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Review

Dual targeting of mitochondrial proteins: Mechanism, regulation and function ☆

Ohad Yogev, Ophry Pines *

Department of Microbiology and Molecular Genetics, IMRIC, Faculty of Medicine, Hebrew University, Jerusalem 91120, Israel

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ABSTRACT

One solution found in evolution to increase the number of cellular functions, without increasing the number of genes, is distribution of single gene products to more than one cellular compartment. It is well documented that in eukaryotic cells, molecules of one protein can be located in several subcellular locations, a phenomenon termed dual targeting, dual localization, or dual distribution. The differently localized proteins are coined in this review "echoforms" indicating repetitious forms of the same protein (echo in Greek denotes repetition) distinctly placed in the cell. This term replaces the term to "isoproteins" or "isoenzymes" which are reserved for proteins with the same activity but different amino acid sequences. Echoforms are identical or nearly identical, even though, as referred to in this review may, in some cases, surprisingly have a totally different function in the different compartments. With regard to mitochondria, our operational definition of dual targeted proteins refers to situations in which one of the echoforms is translocated through/into a mitochondrial membrane. In this review we ask how, when and why mitochondrial proteins are dual localized in the cell. We describe mechanisms of dual targeting of proteins between mitochondria and other compartments of the eukaryotic cell. In particular, we have paid attention to situations in which dual localization is regulated in time, location or function. In addition, we have attempted to provide a broader view concerning the phenomenon of dual localization of proteins by looking at mechanisms that are beyond our simple definition of dual targeting. This article is part of a Special Issue entitled Protein translocation across or insertion into membranes.

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* Corresponding author.
E-mail address: ophryp@ekmd.huji.ac.il (O. Pines).

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1. Introduction

Eukaryotic cells are defined by the existence of subcellular compartments and organelles. This allows the partitioning of various biochemical pathways out of the cytosolic milieu into discrete organelles. For this, each cellular compartment has a specific protein content that is vital for its function. Mitochondria are such organelles, which are responsible for energy production and essential metabolic pathways in the cell, and these organelles have been implicated in other processes such as apoptosis, aging and cancer.

Sorting of proteins to a membrane-sealed organelle, such as mitochondria, involves two main steps: i) Targeting of proteins to and specific recognition by the organelle and ii) Translocation into the organelle, through or into its membranes by an import machinery. This necessitates that the protein includes targeting and translocation information within its sequence that is recognized by the organelle receptors and import machinery. In this review we intentionally do not provide an introduction to protein targeting information within substrates and protein translocation machineries within cells. These are provided by excellent accompanying reviews in this issue dealing with protein targeting and translocation in mitochondria, chloroplasts, peroxisomes and the ER.

It is well documented that in eukaryotic cells molecules of one protein can be located in several subcellular locations, a phenomenon termed dual targeting, dual localization or dual distribution. The differently localized proteins are termed isoproteins. With regard to mitochondria, our conventional definition of dual targeted proteins refers to situations in which one of the isoproteins is translocated through/into a mitochondrial membrane. Thus, dual targeted proteins are recognized by at least one organelle's receptors and translocation machineries.

Dually targeted proteins can be located in two or more compartments, immediately after translation thereby creating a steady state ratio between the different sub-populations. In recent years it has become evident that dual targeting can be regulated; induced or rebalanced in response to cellular signaling or as a response to changing extracellular conditions. Interestingly, these proteins can perform the same or distinct activities and functions in each location. Dual targeting of proteins in eukaryotic cells is a timely topic for which several excellent reviews have been published in the last decade on different aspects of this phenomenon [1–9]. In this review, we describe mechanisms of dual targeting of proteins between mitochondria and other compartments of the eukaryotic cell. We have addressed situations in which dual targeting of proteins is regulated in time, location or function; for instance, changes in targeting under specific cellular conditions, changes in the relative amounts of the isoproteins, and additional new functions performed by one of the isoproteins. Due to the large number of studies dealing with dual targeting we have chosen in each case one or two examples in order to make a point, with the understanding that this choice was oftentimes fortuitous or subjective based on our familiarity and knowledge. In some cases while the data suggests dual localization of a protein, the mechanism by which this occurs is unknown and therefore open questions linger.

The review will deal with dual targeting mechanisms of mitochondrial proteins that are based on single or multiple translation products (Table 1). In addition, we provide a broader view concerning the phenomenon of dual localization of proteins by looking at mechanisms that are beyond our simple definition of dual targeting.

Table 1
Dual targeting mechanisms of mitochondrial protein.

Dual targeting mechanism	Genes	mRNAs	Translation products	Examples in this review[a]	Illustrated in figure
Two genes encode two isoprotein: one that contains a targeting signal which the other lacks.	2	2	2	Mdh^{YM} Aconitase^{M}	1A
Two transcripts are created by alternative transcription initiation or alternative splicing, which removes a targeting signal.	1	2	2	Hts 1^{Y}, Vas 1^{Y}, Renin^{M}	1B
Translation initiation from in-frame start codons, which removes a targeting signal.	1	1	2	Mod5^{Y}, Pol gamma 2^{P}, Hcs 1^{P}	1C
An ambiguous targeting signals is recognized by two organelles.	1	1	1	TyrRS^{P}, LpSod^{P}, NADH-cytochrome b5R^{M}, Fis 1^{M}	2A
Two targeting signals on one protein target it to two organelles.	1	1	1	NAD(P)H dehydrogenase^{M}, CYP2B1^{M}	2B
Targeting sequence is inaccessible in some of the protein molecules.	1	1	1	Aky2^{Y}, Apn 1^{Y}, ERS^{Y}, CYP2B1^{M}, Cyp1A1^{M}	2C
Reverse translocation of some of the protein molecules.	1	1	1	Fumarase^{Y}, NFS1^{Y}, Aconitase^{Y}	2D
Membrane permeabilization, release or export of proteins	1	1	1	Cytochrome C^{M}, HSP60^{M}, Mortalin^{M}	2E

[a] Y, Yeast; M, Mamalian; P, Plants.

2. Dual targeting mechanisms of mitochondrial proteins

2.1. Two (or more) translation products

2.1.1. Two or more genes

The simplest and most basic mechanism for achieving dual targeting is the existence of two genes. In this case, each gene can encode a slightly different isoprotein; one that contains a targeting signal which the other lacks. This allows the creation of two translation products that are targeted to two cellular locations (Fig. 1A). Examples for this mechanism can be found in the yeast malate dehydrogenases (MDH) and alcohol dehydrogenases (ADH). *Saccharomyces cerevisiae* contains 3 highly homologous isozymes of malate dehydrogenase. All three catalyze the interconversion of malate and oxaloacetate. While sharing the same enzymatic activity, each isoenzyme is located in a separate compartment; MDH1 participates in the tricarboxylic acid cycle in mitochondria, MDH2 functions in the cytosol as part of the malate/aspartate shuttle and MDH3 catalyzes the reaction as part of the glyoxylate shunt in peroxisomes [10–13]. The same phenomenon can be found in higher eukaryotes where MDH is encoded by two genes MDHI and MDHII, located in the cytosol and mitochondria respectively [14–16].

A second example, from higher eukaryotic cells, is the protein aconitase. Two proteins are encoded by two genes *ACO1* and *ACO2*; Aco2 is a mitochondrial enzyme of the Krebs cycle and Aco1 is a cytosolic participant in cellular iron regulation. In the case of aconitase, the two isoproteins differ in their location and also in their function. While Aco2 is an enzyme that catalyzes the conversion

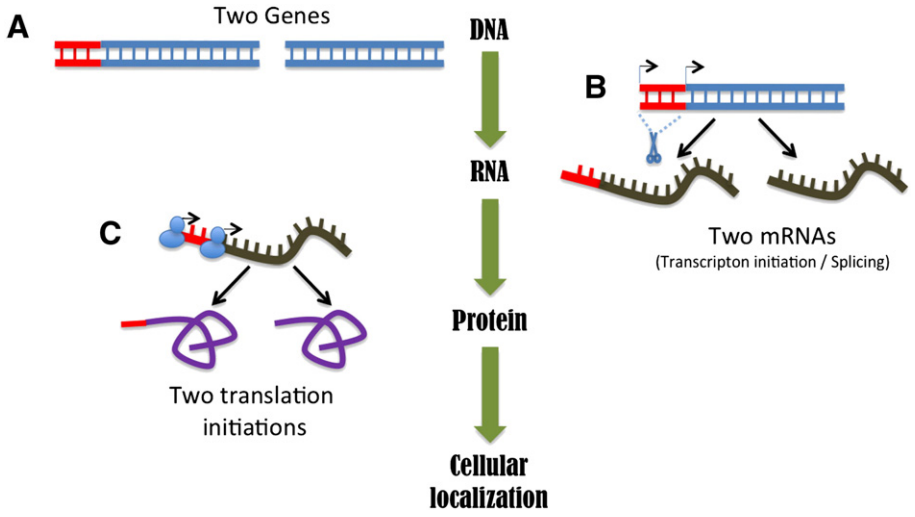

Fig. 1. Dual targeting mechanisms based on two translation products. Mechanisms by which two isoproteins derived from two translation products are dual targeted in the eukaryotic cell. (A) Two genes, of which only one encodes a mitochondrial-targeting signal (MTS). (B) Two mRNAs from a single gene of which only one encodes an MTS; obtained either by alternative transcription initiation (arrows) or by splicing (scissors). (C) Two proteins from a single mRNA of which only one harbors the MTS; obtained by alternative translation initiation (ribosomes attached at initiation codons). The DNA, RNA and polypeptide specifying the MTS are colored in orange.

of citrate to isocitrate, Aco1 is an mRNA binding protein [17]. Worth mentioning is that in yeast the single translation product of aconitase has been shown to also bind mitochondrial DNA, and takes part in stabilization of the mitochondrial genome [18]. Thus in both yeast and human cells aconitase binds nucleic acids as a function distinct from its enzymatic activity.

2.1.2. One gene multiple mRNAs

Dual targeting can occur due to the existence of multiple mRNAs that are derived from a single gene. This can be achieved either by alternative transcription initiation or by mRNA splicing. In both cases two (or more) translation products are made and are targeted to different cellular locations (Fig. 1B).

In yeast the histidine tRNA synthetase (HTS1) and valyl-tRNA synthetase (VAS1) are localized both to the cytosol and to mitochondria. The corresponding genes encode two transcripts: a long one, which includes the mitochondrial-targeting sequence, that is translated into the mitochondrial form of the protein, and a short one that is translated into the cytosolic form that lacks this signal [19–21].

An example involving splicing in higher eukaryotes can be found in the case of the rat renin which is dual targeted to mitochondria and the ER [22]. The renin gene encodes two transcripts: a full-length transcript that encodes an ER N-terminal targeting signal, and a second shorter one, whose alternative transcription initiation and alternative splicing remove the ER targeting signal. This allows a cryptic mitochondrial-targeting sequence found downstream (between amino acids 36 and 50) to function in targeting of renin to mitochondria.

2.1.3. One mRNA multiple translation products

One mRNA can give rise to several proteins by translation initiation from several in-frame start codons. In this case the full-length protein includes an N-terminal targeting sequence while the shorter lacks it and may or may not contain a second targeting sequence (Fig. 1C). Thus the mechanism is based on the ability of the ribosome to skip of the first AUG, which can be affected by the sequence context surrounding the AUG (Kozak) and/or by RNA secondary structures in the vicinity of the AUG.

MOD5, which is encoded by a single gene, was found to localize to the mitochondria, cytosol and nucleus. This gene encodes a single mRNA, which contains two translation initiation sites. Translation from these sites leads to the synthesis of two translation products: a long one (from ATG1) harboring a mitochondrial-targeting signal, and a short one (from ATG12) that lacks the functional signal [23]. Accordingly, each of the translation products was localized differently; the full-length polypeptide is found predominantly in mitochondria while the short translation product, which is absent from mitochondria, is found in the cytosol and the nucleus.

An example in which we can discuss different parameters, which can affect alternative translation initiation that allows dual targeting, is the DNA polymerase POLgamma2 in plants. This gene's translation initiates at four distinct sites, to produce distinct plastid and mitochondria-targeted forms of the protein. The mechanism is based on alternative translation initiation due to leaky ribosome scanning in which the ribosomes skip some of the start codons. In the case of POLgamma2 there are four in-frame translation initiation sites: two AUGs and two upstream non-AUG start codons. At all these translation initiation sites there is only a partial Kozak consensus sequence. This leaky scanning also relies on the less efficient upstream non-AUG start codons, and in fact substitution of these non-AUG sites for AUG eliminates translation from the downstream AUG sites. Moreover, ribosome binding to the upstream non-AUG sites depends on sequences surrounding these start sites; deletion of the non-coding upstream sequence leads to translation only from the AUG sites and to an exclusive plastid localization. Nevertheless translation only from the upstream sites leads to a protein with an ambiguous (see definition below) targeting sequence, while the short version includes an N-terminal exclusive plastid targeting sequence [9,24]. Hence, dual targeting in this case is determined by the nature of the translation initiation codons and surrounding sequences. An interesting example regarding an upstream sequence that can affect translation initiation, is the Arabidopsis HCS1. Splicing out of 101 bases from the untranslated 5′UTR affects AUG choice for translation initiation and results in the production of a full-length protein harboring a chloroplast/mitochondrial-targeting signal (see below) and a shorter cytosolic protein that lacks it [25].

2.2. Single translation product

The most interesting and complicated dual targeting mechanisms are those that allow a single translation product to be localized to more than one compartment. The ability of one protein to be recognized by and translocated into more than one compartment can be achieved by several distinct mechanisms.

2.2.1. Ambiguous targeting signal

Some proteins have an ambiguous targeting sequence that can be recognized by more than one organelle (Fig. 2A). In this case, protein localization will be determined by "competition" between the organelles for the signal. The ratio between the protein sub-populations, in this case is determined by the affinity of the receptors of each organelle for this sequence. Recently there are an increasing number of reports on dual localization of proteins to mitochondria and chloroplasts [1,26,27]. The amino acid composition of mitochondrial and chloroplast targeting sequences is similar, with high abundance of hydroxylated, hydrophobic, and positively charged amino acids and low abundance of negatively charged residues. In this regard the targeting sequence of amino acyl-tRNA synthetases was analyzed in Arabidopsis [28]. For three of the proteins (TyrRS, ValRS, and ThrRS) deletions within this targeting sequence affected targeting to mitochondria and chloroplasts similarly. For two other proteins (ProRS and AspRS) the deletions remove either chloroplast or mitochondrial-targeting information without abolishing the respective alternative targeting signal. While the first two examples are consistent with a true ambiguous signal with overlapping information, the two latter cases are examples of separate targeting signals to chloroplasts and mitochondria placed in tandem.

Ambiguous targeting signals are not restricted to mitochondria and chloroplasts as for example in the case of iron-containing superoxide dismutase (LpSOD). In addition to the mitochondria/ER ambiguous signal its mRNA contains two in-frame AUG start codons. Translation initiation from the first AUG enables the creation of the full-length protein that contains the N-terminus targeting signal. This is an ambiguous signal since it harbors both a weak ER signal peptide and a mitochondrial-targeting signal. If the synthesized polypeptide binds the signal recognition particle (SRP) it will be imported into the ER co-translationally. On the other hand if the polypeptide binds the nascent polypeptide-associated complex (NAC) the protein will be post-translationally imported into mitochondria. Translation initiation from the second AUG gives rise to the synthesis of a shorter version, lacking the N-terminal ambiguous targeting signal. This latter protein is synthesized in the cytosol and then targeted to the peroxisome due to a C-terminal targeting signal [29]. So, this protein is localized to three different subcellular compartments employing two mechanisms: alternative translation initiation and an ambiguous targeting signal.

In some cases the affinity of an ambiguous targeting signal to the different compartments can be changed by modification or binding of other proteins. An example for modification of an ambiguous targeting sequence can be found in the mammalian NADH–cytochrome b(5) reductase (b5R). This protein is localized both to the mitochondrial outer membrane and to the ER membrane. On the ER membrane it participates in different aspects of lipid metabolism via its function as an electron acceptor. On the mitochondrial outer membrane it mediates the regeneration of ascorbate from ascorbate free radical and is involved in transfer of electrons from cytosolic NADH to cytochrome c in the intermembrane space. This protein is translated from a single mRNA to create a single translation product, harboring an N-terminal targeting signal, required for both ER and mitochondrial targeting. This signal contains a moderately hydrophobic stretch of 14 amino acids that is preceded by a myristoylation consensus sequence, which is modified in about half of the molecules. In the absence of myristoylation, the nascent chain is bound to the SRP and is targeted to the ER. Myristoylation of the N-terminus lowers the affinity of the signal for SRP; the nascent chain remains on free polysomes and is imported post-translationally into the mitochondria [30].

Human Fis1 is an example of a C-terminal ambiguous signal, which is affected by the binding of another protein. This tail-anchored membrane protein regulates the membrane fission of both

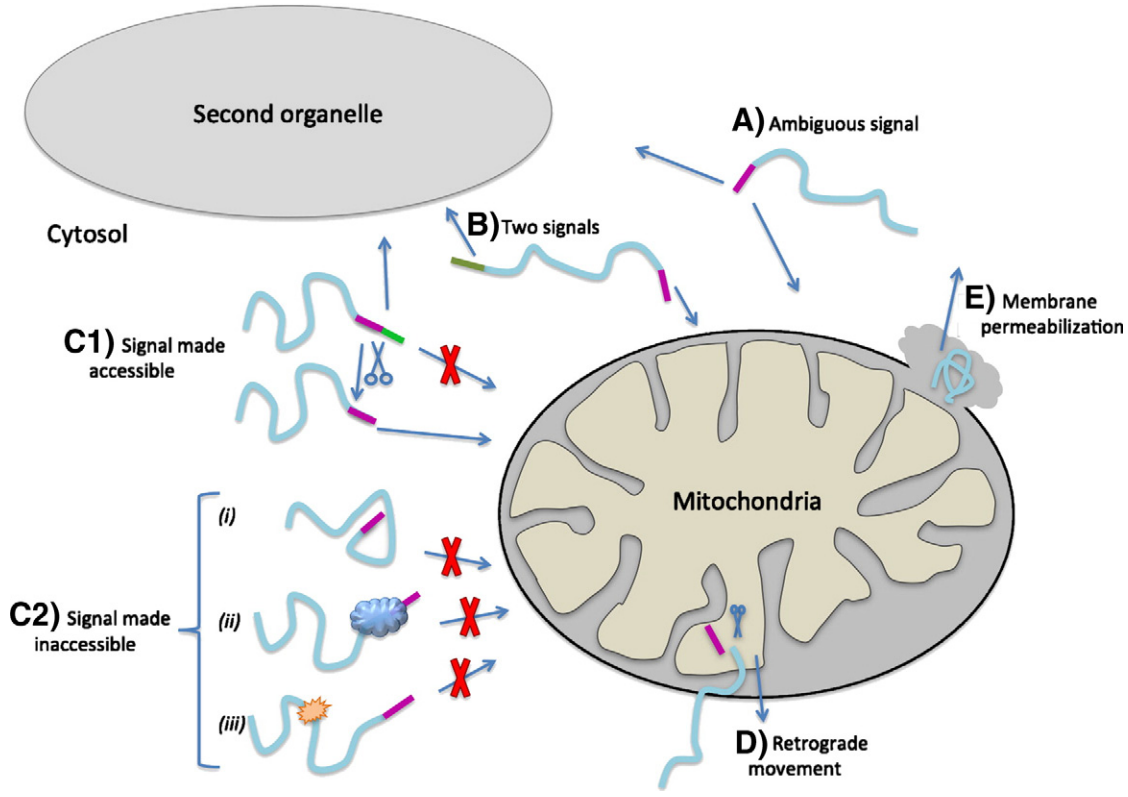

Fig. 2. Dual targeting mechanisms based on a single translation product. Mechanisms by which two isoproteins derived from a single translation product are dual targeted in the eukaryotic cell. (A) An ambiguous targeting signal is recognized by two organelles. (B) Competition between two targeting signals on the same polypeptide. (C) Changes in the targeting signal accessibility. (C1) A cytosolic protease cleaves the nascent chain thereby exposing a cryptic targeting signal. (C2) Inaccessibility of a signal prevents protein targeting; caused by *(i)* folding, *(ii)* binding to cellular factors or *(iii)* modification of the polypeptide. (D) Reverse translocation of the protein; a sub-population of the molecules moves back to the cytosol during import. (E) Release of proteins from mitochondrial intermembrane space by membrane permeabilization.

mitochondria and peroxisomes. The protein has been shown, via its last 26 amino acids, to bind Pex19p, a peroxisomal membrane protein import factor or alternatively function as a mitochondrial tail anchor. Interestingly, down-regulation of Pex19p reduces peroxisome targeting but not targeting to mitochondria, suggesting that targeting to mitochondria and peroxisomes are independent events even though both are dependent on the same C-terminal sequence [31].

2.2.2. Two targeting signals

Two (or more) targeting signals on a single polypeptide can provide the mechanism of dual targeting (Fig. 2B). Here the balance of isoprotein amounts between the different organelles is determined by the affinity of each signal for its target.

In Arabidopsis, 7 genes encode type II NAD(P)H dehydrogenases (ND). These proteins are typically located in the mitochondrial inner membrane where they can oxidise NAD(P)H. Three of these proteins were shown to be dual targeted to mitochondria and peroxisomes. Dual targeting of these three is dictated by two targeting signals: an N-terminal mitochondrial-targeting signal and a C-terminal peroxisomal targeting signal [32].

In some cases, the affinity of each targeting sequence for its target can be altered by protein modification, which in turn can change the relative amounts of the subcellular populations of the protein. The phenobarbital-inducible mitochondrial and microsomal CYP2B1 is targeted both to the ER and mitochondria by an amino-terminal bipartite signal, which includes an ER targeting sequence followed by a cryptic mitochondrial-targeting sequence. Dual targeting of CYP2B1 apoproteins to the ER and to mitochondria is modulated via a cAMP-dependent protein kinase A site. Phosphorylation of ser128 reduces the affinity of the protein for SRP and increases its affinity for the mitochondrial import machinery. This mechanism allows the cell to tilt the balance between two cellular compartments, according to cellular cAMP levels, which is a major constituent of cellular signaling [33].

2.2.3. Accessibility of targeting signals

Single translation products harboring one or more specific targeting signals can be dual targeted due to a situation in which one of the signals is inaccessible. In these cases all of the protein harbors a targeting sequence; however, for a sub-population of the molecules the signal becomes inaccessible, thereby preventing its interaction with its specific receptor (Fig. 2C). Inaccessibility of the targeting sequence can be caused by folding of the protein (i) interaction of the protein with other proteins (ii) or by modification of the polypeptide chain (iii). For example, yeast adenylate kinase 2 (Aky2) is located in the cytosol and the mitochondrial intermembrane space. Its rapid folding hinders the accessibility of the targeting signal to the mitochondrial receptor in some of the molecules and these molecules are localized in the cytosol. In this case competition between folding and targeting determines protein localization [34].

In a previous review we have referred to the protein Apurinic/apyrimidinic endonuclease 1 (Apn1) [2,35] as an example of inaccessibility of a signal caused by protein binding. Briefly, the protein Pir1 binds to Apn1 thereby masking its NLS and thus enhances mitochondrial targeting of the protein. This is an example of a signal whose accessibility is determined by the binding of another protein. Another example for this type of mechanism is Glutamyl-tRNA synthetase (ERS) in yeast which is localized to the cytosol and mitochondria. Import into mitochondria is due to a noncanonical MTS located after the first 190 residues. Binding to Arc1p, a protein that serves as a cytoplasmic anchoring platform, prevents the import and sequesters a major portion of ERS in the cytoplasm (in its absence all molecules are located in mitochondria). Arc1p levels are decreased in response to a switch between fermentation to respiratory metabolism, increasing as a result the mitochondrial ERS and mitochondrial protein synthesis. Thus Arc1 allows the cells to switch between fermentation and respiration by changing the cellular localization of Glutamyl-tRNA synthetase [36].

Examples given in previous sections, myristoylation of b5R and phosphorylation of CYP2B1, may also fall into this category of affecting accessibility of signals by covalent modification of the polypeptide chain (Fig. 2*Ciii*). An interesting example is CYP1A1, in which not only does polypeptide modification make one of the signals inaccessible it actually removes this signal from the protein by cleavage of the protein (Fig. 2C1). CYP1A1 harbors an N-terminal ER targeting signal followed by a cryptic mitochondrial-targeting signal. The primary translation product of the protein, according to its N-terminal signal, is targeted to the ER. While the majority of the molecules are translocated into the ER, 25% of them escape ER membrane insertion and are cleaved by a cytosolic protease, thereby activating a cryptic mitochondrial-targeting sequence [37]. Thus, the distribution of CYP1A1 can be regulated by inducing the protease (e.g. with b-naphthoflavone). Intriguingly the distribution of other proteins such as the glucocorticoid receptor, the retinoid X receptor, and p53, between mitochondria and other cellular compartments, may be dependent on cleavage of the protein by this serine protease and the distribution is probably determined by a mechanism similar to CYP1A1.

2.2.4. Reverse translocation

A unique dual targeting mechanism is based on retrograde movement of the protein during its import (Fig. 2D). The well-documented example of this mechanism is the enzyme fumarase in the yeast *S. cerevisiae*. In this case, all molecules are first targeted to mitochondria, begin their translocation and are processed by MPP. Nevertheless, a sub-population of the molecules moves back to the cytosol. The driving force for this distribution is protein folding; if during import the nascent chain starts to fold in the mitochondrial matrix, it will complete its import and be localized in mitochondria. On the other hand if the nascent chain starts its folding in the cytosol, thereby blocking its forward movement, the protein will withdraw from the import machinery, and will be localized in the cytosol [38]. Intriguingly, metabolic cues originating from the glyoxylate shunt can affect the ratio of mitochondrial versus cytosolic levels of fumarase. Deletions of glyoxylate shunt genes or addition of succinate, (a product of the glyoxylate shunt) to the yeast growth medium causes a shift of fumarase to mitochondria [39]. This suggests an interesting connection between primary metabolism pathway and protein localization.

Recently two other proteins were shown to be dual targeted in *S. cerevisiae* by a reverse translocation mechanism, Nfs1 and Aco1. Aconitase is dual localized to mitochondria and the cytosol, while Nfs is localized to mitochondria and the nucleus [40,41]. In this regard the sequence of the MTS can tilt the balance between the two locations of aconitase (and fumarase). The mechanism suggested is that the mitochondrial-targeting signal affects the translocation rate thereby determining the time (opportunity) required for the protein to fold or bind factors in the cytosol that block import [42].

2.2.5. Membrane permeabilization, release or export of proteins

The mechanism by which mitochondrial proteins, under specific conditions, can be found outside the organelle is not fully understood. This is particularly disturbing since an export apparatus of proteins out of mitochondria has not been described. The breakdown of the outer membrane or both inner and outer membranes could be a source for such proteins following induction of apoptosis or mitophagy (Fig. 2E). Following certain stress conditions such as hypoxia and DNA damage, the intrinsic apoptosis pathway is characterized by recruitment of cytosolic pro-apoptotic proteins to the mitochondrial outer membrane, membrane permeabilization, and release of potent death factors from the intermembrane space into the cytosol (reviewed in refs. [43,44]). A well-known example for this mechanism is the release of cytochrome c from the intermembrane space, during the intrinsic apoptosis pathway [45]. Thus, cytochrome

c, in addition to its function in the respiratory chain, has a crucial role in the caspase activation cascade; cytochrome c binds ARF in the cytosol, which leads to activation of caspase-9 and in turn caspase-3. Whether cytochrome c passes through a channel such as the porin VDAC or whether the outer membrane is disrupted is still under debate [46].

It is hard to explain detection of certain well-known mitochondrial proteins at unexpected extramitochondrial sites in the cell. For example Hsp60 is a mitochondrial chaperone residing in the mitochondrial matrix, which is essential for normal mitochondrial function and biogenesis. In yeast Hsp60 is found only in the mitochondria; however in mammalian cells under certain conditions about 15–20% of the proteins were reported to be outside the mitochondria in proximity with the plasma membrane, endoplasmic reticulum (ER), peroxisomes, and unidentified cytoplasmic granules/vesicles [47]. Thus Hsp60 may participate in other cellular activities apart from being a mitochondrial chaperone. Indeed Hsp60 participates in a number of intra/extracellular activities, with a role in the control of cell survival and cell death. How Hsp60 is localized outside mitochondria is unknown. A recent study suggests that under certain conditions Hsp60 can be released from mitochondria without disruption of either the outer or the inner mitochondrial membrane yet how this may occur has not been determined or even speculated [48].

A similar situation is found for the human mitochondrial mtHsp70 homologue, mortalin (see Review in this issue by Azem et al.). In contrast to normal cells, mortalin was found to be over-expressed and to be localized in the cytosol of Parkinson, Alzheimer and cancerous cells [49]. In fact, mortalin has been suggested to perform extra-mitochondrial functions and has been shown to associate with a number of unrelated cytosolic proteins [49,50]. Again, as in the case of Hsp60, how this matrix localized mortalin ends up in the cytosol is unknown.

3. Rethinking aspects of dual localization

As pointed out in the Introduction, our conventional definition of dual targeted mitochondrial proteins refers to situations in which one of the isoproteins is translocated through/into a mitochondrial membrane and the second isoprotein is localized to a different compartment of the cell. Here we briefly refer to other aspects of dual targeting that may not always fit our initial definition. In this section we chose to present some interesting examples that expand our point of view on the phenomenon of dual localization (Table 2).

3.1. Alternative topogenesis

A protein that is distributed between two subcompartments of the same organelle is considered to display alternative topogenesis. While this does not fit our simple definition of distribution between two separate compartments of a cell, the mechanisms by which this can occur are clearly related to the topic of this review.

The *S. cerevisiae* MCR1 gene encodes two mitochondrial isoforms of NADH–cytochrome b5 reductase: a 34-kDa isoform that is localized to the outer membrane of mitochondria, and a 32-kDa isoform that reaches the intermenbrane space [51]. The first 12 residues of Mcr1 function as a classical MTS, which is followed by a hydrophobic sequence. The small isoform crosses the outer membrane via the translocase of the outer mitochondrial membrane (TOM) complex, and after interaction with the translocase of the inner mitochondrial membrane (TIM) complex, is cleaved by the inner membrane protease (IMP). The other form is tail-anchored in the outer membrane by a process that does not require any of the TOM components of the outer membrane. Thus, there is a competition of the two independent import pathways, which determine the relative amounts of the two forms.

A second protein, which is localized to more than one compartment within mitochondria, is the yeast dynamin-like GTPase Mgm1. This protein is distributed between the inner membrane and the intermembrane space of mitochondria [52]. The presequence of Mgm1 during translocation through the mitochondrial membranes is exposed to the matrix and the N-terminal hydrophobic segment becomes embedded in the inner membrane by a translocation-arrest mechanism. Subsequently, part of this full-length Mgm1 (l-Mgm1) molecules undergoes a second proteolytic cleavage by the mitochondrial rhomboid protease Pcp1. The resulting shorter, small product, s-Mgm1, is released into the intermembrane space.

3.1.1. Tissue-specific targeting

Our simple definition of dual targeting refers to situations in which a protein is localized to more than one compartment within a single cell. Nevertheless, the mechanisms of dual targeting can be employed to accomplish tissue-specific targeting; the protein is localized to a single subcellular compartment but this is a different compartment in different tissues. The enzyme glutamine synthetase in organisms such as dogfish shark and chicken is located in different compartments according to tissue and function. In the liver of these organisms, the protein catalyzes ammonia detoxification, while in neural tissues it functions in the recycling of the neurotransmitter glutamate. Although a single gene encodes this protein, its tissue-specific dual targeting in the different species is achieved by different mechanisms; in dogfish shark two different transcripts are generated by tissue-specific alternative splicing. The liver transcript contains an alternative exon that is not present in the neural one. This exon contains an additional upstream in-frame start codon that leads to the formation of a mitochondrial-targeting signal [53]. In contrast, in chicken the single translation product harbors a “weak” mitochondrial-targeting signal. The suggested mechanism is that targeting and translocation of this “weak” signal is dependent on the more negative mitochondrial membrane potential of the liver versus brain cells. Thus, tissue-specific differences in membrane potential lead to localization of glutamine synthetase in the mitochondria of liver cells and in the cytosol of brain cells [54].

Table 2
Examples of unconventional dual targeting.

Process	Example in this review	Mechanism	Genes	mRNAs	Translation products
Alternative topogenesis	Mcr1	One product is tail-anchored into the OM; the second is stop transfer embedded in IM.	1	1	1
	Mgm1	Full length product is embedded in the IM and a cleaved product is released to the IMS.	1	1	1
Tisssue-specific targeting	GS (DF Shark)	Two transcrpits by alternative splicing; one includes an MTS, and a shorter one lacks it.	1	2	2
	GS (chicken)	The protein harbors a “weak” MTS whose translocation depends on the IM potential.	1	1	1
From external genomes	UL37 (CMV)	Ambiguous targeting signal to the ER and mitochondria.	1	1	1
	US9 (CMV)	Two targeting signals to the ER and mitochondria on the same protein.	1	1	1
	Map (EPEC)	Incomplete import into mitchondria leaves a subpopulation in the cytosol.	1	1	1
Retargeting of a protein	p53	Retargeting of a protein from the cytosol to mitchondria due to binding to Tid1, BclZ or proteolitic cleavage that exposes an MTS.	1	1	1

OM, Outer membrane; IM, Inner membrane; MTS, Mitochondrial targeting sequence.

Cyclic AMP response element-binding protein (CREB) is a member of a family of transcription factors (CREB/ATF family). This transcription factor is important for intracellular signal transduction systems and it plays a role in the plasticity, growth, and survival of neurons. In addition to its function in the nucleus, recently it has been shown that CREB is also localized in the mitochondrial matrix of neurons. CREB can specifically bind mitochondrial DNA and it can affect mitochondrial transcription, leading to expression of mitochondrial genes and neuronal survival [55,56]. Nevertheless the full mechanism allowing the mitochondrial import of the protein only in neurons is still unknown.

3.1.2. Dual targeting of proteins encoded by external genomes

Dual targeting of proteins is one of the evolutionary solutions of eukaryotic cells to expand the quantity of protein functions using the same amount of genes. It is fascinating to see that some pathogens that are constrained by the number of genes their genomes encode, use similar tricks of dual targeting.

The human cytomegalovirus (CMV) encodes two dual targeted proteins. The first is the immediate-early protein UL37 exon 1 (pUL37x1), which is dual localized to mitochondria and ER. An ambiguous signal for targeting is located at the N′ terminus of the protein which is required for targeting to both organelles [57]. The second protein, US9 is also dual targeted and localized to mitochondria and the ER. Targeting of this protein is due to two distinct targeting sequences: an ER targeting signal peptide at the N′ terminus, and a mitochondrial-targeting sequence located at the C′ terminus [58].

The EPEC (enteropathogenic *Escherichia coli*) protein Map is injected directly into the eukaryotic host cytoplasm via a type III secretion system. There are a number of cytoplasmic functions associated with Map such as its ability to trigger Cdc42-dependent actin rearrangements and disruption of the epithelial barrier function. The first 44 residues of Map are essential for targeting to mitochondria in a TOM, mtHsp70 and membrane potential dependent manner. It was suggested that incomplete Map import might be employed to regulate the levels of mitochondrial versus cytosolic isoproteins. This regulation may be required to attenuate Map cytoplasmic functions, which bear toxicity towards the eukaryotic host. However, the identification of a specific effect for Map on yeast mitochondrial morphology indicates a specific function within these organelles [59].

3.1.3. Retargeting of an isoprotein

Is dual or exclusive localization of a protein to specific compartments in the cell a dead end (unchangeable) process? In fact protein relocalization is one of the mechanisms that should allow response to changes within the cell or in its environment. One example is TERT, the catalytic subunit of telomerase, which plays a role in counteracting telomere shortening. Nevertheless, there is evidence for a TERT function that is telomere-length-independent. Recently it had been shown that upon oxidative stress, TERT is excluded from the nucleus and imported into mitochondria. Import of TERT into mitochondria improves mitochondrial function and protects mitochondrial DNA from reactive oxygen species (ROS) [60]. An N-terminal targeting sequence enables import of TERT into mitochondria, but the full mechanism regulating nuclear import (rather than mitochondrial import) and then nuclear export is still unknown.

A second example for protein relocalization is P53, which induces apoptosis both by regulation of its target genes and by signaling pathways that are transcription independent. As a transcription factor p53 mediates apoptosis by transcription activation of pro-apoptotic genes or repression of anti-apoptotic genes. In addition to its transcription it has been shown that p53 under specific cellular conditions is targeted to mitochondria where it forms an inhibitory complex with the protective BclXL and Bcl2 proteins, via its DNA binding domain (Fig. 3*iii*). These results explain how mutations in p53 have tumor suppression

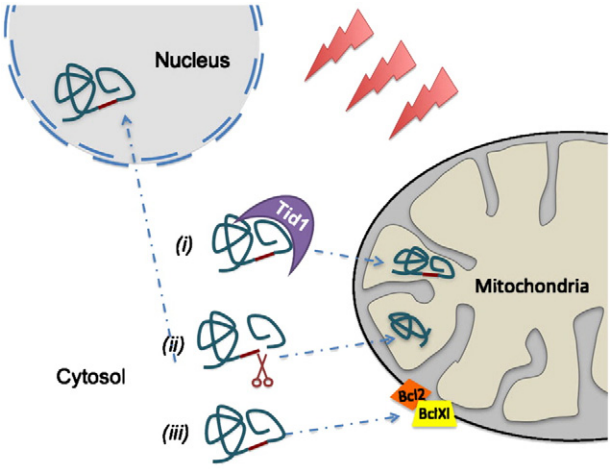

Fig. 3. P53 retargeting to mitochondria. Under stress conditions p53 is stabilized in the cytosol and targeted both to the nucleus and to mitochondria. Targeting and import of p53 to/into mitochondria has been proposed to be due to *(i)* binding to Tid1, which harbors an MTS and can somehow promote p53 import, *(ii)* proteolytic cleavage of p53 that exposes an internal MTS or *(iii)* binding of p53 to the Bcl2 complex on the outer membrane.

activity both via the transcriptional and mitochondrial apoptosis pathways [61]. In addition to this interaction with outer membrane receptors, and most relevant to this review, are recent reports that claim that p53 is translocated into mitochondria, as part of its function as an apoptosis inducer: i) A recent publication has shown that under hypoxic conditions p53 specifically binds to Tid1 (Fig. 3*i*). Tid1 harbors a specific N-terminus mitochondrial-targeting sequence, which under these conditions is suggested to allow translocation of both proteins to mitochondria by an unknown mechanism [62]. ii) Intriguingly p53 also was found to be a substrate of a specific ser endoprotease. This protease, which also cleaves CYP1A1 (as described here earlier), cleaves p53 to a 40 kDa fragment and exposes a cryptic mitochondrial-targeting sequence, suggesting a mechanism for p53 translocation into mitochondria (Fig. 3*ii*). iii) p53 has been suggested to regulate the mitochondrial mtDNA copy number. Although the mechanism which allows p53 translocation into mitochondria under these conditions is not clear, this case suggests a function for p53 in mitochondria [63]. Taken together, p53 may constitute an example of an extramitochondrial protein that under specific conditions is recruited to and translocates into the organelle.

3.2. Perspectives and concluding remarks

We have come a long way from the central dogma of molecular biology postulating the flow of genetic information, “from one gene to one mRNA and one protein” and its derivative assumptions of “one subcellular location and one function”. Today it is clear that a single gene can encode multiple protein products, and as discussed in this review, there is substantial information on the fascinating mechanisms of dual targeting whether these involve multiple or single translation products. As discussed in this review it is intriguing that the isoproteins may have different activities and functions in each location. Moreover, we now understand that dual targeting is a dynamic process and that the relative levels of isoproteins can be rebalanced and that an isoprotein can be retargeted to a completely different compartment. This allows the cells an important flexibility and the ability to rapidly react to varying conditions.

What is surprising is the estimation of high abundance of protein dual targeting in eukaryotic cells ([64] and our unpublished results); about a quarter of the yeast mitochondrial proteome is predicted to be dual targeted. This surge in the identification of dual targeted proteins and their mechanisms of distribution is the result of the development of new and more sensitive methods. In addition to GFP and epitope

tagging approaches, and subsequent impressive genomic screens [65–69], mass spectrometry has led to a wide and more accurate estimation of the whole yeast mitochondrial proteome [70,71]. A good example of the need for more sensitive methods is "eclipsed distribution"; the amount of one of the isoproteins, in one of the locations, is minute and its detection by standard biochemical and visualization methods is masked by the presence of the dominant isoprotein [7]. Split genes and specific isoprotein depletion approaches are examples of methods that have been developed to identify such eclipsed distribution [72,73].

Single gene studies, genomic screens referred to above and novel bioinformatic analysis of mitochondrial genes/proteins, have led to the estimation of dual targeting as a highly abundant phenomenon. These studies, mainly in yeast and plants suggest that the phenomenon of dual targeting is indeed much more abundant than initially estimated and in fact hundreds of mitochondrial proteins are predicted to be dual localized [64,74]. These findings should reshape our opinion regarding protein subcellular localization and our full understanding of gene expression in eukaryotes.

As for the future we certainly need to decipher the mechanism of known dual targeted proteins as for example in the cases of Hsp60 and mtHsp70 (mortalin); are they exported or released from the matrix or do they never fully enter the mitochondrial matrix? In parallel, our expectation is that innovative single gene studies will lead to the identification of novel mechanisms of dual targeting some of which we may have considered improbable. Another expectation is that additional genomic screens, using novel approaches, will be employed in order to get a better grasp on how widespread the phenomenon of dual targeting really is.

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