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Review

The constraints of allotopic expression

Felipe Nieto-Panqueva [a], Diana Rubalcava-Gracia [a,b], Patrice P. Hamel [c,d], Diego González-Halphen [a,*]

[a] Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Mexico City, Mexico
[b] Division of Molecular Metabolism, Department of Medical Biochemistry and Biophysics, Karolinska Institutet, Stockholm, Sweden
[c] Department of Molecular Genetics and Department of Biological Chemistry and Pharmacology, Ohio State University, Columbus, OH, USA
[d] Vellore Institute of Technology (VIT), School of BioScience and Technology, Vellore, Tamil Nadu, India

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ABSTRACT

Allotopic expression is the functional transfer of an organellar gene to the nucleus, followed by synthesis of the gene product in the cytosol and import into the appropriate organellar sub compartment. Here, we focus on mitochondrial genes encoding OXPHOS subunits that were naturally transferred to the nucleus, and critically review experimental evidence that claim their allotopic expression. We emphasize aspects that may have been overlooked before, i.e., when modifying a mitochondrial gene for allotopic expression—besides adapting the codon usage and including sequences encoding mitochondrial targeting signals—three additional constraints should be considered: *(i)* the average apparent free energy of membrane insertion ($\mu\Delta G_{app}$) of the transmembrane stretches (TMS) in proteins earmarked for the inner mitochondrial membrane, *(ii)* the final, functional topology attained by each membrane-bound OXPHOS subunit; and *(iii)* the defined mechanism by which the protein translocator TIM23 sorts cytosol-synthesized precursors. The mechanistic constraints imposed by TIM23 dictate the operation of two pathways through which alpha-helices in TMS are sorted, that eventually determine the final topology of membrane proteins. We used the biological hydrophobicity scale to assign an average apparent free energy of membrane insertion ($\mu\Delta G_{app}$) and a "traffic light" color code to all TMS of OXPHOS membrane proteins, thereby predicting which are more likely to be internalized into mitochondria if allotopically produced. We propose that the design of proteins for allotopic expression must make allowance for $\mu\Delta G_{app}$ maximization of highly hydrophobic TMS in polypeptides whose corresponding genes have not been transferred to the nucleus in some organisms.

1. Endosymbiotic origin of mitochondria and natural transfer of mitochondrial genes to the nucleus

Mitochondria perform oxidative phosphorylation (OXPHOS), a fundamental energy-conversion process (Green and Tzagoloff, 1966; Mitchell, 1961). These organelles arose from the endosymbiosis between an ancestor of an alpha proteobacterium (Andersson et al., 1998; Martin and Müller, 1998; Roger et al., 2017; Martijn et al., 2018) and an ancestor of an archaeon related to extant members of the *phylum* Lokiarchaeota (Zaremba-Niedzwiedzka et al., 2017). After establishing this association, a massive transfer of genetic material from the endosymbiont to the archaeal host occurred (Lynch 1997; Gray, 1989; Gray et al., 1998). Once a protein-encoding gene from the endosymbiont (or protomitochondrion) relocalizes to the nucleus, there is evolutionary pressure for the relocated gene to become functional (Adams and Palmer, 2003), i.e., the transferred gene requires changes in its genetic code and/or codon usage, the acquisition of transcriptional and translational regulatory sequences, and the addition of nucleotide sequences encoding mitochondrial targeting sequences (MTS) which will enable the corresponding protein products to localize to the mitochondria. As a result, contemporary mitochondrial genomes are reduced and encode between 10- and 100-times fewer genes than most alpha proteobacterial genomes (Adams and Palmer, 2003), e.g., the human mitochondrial genome (mtDNA) encodes only 2 rRNAs, 22 tRNAs, and 13 proteins required for OXPHOS (Attardi, and Schatz, 1988; Roger et al., 2017). Despite the ongoing transfer of mitochondrial genes to the nuclear genome, some genes never become activated, and remain as pseudogenes, the so called nuclear-mitochondrial DNA segments (NUMTs)

* Corresponding author at: Departamento de Genética Molecular, Instituto de Fisiología Celular, Universidad Nacional Autónoma de México, Apartado Postal 70-243, México 04510, DF, Mexico.
E-mail address: dhalphen@ifc.unam.mx (D. González-Halphen).

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(Wallace et al., 1997; Bensasson et al., 2001; Woischnik and Moraes, 2002; Ricchetti et al., 2004; Calabrese et al., 2017; Lutz-Bonengel et al., 2021). A recent study shows that over 99 % of 66,083 whole-genome sequences in the human population carry at least one NUMT, and that most of these NUMTs have been inserted after humans diverged from apes (Wei et al. 2022).

Mitochondrial gene transfer to the nucleus has played a central role in the evolution of the organelle (Henze and Martin, 2001). If the relocation of mtDNA to the nuclear genome results in the successful activation of all genes, then it is expected that all coding sequences could be eventually lost from the mitochondrion (Berg and Kurland, 2000). However, mitochondria from virtually all oxygen-respiring eukaryotes possess a mitochondrial genome, except for the parasitic dinoflagellate *Amoebophrya ceratii* (John et al., 2019). In this text, we focus on human mtDNA-encoded genes and their protein products, although we also include seminal discoveries on allotopic expression made using budding yeast as a model system. However, the fundamental differences between mammalian and yeast mtDNA must be considered when describing examples of allotopic expression. Mammalian mtDNA is a small, circular molecule of ~16 kb encoding intron-less genes. The mtDNA of the yeast *Saccharomyces cerevisiae* is larger (~86 kb) and encodes genes interspersed by introns. In contrast to mammalian mtDNA, yeast mtDNA is subject to homologous recombination if it is biparentally inherited after mating (Birky, 2001). Notably, there is no evidence for the functional transfer of mitochondrial tRNA or mitoribosomal RNAs encoding genes to the nucleus (Adams and Palmer, 2003; Telonis et al., 2015). Mitochondria can use nuclear-encoded tRNA genes whose gene products are imported into the mitochondria but there is no support for a mitochondrial ancestry for these genes (Salinas-Giegé et al., 2015).

Here, we review examples of natural and artificial gene transfer of mitochondrial genes to the nucleus. We formulate the main constraints to be considered in the design of constructs for allotopic expression and examine previous reports using this experimental approach.

2. Biogenesis of mtDNA-encoded OXPHOS subunits and topological arrangement in the membrane

OXPHOS results from the concerted activity of five oligomeric protein complexes (I to V) embedded in the inner mitochondrial membrane (IMM) (Saraste, 1999; Papa et al., 2012; Vercellino and Sazanov, 2022). Electron transport through complexes I, III, and IV is tightly linked to protons being vectorially translocated from the matrix into the intermembrane space (IMS), generating an electrochemical potential $\Delta\mu H+$ across the IMM that is harnessed by complex V to generate ATP (Mitchell, 1961). High-resolution 3D structural models of human OXPHOS complexes are now accessible: the membrane-bound region of complex I, the whole complexes III and IV (Guo et al., 2017; Zong et al., 2018), and the complete complex V (Lai et al., 2023). Furthermore, many 3D structures of other mammalian OXPHOS complexes are also available (Iwata et al., 1998; Inaoka et al.,2015; Fiedorczuk et al., 2016; Letts et al., 2016; Gu et al., 2019; Pinke et al., 2020; Spikes et al., 2021). Thus, we now know the exact number and folding architecture of the transmembrane segments (TMS) of all OXPHOS subunits. Based on the experimentally deduced topological arrangement, the human mtDNA encoded subunits can be classified in four groups based on the exposition of its N or C-terminus to the IMS (out) or to the matrix (in): N_{out}-C_{out}, N_{in}-C_{in}, N_{out}-C_{in} and N_{in}-C_{out} (Fig. 1).

The mitochondrial proteome is a mosaic of components with two different genetic origins, with most proteins encoded by nuclear genes (1136 in humans according to Mitocarta 3.0) (Rath et al., 2021), and a small portion by mtDNA (~1.1 %). In general, mitochondrial genomes encode ribosomal RNAs, transfer RNAs, and a subset of proteins required for OXPHOS (Attardi, and Schatz, 1988; Roger et al., 2017). In mammals, 13 out of the 89 genes encoding OXPHOS proteins reside in the mtDNA: *nd1*, *nd2*, *nd3*, *nd4*, *nd4L*, *nd5, nd6* (for complex I), *cox1*, *cox2, cox3* (for complex IV), *cytb or cob* (for complex III), *atp6,* and *atp8* (for complex V) (Anderson et al, 1981; Boore and Fuerstenberg, 1999). Complex II is typically composed only of nuclear DNA-encoded subunits, with some exceptions in land plants (Roger et al., 2017; Huang et al., 2019). Although outnumbered, mtDNA-encoded proteins reside at the core of their respective OXPHOS complexes and are highly hydrophobic, membrane-embedded polypeptides (von Heijne, 1986; Popot and de Vitry,1990, Johnston and Williams, 2016). In this review, we will focus on the human genes mentioned above and their protein products, although the gene *atp9*, which is encoded in the mitochondrial genome of yeast, is briefly discussed.

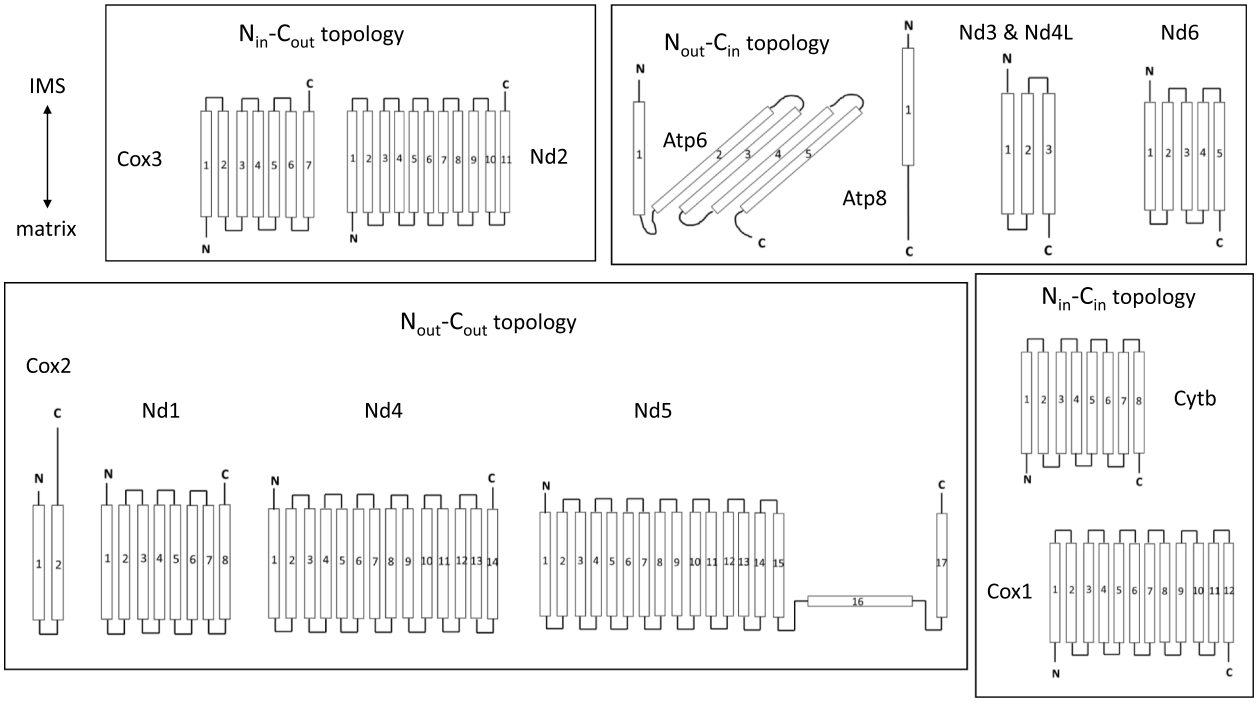

Fig. 1. The 13 human mtDNA encoded OXPHOS proteins can be classified in four different topologies.

Newly made mitochondrial polypeptides cotranslationally insert into the IMM (Dennerlein et al., 2021; Kummer and Ban, 2021), acquire prosthetic groups, and integrate into their OXPHOS complex with the aid of assembly factors (Singh et al., 2020). MtDNA-encoded subunits are synthesized by membrane-tethered mitochondrial ribosomes (mitoribosomes) and then co-translationally integrated into the IMM by Oxa1 (Oxa1L in mammals) (Bonnefoy et al., 1994; Rehling et al., 2003, Szyrach et al., 2003; Preuss et al., 2005; Ott and Herrmann, 2010; Itoh et al. 2021), an insertase belonging to a family of evolutionarily conserved membrane-embedded translocases found in bacteria and in other organelles such as the endoplasmic reticulum (ER) (McDowell et al., 2021; Güngör et al., 2022) and the chloroplasts (Wang and Dalbey, 2011). Oxa1 interacts with the N-termini of nascent polypeptides, looped segments of neighboring TMS (hairpins), and C-terminal segments of polytopic proteins (Hell et al., 2001; Szyrach et al., 2003). Human mitoribosomes are anchored to the IMM through the interaction of Oxa1L and mL45 (Mba1 in yeast), a mitoribosome subunit (Ott et al., 2006). Oxa1L also inserts proteins harboring multiple TMS, which are thought to be integrated into the membrane through hairpins (Engelman and Steitz, 1981; Herrmann et al., 1997; Herrmann and Neupert, 2003) i.e., two antiparallel, hydrophobic alpha-helices linked by a central turn. Except for Nad3 and Nad4L, all OXPHOS subunits follow a different insertion pathway into the IMM. Although details remain to be explored for each protein, we depict how the human mtDNA-encoded OXPHOS subunits may be cotranslationally inserted into the IMM, giving a representative for each of the four topological arrangements described in Fig. 1 (Suppl Figs. S1–S4).

3. Mitochondrial protein import of inner membrane proteins through TIM23

Mitochondria specialized their translocase machinery throughout evolution to allow the import of cytosol-synthesized proteins. The endosymbiont that gave rise to mitochondria probably contained core import components such as the PAM motor complex and the TOM, SAM, and OXA1 translocases at the plasma membrane, although their function was probably involved in exporting proteins (Hewitt et al., 2011; Liu et al., 2011). In addition, primitive forms of the TOM and TIM complexes, capable of importing presequence containing proteins, were most probably present in the last common ancestor of eukaryotes (Fukasawa et al., 2017).

A comprehensive description of all the import pathways of proteins targeted to mitochondrial sub-compartments and the evolution of the mitochondrial import machinery may be found in recent reviews (Pfanner et al., 2019; Grevel et al., 2020; Dimogkioka et al. 2021; Ruiz-Pesini et al., 2021; Schneider 2022). Most proteins imported into mitochondria contain an N-terminal cleavable peptide, known as mitochondrial targeting sequence (MTS), which guides them through the outer and inner membranes, and into the mitochondrial matrix. Although MTS do not have defined sequence motifs, they display some general features: they span 15–50 amino acids, tend to form positively charged, amphiphilic alpha-helices, and are rich in alanine, leucine, serine, glycine, and arginine residues, with few if any acidic residues (von Heijne et al., 1989; Chacinska et al., 2009).

MTS-containing precursors in transit to mitochondria are kept in an import-competent form by cytosolic chaperones (e.g., HSP70) and are recognized and translocated by the translocase of the outer mitochondrial membrane (TOM complex, Hartl et al., 1989; Dimogkioka et al. 2021), while others are cotranslationally imported into mitochondria (Williams et al., 2014). These precursors then cross the inner membrane through the translocase complex of the inner mitochondrial membrane, TIM23 (Callegari et al., 2020).

Proteins targeted to the IMM that lack a cleavable MTS, like the family of carriers containing six TMS, use specialized translocation machinery known as TIM22, the carrier translocase (Sirrenberg et al., 1996; Neupert, 2015; Wiedemann & Pfanner, 2017; Hansen & Herrmann, 2019; Horten et al., 2020). Aided by the membrane potential, this complex distinctively inserts multispanning, inner-membrane proteins that exhibit internal targeting signals (Rehling et al., 2004; Chacinska et al., 2009). TIM22 has a narrow, well-defined substrate repertoire (Chacinska et al., 2009): the members of the mitochondrial carrier's family with 6 TMS; the translocase components Tim23, Tim17, and Tim22 that share the same N_{out}-C_{out} topology; sideroflexins; and the Mpc2 and Mpc3 pyruvate carriers (Gomkale et al., 2020; Rampelt et al., 2020). Thus, there seems to be a clear-cut separation of protein substrates entering through TIM22 or TIM23: while TIM22 inserts hairpins of a specific group of membrane proteins in an IMS-matrix direction, TIM23 sorts MTS containing precursors (Fig. 2). Since protein import through TIM22 is not relevant to the discussion that follows, we only describe here the details of TIM23 function.

4. Mechanisms of protein sorting by TIM23 – Translocation vs. Lateral release

TIM23 exhibits the dual ability to sort TMS using either the "conservative-sorting" or the "stop-transfer" pathway (Mahlke et al., 1990; Bohnert et al. 2010) (Fig. 2). In the latter, the TMS containing a "stop-transfer" signal within their sequence stops their translocation, arrested by a TIM23 conformational state known as $TIM23^{SORT}$, which releases them laterally into the lipid phase of the IMM (Chacinska et al., 2005; van der Laan et al., 2006; 2007). In the "conservative sorting" pathway, alpha helices are fully translocated through TIM23, linked to the motor ATP-dependent presequence translocase-associated protein import motor (PAM), in a conformational state known as $TIM23^{MOTOR}$. Thus, alpha-helices get delivered into the matrix where their MTS is cleaved (by the matrix processing peptidase, MPP). If required, in a subsequent step, TMS may be inserted into the IMM by Oxa1L from the matrix side of the membrane (Omura, 1998; van der Laan et al., 2006; Mossmann et al., 2012).

Although it is not completely clear how the TIM23 complex alternates between its two distinct conformational states, incoming TMS containing stop-transfer signals will be integrated into the IMM (Glick et al., 1992). Hydrophobicity and the location of polar and aromatic residues are strong determinants of the fore-mentioned membrane insertion pathway. Alpha helices with low hydrophobicity and carrying one or more prolines, are prone to be fully translocated by $TIM23^{MOTOR}$. In contrast, TMS exhibiting high average hydrophobicity, low or null proline content, and having positively charged residues (Arg, Lys) flanking the alpha-helix (mainly on the matrix side), tend to be arrested and released laterally by $TIM23^{SORT}$ (Meier et al., 2005; Botelho et al., 2011; Osterberg et al., 2011; Lee et al., 2020). In yeast, Tim23 interacts with the Mgr2 subunit (ROMO1 in humans), which functions as a lateral gatekeeper providing quality control while binding the Tim21 subunit forming all together the $TIM23^{SORT}$ arrangement (Gebert et al., 2012; Matta et al., 2020; Richter et al. 2019; Needs et al., 2021). Since Mgr2 monitors the hydrophobicity of transiting alpha-helices, *mgr2*-null yeast strains display increased lateral release of proteins by $TIM23^{SORT}$ to the IMM (Ieva et al., 2014). Conservative sorting and stop-transfer are not mutually exclusive pathways, they represent mechanisms that may cooperate in the membrane integration of a protein (Bohnert et al., 2010). Although structurally different, $TIM23^{SORT}$ and $TIM23^{MOTOR}$ are in dynamic exchange during precursor translocation and sorting (Chacinska et al., 2010).

Pioneer work revealed that TIM23 behaves as a voltage-gated channel with an estimated diameter of 13 Å (Schwartz and Matouschek 1999; Truscott et al. 2001). An NMR-derived structure revealed dimeric Tim23 forms a pore with a width of ~12 Å at its narrowest, and ~22 Å at its widest (Zhou et al., 2020). More recently, a cryo-EM structure at 2.9 Å resolution of the yeast core TIM23 complex (formed by subunits Tim17, Tim23 and Tim44) shows that Tim23 and Tim17 do not form a water-filled channel (Sim et al., 2023). Instead, these two subunits interact back-to-back, and exhibit separate, membrane-exposed

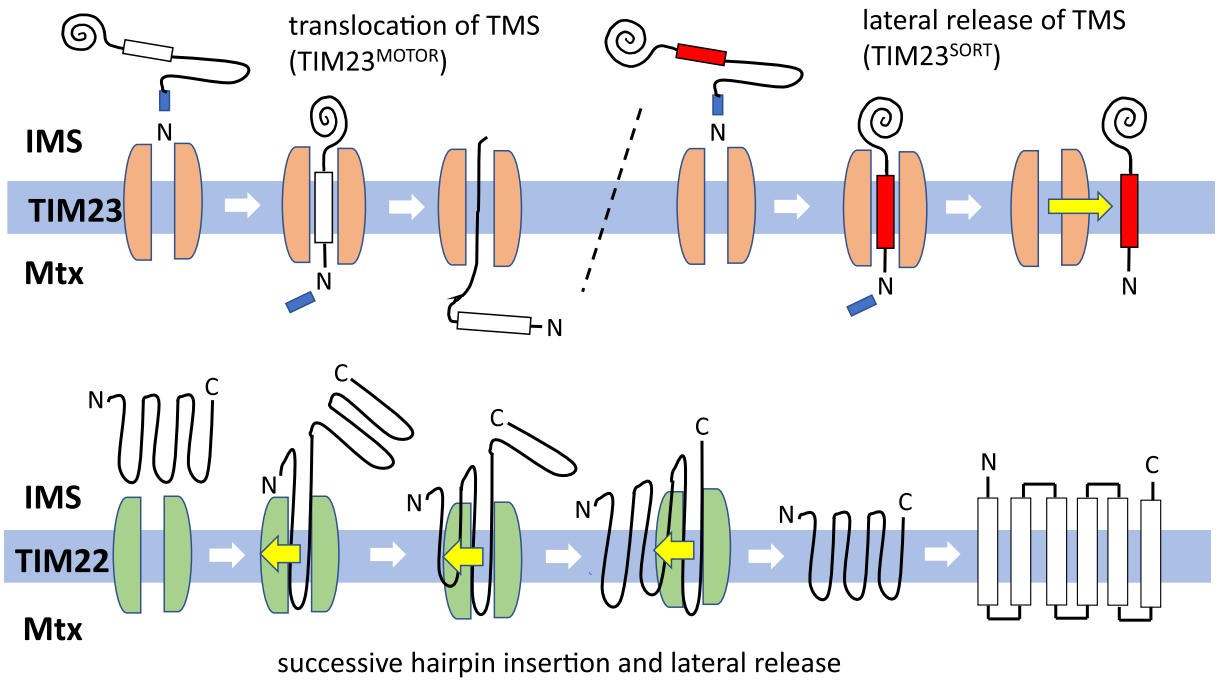

Fig. 2. Protein import mechanisms of the IMM translocators TIM23 and TIM22. For simplicity, the Outer Mitochondrial Membrane is not depicted. Top: TIM23 (pink) exhibits the dual ability to distribute proteins into two different pathways. When following the "conservative sorting" pathway, alpha-helices of relatively low hydrophobicity (white) are translocated by the $TIM23^{MOTOR}$ into the matrix. By contrast, when following the "stop-transfer" pathway, highly hydrophobic alpha-helices (red) usually lacking prolines and flanked by positively charged amino acids in their matrix-exposed end, are arrested by $TIM23^{SORT}$ and released laterally into the IMM. Bottom: the TIM22 complex (green) inserts successively hairpins composed of a pair of TMS into the IMM. Although not exclusively, TIM22 is the main import system for carrier proteins containing six TMS. The mitochondrial inner membrane is represented in light blue. IMS: intermembrane space; Mtx: matrix. N and C termini of protein substrates are indicated, and the blue rectangle indicates the mitochondrial targeting sequence that is cleaved by the matrix peptidase (top panel).

concave cavities that are facing away from each other. Crosslinking experiments suggest that Tim23 probably plays a structural role, while protein translocation occurs through the Tim17 cavity, which appears to be in close contact with the Mgr2 subunit. A cryo-EM structure of another TIM23 subcomplex (formed by subunits Tim17, Tim23 and Mgr2) revealed how Tim23 and Mgr2 shield the polypeptide substrates from the lipid environment (Zhou et al., 2023). Furthermore, both recent 3D structures suggest that the cavity formed by Tim17 is highly conserved, exhibiting a patch of negatively charged residues at the entrance (close to the IMS) and a 10–15 Å long hydrophobic patch in the middle of the translocation pathway (Sim et al., 2023; Zhou et al., 2023). Altogether this novel structural data, along with previous biochemical results (Rehling et al., 2004), strongly indicate that TIM23 handles the translocation of a single TM at a given time.

5. Mitochondrial diseases and their therapeutics

Mitochondrial diseases are prevalent genetic disorders characterized by defects in OXPHOS (La Morgia et al., 2020; Gorman et al., 2016). These diseases can result from mutations in either the nuclear or mitochondrial genomes, with mtDNA mutations accounting for approximately 75 % of the cases in adults and 25 % in children. The nuclear DNA mutations that cause mitochondrial dysfunction (Gorman et al., 2016) are not addressed here. Most patients with mitochondrial diseases harbor heteroplasmic mtDNA mutations, where both mutant and wild type mtDNA versions coexist. The disease phenotype is manifested when the ratio of mutant versus wild type mtDNA copies exceed a certain threshold that depends on the type of mutation, the tissue, and the patient (Russell et al., 2020). Over 300 pathogenic mtDNA mutations have been reported in the well-curated MITOMAP database (https://www.mitomap.org).

Mitochondrial diseases exhibit heterogeneous clinical symptoms and tissue-specificity but typically affect multiple organ systems. The link between a mitochondrial mutation and its pathophysiology is not well understood. Tissues with high energy demands, such as nerves and muscles, are often the most affected. Clinical syndromes range from the muscle disorder Progressive External Ophthalmoplegia (PEO) to multisystemic syndromes such as Myoclonus Epilepsy with Ragged-Red Fibers (MERRF) and Mitochondrial Myopathy, Encephalopathy, Lactic Acidosis, and Stroke-like episodes (MELAS). Leber Hereditary Optic Neuropathy (LHON) is a maternally inherited mitochondrial disorder that affects retinal ganglion cells, causing visual impairment and potential blindness (Carelli et al., 2004). Over the 19 mtDNA mutations associated with LHON, the three most prevalent (G3460A in Nd1, G11778A in Nd4 and T14484C in Nd6) affect subunits of complex I (Wallace et al., 1988; Howell et al., 1991; Jun et al., 1994).

MtDNA mutations fall into three categories: mutations in protein-encoding genes, mutations in structural RNAs (tRNAs and rRNAs), and large-scale mtDNA rearrangements (deletions or duplications). Here, we focus on the 13 genes encoding OXPHOS proteins for which mutations result in severe mitochondrial diseases that currently lack effective treatments. While direct manipulation of human mtDNA is still in development, other strategies for mitochondrial disease therapies have been put forward (DiMauro et al., 2006; Wallace, 2018; Russell et al., 2020; Ng et al., 2021), including mitochondrial delivery of nucleic acids, manipulation of mtDNA heteroplasmy, direct mtDNA editing, and allotopic expression (Di Donfrancesco et al., 2022).

Mitochondrial delivery of nucleic acids involves the use of an adenoassociated virus capsid VP2 fused to an MTS (Yu et al., 2012) or the generation of mitochondrial-targeted transcripts produced in the cytosol and competent in protein translation inside the mitochondrial matrix (Wang et al., 2015). This approach was successfully tested in *Drosophila melanogaster* with the Atp6 subunit encoding transcript (Markantone et al., 2018). Future approaches involve silencing of mitochondrial

translation by importing precursor-morpholino hybrids that could target individual mRNAs at the mitochondrial matrix (Cruz-Zaragoza et al., 2021). Another strategy for manipulating mtDNA heteroplasmy involves the targeted elimination of mutated mtDNA copies using nucleases (Srivastava and Moraes 2001; Bacman and Moraes, 2007; Alexeyev et al., 2008; Xu et al., 2008; Bacman et al., 2013; Gammage et al., 2014; Hashimoto et al, 2015; Bacman et al., 2018; Barrera-Paez and Moraes, 2022). CRISPR-based methods have been used to edit mtDNA (Hussain et al., 2021), although they remain to be substantiated by other studies (Gammage et al., 2018). In addition, advances have been achieved with CRISPR-free mitochondrial base editing of cytosines (Mok et al., 2020; Chen et al., 2022; Nakazato et al., 2022), although this alternative approach also induces undesirable editions in the nuclear genome (Lei et al., 2022). Even though all these approaches show promise, they are still in early stages of development and have not reached mainstream use in gene therapies. Here, we concentrate on allotopic expression, i.e., overcoming mutations in mtDNA-encoded proteins by introducing a wild-type copy of the affected gene in the nucleus and targeting the corresponding cytosol-synthesized protein to mitochondria, thus replacing the defective mitochondrial counterpart (de Grey, 2000; Zullo, 2001; González-Halphen et al., 2004; DiMauro et al., 2006; Kyriakouli et al., 2008).

6. Allotopic expression

Allotopic expression is a strategy to reroute the biogenesis and intracellular transport of a protein (Nagley and Devenish,1989), thus involving the functional activation of an organellar gene in the nucleus (de Grey, 2000). Allotopic expression as therapeutics is intended to overcome mutations in mtDNA-encoded proteins, by placing a modified wild-type copy of the affected gene in the nucleus. If successful, the corresponding protein product can reach the mitochondrion, thereby replacing its dysfunctional counterpart. To achieve this, changes in codon usage must be considered. While the UGA codon is interpreted as tryptophan in mitochondria, it is a termination codon in the nucleus (Macino et al., 1979). Likewise, the AUA codon directs the incorporation of methionine in mitochondria but isoleucine in the nucleus (Takemoto et al., 2009). Prior pioneering research on allotopic expression was carried out in baker's yeast using mitochondrial genes encoding soluble proteins. The first example involved a nuclear version of a cytochrome *b* gene intron encoding the soluble mitochondrial bI4 maturase, which was able to restore respiratory competence to a strain defective in the mtDNA-encoded version of the maturase protein (Banroques et al., 1986; 1987). A similar approach was used in yeast for the soluble Var1 protein, a subunit of the mitoribosome encoded in a mitochondrial gene (Sanchirico et al. 1995). In the plant *Nicotiana sylvestris,* another successful allotopic production of a soluble protein was achieved by targeting the cytosol-synthesized subunit Nd7 (subunit 7 of complex I) to its mitochondrion. Such expression restored complex I biosynthesis in plants lacking a functional mitochondrial *nd7* gene (Pineau et al., 2005). Allotopic expression of genes encoding soluble proteins can be considered trivial, since it is usually achieved after codon optimization and the addition of a sequence encoding an adequate MTS. Undeniably, attempts to direct cytosol-synthesized membrane-bound proteins to mitochondria represents a more significant challenge. A successful allotopic expression requires that the cytosol-synthesized membrane protein gets internalized into mitochondria, inserted in the IMM in its correct topology, and functionally assembled within its corresponding OXPHOS complex. Before describing the specific strategies to achieve allotopic expression in different experimental systems, we discuss examples of mitochondrial genes, encoding membrane proteins, which have been transferred to the nucleus in nature.

7. Examples of mitochondrial gene transfer to the nucleus

Several hypotheses on why mitochondria have retained a genome have been formulated (Daley and Whelan, 2005; Wang, 2012; Allen, 2015). One pioneering hypothesis suggested that, since the protein export machinery of the ER was already in existence when mitochondria appeared, only those genes whose protein products were not highly hydrophobic could have been successfully transferred to the nucleus (von Heijne, 1986). Indeed, hydrophobic stretches, especially those located at the N-terminus of proteins, act as export signals because they are recognized by the Signal Recognition Particle (SRP) and target the protein to the ER (Björkholm et al., 2015). As demonstrated by selective ribosome profiling experiments carried out in *Escherichia coli*, SRP sometimes fails to bind the first TMS and binds instead a TMS located more closely to the C-terminus of the nascent protein. When this happens, the second TMS tends to exhibit a higher hydrophobicity (i.e., a more negative Gibbs free energy difference of membrane insertion ΔG_{app}) (Schibich et al., 2016). So even when the N-terminal TMS is skipped, there will be another one recognized by SRP more internally, and at the end SRP will triage the protein to the ER. Experimental support for the hypothesis that cytosol-synthesized mitochondrial proteins may be mis-targeted to the ER via the SRP was provided with the finding that synthesis of all 13 human mitochondrial-encoded OXPHOS subunits with strong mitochondrial-targeting sequences in the cytosol of human cells, resulted in their transport to the ER, except for Atp8 (Björkholm et al., 2015). Thus, the hydrophobicity of the TMS in the mitochondrial proteins was hypothesized to be a critical limiting factor. In general, mitochondrial proteins whose genes have been relocated to the nucleus exhibit a diminished average hydrophobicity relative to their mtDNA-encoded counterparts (Funes et al., 2002a). A diminished hydrophobicity of proteins synthesized in the cytosol and destined to mitochondria may be a key feature to avoid misrouting to the ER and enable the internalization in the mitochondria (Claros et al., 1995; Daley et al., 2002a,2002b; González-Halphen et al., 2004; Cardol et al., 2006).

In some organisms, mitochondrial genes encoding membrane proteins have been transferred to the nucleus. For example, the genes *atp6*, *cox2*, *cox3, nd3,* and *nd4L*, are absent in the mtDNA of *Chlamydomonas reinhardtii* and related algae (Vahrenholz et al., 1993; Denovan-Wright et al., 1998; Fan and Lee, 2002), and have been functionally relocated to the nucleus. Their corresponding protein products are synthesized by cytosolic ribosomes, post-translationally or co-translationally imported into mitochondria, and functionally assembled into the appropriate OXPHOS complex (Pérez-Martínez et al., 2000; Pérez-Martínez et al., 2001; Funes et al., 2002a). Through evolutionary time the transferred genes have undergone extensive sequence modification to become functional, including mutagenesis, capture of introns, promoters, 5′ and 3′ UTRs, and nucleotide sequences encoding MTS. Addition of MTS may occur by duplication of already existing targeting sequences in instances where functional transfer has already taken place (Kadowaki et al., 1996). In certain lineages the acquired MTS are atypically long, ranging from 100 to 140 residues (Pérez-Martínez et al., 2002; González-Halphen et al., 2004). These long MTS could either promote the binding of precursors to the mitochondrial import machinery or facilitate protein unfolding to enhance its importability (Claros and Vincens, 1996; Claros et al., 1995). When the import rates of artificial precursors containing MTS of varying lengths were measured, it was found that the unfolding of a precursor at the mitochondrial surface is accelerated if the MTS is long enough to span both the OMM and the IMM, enabling the MTS to interact with mtHsp70 in the mitochondrial matrix (Matouschek et al., 1997). Moreover, the synergy between two distinct alpha-helices in a long MTS may stimulate the internalization of some precursors, as demonstrated for the mammalian glutamate dehydrogenase (Kalef-Ezra et al., 2016). In the sections below, we describe examples of natural gene transfer of genes encoding OXPHOS subunits in different organisms.

7.1. The atp6 gene

A gene that is mtDNA-localized in almost all eukaryotic taxa is *atp6*, which encodes subunit 6 of F_1F_o-ATP synthase (Atp6 or subunit a).

Prominent exceptions are found in the malarial parasite *Plasmodium* and the ciliates *Tetrahymena* and *Paramecium*, where the *ATP6* gene is localized in the nucleus (Feagin et al., 1992; Pritchard et al., 1990).

The chlorophycean green alga *Chlamydomonas reinhardtii* also has a nucleus encoded *ATP6* gene (Funes et al., 2002a). The Atp6 precursor with a large MTS of 107 amino acids is synthesized in the cytosol. The algal Atp6 subunit differs from its bovine homolog in that it lacks the first TMS, as observed when comparing the 3D structures of both proteins (Spikes et al., 2021; Murphy et al., 2019) (Suppl Fig. 5). In mammals, Atp6 is mitochondria-synthesized and has five TMS, the first of which is proposed to be cotranslationally inserted into the IMM, followed by the subsequent insertion of two hairpins (Suppl Fig. 1), a process most probably mediated by the Oxa1 insertase. In contrast, during the biogenesis of a cytosol synthesized, algal Atp6 subunit, it is proposed that four TMS are fully translocated into the matrix using $TIM23^{MOTOR}$. Once inside, they are embedded in the IMM as two hairpins via an Oxa1-dependent process (Suppl Fig. 6). It is assumed that the lack of TMS1 in the algal cytosol synthesized Atp6 subunit may facilitate its import into mitochondria.

Subunit *a* (ATP6) is also encoded in the nuclear genome of the apicomplexan parasite *Toxoplasma gondii* (Mühleip et al., 2021). In most eukaryotes, Atp6 typically exhibits a structure with six helices. However, in the case of *T. gondii*, this subunit is distinct, since it lacks helices 1–4 (aH1-4), as revealed by its 3D structure (Suppl Fig 5). Only the helices aH5 and aH6 were found to interact with the *c*-ring (an oligomer of Atp9 subunits), which serves as one of the rotary components in all ATP synthases. The absence of helices 1–4 appears to be compensated by the independent subunits ATPTG16 and ATPG17. Consequently, *T. gondii* Atp6 exhibits a simplified structure with only two TMS. This structural divergence may explain why the cytosol synthesized Atp6 subunit of this parasite can be imported into the mitochondria. Split ATP6 subunits have been also identified in *Trypanosoma brucei* and in other members of the Euglenozoa lineage (Wong et al., 2023).

7.2. The atp8 gene

The *atp8* gene usually resides in the mtDNA, although some exceptions have been reported. The absence of *atp8* is common in rotifers, commonly called wheel animals (Nie et al., 2016). Although the *atp8* gene was originally thought to be absent in some bivalve species, this may not be the case (Breton et al., 2010). In addition, *atp8* seems to be absent from the mtDNA of the chlorarachniophyte alga *Lotharella oceanica* as well, albeit not detected in the nuclear genome either (Tanifuji et al., 2016). In parasitic red algae, the *atp8* gene has either become a pseudogene or disappeared completely from the mtDNA (Hancock et al., 2010). In general, *atp8* may be absent from the mitochondrial genomes because it has migrated to the nucleus or because its encoded protein is no longer a constituent of mitochondrial ATP synthase (i.e., the enzymes from chlorophycean algae typically lack the Atp8 subunit). Nevertheless, to the best of our knowledge, no *atp8* gene that was functionally transferred to the nucleus has been reported.

7.3. The cox1 gene

The *cox1* gene seems to be invariably located in the mtDNA. However, in some organisms, a small fragment of the gene has been transferred to the nucleus. In the amoeboid *Acanthamoeba castellanii* and some other protists, a small, hydrophilic C-terminal portion of Cox1 of only 36 residues, lacking a TMS, has been found to be encoded in the nucleus. The mtDNA-encoded hydrophobic Cox1 large fragment retaining 12 TMS, lacks this C-terminus, but is still encoded in mtDNA (Gawryluk and Gray, 2010). Thus, at some point in evolution, the protist mitochondrial *cox1* gene was fragmented, with the nucleotide sequence encoding the C-terminus of Cox1 functionally integrated into the nuclear genome. This example shows that hydrophobicity limits the transfer of mitochondrial genes to the nucleus, as only the gene encoding the hydrophilic part of Cox1 was successfully relocated.

7.4. The cox2 gene

The Cox2 subunit is usually synthesized in the mitochondrial matrix, (Suppl Fig 2) and embedded in the IMM by the concerted action of the Oxa1 insertase (He and Fox, 1997) and the peripheral IMM protein Mss2 (Broadley et al., 2001). In some species, the *cox2* gene has been transferred to the nucleus, either as a full-length gene (as in leguminous plants) or as a split gene (*COX2A* and *COX2B* in green algae and apicomplexan parasites) (Nugent and Palmer, 1991; Covello et al., 1992; Adams et al., 1999; Pérez-Martínez et al., 2001; Gardner et al., 2002; Funes et al., 2002b). Legumes provide an illustrative example that gene transfer from mitochondria to the nucleus is still occurring. While many species in this clade contain orthodox, mitochondrial *cox2* genes, others show nucleus-localized *cox2* genes, and yet others possess both the mitochondrial and the nuclear versions of the gene (Adams et al., 1999). While in certain instances only one of the versions is active, in others both the nuclear and mitochondrial copies are expressed, strongly suggesting that transfer of mitochondrial genes to the nucleus is an ongoing evolutionary process.

In several chlorophycean algae, the mitochondrial *cox2* gene underwent a fragmentation event, giving rise to the *COX2A* and *COX2B* genes (encoding the hydrophobic domain of Cox2 with its two TMS, and the hydrophilic domain that binds the binuclear copper center, respectively). In some of these green algae, like *Scenedesmus obliquus*, the *cox2a* gene was retained in the mtDNA, while the *COX2B* migrated to the nucleus. In other algal species, like *C. reinhardtii*, *Volvox carteri*, and *Polytomella parva*, both genes were relocated to the nucleus and integrated into different chromosomes (Pérez-Martínez et al., 2001). A similar split in *COX2A* and *COX2B* genes has also been described in apicomplexan parasites (Funes et al., 2002a), and the genes were found to be present in the nuclear genome. All examples of natural, cytosol synthesized Cox2 subunits, i.e., in legumes, chlorophycean algae, and apicomplexan parasites, exhibit low hydrophobicity in their TMS1 and a slightly increased hydrophobicity in their TMS2. Thus, it is suggested that TIM23 may allow the transit of TMS1 toward the matrix (through the conservative sorting pathway) while retaining TMS2 and subsequently releasing it laterally into the IMM (through the stop-transfer pathway) (Daley et al. 2002a; Jiménez-Suárez et al. 2012; Rubalcava-Gracia et al., 2018). The proposed mechanism is the only one that provides a suitable explanation as to why the cytosol-synthesized Cox2 acquires the same final topology as its mitochondria-synthesized counterpart, with both its N- and C-termini exposed to the IMS (Fig. 1 and Suppl Fig. S2).

7.5. The cox3 gene

In several chlorophycean species, closely related to the green alga Chlamydomonas, the *cox3* gene is absent from the mtDNA (Vahrenholz et al., 1993; Denovan-Wright et al., 1998; Fan and Lee, 2002) and resides in the nucleus instead (Pérez-Martínez et al., 2000, 2001, 2002; Watanabe and Ohama, 2001; Merchant et al., 2007; Prochnik et al., 2010). Therefore, the algal Cox3 subunit is synthesized in the cytosol as a precursor containing an unusually large MTS of 98 residues that directs it to mitochondria, where it gets internalized, and functionally assembled into cytochrome *c* oxidase. The mature Cox3 subunit, lacking its MTS, is present in the isolated complex IV of the chlorophycean alga *Polytomella* sp. (Pérez-Martínez et al., 2000). Since Cox3 is a component of the catalytic core of cytochrome oxidase, the knock-down of the nuclear *COX3* gene leads to loss of complex IV assembly, as demonstrated in *C. reinhardtii* (Remacle et al., 2010). *In vitro* experiments have shown that the Cox3 precursor is also readily internalized in isolated *Polytomella* mitochondria and assembled into cytochrome *c* oxidase (Vázquez-Acevedo et al., 2014). In ciliates, for a long time it was considered that their mtDNA did not contain a *cox3* gene, but upon re-

examination it was found that the gene was present but fused to a *cox1* gene that has undergone many variations in its sequence (Degli Esposti et al, 2014).

7.6. The nd3 and nd4L genes

The *ND3 and ND4L* genes are localized in the nucleus in *Chlamydomonas* and other closely related chlorophycean algae. The Nd3 and Nd4L subunits, exhibiting three TMS each, are synthesized in the cytosol as precursors containing MTS of 160 and 130 residues respectively. Both polypeptides show lower hydrophobicity compared to their mitochondrion-encoded counterparts found in other organisms (Cardol et al., 2006). These subunits are eventually processed by the MPP in the mitochondrial matrix, giving rise to the mature subunits that will ultimately integrate into complex I.

7.7. The nd4, nd5 and nd6 genes

To the best of our knowledge, no report of nucleus encoded Nd4, Nd5, and Nd6 exists. This is scarcely surprising, considering the number of TMS in these subunits and their overall high hydrophobicity.

8. Which cytosol synthesized OXPHOS proteins may integrate and reach their correct topology in the IMM? The $\mu\Delta G_{app}$ of each TMS may be the limiting factor

To date, all designs of OXPHOS subunits allotopically produced for import into mitochondria include adding an MTS, since these engineered precursors are expected to be sorted by the TIM23 translocon. As discussed above, a large core of data suggests that TIM23 manages a single alpha-helix at a time, which can either be fully translocated into the matrix or arrested and then released laterally into the IMM. Thus, the average hydrophobicity must be a key parameter that determines through which pathway (conservative or stop-transfer) TIM23 directs a given alpha-helix in an imported protein substrate. To quantify how the hydrophobicity of each alpha-helix governs the insertion of membrane proteins by TIM23, Botelho et al., (2011) took advantage of the nucleus-encoded yeast Mgm1, a membrane protein with two TMS (TMS1 and TMS2) that exhibits an alternative topogenesis and produces two isoforms (large and small). Upon import of the Mgm1 precursor into the mitochondria, TIM23 may laterally release TMS1 into the IMM, giving rise to the large isoform of Mgm1 which remains membrane bound. Alternatively, TMS1 is translocated into the matrix and TMS2 is laterally released into the IMM, where it will be proteolytically processed, giving rise to the small isoform of Mgm1, which will remain as a soluble protein in the IMS (Herlan et al., 2003). For a given population of cytosol synthesized Mgm1 precursors, a mixture of membrane-anchored and soluble isoforms will be formed, where nearly 30–40 % will represent the large isoform of Mgm1 (Botelho et al., 2013). Both isoforms can be resolved according to their different apparent molecular masses in denaturing electrophoresis (SDS-PAGE). By replacing the single TMS of Mgm1 with sequences of varying hydrophobicity, followed by quantification of the ratios of the two Mgm1 isoforms, an apparent free energy of membrane insertion ΔG^{X}_{app} was estimated for each of the 20 amino acids. Thus, for yeast mitochondria, a "biological scale" was constructed, providing ΔG^{X}_{app} values for each residue, ranging from −0.20 (leucine, highly hydrophobic) to +3.01 kcal/mol (arginine, highly hydrophilic). Making use of this tool, the average apparent free energy of membrane insertion $\mu\Delta G_{app}$ can be estimated for any TMS based on its amino acid composition and used as a prediction for TIM23-dependent translocation into the matrix or arrest and lateral release into the IMM.

Table1 lists the calculated $\mu\Delta G_{app}$ values for the TMS of three proteins that are known to be in this threshold scenario: the TMS1 of the yeast Mgm1 protein (Botelho et al., 2013); the TMS1 of the yeast Cox2 subunit (Supekova et al., 2010); and the TMS1 of soybean Cox2 subunit (Daley et al., 2002a). Based on Table 1, we can predict that TMS with $\mu\Delta G_{app}$ values below 0.54 kcal/mol will probably be arrested and released laterally by TIM23, those with $\mu\Delta G_{app}$ values above 0.62 kcal/mol will probably be translocated, and those with $\mu\Delta G_{app}$ values between 0.54 and 0.62 kcal/mol are in a threshold zone and can be either translocated or arrested like the TMS1 in Mgm1. For simplicity, we developed a "traffic-light" color code for each TMS, intended to graphically illustrate which one may be fully transferred (green light), which ones will be presumably arrested and released laterally (red light), and the ones which are in the threshold zone (yellow-light) (Fig. 3). It must be underscored that besides this "traffic-light" color, the choice of pathway through which each alpha-helix in the TMS will be directed does not depend exclusively on its hydrophobicity, but also on other determinants such as its proline content and the presence/absence of charged residues flanking the TMS. Here, we used the biological scale to estimate the $\mu\Delta G_{app}$ values of all TMS present in the 13 mitochondria encoded OXPHOS proteins (Suppl Table S1) and of all the naturally nucleus encoded, membrane-embedded subunits of complexes I, III, IV and V (Suppl Table S2). All 13 mitochondria encoded OXPHOS proteins were found to contain from 1 to 15 "red light" TMS in their sequence (Fig. 4). This result indicates that if allotopically produced, the transfer into the matrix through TIM23 will be hampered and ultimately impede that the proteins reach their final, functional topology. In sharp contrast, the 34 naturally occurring, nucleus-encoded, membrane-embedded subunits of OXPHOS complexes exhibit mainly "green light" TMS, and few of them "red" or "yellow light" (Suppl Table S2). Altogether, the data suggest that the TMS of the nucleus encoded subunits are on average less hydrophobic than the TMS of their mitochondria encoded counterparts (Fig. 3). Although the traffic-light color is a good predictor of the feasibility of TIM23 to sort a given TMS, it reveals some outliers. Of the six nucleus-encoded proteins exhibiting TMS with "red lights", four consist either of a sole TMS or harbor the TMS exhibiting high hydrophobicity as the last one. Conceivably, such TMS would not pose challenge for their insertion into the membrane. The two remaining proteins, have two predicted arrested TMS indicating that our prediction is not absolute. Alternatively, it is possible that these proteins rely on specialized factors that could facilitate their insertion. As illustrated in Fig. 5, the $\mu\Delta G_{app}$ of each TMS must be considered when designing a

Table 1
$\mu\Delta G_{app}$ values for TMS that are in the threshold of being either translocated by TIM23 or arrested and released laterally.

SOURCE	PROTEIN	SEQUENCE OF TMS1	$\mu\Delta G_{app}$ (kcal/mol)	TRAFFIC LIGHT
YEAST	nu Mgm1 (CAA99426.1)	**IISKIIRLPIYVGGGMAAAGSYIAYKM**	0.61	*TRANSLOCATED 40 %*
YEAST	mt Cox2 (NP_009326.1)	**GILELHDNIMFYLLVILGLVSWMLYTIVMTYS**	**0.51**	**ARRESTED**
YEAST	Allotopic Cox2 (W56R mutant)	**GILELHDNIMFYLLVILGLVSRMLYTIVMTYS**	0.57	*TRANSLOCATED*
SOYBEAN	mt Cox2 (NP_001276209.2)	**MMQGIIDLHHDIFFFLILILVFVSWILVRALWHF**	**0.53**	**ARRESTED**
SOYBEAN	nu Cox2 (YP_007516925.1)	**IMQGIIDLHHDIFFFVIQIGVFVSWVLLRALWHF**	0.63	*TRANSLOCATED*
SOYBEAN	Allotopic Cox2 (L169Q/L171G mutant)	**MMQGIIDLHHDIFFFLIQIGVFVSWILVRALWHF**	0.62	*TRANSLOCATED*

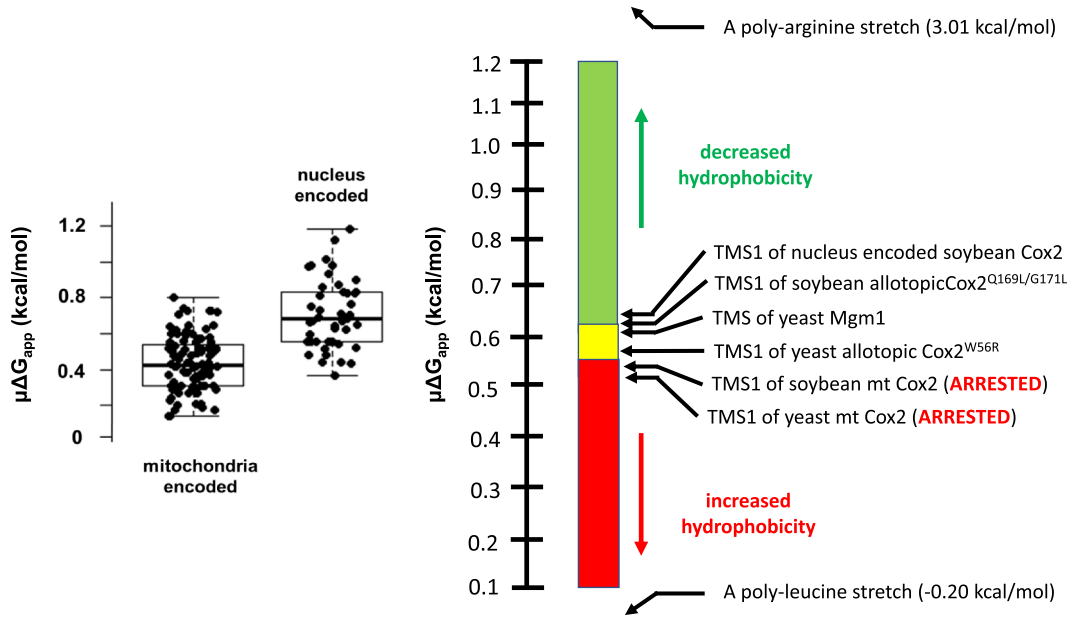

Fig. 3. $\mu\Delta G_{app}$ values for TMS of membrane embedded OXPHOS proteins. The $\mu\Delta G_{app}$ values of various TMS were calculated using the biological scale (Botelho et al., 2011). Boxplots on the left show $\mu\Delta G_{app}$ values for all the TMS of OXPHOS subunits listed on Suppl Table 1 (mitochondria-encoded) and Suppl Table 2 (nucleus-encoded). TMS from mitochondrial encoded subunits exhibit $\mu\Delta G_{app}$ values between 0.14 and 0.80 kcal/mol, with a mean value of 0.44, a median of 0.43 and a mode of 0.31 kcal/mol. In contrast, the TMS from nucleus encoded OXPHOS components tend to be less hydrophobic, exhibiting $\mu\Delta G_{app}$ values between 0.36 and 1.2 kcal/mol, with a mean of 0.70, a median of 0.68 and a mode of 0.55 kcal/mol. Shown on the right is an expanded view of the same biological scale. The upper limit with an $\mu\Delta G_{app}$ value of 3.01 kcal/mol, corresponds to a highly hydrophilic stretch of arginines, whereas the lower limit with an $\mu\Delta G_{app}$ value of −0.20 kcal/mol would represent a highly hydrophobic stretch of leucines. Based on their calculated $\mu\Delta G_{app}$ values, TMS were assigned a "traffic light" color: red, for highly hydrophobic ($\mu\Delta G_{app}$ values below 0.54 kcal/mol), yellow for moderately hydrophobic ($\mu\Delta G_{app}$ values between 0.54 and 0.62 kcal/mol) and green for marginally hydrophobic or highly hydrophilic ($\mu\Delta G_{app}$ values above 0.62 kcal/mol). Arrows indicate $\mu\Delta G_{app}$ values for the single TMS of Mgm1, TMS1 of the wild-type yeast Cox2 subunit, TMS1 of the yeast $Cox2^{W56R}$ subunit variant, TMS1 of the wild-type soybean Cox2 subunits (mitochondria and nucleus encoded) and TMS1 of the soybean $Cox2^{Q169L/G171L}$ subunit variant. Boxplots were built using the R graphics package (version 4.1.0, Vienna, Austria) (R Core Team, 2021).

protein precursor that will be allotopically synthesized.

Our rationale here assumes that the biological scale of Botelho, experimentally determined for yeast mitochondria, also applies to mitochondria from other eukaryotes (note for example, that the $\mu\Delta G_{app}$ values for TMS1 of the wild-type and mutant yeast Cox2 are very similar to the ones of soybean Cox2, as shown in Table 1). Our demonstration that mimicking the naturally occurring mutations that lower the hydrophobicity of soybean Cox2 in its yeast counterpart allows the allotopic expression of Cox2, suggest that $\mu\Delta G_{app}$ extends to mitochondria in other organisms (Rubalcava-Gracia et al. 2019). However, this case may not be generalized, and further experimentation is required considering the variation from organism to organism. For example, the yeast m-AAA protease, that is known to dislocate substrate proteins from the IMM (Korbel et al., 2004), can modulate the threshold hydrophobicity of a given hydrophobic segment being translocated through TIM23. Thus, the pulling force exerted by the m-AAA protease during protein biogenesis may determine whether a hydrophobic segment will form a TMS in the IMM or not (Botelho et al., 2013).

9. Allotopic expression of human OXPHOS related genes

Allotopic expression is considered as one of the promising strategies to develop gene therapies for diseases linked to mtDNA mutations in protein-encoding genes (Zullo, 2001; González-Halphen et al., 2004; DiMauro et al., 2006; Kyriakouli et al., 2008; Scarpelli et al., 2010; Cwerman-Thibault et al., 2010; Tischner and Wenz 2015). To date, all reports of allotopic expression in human cells share two minimal requirements: *i)* the recoding of mitochondrial genes to mirror the genetic code and the codon bias of nuclear genes (Lewis et al., 2020) and *ii)* the addition of a nucleotide sequence encoding an MTS, usually from a precursor protein already known to be targeted to mitochondria (Artika, 2019). In principle, these two modifications should suffice to successfully target, import, and restore OXPHOS activity by any gene expressed allotopically (Gearing and Nagley, 1986; Manfredi et al., 2002; Bonnet et al., 2007; Ojaimi et al., 2002). The choice of an adequate MTS sequence is relatively simple, i.e., the fusion of the MTS of either the beta subunit of ATP synthase (Atp2), the iron-sulfur Rieske protein (ISP) of complex III, or subunit IV of cytochrome *c* oxidase (Cox4) were sufficient to provide access to the allotopically produced bacteriorhodopsin of the archaeon *Halobacterium salinarum* into the mitochondria of the yeast *Schizosaccharomyces pombe* (Hoffmann et al., 1994). In other cases, MTS duplication may increase import efficiency (Galanis et al.,1991; Chin et al., 2018). The addition of untranslated regions, e.g., a 3′-UTR sequence, has been considered as a parameter to enhance allotopic expression (Kaltimbacher et al. 2006). However, an unbiased screening comparing a wide variety of 3′-UTR sequences has shown that their role is not as critical as that of the MTS (Chin et al., 2018).

Here, we underscore the concept that the key constraint for the allotopic expression of a given mitochondrial gene is the hydrophobicity of its encoded protein precursor. There is a large body of evidence suggesting that the high hydrophobic nature of the proteins whose final destination is the IMM hinders the functional, nuclear relocalization of their cognate mitochondrial genes, either by natural gene transfer or by experimental design (Popot & de Vitry 1990; Claros et al. 1995; Claros & Vincens 1996; Adams & Palmer 2003; Ojaimi et al. 2002; Daley et al., 2002; Oca-Cossio et al. 2003; González-Halphen et al. 2004; Figueroa-Martínez et al. 2011; Neupert 2015; Björkholm et al. 2015; Johnston & Williams 2016). As shown in some instances, increasing the $\mu\Delta G_{app}$ (which indicates a decrease of the hydrophobicity) of certain TMS may facilitate the import of proteins into mitochondria (Claros et al., 1995; González-Halphen et al., 2004). Indeed, changing one or two residues in the Cox2 protein was shown to be sufficient to allow its *in vivo* import in yeast (Supekova et al., 2010) or its *in vitro* import into isolated soybean

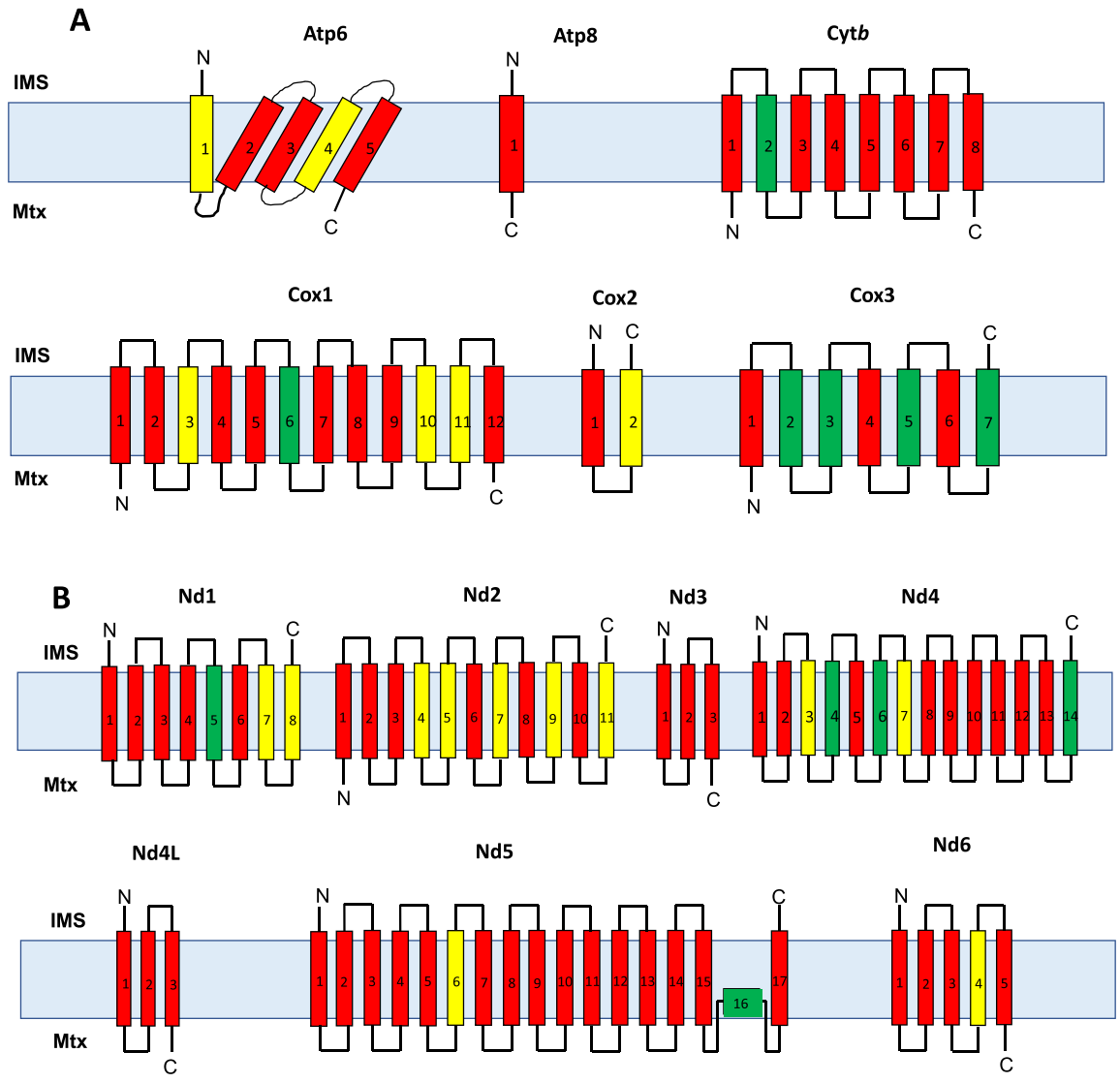

Fig. 4. "Traffic light" color code for all the TMS of the 13 mitochondria encoded OXPHOS subunits. (A) Topological arrangement of the mitochondria-encoded subunits corresponding to complexes III, IV and V, and the "traffic-light" color code assigned to each TMS. (B) Topological arrangement of the mitochondria-encoded subunits of complex I with TMS colored accordingly. Red TMS are predicted to be arrested and released into the IMM by TIM23^{SORT}. The $\mu\Delta G_{app}$ values used to assign the "traffic-light" colors (Fig. 3) were taken from Table S1. The mitochondrial inner membrane is represented as a light blue rectangle. IMS: intermembrane space; Mtx: matrix. N and C termini of each represented protein are indicated.

mitochondria (Daley et al., 2002a). Reports of allotopic expression of genes encoding OXPHOS components have been listed in a recent comprehensive review (Saravanan et al., 2022) and are critically discussed here.

9.1. *The atp6 gene*

More than 12 pathogenic mutations identified in the *atp6* gene have been implicated in several mitochondrial diseases, including LHON, Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP), Parkinson's disease, multiple sclerosis (MS), and systemic lupus erythematosus (SLE) (Ganetzky et al, 2019). Moreover, the protein product of *atp6* is part of the proton channel of complex V and is thus essential for ATP synthesis. Hence, several efforts have been directed to accomplish the allotopic expression of this gene (Kucharczyk et al., 2019). However, the highly hydrophobic nature of Atp6 makes its allotopic production challenging. The proposed mechanism for the internalization into mitochondria of a cytosol synthesized Atp6 subunit (N_{out}-C_{in}) implies the complete translocation of five TMS (Fig. 4) followed by the insertion of two hairpins and an additional TMS into the IMM, probably mediated by the Oxa1 insertase (Suppl Fig. 7).

Allotopic expression of the *atp6* gene in human cells was described in several reports (Manfredi et al., 2002; Zullo et al., 2005; Kaltimbacher et al., 2006; Bonnet et al., 2007). The *atp6* gene was re-designed for nuclear expression, and several MTS were tested, such as the one from Cox8, a nucleus-encoded subunit of complex IV, or from the P1 isoform of subunit *c* of ATP synthase (Manfredi et al., 2002; Oca-Cossio et al., 2003). In other constructs, the MTS of superoxide dismutase 2 (SOD2) was used, along with the addition of the 3′-UTR from the same gene inserted at the end of the transcriptional unit (Kaltimbacher et al., 2006; Bonnet et al., 2007). In these reports, the presence of the Atp6 subunit inside mitochondria was detected by immunofluorescence or immunodetection of a FLAG epitope engineered in the allotopic construct. However, no direct evidence for the allotopically produced subunit assembling into complex V was provided.

Restoration of ATPase activity was reported in cells transformed with the *atp6* gene from human and from the green alga *C. reinhardtii* (Ojaimi et al., 2002) using transiently transfected HEK293T cells, and JCP213 or

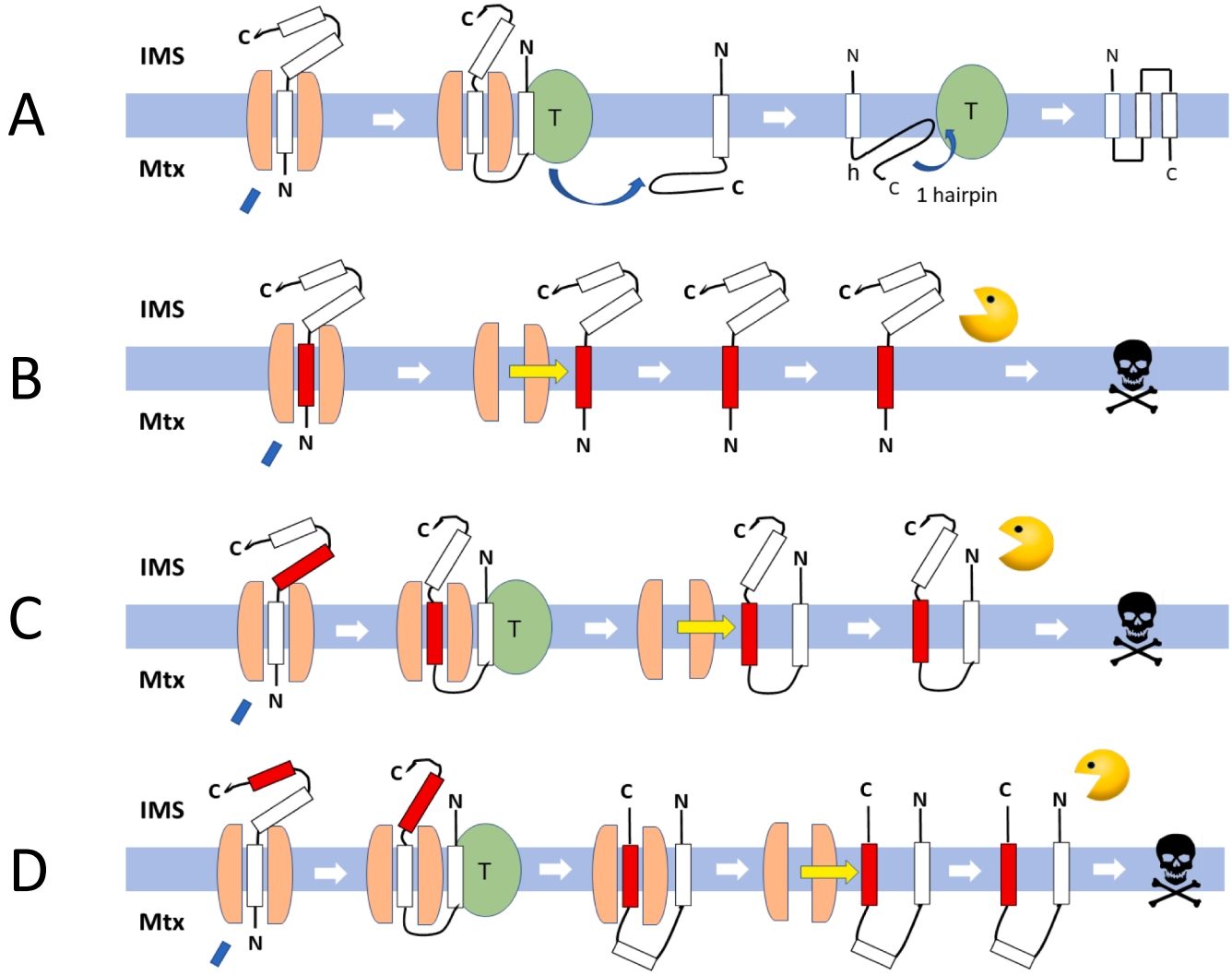

Fig. 5. When things go wrong with allotopic expression and protein import. (A) Diagram representing the import by TIM23 of a cytosol-synthesized membrane protein containing three TMS (that could correspond to Nd3 or Nd4L subunits). For the allotopic protein to adopt a topology identical to the one reached by its mitochondria encoded counterpart, the three TMS must be initially fully translocated into the matrix. If the first (B), second (C) or third TMS (D) is highly hydrophobic (red), TIM23 will laterally release the corresponding alpha-helix into the IMM, and the protein will acquire an anomalous topology. Proteins with an improper folding in the bilayer are recognized by mitochondrial proteases and degraded. The higher the number of red traffic light TMS in a protein, the greater the probability that it will be folded into an inappropriate conformation. The mitochondrial inner membrane is represented as a light blue rectangle. IMS: intermembrane space; Mtx: matrix. N and C termini of each represented protein are indicated.

JCP216 cybrids for stable transfection. The Atp6 subunits were immunodetected as part of a high molecular weight species that was interpreted to be complex V following blue native electrophoresis (BN-PAGE). However, independent experiments by other investigators carried out with the same human and *C. reinhardtii atp6* constructs, showed that both subunits failed to integrate into ATP synthase, and aggregated instead into a high molecular weight species (Bokori-Brown and Holt, 2006). Also, in this report, human and algal constructs were optimized for import into mitochondria of human cell lines (i.e., HEK293T for transient transfection, and JCP2116 cybrid and human osteosarcoma cell line 143B for stable transfection) using the human and the algal MTS of the beta subunit from ATP synthase.

In an independent approach, the human *atp6* gene was engineered as a chimeric human-*C. reinhardtii* construct including the algal MTS and several amino acid substitutions in the TMS to mimic the algal sequence. This chimeric construct was expressed in homoplasmic human mutant cells carrying the T8993G mutation in the mitochondrial *atp6* gene (Figueroa-Martínez et al., 2011). Although the Atp6 subunit chimera was successfully internalized into mitochondria as judged by fluorescence microscopy, the construct failed to increase ATP synthesis.

Zullo et al. (2005) reported that the Atp6 subunit from Chinese Hamster Ovary (CHO) cells could be imported *in vitro* into isolated yeast mitochondria. The construct used in this work was modified to enable the usage of the nuclear genetic code and included a sequence coding the MTS of ornithine transcarbamylase, which was known to direct the import of dihydrofolate reductase (Horwich et al.,1985). A mature form of Atp6 was assumed to be internalized into mitochondria because it exhibited resistance to protease treatments. Nonetheless, these authors did not provide evidence that mitochondrial import was dependent on the electrochemical gradient, i.e., that it could be suppressed by uncouplers or OXPHOS inhibitors; and proof that the allotopic Atp6 was incorporated into Complex V was not given.

The pathogenic human T8993G mutation in *atp6* (NARP mutation) was used as a case study with transgenic mice (Dunn and Pinkert, 2012; Dunn and Pinkert, 2021). Two constructs were designed, one carrying the human EF1α promoter plus sequences encoding the MTS of human Cox8 followed by either the human wild-type *atp6* gene or the one carrying the T8993G mutation, plus a region encoding a C-terminal myc

epitope tag. Transgenic mice expressing either construct were subjected to several neuromuscular and motor tests that yielded mixed results. Mutant mice exhibited motor deficiencies like those observed in NARP patients in some tests, but enhanced performance in others. Biochemical assays to assess the restoration of OXPHOS included quantification of serum lactate concentrations, mitochondrial Manganese superoxide dismutase (MnSOD) levels, ATP synthesis rate, and oxygen uptake rates. Mice from both transgenic lines did not differ substantially from non-transgenic controls, except for lower lactate concentrations in the mice expressing wild-type human *atp6*. Although the presence of the allotopic proteins in mitochondria was assayed by immunostaining striatum sections with gold particles, the delivery of proteins to mitochondria was not quantified, and no evidence was provided that the labeled, allotopically produced Atp6 subunits were assembled into the ATP synthase complex. In these systems, the mitochondrial Atp6 subunit is still efficiently synthesized inside mitochondria, competing against incoming allotopic proteins from the cytosol.

An automated, quantitative, and unbiased screening platform was set up to evaluate protein localization and mitochondrial morphology. This approach entailed generating 124 plasmids with different combinations of 31 MTS and 15 3′-UTRs that were assayed for their ability to localize OXPHOS subunits Atp6, Atp8, Cox2, Cox3, Nd1, Nd3, Nd4 and Nd6 to the mitochondrion (Chin et al., 2018). While the different 3′-UTR sequences did not significantly alter the localization of these proteins, distinct MTS exhibited a superior capability to direct these proteins into mitochondria. In particular, the MTS of maize 4-hydroxybenzoate polyprenyltransferase (encoded by the LOC100282174 gene), either by itself or combined in tandem with another MTS, yielded the highest localization for the allotopically expressed proteins. Chemically modified mRNAs with enhanced stability encoding an optimized allotopic expression construct for *atp6* carrying the MTS of LOC100282174 could functionally rescue a cell line harboring the T8993G point mutation in the mitochondrial *atp6* gene. Although no biochemical evidence showing the presence of the allotopic Atp6 subunit in ATP synthase was provided, expression of the construct in transiently transfected HeLa cells restored respiratory function and ATP synthase functionality. The report by Chin et al. highlights the importance of MTS selection in designing constructs for the allotopic expression of OXPHOS-related genes.

9.2. The atp8 gene

The mitochondrial *atp8* gene encodes subunit Atp8 (also known as subunit A6L in the bovine enzyme), a small hydrophobic polypeptide of 48 amino acids with a single TMS (Fig. 4) that is an essential component of the F_0 sector of the mitochondrial F_1F_0-ATP synthase (Hong and Pedersen, 2004). The group of Philip Nagley in Australia led pioneering efforts in the field achieving the allotopic expression of the yeast *atp8* gene, after optimizing its codon usage for nuclear expression. These experiments made use of two different MTS-encoding sequences, one from the fungus *Neurospora crassa* Atp9 subunit (NcAtp9) and the other from the yeast Cox6 subunit. Whereas the MTS of NcAtp9 (66 residues) promoted the import of Atp8, the MTS of subunit Cox6 did not (Gearing and Nagley, 1986). Furthermore, when the MTS of the NcAtp9 subunit was duplicated in tandem, the import efficiency of the yeast Atp8 subunit was notably increased (Galanis et al., 1991). The successful import into mitochondria of the Atp8 subunit was corroborated by direct sequencing of radiolabeled methionine residues in the imported protein. In another study, the role of the three positively charged residues at the C-terminus of Atp8 was explored using a series of truncated variants, in which the most distal K47 residue was found to be dispensable for Atp8 importability (Nero et al., 1990). This time, the structural and functional assembly of Atp8 and its truncated K47 variant into complex V was demonstrated by its ability to restore respiratory growth to an *atp8*-null yeast strains and by the immunochemical detection of the produced proteins (Grasso et al, 1991; Nagley et al., 1988; Law et al. 1990). Moreover, expression of the *atp8* gene in the nucleus of yeast *atp8* null mutants restored respiration to levels like that of the wild-type strain (Roucou et al., 1999). Altogether, these results show the successful allotopic expression of the yeast *atp8* gene.

The human mitochondrial *atp8* gene was optimized for nuclear codon usage and expressed allotopically in human cells (Oca-Cossio et al., 2003). For this purpose, a recombinant sequence harboring the Atp8 subunit encoding sequence appended with the sequences corresponding to the MTS of Cox8 and a hemagglutinin (HA) C-terminal tag was designed. Although the incorporation of Atp8 into mitochondrial ATP synthase was not examined, the subunit was successfully targeted to mitochondria after transfection of the COS and HeLa cells with the engineered construct, as evidenced by the colocalization of Mitotracker fluorescence and the HA tag detected via immunofluorescence.

In a separate report, all 13 human mitochondrial OXPHOS proteins with sequences carrying the strong MTS of Cox8 were synthesized in the cytosol of human HeLa cells when produced from recoded gene constructs. A FLAG tag sequence at the 3′-terminal end of each gene construct was introduced to enable localization of the synthesized proteins by immunofluorescence confocal microscopy (Björkholm et al., 2017). Only Atp8 was successfully internalized into mitochondria, while the other 12 OXPHOS proteins were all found in the ER, indicating that subcellular protein mistargeting was the reason for lack of functional allotopic expression.

Furthermore, the mouse Atp8 recoded and bearing the MTS of the mouse ATP6G1 subunit and a Myc-FLAG epitope at the C-terminus was produced. A doxycycline dependent induction system was used in C57/BL6$^{(mtFVB)}$ mouse fibroblast cybrids bearing the G7778T mutation in the mitochondrial *atp8* gene. BN-PAGE showed that the allotopically-expressed Atp8 subunit integrated into Complex V and increased the viability of the cybrids (Lewis et al., 2020).

Boominathan et al. (2016) used an homoplasmic cell line harboring the G8529A mutation in the overlap region of the *atp8* and *atp6* genes. This mutation causes premature truncation of the Atp8 subunit but also reduced levels of Atp6 and yields complex V deficiency. Mutant cells were transfected with constructs enabling the synthesis of either Atp8 or Atp6 subunits carrying the MTS of human ATP6G1 fused to a FLAG-tag at their C-termini. Transfections were also carried out with a modified construct containing both genes (*atp8* and *atp6*) in tandem. While the allotopic expression of either the *atp6* or the *atp8* gene had negligible effects, the simultaneous expression of both genes partially recovered complex V assembly and around 55 % of its enzymatic activity.

Overall, there is a large body of experimental evidence indicating that Atp8 subunit has been allotopically expressed successfully in various systems. Since the Atp8 subunit has a single TMS (N_{out}-C_{in}) (Fig. 1), the proposed mechanism for the internalization into mitochondria is relatively simple, involving the complete translocation of this TMS into the matrix followed by its insertion into the IMM, allowing the protein to expose its N-terminus to the IMS.

9.3. The cox1 gene

Although the *cox1* gene, which encodes subunit I of cytochrome *c* oxidase (with 12 TMS) is invariably found in mtDNA, several claims of successful allotopic production have been made. The bovine *cox1* gene was modified to encode Cox1 carrying the MTS of the nucleus-encoded subunit Cox4 and an hexahistidine (6xHis) tag at the C-terminus. The genes encoding wild type and variant versions (D51N) of Cox1 were cloned in a mammalian expression vector under the control of the human cytomegalovirus promoter, which was then used to transfect HeLa cells to obtain stable transfectants. The presence of the Cox1 subunit in complex IV was demonstrated by immunodetection, asserting that both wild-type and mutant subunits formed hybrid enzymes along with the other 12 human subunits of cytochrome *c* oxidase in the transfected HeLa cells (Tsukihara et al., 2003; Shimokata et al., 2007). It is unlikely that the hydrophobicity barrier was overcome by the

allotopically produced Cox1 subunit (N_{in}-C_{in}) (Fig. 1) in these experiments, since the mechanism by which this cytosol synthesized subunit is imported into mitochondria must involve the entire translocation of twelve TMS by TIM23 followed by the insertion of six hairpins by Oxa1 (Suppl Fig. 8). Allotopic expression would probably require reducing the hydrophobicity of at least eight "red light" TMS (Fig. 4). Finally, as part of another approach, the human *cox1* gene, including both the wild-type and the mutant version W494R responsible for a mitochondrial disease, were allotopically expressed in HEK293T cells. Even though the allotopically produced wild-type and variant Cox1 subunits were reported to be located to mitochondria, the allotopically produced proteins appeared to aggregate, and no evidence for their functional integration into complex IV was obtained (Singh et al., 2019).

9.4. The cox2 gene

As discussed above (see section 7.4), the *cox2* gene has been naturally transferred to the nucleus in several legumes, i.e., soybean contains both mitochondria-encoded and nucleus-encoded versions of the Cox2 subunit. In soybean, the nucleus encoded Cox2 precursor exhibits a long MTS of 136 residues, which is required for internalizing the protein into mitochondria. Comparison of the sequences of mitochondria and nucleus encoded Cox2 sequences unveiled significant differences in residues present in the first TMS of the protein. With an MTS and two-point mutations (L169Q and L171G) that together lower the hydrophobicity of the first TMS, the mitochondrial version of the Cox2 protein could be imported into isolated soybean mitochondria (Daley et al., 2002a). This result was a proof-of-concept that hydrophobicity of TMS1 is a key parameter for the allotopic expression of mitochondrial OXPHOS subunits. This is further substantiated by the fact that allotopically produced Cox2 subunit successfully complemented a respiration deficient yeast *cox2*-null mutant when tryptophan was replaced with arginine at position 56 in the first TMS of the protein (Supekova et al., 2010). The W56R mutation was isolated following PCR-prone mutagenesis of a recoded *cox2* gene encoding an MTS from either Atp9 or Oxa1 and shown to restore respiratory growth of a *cox2*-null strain. Also, the W56R mutation increases the $\mu\Delta G_{app}$ of the first TMS of $COX2^{W56R}$, thereby allowing its import into mitochondria and its assembly into complex IV (Fig. 3). Subsequent experiments have shown that other variants of the Cox2 yeast subunit like $Cox2^{W56K}$ and $Cox2^{W56Q}$ are also imported into mitochondria, albeit less efficiently than $Cox2^{W56R}$ (Rubalcava-Gracia et al., 2018). It is assumed that the higher $\mu\Delta G_{app}$ of $COX2^{W56R}$ TMS1 enables its transfer into the mitochondrial matrix by the TIM23 translocon, while $COX2^{W56R}$ TMS2, exhibiting a lower $\mu\Delta G_{app}$, will be retained by TIM23 and released laterally into the IMM (Qualmann et al., 2003; Rubalcava-Gracia et al., 2018) (Suppl Fig. 9). Complementing the *Δcox2* mutant with a cytosol synthesized $Cox2^{W56R}$ (N_{out}-C_{out}) only yields partial recovery of the respiratory proficiency (Supekova et al., 2010). This partial complementation could be due to the lower activity of cytochrome *c* oxidase carrying the W56R mutation or reduced steady-state accumulation levels of this enzyme. The specific enzymatic activity of the purified complex IV from both the wild-type strain and the $Cox2^{W56R}$ mutant was almost equivalent, suggesting that the W56R mutation does not affect the intrinsic complex IV activity. However, in-gel activity and spectroscopic quantification revealed that only 40–60 % of cytochrome *c* oxidase accumulates in the strain producing the $Cox2^{W56R}$ protein (Cruz-Torres et al., 2012). This suggests that the W56R mutation affects complex IV biogenesis, leading to lower steady state levels of the enzyme.

In yeast, defects in mitochondrial Cox2 synthesis impact Cox1 synthesis (Naithani et al., 2003; Barrientos et al., 2004; Pérez-Martinez et al., 2009; García-Villegas et al., 2017), but the allotopically expressed $Cox2^{W56R}$ precursor subunit can restore the mitochondrial synthesis of Cox1 in a *Δcox2* mutant to wild-type levels. In addition, a strain synthesizing $Cox2^{W56R}$ from a mitochondrial *cox2W56R* gene which followed a canonical biogenesis of subunit Cox2, exhibited normal levels of functional cytochrome *c* oxidase (Rubalcava-Gracia et al., 2018). This suggests that the W56R substitution does not affect complex IV activity. Instead, the import of the $Cox2^{W56R}$ precursor through TIM23 could be the limiting step of complex IV biogenesis. Furthermore, when both the cytosolic $Cox2^{W56R}$ and the mitochondrial Cox2 were co-expressed, each protein assembled into complex IV independently from its genetic origin, resulting in a mixed population of this complex, most of which contained the mitochondria-synthesized version (Rubalcava-Gracia et al., 2018).

9.5. The cox3 gene

The human *cox3* gene was re-designed to facilitate the mitochondrial import of its gene product by emulating the structural properties of the corresponding nucleus-encoded algal proteins from *C. reinhardtii.* The algal MTS from Cox3 was used, including multiple algal-like amino acid substitutions to reduce TMS hydrophobicity. The resulting human-algal chimeric *cox3* gene was expressed in a CHO cell line carrying an homoplasmic 15 bp deletion in the mitochondrial gene (Figueroa-Martínez et al., 2011). The chimeric Cox3 protein re-designed for nuclear expression was targeted to the mitochondria but failed to integrate into complex IV. To our knowledge, no further attempts to achieve allotopic expression of the *cox3* gene have been described. The predicted insert pathway of an allotopic Cox3 subunit (N_{in}-C_{out})(Fig. 1) requires the full translocation of six TMS (Fig. 4) by TIM23 (three of which are "red light") followed by the lateral release of TMS7 into the IMM and insertion of three hairpins into the IMM.

9.6. The cytb gene

Cytochrome *b* of complex III, a subunit with six TMS (Fig. 4), is invariably encoded in the mitochondrial genome. Several attempts to engineer the *cytb* gene for allotopic expression have been documented. In a pioneering study, hybrid proteins containing different combinations of the cytochrome *b* transmembrane domains fused to both the MTS of the *N. crassa* Atp9 subunit at the N-terminus and a cytoplasmic version of the bI4 RNA maturase at the C-terminus were produced. The bI4 RNA maturase was used as a reporter to assess the efficiency of mitochondrial import of the different combination of TMS. All TMS from Cyt*b*, either alone or in combination, were found to be internalized into mitochondria, but no more than four TMS could be imported at once (Claros et al., 1995), an indication that overall hydrophobicity was a constraint. It was proposed that the entire protein hydrophobicity and the local TMS hydrophobicity were the limiting factors for Cyt*b* import into mitochondria (Lewis et al., 2020).

In other experiments, different MTS (from the Cox8 subunit, P1 isoform of subunit *c* of ATP synthase, and Nicotinamide Nucleotide Transhydrogenase) were attached to the full-length cytochrome *b* precursor to target the protein to the mitochondria. The resulting allotopically synthesized cytochromes *b* were found to aggregate in fiber-like structures in the periphery of mitochondria, indicating that hydrophobicity was a physical impediment for internalization into the organelle (Oca-Cossio et al., 2003). The case of cytochrome *b* illustrates the difficulties in achieving the correct import and functional integration of a highly hydrophobic membrane protein into its appropriate mitochondrial complex. Indeed, the proposed theoretical mechanism for the import of a cytosol synthesized Cyt*b* subunit (N_{in}-C_{in}) (Fig. 1) involves the complete translocation of its eight TMS (Fig. 4) into the mitochondrial matrix by TIM23, seven of which are "red-light", followed by the insertion of four hairpins by Oxa1.

9.7. The nd1 gene

The human *nd1* gene, encoding subunit Nd1 of complex I (8 TMS), was claimed to be allotopically produced (Bonnet et al., 2008). A construct containing the *nd1* gene with an appended sequence encoding

the MTS from Cox10 protein was used to stably transfect cultured skin fibroblasts from LHON patients (carrying the mutation G3460A). The transfected fibroblasts were restored for growth on galactose, complex I activity, and the rate of ATP synthesis.

A similar construct with additional 3′-UTR from the *COX10* gene was used to transform 43B osteosarcoma-derived cybrids harboring a mostly homoplasmic mutation in the *nd1* gene. Immunoblotting analysis revealed the presence of the Nd1 subunit in transfected cells that also exhibited partial recovery of bioenergetic competence (Calabrese et al., 2013; Iommarini et al., 2018). Nonetheless, since only 8 % of the ATP synthesis was recovered, this suggests that only a small proportion of the imported subunit reaches its final compartment or is functionally incorporated into complex I.

In a recent study the *nd1* gene was engineered for codon optimization to obtain a robust and efficient expression (Lewis et al., 2020). A homoplasmic cybrid cell line containing the mutation m.3571insC which is an insertion of an additional cytosine resulting in a premature stop codon, truncation of the Nd1 subunit, and failure of complex I to assemble, was used as recipient cell line. This recipient cell-line was used for the allotopic production of a FLAG-tagged human ND1 protein fused to the MTS of human ATP5G1 subunit. Allotopically produced Nd1 was immunodetected using anti-FLAG antibodies in a high molecular weight complex following BN-PAGE. However, the integration efficiency of the allotopic Nd1 subunit was suboptimal and insufficient to restore complex I activity to wild-type levels.

The proposed mechanism for a cytosol-synthesized Nd1 subunit (N_{out}-C_{out}) (Fig. 1) implies translocation of the protein first TMS followed by its insertion into the IMM, such that the N-terminus is exposed to the IMS. After that, full translocation of the seven remaining TMS through TIM23 must occur. Finally, three hairpins will be inserted into the IMM, followed by the insertion of TMS8 (Suppl Fig. 10).

9.8. The nd2 gene

Despite the lack of reports describing the allotopic expression of the *nd2* gene, the proposed mechanism for the Nd2 subunit (N_{in}-C_{out}, 11 TMS, Fig. 4) internalization involves the full translocation of ten TMS (six "red-light", Fig. 4) and the lateral release of TMS11 into the IMM, followed by the insertion of five hairpins.

9.9. The nd3 and nd4L genes

Although no claims for the allotopic expression of the *nd3* and *nd4L* genes have been put forth, the theoretical insertion pathway of Nad3 and Nad4L (N_{out}-C_{in}, each one carrying three "red-light" TMS, Fig. 4) involves the full translocation of the three TMS, followed by the insertion of TMS1 and 1 hairpin into the IMM.

9.10. The nd4 gene

The *nd4* gene encoding the membrane-bound subunit Nd4 of complex I has been the subject of efforts to achieve its allotopic expression, mostly because a point mutation in the *nd4* gene (G11778A) is responsible for about 70 % of LHON cases (Manickam et al., 2017).

Pioneering attempts involved expressing constructs encoding the Nd4 subunit fused to the MTS of the Cox8 subunit and tagged either with a HA or a green fluorescent protein (GFP) in COS-7 or HeLa cells. As a result, the Nd4 protein (14 TMS) aggregated, forming fiber-like structures around mitochondria. Similar results were reported when C-terminal truncated forms of the Nd4 subunit (with only 6 or 8 TMS) were allotopically produced (Oca-Cossio et al., 2003). In contrast, successful allotopic expression in human cells has been reported for the *nd4* gene (Bonnet et al., 2007; Bonnet et al., 2008; Guy et al., 2002; Ellouze et al., 2008). In a set of experiments, the recoded *nd4* gene attached to a sequence encoding the MTS of the P1 isoform of subunit c of human ATP synthase (Atp9) and a sequence encoding a short FLAG epitope tag for import detection was used. Complementation of G11778A cybrids with this construct showed a threefold increase in ATP synthesis (Guy et al., 2002). In other independent constructs, the MTS from either mitochondrial SOD2 or COX10 were added to the Nd4 subunit. Immunodetection of the FLAG epitope confirmed the presence of the Nd4 subunit inside mitochondria, but no direct evidence for the allotopically produced subunit assembling into complex I was provided (Bonnet et al., 2007). Also, a human variant Nd4 subunit was expressed allotopically in a murine model. Constructs encoding a synthetic wildtype human Nd4 subunit and a variant Nd4 subunit corresponding to the LHON R340H mutation, carrying the MTS of the Atp9 subunit, were delivered to the mouse visual system. The effects of the variant Nd4 subunit on the optic nerve were assessed by electron microscopy, magnetic resonance imaging, and immunohistochemistry. While the variant Nd4 subunit disrupted mitochondrial architecture and elevated reactive oxygen species, severely affecting the retina ganglion cells, the wild-type Nd4 subunit had no apparent deleterious effects (Qi et al., 2007). Subsequent works used the nuclear-encoded human Nd4 subunit fused to the P1 isoform of subunit c (Atp9) of ATP synthase MTS and FLAG epitope with a self-complementary adeno-associated virus to induce an efficient synthesis of the human Nd4 subunit in the mouse visual system, although no evidence for integration of Nd4 into complex I was provided (Koilkonda et al., 2010).

More recently, efficient delivery of the Nd4 subunit into mitochondria was claimed after ocular administration of a recombinant adeno-associated viral vector containing the human *nd4* gene sequence to adult rats (Cwerman-Thibault et al., 2015). The construct expressed the Nd4 subunit attached to the MTS of the Cox10 subunit and a triple HA tag. An electrophoretically resolved species of about 54 kDa, present in treated retinas but not in the untreated, was identified using an anti-HA antibody. This species was assumed to be the Nd4 subunit (51.7 kDa) fused to a 27-residue segment corresponding to three contiguous HA1 epitopes (about 3 kDa). However, in preparations of complex I from bovine heart mitochondria, Nd4, which is a highly hydrophobic subunit, migrates in SDS-PAGE with a substantial lower apparent molecular mass of about 39 kDa, due to its high hydrophobicity (Fearnley et al., 1991). The discrepancy in apparent molecular masses deserves further experimentation to establish unambiguously if the observed human 51.7 kDa band indeed corresponds to the allotopically-produced Nd4.

It has been claimed that sustained expression of the human *nd4* gene does not lead to harmful effects and that the human Nd4 subunit is efficiently imported into mitochondria and assembled into complex I (Vignal et al., 2018; Vignal-Clermont et al., 2021). Furthermore, in this experimental model of LHON retinal ganglion cell degeneration was prevented, while visual function and complex I activity in optic nerves were preserved. Surprisingly, m.11778G > A LHON patients treated with rAAV2/2-ND4 exhibited an improvement in visual acuity for more than 4 years after vision loss (Newman et al., 2021; 2023). In this regard, GenSight Biologics (Paris/New York) is currently conducting clinical trials using Nd4 in an allotopic expression strategy to treat LHON patients. Since the proposed mechanism for import of the Nd4 subunit (N_{out}-C_{out}) into mitochondria involves the full translocation of 14 TMS (nine of which are "red light"), followed by insertion of TMS1 and 6 hairpins into the IMM, probably mediated by Oxa1, such results in patients are unexpected, and unambiguous proof for successful allotopic production of the subunit must be provided.

9.11. The nd5 gene

To date, the allotopic expression of the *nad5* gene has not been reported. The proposed theoretical mechanism for the import into mitochondria and insertion into the IMM of an allotopic Nd5 subunit (N_{out}-C_{out}) (Fig. 1) involves the entire translocation of 17 TMS (15 of which are "red-light", Fig. 4), the insertion of TMS1 and 7 hairpins, followed by the insertion of TMS16 and the translocation of TMS17.

9.12. The nd6 gene

The mouse mitochondrial *nd6* gene was optimized for nuclear expression and sequences encoding the MTS from Cox8, and an HA epitope were integrated in a construct designed for allotopic production. Transfection of a mice KO mutant line exhibiting a cytosine 13887deletion in mtDNA with the construct showed that the Nd6 subunit was mainly located outside of mitochondria, although complex I activity seemed to be restored. Further experimentation showed that the selected clones were revertants for the mitochondrial *nd6* mutation (Perales-Clemente et al., 2010). No further reports claiming the allotopic expression of the *nd6* gene are currently available. The proposed mechanism for the import of a cytosol synthesized Nd6 (N_{out}-C_{in}) (Fig. 1) involves the full translocation of 5 TMS (4 of which are "red-light", Fig. 4) and the insertion of TMS1 and two hairpins.

10. Allotopic expression of the atp9 gene, a gene not present in human mtDNA

Although the *atp9* gene (encoding *c* subunit, a constituent of ATP synthase rotary *c*-ring) is absent from the mtDNA of mammals, we will briefly discuss its allotopic expression in yeast as the results are relevant here. In early experiments, the yeast *atp9* gene recoded for nuclear expression fused to a sequence encoding the MTS of the *atp9* gene from *N. crassa* was tested (Law et al., 1988). The resulting fusion protein was imported in purified yeast mitochondria and adequately processed. The import of yeast Atp9 was less efficient than the import observed for the yeast Atp8 subunit attached to the same MTS in parallel import experiments (Farrell et al., 1988). Furthermore, duplication in tandem of *N. crassa* Atp9 MTS notably increased its import efficiency (Galanis et al., 1991).

More recently, Bietenhader et al. (2012), developed a strategy that involved relocating the mitochondrial *atp9* gene of *S. cerevisiae* by using the version of *atp9* from the fungus *Podospora anserina*, which naturally carries the *ATP9* gene in its nuclear genome. A notable difference between *P. anserina* and yeast Atp9 proteins is the reduced hydrophobicity of TMS1 in the former, as indicated both by hydropathy plots and its diminished resistance to extraction by detergents. *In vivo*, the *P. anserina* Atp9 subunit was successfully imported into mitochondria and restored the respiratory function of a yeast *atp9*-null strain, restoring fully functional OXPHOS. Nevertheless, these experiments also illustrated other limitations of allotopic expression: the production of Podospora Atp9 in yeast perturbed cellular morphology and activated the heat shock response, as revealed by transcription profiling.

11. Discussion

While several allotopic expressions of mitochondrial genes in human or mammalian cell lines have been reported (Manfredi et al., 2002, Ojaimi et al., 2002; Guy et al., 2002), some of the initially described reports could not be reproduced by other investigators (Oca-Cossio et al., 2003; Bokori-Brown and Holt, 2006). In some of these studies, the apparent improvements in OXPHOS performance may not have resulted from allotopic expression. This is due to the inherent difficulties of working with cultures in which cells with apparently identical genotypes can exhibit clone-to-clone variability (King and Attardi, 1989). In one case, the apparent complementation of a mtDNA mutation by an allotopically produced subunit of ATP synthase was due to a reversion of the original mutation in the cell line used for the experiment (Perales-Clemente et al., 2010). In addition, experimentalists must be mindful of the fact that nonmitochondrial tags could hinder mitochondrial proteins from reaching their correct topology (Sickmann et al., 2003). In our view, the publication of negative data has been instrumental in advancing the field since it has allowed researchers to avoid repeating unsuccessful experimental approaches.

Here, we discussed the factors that limit the import of cytosol-synthesized polytopic membrane proteins impacting the allotopic expression of OXPHOS-related genes, underscoring the importance of the $\mu\Delta G_{app}$ of each TMS. Synthesis of membrane proteins inside mitochondria and their incorporation into the IMM is undoubtedly, on the surface, less complicated than that of their cytosol-synthesized counterparts. The latter follow a multi-step and seemingly more complex biogenesis: import through the TOM/TIM23 translocators, protease-dependent maturation, incorporation into the IMM, and assembly into a multi-subunit complex. When the original mitochondrial gene is still functional, allotopically expressed proteins must outcompete, at least to a functional threshold, the defective endogenous proteins which are continuously synthesized inside mitochondria and that follow their canonical biogenesis pathway. Therefore, complete recovery of OXPHOS after allotopic expression, as reported for the Atp6 and Nad4 subunits (Bonnet et al., 2007,2008), is not necessarily expected, since it implies that all ATP synthase or complex I enzymes preferably incorporate allotopically produced subunits, entirely replacing all endogenous, mutant ones. More likely, a mixed population of OXPHOS complexes coexists, some carrying the allotopically-produced protein and others bearing the mitochondria-synthesized mutant subunit. Indeed, when mtDNA-encoded and allotopically expressed Cox2 proteins were coexpressed in yeast, each protein could assemble into a complex, resulting in a mixed population of complex IV, with most complexes containing the mtDNA-encoded version (Rubalcava-Gracia et al., 2018). In the case of biological models with null mutations (or equivalent to null) in their mtDNA, this competition problem is circumvented.

Another challenge in allotopic expression is selecting appropriate promoters and genome loci to control protein levels. High doses of allotopically expressed proteins could be counterproductive, since they may aggregate outside the organelle or clog the mitochondrial import machinery (Boos et al., 2019; Rubalcava-Gracia et al., 2019).

As suggested before, claims of allotopic production of respiratory chain components should be ideally supported by biochemical evidence showing that imported proteins were fully integrated and functionally assembled into their appropriate complex (Kyriakouli et al., 2008; Perales-Clemente et al., 2010), especially for membrane proteins with two or more TMS. Demonstration should be unambiguous and include multiple experimental approaches such as immunodetection, regain of function, and if possible, biochemical purification and identification of the allotopic subunit by mass spectrometry. This is of utmost importance, especially in instances where allotopic expression approaches are used in human clinical trials to treat mitochondrial diseases (Lam et al., 2010,2022; Newman et al., 2023). It is our opinion that in non-human species, only three reports provide unequivocal functional and biochemical evidence of incorporation of allotopically expressed OXPHOS proteins into their respective complexes, namely Atp8, Atp9, and Cox2 in yeast (Law et al., 1990; Supekova et al., 2010; Bietenhader et al., 2012). These three proteins share some crucial features: *(i)* they are membrane proteins with just one or two TMS, *(ii)* they are products of genes for which examples of naturally re-localized gene versions to the nuclear genome exist in several species, and *(iii)* the allotopic expression was carried out in a mutant organism where the original mitochondrial gene was inactivated entirely, thus avoiding competition between the assembly of the nucleus-encoded version and its mitochondrial counterpart. As for humans, several lines of evidence suggest a successful allotopic expression for the Atp8 subunit (Oca-Cossio et al., 2003; Björkholm et al., 2017; Lewis et al., 2020). In addition, a report describing the expression of an Atp6 construct in cultured mutant HeLa cells carrying the T8993G mutation, showed an evident partial restoration of coupled respiration, which strongly suggests that the allotopic Atp6 subunit was successfully integrated into complex V (Chin et al., 2018).

From a large scale bioinformatic analysis using 150,000 organellar DNA sequences and over 300 whole nuclear genome sequences (Giannakis et al., 2022), four universal features that seem to determine gene retention in organelles were postulated: *(i)* hydrophobicity of the

organelle-encoded protein; *(ii)* the centrality of the encoded subunit within its protein complex; *(iii)* enrichment of residues with a higher carboxyl pKa in the organelle-encoded protein; and *(iv)* the GC content of its cognate gene. Hydrophobicity of the protein product was therefore confirmed to be a reliable predictor for gene retention in mitochondria, and as an inevitable corollary, hindering both transfer to the nucleus and successful allotopic expression of certain organellar genes. We conclude that several mitochondrial genes like *cox1*, *cytb*, *nd1, nd2, nd4, nd5 and nd6*, whose protein products are highly hydrophobic and mistargeted to the ER when synthesized in the cytosol (Björkholm et al., 2017), do not lend themselves to allotopic expression, unless extensive modifications designed to increase the $\mu\Delta G_{app}$ of certain TMS are incorporated in the original design of their expression constructs. In addition, even if precursors are targeted to the mitochondrion, the presence of one or more TMS exhibiting a low $\mu\Delta G_{app}$, may cause the TIM23 translocator to misguide them into an inappropriate sorting route, thus blocking their ability to reach their final functional topology (Fig. 4).

The existence of hydrophilic pathways with lateral opening gates, representing potential paths for preprotein sorting, is a common feature of many protein translocons, such as SecY on the bacterial inner membrane (Ma et al., 2019), Sec61 and Hrd1 complex on the ER membrane (Wu et al., 2019, 2020), TIM22 on the mitochondrial inner membrane (Qi et al., 2021) and the chloroplast TIC complex (Jin et al., 2022; Liu et al., 2023). As discussed above, TIM23 sorts alpha helices either translocating them into the matrix or releasing them laterally into the IMM. Constrained by this sorting mechanism, all the TMS of the allotopically produced membrane proteins ***must*** be fully translocated into the mitochondrial matrix (by $TIM23^{MOTOR}$) and ***then*** incorporated into the IMM (except for the last TMS, which in certain proteins could be laterally released into the IMM (by $TIM23^{SORT}$). Inevitably, physical limitations in importing a cytosol-synthesized membrane protein must augment as the number of TMS increases (Claros et al., 1995). Hence it is expected that major hurdles may be encountered when the Nd6 (5 TMS), Cyt*b* (8 TMS), Nd1 (8 TMS), Nd2 (11 TMS), Cox1 (12 TMS), Nd4 (14 TMS), and Nd5 (17 TMS) subunits are expressed allotopically (Fig. 1). The key challenge in designing constructs for allotopic expression is to include mutations that increase the $\mu\Delta G_{app}$ of certain TMS without altering protein function, as exemplified by the yeast Cox^{W56R} allotopic subunit. While the W56R mutation allows the internalization of the protein into mitochondria, its effect on cytochrome *c* activity is negligible (Rubalcava-Gracia et al., 2018). By contrast, other OXPHOS genes, mainly those that have been transferred to the nucleus in some organisms and encode relatively few "red-light" TMS (Fig. 4), may be viable candidates to attempt allotopic expression in humans, namely: *atp8, cox2 and cox3*. As a general strategy, it might be conceivable to mimic the structural changes that the protein products have undergone, i.e., point mutations replacing hydrophobic amino acids with more polar residues in the first TMS of the protein as in the yeast and algal Cox2, or the loss of a TMS as in the algal Atp6 and Cox3 proteins. Alternatively, the sequence of a complete TMS could be exchanged by the TMS of the same protein pertaining to a closely related organism whose $\mu\Delta G_{app}$ exhibits a higher value. The fragmentation of genes, as illustrated above for Cox2a and Cox2b, each encoding one of the two complementary subunits, may also represent an alternative strategy. Split subunits, each one containing a small number of TMS, may enter mitochondria more efficiently and functionally associate in the IMM after import. There are several examples of genes encoding OXPHOS components that have undergone natural fragmentation including the *cox1*, *cox2, nad1*, *nad2*, and *sdhB* genes (Szafranski, 2017). Alternative approaches could also involve mutations in the TIM/TOM import apparatus or overexpression of certain factors that could enhance the import of allotopic proteins into mitochondria.

The arguments presented above largely rest upon the available data on 3D structures of OXPHOS proteins and the known mechanisms of protein import into mitochondria. In this work, one critical assumption was made: that the biological hydrophobicity scale of Botelho et al. (2011), originally determined in yeast mitochondria, may be extrapolated to other organisms including humans. Further experimentation in human cells will clarify this issue, but at present, we find it to be a reasonable assumption. Two of the approaches used in the past to modify genes for allotopic expression, i.e., codon optimization and the addition of a sequence encoding an MTS, are necessary but not sufficient to ensure a successful outcome. Here, we have argued that three additional elements should be considered: *i)* the $\mu\Delta G_{app}$ of the TMS of proteins destined to reside in the IMM; *ii)* the final topology of each OXPHOS subunit and the number of TMS they contain; and *iii)* the mechanism by which TIM23 distributes cytosol-synthesized precursors. The last two factors are intimately related, since the $\mu\Delta G_{app}$ value will determine the biogenesis pathway by which TIM23 will direct a given TMS. Despite all its caveats, allotopic expression remains a promising, intellectually challenging strategy to develop gene-based therapies for mitochondrial diseases.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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