Mitochondrial Protein Synthesis, Import, and Assembly

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ABSTRACT The mitochondrion is arguably the most complex organelle in the budding yeast cell cytoplasm. It is essential for viability as well as respiratory growth. Its innermost aqueous compartment, the matrix, is bounded by the highly structured inner membrane, which in turn is bounded by the intermembrane space and the outer membrane. Approximately 1000 proteins are present in these organelles, of which eight major constituents are coded and synthesized in the matrix. The import of mitochondrial proteins synthesized in the cytoplasm, and their direction to the correct soluble compartments, correct membranes, and correct membrane surfaces/topologies, involves multiple pathways and macromolecular machines. The targeting of some, but not all, cytoplasmically synthesized mitochondrial proteins begins with translation of messenger RNAs localized to the organelle. Most proteins then pass through the translocase of the outer membrane to the intermembrane space, where divergent pathways sort them to the outer membrane, inner membrane, and matrix or trap them in the intermembrane space. Roughly 25% of mitochondrial proteins participate in maintenance or expression of the organellar genome at the inner surface of the inner membrane, providing 7 membrane proteins whose synthesis nucleates the assembly of three respiratory complexes.

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TO think about how mitochondrial proteins are synthesized, imported, and assembled, it is useful to have a clear picture of the organellar structures that they, along with membrane lipids, compose and the functions that they carry out. As almost every schoolchild learns, mitochondria carry out oxidative phosphorylation, the controlled burning of nutrients coupled to ATP synthesis. Since *Saccharomyces cerevisiae* prefers to ferment sugars, respiration is a dispensable function and nonrespiring mutants are viable [although they cannot undergo meiosis (Jambhekar and Amon 2008)]. However, mitochondria themselves are *not* dispensable. A substantial fraction of intermediary metabolism occurs in mitochondria (Strathern *et al.* 1982), and at least one of these pathways, iron–sulfur cluster assembly, is essential for growth (Kispal *et al.* 2005). Thus, any mutation that prevents the biogenesis of mitochondria by, for example, preventing the import of protein constituents from the cytoplasm, is lethal (Baker and Schatz 1991).

The mitochondria of *S. cerevisiae* are tubular structures at the cell cortex. While the number of distinct compartments can range from 1 to ~50 depending upon conditions (Stevens 1981; Pon and Schatz 1991), continual fusion and fission events among them effectively form a single dynamic network (Nunnari *et al.* 1997). The outer membrane surrounds the tubules. The inner membrane has a boundary domain closely juxtaposed beneath the outer membrane and cristae domains that project internally from the boundary into the matrix (Figure 1A). The matrix is the aqueous compartment surrounded by the inner membrane. The aqueous intermembrane space lies between the membranes and is continuous with the space within cristae.

Inner membrane cristae are often depicted as baffles emanating from the boundary domain. However, electron tomography of mitochondria from several species, including yeast, shows that cristae actually emanate from the boundary membrane as narrow tubular structures at sites termed "crista junctions" and expand as they project into the matrix (Frey and Mannella 2000; Mannella *et al.* 2001) (Figure 1B). It seems clear that the boundary and cristae domains of the inner membrane have distinct compositions with respect to the respiratory complexes that are embedded preferentially in the cristae membrane domains, as well as other components (Vogel *et al.* 2006; Wurm and Jakobs 2006; Rabl *et al.* 2009; Suppanz *et al.* 2009; Zick *et al.* 2009; Davies *et al.* 2011).

The outer and inner boundary membranes are connected at multiple contact sites, at least some of which are involved in protein translocation and may be transient (Pon and Schatz 1991). In addition, there appear to be firm contact sites, not directly involved with protein translocation, preferentially colocalized with crista junctions (Harner *et al.* 2011a).

Overall, there appear to be ~1000 distinct proteins in yeast mitochondria (Premsler *et al.* 2009). One series of proteomic studies on highly purified organelles identified 851 proteins thought to represent 85% of the total number of species (Sickmann *et al.* 2003; Reinders *et al.* 2006; Zahedi *et al.* 2006). Another study identified an additional 209 candidates (Prokisch *et al.* 2004). A computationally driven search for candidates involved in yeast mitochondrial function, coupled with experiments to assay respiratory function and maintenance of mitochondrial DNA (mtDNA), identified 109 novel candidates, although many of these may not be mitochondrial *per se* (Hess *et al.* 2009). Taking the boundary and cristae domains together, the inner membrane is the most protein-rich mitochondrial compartment, followed by the matrix (Daum *et al.* 1982).

Only eight of the yeast mitochondrial proteins detected in proteomic studies are encoded by mtDNA and synthesized within the organelle. They are hydrophobic subunits of

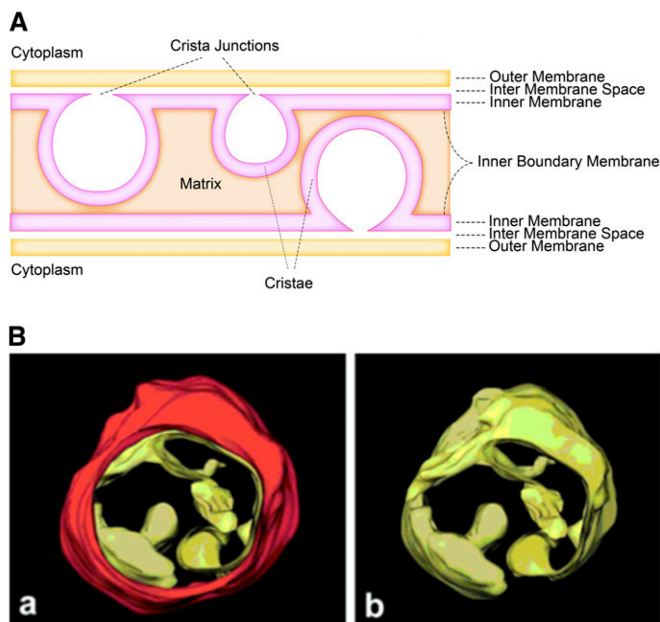

Figure 1 Overview of mitochondrial structure in yeast. (A) Schematic of compartments comprising mitochondrial tubules. The outer membrane surrounds the organelle. The inner membrane surrounds the matrix and consists of two domains, the inner boundary membrane and the cristae membranes, which are joined at cristae junctions. The intermembrane space lies between the outer membrane and inner membrane. (B) Electron tomograph image of a highly contracted yeast mitochondrion observed en face (a) with the outer membrane (red) and (b) without the outer membrane. Reprinted by permission from John Wiley & Sons from Mannella *et al.* (2001).

respiratory complexes III (bc_1 complex or ubiquinol-cytochrome *c* reductase), IV (cytochrome *c* oxidase), and V (ATP synthase), as well as a hydrophilic mitochondrial small subunit ribosomal protein. The remaining ~99% of yeast mitochondrial proteins are encoded by nuclear genes, synthesized in cytoplasmic ribosomes, and imported into the organelle.

An overview of known nuclearly encoded mitochondrial protein functions (Figure 2) reveals that ~25% of them are involved directly in genome maintenance and expression of the eight major mitochondrial genes (Schmidt *et al.* 2010). The functions of ~20% of the proteins are not known. Fifteen percent are involved in the well-known processes of energy metabolism. Protein translocation, folding, and turnover functions occupy ~10% of mitochondrial proteins.

The following discussion reviews our understanding of the biogenesis of mitochondria starting on the outside, the cytoplasm, and working inward through the mitochondrial compartments.

Cytoplasmic Synthesis of Mitochondrial Proteins

Localization of some cytoplasmic messenger RNAs to mitochondria promotes import of the proteins that they encode

Expression of nuclear genes coding mitochondrial proteins begins with the transcription of messenger RNAs (mRNAs). (Mechanisms controlling the synthesis of these mRNAs are beyond the scope of this review.) It has been known for some time that the synthesis of proteins destined to reside in mitochondria can occur on polysomes bound to mitochondria or on other polysomes, usually referred to as "free polysomes" (Kellems *et al.* 1975; Ades and Butow 1980; Suissa and Schatz 1982). More recently, surveys of the intracellular locations of specific mRNAs encoding the bulk of the mitochondrial proteome have indicated a range, with approximately half of them selectively translated at the surface of the outer membrane, while translation of others occurs selectively on free polysomes or is not biased between mitochondrial and cytoplasmic locations (Corral-Debrinski *et al.* 2000; Marc *et al.* 2002; Garcia *et al.* 2007a; Saint-Georges *et al.* 2008; Gadir *et al.* 2011) (Figure 3). It is easy to imagine that the biological rationale for localized synthesis of organellar proteins is to promote their efficient import and assembly. The rationale for synthesizing roughly half of mitochondrial proteins on free cytoplasmic polysomes remains to be discerned.

What directs and tethers so many mitochondrially bound mRNAs to the outer surface of the organelles? Current evidence indicates the involvement of nucleotide signals in mRNA 3′-untranslated regions (3′-UTRs) that function prior to translation. In addition, the familiar (Pon and Schatz 1991) mitochondrial targeting signals in the amino acid sequences of the precursor proteins that the mRNAs encode also appear to contribute mRNA localization by mechanisms that are at least partially redundant—and poorly understood (Lithgow 2000).

The *ATM1* mRNA, which encodes an essential inner membrane transporter protein, is among those that are highly enriched on mitochondrial-bound polysomes (Corral-Debrinski *et al.* 2000). By examining the localization of chimeric mRNAs, it was shown that the *ATM1* 3′-UTR was sufficient to direct mitochondrial localization of a reporter mRNA lacking any other mitochondria-related signals, although this did not lead to import of the GFP reporter protein itself into mitochondria. The 3′-UTR of the *PGK1* mRNA, which encodes a soluble cytoplasmic protein, did not direct localization of the mRNA to mitochondria (Corral-Debrinski *et al.* 2000).

The physiological significance of mRNA localization signals in 3′-UTRs was demonstrated by a study in which the 3′-UTR of the *ATP2* mRNA, which encodes the β-subunit of the F_1 ATP synthase, was replaced by 3′-UTR of the *ADH1* mRNA by alteration of the chromosomal *ATP2* locus (Margeot *et al.* 2002). This alteration prevented normal growth on nonfermentable carbon sources, presumably due to decreased ATP synthase activity. The swap of 3′-UTRs did not affect the overall steady-state level of *ATP2* mRNA. However, it did cause a large reduction in the fraction of *ATP2* mRNA associated with mitochondria and a large increase of this mRNA in the free polysomal fraction.

The behavior of the Atp2 precursor protein translated from the altered mRNA was particularly interesting: while

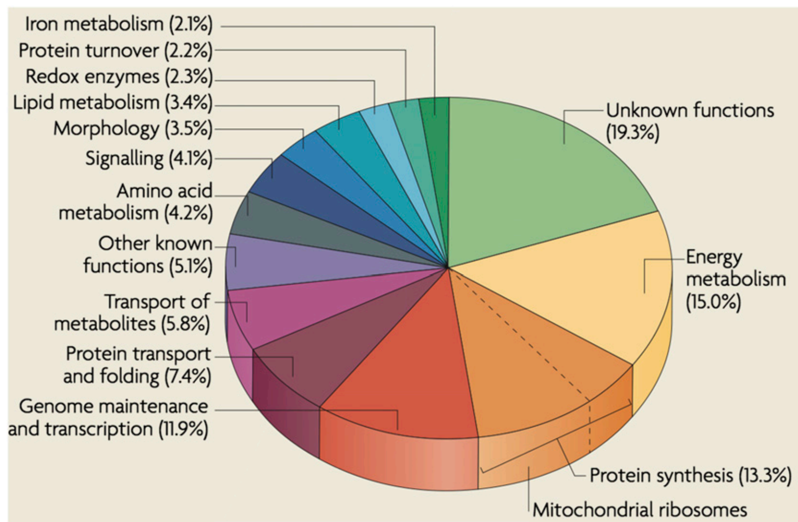

Figure 2 Classification of identified mitochondrial proteins according to function. Reprinted by permission from Nature Publishing Group from Schmidt *et al.* (2010).

normal levels of protein were associated with mitochondria, it was overwhelmingly in the larger precursor form, retaining the 34-amino-acid N-terminal targeting signal (Margeot *et al.* 2002). This contrasted with Atp2 translated from the wild-type mRNA, which was overwhelmingly in the mature processed form. Furthermore, the pre-Atp2 protein translated from the altered mRNA appeared to be on the outside of the outer membrane. Taken together, these results suggest that localization of wild-type *ATP2* mRNA to mitochondria via signals in its 3′-UTR promotes synthesis of an import-competent pre-Atp2 polypeptide. Synthesis of pre-Atp2 from an mRNA lacking this 3′-UTR on free polysomes yields a protein that binds the outer surface of mitochondria but fails to be imported efficiently, presumably due to altered structure or interactions with other proteins. Thus, mRNA localization directed by a signal in the *ATP2* mRNA 3′-UTR promotes efficient translocation of the protein into mitochondria.

Based on this observation, one should use caution when conducting genetic experiments involving alteration of mitochondrial proteins by methods that also alter the 3′-UTRs of their mRNAs.

A large-scale survey of mRNA abundance in mitochondrially bound polysomes *vs.* free polysomes was carried out by hybridization to microarrays. After corrections for the amount of mRNA in each fraction, and for cross-contamination of fractions, the degree to which mRNAs encoding the yeast proteome are selectively localized to mitochondria was determined (Garcia *et al.* 2007b). The *ATP2* mRNA, for example, ranks among the most highly localized to mitochondria, with 50% of the total bound to the organelles. At the other extreme of the distribution, none of the *COX4* mRNA encoding cytochrome *c* oxidase subunit IV was found among mitochondria-bound polysomes (Figure 3). The degree of mitochondrial association for each mRNA was then scored by the rank of its ratio among all mRNAs (Garcia *et al.* 2007b). About half of the 423 mRNAs known to encode mitochondrial proteins at the time of this study were found to be preferentially associated with mitochondria, while relatively few mRNAs encoding known non-mitochondrial proteins were in this group (Marc *et al.* 2002). The subcellular mRNA distributions observed using genomic microarrays were confirmed by a focused study on 112 mRNAs encoding protein components of known mitochondrial complexes, using quantitative PCR to assay the fractions (Garcia *et al.* 2007a).

The notion that the strong association of specific mRNAs with mitochondria is correlated with the behavior of the proteins that they encode received striking support from a careful proteomic study of highly purified vesicles derived from the mitochondrial outer membrane (Zahedi *et al.* 2006). Forty-nine proteins, with a wide range of abundance, were found to be exposed on the outer surface of mitochondria. Surprisingly, 36 of these surface proteins were already well known to actually reside in internal mitochondrial compartments. Two such anomalous surface proteins, Atp2 and Cyb2, were experimentally shown to be unprocessed precursors of the mature internal proteins, and it is likely that at least several others are as well (Zahedi *et al.* 2006). Thus, a significant group of precursors, possibly awaiting import to internal destinations and processing, are bound to the surface of mitochondria. Significantly, 16 of these proteins are encoded by the 25 mRNAs most selectively localized to mitochondrial-bound polysomes as determined by Marc *et al.* (2002). Zahedi *et al.* (2006) performed comparisons of the entire yeast proteome, the mitochondrial proteome, and the internal mitochondrial proteins detected on the surface of the outer membrane, with the degree of mitochondrial localization of all yeast mRNAs as scored by rank (Marc *et al.* 2002). These distributions demonstrated a strong bias for localized mRNAs to encode internal proteins that were also detected at the surface.

Taken together, these findings appear to be paradoxical. If the purpose of localized translation at the surface of mitochondria is to promote efficient cotranslational import of proteins, then why should the locally synthesized proteins

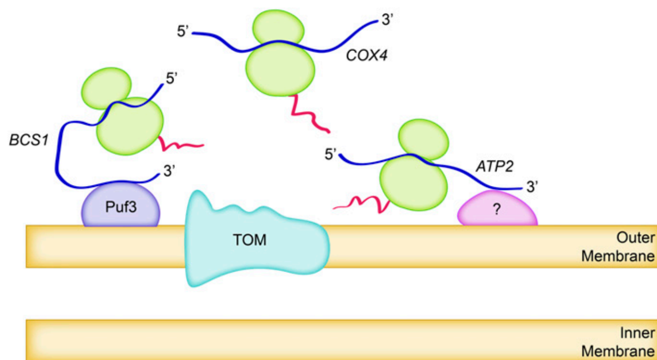

Figure 3 Cytoplasmic synthesis of some mitochondrial proteins is localized to the organelles, while the synthesis of others is not. The figure depicts three examples: (1) The *ATP2* mRNA is highly localized to mitochondria-bound polysomes (Garcia *et al.* 2007b), although factors required for this localization are unknown. (2) The *BCS1* mRNA is also selectively found in mitochondria-bound polysomes, and its localization is partially dependent upon the mitochondrially localized RNA-binding protein Puf3 and the Puf3-binding sites in its 3′-UTR (Saint-Georges *et al.* 2008). (3) The *COX4* mRNA is exclusively found on free polysomes, unassociated with mitochondria (Garcia *et al.* 2007b). The Atp2, Bcs1, and Cox4 proteins all traverse the outer membrane via the TOM complex pore.

be preferentially found among the full-length unimported and unprocessed species detected on the organellar surface? Such molecules were clearly not cotranslationally imported. Perhaps these proteins, as a group, tend to rapidly adopt folded conformations that inhibit translocation. In this case, localized synthesis could be an adaptation that alleviates this problem, albeit incompletely, by facilitating cotranslational import of a significant fraction of molecules. It is currently unknown whether the fully synthesized precursor proteins bound to the mitochondrial outer surface are destined to be imported or degraded (Zahedi *et al.* 2006). The observation that unprocessed pre-Atp2 accumulates to an abnormally high level outside of mitochondria in cells translating a chimeric *ATP2* mRNA lacking the localization signal in its 3′-UTR (Margeot *et al.* 2002) is consistent with the possibility that post-translational import of pre-ATP2 is inefficient *in vivo*, despite the fact that it occurs *in vitro* (Maccecchini *et al.* 1979). Perhaps the pre-ATP2 molecules detected on the surface of mitochondria were actually translated from those *ATP2* mRNA molecules, ~50% of the total, that were *not* localized to mitochondria-bound polysomes. In any event, it is clear that localized synthesis of pre-ATP2 somehow facilitates its import.

Another fascinating but imperfect correlation emerged from the ranking of mRNAs by their propensity to be mitochondrially localized. Those mRNAs found most selectively in mitochondria-bound polysomes tend to encode proteins whose evolutionary origins can be clearly traced to Bacteria and/or Archaea. Conversely, those mRNAs found most selectively in free polysomes tend to encode proteins lacking clear homologs in those phylogenetic domains and are therefore likely to be more recently evolved inventions of Eukarya (Marc *et al.* 2002; Garcia *et al.* 2007a). This correlation runs in parallel with the observation that the more locally synthesized proteins tend to be either ancient, conserved components of the mitochondrial genetic system or respiratory complexes or conserved proteins with roles in assembly of those core components (Margeot *et al.* 2005). In the case of cytochrome *c* oxidase, for example, proteins with bacterial orthologs that assemble mitochondrially coded core subunits in the inner membrane, insert metal cofactors, and synthesize the specific heme A cofactor are all selectively translated at the mitochondrial surface (although they do not all have clear bacterial or archaeal ancestors). In contrast, the eukaryotic-specific subunits of cytochrome *c* oxidase that surround the catalytic core of the enzyme are all selectively translated on free polysomes.

There are no obvious structural or chemical similarities among the set of proteins most selectively synthesized at the mitochondrial surface (Marc *et al.* 2002). So, what selective constraints maintain the localized translation of more anciently evolved proteins? It has been argued that synthesis localized to mitochondria may promote efficient assembly of core components of complexes by directing import of proteins to specific regions (Margeot *et al.* 2005; Garcia *et al.* 2007a). While this is an attractive hypothesis, there is no strong evidence that localized synthesis of any mitochondrial protein is spatially organized on the organellar surface. Nor is it obvious why nonlocalized translation of other essential but peripheral subunits of complexes would be advantageous. Interestingly, the mitochondria-bound mRNAs tend to be synthesized early during the yeast metabolic cycle (Tu *et al.* 2005; Lelandais *et al.* 2009).

Complex mechanisms for mRNA localization

Regardless of why some mRNAs are localized to mitochondria while others are not, the example of *ATP2* demonstrates the importance of mRNA targeting for mitochondrial biogenesis. How are localized mRNAs brought and tethered to the organelles? mRNA 3′-UTRs contain information for localization in at least eight cases that have been experimentally examined (Corral-Debrinski *et al.* 2000; Marc *et al.* 2002; Margeot *et al.* 2002).

One factor with apparent roles in localization of many mRNAs encoding mitochondrial proteins is Puf3, a member of the Pumilio-homology domain family (PUF) of RNA-binding proteins. PUF family proteins are found in a wide variety of eukaryotes and carry out a wide variety of functions through their ability to mediate interactions between target RNAs and other proteins (Quenault *et al.* 2011). An initial survey of yeast PUF protein functions (Olivas and Parker 2000) revealed that a *puf3*Δ mutation strongly affected the *COX17* mRNA, which encodes a (Eukarya-specific) mitochondrial copper-binding protein required for cytochrome *c* oxidase assembly (Glerum *et al.* 1996). The presence of Puf3 was shown to stimulate deadenylation and degradation of *COX17* mRNA, but did not affect its translation as measured by the degree of polysome association or the level of accumulated Cox17 protein.

A genomic investigation of RNAs bound to Puf3 (as well as other members of the yeast Puf protein family) revealed a striking specificity: among 154 Puf3-binding mRNAs of known function, 135 encoded mitochondrial proteins (Gerber *et al.* 2004). Furthermore, mitochondrial proteins coded by 80 of the Puf3-binding mRNAs have roles in organellar translation (*e.g.*, mitochondrial ribosomal proteins) while most of the rest participate in post-translational assembly functions. A Puf3-binding sequence was identified in the 3′-UTRs of these mRNAs (Gerber *et al.* 2004). This site occurs twice in the *COX17* mRNA, and those sites are necessary for Puf3-dependent mRNA destabilization (Jackson *et al.* 2004).

The Puf3 protein itself was found to be located on the outer surface of mitochondria and visualized in puncta largely associated with mitochondrial tubules (Garcia-Rodriguez *et al.* 2007). Puf3 was also associated with Mdm12, a protein component of the tether that connects distinct sites on mitochondria with the endoplasmic reticulum (Garcia-Rodriguez *et al.* 2007; Kornmann *et al.* 2009). Consistent with a role in promoting degradation of mRNAs required for respiratory metabolism, and thereby affecting the production of respiratory complexes, overproduction of Puf3 caused a modest reduction in the growth of cells on the nonfermentable carbon source glycerol (Garcia-Rodriguez *et al.* 2007) and oxygen consumption (Chatenay-Lapointe and Shadel 2011). Log-phase cells lacking Puf3 contained elevated levels of respiratory complex subunits and exhibited increased rates of oxygen consumption (Chatenay-Lapointe and Shadel 2011). However, Puf3 appears to be more than simply a post-transcriptional repressor of mitochondrial functions since a *puf3*Δ also produces a very modest defect in growth on glycerol (Gerber *et al.* 2004).

Taken together, these findings suggest the possibility that Puf3 could have a direct role in localizing a number of mRNAs to the mitochondrial surface. Consistent with this possibility, a majority of those Puf3-binding mRNAs that encode known mitochondrial proteins (Gerber *et al.* 2004), and were examined for subcellular distribution (Marc *et al.* 2002), were among those selectively localized to mitochondria. Overall, it appears that about half of the mRNAs selectively localized to mitochondria contain Puf3-binding sites in their 3′-UTRs, and the localization of about half of those is significantly decreased in the absence of Puf3 (Saint-Georges *et al.* 2008). In addition, mutation of the Puf3-binding site in one such mRNA (*BCS1*) reduced its selective association with mitochondria by a factor of two as assayed both by quantitative PCR of the two polysome fractions and by quantitation of RNA granule location cytologically in FISH images (Saint-Georges *et al.* 2008) (Figure 3).

Similar results were obtained for a set of 24 mRNA-encoding mitochondrial proteins that were tagged with binding sites for an RNA-binding GFP fusion protein and visualized in granules (Gadir *et al.* 2011). These images suggest the possibility that mRNAs bound to the surface of mitochondria may not be evenly distributed on the organellar surface. Experiments to test whether these RNA granules colocalize with Puf3 puncta on mitochondria have not been reported. However, if they do, the fact that Puf3 associates with the mitochondrial-ER tether protein Mdm12 (Garcia-Rodriguez *et al.* 2007; Kornmann *et al.* 2009) would be consistent with reported partial colocalization of mitochondrial mRNA granules with ER (Gadir *et al.* 2011). Such localization of protein synthesis and presumably import could facilitate assembly of mitochondrial complexes, for example, mitochondrial ribosomes (Saint-Georges *et al.* 2008).

It is important to bear in mind that Puf3 promotes degradation of at least some mitochondrially localized mRNAs (Olivas and Parker 2000; Jackson *et al.* 2004; Foat *et al.* 2005). Thus, even if Puf3 were not directly involved in localization, an mRNA stabilized in the absence of Puf3, or by mutation of its Puf3-binding site, could appear to be less selectively bound to mitochondria as measured by the ratio of its presence in bound *vs.* free polysomes. This would occur if an RNA's abundance increased sufficiently to saturate other limiting localization factors on the organelle surface. The extent to which altered RNA stability may contribute to Puf3 dependence of localization has not been systematically explored. Nevertheless, it seems likely that Puf3 binding contributes directly to localization of those mRNAs bearing its binding site in addition to influencing their rates of degradation. How the interplay between these two activities influences protein import and assembly of mitochondrial complexes remains an open question (Quenault *et al.* 2011).

The existence of distinct mechanisms for RNA sequence-based mRNA recognition is indicated by the fact that the *ATM1* and *ATP2* mRNAs, whose 3′-UTRs clearly cause mitochondrial localization, lack Puf3-binding sites (Corral-Debrinski *et al.* 2000; Margeot *et al.* 2002; Saint-Georges *et al.* 2008). Selection of variant sequences derived from the *ATP2* 3′-UTR that functionally localize the mRNA suggest that both nucleotide sequence and secondary structural features play a role in its recognition (Liu and Liu 2007). However, no protein or other species that interact with this RNA element have been identified (Figure 3). Interestingly, the absence of Puf3 may reduce mitochondrial localization of the *ATP2* mRNA, presumably by an indirect mechanism (Gadir *et al.* 2011), although this observation is inconsistent with an earlier report (Saint-Georges *et al.* 2008).

Tethering of mRNAs to mitochondria via nascent polypeptide chains

Actively translated mRNAs can be tethered to membranes via nascent polypeptide chains undergoing cotranslational membrane translocation. This appears to occur in the case of at least some mRNAs localized to mitochondria. A chimeric mRNA encoding the Atm1 N-terminal mitochondrial targeting signal fused to GFP, but with the *ATM1* 3′-UTR replaced by the *PGK1* mRNA 3′-UTR, localized to mitochondria (Corral-Debrinski *et al.* 2000). Thus, the wild-type *ATM1* mRNA on mitochondria appears to be localized both by an untranslated signal in its 3′-UTR and by the interaction of the polypeptide

Table 1 Components of the TOM complex: transport of proteins through the outer membrane

Protein	ORF	Known function	Null phenotype
Mim1	YOL026C	Insertion of transmembrane helix proteins into the outer membrane	Inviable
Mim2	YLR099W-A	Insertion of transmembrane helix proteins into the outer membrane	Inviable
Tom5	YPR133W-A		Viable, various defects
Tom6	YOR045W		Viable, various defects
Tom7	YNL070W		Viable, various defects
Tom20	YGR082W	Receptor for substrates with presequences	Viable, various defects
Tom22	YNL131W	Central receptor facing cytoplasm and IMS; interaction with TIM23 complex	Viable, various defects[a]
Tom40	YMR203W	Translocation channel–β-barrel structure	Inviable
Tom70	YNL121C	Receptor for substrates lacking presequences	Viable, various defects
Tom71	YHR117W	Receptor	Viable, various defects

[a] Null was inviable in large-scale studies, but is viable if obtained by loss of *TOM22* plasmid during mitotic growth (van Wilpe *et al.* 1999).

targeting signal with receptors on the outer mitochondrial surface and the protein import machinery.

In the case of an *ATP2* mRNA lacking its normal 3′-UTR, residual localization to mitochondria required translation of both the N-terminal targeting sequence and sequences within the mature protein itself (Garcia *et al.* 2010). Normal association of the wild-type *ATP2* mRNA also required one of the three Translocase of the Outer Membrane (TOM) complex outer membrane import receptor proteins, Tom70 (Table 1), and was reduced by mutation of the *ATP2* translation initiation codon (Gadir *et al.* 2011).

Deletion of another outer membrane import receptor protein, Tom20, was found to lower but not eliminate selective localization of most mRNAs associated with mitochondria (Eliyahu *et al.* 2010). While *tom20*Δ mutants are viable with a modest respiratory defect, and *puf3*Δ mutants are viable and almost wild type with respect to respiratory growth, a *tom20*Δ, *puf3*Δ double mutant was viable with a very tight respiratory defect. This synthetic respiratory phenotype is consistent with the picture of synergy in targeting of mRNAs to mitochondria by factors recognizing mRNA 3′-UTRs and the protein import machinery acting on nascent chains to promote efficient assembly of functional mitochondrial complexes. At the same time, the viability of the *tom20*Δ, *puf3*Δ double mutant demonstrates that protein import to mitochondria remains active by the action of partially redundant pathways for mRNA localization and precursor recognition.

Translocation and Membrane Insertion of Cytoplasmically Synthesized Mitochondrial Proteins

The distant ancestors of mitochondria were bacteria from the α-proteobacterial lineage (Gray *et al.* 2001). While the origins of all known extant eukaryotes trace back to organisms that contained both mitochondria and nucleo-cytoplasmic genetic systems related to Archaea, the events leading to endosymbiosis and the subsequent evolution of mitochondria as integrated cellular organelles have not been clearly discerned (Embley and Martin 2006). However, since bacteria are not known to import large polypeptides, their evolution into mitochondria apparently required the evolution of new mechanisms for the transport of cytoplasmically synthesized proteins across one or both of the mitochondrial (formerly bacterial) membranes. Some components of the present-day protein import machinery are clearly of bacterial origin. However, most appear to have distant bacterial homologs that do not participate in protein translocation or to have evolved *de novo* as endosymbionts became organelles (Dolezal *et al.* 2006; Kutik *et al.* 2009; Hewitt *et al.* 2011).

Transport of cytoplasmically synthesized mitochondrial proteins or their precursors across or into the outer membrane is carried out by the TOM complex, which includes both receptor proteins facing the cytoplasm and a pore in the membrane (Table 1). It is widely believed that the precursor proteins arrive at the organelle bound by chaperones and, in that state, are recognized by receptors of the TOM complex, although this has been demonstrated in only a few cases (Gautschi *et al.* 2001; Young *et al.* 2003).

Depending upon the nature of their targeting signals, proteins may be inserted into the outer membrane, translocated into the intermembrane space (IMS), or delivered to one of the two Translocase of the Inner Membrane (TIM) complexes for insertion into the inner membrane or translocation into the matrix (Pon and Schatz 1991; Neupert 1997; Voos *et al.* 1999). (While the nature of targeting signals for different compartments has been investigated intensively, it is important to note that they cannot be predicted solely from sequence information with a high degree of certainty.) A wide variety of different translocation and sorting events must be completed prior to, or concomitant with, the assembly of imported proteins into functional multimeric enzymes and higher-order complexes.

The literature on import of proteins into yeast mitochondria is extensive and has been extensively reviewed. Recent reviews present detailed descriptions of the components of import complexes and their functions (Young *et al.* 2003; Neupert and Herrmann 2007; Chacinska *et al.* 2009; Koehler and Tienson 2009; Mokranjac and Neupert 2009; Walther and Rapaport 2009; Endo and Yamano 2010; Schmidt *et al.* 2010; Dukanovic and Rapaport 2011; Gebert *et al.* 2011; Hewitt *et al.* 2011; Marom *et al.* 2011a; Riemer *et al.* 2011; Yogev and Pines 2011). Molecular structures of hydrophilic domains of proteins composing the import machinery are emerging, but as yet no full structures of

mitochondrial translocation complexes are available (Endo *et al.* 2011), precluding, for the most part, precise biochemical descriptions of mechanisms. Outlined below are the routes taken by cytoplasmically synthesized proteins destined for the outer membrane, the intermembrane space, the inner membrane, and the matrix. The known pathways to these compartments overlap for most proteins as they traverse the outer membrane, but then become distinct.

Insertion of proteins into the outer membrane

All proteins entering mitochondria first encounter pre-existing outer membrane proteins and lipids. Thus, outer membrane proteins are crucial for all import, including the biogenesis of the outer membrane itself. All outer membrane proteins are synthesized in the cytoplasm, and none are known to be proteolytically cleaved during import or assembly (Schmidt *et al.* 2010). The signals that target these proteins are poorly understood but appear to reside in transmembrane domains (Mokranjac and Neupert 2009; Walther and Rapaport 2009).

Import and insertion of β-barrel proteins: The overwhelming majority of cytoplasmically synthesized proteins that become incorporated into mitochondrial structures first associate with the organelles by interaction with the TOM complex in the outer membrane (Endo and Yamano 2010). Among them are the integral proteins of the mitochondrial outer membrane with β-barrel structures, including the most abundant, Por1 (porin) (Riezman *et al.* 1983), a voltage-gated anion channel. Another key β-barrel protein is Tom40 (Baker *et al.* 1990), which forms the TOM complex pores in the outer membrane through which most imported proteins pass (Hill *et al.* 1998; Künkele *et al.* 1998).

Both of these β-barrel proteins are translated on free cytoplasmic polysomes (Saint-Georges *et al.* 2008) and directed to mitochondria by unknown signals and mechanisms (Mokranjac and Neupert 2009). It is also unknown whether they arrive at the TOM complex associated with cytoplasmic chaperones. In any event, these apparently unfolded β-barrel proteins pass through the Tom40 pore of the TOM complex after interaction with the TOM receptor subunits Tom20 and Tom22 (Krimmer *et al.* 2001; Model *et al.* 2001) (Figure 4).

At this point, the β-barrel proteins are present in the IMS, which is homologous to the periplasm of Gram-negative bacteria. In this soluble milieu, they are bound by heterohexameric chaperone complexes composed of the small proteins (∼100 amino acids each) Tim9-Tim10 and of Tim8-Tim13 (Hoppins and Nargang 2004; Wiedemann *et al.* 2004) (Table 2). The crystal structure of the Tim9-Tim10 hexamer reveals a propeller arrangement with 12 mobile α-helical tentacles descending from a core stabilized by intramolecular disulfide bonds in each subunit (Baker *et al.* 2009). The N-terminal tentacle of Tim9 is especially important for substrate binding *in vivo*. These chaperones accompany the β-barrel proteins back to the inner surface of the outer membrane where they are delivered to the

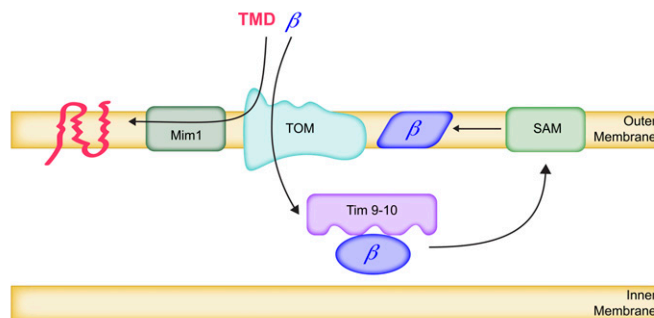

Figure 4 Insertion of proteins into the outer membrane. β-Barrel proteins are imported through the pores of the TOM complex in the outer membrane and then bound by IMS chaperone complexes comprising Tim9 and Tim10. The β-barrel-Tim9-Tim10 complexes bind to the inner surfaces of SAM complexes in the outer membrane, leading to insertion of β-barrel proteins into the outer membrane lipid bilayer. Some integral outer membrane proteins with multiple transmembrane domains (TMD) contact the Tom70 receptor and are then inserted into the bilayer from the outside through their interaction with multimeric complexes of Mim1.

Sorting and Assembly Machinery (SAM) complex, embedded in the outer membrane (also known as TOB for Topogenesis of β-Barrel proteins) (Paschen *et al.* 2003; Wiedemann *et al.* 2003a) (Table 3). The Sam35 component of the SAM complex recognizest β-barrel proteins by virtue of an amino acid sequence near their C termini (Wiedemann *et al.* 2003a; Kutik *et al.* 2008). The β-barrel proteins associate with the SAM complex from the IMS side and are then inserted laterally into the outer membrane lipid bilayer by an as-yet-unknown mechanism, where they assume β-barrel structure (Stroud *et al.* 2011) (Figure 4).

The essential core component of the SAM complex, Sam50, is itself a β-barrel protein. Thus, the assembled and functional β-barrel proteins Tom40 and Sam50 are necessary for import and assembly of newly synthesized Tom40 and Sam50, as well as that of other outer membrane β-barrel proteins. Sam50 is homologous to the bacterial outer membrane protein Omp85, which has a similar function in the insertion of β-barrel proteins in the outer membrane of Gram-negative bacteria (Paschen *et al.* 2003; Gentle *et al.* 2004).

Insertion of other integral proteins into the outer membrane: In addition to β-barrel proteins, the outer membrane contains integral proteins anchored in the lipid bilayer by one or more individual transmembrane domains. There appear to be multiple pathways for such proteins, and they are not well understood at present. At least some integral outer membrane proteins are exceptional in that they do not traverse the membrane via the Tom40 pore of the TOM complex. In the case of proteins with multiple membrane-spanning helices, the newly synthesized polypeptides first contact the outer membrane via the Tom70 receptor, but are then inserted into the bilayer from the outside, independently of Tom40, through their interaction with the multimeric complexes of Mim1 (Becker *et al.* 2011; Papic *et al.* 2011). Mim1 is a short (113 amino acids) single-spanning outer

Table 2 IMS import chaperones: delivery of hydrophobic proteins to the SAM complex (outer membrane) or TIM22 complex (inner membrane)

Protein	ORF	Known function	Null phenotype
Tim8	YJR135W-A	Complexed with Tim13	Viable, various defects
Tim9	YEL020W-A	Complexed with Tim10	Inviable
Tim10	YHR005C-A	Complexed with Tim9	Inviable
Tim12	YBR091C	Associated with Tim22 complex	Inviable
Tim13	YGR181W	Complexed with Tim8	Viable, various defects

membrane protein (Ishikawa *et al.* 2004; Waizenegger *et al.* 2005) that forms dimers that organize into higher-order complexes (Popov-Čeleketić *et al.* 2008b) that were recently also shown to contain a second protein, Mim2 (YLR099W-A) (Dimmer *et al.* 2012). These Mim1-Mim2 complexes appear to have a membrane insertase function (Figure 4).

Mim1 is also required for insertion of at least some proteins with a single transmembrane domain near the their N termini, often termed signal anchored proteins. These include the outer membrane receptor proteins Tom20 and Tom70 (Becker *et al.* 2008; Hulett *et al.* 2008; Popov-Čeleketić *et al.* 2008b). Interestingly, insertion of these receptors does not depend upon their own receptor function (Ahting *et al.* 2005).

Mim1 itself has a conserved, centrally located transmembrane domain that is partially functional even in the absence of both flanking hydrophilic domains (Popov-Čeleketić *et al.* 2008b). The Mim1 C-terminal domain is exposed to the cytoplasm (Lueder and Lithgow 2009; Walther and Rapaport 2009). The pathway that Mim1 takes into the outer membrane has not yet been studied.

There is apparently at least one additional pathway into the outer membrane employed by proteins anchored in the membrane by a single transmembrane domain at their C termini, the so-called "tail-anchored proteins." In the case of the tail-anchored protein Fis1, required for normal mitochondrial fission, outer membrane insertion is independent of all known components of the TOM and SAM complexes (Kemper *et al.* 2008). Furthermore, the insertion of Fis1 into lipid vesicle membranes with a low ergosterol content resembling the mitochondrial outer membrane indicates that lipid content may play a role in specificity *in vivo*. A possible role for Mim1 in Fis1 insertion was not tested. Another tail-anchored protein, the essential receptor Tom22, enters the membrane through the direct or indirect action of the SAM complex, after being recognized on the surface by TOM receptors (Stojanovski *et al.* 2007). Thus, the SAM complex may not be specific for the insertion of β-barrel proteins and may recognize substrates on either side of the membrane. The insertion of Tom22 is not dependent upon Mim1 (Becker *et al.* 2008).

Recently, evidence indicating the possibility of lateral diffusion of transmembrane domains out of the TOM complex has been reported (Harner *et al.* 2011b). Chimeric fusion proteins were trapped across the outer membrane by the folded structure of GFP on the outside and the multi-spanning inner membrane protein Tim23 inserted in the inner membrane. Fusion proteins with transmembrane domains accessible to the outer membrane were released from the TOM complex by an unknown mechanism. It remains to be determined whether any endogenous mitochondrial proteins employ this route into the outer membrane.

Import of proteins into the IMS

There are at least three mechanisms by which proteins are localized to the intermembrane space. Two involve covalent modifications of precursors after transit across the outer membrane by enzymes located in the intermembrane space there. The modifications stabilize folded structures that prevent retrograde transport out of the organelle. Trapping by noncovalent bonds may also occur in some cases. Finally, as discussed below in conjunction with transport to the inner membrane, some proteins are first targeted to the inner membrane and then released into the IMS by proteolytic cleavage.

Covalent attachment of heme: Cytochrome *c* (Cyc1 and Cyc7), which is located in the IMS, is perhaps the most intensively genetically analyzed *S. cerevisiae* protein (Sherman 2005). Surprisingly, the import of cytochrome *c* to the IMS is still relatively poorly understood. Cyc1 is largely synthesized on mitochondria-bound polysomes (Saint-Georges *et al.* 2008) and requires the TOM complex to traverse the outer membrane in a reaction that does not require ATP or an inner membrane potential (Diekert *et al.* 2001; Wiedemann

Table 3 Components of the SAM complex: insertion of β-barrel proteins into the outer membrane

Protein	ORF	Known function	Null phenotype
Mdm10	YAL010C		Viable, various defects
Sam35	YHR083W	Receptor	Inviable
Sam37	YMR060C		Viable, various defects
Sam50	YNL026W	β-Barrel protein of SAM complex	Inviable

et al. 2003b). However, the mechanism by which the TOM complex translocates apo-cytochrome *c* remains enigmatic. Blockage of the Tom40 pores used by other TOM substrates does not prevent import of apo-cytochrome *c* into membrane vesicles containing purified TOM complexes. Furthermore, removal of cytosolic domains of the TOM receptor subunits did not affect apo-cytochrome *c* import into mitochondria (Wiedemann *et al.* 2003b; Yamano *et al.* 2008). Nevertheless, complete removal of the Tom22 receptor did prevent import (Wiedemann *et al.* 2003b). Thus, Tom22 domains within the outer membrane or exposed on its inner surface may play a role in this as-yet-enigmatic translocation process. This behavior of apo-cytochrome *c* is unique among studied proteins.

Once in the IMS, apo-cytochrome *c* binds with the cytochrome *c* heme lyase, Cyc3, which is itself bound peripherally to the outer surface of the inner membrane (Dumont *et al.* 1991; Steiner *et al.* 1995; Bernard *et al.* 2005). Apo-cytochrome *c* is then irreversibly trapped by the covalent attachment of heme, which forms mature cytochrome *c* (Dumont *et al.* 1991) (Figure 5). The first 27 amino acids of cytochrome *c* contain the residues required for heme attachment, and they appear to be required for import (Wang *et al.* 1996). It is not clear whether this region is required for interaction with the TOM complex in addition to the heme lyase. In any event, apo-cytochrome *c* does not selectively partition to mitochondria in the absence of Cyc3, or if *cyc1* mutations block the heme lyase reaction, although small amounts are associated with the organelles (Dumont *et al.* 1991). Interestingly, even when *cyc1* mutations block heme attachment, overexpression of heme lyase increases partitioning of the mutant apo-cytochrome *c* to mitochondria, suggesting that protein–protein interactions alone initially sequester it (Dumont *et al.* 1991).

The cytochrome *c* heme lyase Cyc3 is itself imported into the intermembrane space via the TOM complex (Steiner *et al.* 1995). Based on studies with *Neurospora crassa*, a conserved targeting signal for heme lyases has been identified within its amino acid sequence, and this 60-residue region can target passenger proteins to the IMS (Diekert *et al.* 1999). This signal is believed to interact with *cis* and *trans* sites of the TOM complex and may also direct binding to unknown components of the inner membrane that could anchor it in the IMS.

Oxidation of paired cysteine residues to form disulfide bonds: A second form of covalent modification that sequesters some IMS proteins is the generation of internal disulfide bonds between paired cysteine residues (CX_3C or CX_9C motifs) after import. There are at least 24 such proteins (Koehler and Tienson 2009), including the small chaperone proteins Tim8, Tim9, Tim10, Tim12, and Tim13 whose folded structures are known to be stabilized by intramolecular disulfide bonds (Baker *et al.* 2009). Like the Gram-negative bacterial periplasmic space (Messens and Collet 2006), the IMS is a more oxidizing environment than the cytoplasm

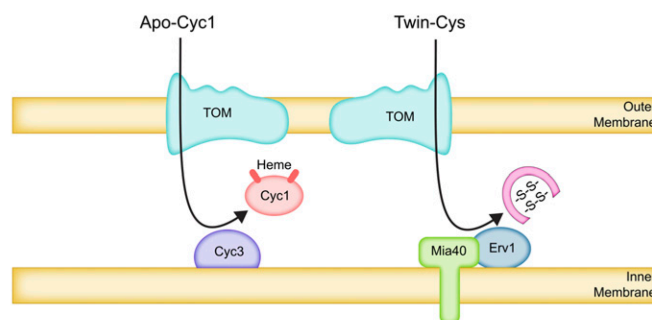

Figure 5 Trapping of proteins in the IMS by covalent modification. Apo-cytochrome *c* (Cyc1) traverses the outer membrane via the TOM complex by an unusual and poorly understood mechanism (see text). Covalent attachment of heme by the lyase (Cyc3), bound to the outer surface of the inner membrane, generates holo-cytochrome *c*. Holo-cytochrome *c* cannot translocate through the TOM complex and remains in the IMS. In an analogous mechanism, IMS proteins with twin-Cys residue pairs in reduced form are imported through the TOM complex and then oxidized by the Mia40-Erv1 disulfide relay system bound to the inner membrane. The internal disulfide bonds formed in the twin-Cys proteins prevent reverse translocation.

(Hu *et al.* 2008) and contains enzymatic machinery for the controlled generation of intramolecular disulfide bonds (Koehler and Tienson 2009; Herrmann and Riemer 2012).

Some IMS proteins with paired Cys residues are synthesized on mitochondria-bound polysomes (*e.g.*, Pet191, Cox23, Cox17), while others (*e.g.*, Tim9, Tim13) are not (Saint-Georges *et al.* 2008). It is not clear what directs these proteins to mitochondria (Riemer *et al.* 2011). Import of Tim13 does not depend upon surface receptors of the TOM complex, but it apparently does enter mitochondria through the Tom40 pore of the TOM complex (Lutz *et al.* 2003). The Tom5 subunit of the TOM complex is also required (Kurz *et al.* 1999). Chemical modification or mutation of the Cys residues prevented accumulation of Tim13 in the IMS (Lutz *et al.* 2003). Uptake of Tim10 was blocked if Cys residues were oxidized prior to import (Lu *et al.* 2004).

Mia40 is an essential protein bound to the outer surface of the inner membrane that is required for import of the essential Tim9-Tim10 chaperones and other twin-Cys proteins to the IMS (Chacinska *et al.* 2004; Naoe *et al.* 2004). These imported proteins associate with Mia40 via disulfide bonds. As one would expect, import of cytochrome *c* does not require Mia40 (Chacinska *et al.* 2004). A short peptide sequence containing a single Cys residue has been identified in several twin-Cys proteins that directs them to Mia40 in the IMS and binds covalently to it via a disulfide bond (Milenkovic *et al.* 2009; Sideris *et al.* 2009). This signal may also promote passage from the cytoplasm to the IMS, but it is not clear what outer membrane surface component could be involved in this recognition. The interaction of newly imported reduced twin-Cys substrates with oxidized Mia40 promotes folding of the substrate proteins and the formation of disulfide bonds, trapping the folded proteins in the intermembrane space (Banci *et al.* 2010) (Figure 5).

Reduced Mia40 is oxidized in turn by the essential intermembrane space protein Erv1, a conserved flavin-linked sulfhydryl oxidase (Mesecke *et al.* 2005). Electrons from the resulting reduced Erv1 can be accepted by cytochrome *c* and enter the respiratory chain or be accepted by molecular oxygen to form hydrogen peroxide that is metabolized by cytochrome *c* peroxidase (Bihlmaier *et al.* 2007; Dabir *et al.* 2007). This disulfide relay system has been reconstituted *in vitro* (Tienson *et al.* 2009).

Although Erv1 does not contain the CX_3C or CX_9C motifs present in the other substrates of this system, its import does depend upon Mia40 action following TOM-dependent passage through the outer membrane (Terziyska *et al.* 2007). Interestingly, the import of another protein located partially in the IMS and lacking the closely paired Cys residue, Ccs1, depends upon Mia40 to form a disulfide bond, but that bond is not necessary for enzymatic activity (Gross *et al.* 2011; Klöppel *et al.* 2011). The import pathway followed by Mia40, and other proteins anchored in the inner membrane with hydrophilic domains in the IMS, will be described below.

Import of proteins into the inner membrane

The mitochondrial inner membrane contains a very wide variety of integral proteins. All studied cytoplasmically synthesized inner membrane proteins are recognized by receptors of the TOM complex and imported through its pores. They are then inserted into the inner membrane by one of three mechanisms, or combinations of them, depending upon the signals that they contain and their ultimate topology.

Insertion of metabolite carriers and other multispanning inner membrane proteins by the TIM22 insertase/translocase complex: A major class of inner membrane proteins are imported and assembled into multispanning topologies without being proteolytically processed. At least 34 of these proteins are members of the metabolite carrier family (Palmieri *et al.* 2006), which includes the ATP/ADP carriers. Two other such proteins are Tim22 and Tim23, the essential pore-forming subunits of the TIM complexes described below.

These multispanning membrane proteins contain multiple internal mitochondrial targeting signals that generally flank transmembrane domains (Neupert and Herrmann 2007; Chacinska *et al.* 2009). Newly synthesized yeast carrier proteins have been shown to associate with the cytoplasmic chaperone Hsp70, which participates in their recognition by the Tom70 receptor subunit of the TOM complex (Young *et al.* 2003; Endo and Yamano 2010). ATP-dependent release from the Hsp70 chaperones allows the carrier proteins to enter the TOM complex pore (Figure 6). The N and C termini initially remain on the outside while internal regions containing recognition signals traverse the TOM complex as looped polypeptide chains through the Tom40 pore in the outer membrane (Wiedemann *et al.* 2001; Neupert and Herrmann 2007; Chacinska *et al.* 2009). The Tom40 pore appears to have

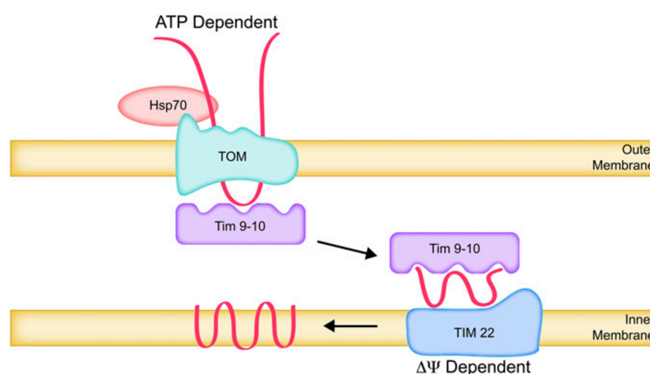

Figure 6 Insertion of multi-spanning carrier proteins into the inner membrane. Newly synthesized multi-spanning carrier proteins, complexed with cytoplasmic Hsp70, are recognized by the Tom70 receptor subunit of the TOM complex. ATP-dependent release from cytoplasmic Hsp70 leads to translocation through the TOM complex in a looped configuration and binding to the Tim9-Tim10 IMS chaperone complex. The multi-spanning proteins are delivered to the TIM22 insertase complex in the inner membrane, released from Tim9-Tim10, and inserted into the inner membrane by reactions that depend upon the $\Delta\psi$ potential across the inner membrane.

specific interactions with different imported proteins and thus may also play an active role in substrate recognition (Gabriel *et al.* 2003; Sherman *et al.* 2006).

On the inside of the outer membrane, incoming carrier proteins are removed from the TOM complex by binding to the soluble essential Tim9-Tim10 chaperone complexes (Koehler *et al.* 1998; Sirrenberg *et al.* 1998; Curran *et al.* 2002; Vasiljev *et al.* 2004). Incoming Tim23 is preferentially bound by the homologous but dispensable Tim8-Tim13 chaperone complex (Davis *et al.* 2000; Paschen *et al.* 2000). The hydrophobic client proteins are thus transported through the aqueous intermembrane space (Figure 6). Their destination in the inner membrane is the TIM22 insertase/translocase complex (Sirrenberg *et al.* 1996; Kerscher *et al.* 1997) (Table 4). This complex has at its core a voltage-gated pore formed by the essential Tim22 protein (Kovermann *et al.* 2002), which is required for its own membrane insertion (Sirrenberg *et al.* 1998).

Carrier proteins, or other substrates, associated with the IMS chaperones Tim9-Tim10 or Tim8-Tim12 bind to the TIM22 complex on the outer surface of the inner membrane. The substrates are dissociated from the chaperones, a process that may involve redox reactions (Curran *et al.* 2004). Uptake of the substrates into the TIM22 complex, translocation of substrate domains through the membrane, and insertion of transmembrane domains into the membrane require the $\Delta\psi$-membrane potential but not ATP (Rehling *et al.* 2003; Peixoto *et al.* 2007). The mechanistic details of these reactions are not well understood.

Functional carrier protein dimers appear to self-assemble in the membrane rapidly following insertion (Dyall *et al.* 2003). Tim22 is assembled into the TIM22 complex along with subunits that are inserted into the inner membrane by the pathway described below for proteins with cleavable presequences (Wagner *et al.* 2008).

Table 4 Components of the TIM22 complex: insertion of multispanning carrier proteins into the inner membrane

Protein	ORF	Known function	Null phenotype
Tim18	YOR297C		Viable, various defects
Tim22	YDL217C	Core insertase of the complex	Inviable
Tim54	YJL054W		Viable, various defects

Insertion of inner membrane spanning proteins with cleavable presequences by the TIM23 insertase/translocase complex: Sixty percent or more of all yeast mitochondrial proteins are synthesized as precursors whose N termini are cleaved during import (Vögtle *et al.* 2009). The N-terminal presequences typically contain targeting signals comprising amphipathic α-helices with positively charged and hydrophobic surfaces, although there is no consensus sequence. These "classical" targeting signals, which were reviewed in the previous edition of YeastBook (Pon and Schatz 1991) and elsewhere (Neupert 1997; Voos *et al.* 1999), are sufficient on their own to target proteins to the matrix, as discussed in the section below. However, these amphipathic α-helices can also be combined with nearby downstream hydrophobic sorting signals to form bipartite signals that direct proteins to the inner membrane (Figure 7A). Mia40 is such a protein, anchored in the inner membrane by the N-terminal hydrophobic sorting signal with its hydrophilic domains facing the IMS (Naoe *et al.* 2004; Neupert and Herrmann 2007). In addition, at least two well-studied IMS proteins, cytochrome b_2 (Cyb2) and cytochrome c_1 (Cyt1), adopt the same topology before being released from their N-terminal membrane anchors by the inner membrane protease (IMP: Imp1, Imp2, Som1) (Glick *et al.* 1992; Nunnari *et al.* 1993; Jan *et al.* 2000). In the case of Cyt1, a second internal sorting sequence near the C terminus inserts into the inner membrane by an unknown mechanism anchoring the hydrophilic N-terminal domain on the intermembrane space side (Arnold *et al.* 1998; Lange and Hunte 2002).

Different presequence-containing proteins are synthesized on free or mitochondria-bound polysomes (the precursors of Mia40, Cyb2, and Cyt1 are all synthesized on mitochondria-bound polysomes) (Saint-Georges *et al.* 2008). There is relatively little information on the binding of cytoplasmic chaperones to presequence-containing precursors. However, in at least some cases, cytoplasmic Hsp70 (Ssa1–4) is required for import. This is thought to reflect the ability of Hsp70 to maintain precursors in partially unfolded states (Deshaies *et al.* 1988; Gautschi *et al.* 2001; Sass *et al.* 2003; Endo and Yamano 2010). While the pathways taken by these precursors to the outer surface of mitochondria are poorly understood, their pathways into the organelle have been the subject of intensive research.

A domain of the TOM receptor subunit Tom20 on the cytoplasmic side of the outer membrane recognizes the hydrophobic surfaces of presequence amphipathic α-helices (Abe *et al.* 2000; Yamamoto *et al.* 2011). The presequences are in turn bound by the Tom22 receptor subunit via electrostatic interactions and directed into the pore formed by Tom40 (Schmidt *et al.* 2010; Shiota *et al.* 2011). The Tom70 subunit is not thought to play a major role in recognition of presequence-containing precursors. However, while yeast cells survive without either Tom70 or Tom20, deletion of both is lethal, indicating that they can carry out redundant functions (Ramage *et al.* 1993).

The IMS side of the TOM complex interacts transiently with the major TIM complex, whose essential pore-forming subunit is Tim23 (Chacinska *et al.* 2009; Mokranjac and Neupert 2009; Marom *et al.* 2011a). This TIM23 complex (Table 5) has an essential receptor, Tim50, that seals the Tim23 pore in the absence of a substrate protein, preserving the inner membrane potential (Meinecke *et al.* 2006). Tim50 recognizes presequences emerging from the TOM complex and facilitates their transit to the pore (Yamamoto *et al.* 2002; Mokranjac *et al.* 2009; Tamura *et al.* 2009; Marom *et al.* 2011b; Schulz *et al.* 2011) in a reaction that must occur at translocation contact sites between outer and inner membranes (Pon *et al.* 1989).

Passage of the presequence into and through the TIM23 complex pore is electrophoretically driven by the inner membrane electrical potential, $\Delta\psi$, which is negative inside (Pon and Schatz 1991; Chacinska *et al.* 2009; Mokranjac and Neupert 2009; Marom *et al.* 2011a). It is independent of ATP hydrolysis (Glick *et al.* 1992). This transit of the positively charged presequence through the TIM23 complex can bring the downstream hydrophobic sorting signal into the TIM23 pore if the distance between them is short (Figure 7A). The presence of the hydrophobic sorting signal in the TIM23 channel prevents further translocation of the polypeptide chain. The presequence, now located in the matrix, is removed by sequence-specific activity of the soluble mitochondrial processing protease (MPP) (Pon and Schatz 1991; Taylor *et al.* 2001; Vögtle *et al.* 2009).

The "stop-transfer" activity of the sorting signal also triggers a lateral release of the polypeptide from the TIM23 complex, resulting in its insertion into the lipid bilayer of the inner membrane (Neupert and Herrmann 2007; Chacinska *et al.* 2009; Marom *et al.* 2011a) (Figure 7A). This lateral insertion reaction can be reconstituted *in vitro* with purified TIM23 complex components in lipid vesicles containing the mitochondria-specific lipid cardiolipin (van der Laan *et al.* 2007). Once embedded in the membrane, the sorting signal functions as a membrane anchor that eventually sequesters the rest of the polypeptide in the IMS after its passage through the TOM complex.

In the case of inner membrane proteins whose stop-transfer hydrophobic sorting signal is far downstream of the positively charged presequence, $\Delta\psi$-dependent translocation of the presequence alone will not bring the sorting signal into the TIM23 translocase. For such proteins, the intervening residues must be pulled into the matrix by the ATP-driven presequence translocase-associated motor (PAM) until the stop-transfer sequence enters the TIM23 channel (Figure 7B).

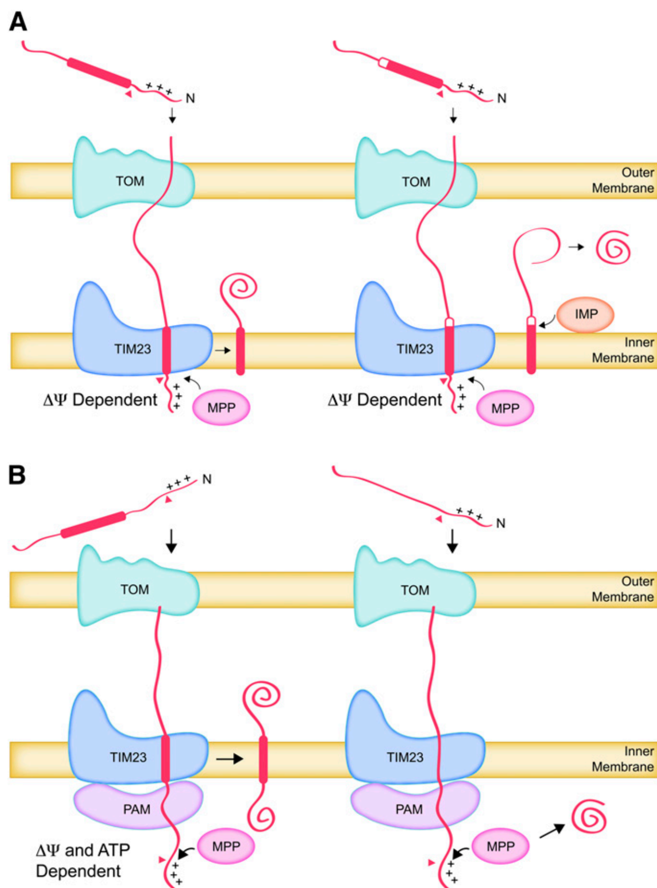

Figure 7 Import of proteins with amphipathic positively charged cleavable presequences. (A) $\Delta\Psi$-Dependent transport of proteins containing hydrophobic sorting signals (thick bars) to the inner membrane and IMS. The presequences are recognized by the Tom22 receptor of the TOM complex, pass through the TOM complex, and are recognized by the Tim50 subunit of the TIM23 complex. The presequence translocates through the TIM23 complex, driven electrophoretically by $\Delta\Psi$. This brings the hydrophobic sorting signal, located immediately downstream of the presequence, into the TIM23 complex. The presequence is removed by proteolysis and the sorting signal is inserted laterally into the inner membrane where it can function as a membrane anchor (left). Proteolytic cleavage at the outer surface of the inner membrane can release a soluble protein into the inner membrane space (right). (B) $\Delta\Psi$- and ATP-dependent transport to the inner membrane and the matrix. Presequences traverse the TOM and TIM23 complexes and are removed by proteolysis, as in A. If there is no hydrophobic sorting signal immediately downstream of the presequence, the incoming polypeptide is engaged by the PAM complexed with the inner surface of the TIM23 translocon. ATP hydrolysis by the Hsp70 (Ssc1) subunit of PAM translocates the polypeptide into the matrix. If a downstream hydrophobic sorting signal enters the TIM23 complex, it is released laterally into the inner membrane and translocation ceases (left). If there is no sorting signal, the entire polypeptide is translocated into the matrix (right).

The catalytic heart of the PAM complex (Table 6) is the essential mitochondrial Hsp70 protein Ssc1 (mtHsp70) (Kang *et al.* 1990; Manning-Krieg *et al.* 1991; Chacinska *et al.* 2009; Marom *et al.* 2011a). This ATP-hydrolyzing chaperone is a very abundant soluble constituent of the matrix with diverse roles in protein folding and assembly (Craig 1993; Hartl 1996; Voos and Röttgers 2002; Fontanesi *et al.* 2010b; Marom *et al.* 2011a). A fraction of the mtHsp70 molecules are bound to the TIM23 complex on the matrix side via an essential subunit, Tim44, which also contacts the incoming polypeptide (Slutsky-Leiderman *et al.* 2007; Marom *et al.* 2011b). Hydrolysis of ATP by mtHsp70 bound to the incoming chain provides the energy for translocation, although the molecular mechanism by which the released energy causes movement of the polypeptide chain remains a matter of debate (Chacinska *et al.* 2009; Marom *et al.* 2011a). In any event, repeated cycles of mtHsp70 molecules binding to the incoming chain, ATP hydrolysis, and ADP release effectively pull the chain through the TIM23 complex (Figure 7B). These cycles require the action of the essential nucleotide exchange factor Mge1 and the essential J-domain co-chaperone protein Pam18 that promotes ATP hydrolysis and thus stabilize interaction between mtHsp70 and the incoming polypeptide chain. An essential J-domain-like protein, Pam16, stabilizes association of Pam18 with the TIM23 complex (Frazier *et al.* 2004; Pais *et al.* 2011) and may regulate Pam18 activity (Marom *et al.* 2011a).

Entry of a trailing stop-transfer sequence into the TIM23 channel prevents further PAM-dependent uptake of the polypeptide chain. It is not known whether this is due to a signal transduced from the channel to the PAM motor to cease the ATP-driven cycle or to the generation of an energy barrier that prevents further import. Lateral movement of the stop-transfer sequence from the TIM23 channel into the membrane can then anchor the inner membrane to a protein with hydrophilic domains in the matrix (Gärtner *et al.* 1995) (Figure 7B).

Insertion of multispanning inner membrane proteins with presequences: Relatively few studies have focused on this class of proteins. Presequences that are present on precursors of three multispanning inner membrane proteins—Mdl1, Oxa1, and Cox18—enter mitochondria through the TOM complex and pass through the TIM23 complex in a $\Delta\psi$-dependent fashion (Herrmann *et al.* 1997; Frazier *et al.* 2003; Reif *et al.* 2005; Bohnert *et al.* 2010). Completed import and topogenesis also requires ATP and the PAM motor complex. However, different mechanisms appear to be responsible for insertion of different transmembrane domains, and they are not well characterized.

Mdl1 is an ABC-cassette transporter in the inner membrane that has six transmembrane domains and is oriented with its N and C termini on the matrix side (Young *et al.* 2001). Its import and completed topogenesis depends upon the TOM and TIM23 complexes as well as upon the pulling action of the PAM motor complex (Reif *et al.* 2005). The insertion of its first two transmembrane domains appears to depend upon the stop transfer mechanism since their topogenesis requires the inner membrane $\Delta\psi$, but does not require the pulling action of the PAM motor complex. However, insertion of the third and fourth transmembrane domains is more complex. These regions are apparently pulled across the inner membrane by the PAM motor and then

Table 5 Components of the TIM23 complex: transport of polypeptides through the inner membrane and lateral insertion of membrane anchors into the inner membrane

Protein	ORF	Known function	Null phenotype
Tim17	YJL143W		Inviable
Tim21	YGR033C		Viable various defects
Tim23	YNR017W	Pore formation	Inviable
Tim44	YIL022W	Tethers PAM to TIM23 complex	Inviable
Tim50	YPL063W	Substrate receptor and pore gating	Inviable

inserted into the membrane from the inside by the action of Oxa1 (Reif *et al.* 2005). Oxa1 is an inner membrane translocase/insertase known to export mitochondrially synthesized protein domains from the matrix (Bonnefoy *et al.* 2009). Since Oxa1 is homologous and functionally similar to bacterial YidC proteins, the insertion of imported domains back into the inner membrane from the inside is often referred to as "conservative sorting."

Oxa1 has five transmembrane domains and is oriented in the inner membrane with its N terminus in the IMS and its C terminus in the matrix (Bonnefoy *et al.* 2009). During its import, the first Oxa1 transmembrane domain appears to cross the inner membrane, following the presequence. In a second step, the N-terminal domain is re-exported, concomitant with insertion of the first transmembrane domain by the translocase activity of pre-existing Oxa1 itself (Herrmann *et al.* 1997). It is not clearly established whether the other transmembrane domains are imported and then inserted from inside, transferred laterally into the membrane from the TIM23 complex, or perhaps inserted via some other pathway (Herrmann *et al.* 1997). The bacterial homolog of Oxa1, YidC, can promote lateral insertion of transmembrane domains from the Sec translocase into the bilayer (Dalbey and Kuhn 2004). This suggests the possibility that Oxa1 could carry out an analogous function with some substrates, in conjunction with the TIM23 complex (Reif *et al.* 2005). It is clear that Oxa1 cannot be absolutely required for its own topogenesis since nonrespiring *oxa1*Δ mutants can be restored to normal phenotype by reintroduction of a wild-type *OXA1* gene (Bonnefoy *et al.* 1994).

Import of presequence-containing proteins to the matrix

A large fraction of presequence-containing precursors are targeted to the innermost mitochondrial compartment, the matrix. They contain amphipathic α-helices in their presequences but no stop-transfer sorting signals (Pon and Schatz 1991). Following synthesis on either bound or free polysomes, they traverse the TOM and TIM23 complexes as described above. After Δψ-dependent uptake of the presequence, ATP-dependent action of the PAM complex pulls the entire polypeptide into the matrix.

The pulling of entire proteins into the matrix by PAM depends upon at least partial unfolding of the C-terminal domains that are often still on the cytoplasmic side of the TOM complex when PAM engages the N-terminal end. This has been clearly demonstrated in *in vitro* reactions (Pon and Schatz 1991). The importance of this *in vivo* is demonstrated by the import of Fum1, the precursor of fumarase (Sass *et al.* 2003; Karniely *et al.* 2006). Wild-type Fum1 has the ability to fold rapidly into a stable conformation while the presequence is imported into the matrix and processed. Molecules that achieve this state fail to import and are released back into the cytoplasm in mature form by retrograde movement of their N termini. On the other hand, molecules whose C-terminal domains do not fold rapidly are pulled into the matrix. This is one of several mechanisms by which proteins can be localized both in mitochondria and in the cytoplasm (Yogev and Pines 2011).

Presequences of imported precursors are typically removed by the soluble MPP (Pon and Schatz 1991; Taylor *et al.* 2001). Many matrix proteins are further processed at their N termini by removal of a single residue or eight residues by the proteases Icp55 and Oct1, respectively (Vögtle *et al.* 2009, 2011). These alterations apparently serve to generate mature products with increased stability, following the bacterial N-end rules.

The folding of imported matrix proteins must be largely coupled to their interaction with, and release from, mtHsp70 associated with the PAM motor. Mitochondria also contain

Table 6 Components of the PAM complex: ATP-dependent pulling of proteins through the TIM23 complex into the matrix

Protein	ORF	Known function	Null phenotype
Mdj2	YNL328C		Viable, various defects
Mge1	YOR232W	Nucleotide release factor for Ssc1	Inviable
Pam16	YJL104W		Inviable
Pam17	YKR065C	Interaction between TIM23 and PAM complexes	Viable
Pam18	YLR008C	J protein co-chaperone for Ssc1	Inviable
Ssc1	YJR045C	Hsp70; ATP hydrolysis drives import of substrates into matrix	Inviable

the essential chaperonin complex Hsp60-Hsp10 (Pon and Schatz 1991; Voos and Röttgers 2002), which is characteristic of bacteria and energy-transducing organelles. These chaperones, together with the dispensable Hsp78 and Pim1, homologs of bacterial ClpB and lon ATP-dependent proteases, assist in folding and maintenance of imported matrix proteins (Leonhardt *et al.* 1993; Suzuki *et al.* 1997; Bender *et al.* 2011).

Spatial distributions and regulation of import complexes

The import of presequence-containing proteins to the inner membrane and matrix requires at least transient interaction of some TOM complexes and TIM23 complexes at translocation contact sites between the membranes. By incubating isolated mitochondria with saturating amounts of an artificial presequence-containing protein that become trapped both outside the outer membrane and in the inner membrane, TOM complexes bound to TIM23 complexes can be detected. Using such a trap, it could be shown that all of the TIM23 complexes were associated with $\sim$25% of the TOM complexes (Dekker *et al.* 1997). Thus, at least under these conditions, the TIM23 complex must be located almost exclusively in the boundary domain of the inner membrane and form translocation contact sites with the outer membrane TOM complexes. However, in respiring wild-type cells, Tim23 appears to be only moderately enriched in the inner membrane boundary domain relative to cristae (Vogel *et al.* 2006).

The TIM23 complex must respond to signals in the substrate precursors that direct either their lateral insertion into the inner membrane or their complete translocation into the matrix. There is currently some dispute about whether the lateral insertion and matrix translocation functions are carried out by a single multi-functional TIM23-PAM complex with different conformations (Popov-Čeleketić *et al.* 2008a, 2011; Mokranjac and Neupert 2009) or by two forms of the TIM23 complex in dynamic equilibrium with each other (Chacinska *et al.* 2009, 2010; Schmidt *et al.* 2010). Two forms of static TIM23 complex were detected in solubilized extracts of mitochondria, depending upon whether they were trapped importing a precursor targeted for sorting to the outer surface of the inner membrane, which does not require the ATP-driven PAM motor, or for import into the matrix, which does require the motor (Chacinska *et al.* 2005, 2010). The isolated TIM23-sorting complexes contained the protein Tim21, but not PAM complex subunits. In contrast, the isolated TIM23 motor complexes contained PAM subunits but only low levels of Tim21. It has been suggested that the PAM motor could associate with the TIM23 channel if and when Tim21 is ejected preceding import into the matrix (Wiedemann *et al.* 2007).

Surprisingly, the TIM23 complex has been found to associate with proton-pumping supercomplexes of the bc_1 complex and cytochrome *c* oxidase. These interactions are facilitated by the Tim21 subunit (van der Laan *et al.* 2006), which also has a role in connecting the TOM complex to the TIM23 complex (Chacinska *et al.* 2005; Mokranjac *et al.* 2005), and by Pam16 and Pam18 (Wiedemann *et al.* 2007). The physiological relevance of this association is suggested by the observation that Tim21 accelerates $\Delta\psi$-dependent precursor translocation only when the respiratory complexes are active (van der Laan *et al.* 2006). Thus, it appears that local increases in $\Delta\psi$ may affect the rate of presequence translocation through TIM23. Active TIM23 complexes must be located in the boundary domain of the inner membrane, while the respiratory complexes are selectively, but presumably not completely, located in the cristae domains (Vogel *et al.* 2006; Wurm and Jakobs 2006; Rabl *et al.* 2009; Zick *et al.* 2009; Davies *et al.* 2011).

A complex of inner membrane proteins present at the crista junctions, and required to form them, has been identified in several labs and named MINOS, MitOS, and MICOS (Harner *et al.* 2011a; Hoppins *et al.* 2011; von der Malsburg *et al.* 2011; Alkhaja *et al.* 2012). A core component of this complex, Fcj1, was previously shown to be required for crista junction formation (Rabl *et al.* 2009). The crista junctions are located at firm contact sites between the outer and inner membranes (Harner *et al.* 2011a). These firm contact sites apparently do not correspond to translocation contact sites between TOM and TIM23 complexes since TIM23-dependent precursor translocation is not directly affected by the absence of Fcj1 (von der Malsburg *et al.* 2011). However, Fcj1 interacts with Mia40 and with TOM complexes not associated with TIM23, facilitating Mia40-dependent import of soluble twin-Cys proteins to the IMS (von der Malsburg *et al.* 2011). This suggests that import of substrates dependent upon the Mia40-Erv1 disulfide relay system is selectively located near crista junctions. Cells lacking Fcj1 or other components of MINOS/MitOS/MICOS have respiratory defects, but are viable. Thus, this complex does not appear to be absolutely required for any essential import pathway.

Is the mitochondrial protein import machinery simply a conduit for any targeted protein that arrives at the outer membrane, or is its activity *per se* subject to modulation? Recent evidence indicates that TOM complex activity is regulated by phosphorylation of the Tom70 receptor by cAMP-dependent protein kinase A (PKA) (Schmidt *et al.* 2011). PKA activity is controlled such that its activity is high in the presence of glucose but low in nonfermentable carbon sources (Zaman *et al.* 2008). In the presence of glucose, PKA phosphorylates Tom40 residue Ser174 (among a wide variety of targets). This modification decreases the ability of Tom40 to interact with cytoplasmic Hsp70, the chaperone that delivers metabolite carrier proteins to the TOM complex (Young *et al.* 2003), decreasing the efficiency with which carrier proteins are imported to the inner membrane (Schmidt *et al.* 2011). Constitutive activation of PKA results in decreased levels of carrier proteins relative to mitochondrial proteins whose import is not affected by decreased Tom70 activity. Thus, this regulatory pathway has significant physiological effects on mitochondria.

Interestingly, another kinase, casein kinase 2, quantitatively phosphorylates two Ser residues of the central TOM receptor Tom22 and two residues of Mim1 (Schmidt *et al.* 2011). These modifications are required for normal assembly and activity of both proteins, and thus for the activity of the TOM complex. It is not clear whether these phosphorylations are modulated in response to environmental conditions.

Assembly of Complexes Containing Mitochondrially Synthesized Proteins

Mitochondrial gene expression in *S. cerevisiae* is required for active respiratory complexes located in the inner membrane, but is not required for any other physiological processes (Pon and Schatz 1991; Chacinska and Boguta 2000; Lipinski *et al.* 2010). The organellar genetic system produces only eight major proteins in *S. cerevisiae*. Seven are hydrophobic integral inner membrane proteins: Cob (Apo-cytochrome *b*), a subunit of the proton-pumping bc_1 complex (respiratory complex III); Cox1, Cox2, and Cox3, subunits of proton-pumping cytochrome *c* oxidase (respiratory complex IV); and Atp6, Atp8, and Atp9, subunits of the F_0 component of ATP synthase (respiratory complex V). These mitochondrially synthesized subunits are assembled with imported subunits to form the active enzymes. (*S. cerevisiae* does not have respiratory complex I, the proton-pumping NADH dehydrogenase found in most other eukaryotes. Succinate dehydrogenase, respiratory complex II, does not contain mitochondrial gene products.)

The eighth major mitochondrial gene product, Var1, is a small mitochondrial ribosomal subunit protein. Aside from Var1, all proteins known to be required for yeast mitochondrial gene expression are imported from the cytoplasm [with the exception of some minor proteins encoded by mitochondrial introns that facilitate intron splicing (Pon and Schatz 1991; Saldanha *et al.* 1993)]. Var1 is assembled inside the organelle with mitochondrially coded 15S rRNA and at least 33 imported ribosomal proteins coded by nuclear genes (Groot *et al.* 1979; Terpstra *et al.* 1979; Gan *et al.* 2002; Williams *et al.* 2005). The large mitochondrial ribosomal subunit is assembled from the mitochondrially coded 21S rRNA and at least 44 imported proteins (Gan *et al.* 2002). These organellar ribosomes do not share any known components with cytoplasmic ribosomes.

The assembly of yeast cytochrome *c* oxidase has been studied and reviewed extensively (Barrientos *et al.* 2002a, 2009; Carr and Winge 2003; Herrmann and Funes 2005; Khalimonchuk and Rodel 2005; Cobine *et al.* 2006; Fontanesi *et al.* 2006, 2008; Horn and Barrientos 2008; Mick *et al.* 2011; Soto *et al.* 2011). A striking fact to emerge from these studies is the specific involvement of >30 nuclear gene products that are not constituents of cytochrome *c* oxidase. They are required to produce the active enzyme by promoting specific reactions in the expression of genes; insertion of proteins into the inner membrane; insertion of heme, copper, zinc, and magnesium; and chaperoning assembly intermediates (Soto *et al.* 2011). The assembly of the cytochrome bc_1 complex (Zara *et al.* 2009; Conte *et al.* 2011; Wagener *et al.* 2011; Smith *et al.* 2012) and the ATP synthase (Ackerman and Tzagoloff 2005; Rak *et al.* 2009) also requires similar assistance. This appears to contrast with the assembly of the TOM complex, for example. Its constituents are inserted into the outer membrane by general mechanisms and are believed to self-assemble once in the membrane (Becker *et al.* 2008, 2010). Furthermore, as discussed below, the mitochondrial synthesis of several respiratory complex subunits is coupled to assembly of those complexes.

At a higher level of assembly, the cytochrome bc_1 complex and cytochrome *c* oxidase are organized together into supercomplexes that appear to facilitate efficient electron transport between them via cytochrome *c* (Heinemeyer *et al.* 2007; Stuart 2008), although this rationale has been questioned (Trouillard *et al.* 2011). Assembly of these supercomplexes has recently been shown to depend upon two supercomplex subunits, Rcf1 and Rcf2 (Chen *et al.* 2012; Strogolova *et al.* 2012; Vukotic *et al.* 2012).

Mitochondrial protein synthesis is membrane bound

Components of the mitochondrial genetic system have been identified chiefly through the isolation of mutants and the study of their phenotypes and of the proteins affected by their mutations (Tzagoloff and Dieckmann 1990). While mitochondrial protein synthesis can be studied in isolated intact organelles, no true *in vitro* translation system has been developed from yeast mitochondria. It appears that the mitochondrial genetic system is largely organized on the two-dimensional inner surface of the inner membrane, and an intact membrane [although not the membrane $\Delta\Psi$ (Clarkson and Poyton 1989; He and Fox 1997)] may be required for protein synthesis. Indeed, the lipid composition of the inner membrane appears to affect mitochondrial protein synthesis (Marzuki and Hibbs 1986; Ostrander *et al.* 2001).

Mitochondrial ribosomes are bound to the inner membrane, preferentially to the cristae domains, apparently to facilitate cotranslational protein insertion (Vogel *et al.* 2006; Ott and Herrmann 2010). To date, three inner membrane proteins are known to interact with large mitochondrial ribosomal subunits and are thought to tether them to the membrane independently of nascent chains: they are Oxa1 (Jia *et al.* 2003, 2009; Szyrach *et al.* 2003), Mba1 (Ott *et al.* 2006; Gruschke *et al.* 2010), and Mdm38 (Frazier *et al.* 2006; Lupo *et al.* 2011). Oxa1 functions as a translocase/insertase-promoting export of mitochondrially coded hydrophilic domains, particularly those of Cox2, to the intermembrane space and insertion of transmembrane domains (He and Fox 1997; Hell *et al.* 1997, 2001). MbaI, a peripheral membrane protein, appears to be partially redundant in function with Oxa1 (Preuss *et al.* 2001; Ott *et al.* 2006). Similarly, Mdm38 promotes export of Cob and Atp6 domains (Frazier *et al.* 2006). Interestingly, Mdm38 is a bifunctional

protein that also participates in K^+/H^+ exchange across the inner membrane (Nowikovsky *et al.* 2004; Froschauer *et al.* 2005; Zotova *et al.* 2010; Lupo *et al.* 2011).

The synthesis of mitochondrially coded proteins at the inner membrane surface appears to limit their contact with matrix chaperones. Membrane proteins emerging from mitochondrial ribosomes do not interact with mtHsp70 (Ssc1) (Ott *et al.* 2006). Furthermore, soluble reporter proteins synthesized inside mitochondria appear to fold more slowly than identical polypeptides synthesized in the cytoplasm and imported into the matrix via the mtHsp70-dependent TIM23/PAM machinery (Demlow and Fox 2003).

Channeling of mRNAs to the inner membrane

Mitochondrial DNA molecules are present in nucleoprotein aggregates, termed "nucleoids" (Meeusen *et al.* 1999; Kaufman *et al.* 2000; Kucej *et al.* 2008). A fraction of the ~40 nucleoids per cell are tethered to the inner and outer membranes by a membrane-spanning protein complex (Aiken Hobbs *et al.* 2001; Boldogh *et al.* 2003; Meeusen and Nunnari 2003; Chen and Butow 2005). While the distribution of the mitochondrial RNA polymerase, Rpo41, among the nucleoids has not been reported, transcription likely occurs adjacent to the inner membrane. Furthermore, strong evidence suggests that mRNAs are channeled from RNA polymerase to the membrane-bound translation apparatus. Rpo41 interacts through its amino terminal domain with the soluble protein Mtf2 (Nam1), which in turn appears to chaperone processing of primary transcripts and delivery of mRNAs to the inner membrane surface, together with the membrane-associated proteins Sls1 and Rmd9 (Dieckmann and Staples 1994; Wallis *et al.* 1994; Rouillard *et al.* 1996; Rodeheffer *et al.* 2001; Bryan *et al.* 2002; Rodeheffer and Shadel 2003; Nouet *et al.* 2007; Williams *et al.* 2007) (Figure 8). Mtf2 (Nam1), Sls1, and Rmd9 are all required for normal mitochondrial translation.

The physiological importance of mitochondrial mRNA channeling from synthesis to translation is most clearly demonstrated by the fact that mutations in the mitochondrial RNA polymerase amino terminal domain that prevent interaction with Mtf2 (Nam1) severely reduce translation but do not prevent mRNA synthesis and accumulation (Rodeheffer and Shadel 2003). Thus, new primary transcripts appear to be directly transferred to Mtf2 (Nam1) from RNA polymerase for efficient gene expression, in conjunction with the interacting membrane protein Sls1 and perhaps Rmd9 among other factors. mRNAs do not, apparently, diffuse freely through the matrix.

Localization of protein synthesis by mRNA-specific translational activators

A peculiar feature of mitochondrial translation in *S. cerevisiae* and other ascomycetes is its dependence on translational activators for individual mRNAs (Fox 1996a; Coffin *et al.* 1997; Costanzo *et al.* 2000; Towpik 2005; Kühl *et al.* 2011) (Table 7). The roles of mRNA-specific translational

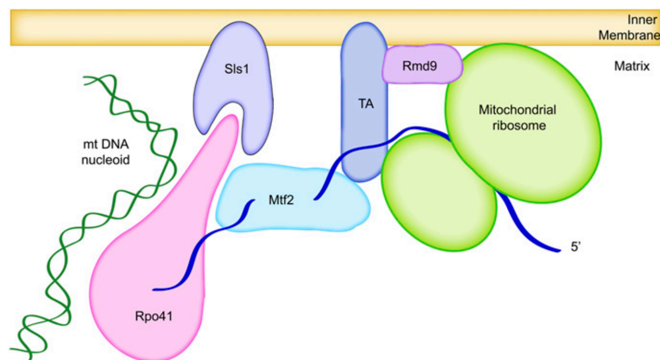

Figure 8 Channeling of mitochondrially coded mRNAs from RNA polymerase (Rpo41) to membrane-bound ribosomes by Sls1, Mtf2, Rmd9, and mRNA-specific translational activators (TA). (The figure is not intended to suggest that mRNAs are translated while still emerging from RNA polymerase.)

activators have been most thoroughly explored for the expression of the *S. cerevisiae* mitochondrial genes encoding the three core cytochrome *c* oxidase subunits and apocytochrome *b*. The best-studied translational activators are membrane-bound proteins that recognize targets in the mitochondrial mRNA 5′-UTRs and interact with mitochondrial ribosomes, apparently promoting translation initiation (Fox 1996a; Rödel 1997; Towpik 2005). The translational activators for the *COX1*, *COX2*, and *COX3* mRNAs are present at low levels that are rate limiting for gene expression (Fox 1996b; Steele *et al.* 1996; Green-Willms *et al.* 2001; Naithani *et al.* 2003; Perez-Martinez *et al.* 2009). Upsetting the balance of translational activators can interfere with cytochrome *c* oxidase biogenesis (Fiori *et al.* 2005).

Mtf2 (Nam1) interacts physically with the activator Pet309, a pentatricopeptide repeat (PPR) protein that binds both the *COX1* mRNA 5′-UTR and mitochondrial ribosomes (Manthey and Mcewen 1995; Naithani *et al.* 2003; Tavares-Carreon *et al.* 2008; F. Tavares-Carreon, A. Zamudio-Ochoa, Y. Camacho-Villasana, and X. Perez-Martinez; personal communication). PPR motifs have been identified in many organellar RNA-binding proteins with sequence-specific functions (Delannoy *et al.* 2007) and have been identified in eight mRNA-specific translational activators as well as Rmd9 and the Rpo41 amino terminal domain (Lipinski *et al.* 2011). Thus, it is likely that the *COX1* mRNA is transferred from the RNA polymerase Rpo41 to Mtf2 (Nam1)/Sls1 and Rmd9 to Pet309 for translation by the ribosome. Indeed, Pet309 and at least two ribosomal proteins copurify with Rpo41 (Markov *et al.* 2009). Mtf2 (Nam1) also interacts with the PPR-containing *COX2* mRNA-specific activator Pet111, and with Pet494, which activates *COX3* mRNA translation, suggesting a similar pathway for those mRNAs (Naithani *et al.* 2003; Lipinski *et al.* 2011) (Figure 8).

The translational activators promoting synthesis of cytochrome *c* oxidase subunits have been shown to interact with each other (Brown *et al.* 1994; Naithani *et al.* 2003). Furthermore, a large protein complex can be isolated from mitochondria

Table 7 Proteins that activate translation of specific mitochondrially coded mRNAs by various mechanisms

Mitochondrial mRNA	Activator proteins	Activator protein ORFs
ATP8-ATP6	Aep3, Assembled F_1 ATPase	YPL005W,YBL099W,YJR121W,YBR039W,YPL271W
ATP6	Atp22	YDR350C
ATP9	Aep1, Aep2, Atp25	YMR064W, YMR282C, YMR098C
COB	Cbp1, Cbp3, Cbp6, Cbs1, Cbs2	YJL209W, YPL215W, YBR120C, YDL069C, YDR197W
COX1	Pet54, Pet309, Mss51	YGR222W, YLR067C, YLR203C
COX2	Pet111	YMR257C
COX3	Pet54, Pet122, Pet494	YGR222W, YER153C, YNR045W
VAR1	Sov1	YMR066W

that contains Pet309 and the *COB* mRNA-specific activator Cbp1, as well as several unidentified proteins (Krause *et al.* 2004). These findings suggest that clusters of translational activators promote colocalized synthesis of mitochondrially coded respiratory complex subunits at distinct locations. Based on the levels of translational activator proteins, there appear to be at most a few hundred such sites per cell (Marykwas and Fox 1989; Fox 1996b; Ghaemmaghami *et al.* 2003; Naithani *et al.* 2003). It seems likely that these sites are adjacent to nucleoids.

The physiological significance of distinct sites or topologies for mitochondrial translation is supported by experiments in which Cox2 was translated *in vivo* from a chimeric mRNA with the 5′- and 3′-UTRs of the *VAR1* mRNA, which normally flank the ORF encoding a ribosomal protein. While the rate of Cox2 synthesis was normal, Cox2 accumulation and cytochrome *c* oxidase activity were severely reduced (Sanchirico *et al.* 1998). The putative translational activator for the *VAR1* mRNA is Sov1, a membrane-bound PPR protein (Sanchirico 1998; Lipinski *et al.* 2011; M. E. Sanchirico, A. R. Wiffen, T. D. Fox, and T. L. Mason; unpublished results), and translation of the chimeric mRNA appears to be membrane bound (Fiori *et al.* 2003). However, the *VAR1* mRNA 5′-UTR apparently mislocalizes or misorients Cox2 synthesis on the inner membrane such that Cox2 cannot be efficiently assembled with other cytochrome *c* oxidase subunits and is largely degraded.

Interestingly, bacteria have also recently been found to employ signals embedded in mRNA sequences to localize translation of membrane proteins (Nevo-Dinur *et al.* 2011).

It has been suggested that localization of mitochondria-bound cytoplasmic protein synthesis and import could be coordinated with localization of mitochondrial protein synthesis to promote assembly of complexes containing both kinds of proteins (Garcia *et al.* 2007a). There is at present no evidence directly bearing on this interesting hypothesis.

Assembly of cytochrome c oxidase

The three mitochondrially coded subunits of yeast cytochrome *c* oxidase form its catalytic core (Tsukihara *et al.* 1996). They are surrounded by eight subunits imported from the cytoplasm (Taanman and Capaldi 1992; Soto *et al.* 2011). The order in which these subunits are assembled is not known, despite efforts to analyze subassemblies detected in mutants (Church *et al.* 2005; Horan *et al.* 2005). However, assembly is believed to be nucleated by mitochondrial synthesis of the core subunits, in particular Cox1 (Herrmann and Funes 2005; Fontanesi *et al.* 2008; Mick *et al.* 2011; Soto *et al.* 2011)

Cox1 is highly hydrophobic, with 12 transmembrane domains, and both termini are on the inside of the inner membrane. In the assembled enzyme Cox1 contains two heme A moieties and a copper atom that participate in electron transport (Tsukihara *et al.* 1995). Cox1 is believed to be cotranslationally inserted into the inner membrane by Oxa1 (Hell *et al.* 2001).

The synthesis of Cox1 is coupled to the formation of early assembly intermediates and is downregulated if assembly is artificially blocked by a variety of mutations (Barrientos *et al.* 2004; Shingu-Vazquez *et al.* 2010). This assembly-feedback regulation may prevent overproduction of Cox1 to protect yeast cells from damage due to pro-oxidant activity of unassembled Cox1 (Khalimonchuk *et al.* 2007). Mss51, a mitochondrial inner membrane protein with at least two functions, is a key factor in this regulatory circuit. Mss51 activates *COX1* mRNA translation via its 5′-UTR, in conjunction with Pet309, and also binds to newly synthesized Cox1 (Decoster *et al.* 1990; Perez-Martinez *et al.* 2003, 2009; Zambrano *et al.* 2007) (Figure 9).

Newly synthesized Cox1 nucleates the formation of an early pre-assembly complex with Mss51 and two short membrane proteins, Cox14 and Coa3 (Cox25) (Perez-Martinez *et al.* 2009; Fontanesi *et al.* 2010a; Mick *et al.* 2010). In addition, whether complexed with Cox1 or not, Mss51 is stoichiometrically associated with mtHsp70 (Ssc1) (Fontanesi *et al.* 2010b) (Figure 9). Both *cox14* and *coa3* deletions prevent association of Mss51 with Cox1 and allow uncontrolled Cox1 synthesis despite the fact that they prevent cytochrome *c* oxidase assembly (Perez-Martinez *et al.* 2009; Fontanesi *et al.* 2010a; Mick *et al.* 2010). Thus, sequestration of Mss51 in assembly intermediates containing Cox1 appears to prevent it from activating *COX1* mRNA translation. Truncation of the C-terminal 11 residues of Cox1, normally exposed in the matrix, destabilizes this pre-assembly complex and disrupts assembly-feedback regulation, but does not prevent cytochrome *c* oxidase assembly (Shingu-Vazquez *et al.* 2010).

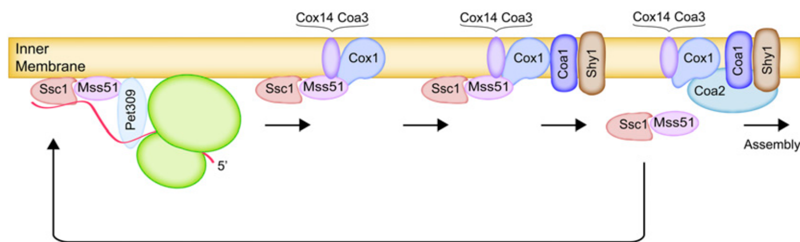

Figure 9 Assembly feedback control of Cox1 synthesis by Mss51 activities as a translational activator and assembly factor. Mitochondria translation of the *COX1* mRNA is activated by the mRNA-specific activators Pet309 and Mss51 [Mss51 is associated with Hsp70 (Ssc1) throughout]. Synthesis of a new Cox1 polypeptide nucleates an early assembly intermediate containing Mss51 and the assembly factors Cox14 and Coa3. As additional assembly factors associate with newly synthesized Cox1, Mss51 is released from the assembly intermediates and is then available to initiate additional Cox1 synthesis.

Assembly of Cox1 proceeds by the addition of the assembly factor Coa1 to the pre-assembly complex nucleated by Cox1, which in turn allows the addition of Shy1 (Mick *et al.* 2007, 2010; Pierrel *et al.* 2007; Khalimonchuk *et al.* 2010). Neither Coa1 nor Shy1 are completely essential for production of active cytochrome *c* oxidase [both are virtually dispensable in the D273-10B strain background (L. S. Burwell, Z. W. Via, and T. D. Fox, unpublished results)]. Mss51 is thought to be released from assembly intermediates in a step after Shy1 association, which may involve the addition of the imported cytochrome *c* oxidase subunits Cox5 (encoded by *COX5A* and *COX5B*) and Cox6 (Barrientos *et al.* 2002b; Mick *et al.* 2007; Shingu-Vazquez *et al.* 2010), although some evidence indicates that this occurs farther upstream (Khalimonchuk *et al.* 2010). The released Mss51 becomes free to activate another round of Cox1 synthesis, completing the regulatory circuit (Figure 9).

Cox1 assembly proceeds by the addition of redox cofactors. Shy1, together with Coa2, appears to promote the insertion of heme A into Cox1 (Pierrel *et al.* 2008; Bestwick *et al.* 2010). The copper-binding membrane protein Cox11 receives copper from the soluble shuttle protein Cox17 and inserts it into assembling Cox1 from the IMS side (Beers *et al.* 1997; Banting and Glerum 2006; Cobine *et al.* 2006; Khalimonchuk *et al.* 2010). The subunits Cox5 and Cox6, which directly contact Cox1, are believed to be associated at this stage with Cox1, Cox14, Shy1, Coa1, and Coa3 in a subassembly that awaits the addition of mitochondrially synthesized Cox2 and Cox3 (Horan *et al.* 2005; Fontanesi *et al.* 2010a; Khalimonchuk *et al.* 2010; Mick *et al.* 2010).

Cox2 has two transmembrane domains and acidic N- and C-terminal domains that are exported to the IMS side of the inner membrane prior to assembly (Tsukihara *et al.* 1996). The C-terminal domain binds two copper atoms. In *S. cerevisiae*, Cox2 is synthesized as a precursor with a 15-residue leader peptide (Pratje *et al.* 1983) whose length and sequence can be altered dramatically without destroying function (Bonnefoy *et al.* 2001). However, the mRNA sequence encoding the leader peptide contains a positive element that is required to antagonize negative elements downstream in the mRNA that otherwise prevent completed translation (Bonnefoy *et al.* 2001; Williams and Fox 2003). Taken together with the functional interactions of these elements with mitochondrial ribosomes (Williams *et al.* 2004, 2005; Prestele *et al.* 2009), these findings suggest the possibility that regulation of translation elongation could be coupled to steps in membrane topogenesis of pre-Cox2.

The pre-Cox2 N-terminal domain is cotranslationally exported by Oxa1 (He and Fox 1997; Hell *et al.* 1998; Bonnefoy *et al.* 2009). The leader peptide is rapidly processed in the IMS by the inner membrane protease in a reaction chaperoned by Cox20, an inner membrane protein whose topology resembles that of Cox2 (Nunnari *et al.* 1993; Hell *et al.* 2000; Jan *et al.* 2000).

Export of the acidic Cox2 C-terminal domain also requires Oxa1, but appears to be post-translational (He and Fox 1997; Fiumera *et al.* 2007). Its export also depends specifically upon another highly conserved inner membrane translocase, Cox18, which is paralogously related to Oxa1, and on two inner membrane proteins, Mss2 and Pnt1, which interact with Cox18 (He and Fox 1999; Broadley *et al.* 2001; Saracco and Fox 2002; Funes *et al.* 2004). The Cox20 chaperone also functions in C-tail export, probably by removing the exported protein from Cox18 (Fiumera *et al.* 2009; Elliott *et al.* 2012). Mature but unassembled Cox2 remains associated with Cox20 (Hell *et al.* 2000). Metallation of the Cox2 C-terminal domain is catalyzed by membrane-bound Sco1, which receives copper from the soluble Cox17 shuttle and then directly interacts with Cox2 (Lode *et al.* 2000; Cobine *et al.* 2006; Rigby *et al.* 2008). Metallation is believed to precede Cox2 addition to the Cox1 subassembly complex (Soto *et al.* 2011).

Insertion of the seven transmembrane domains of Cox3, whose amino terminal residues remain on the matrix side, is assisted by Oxa1 (Tsukihara *et al.* 1996; Hell *et al.* 2001). There are no known specific chaperones involved in further assembly of Cox3 into the catalytic core of cytochrome *c* oxidase. Nor are there data on the addition of imported subunits to form the active enzyme.

Assembly of the bc_1 complex

The catalytic core of the bc_1 complex comprises three subunits. Cob (cytochrome *b*) is mitochondrially synthesized and embedded in the membrane with its two noncovalently bound heme B moieties. The imported subunits Cyt1 (cytochrome c_1) and Rip1 (Rieske 2Fe-2S protein) are anchored to the membrane with their hydrophilic domains bearing heme C and 2Fe-2S cluster redox cofactors, respectively, in the IMS (Solmaz and Hunte 2008; Zara *et al.* 2009; Smith *et al.* 2012). Seven additional imported proteins that do not directly participate in electron transfer complete the bc_1 complex structure, which forms a dimer. Analysis of subassemblies detected in mutants indicates that Cob may initially associate

with two imported subunits, Qcr7 and Qcr8, while Cyt1 associates with Cor1 and Cor2 (Crivellone *et al.* 1988; Zara *et al.* 2004, 2007). Association of these subcomplexes, facilitated by the assembly factor Bca1 (Mathieu *et al.* 2011), forms a nearly complete species that subsequently accepts Rip1.

Cob has eight transmembrane domains, and both termini are on the inside of the inner membrane. Its two heme B moieties are inserted by an unknown mechanism. Oxa1 participates in its membrane insertion (Hell *et al.* 2001). Translation of the *COB* mRNA is specifically activated through its 5′-UTR by Cbs1, Cbs2, and Cbp1 (Rödel 1997; Islas-Osuna *et al.* 2002). In addition, synthesis of Cob is promoted by two proteins, Cbp3 and Cbp6, that bind to mitochondrial ribosomes, associate with newly synthesized Cob after release from the ribosome, and together with Cob form a pre-assembly complex with the assembly factor Cbp4 (Gruschke *et al.* 2011). While this mechanism couples translation to assembly, Cob synthesis is not reduced by a feedback mechanism when assembly of the bc_1 complex is disrupted.

The complex import pathway of Cyt1, resulting in a hydrophilic N-terminal domain in the IMS with a C-tail anchor, was described above. Covalent attachment of heme to this hydrophilic domain is catalyzed by the lyase Cyt2 either during or after maturation of Cyt1 (Bernard *et al.* 2003).

The maturation of Rip1 is particularly interesting. This protein is initially fully imported into the matrix (Hartl *et al.* 1986). Since Fe-S cluster biogenesis occurs in the matrix, it is believed that assembly of the Rip1 polypeptide with its 2Fe-2S cluster occurs after this initial import reaction (Kispal *et al.* 1999; Lill 2009; Wagener *et al.* 2011). In any event, the C-terminal domain of Rip1 is subsequently exported through the inner membrane to the IMS. This export reaction specifically requires Bcs1, a member of the AAA family of ATPases that is tethered to the inner surface of the inner membrane (Nobrega *et al.* 1992; Wagener *et al.* 2011). Most mitochondrial AAA proteins are hexameric complexes with a central compartment capable of ATP-dependent unfolding, proteolysis, and chaperone-like activity (Fiumera *et al.* 2009; Tatsuta and Langer 2009; Truscott *et al.* 2010). In the case of Bcs1, it appears that an enlarged central compartment translocates the folded C-terminal domain of Rip1 bearing its 2Fe-2S cluster across the membrane and releases the Rip1 N-terminal transmembrane domain laterally into the membrane (Wagener *et al.* 2011). The matrix protein Mzm1 facilitates this export of Rip1 from the matrix (Atkinson *et al.* 2010, 2011), which must occur prior to its assembly into the bc_1 complex.

Assembly of the ATP synthase

The raison d'être of the respiratory chain is the synthesis of ATP. This reaction is carried out by the well-known F_1F_0 ATP synthase. The F_0 sector in the inner membrane captures energy of the proton gradient as rotary motion and turns the central stalk of the F_1 sector, which projects into the matrix. Rotation of the central stalk relative to the catalytic sites in the $Atp1_3$-$Atp2_3$ ($\alpha_3\beta_3$) hexamer of F_1, which is fastened to nonrotating proteins in F_0 by the peripheral stalk or stator, drives the synthesis of ATP from ADP and inorganic phosphate (Stock *et al.* 1999; von Ballmoos *et al.* 2009). In *S. cerevisiae*, all subunits of F_1 and of the stator are imported (Velours and Arselin 2000; Ackerman and Tzagoloff 2005). Three hydrophobic core proteins of F_0,—Atp6, Atp8, and Atp9—are mitochondrial gene products, while two others are imported. Current evidence indicates that the complete ATP synthase is assembled from modular subassemblies, such that the proton-conducting pore of F_0, formed by the interface between Atp6 and Atp9 (Fillingame and Dmitriev 2002), is formed at the last step (Rak *et al.* 2009, 2011) (Figure 10). This hypothesis predicts that the proton pore is immediately coupled to the catalytic machinery at the moment that it is formed, avoiding the deleterious effects of unregulated proton leakage across the inner membrane.

One pre-assembly module is F_1 itself, which assembles in the absence of F_0 or the stator (Schatz 1968; Tzagoloff 1969; Velours and Arselin 2000). The newly imported subunit Atp1 is bound stoichiometrically in a dimeric assembly intermediate with the specific chaperone Atp12, while the newly imported Atp2 subunit is similarly bound by its chaperone Atp11 (Ackerman and Tzagoloff 1990; Ludlam *et al.* 2009). Release of these F_1 subunits from their chaperones to allow the formation of Atp1-Atp2 dimers and hexamers requires the presence of the subunit Atp3 (γ), a component of the central stalk. In the case of Atp1, structural evidence strongly suggests that Atp3 binding to Atp1 directly displaces the chaperone Atp12 (Ludlam *et al.* 2009). An additional protein, Fmc1, is required to generate Atp1-Atp2 dimers at high temperature (Lefebvre-Legendre *et al.* 2001), and Hsp90 chaperones have also been implicated in this process (Francis and Thorsness 2011). Addition of the central stalk proteins Atp16 (δ) and Atp15 (ε) has not been examined.

A second pre-assembly module is the rotating component of F_0, comprising a decameric ring of the highly hydrophobic mitochondrially coded Atp9 (subunit c) embedded in the membrane (Stock *et al.* 1999; Rak *et al.* 2011). [The assembled ring structure is not dissociated by treatment with SDS (Herrmann *et al.* 1994; Rak *et al.* 2011).] Translation and/or stability of the *ATP9* mRNA requires the specific activators Aep1, Aep2, and Atp25 (Ackerman *et al.* 1991; Finnegan *et al.* 1991; Payne *et al.* 1993; Ziaja *et al.* 1993; Ellis *et al.* 1999; Zeng *et al.* 2008; Rak *et al.* 2009). Atp25 is particularly interesting as the 60-kDa protein is cleaved roughly in half to yield a C-terminal fragment that is required for *ATP9* mRNA stability and/or translation and an N-terminal fragment that is not required for Atp9 synthesis but appears to promote assembly of the Atp9 ring (Zeng *et al.* 2008). Insertion of Atp9 into the inner membrane and assembly of the ring structure does not require Oxa1 (Lemaire *et al.* 2000; Jia *et al.* 2007; Mathieu *et al.* 2010) or the other mitochondrially coded F_0 subunits Atp6 and Atp8 (Rak *et al.* 2011). However, Oxa1 does have a role in promoting association of the Atp9 ring with a single Atp6 molecule late in the assembly of ATP synthase (Jia *et al.* 2007)

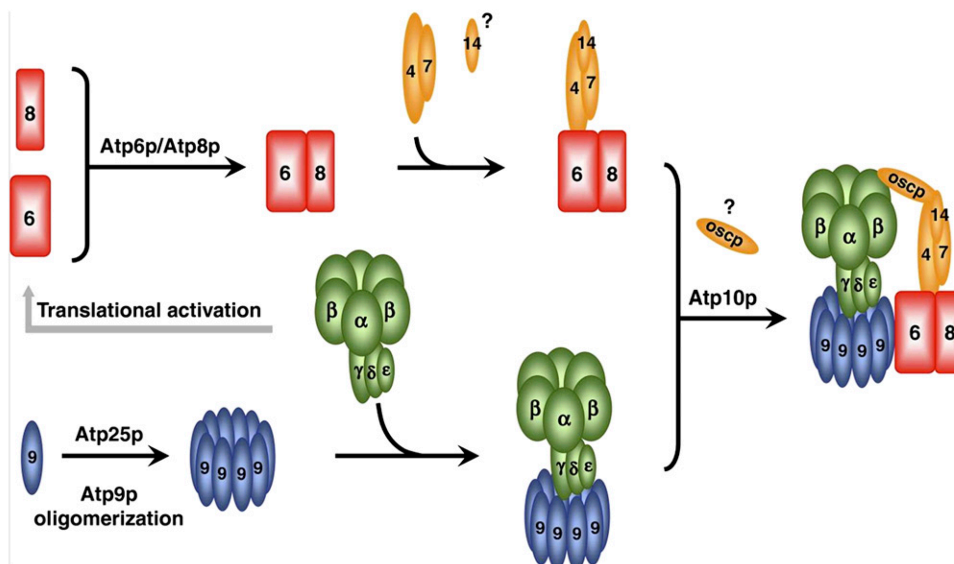

Figure 10 Assembly of ATP synthase from modular subassemblies. The imported subunits of the F_1 complex (green) are assembled in the matrix with the help of specific assembly chaperones (see text for details). Assembled F_1 activates mitochondrial translation of the dicistronic mRNA encoding Atp8 and Atp6, which nucleate the assembly of imported subunits into the stator module (red and tan). The Atp9 ring (blue) is assembled from monomers and then joined with F_1. Finally, association of the stator with the F_1-Atp9 ring subassembly generates the ATP synthase proton pore at the same time that ATPase activity is coupled to the inner membrane proton gradient. Reprinted by permission from Macmillan Publishers Ltd. from Rak *et al.* (2011).

The assembled F_1 and Atp9 ring modules associate to form a larger pre-assembly complex independently of other F_0 subunits through interaction of the central stalk subunits Atp3 and Atp15 with the ring (von Ballmoos *et al.* 2009; Rak *et al.* 2011).

A third pre-assembly module has at its core the mitochondrially synthesized F_0 components Atp6 and Atp8. Both of these two proteins are translated from the only dicistronic yeast mitochondrial mRNA, ensuring that their synthesis is colocalized (Dieckmann and Staples 1994). Translation of the downstream coding sequence, *ATP6*, requires the specific activator Atp22 acting on a region upstream of the coding sequence (Zeng *et al.* 2007c). However, translation of the upstream *ATP8*-coding sequence does not require Atp22. Interestingly, translation of both coding sequences requires the presence of F_1 or an F_1 pre-assembly complex, but does not depend upon F_1 ATPase catalytic activity (Rak and Tzagoloff 2009). The target upon which F_1 acts is in an untranslated regions(s) of the dicistronic mRNA, but has not been further localized. Whatever the mechanism of this translational control, it couples the production of the Atp6-Atp8 preassembly module to the presence of the imported subunits of one of its assembly partners.

Translation of the *ATP6* sequence yields pre-Atp6, with an N-terminal leader peptide (Michon *et al.* 1988). While partially dispensable, this leader peptide does promote efficient assembly of ATP synthase (Zeng *et al.* 2007a). After the N terminus is translocated through the inner membrane by an unknown mechanism, the Pre-Atp6 leader peptide is specifically cleaved by Atp23, a metalloprotease (Osman *et al.* 2007; Zeng *et al.* 2007b).

Immediately after their synthesis, Atp6 and Atp8 associate in the inner membrane with each other, forming a pre-assembly module that lacks Atp9 and F_1 subunits (Rak *et al.* 2011). This complex also contains at least two proteins that comprise the peripheral stator stalk (Atp4 and Atp7) and Atp10, a specific chaperone protein known to bind Atp6 and promote its final assembly with the Atp9 ring (Tzagoloff *et al.* 2004; Rak *et al.* 2011). Additional stator subunits were not identified in this pre-assembly complex (or group of similarly sized complexes) nor was the Atp23 protease, which, like Atp10, binds to Atp6 and promotes association of Atp6 and Atp9 by a mechanism independent of its catalytic activity in cleaving the pre-Atp6 leader peptide (Osman *et al.* 2007; Zeng *et al.* 2007b). Further work will be required to understand the assembly of the stator and the nature of the pre-assembly module containing Atp6, Atp8, and, presumably, the imported subunits of F_0.

Despite current uncertainties, final assembly of the complete F_1F_0 ATP synthase appears to involve the joining of two pre-assembly complexes produced by independent pathways: the F_1-Atp9 ring complex and an Atp6-Atp8-stator complex (Figure 10). Such a mechanism would form, in a single step, both the complete F_0 rotary motor and the stator bridge between the F_1 and F_0 sectors, coupling the proton-motive force to ATP synthesis (Rak *et al.* 2011).

Assembled ATP synthase monomers associate with each other via contacts between their single Atp6 subunits and other components of their F_0 sectors and stators within the membrane (Wagner *et al.* 2010; Velours *et al.* 2011). The ATP synthase dimers in turn form larger chain-like oligomeric structures that apparently help to maintain membrane curvature of cristae (Stuart 2008; Rabl *et al.* 2009; Velours *et al.* 2009; Zick *et al.* 2009).

Perspective

There is no gainsaying the utility of *Saccharomyces* as a system for exploring biological mechanisms. The importance of this is compounded by the evolutionary conservation of so many cellular mechanisms among eukaryotes, including humans. The synthesis, import, and assembly of

mitochondrial proteins can be largely included with other such conserved processes.

The TOM, SAM, TIM22, and TIM23 complexes appear to be nearly universally present in eukaryotes (Paschen *et al.* 2003; Dolezal *et al.* 2006; Kutik *et al.* 2009; Hewitt *et al.* 2011; Rada *et al.* 2011) [the TIM22 complex appears to be absent in some, such as Trypanosomes (Schneider *et al.* 2008)]. The core components of these complexes have maintained significant sequence conservation. Furthermore, expression of a mitochondrially targeted protein from one species in cells of another typically results in correct protein localization by the host import machinery.

Similarly, many of the accessory proteins required to assemble respiratory complexes have apparent or confirmed orthologs in humans (Ackerman and Tzagoloff 2005; Soto *et al.* 2011; Wagener *et al.* 2011). Particularly striking is the fact that the human orthologs of Oxa1 (Bonnefoy *et al.* 1994) and Cox18 (Gaisne and Bonnefoy 2006) can support assembly of cytochrome *c* oxidase and respiratory growth in the corresponding yeast mutants. Furthermore, the ability of the human *OXA1* mRNA to direct synthesis of the functional mitochondrial protein depends upon interaction of the human mRNA 3′-UTR with yeast mitochondria, suggesting conservation in the mechanisms of mRNA targeting (Sylvestre *et al.* 2003).

In contrast to the clear conservation of protein import and assembly mechanisms, mitochondrial genome structures and their expression systems are highly divergent among eukaryotic phyla (Burger *et al.* 2003). Nevertheless, the strategy of linking transcription and translation together at the inner membrane, identified in yeast, appears to be employed by mammals for the synthesis and assembly of highly hydrophobic mitochondrial gene products (Weraarpachai *et al.* 2009; Sasarman *et al.* 2010; Brown *et al.* 2011; He *et al.* 2012).

Given the increasingly evident role of a broad array of mitochondrial functions in the maintenance of healthy human cells, tissues, and bodies (Nunnari and Suomalainen 2012), detailed studies of mechanisms underlying mitochondrial activities in yeast should remain a high priority.

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