REVIEW ARTICLE

Allotopic expression of mitochondrial genes: Basic strategy and progress

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Abstract   Allotopic expression of mitochondrial genes is a deliberate functional relocation of mitochondrial genes into the nucleus followed by import of the gene-encoded polypeptide from the cytoplasm into the mitochondria. For successful allotopic expression of a mitochondrial gene, several key aspects must be considered. These include the different codon dictionary used by the mitochondrial and nuclear genomes, different codon preferences between mitochondrial and nuclear-cytosolic translation systems, and the provision of an import signal to ensure that the newly translated protein in the cytosol is successfully imported into mitochondria. The allotopic expression strategy was first developed in yeast, a useful model organism for studying human and other eukaryotic cells. Currently, a number of mitochondrial genes have been successfully recoded and nuclearly expressed in yeast and human cells. In addition to its use in evolutionary and molecular biology studies, the allotopic expression strategy has been developed as a potential approach to treat mitochondrial genetic disorders. Substantial progress has been recently achieved, and the development of this technique for therapy of the mitochondrial disease Leber's hereditary optic neuropathy (LHON) has entered phase III clinical trials. However, a number of challenges remain to be overcome to accelerate the successful application of this technique. These include improvement of nuclear gene expression, import into mitochondria, processing, and functional integration of the allotopically expressed polypeptides into mitochondrial protein complexes. This review discusses the current basic strategy, progress, challenges, and prospects of the allotopic expression strategy for mitochondrial genes.

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Basic strategy for allotopic expression of mitochondrial genes

Mitochondrial genomes are believed to be remnants of the genomes of an α-proteobacterium engulfed by a eukaryotic cell. During the course of eukaryotic evolution, the size of the mitochondrial genome has been considerably reduced due to massive gene transfer into the nucleus, and other factors. The main function of the retained mitochondrial genomes in the present day is to produce several proteins functioning as subunits of enzyme complexes involved in oxidative phosphorylation. Mitochondrial genomes are also responsible for coding rRNA and tRNA that function exclusively within the mitochondria.[1–3]

Mitochondrial genes are normally expressed within the mitochondria. Following their transcription, the mRNAs generated are translated into proteins on mitochondrial ribosomes. The proteins are then directed into their functional submitochondrial locations. Allotopic expression of a mitochondrial gene is the deliberate functional relocation of the mitochondrial genes into the nucleus, followed by import of the coded proteins synthesized in the cytoplasm into the mitochondria.[4]

For successful expression of mitochondrial genes in the nuclear-cytosolic compartment, several basic requirements must be met. First, the mitochondrial genes must be compatible with the nuclear expression system. Differences related to codon dictionary and codon preferences between the mitochondrion and the nucleus must be considered in recoding these genes. Second, a means to deliver the nuclear-cytoplasmically-expressed proteins into the mitochondria must be established. This can be carried out by incorporating a mitochondrial targeting sequence (MTS) into the protein. Third, a system to maintain and replicate the mitochondrial genes in the nucleus must be developed.[4]

An allotopic expression system for mitochondrial genes was first developed to allotopically express the subunit 8 gene of the yeast mitochondrial ATP synthase in yeast cells lacking an endogenous subunit 8 gene. For this purpose, — a novel DNA sequence of the subunit 8 gene was designed to be expressed in the nuclear-cytosolic system. In the nuclear version of the subunit 8 gene, as many as 31 out of 48 codons of the natural mitochondrial subunit 8 gene were changed. One codon for Thr22 needed to be altered to comply with the nuclear codon dictionary; otherwise, the mitochondrial CTA codon for threonine would be translated to leucine in the cytosolic ribosomes. The other 30 codon changes were made basically to optimize the expression of the subunit 8 gene in the nuclear-cytosolic environment. This alteration was considered to be critical because, although the mitochondrial codon version would be translated correctly, the translation efficiency was expected to be very low since the altered codons are rarely used in highly expressed nuclear genes. For instance, the 12 leucine residues in the natural subunit 8 gene are encoded by the codon TTA, while in highly expressed yeast nuclear genes, leucine is mostly encoded by the codon TTG. Since extensive codon changes needed to be made, the nuclear version of the subunit 8 gene was chemically synthesized.[5]

Conversion of a mitochondrial codon into a nuclear codon, and optimization of codon preference, have also been carried out for allotopic expression of the human ATP synthase subunit 8 gene,[6] and the human ATP synthase subunit 6.[7] Codon optimization for nuclear expression was also required for allotopic expression of yeast *Saccharomyces cerevisiae* mitochondrial ATP synthase subunit 9 using the naturally nuclear subunit 9 gene of *Podospora anserine*.[1] Another emerging strategy for optimizing the allotopic expression of mitochondrial genes is the use of chemically synthesized modified mRNAs that harbor modified nucleotides designed to protect the mRNAs from degradation by the innate immune response without reducing the efficiency of their translation process. Generally the modifications are in the form of alteration of uridine to pseudouridine and cytosine to 5-methyl-cytosine. This approach has been successfully used to allotopically express human mitochondrial ATP synthase subunit 6 in human cells.[3]

Import into mitochondria of allotopically expressed proteins

Naturally, most of the proteins functioning in the mitochondria are nuclearly encoded, synthesized in the cytoplasm and imported into mitochondria. The majority of the mitochondrial precursor proteins are synthesized with mitochondrial targeting sequences that direct the proteins to mitochondria and then into the correct location in the mitochondrial compartment.[8] Yeast has been used as a model organism for the identification of components involved in driving the translocation of nuclearly-encoded mitochondrial proteins. In general, the machinery components are evolutionarily conserved among eukaryotes.[9] Molecular chaperones such as heat shock protein 70 (Hsp70) and ATP-dependent unfolding enzymes play important roles in maintaining the precursor proteins in an import-competent state. Hsp70s are present in both cytosolic and mitochondrial compartments. They interact directly with the translocating proteins. ATP-dependent release of precursor proteins from the cytoplasmic Hsp70 stimulates the translocation of the proteins through the translocase of the outer membrane (TOM) complex, which consists of several proteins. Three proteins, Tom20, Tom22, and Tom70 function as receptors that recognize and direct the MTS-containing protein to the translocation pore formed by Tom40. The MTS of the translocated protein is then handed over to the inner membrane translocase, the TIM23 complex, which is made up of a number of proteins, including Tim23, Tim17, and Tim44. Tim23 forms a protein-conducting pore, whereas Tim17 has been suggested to function in regulating the Tim23 channel. Tim44 serves as a scaffold for the association of translocating proteins with the import motor component, which completes protein translocation into the mitochondrial matrix. In the matrix, the MTS-containing proteins interact with mitochondrial Hsp70, and the MTS is cleaved away by the mitochondrial processing peptidase. Following ATP-dependent release from the mitochondrial Hsp70, the majority of the proteins require Hsp60 for refolding and assembly into functional enzyme complexes.[10–12] The general import mechanism of proteins into mitochondria using the MTS pathway is depicted in Fig. 1.
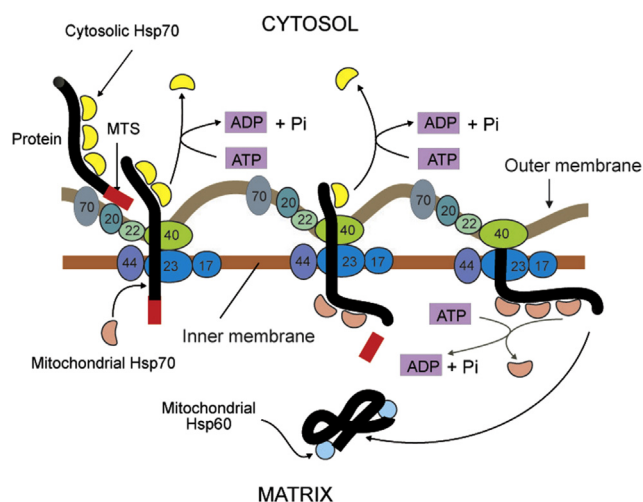
Figure 1 Protein import into mitochondria using the MTS pathway. After the initial insertion of the mitochondrial targeting sequence (MTS) and of the adjacent portions of the polypeptide chain, the unfolded protein slides in a channel that spans both membranes. The import process requires Hsp70 on both sides of the mitochondrial double membrane. Bound cytosolic Hsp70 is released from the protein in a step that depends on ATP hydrolysis. The proteins Tom20, Tom22, and Tom70 function as receptors that recognize and direct the MTS-containing protein to the translocation pore formed by Tom40. The MTS of the translocated protein is then delivered to the translocase of the inner membrane, where the Tim 23 complex consists of a number of proteins, including Tim23, Tim17, and Tim44. In the matrix, the MTS-containing proteins interact with mitochondrial Hsp70, and the MTS is cleaved off by the mitochondrial processing peptidase. Following ATP-dependent release from the mitochondrial Hsp70, the majority of the proteins require Hsp60 for refolding and assembly into functional enzyme complexes.[10–12]

The mitochondrial import of the allotopically expressed proteins is designed to follow the MTS pathway (Fig. 1), in which the proteins are synthesized in the cytosol and their import is dependent on the MTS and the TOM/TIM import machinery. This biogenesis pathway is different from the biogenesis pathway of their mitochondrial counterparts, which are synthesized in the mitochondrial matrix.[13] To ensure the mitochondrial import of allotopically expressed subunit 8 protein of the yeast mitochondrial ATP synthase, a sequence encoding an MTS derived from a precursor of subunit 9 of *Neurospora crassa* mitochondrial ATP synthase was fused to the N-terminal end of the subunit 8 gene.[14] Following insertion of the fusion gene into a suitable plasmid vector, the gene was expressed *in vitro*. The results showed that the allotopically expressed subunit 8 was successfully imported into isolated yeast mitochondria.[14] Further experiments revealed that allotopic expression of the subunit 8 gene in yeast host cells lacking endogenous subunit 8 led to its successful assembly into functional ATP synthase complexes. This was demonstrated by the ability of the allotopically expressed subunit 8 protein to complement the ethanol-negative growth phenotype of the host cells.[15]

A number of factors have been indicated to affect the mitochondrial import efficiency of allotopically expressed mitochondrial proteins. These include mRNA localization, rate of protein delivery to the mitochondrial receptor, hydrophobicity of the protein, compatibility of the protein with its MTS, etc. The import efficiency of allotopically expressed mitochondrial polypeptides can be improved by targeting the mRNA of the corresponding polypeptide to the mitochondrial surface. The sorting of several proteins destined for mitochondrial import involves mRNA localization. Mitochondrial localization and sorting of mRNAs encoding mitochondrial proteins might occur cotranslationally to assist in the import of the protein. In yeast, almost half of the mRNAs encoding mitochondrial proteins are transported to the mitochondrial surface. For example, the ATP2 mRNA, which encodes the β-subunit of ATP synthase, mostly localizes to the mitochondrial surface. Two sequences within mRNAs are responsible for the specific localization to the mitochondrial surface, the regions coding for the MTS and the 3′UTR. This phenomenon is conserved in human cells. When the gene coding for human ATP synthase subunit 6 was associated with the MTS and the 3′UTR of the nuclear Cox 10 gene[16] or SOD2 gene,[7] the mRNAs of which are enriched at the mitochondrial surface, the hybrid mRNA localized to the mitochondrial surface in human cells. This strategy was successfully employed to allotopically express the very hydrophobic subunit 6 of human mitochondrial ATP synthase in human cells.[7] By forcing its mRNA localization to the mitochondrial surface, the allotopically expressed subunit 6 gene lead to a long-lasting and complete rescue of mitochondrial dysfunction in human fibroblast cells carrying a mutation in the mitochondrial ATP synthase subunit 6 gene.[17]

In other studies, tandem duplication of MTS was found to improve the mitochondrial import efficiency of allotopically expressed subunit 8 of yeast mitochondrial ATP synthase. The duplicate MTS might accelerate precursor protein delivery to the outer mitochondrial membrane receptors and therefore enhance the rate of insertion and translocation across the mitochondrial membranes. In addition, duplication of MTS may also increase the overall hydrophilicity of the precursor protein and hence reduce the possibility of its nonspecific binding with the outer membrane. Thus, inefficient binding to the import receptor due to random membrane association would be reduced. The duplicate leader might provide extra hydrophilic domain helpful to maintain the precursor protein in a more import-competent state.[18] Following import of the subunit 8 precursor protein into mitochondria, the N-terminal leader sequence is proteolytically cleaved off by the matrix protease. The sites where the processing enzyme acts were found to depend on the nature of the fusion between subunit 8 and the leader sequence.[19]

It is important to note that the high hydrophobicity of the nascent polypeptides has been indicated to be one of the limiting constraints for successful mitochondrial import of allotopically expressed mitochondrial proteins. This can be overcome by reducing the hydrophobicity of the proteins which are to be allotopically expressed. This strategy was successfully applied by Daley et al[20] for the allotopic expression of subunit 2 of cytochrome *c* oxidase (Cox2) of

soybean in which they altered two amino acid residues L169Q and L171G in the first transmembrane region of the protein. Selection of this particular region for local hydrophobicity reduction was based on analysis of levels of hydrophobicity which indicated this region was a potential mitochondrial import barrier due to its high hydrophobicity. These experiments unequivocally demonstrated that alteration of only two amino acid residues can dramatically change the import ability into mitochondria of the allotopically-expressed highly-hydrophobic soybean Cox2 protein.[20] Recent experiments also showed that the mutation W56R is required for successful import of allotopically expressed subunit 2 of yeast cytochrome *c* oxidase. The W56R mutation, which is located in the first transmembrane segment of the protein, diminishes the hydrophobicity of the first transmembrane stretch and permits its import into mitochondria.[13] Similarly, the high hydrophobicity of subunit 9 of the yeast mitochondrial ATP synthase has been indicated to hamper its allotopic expression because the protein cannot cross the mitochondrial inner membrane and is therefore proteolytically degraded in the mitochondrial intermembrane space. These obstacles were successfully overcome by reducing the hydrophobicity of the subunit 9 protein.[1] In addition, the compatibility of a particular polypeptide with the MTS used to direct its mitochondrial import dictates the success of its allotopic expression. For example, the W56R mutant of the yeast cytochrome *c* oxidase could be allotopically expressed using an MTS derived from the *S. cerevisiae*—OXA1 gene or *Neurospora crassa* mitochondrial ATP synthase subunit 9 gene, both of which code for hydrophobic mitochondrial proteins, but not with an MTS derived from the COX4 gene, which codes for a hydrophilic protein, the yeast cytochrome *c* oxidase subunit 4.[21] Recently, Chin et al[3] developed an unbiased screening platform to compare different MTSs and 3′UTRs in their ability to target different allotopically expressed proteins to the mitochondria. They found that the choice of MTS was more critical for successful mitochondrial protein import compared with the 3′UTR. This strategy has been employed to allotopically express the mitochondrial ATP synthase subunit 6 gene in human cells.

Progress and challenges

The allotopic expression strategy for mitochondrial genes was first employed to transfer the mitochondrial subunit 8 gene of the yeast mitochondrial ATP synthase to the nucleus in yeast cells lacking the endogenous subunit 8 gene.[14] This technique proved to be powerful for elucidating the biogenesis, evolution, and relationship between the structure and function of the subunit.[15,22] The technique has permitted the identification of domains that are critical for subunit 8 function as part of the stator stalk of the ATP synthase complex.[23] In other experiments, the allotopic expression strategy was used to determine the topology and proximity relationships of the subunit 8 protein.[24] A nuclear version of the subunit 8 gene designed to encode FLAG-tagged subunit 8 fused with a mitochondrial signal peptide was also successfully expressed in the nuclear-cytosolic compartment of yeast cells lacking

endogenous subunit 8. The FLAG-tagged subunit 8 protein was imported into mitochondria and then assembled into a functional mitochondrial ATP synthase complex. Furthermore, the subunit 8 protein could be detected using an anti-FLAG monoclonal antibody.[25] This has allowed confirmation of the necessity for subunit 8 protein to maintain its transmembrane topology for its function.[26] To reveal whether subunit 8 of mitochondrial ATP synthase from another organism could function in yeast, Straffon et al[27] allotopically expressed the subunit 8 gene of *Aspergillus nidulans* in a strain of *S. cerevisiae* lacking expression of the endogenous subunit 8 gene. It was found that subunit 8 of *A. nidulans* was functional in *S. cerevisiae* despite significant differences in amino acid residues between the two subunits. The two polypeptides have the same number of amino acid residues and share similar structural motifs; however, they share only approximately 50% identical amino acids. This has led to the conclusion that the common structural motifs shared between the two homologous subunits are essential for function.[27] A dual-control allotopic expression strategy to allow allotopic expression of two different forms of yeast subunit 8 gene has also been developed. The strategy involving the maintenance of two different compatible yeast expression vectors was developed to potentially permit accurate assessment of the assembly behavior of functionally-defective subunit 8 variants *in vivo*.[28] Apart from its successful application in the nuclear expression of the yeast mitochondrial ATP synthase subunit 8 gene,[14] the technique has been employed to allotopically express other yeast mitochondrial genes, such as those that encode mitochondrial RNA maturase,[29] subunit 9 of mitochondrial ATP synthase[1] and subunit 2 of cytochrome *c* oxidase.[13]

In spite of the successful demonstration of allotopic expression in yeast, experiments in mammalian cells have given contradictory results. For example, in 2003, Oca-Cossio and coworkers[30] allotopically expressed three different mitochondrial genes (encoding subunit 8 of ATP synthase, apocytochrome *b* and ND4) in COS-7 and HeLa cells; only subunit 8 of ATP synthase was correctly expressed and targeted into mitochondria. The apocytochrome *b* and ND4 (full-length and truncated forms) were found to decorate the surface of the mitochondria. The polypeptides were also found to aggregate and associate with the cytoskeleton components, tubulin and vimentin. In addition, allotopic expression of apocytochrome *b* and ND4 was found to cause cellular toxicity and death of the transfected cells due to a loss of mitochondrial membrane potential.[30] Similarly, in 2010, Perales-Clemente and colleagues[31] allotopically expressed the mitochondrial ND6 gene in mouse cell lines harboring a mutated endogenous mitochondrial ND6 gene. Although partial rescue of complex I activity was achieved, detailed analysis showed that it was not because of the allotopic expression of the wild-type ND6 gene, but rather because of reversion of the endogenous mutated mitochondrial ND6 gene. These results implied the importance of caution in interpreting the positive results of allotropic expression of mitochondrial genes.[31] In other studies the technique was successfully used to express human mitochondrial genes in animal or human cells. A number of human mitochondrial genes have been allotopically expressed, including the

ATP6 gene, which encodes mitochondrial ATP synthase subunits 6,[3,6,32] the ATP8 gene for mitochondrial ATP synthase subunit 8,[6] the ND1 gene which encodes subunit 1 of NADH dehydrogenase,[33] and the ND4 gene which encodes subunit 4 of NADH dehydrogenase.[34−36]

The allotopic expression technique has been considered an attractive approach for the genetic treatment of mitochondrial genetic disorders, and substantial progress towards this application has been achieved. Mitochondrial genetic disorders are among the most common inherited metabolic diseases, and no established treatment is currently available. These diseases may be caused by rearrangements (large-scale partial deletions and duplications) or point mutations in mitochondrial DNA. Rearrangements of mitochondrial DNA can cause diseases such as chronic progressive external ophthalmoplegia (CPEO), Kearns−Sayre syndrome, diabetes and deafness, Pearson marrow-pancreas syndrome, and sporadic tubulopathy. Similarly, point mutations in mitochondrial DNA can result in LHON, Leigh syndrome, MELAS, MERRF myopathy, cardiomyopathy, diabetes and deafness, encephalomyopathy, nonsyndromic sensorineural deafness, and aminoglycoside-induced nonsyndromic deafness.[37]

One of the mitochondrial genetic diseases for which therapy based on an allotopic expression strategy is being developed is LHON. A French company, GenSight Biologics, is currently conducting phase III clinical trials on allotopic expression of ND4 to treat blindness in LHON patients.[6] LHON, a maternally inherited mitochondrial disorder, leads to severe visual impairment or even blindness due to death of retinal ganglion cells. The disease is associated with three primary mutations in the mitochondrial DNA: G3460A, G11778A and T14484C which affect subunits of respiratory enzyme complex I (ND1 Ala52Thr, ND4 Arg340His, and ND6 Met64Val, respectively). Two additional mtDNA mutations, G14459A and G15257A resulting in the ND6 Ala72Val and Cytb Asp171Asn amino acid alterations, respectively, are also considered to be primary LHON mutations. In Southeast Asia, the G11778A mutation is the main underlying mutation for LHON. Similarly, this mutation is the cause of approximately 50% of LHON cases in the European population, and approximately 95% in the Japanese population.[38,39]

Despite recent exciting progress in the allotopic expression of mitochondrial genes, a number of obstacles remain to be solved to allow widespread and effective applications. The efficiency of allotopic expression needs to be improved. The recoded mitochondrial genes need to be expressed stably in the nuclear-cytosolic system. This is important as one the major challenges in the application of the allotopic expression strategy for gene therapy is the ability to maintain stable, long-term, gene expression. A system for stable allotopic co-expression of subunits 8 and 6 of human mitochondrial ATP synthase was successfully developed by Boominathan and co-workers[6] which led to proper assembly of the two subunit proteins into a functional mitochondrial ATP synthase complex. In this system, the recoded subunits 8 and 6 gene sequences, in the form of a fusion with the MTS of the ATP5G1 (one of three genes encoding subunit 9 of the human mitochondrial ATP synthase) were cloned into a plasmid vector (pCMV6). The cloned genes were then allotopically expressed in a human transmitochondrial cytoplasmic hybrid (cybrid) cell line having no mitochondrial ATP synthase activity due to absence of functional subunit 8 protein and having significantly reduced subunit 6 levels. This stable nuclear expression of both subunits 8 and 6 successfully rescued a disease borne phenotype arising from a nonsense G8529A point mutation located in the overlap region between the subunit 8 and subunit 6 genes in the human mitochondrial genome.[6] A patient having this mutation, from which the cybrid cell line was derived, suffered from apical hypertrophic cardiomyopathy and neuropathy.[40]

In addition, successful allotopic expression requires that the protein be efficiently translocated into mitochondria, and then properly processed. In the mitochondria, the protein needs to be integrated into the mitochondrial inner membrane with the same topology as the mitochondrially synthesized version.[13] One of the critical steps dictating the success of the allotopic expression approach is the protein import process into mitochondria. Hydrophobicity of the proteins has been considered to be the primary barrier. This may be overcome by reducing the hydrophobicity of the allotopically expressed proteins and selecting the appropriate MTS. In particular, hydrophobicity reduction can be directed at the protein regions that are not involved in the subunit-subunit interaction or that contain critical residues for enzyme activity.[41] In addition, a more detailed understanding of the mechanism of import and assembly of nuclearly encoded mitochondrial proteins is needed.[2] Furthermore, it is crucial to confirm the successful functional integration of the allotopically expressed protein in an assembled enzyme complex, especially when the strategy is to be used to start clinical trials in humans.[31]

Conclusion

Gene transfer from mitochondria to the nucleus has been occurring throughout mitochondrial evolution. This phenomenon can be mimicked by using the allotopic expression strategy in which the mitochondrial gene is deliberately recoded and relocated into the nucleus and the encoded polypeptide is imported back into the mitochondrion. The technique has proven powerful for molecular and evolutionary studies of mitochondrially encoded proteins. Although the general implementation of allotopic expression in yeast has been successful, some experiments in mammalian cells have given unclear results, indicating that the technique has important limitations for use as a therapeutic approach. Optimization of the technique to overcome the above hurdles has led to more reports on successful allotopic expression of mitochondrial genes in both yeast and human cells. This strategy has been postulated to be one of the potential approaches for treating mitochondrial diseases arising from mutations of mitochondrial genomes. Considerable progress has been achieved in applying the strategy to treat mitochondrial genetic diseases. However, to successfully implement this strategy as a form of gene therapy, a number of obstacles remain to be overcome. The allotopically expressed mitochondrial protein needs to be efficiently synthesized, imported into mitochondria, properly processed, and successfully integrated into the enzyme complex at the site

where that particular subunit functions. Future research should be directed towards the development of a system that ensures stable allotopic expression of the mitochondrial gene because stable, long-term allotopic expression must be achieved for the successful application of prospective gene therapy. As hydrophobicity of proteins has been considered a primary barrier for efficient protein import into mitochondria, a strategy to effectively reduce the hydrophobicity level of allotopically expressed proteins, needs to be developed. Detailed understanding of the mechanism of import and assembly of nuclearly encoded mitochondrial proteins is crucial. In addition, the mechanism of processing and assembly of allotopically expressed proteins needs to be elucidated to develop strategies that allow correct processing and integration of nascent proteins following import into mitochondria.

Conflict of interest

The author declares that he has no conflict of interest.

Acknowledgements

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