

# Modifications and Innovations in the Evolution of Mitochondrial Protein Import Pathways

Victoria Hewitt, Trevor Lithgow, and Ross F. Waller

**Abstract** Eukaryotic cells are defined by their mitochondria, organelles that were derived through endosymbiosis. The development of this organelle from a bacterial endosymbiont required establishment of effective protein import pathways so that much of the genetic capacity of the bacterium could be relocated to the host cell. Two realms of study have delivered insight into the early evolution of these mitochondrial pathways: (1) considering the “starting material” based on what can be observed of protein trafficking pathways in extant species of bacteria and (2) analysing the protein import pathways of parasites whose mitochondria have undergone secondary reduction and now offer insight into minimal functional pathways. These approaches have illuminated what components of bacterial trafficking pathways were co-opted in the developing mitochondrion and what further innovations occurred within the eukaryote host. Now comparative analysis of model mitochondrial systems, with organelles found in a broad diversity of eukaryotes (namely protists), shows when in eukaryotic radiation these major innovations took place and what lineage-specific changes have since occurred to mitochondrial import systems in eukaryotes.

---

V. Hewitt

Department of Biochemistry and Molecular Biology, Monash University, Clayton Campus, Melbourne 3800, Australia

T. Lithgow (✉)

Department of Microbiology, Monash University, Clayton Campus, Melbourne 3800, Australia

e-mail: [trevor.lithgow@monash.edu](mailto:trevor.lithgow@monash.edu)

R.F. Waller (✉)

School of Botany, University of Melbourne, Melbourne 3010, Australia

e-mail: [r.waller@unimelb.edu.au](mailto:r.waller@unimelb.edu.au)

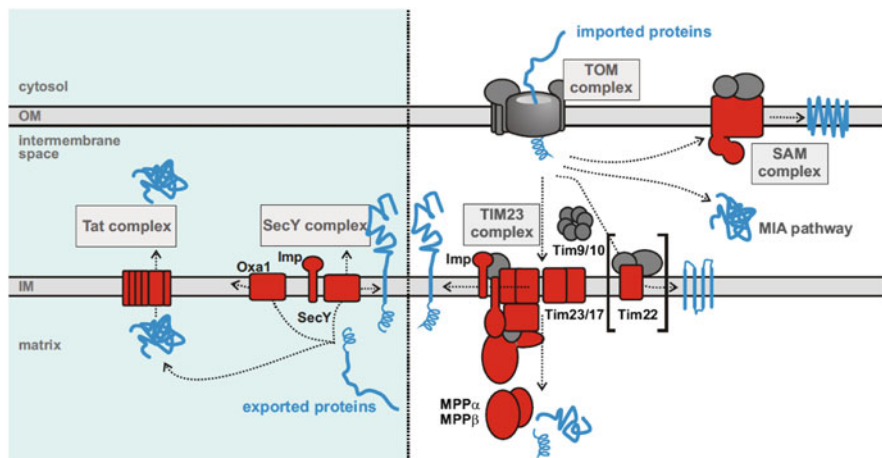
## Contents

Introduction .....	20
An Ancestral System Doing (More or Less) What It Has Always Done .....	21
Ancestral Machines Have Been Modified and Recombined in the Course of Evolution .....	23
New Machines—Without a (Bacterial) Trace .....	25
New Insights into Organelle Evolution from Mitochondrial Diversity .....	26
References .....	30

## Introduction

Like metabolic and signal transduction pathways, protein transport pathways have been revealed component by component through diverse experimental approaches and using diverse model organisms. In many cases, including the protein import pathway into mitochondria, we now have sufficient understanding to address holistic questions about protein transport pathways, such as how transport along a pathway is regulated, how the flux of a protein transport pathway impacts on other (metabolic, signal transduction, gene regulatory) pathways and, the subject of this review, how the pathways evolved. The conversion of an ancestral endosymbiont to a mitochondrion involved the transfer of genes from the bacterial population to the host cell genome (Andersson and Kurland 1999; Cavalier-Smith 2002, 2006; de Duve 2007; Embley and Martin 2006; Gray et al. 1999, 2001; Lang et al. 1999; Timmis et al. 2004). For this process to take effect the gene products translated in the cytosol had to be recognised and imported into mitochondria. The changes to the endosymbiont in its conversion to an organelle could not have occurred simultaneously and, accordingly, our understanding of the evolution of protein import pathways is developing through the characterisation of key events in this stepwise evolutionary process.

Mitochondrial protein import depends on the activity of a series of molecular machines. There are three themes that emerge when investigating the evolution of the mitochondrial protein transport machinery. Firstly, some pre-existing protein transport machines derived from bacteria were used directly, maintaining their ancestral function in mitochondria. Secondly, some parts of the bacterial machinery have been modified and recombined so that their ancestral biochemical function is adapted to perform new functions in a protein transport pathway. Thirdly, some machines bear no sequence similarity to bacterial proteins suggesting a non-bacterial origin, and examples from parasites give clues as to where and how these factors arose. Here we review how the endosymbiont's own protein transport machinery was adapted to new roles, how entirely novel machinery arose from pre-existing component parts and how embellishments unique to this particular host-symbiont relationship were set in place in the evolution of mitochondria. We will also consider how an increasingly expanded view of mitochondrial diversity in eukaryotes is shaping our understanding of the development of these pathways.



**Fig. 1** Ancestral and derived import machines used in extant mitochondria. Membrane-located translocation machines are positioned in the outer and inner mitochondrial membranes, where they effect protein import into the organelle and assembly of integral proteins into the mitochondrial membranes. The details of the components of these machines have been recently reviewed (Chacinska et al. 2009; Neupert and Herrmann 2007). The *blue panel* summarises the machinery required for assembly of matrix-encoded membrane proteins into the inner membrane. *Red shading* denotes proteins for which bacterial proteins have been identified as ancestral; *grey shading* denotes components for which no bacterial ancestor has been identified

## An Ancestral System Doing (More or Less) What It Has Always Done

The major biosynthetic route for bacterial membrane and periplasmic (intermembrane space) proteins is outward, from their synthetic origin in the cytoplasm to their peripheral destination. The bacterial protein trafficking pathways conserved in mitochondria, therefore, reflect this outward orientation. Initially these pathways would have supported only organelle-encoded gene products. Yet as more genes moved to the nucleus, some of these proteins would come to journey first across the mitochondrial membranes (either one or two) and then be redirected into their correct destination by these relic bacterial systems. Despite differences in nomenclature, four such “bacterial” systems are remnant in mitochondria—SecYEG, YidC (Oxa1), BAM (SAM) and Tat (discussed in section “New Insights into Organelle Evolution from Mitochondrial Diversity”).

SecYEG and YidC translocases function in protein transport across bacterial inner membranes, are universally found in bacteria and therefore would have been present in the ancestral endosymbiont to mediate protein assembly into the inner membrane. The mitochondrial OXA (Oxidase Assembly) translocase was derived from the endosymbiont’s YidC (Kiefer and Kuhn 2007; Ott and Herrmann 2010; Pohlschroder et al. 2005; Stuart 2002; van der Laan et al. 2005) and fulfils a functionally homologous role in mitochondria (Fig. 1). The mitochondrial core

subunit Oxa1 can complement the lack of *yidC2* from *Streptococcus mutans*; YidC2 from this bacterium can also function in place of Oxa1 in *Saccharomyces cerevisiae* (Funes et al. 2009). The Oxa1 protein is widely found in eukaryotes (Dolezal et al. 2006), though it has been lost from organisms such as *Cryptosporidium parvum* that lack a mitochondrial genome and therefore do not assemble membrane proteins from the internal face of the inner membrane (Alcock et al. 2012). The SecYEG complex can function alone to transfer proteins into the bacterial periplasm (equivalent to the mitochondrial intermembrane space) and to insert membrane proteins with simple topologies into the inner membrane (Driessen et al. 2001) (Fig. 1). The SecYEG complex can also function in concert with YidC to assemble membrane proteins with more complicated topologies (Samuelson et al. 2000). SecYEG would have been present in the early protomitochondria: even today some jakobid protists, such as *Reclinomonas americana*, encode a SecY translocase in their mitochondrial DNA [see section “New Machines—Without a (Bacterial) Trace” for discussion] (Lang et al. 1997; Tong et al. 2011). In most mitochondria the SecYEG has been lost, but strikingly, for proteins of complex membrane topology, the inner membrane TIM complex cooperates with the OXA complex just as the bacterial SecYEG and YidC complexes do (van der Laan et al. 2005; Webb and Lithgow 2010).

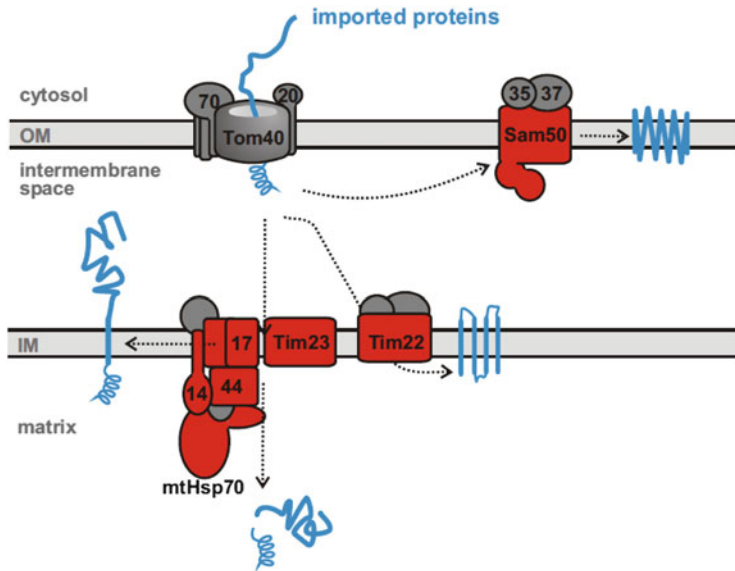
The bacterial origin of the SAM (Sorting and Assembly Machinery) complex in the mitochondrial outer membrane is even clearer; it evolved from the BAM complex, found in the outer membrane of all Gram-negative bacteria (Fig. 1) (Cavalier-Smith 2006; Dolezal et al. 2006; Gentle et al. 2004; Gross and Bhattacharya 2009). The SAM and the BAM complexes fulfil the same function: inserting beta-barrels from the internal face of the outer membrane, although in the case of mitochondria these proteins are first imported across this outer membrane. The core components of the SAM and BAM complexes have a common ancestry and are known as Sam50 in mitochondria and BamA (also known as Omp85) in bacteria (Gatsos et al. 2008; Gentle et al. 2004, 2005; Knowles et al. 2009; Ruiz et al. 2006). While the SAM and BAM complexes are functionally homologous, significant evolution is evident in the mitochondrial SAM complex. Mitochondria have lost whole aspects of bacterial envelope biogenesis including the ability to synthesise lipoproteins (Gabaldon and Huynen 2007) determining the loss of the lipoprotein partners: BamB, BamC, BamD and BamE from the endosymbiont's BAM complex (Gatsos et al. 2008; Knowles et al. 2009; Ruiz et al. 2006). These have been replaced, either during or subsequent to this period of lipoprotein loss, by key proteins of uncertain ancestry. From functional studies we know that the metaxins, and perhaps other proteins too, serve as “modules” of the SAM complex (discussed further below). Further analysis of these components promises a fuller understanding of how the mitochondrial SAM complex evolved. It is reasonable to imagine that there was a relatively seamless transition between the two forms of the essential Omp85 protein at the core of the complex (BamA/Sam50). Further mechanistic or structural insights are needed before we can fully understand how these proteins acted as the drivers of outer membrane protein assembly throughout this evolutionary scenario.

## Ancestral Machines Have Been Modified and Recombined in the Course of Evolution

As the number of genes relocated from the endosymbiont to the host cell nucleus increased from tens to hundreds (and up >1,000), the need to translocate this vast repertoire of proteins would have substantially changed the demands placed on these former bacterial membranes. Novel translocation machinery therefore demanded innovation beyond the means of modified bacterial machinery. One way that mitochondria responded to this demand was to tinker with existing machinery, deploying it in new ways.

In many eukaryotes, there are two inner membrane translocases in mitochondria: TIM22 and TIM23 (Fig. 1). These translocases have divergent functions, with TIM22 responsible for delivering the many poly-topic membrane proteins of the inner membrane, and TIM23 handling the bulk of the matrix destined proteins as well as select inner membrane proteins. The channel components of TIM23 and TIM22 are related to each other by sequence and were derived from a gene duplication event (Cavalier-Smith 2006; Gross and Bhattacharya 2009; Jensen and Dunn 2002; Schneider et al. 2008). It can therefore be readily argued that the first mitochondria had a single TIM complex. At least two diverse sets of parasites, the Trypanosomes and the Microsporidia, contain only one TIM complex. While it is unclear at this point if Trypanosomes ancestrally possessed two TIMs, Microsporidia are related to Fungi providing clear evidence that in this case one of the duplicated TIMs has been secondarily lost. These parasites serve as an excellent example of how in an ancestral mitochondrion such a single TIM complex could provide both translocation and membrane assembly functions.

The core of the TIM23 complex is the Tim23 subunit, a poly-topic membrane protein that forms the protein import channel (Alder et al. 2008; Truscott et al. 2001). In a groundbreaking paper, Rassow et al. (1999) suggested that the Tim23 channel was derived from an amino acid transporter called LivH. While this ancestral LivH relationship has been questioned (Gross and Bhattacharya 2009), the model proposed by Rassow and colleagues remains enlightening: that a channel capable of transporting bulky, hydrophobic amino acids across the inner membrane would require relatively little adaptation in order to transfer polypeptides. The crux would be providing sufficient energy to “pull” a polymer of these amino acids through the channel. Even though the pair-wise sequence conservation between LivH family members and Tim23 proteins is very low (Cavalier-Smith 2006; Gross and Bhattacharya 2009; Murcha et al. 2007; Rassow et al. 1999), a signature PRAT (PReprotein and Amino acid Transporters) motif found in the Tim23 family of mitochondrial translocases is also present in the LivH protein of bacteria (Cavalier-Smith 2006; Murcha et al. 2007; Rassow et al. 1999). Systematic analysis of the family in *Arabidopsis thaliana* has also shown that plants retain mitochondrial versions of this protein family that transport amino acids as well as proteins (Murcha et al. 2007).



**Fig. 2** Components of the major protein translocases in the mitochondrial inner and outer membranes. The details of the components of these machines have been recently reviewed (Chacinska et al. 2009; Neupert and Herrmann 2007). The major, essential component of each translocase is labelled (e.g. Tom40, Sam50, Tim23 and Tim22) and other subunits are given only numbers (e.g. “20” and “70” in the TOM complex refers to Tom20 and Tom70). For consistency, the *red* and *grey shading* denotes proteins that have or do not have identifiable bacterial ancestors, respectively. The functions of the various components are discussed in the text

The work required to drive vectorial polypeptide transport is provided by the import motor mtHsp70 (Fig. 2). This mtHsp70 is derived from a bacterial Hsp70 called DnaK, a protein found ubiquitously in bacteria (Boorstein et al. 1994). The import motor is docked to the TIM23 translocase by the Tim44 subunit (Rassow et al. 1994; Schneider et al. 1994) and a protein called Pam18/Tim14 regulates motor ATPase activity (D’Silva et al. 2003; Mokranjac et al. 2003; Truscott et al. 2003). Recent work has shown that  $\alpha$ -proteobacteria possess inner membrane proteins with strong sequence similarity to the Tim44 (TimA) and Tim14 (TimB) core subunits of the TIM23 complex (Clements et al. 2009; Dolezal et al. 2006; Kutik et al. 2009) and a single point mutation in the *Caulobacter crescentus* Tim14 homologue is sufficient to convert it to a functional yeast TIM23 translocase subunit (Clements et al. 2009). These components were all available to evolution and, with even a rudimentary TIM complex in place [in the continued presence of both SecYEG and YidC translocases (Dolezal et al. 2006)], the proto-mitochondrion would have had a functional system for import of both matrix and inner membrane proteins. A primitive system such as this would provide the basis for the evolution of the highly specialised TIM translocases in extant organisms.

## New Machines—Without a (Bacterial) Trace

The translocase of the outer membrane, or TOM complex, provides the general pore through which all proteins for the inner compartments of the mitochondrion (and also some outer membrane proteins) must pass (Fig. 1). Its development was key to the original bacterial endosymbiont transitioning into a genuine organelle. It has been suggested that the first protein translocase system in the “proto-mitochondrion” would have involved a primitive set-up: a  $\beta$ -barrel protein in the outer membrane and substrates in the host cytosol predisposed for targeting to mitochondria (Clements et al. 2009; Lucattini et al. 2004). Could such a simple pore have provided for the early needs of this primitive organelle? Microsporidians provide a true proof-of-principle example of such a simple TOM complex. These relatives of fungi possess readily identified TOM and TIM homologues (Burri et al. 2006; Waller et al. 2009). Only two TOM proteins are encoded in the complete genome of microsporidian *Encephalitozoon cuniculi*: Tom70 and Tom40. Given the function of Tom70 as a receptor, acting prior to the translocation reaction, this says that Tom40 alone can form a functional protein translocation pore. While this reduced TOM is clearly a result of secondary gene loss in microsporidians, it demonstrates the feasibility of a simpler primitive TOM complex in the ancestral endosymbiont.

Current phylogenetic analysis does not establish the ancestry of Tom40. Based on its predicted  $\beta$ -barrel topology it is broadly accepted that Tom40 was derived from the genome of the endosymbiont (Alcock et al. 2010; Bains and Lithgow 1999; Cavalier-Smith 2006; Gabriel et al. 2001; Herrmann 2003; Kutik et al. 2009; Mannella et al. 1996; Neupert and Herrmann 2007). Like all bacteria with two membranes, the endosymbiont would have had a range of  $\beta$ -barrel outer membrane proteins. Initially synthesised within the endosymbiont, a primitive TOM translocase could have been transported to the periplasm using the bacterial export pathway. If Tom40 was derived from a bacterial protein export channel it now imports, rather than exports, proteins but even this difference need not be problematic: biochemical analysis of purified mitochondrial outer membrane vesicles has shown that purified proteins can move in either direction through the TOM channel (Mayer et al. 1995).

The small TIM chaperones are found only in eukaryotes (Gentle et al. 2007), where they transfer precursors of both inner and outer mitochondrial membrane proteins from the TOM complex to the appropriate downstream machinery (either TIM22 or SAM, Fig. 1) (Hoppins and Nargang 2004; Jarosch et al. 1996; Koehler et al. 1998a, b; Wiedemann et al. 2004). Comparative analysis of the analogous but non-homologous prokaryotic chaperone (SurA) with the small TIMs (e.g. Tims 9 and 10) shows that while both chaperones can bind similar substrates, only the small TIM chaperones can transfer mitochondrial inner membrane proteins to the TIM22 translocase for insertion (Alcock et al. 2008). The small TIM family may therefore have arisen to enhance transport of inner membrane proteins, and also proved competent in transfer of outer membrane precursors,

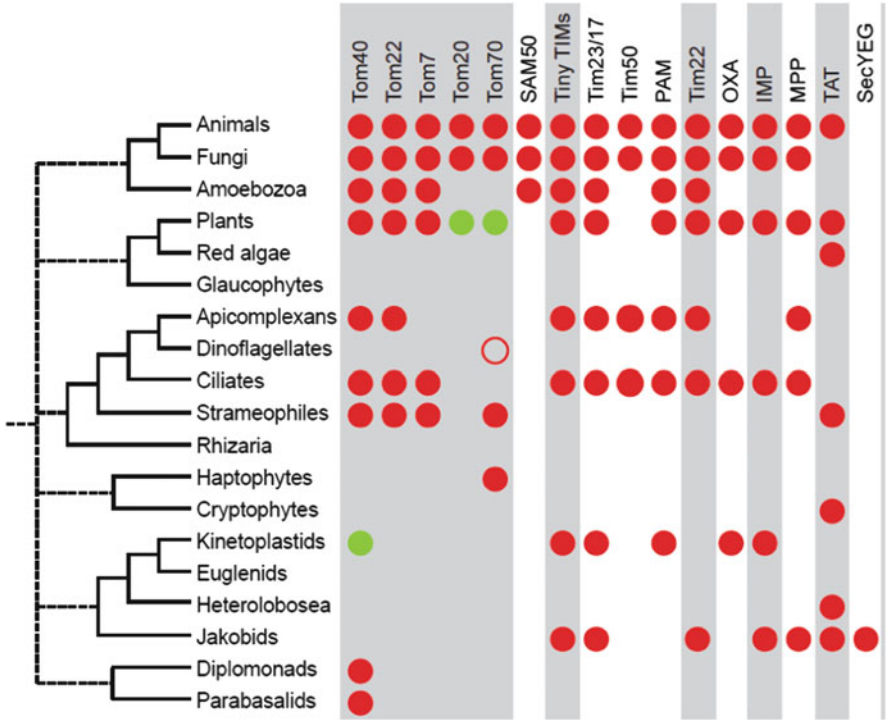
leaving bacterial chaperones like SurA redundant. But how does a chaperone like the small TIM evolve, where apparently four distinct protein subunits are required? It starts from one. Again, an investigation of a parasite and its secondary reduction in genes provides the proof of principle for how a single gene product could give rise to the small TIM systems seen today. *C. parvum* is a single-celled human parasite possessing simple mitochondria with and a single small TIM protein that forms a homo-hexameric chaperone (Alcock et al. 2012).

As mentioned earlier in this review, the SAM complex can engage with two outer membrane proteins found only in fungi: Mdm10 and Mim1 (Thornton et al. 2010). Mdm10 is a modular component of two complexes which seem to function in distinct pathways for assembly of outer membrane proteins. A SAM-Mdm10 complex assists in assembly of the TOM complex. Mim1 is another modular subunit that can engage with the SAM complex (Becker et al. 2008). Mim1 functions in assembly of integral membrane proteins (Becker et al. 2011; Hulett et al. 2007; Ishikawa et al. 2004; Lueder and Lithgow 2009; Meisinger et al. 2004; Thornton et al. 2010). Mim1 and Mdm10 are each required for integration of different subunits into the TOM complex, demonstrating substrate specificity for each module, and Mim1 at least is involved in assembling other membrane proteins, including poly-topic proteins, into the outer membrane (Becker et al. 2011). Despite their functionally important and fundamental roles in assembling membrane proteins, neither Mdm10 nor Mim1 appears to be conserved outside the fungal lineage. This highlights the need to characterise protein import in organisms other than yeast: something analogous would likely function in place of Mdm10 and Mim1 in other organisms.

## New Insights into Organelle Evolution from Mitochondrial Diversity

While clear insights into the adaptation of protein import pathways in the nascent mitochondrial organelle have been gained by studies of equivalent bacterial systems and minimalist mitochondria in parasites, to truly understand the evolution of mitochondrial import systems we must graft these insights onto the full diversity of the tree of eukaryotic life and test their universality. This is already providing new insights into, and questions regarding, mitochondrial import evolution. Figure 3 summarises the current state of knowledge of the presence of major components of mitochondrial import systems across the diversity of eukaryotes (Table 1 indicates module function with accessory/regulatory components shaded in grey). This snapshot considers detectable presence, rather than verifiable absence. The presence of a common core of import-related proteins found throughout eukaryotes points to the earliest innovations of this system after the establishment of the mitochondrion as a true organelle but before the radiation of present lineages





**Fig. 3** Phylogeny of eukaryotes and known presences of mitochondrial import proteins and machines. *Red circles* indicate presence of homologues, *green circles* indicate presence of analogous proteins and *open circle* indicates indirect evidence based on function. *Dashed lines* indicate uncertainty in the eukaryotic phylogeny. Note: absences are generally not verified, and often represent missing data [references: Dagley et al. (2009), Danne and Waller (2011), Likic et al. (2010), Lister et al. (2003), Lithgow and Schneider (2010), Macasev et al. (2004), Perry et al. (2008), Pusnik et al. (2009), Schneider et al. (2008), Smith et al. (2007), Tong et al. (2011), Tsaousis et al. (2011), van Dooren et al. (2006), Wang and Lavrov (2007) and Yen et al. (2002)]

(Dolezal et al. 2006). Some newer insights challenging aspects of our views of mitochondrial import diversity and conservation are discussed in this section.

The TOM component Tom70 has been considered a specific receptor of the animal/fungal lineage where it handles inner membrane proteins with internal targeting sequences (Chan et al. 2006). The notion of Tom70 as a more recent mitochondrial innovation has been previously bolstered by failure to find Tom70 in the Amoebozoa (sister to animals/fungi), and the presence of an analogous receptor in plants, mtOM64, that is apparently derived from a chloroplast translocase in the absence of Tom70 (Chew et al. 2004; Perry et al. 2008). Unexpectedly, however, Tom70 homologues have recently been identified within distantly related groups to animals and fungi, the Stramenopiles and Haptophytes, including verification of heterologous function of one homologue in yeast (Tsaousis et al. 2011). Furthermore, studies of targeting signals in dinoflagellates indicate the functional

**Table 1** Major known components of mitochondrial protein sorting machinery

	<b>Modules</b>	<b>Subunits in module (yeast)</b>	<b>Function of module</b>
<b>TOM complex</b>	Core translocase	Tom40, Tom22, Tom7	Translocation channel
	small subunits	Tom6, Tom5	Assists substrate transfer
	receptors	Tom70, Tom20	Promote substrate binding
<b>SAM complex</b>	Core translocase	Sam50	Membrane protein assembly
	metaxins	Sam35, Sam37	Assist protein assembly?
	Mdm10	Mdm10 (others?)	Assists protein assembly?
<b>Tiny TIMs</b>	Core complexes	Tim9, Tim10 and Tim8, Tim13	Transfer of substrates to TIM22 or SAM complexes
<b>TIM22 complex</b>	Core translocase	Tim22	Assembly of proteins into inner membrane.
	peripheral Tim accessory subunits	Tim12 Tim54, Tim18	Docking of tiny TIMs Assists protein assembly?
<b>TIM23 complex</b>	Core translocase	Tim23, Tim17	Translocation channel
	Tim50	Tim50	Regulates channel opening
	PAM complex	Pam18, Pam16, Tim44, mHsp70	Transfer of substrates into the matrix
	Tim21	Tim21	Regulates module docking
<b>OXA complex</b>	Core chaperone	Oxa1	Assembly of proteins into inner membrane.
	ribosome receptors	Mba1, Mdm38, Ylh47	Docking of mitochondrial ribosomes
<b>IMP complex</b>	Core peptidase	Imp1, Imp2	Processing of transfer-type sequences
	substrate binding	Som1	Modulates recognition
<b>MPP</b>	Core peptidase	Mas1, Mas2	Processing of N-terminal presequences in matrix
<b>TAT</b>	Core translocase	TatA	Translocation channel
	substrate binding	TatC	Promotes substrate binding
<b>SecYEG</b>	Core translocase	SecY	Translocation channel

conservation of the internal targeting signals between fungi and dinoflagellates, which further suggests that Tom70 function occurs very broadly, even though homologues are yet to be identified (Danne and Waller 2011). These observations suggest that Tom70 developed very early in mitochondrial evolution and has potentially been lost in several eukaryotic groups.

Two further examples of loss of early acquired targeting pathways reinforce that complexity gained during organelle evolution can also be reversed. As discussed above, there is compelling evidence of only a single TIM23/22 in both trypanosomatids and microsporidia (Schneider et al. 2008; Waller et al. 2009). It is unlikely that either of these two parasite groups have simply eliminated the substrates for one of these major complexes as both continue to import both polytopic membrane proteins and matrix proteins. Thus either TIM23 or TIM22 has apparently broadened its substrate range more recently in these lineages such that at least one of these inner membrane translocases became redundant and could be lost.

The bacterial SecYEG secretion route, on the other hand, is an ancestral pathway that likely played an early role in mitochondrial evolution but was then generally lost. Curiously it has been retained only in one group, the Jakobids (Lang et al. 1997). Why this group has continued to employ the SecY pathway, presumably for insertion of one or more proteins into the intermembrane space from the matrix, is unclear. Jakobids possess the most gene rich mitochondrial genomes of any mitochondria, and clues to SecYEG retention may be indicated by the presence of some of these genes. The *cox11* gene product, for example, has been suggested as a possible substrate for the SecY complex (Tong et al. 2011); however, this gene is present on at least one other mitochondrial genome, i.e. of the Heterolobosean, *Naegleria* (Gray et al. 2004). Thus, mitochondrial location of this gene alone does not necessarily require the maintenance of SecYEG.

A further observation that can be made from the diversity of mitochondrial targeting systems, and one that the models of fungi and animals alone have not illuminated, is the broad retention of the twin-arginine translocation (Tat) pathway. The Tat pathway is a bacterial secretion system that can translocate folded proteins as well as proteins complexed with cofactors or even other proteins (Berks et al. 2005; Natale et al. 2008). In bacteria, Tat substrates include many redox-related proteins such as respiratory proteins that contain cofactors necessary for electron transport processes. The Tat pathway has also been inherited by plastids where it is involved in protein transport into the internal thylakoid membranes (and presumed not to function in the organelle envelope membranes) (Müller and Klös gen 2005). A Tat pathway has not been investigated in mitochondria, although the presence of genes for Tat translocator components has often been noted in mitochondrial genomes. Figure 3 indicates major eukaryotic groups where Tat genes (also known as *ymf16* and *mttB*) are found, and this includes basal animal lineages (choanoflagellates and some sponges), plants and several protist groups (Bogsch et al. 1998; Burger et al. 2003; Gray et al. 1999; Lang et al. 1997; Wang and Lavrov 2007, 2008; Yen et al. 2002). TatC is the most common gene indicator of this pathway in mitochondrial genomes, but Jakobids possess both TatC and TatA that potentially encode a minimal functional translocon. Presumably, TatA genes have relocated to the nucleus of most groups, and TatC has also been found in the nuclei of some basal animals (Wang and Lavrov 2007), indicating that nucleus-encoded genes might reveal an even wider occurrence of mitochondrial Tat.

The questions of what substrates might use a Tat pathway in mitochondria, and why or how (or even if) some groups have eliminated this pathway, are now in need of attention. There is clear scope for several cofactor containing redox-related proteins to use this pathway in mitochondria, potentially including substrates directly inherited from the bacterial progenitor of this organelle. These substrates might be proteins either still coded for on mitochondrial genomes, or for which the genes now occur in the nucleus as is also the case for many plastidal Tat substrates (Müller and Klös gen 2005). A possible way to identify them is to search mitochondrial proteins for the Tat pathway sorting signal, which resembles the Sec-type cleavable N-terminal signal sequence but also including a double arginine residue typically in the context Z-R-R-x-Φ-Φ (where Z stands for any polar residue and Φ

for hydrophobic residues) (Berks et al. 2005; Natale et al. 2008). While these bacterial signals are generally well conserved in the plastid system, some substrates are known to have more cryptic signals, and it is possible that a mitochondrial system has also diverged (Müller and Klösken 2005). In plastids one known Tat substrate is the Fe–S containing Rieske protein of the cytochrome *b<sub>6</sub>f*-complex (Rip1), and this protein also occurs in the equivalent mitochondrial cytochrome *bc<sub>1</sub>* complex (Molik et al. 2001). Intriguingly, in yeast this mitochondrial protein contains two N-terminal pre-peptides, one that acts as a mitochondrial matrix-targeting peptide, and the second one that is removed upon final delivery back across the inner membrane to the intermembrane space (Conte and Zara 2011). The significance of this second peptide, and the route of this final targeting event, is unknown.<sup>1</sup> Such a protein might offer scope to explore the role of the mitochondrial Tat pathway in either this or another experimentally tractable system.

The broad themes of re-development and re-deployment discussed in sections “An Ancestral System Doing (More or Less) What It Has Always Done” and “Ancestral Machines Have Been Modified and Recombined in the Course of Evolution” showcase the first chapters in our understanding of the evolution of the mitochondrial import machinery (Hewitt et al. 2011). Where details of the transition from endosymbiont to organelle have been obscured, section “New Machines—Without a (Bacterial) Trace” shows how minimalist mitochondrial systems of parasites provide a valuable testing ground for hypotheses regarding the earliest stages of mitochondrial evolution. Ultimately our understanding of organelle evolution should encompass the whole eukaryotic tree, and with the advent of next-gen sequencing methodologies, genome sequencing is economically and practically achievable for even unculturable and otherwise obscure organisms. The re-evaluation of Tom70 in light of newly identified homologues is an example of how bioinformatics alone can reshape our understanding of the evolutionary history of protein transport machinery. However, as with most of the insights discussed here, the full power of these bioinformatics discoveries is realised through biochemical verification and further investigation of the questions raised by an expanding view of mitochondrial diversity.

## References

- Alcock FH, Grossmann JG, Gentle IE, Likic VA, Lithgow T, Tokatlidis K (2008) Conserved substrate binding by chaperones in the bacterial periplasm and the mitochondrial intermembrane space. *Biochem J* 409:377–387

---

<sup>1</sup> Note added in proof: The assembly of Rip1 was recently investigated and found to depend on the AAA-ATPase Bcs1 (Wagener et al. 2011). This raises the intriguing possibility that the Tat pathway has been replaced by a functionally homologous system that translocates the folded Fe–S containing domain, but uses an entirely different protein3 machinery to do so.

- Alcock F, Clements A, Webb C, Lithgow T (2010) Tinkering inside the organelle. *Science* 327:649–650
- Alcock F, Webb CT, Dolezal P, Hewitt V, Shingu-Vasquez M, Likic VA, Traven A, Lithgow T (2012) A small Tim homohexamer in the relic mitochondrion of *Cryptosporidium*. *Mol Biol Evol* 29:113–122
- Alder NN, Jensen RE, Johnson AE (2008) Fluorescence mapping of mitochondrial TIM23 complex reveals a water-facing, substrate-interacting helix surface. *Cell* 134:439–450
- Andersson SG, Kurland CG (1999) Origins of mitochondria and hydrogenosomes. *Curr Opin Microbiol* 2:535–541
- Bains G, Lithgow T (1999) The Tom channel in the mitochondrial outer membrane: alive and kicking. *Bioessays* 21:1–4
- Becker T, Pfannschmidt S, Guiard B, Stojanovski D, Milenkovic D, Kutik S, Pfanner N, Meisinger C, Wiedemann N (2008) Biogenesis of the mitochondrial TOM complex: Mim1 promotes insertion and assembly of signal-anchored receptors. *J Biol Chem* 283:120–127
- Becker T, Wenz L-S, Krüger V, Lehmann W, Müller JM, Goroncy L, Zufall N, Lithgow T, Guiard B, Chacinska A, Wagner R, Meisinger C, Pfanner N (2011) The mitochondrial import protein Mim1 promotes biogenesis of multispanning outer membrane proteins. *J Cell Biol* 194:387–395
- Berks BC, Palmer T, Sargent F (2005) Protein targeting by the bacterial twin-arginine translocation (Tat) pathway. *Curr Opin Microbiol* 8:174–181
- Bogsch EG, Sargent F, Stanley NR, Berks BC, Robinson C, Palmer T (1998) An essential component of a novel bacterial protein export system with homologues in plastids and mitochondria. *J Biol Chem* 273:18003–18006
- Boorstein WR, Ziegelhoffer T, Craig EA (1994) Molecular evolution of the HSP70 multigene family. *J Mol Evol* 38:1–17
- Burger G, Forget L, Zhu Y, Gray MW, Lang BF (2003) Unique mitochondrial genome architecture in unicellular relatives of animals. *Proc Natl Acad Sci USA* 100:892–897
- Burri L, Williams BA, Bursac D, Lithgow T, Keeling PJ (2006) Microsporidian mitosomes retain elements of the general mitochondrial targeting system. *Proc Natl Acad Sci USA* 103:15916–15920
- Cavalier-Smith T (2002) The phagotrophic origin of eukaryotes and phylogenetic classification of Protozoa. *Int J Syst Evol Microbiol* 52:297–354
- Cavalier-Smith T (2006) Rooting the tree of life by transition analyses. *Biol Direct* 1:19
- Chacinska A, Koehler CM, Milenkovic D, Lithgow T, Pfanner N (2009) Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138:628–644
- Chan NC, Likic VA, Waller RF, Mulhern TD, Lithgow T (2006) The C-terminal TPR domain of Tom70 defines a family of mitochondrial protein import receptors found only in animals and fungi. *J Mol Biol* 358:1010–1022
- Chew O, Lister R, Qbadou S, Heazlewood JL, Soll J, Schleiff E, Millar AH, Whelan J (2004) A plant outer mitochondrial membrane protein with high amino acid sequence identity to a chloroplast protein import receptor. *FEBS Lett* 557:109–114
- Clements A, Bursac D, Gatsos X, Perry AJ, Covicristov S, Celik N, Likic VA, Poggio S, Jacobs-Wagner C, Strugnelli RA, Lithgow T (2009) The reducible complexity of a mitochondrial molecular machine. *Proc Natl Acad Sci USA* 106:15791–15795
- Conte L, Zara V (2011) The rieske iron-sulfur protein: import and assembly into the cytochrome bc(1) complex of yeast mitochondria. *Bioinorg Chem Appl* 2011:363941
- D'Silva PD, Schilke B, Walter W, Andrew A, Craig EA (2003) J protein cochaperone of the mitochondrial inner membrane required for protein import into the mitochondrial matrix. *Proc Natl Acad Sci USA* 100:13839–13844
- Dagley MJ, Dolezal P, Likic VA, Smid O, Purcell AW, Buchanan SK, Tachezy J, Lithgow T (2009) The protein import channel in the outer mitochondrial membrane of *Giardia intestinalis*. *Mol Biol Evol* 26:1941–1947

- Danne JC, Waller RF (2011) Analysis of dinoflagellate mitochondrial protein sorting signals indicates a highly stable protein targeting system across eukaryotic diversity. *J Mol Biol* 408:643–653
- de Duve C (2007) The origin of eukaryotes: a reappraisal. *Nat Rev Genet* 8:395–403
- Dolezal P, Likic V, Tachezy J, Lithgow T (2006) Evolution of the molecular machines for protein import into mitochondria. *Science* 313:314–318
- Driessen AJ, Manting EH, van der Does C (2001) The structural basis of protein targeting and translocation in bacteria. *Nat Struct Biol* 8:492–498
- Embley TM, Martin W (2006) Eukaryotic evolution, changes and challenges. *Nature* 440:623–630
- Funes S, Hasona A, Bauerschmitt H, Grubbauer C, Kauff F, Collins R, Crowley PJ, Palmer SR, Brady LJ, Herrmann JM (2009) Independent gene duplications of the YidC/Oxa/Alb3 family enabled a specialized cotranslational function. *Proc Natl Acad Sci USA* 106:6656–6661
- Gabalidon T, Huynen MA (2007) From endosymbiont to host-controlled organelle: the hijacking of mitochondrial protein synthesis and metabolism. *PLoS Comput Biol* 3:e219
- Gabriel K, Buchanan SK, Lithgow T (2001) The alpha and the beta: protein translocation across mitochondrial and plastid outer membranes. *Trends Biochem Sci* 26:36–40
- Gatsos X, Perry AJ, Anwari K, Dolezal P, Wolyne PP, Likic VA, Purcell AW, Buchanan SK, Lithgow T (2008) Protein secretion and outer membrane assembly in Alphaproteobacteria. *FEMS Microbiol Rev* 32:995–1009
- Gentle I, Gabriel K, Beech P, Waller R, Lithgow T (2004) The Omp85 family of proteins is essential for outer membrane biogenesis in mitochondria and bacteria. *J Cell Biol* 164:19–24
- Gentle IE, Burri L, Lithgow T (2005) Molecular architecture and function of the Omp85 family of proteins. *Mol Microbiol* 58:1216–1225
- Gentle IE, Perry AJ, Alcock FH, Likic VA, Dolezal P, Ng ET, Purcell AW, McConnville M, Naderer T, Chanez AL, Charriere F, Aschinger C, Schneider A, Tokatlidis K, Lithgow T (2007) Conserved motifs reveal details of ancestry and structure in the small TIM chaperones of the mitochondrial intermembrane space. *Mol Biol Evol* 24:1149–1160
- Gray MW, Burger G, Lang BF (1999) Mitochondrial evolution. *Science* 283:1476–1481
- Gray MW, Burger G, Lang BF (2001) The origin and early evolution of mitochondria. *Genome Biol* 2:Reviews1018
- Gray MW, Lang BF, Burger G (2004) Mitochondria of protists. *Annu Rev Genet* 38:477–524
- Gross J, Bhattacharya D (2009) Mitochondrial and plastid evolution in eukaryotes: an outsiders' perspective. *Nat Rev Genet* 10:495–505
- Herrmann JM (2003) Converting bacteria to organelles: evolution of mitochondrial protein sorting. *Trends Microbiol* 11:74–79
- Hewitt V, Alcock F, Lithgow T (2011) Minor modifications and major adaptations: the evolution of molecular machines driving mitochondrial protein import. *Biochim Biophys Acta* 1808:947–954
- Hoppins SC, Nargang FE (2004) The Tim8-Tim13 complex of *Neurospora crassa* functions in the assembly of proteins into both mitochondrial membranes. *J Biol Chem* 279:12396–12405
- Hulett JM, Walsh P, Lithgow T (2007) Domain stealing by receptors in a protein transport complex. *Mol Biol Evol* 24:1909–1911
- Ishikawa D, Yamamoto H, Tamura Y, Moritoh K, Endo T (2004) Two novel proteins in the mitochondrial outer membrane mediate beta-barrel protein assembly. *J Cell Biol* 166:621–627
- Jarosch E, Tuller G, Daum G, Waldherr M, Voskova A, Schweyen RJ (1996) Mrs5p, an essential protein of the mitochondrial intermembrane space, affects protein import into yeast mitochondria. *J Biol Chem* 271:17219–17225
- Jensen RE, Dunn CD (2002) Protein import into and across the mitochondrial inner membrane: role of the TIM23 and TIM22 translocons. *Biochim Biophys Acta* 1592:25–34
- Kiefer D, Kuhn A (2007) YidC as an essential and multifunctional component in membrane protein assembly. *Int Rev Cytol* 259:113–138
- Knowles TJ, Scott-Tucker A, Overduin M, Henderson IR (2009) Membrane protein architects: the role of the BAM complex in outer membrane protein assembly. *Nat Rev Microbiol* 7:206–214

- Koehler CM, Jarosch E, Tokatlidis K, Schmid K, Schweyen RJ, Schatz G (1998a) Import of mitochondrial carriers mediated by essential proteins of the intermembrane space. *Science* 279:369–373
- Koehler CM, Merchant S, Oppliger W, Schmid K, Jarosch E, Dolfini L, Junne T, Schatz G, Tokatlidis K (1998b) Tim9p, an essential partner subunit of Tim10p for the import of mitochondrial carrier proteins. *EMBO J* 17:6477–6486
- Kutik S, Stroud DA, Wiedemann N, Pfanner N (2009) Evolution of mitochondrial protein biogenesis. *Biochim Biophys Acta* 1790:409–415
- Lang BF, Burger G, O’Kelly CJ, Cedergren R, Golding GB, Lemieux C, Sankoff D, Turmel M, Gray MW (1997) An ancestral mitochondrial DNA resembling a eubacterial genome in miniature. *Nature* 387:493–497
- Lang BF, Gray MW, Burger G (1999) Mitochondrial genome evolution and the origin of eukaryotes. *Annu Rev Genet* 33:351–397
- Likic VA, Dolezal P, Celik N, Dagley M, Lithgow T (2010) Using hidden Markov models to discover new protein transport machines. *Methods Mol Biol* 619:271–284
- Lister R, Murcha MW, Whelan J (2003) The mitochondrial protein import machinery of plants (MPIMP) database. *Nucleic Acids Res* 31:325–327
- Lithgow T, Schneider A (2010) Evolution of macromolecular import pathways in mitochondria, hydrogenosomes and mitosomes. *Philos Trans R Soc Lond B Biol Sci* 365:799–817
- Lucattini R, Likic VA, Lithgow T (2004) Bacterial proteins predisposed for targeting to mitochondria. *Mol Biol Evol* 21:652–658
- Lueder F, Lithgow T (2009) The three domains of the mitochondrial outer membrane protein Mim1 have discrete functions in assembly of the TOM complex. *FEBS Lett* 583:1475–1480
- Macasev D, Whelan J, Newbigin E, Silva-Filho MC, Mulhern TD, Lithgow T (2004) Tom22’, an 8-kDa trans-site receptor in plants and protozoans, is a conserved feature of the TOM complex that appeared early in the evolution of eukaryotes. *Mol Biol Evol* 21:1557–1564
- Mannella CA, Neuwald AF, Lawrence CE (1996) Detection of likely transmembrane beta strand regions in sequences of mitochondrial pore proteins using the Gibbs sampler. *J Bioenerg Biomembr* 28:163–169
- Mayer A, Neupert W, Lill R (1995) Mitochondrial protein import: reversible binding of the presequence at the trans side of the outer membrane drives partial translocation and unfolding. *Cell* 80:127–137
- Meisinger C, Rissler M, Chacinska A, Szklarz LK, Milenkovic D, Kozjak V, Schonfisch B, Lohaus C, Meyer HE, Yaffe MP, Guiard B, Wiedemann N, Pfanner N (2004) The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane. *Dev Cell* 7:61–71
- Mokranjac D, Sighting M, Neupert W, Hell K (2003) Tim14, a novel key component of the import motor of the TIM23 protein translocase of mitochondria. *EMBO J* 22:4945–4956
- Molik S, Karnauchov I, Weidlich C, Herrmann RG, Klosgen RB (2001) The Rieske Fe/S protein of the cytochrome b6/f complex in chloroplasts: missing link in the evolution of protein transport pathways in chloroplasts? *J Biol Chem* 276:42761–42766
- Müller M, Klösigen RB (2005) The Tat pathway in bacteria and chloroplasts (review). *Mol Membr Biol* 22:113–121
- Murcha MW, Elhafez D, Lister R, Tonti-Filippini J, Baumgartner M, Philippar K, Carrie C, Mokranjac D, Soll J, Whelan J (2007) Characterization of the preprotein and amino acid transporter gene family in *Arabidopsis*. *Plant Physiol* 143:199–212
- Natale P, Bruser T, Driessen AJ (2008) Sec- and Tat-mediated protein secretion across the bacterial cytoplasmic membrane – distinct translocases and mechanisms. *Biochim Biophys Acta* 1778:1735–1756
- Neupert W, Herrmann JM (2007) Translocation of proteins into mitochondria. *Annu Rev Biochem* 76:723–749
- Ott M, Herrmann JM (2010) Co-translational membrane insertion of mitochondrially encoded proteins. *Biochim Biophys Acta* 1803:767–775

- Perry AJ, Rimmer KA, Mertens HD, Waller RF, Mulhern TD, Lithgow T, Gooley PR (2008) Structure, topology and function of the translocase of the outer membrane of mitochondria. *Plant Physiol Biochem* 46:265–274
- Pohlschroder M, Gimenez MI, Jarrell KF (2005) Protein transport in Archaea: Sec and twin arginine translocation pathways. *Curr Opin Microbiol* 8:713–719
- Pusnik M, Charriere F, Maser P, Waller RF, Dagley MJ, Lithgow T, Schneider A (2009) The single mitochondrial porin of *Trypanosoma brucei* is the main metabolite transporter in the outer mitochondrial membrane. *Mol Biol Evol* 26:671–680
- Rassow J, Maarse AC, Krainer E, Kubrich M, Muller H, Meijer M, Craig EA, Pfanner N (1994) Mitochondrial protein import: biochemical and genetic evidence for interaction of matrix hsp70 and the inner membrane protein MIM44. *J Cell Biol* 127:1547–1556
- Rassow J, Dekker PJ, van Wilpe S, Meijer M, Soll J (1999) The preprotein translocase of the mitochondrial inner membrane: function and evolution. *J Mol Biol* 286:105–120
- Ruiz N, Kahne D, Silhavy TJ (2006) Advances in understanding bacterial outer-membrane biogenesis. *Nat Rev Microbiol* 4:57–66
- Samuelson JC, Chen M, Jiang F, Moller I, Wiedmann M, Kuhn A, Phillips GJ, Dalbey RE (2000) YidC mediates membrane protein insertion in bacteria. *Nature* 406:637–641
- Schneider HC, Berthold J, Bauer MF, Dietmeier K, Guiard B, Brunner M, Neupert W (1994) Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature* 371:768–774
- Schneider A, Bursac D, Lithgow T (2008) The direct route: a simplified pathway for protein import into the mitochondrion of trypanosomes. *Trends Cell Biol* 18:12–18
- Smith DG, Gawryluk RM, Spencer DF, Pearlman RE, Siu KW, Gray MW (2007) Exploring the mitochondrial proteome of the ciliate protozoon *Tetrahymena thermophila*: direct analysis by tandem mass spectrometry. *J Mol Biol* 374:837–863
- Stuart R (2002) Insertion of proteins into the inner membrane of mitochondria: the role of the Oxa1 complex. *Biochim Biophys Acta* 1592:79–87
- Thornton N, Stroud DA, Milenkovic D, Guiard B, Pfanner N, Becker T (2010) Two modular forms of the mitochondrial sorting and assembly machinery are involved in biogenesis of alpha-helical outer membrane proteins. *J Mol Biol* 396:540–549
- Timmis JN, Ayliffe MA, Huang CY, Martin W (2004) Endosymbiotic gene transfer: organelle genomes forge eukaryotic chromosomes. *Nat Rev Genet* 5:123–135
- Tong J, Dolezal P, Selkig J, Crawford S, Simpson AG, Noinaj N, Buchanan SK, Gabriel K, Lithgow T (2011) Ancestral and derived protein import pathways in the mitochondrion of *Reclinomonas americana*. *Mol Biol Evol* 28:1581–1591
- Truscott KN, Kovermann P, Geissler A, Merlin A, Meijer M, Driessen AJ, Rassow J, Pfanner N, Wagner R (2001) A presequence- and voltage-sensitive channel of the mitochondrial preprotein translocase formed by Tim23. *Nat Struct Biol* 8:1074–1082
- Truscott KN, Voos W, Frazier AE, Lind M, Li Y, Geissler A, Dudek J, Muller H, Sickmann A, Meyer HE, Meisinger C, Guiard B, Rehling P, Pfanner N (2003) A J-protein is an essential subunit of the presequence translocase-associated protein import motor of mitochondria. *J Cell Biol* 163:707–713
- Tsaousis AD, Gaston D, Stechmann A, Walker PB, Lithgow T, Roger AJ (2011) A functional Tom70 in the human parasite *Blastocystis* sp.: implications for the evolution of the mitochondrial import apparatus. *Mol Biol Evol* 28:781–791
- van der Laan M, Nouwen NP, Driessen AJ (2005) YidC – an evolutionary conserved device for the assembly of energy-transducing membrane protein complexes. *Curr Opin Microbiol* 8:182–187
- van Dooren GG, Stimmler LM, McFadden GI (2006) Metabolic maps and functions of the *Plasmodium* mitochondrion. *FEMS Microbiol Rev* 30:596–630
- Wagener N, Ackermann M, Funes S, Neupert W (2011) A pathway of protein translocation in mitochondria mediated by the AAA-ATPase Bcs1. *Mol Cell* 44:191–202



- Waller RF, Jabbour C, Chan NC, Celik N, Likic VA, Mulhern TD, Lithgow T (2009) Evidence of a reduced and modified mitochondrial protein import apparatus in microsporidian mitosomes. *Eukaryot Cell* 8:19–26
- Wang X, Lavrov DV (2007) Mitochondrial genome of the homoscleromorph *Oscarella carmela* (Porifera, Demospongiae) reveals unexpected complexity in the common ancestor of sponges and other animals. *Mol Biol Evol* 24:363–373
- Wang X, Lavrov DV (2008) Seventeen new complete mtDNA sequences reveal extensive mitochondrial genome evolution within the Demospongiae. *PLoS One* 3:e2723
- Webb CT, Lithgow T (2010) Mitochondrial biogenesis: sorting mechanisms cooperate in ABC transporter assembly. *Curr Biol* 20:R564–R567
- Wiedemann N, Truscott KN, Pfannschmidt S, Guiard B, Meisinger C, Pfanner N (2004) Biogenesis of the protein import channel Tom40 of the mitochondrial outer membrane: intermembrane space components are involved in an early stage of the assembly pathway. *J Biol Chem* 279:18188–18194
- Yen MR, Tseng YH, Nguyen EH, Wu LF, Saier MH Jr (2002) Sequence and phylogenetic analyses of the twin-arginine targeting (Tat) protein export system. *Arch Microbiol* 177:441–450

Endosymbiosis

Löffelhardt, W. (Ed.)

2014, XI, 330 p. 43 illus., 32 illus. in color., Hardcover

ISBN: 978-3-7091-1302-8