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Biol. Chem. 2025; 406(10–12): 413–421

**Review**

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# Manipulating mitochondrial gene expression

**Abstract:** Mitochondria are essential for cellular metabolism, serving as the primary source of adenosine triphosphate (ATP). This energy is generated by the oxidative phosphorylation (OXPHOS) system located in the inner mitochondrial membrane. Impairments in this machinery are linked to serious human diseases, especially in tissues with high energy demands. Assembly of the OXPHOS system requires the coordinated expression of genes encoded by both the nuclear and mitochondrial genomes. The mitochondrial DNA encodes for 13 protein components, which are synthesized by mitochondrial ribosomes and inserted into the inner membrane during translation. Despite progress, key aspects of how mitochondrial gene expression is regulated remain elusive, largely due to the organelle's limited genetic accessibility. However, emerging technologies now offer new tools to manipulate various stages of this process. In this review, we explore recent strategies that expand our ability to target mitochondria genetically.

**Keywords:** gene expression; genetic tools; mitochondria; RNA

## 1 Introduction

Mitochondria are essential cellular organelles that fulfill key functions in cellular metabolism. Among these functions, the production of ATP by oxidative phosphorylation is central to driving cellular energy demands. The OXPHOS system in the inner mitochondrial membrane (IMM) is composed of subunits that are either encoded by the nuclear or mitochondrial genome (Isaac et al. 2018; Kummer and Ban 2021; Ott et al. 2015; Priesnitz and Becker 2018; Richter-Dennerlein et al. 2015; Tang et al. 2020). The human mitochondrial genome is a 16,569 bp double-stranded circular DNA molecule that encodes 13 OXPHOS system subunits, 22 transfer RNAs (tRNAs), and two ribosomal RNAs (rRNAs) (Figure 1). The double-stranded genome consists of a guanine-rich heavy (H) strand and a cytosine-rich light (L) strand (Anderson et al. 1981; Falkenberg et al. 2024).

Mitochondria contain multiple copies of their genome (mtDNA), the number of which differs significantly between different cell types (Stewart and Chinnery 2021). Mutations of the mitochondrial DNA leads to dysfunction of the OXPHOS system and concomitantly severe human disorders (Smeitink et al. 2001). Mitochondrial DNA mutation syndromes encompass a broad range of disorders, each linked to specific genetic variants. For example, MELAS (Mitochondrial Encephalopathy, Lactic Acidosis, and Stroke-like Episodes) is predominantly caused by the $m.3243A > G$ mutation in the mitochondrial tRNA$^{Leu}$ gene ($MT$-$TL1$), affecting translation and energy production; MERRF (Myoclonic Epilepsy with Ragged Red Fibers) commonly arises from $m.8344A > G$ in the tRNA$^{Lys}$ gene ($MT$-$TK$), impairing mitochondrial protein synthesis; and LHON (Leber Hereditary Optic Neuropathy) is primarily associated with three mutations-$m.3460G > A$ ($MT$-$ND1$), $m.11778G > A$ ($MT$-$ND4$), and $m.14484T > C$ ($MT$-$ND6$)-which affect complex I of the OXPHOS system and cause optic nerve atrophy (Fu et al. 2021; Gusic and Prokisch 2021). Underlying these disorders and impacting their phenotype and progression is the heteroplasmy of mitochondrial DNA mutations (mtDNA that is present in multiple copies displays a mixture of mutant and wild-type molecules within a cell). The clinical manifestations of mitochondrial diseases depend on the mutant-to-wild-type mtDNA ratio, with symptoms emerging when the mutant mtDNA exceeds tissue-specific thresholds. Tissues with high energy demands, such as the brain, heart, and skeletal muscle, are particularly vulnerable due to their reliance on oxidative phosphorylation for energy production (Stewart and Chinnery 2015). Moreover, heteroplasmy

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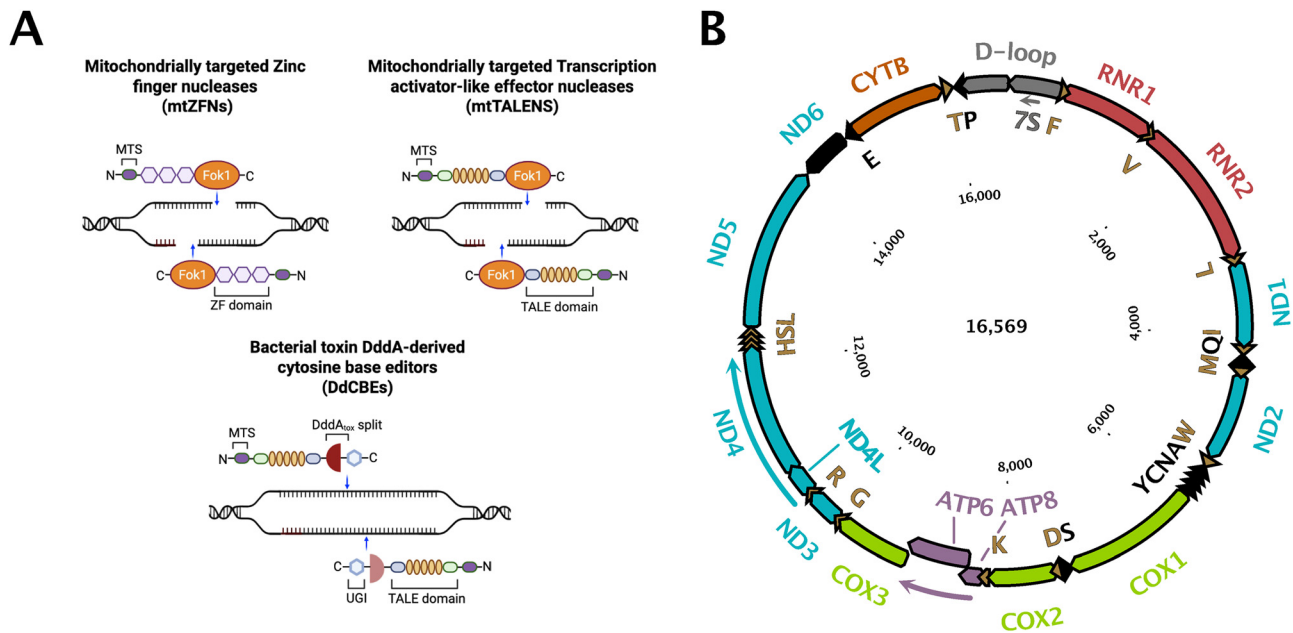
**Figure 1:** Overview of various tools to manipulate mitochondrial DNA and graphical illustration of mitochondrial genome (A). Mitochondrially targeted nucleases, including mitochondrially targeted zinc finger nucleases (mtZFNs), transcription activator-like effector nucleases (mtTALENs), and DddA-derived cytosine base editors (DdCBEs), can be directed into mitochondria via a mitochondrial targeting sequence (MTS). mtZFNs consist of DNA sequence-specific zinc finger (ZF) domains fused to the FokI endonuclease, enabling targeted double-strand breaks (DSBs) in mtDNA. Similarly, mtTALENs are composed of sequence-specific TALE DNA-binding domains conjugated to FokI, also generating site-specific DSBs. In contrast, DdCBEs employ split DddA cytidine deaminase fused to TALE domains, conferring sequence-specific cytosine deaminase activity that enables precise C- G-to-T- A base editing without introducing DSBs. Importantly, DdCBEs also consist of an uracil glycosylase inhibitor (UGI), which stabilizes the edited base by inhibiting the base excision repair pathway, thereby preventing the removal of uracil and ensuring efficient and permanent C-to-T conversion (B) map of human mito-chondrial DNA (mtDNA). Protein-coding genes encoded on the heavy strand (H) are depicted with distinct colors and a black border, while those on the light strand (L) are shown in black. Transfer RNA (tRNA) genes are represented as triangles. Subunits of respiratory complex I are labeled in blue, complex III subunits in orange, and complex IV subunits in green. Ribosomal RNA (rRNA) genes are highlighted in red, and the D-loop region is indicated in grey.

levels can vary between cells, organs, and individuals, and contribute to the variable disease severity and tissue involvement observed in mitochondrial disorders (Stewart and Chinnery 2021).

Mitochondrial gene expression is a highly orchestrated process involving DNA replication, transcription, RNA processing, and translation. mtDNA is tightly packed into nucleoids by the mitochondrial transcription factor A (TFAM), thereby regulating DNA availability for replication and transcription (Kaufman et al. 2007; Kukat et al. 2015; Ngo et al. 2014). DNA replication is executed by DNA polymerase $\gamma$ (POL$\gamma$), which binds to the heavy-strand origin (OH) and extends RNA primers synthesized by mitochondrial RNA polymerase (POLRMT) (Falkenberg et al. 2024; Montoya et al. 1982; Robberson et al. 1972). As POL$\gamma$ synthesizes the new heavy strand, the TWINKLE helicase unwinds the double-stranded DNA ahead of the replication fork, enabling strand separation (Ikeda et al. 2015; Korhonen et al. 2003; Korhonen et al. 2008; Milenkovic et al. 2013; Tyynismaa et al. 2004). The displaced parental heavy strand is stabilized by the mitochondrial single-stranded DNA-binding protein (mtSSB),

which prevents secondary structure formation and unscheduled primer synthesis (Curth et al. 1994; Kuznetsov et al. 2006; Montoya et al. 1982; Tiranti et al. 1993). When the replication fork reaches the light-strand origin (OL), the displaced heavy strand forms a stem-loop structure that excludes mtSSB, allowing POLRMT to synthesize a new RNA primer. POL$\gamma$ then initiates light-strand synthesis using this primer (Fusté et al. 2010; Kühl et al. 2016; Wanrooij et al. 2008). Apart from synthesizing primers for DNA replication, POLRMT recruited by TFAM also performs transcription of mtDNA into two polycistronic primary RNAs from the heavy (HSP) and light strand promoters (LSP) (Fisher and Clayton 1985; Hillen et al. 2017; Kühl et al. 2016; Minczuk et al. 2011; Morozov et al. 2015; Ngo et al. 2014). The heavy strand encodes 12 OXPHOS proteins (subunits of complexes I, III, IV, V), two rRNAs, and 14 tRNAs, while the light strand codes for *MT-ND6* and eight tRNAs (Falkenberg et al. 2024; Mercer et al. 2011). These primary transcripts are processed into individual mRNAs, tRNAs, and rRNAs by endonucleases such as RNase P and ELAC2 (Bhatta et al. 2021; Brzezniak et al. 2011; Holzmann et al. 2008; Sanchez et al. 2011). FASTKD5

performs *MT-CO1*, *MT-CO3* and *MT-CYB* mRNA processing at the non-canonical junctions which lack tRNA punctuation (Antonicka et al. 2025). Mature mRNAs are stabilized by LRPPRC/SLIRP complexes (Chujo et al. 2012; Lagouge et al. 2015; Mootha et al. 2003; Ruzzenente et al. 2012; Sasarman et al. 2010; Singh et al. 2024). Translation of mature mRNAs occurs on membrane-tethered mitoribosomes, which directly dock to the inner membrane, enabling the co-translational insertion of nascent OXPHOS subunits into the lipid bilayer via the OXA1L insertase (Homberg et al. 2023; Itoh et al. 2021; Jia et al. 2003; Ott et al. 2015; Szyrach et al. 2003). Despite the importance of mitochondrial gene expression for cellular energy metabolism and physiology, there still are gaps in our knowledge of the mechanisms and their coordination. A lack of genetic access to mitochondria has hampered experimental access to address these questions. However, in recent years, new technologies have been established that enable us to manipulate each level of mitochondrial gene expression to elucidate mechanistic details of mitochondrial gene expression. In this review, we aim to detail tools to manipulate both mtDNA and mtRNA, which not only provides technologies for studying mitochondrial gene expression but might also pave the way to potential therapeutic options.

## 2 Targeting mitochondrial DNA

### 2.1 Biolistic transformation of mitochondria

Biolistic transformation, also known as particle bombardment, is one of the most established and effective methods for introducing genetic modifications into the mitochondrial DNA (mtDNA) of *Saccharomyces cerevisiae* (Bonnefoy and Fox 2001; Fox et al. 1988; Johnston et al. 1988). In this approach, DNA-coated tungsten or gold microprojectiles are accelerated into yeast cells using a gene gun, physically breaching cellular and mitochondrial membranes to deliver exogenous DNA directly into the organelle (Bonnefoy and Fox 2007) (Figure 2). This method circumvents the natural barriers that typically prevent nucleic acid uptake into mitochondria, making it a valuable tool for mitochondrial genome engineering.

Several variations of the approach have been reported in the literature. Since the transformation efficiency is low, the approach usually utilizes a selection strategy to identify those cells in which mitochondria have received the exogenous DNA molecule. The original work on this method used a yeast strain that was defective in the mitochondrial COX1 gene. DNA transfer into mutant mitochondria rescued the respiratory defect enabling selection on non-fermentable
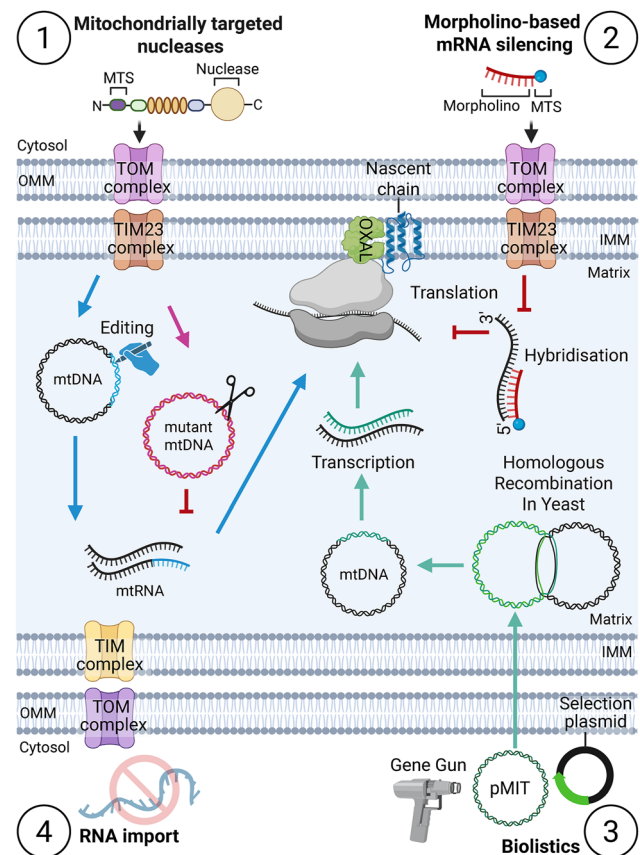


**Figure 2:** Tools for manipulating mitochondrial gene expression: (1) mitochondria-targeted nucleases are directed to the mitochondrial matrix, enabling targeted degradation of mtDNA or nucleotide base editing. This approach prevents the expression of mutant mitochondrial genes or facilitates the expression of edited gene sequences. (2) morpholino-based mRNA silencing tools utilize mRNA sequence-specific morpholinos targeted to the mitochondrial matrix; these inhibit mitochondrial translation by blocking ribosome-mRNA interactions through hybridization with mRNA. (3) in yeast, the pMIT (mitochondrially targeted plasmid) system allows integration of an exogenous gene of interest alongside a selection plasmid, exploiting homologous recombination within yeast mitochondria for stable genetic manipulation. (4) no efficient tool for enabling RNA import into mitochondria is currently available.

medium (Johnston et al. 1988). In standard protocols, yeast strains that lack functional mtDNA are transformed with plasmids containing the desired mitochondrial. Further, mating with a rho⁺ strain is carried out to introduce desired gene into the target mtDNA via homologous recombination. This approach was utilized for the first time to transfer a plasmid borne COX2 (Bonnefoy and Fox 2007). In addition to mitochondria-specific genes, plasmids that harbor reporter genes recoded to the mitochondrial genetic code have been introduced by the bombardment technology and can be used to select for transformed mitochondria (Cohen and Fox 2001; Franco and Barros 2023; Hall et al. 2012; Mireau et al. 2003; Suhm et al. 2018).

Despite its utility, biolistic transformation presents several limitations. The potential for off-target integration of exogenous DNA into the nuclear genome cannot be entirely excluded, raising concerns about unintended genetic alterations. Additionally, the efficiency of transformation and, if desired, recombination between the introduced and endogenous genomes may be insufficient to surpass the heteroplasmy threshold required for phenotypic manifestation of the edited mtDNA, necessitating screening and selection procedures. Moreover, the reliance on homologous recombination restricts the applicability of this method to organisms like yeast, whose mitochondrial genomes are amenable to recombination. In contrast, higher eukaryotes generally lack efficient mitochondrial homologous recombination mechanisms, limiting the broader utility of biolistic transformation for mitochondrial genome editing in complex organisms.

## 2.2 Mitochondrially targeted nucleases

The development of programmable nucleases, particularly CRISPR-Cas9, has revolutionized genetic engineering by enabling highly specific and efficient editing of nuclear genomes in a wide range of organisms. CRISPR-Cas9 has been instrumental in gene therapy, functional genomics, and disease modeling. However, its application to mitochondrial genomes remains challenging due to the unique structural and functional properties of mitochondria. The double-membrane architecture of mitochondria, coupled with the lack of established mechanisms for importing guide RNAs and other nucleic acids into the organelle, has hindered the direct use of CRISPR-Cas9 for mitochondrial genome editing (Schmiderer et al. 2022). To overcome these obstacles, researchers have developed mitochondrially targeted nucleases, such as mitochondria-targeted zinc finger nucleases (mtZFNs) and transcription activator-like effector nucleases (mtTALENs) (Figure 1). These engineered proteins are equipped with mitochondrial targeting sequences that direct them to the organelle and into the matrix, where they can induce site-specific double-strand breaks in mtDNA. By selectively cleaving mutant or undesired mtDNA molecules, these nucleases facilitate the degradation of targeted genomes, thereby promoting a shift toward wild-type mtDNA and enabling the study of mitochondrial gene function and disease-associated mutations.

Zinc finger proteins are characterized by a distinctive DNA-binding motif in which a zinc ion stabilizes a ββα structural fold. Each zinc finger domain typically recognizes a DNA sequence of 3–4 base pairs, and engineered proteins containing tandem arrays of zinc finger domains can achieve sequence specificity ranging from 9 to 18 base pairs. Zinc finger nucleases (ZFNs) are created by fusing these DNA-binding domains to the FokI endonuclease, thereby enabling the generation of targeted double-strand breaks in mitochondrial DNA. Despite their utility, ZFNs exhibit relatively modest editing efficiencies in mitochondrial DNA, with reported rates ranging from 3 % to 30 %. Additionally, their modular protein interactions can lead to off-target effects, limiting their precision and safety in genome editing applications (Gammage et al. 2016; Willis et al. 2022).

TALENs employ arrays of 33–34 amino acid repeats, known as TALE arrays, which confer single-nucleotide recognition specificity. These arrays are also fused to the FokI nuclease domain. Compared to ZFNs, TALENs demonstrate enhanced specificity due to reduced context-dependent effects on DNA binding. However, their large molecular size poses challenges for viral vector packaging, and the requirement for complete redesign of the TALE array when targeting new sequences adds complexity to their application (Bacman et al. 2013).

Mitochondrially targeted endonucleases, such as restriction endonucleases (REs) like SmaI and XmaI, have historically been utilized to introduce double-strand breaks in mutant mtDNA (Tanaka et al. 2002). Notably, mitochondrially targeted ApaLI-based restriction enzymes (mitoREs) were successfully used to shift heteroplasmy from 60 % to 15 % in cells derived from patients with Leber's hereditary optic neuropathy (LHON) (Reddy et al. 2015). However, the utility of REs is constrained by their inability to be reprogrammed for novel restriction sites. In contrast, homing endonucleases such as mitoARCUS can selectively cleave mtDNA at specific mutation sites (e.g., m.5024C > T), achieving single-nucleotide resolution in targeting (Zekonyte et al. 2021; Shoop et al. 2023). This represents a significant advancement in the precision of mitochondrial genome editing.

Collectively, these engineered nucleases provide diverse strategies for targeted manipulation of the mitochondrial genome, each with unique advantages and limitations regarding specificity, efficiency, and adaptability to new target sequences (Figure 2). Ongoing innovations in protein engineering and delivery methods are expected to further enhance the therapeutic potential of these genome editing tools for mitochondrial diseases.

## 2.3 Base editors for mitochondrial genome engineering

The development of base editors represents a significant advancement in the field of mitochondrial genome engineering, offering a robust and precise alternative to

traditional genome editing methods. One example is the Cytosine Base Editors (CBEs). DddA-derived cytosine base editors (DdCBEs) utilize a split bacterial deaminase, DddA-tox, in conjunction with transcription activator-like effector (TALE) DNA-binding domains to facilitate site-specific cytosine-to-thymine (C-to-T) conversions within the double stranded mitochondrial DNA. These editors have demonstrated editing efficiencies ranging from 5 % to 50 %, making them highly effective tools for targeted mtDNA modifications (Mok et al. 2020). The advent of high-fidelity DdCBEs (HiFi-DdCBEs) has further improved the specificity of these systems by substantially reducing off-target editing events, thereby enhancing their safety profile for potential therapeutic applications (Lee et al. 2023).

A comprehensive collection of DdCBEs has been engineered to create the mitoKO library, which enables the precise ablation of all protein-coding genes in the mouse mitochondrial genome (Silva-Pinheiro et al. 2023). This system operates by introducing premature stop codons into mtDNA, thereby facilitating the knockout of each of the mtDNA-encoded proteins. The approach relies on TALE-based DNA-binding domains fused to split DddA deaminase halves, allowing for highly specific, targeted C-to-T base editing. Through iterative cycles of transfection and selection, researchers have successfully established near-homoplasmic knockout cell lines and generated mouse models characterized by high levels of heteroplasmy and minimal off-target modifications (Silva-Pinheiro et al. 2023). These advances underscore the versatility and precision of DdCBEs for mitochondrial functional genomics.

Adenine base editors, including TALE-linked deaminases (TALEDs) and mitochondrial adenine base editors (mtABEs), have expanded the toolkit for mitochondrial genome editing by enabling targeted adenine-to-guanine (A-to-G) conversions. These editors employ the engineered deaminase TadA8e and are mitochondrially targeted (Cho et al. 2024). Moreover, an advanced tool now combines TALE-fused nickase (MutH or Nt.BspD6I) with adenine or cytosine deaminases to perform single stranded base editing of mitochondrial DNA. The tools has been reported to be up to 77 % efficient and highly specific (Yi et al. 2024) The development of TALEDs and mtABEs has opened new avenues for correcting pathogenic mtDNA mutations and for functional studies of mitochondrial genes, complementing the capabilities of cytosine base editors (Figure 2).

In summary, the development of both cytosine and adenine base editors has enabled new ways of mitochondrial genome engineering, providing researchers with precise, efficient, and versatile tools for studying mitochondrial biology and developing potential therapeutic strategies.

# 3 Targeting mitochondrial RNA

## 3.1 RNA import into mitochondria: mechanisms and functional implication

In *Trypanosoma brucei*, all mitochondrial tRNAs are nuclear-encoded and subsequently imported into the organelle. Accordingly, RNA molecules can in principle pass the inner mitochondrial membrane. Recent studies have elucidated that tandem tRNA precursors are imported into the organelle in a manner dependent on both ATP and the mitochondrial membrane potential (Shikha et al. 2020; Tschopp et al. 2011). Notably, the translocase of the inner membrane (TIM) complex has been identified as essential for tRNA import. Interestingly, this process operates independently of the presequence-associated import motor, supporting the so-called "alternate import model" (Shikha et al. 2020). According to this model, tRNA and protein import utilize common translocation machineries but are mediated through distinct mechanisms, ensuring the specificity and regulation of mitochondrial import pathways.

In the budding yeast *S. cerevisiae*, most mitochondrial tRNAs are encoded by the mitochondrial genome. However, an exception exists for tRNA$^{Lys}$ (CUU), which is imported from the cytosol. The import of this tRNA is facilitated by interactions with specific cytosolic proteins, including the precursor of mitochondrial lysyl-tRNA synthetase (preMsk1p) (Entelis et al. 1998). These interactions are critical for the efficient delivery of tRNA$^{Lys}$ into the mitochondrial matrix. The import machinery responsible for this process involves components of both the TOM and TIM23 complex, suggesting functional overlap with the canonical protein import machinery (Entelis et al. 1998; Sepuri et al. 2012).

Despite these examples, the transport of RNA into mitochondria appears to be an exception (Figure 2). This is supported by the composition of the mitochondrial ribosome in which the RNA content is markedly reduced compared to its cytosolic counterparts and the rRNAs are encoded on mtDNA (Amunts et al. 2015; Itoh et al. 2021; Lavdovskaia et al. 2024; Metodiev et al. 2009; Ott et al. 2015). A second example is the loss of the RNA component in mitochondrial RNase P (Bhatta et al. 2021; Holzmann et al. 2008), highlighting that the mitochondrion reduced the demand for RNA compounds during evolution. It appears that mitochondria have very limited capacity for RNA import. As of today, no robust import system that enables RNA transport into mitochondria has been established.

## 3.2 Polymorpholino-mediated gene silencing

Small interfering RNA (siRNA)-mediated RNA interference (RNAi) is a widely used tool for silencing cytosolic mRNA that revolutionized molecular biology by enabling sequence-specific gene silencing, yet its application remains constrained to cytosolic targets. While cytosolic siRNA exploits the RNA-induced silencing complex (RISC), mitochondrial transcripts lack comparable endogenous silencing pathways. Moreover, the delivery of custom RNA molecules into the mitochondrial matrix represents a significant challenge (Phan et al. 2023; Silva-Pinheiro and Minczuk 2022). To address this limitation, phosphorodiamidate morpholino oligonucleotides (PMOs) have emerged as a promising and powerful tool for achieving precise mitochondrial gene silencing (Figure 2). PMOs are synthetic antisense molecules characterized by a morpholine ring backbone and neutral phosphorodiamidate linkages, features that confer high resistance to nucleases and high stability within biological systems. Nevertheless, conventional PMOs are unable to passively traverse cellular or mitochondrial membranes, which restricts their utility for targeting mitochondrial transcripts. To overcome this barrier, researchers have engineered mitochondrially targeted protein/peptide-PMO chimeras using click chemistry, thus enabling specific binding to individual mitochondrial mRNAs (mt-mRNAs) (Cruz-Zaragoza et al. 2021, 2025). In a recent study, a mitochondrially targeted mitochondrial matrix protein conjugated to a PMO was successfully imported into isolated mitochondria, where it demonstrated transcript-specific and efficient silencing activity (Cruz-Zaragoza et al. 2021; Koschel and Cruz-Zaragoza 2024). Building on these findings, further optimization efforts focused on reducing the size of the chimera. This was achieved by replacing the full-length protein with only a mitochondrial targeting sequence (MTS, presequence), which was directly linked to the PMO. The improved presequence-PMO chimera exhibited enhanced silencing efficiency in isolated mitochondria. Importantly, the presequence-PMO chimera could be transfected into cells where they facilitate robust and specific mitochondrial mRNA silencing (Cruz-Zaragoza et al. 2025). This marked the first successful demonstration of this approach in living cells, representing a significant advance in the field of mitochondrial gene regulation. The chimeras used in these studies, typically comprising 19–25 nucleotides, are imported into the mitochondrial matrix, where they bind complementary mt-mRNAs and sterically block mitochondrial ribosome interactions at the 5′ end of the target mRNA. Thereby, they effectively inhibit translation and achieve transcript-specific silencing (Cruz-Zaragoza et al. 2021, 2025). This strategy highlights the potential of targeted PMO chimeras as a versatile platform for mitochondrial gene manipulation and functional studies.

## 4 Conclusions

Despite decades of research in mitochondrial biology, significant gaps remain in our understanding of mitochondrial gene expression processes and their modulation in response to cellular requirements. Progress in this field has been hampered by the lack of suitable techniques for analyzing gene expression within mitochondria. However, the development of new technologies in recent years has opened promising avenues to address key questions regarding the mechanisms and regulation of mitochondrial gene expression. While these technologies have enabled advances in basic research, their application in the treatment of mitochondrial disorders remains limited. Nevertheless, it is anticipated that continued progress in this area will not only deepen our understanding of fundamental biological processes but also shed light on the pathological mechanisms underlying mitochondrial diseases. Ultimately, the ongoing development of innovative techniques holds the promise of paving the way toward future clinical strategies.

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