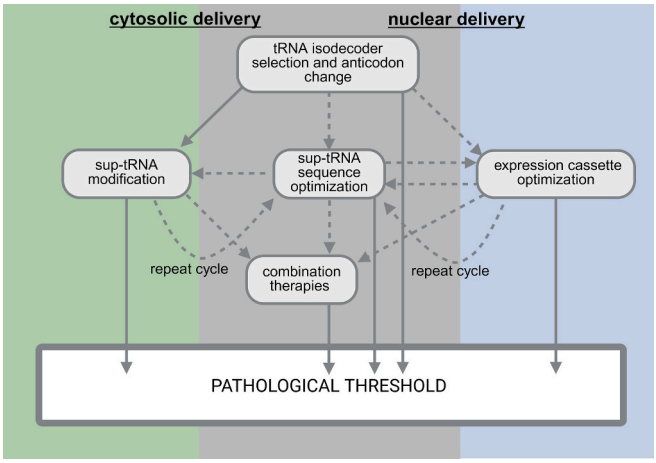

Fig. 5. Scheme for optimizing sup-tRNAs efficacy. Both delivery routes require common (gray box) and different optimization steps (green box for cytosolic and blue box for nuclear delivery). At each step, it is determined whether the desired pathological threshold has been reached. Continuous lines indicate that sup-tRNA activity exceeds pathological threshold, whereas dashed lines represent alternative steps to follow in case of insufficient efficacy. Depending on the desired pathological threshold, many of the steps may need to be repeated. The first step is the choice of a suitable isodecoder whose anticodon is changed to match the target PTC. For example, in the case of cytosolic delivery, if sup-tRNA activity is sufficient, the next step would be to introduce modifications to minimize immunogenicity. In turn, if suppression activity is insufficient, the sup-tRNA should undergo one or more sequence optimization cycles. For nuclear delivery, improving efficacy should be coupled with refining the expression cassette to ensure that expression levels are balanced with functional activity. For both delivery routes, in cases of low sup-tRNA efficacy, combination therapies with various adjuvants (e.g., translational modulators, protein-specific folding agents) might be considered. Created with BioRender.com.

5.2. Tissue- and disease-specific requirements in sup-tRNA development

Nonsense mutations impact genes expressed across a wide range of tissues, with either tissue-specific or systemic effects depending on the function and expression pattern of the affected gene(s) (Table 2). Thus, to develop efficacious sup-tRNA-based therapies, it is essential to specifically target the tissue(s) affected by the nonsense mutation.

The identification of an appropriate delivery modality and administration regimen is a critical early step in the development of sup-tRNA therapeutics, as it directly informs the subsequent optimization strategies for sup-tRNA efficacy (Fig. 5).

During the optimization, it is advisable to assess the percentage of wild-type protein expression and functional restoration as a readout of sup-tRNA efficacy. However, more often a fold-change metric is used, namely the relative readthrough efficiency expressed as a ratio between engineered sup-tRNA and anticodon-modified variant. Fold-change values can be misleading, particularly when the baseline readthrough by the anticodon-edited sup-tRNAs (or ACE-tRNA) is very low (Lueck et al., 2019; Pavlíková et al., 2024). In such cases, even substantial fold improvements may remain insufficient to reach the therapeutic threshold.

The effectiveness of the different delivery systems is restricted by tissue-specific barriers, and sup-tRNA-based therapy should consider such specific modalities. The widespread application of AAV vectors in clinical trials targeting diseases of the brain, eye, and muscle, along with the approval of AAV-based drugs (Luxturna, Zolgensma), highlights their suitability as delivery vehicles for sup-tRNA administration in those tissues (Huang et al., 2021; Wang, Gessler, et al., 2024). To circumvent non-specific enrichment in the liver, following systemic administration, a localized delivery could be considered when feasible.

For CNS disorders, intracranial injection enables localized transduction; however, given that many CNS pathologies affect multiple brain regions, systemic delivery may ultimately be more appropriate (Guhasarkar et al., 2017; Huang et al., 2021; Pozsgai et al., 2017). AAV vectors face several limitations in specific tissues (e.g., the lung), where pre-existing neutralizing antibodies can compromise the efficacy and provoke inflammation. In these tissues, or in proliferative tissues, where episomal plasmids would be progressively diluted during cell division, LNP-mediated delivery with repeated dosing of sup-tRNAs may offer a more viable alternative.

6. Conclusion and future perspectives

Sup-tRNA-based therapies hold promise for addressing a broad range of genetic diseases, with a common molecular mechanism linked to nonsense mutation. Rapid progress in tRNA biology and modification chemistry, computational and AI-based approaches, and expression cassettes is driving forward the development of sup-tRNAs towards efficient therapeutic applications. Given the small patient populations with nonsense mutation-associated disorders, the advancement of sup-tRNA therapeutics is closely supported by the developments in other nucleic acid-based gene therapies. Innovations in tissue-specific delivery platforms will be instrumental in enhancing the targeting efficiency of sup-tRNAs.

Despite the progress, several challenges remain and must be thoroughly addressed to enable the successful clinical translation of sup-tRNA therapies. The targeted pathological condition dictates the choice of administration route (e.g., nuclear or cytosolic), which in turn determines the specific optimization strategies required to achieve the desired therapeutic outcome. Another key aspect that requires careful consideration is the long-term safety of sup-tRNAs. Each delivery route presents distinct safety considerations, e.g., sustained long-term expression by nuclear delivery, immunological compatibility, and long-term tolerability of durable re-administration regimens of IVT sup-tRNAs by cytosolic delivery.

The concept of a basket approach, or the grouping of multiple indications under a common therapeutic strategy, will significantly accelerate the clinical establishment of sup-tRNA therapies, despite the small number of individuals affected by each condition. A first-in-class approval of a single sup-tRNA for one indication, with all safety requirements thoroughly addressed, would serve as a strong precedent and catalyst for broader clinical adoption. A detailed understanding of tissue-specific delivery constraints, safety considerations, and dosing regimens required to meet multiple therapeutic thresholds is essential for the rational design and success of the basket strategies.

Declaration of generative AI and AI-assisted technologies in the writing

During the preparation of this work, the authors used Grammarly to refine syntax and orthography. After using this tool, the authors reviewed and edited the content as needed and take the full responsibility for the content of the published article.

CRediT authorship contribution statement

Zoya Ignatova: Writing – review & editing, Writing – original draft, Conceptualization. **Suki Albers:** Writing – review & editing, Writing – original draft, Conceptualization.

Declaration of competing interest

The authors declare competing interests. Z.I. and S.A. are inventors on patents related to tRNA designs for PTC correction. Z.I. is also a scientific advisor for Tevard Biosciences.

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Data availability

No data was used for the research described in the article.

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