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The Plant Mitochondrial Protein Import Apparatus – the differences make it interesting

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Abstract

Background: Mitochondria play essential roles in the life and death of almost all eukaryotic cells, ranging from single-celled to multi-cellular organisms that display tissue and developmental differentiation. As mitochondria only arose once in evolution, much can be learned from studying single celled model systems such as yeast and applying this knowledge to other organisms. However, two billion years of evolution has also resulted in substantial divergence in mitochondrial function between eukaryotic organisms.

Scope of Review: Here we review our current understanding of the mechanisms of mitochondrial protein import between plants and yeast (*Saccharomyces cerevisiae*) and identify a high level of conservation for the essential subunits of plant mitochondrial import apparatus. Furthermore we investigate examples whereby divergence and acquisition of functions has arisen and highlight the emerging examples of interactions between the import apparatus and components of the respiratory chain.

Major Conclusions: After more than three decades of research into the components and mechanisms of mitochondrial protein import of plants and yeast, the differences between these systems are examined. Specifically, expansions of the small gene families that encode the mitochondrial protein import apparatus in plants are detailed, and their essential role in seed viability is revealed.

General Significance: These findings point to the essential role of the inner mitochondrial protein translocases in Arabidopsis, establishing their necessity for seed viability and the crucial role of mitochondrial biogenesis during germination.

Keywords: protein import; mitochondrial biogenesis; TOM; TIM; plant mitochondria; gene families

Abbreviations: TOM - Translocase of the Outer Mitochondrial membrane, OM - Outer Membrane, SAM - Sorting and assembly machinery, TIM - Translocase of the Inner Mitochondrial membrane MIA - Mitochondrial Inter membrane space import and Assembly, ERV - Essential for Respiration and Vegetative growth, MPP Mitochondrial Processing Peptidase, PreP – Presequence Protease

1. Introduction

Mitochondria are membrane bound organelles that play essential roles in metabolism, energy production and biosynthesis of a variety of compounds in almost all eukaryotic cells. They are endosymbiotic in origin, and over time the majority of genes in the endosymbiont were lost or transferred to the host nucleus [1]. Thus, the majority of the 1000+ proteins located in mitochondria are encoded by nuclear genes, translated in the cytosol and imported into mitochondria [2]. *Saccharomyces cerevisiae* (yeast) has long been established as the pre-eminent model for the study of mitochondrial protein import [3]. However, many of the components involved in mitochondrial protein import are also well conserved across different species and while they have not been characterized in plants to the extent they have been in yeast, the presence of orthologous genes and complexes is well established across plants, animals, fungi [4-6] and even protists to a slightly lesser extent [7, 8]. Figure 1 summarizes the various pathways and complexes involved in protein import in plant mitochondria.

A multi-subunit protein complex on the mitochondrial outer membrane, termed the Translocase of the Outer Membrane (TOM), recognizes mitochondrial precursor proteins and passes them to one of two inner membrane multi-subunit protein complexes, termed the Translocases of the Inner Membrane (TIM) (Figure 1). One of these is TIM17:23, which is responsible for the import of proteins via the general import pathway, i.e. for proteins that contain N-terminal targeting signals (Figure 1). Alternatively, TIM22 is responsible for the import of proteins via the carrier import pathway, which is specific for the import of inner membrane proteins containing internal mitochondrial targeting signals (Figure 1). Along with the Sorting and Assembly Machinery (SAM) complex on the outer membrane and the Mitochondrial Intermembrane space Assembly (MIA) in the intermembrane space, these complexes are responsible for the import of the majority of proteins into the mitochondria [3, 9]. In the last few years, studies on the mechanisms of protein import into mitochondria have revealed interactions between the protein import complexes with other multi-subunit protein complexes. Many of the studies revealing these interactions have been carried out in yeast [10, 11]. Additionally, there have been emerging reports of interactions between the TIM17:23 complex and

respiratory chain in both yeast and plants, although the biological implications of these interactions is not yet known [12, 13].

While most mitochondrial import components are highly conserved between yeast and plants, there are several areas of divergence that have arisen. These include the presence of plant specific mitochondrial import components, as well as the expansion of the gene families encoding these components, resulting in sub-functionalisation and/or neo-functionalisation. Studies characterizing the function of mitochondrial import components in yeast have revealed that several are essential for viability [14]. Interestingly, studies in plants have revealed a requirement for mitochondrial import components very early during germination in *Arabidopsis* (*Arabidopsis thaliana* - At) and rice (*Oryza Sativa*), when organelle biogenesis is actively occurring [15-17]. This early requirement is also supported by the findings that knocking-out certain import components, such as AtTIM50, results in embryo lethality [18]. To date, over 400 genes have been defined as essential in *Arabidopsis* [18]. In this review, the plant mitochondrial import components are explored and several inner membrane components are shown to be essential, whereby knocking out these genes results in an embryo lethal phenotype in *Arabidopsis*.

2. Outer Membrane

The recognition of mitochondrial preproteins and the commencement of translocation occurs via protein complexes on the outer membrane, namely the TOM complex and the SAM complex. Characterization of the mitochondrial protein import machinery on the outer membrane reveals that while the overall process is conserved in both yeast and plants, there are important mechanistic differences [6].

2.1 The TOM complex

The TOM complex consists of the cytosolic facing receptor subunits Tom20 and Tom70, the 'convergent' receptor Tom22 and the pore forming Tom40 channel. Preprotein recognition generally occurs via the N-terminal presequence, present on ~70 % of mitochondrial proteins that ranges from 6-90 amino acids [19]. Tom20 exhibits substrate specificity with N-terminal presequences containing proteins whilst Tom70 binds proteins with internal non-cleavable targeting signals [20, 21]. Tom40,

a β -barrel protein, constitutes the translocation pore for almost all mitochondrial proteins, it was the first mitochondrial membrane protein shown to be essential for yeast viability. Similarly, in Arabidopsis, T-DNA insertional knock-out lines for *AtTom40-1*, the highest expressed isoform [22] are not viable, suggesting that whilst there are two isoforms, *AtTom40-2* cannot compensate for the loss of *AtTom40-1*.

No direct orthologs to yeast and mammalian Tom20 and Tom70 can be identified in plants. Instead, the plant Tom20 receptor is likely to have an independent genetic origin, as it is anchored to the membrane via the C-terminus (the reverse of yeast, mammalian and fungal systems) and displays a unique bidendate binding site for precursor proteins [23] (Figure 1). Given these significant differences, this cautions the use of mitochondria or precursor proteins from other systems to study protein import into plant mitochondria, as the recognition mechanism for precursor proteins, while similar, arose independently. It is not yet known whether this took place before or after the endosymbiotic event that gave rise to plastids in plants cells. The removal of all three isoforms of Tom20 in Arabidopsis resulted in a decrease in protein import ability to around 70% of that seen in wild-type plants, yet only small phenotypic differences were observed under normal or optimal growing conditions [24].

Following transfer from the Tom20 and 70 receptors, preproteins are subsequently transferred to Tom22 [25]. In yeast, Tom22 is essential for viability [26] and initiates the transfer of preproteins to the Tom40 channel [21, 25]. Tom22 has also been implicated in the assembly of the TOM complex itself and for further transfer of preproteins into the IMS to the TIM17:23 complex via its extended intermembrane space domain [27-29]. The plant ortholog to Tom22 is termed Tom9, being significantly shorter, as it lacks the cytosolic domain of Tom22, and is highly conserved in a variety of plant species [5, 30, 31]. It has been suggested that Tom22 was present prior to the divergence event and that plants either selectively lost the receptor domain of Tom22 or acquired a novel Tom9 to functionally replace Tom22. Furthermore it has been suggested that the absence of the cytosolic domain may prevent the mis-targeting of chloroplast precursor proteins to plant mitochondria [5, 30, 31]. In Arabidopsis, two orthologs exist for *AtTom9*, both identified in the outer membrane TOM complex [32], though no further characterization has been carried out. Given the essential nature of Tom22 it would be of interest to establish if

AtTom9 has equivalent functions to yeast Tom22 in terms of viability.

Additional small non-essential Tom5, 6 and 7 proteins have also been identified to be associated with the TOM40 complex in yeast. These small proteins have been shown to be involved in regulating the assembly of the complex, maintaining its stability and function to transfer precursor proteins to the Tom40 insertion pore [33-36]. Arabidopsis also contains small Toms [37], with AtTom7 identified as part of the TOM40 complex proteome [32] and AtTom5 and 6 identified by proteomic analysis of enriched outer membrane proteome fractions [38]. However apart from Tom7, the small size of these proteins makes it difficult to determine if they are orthologous or analogous.

Whilst Tom70 appears to be absent in plants, a novel plant specific outer membrane receptor has been identified termed Outer Membrane protein 64 (OM64), a paralog of the Toc64 protein import receptor found in plastids [5, 24]. BN-PAGE analysis, did not find OM64 to associate with any protein complex, though does not rule out the possibility of transient interactions with the TOM40 complex [24]. Whilst functional studies have shown that the depletion of OM64 affects the import of selected mitochondrial proteins, mutants exhibited minimal phenotypic abnormalities [24]. Thus, while TOM20 and OM64 proteins may be unique in plants, removal of each is clearly not deleterious [24]. However, the generation of a quadruple knock-out line, consisting of deletions of all three Tom20 isoforms and OM64 has been shown to result in an embryo lethal phenotype in Arabidopsis, indicating an essential role for at least one of these at a time and the inability of putative additional receptors to compensate for the combined loss of all Tom20 and OM64 subunits [6].

2.2 The SAM complex

In yeast, the SAM complex is composed of several subunits termed Sam50, Sam35 and Sam37/metaxin, involved in the assembly of β -barrel proteins into the outer membrane. Sam50 is integrated in the outer membrane while Sam35 and 37 are peripheral subunits, with cytosolic domains facing the cytosol that bind and release substrates respectively [39, 40]. Additional components that have been shown to interact with the SAM complex are Mdm10, Mdm12, Mmm1 and Mmm2, all

previously identified in maintaining mitochondrial distribution and morphology [41-44], have been determined to specifically interact with SAM complex and involved in the assembly of Tom40 [45-48]. Plants contain orthologs to Sam50, and Sam37/metaxin only, with AtSam50 confirmed to be located in the outer membrane [38] and AtSam37/metaxin implicated in the import of β -barrel proteins VDAC and TOM40. Furthermore T-DNA insertional knock-out of metaxin in plants exhibited severe phenotypic abnormalities with decreased import ability of all precursor proteins tested suggesting that whilst not essential, AtSam37 plays a vital role in mitochondrial biogenesis in Arabidopsis [24].

2.3 The ERMES complex

An important development with regards to the TOM and SAM complexes is their functional connections with a newly identified ERMES network (Endoplasmic Reticulum Mitochondrial Encounter Structure). The ERMES complex was first identified by a genetic screen in yeast and was found to tether the ER and mitochondria and proposed to aid in the exchange of phospholipids and calcium [49]. This complex is composed of four proteins, Mmm2 and Mdm10 being located on the mitochondria, Mdm12 forming a cytosolic bridge and Mmm1 located on the ER [49], components previously identified to associate with the TOM and SAM complex. Therefore, Mdm10 has a dual-location in both SAM and ERMES complex [46, 47, 50]. Furthermore, interactions of Mdm10 have also been reported with TOM subunits and mutants of Mdm10, Mdm12, Mmm1 and Mmm2 exhibit defects in β -barrel and Tom22 assembly [45-48]. These multiple interactions with protein complexes and the many phenotypes exhibited by ERMES mutants [51] have resulted in difficulty dissecting the specific functional roles of these proteins. The ERMES complex has yet to be identified in plants. Homology based searches against the Arabidopsis genome identifies candidate orthologs that exhibit some, albeit low identity (29-42%). Furthermore, bioinformatic analysis of Mdm10, Mdm10, Mmm1 and Mmm2 has identified conserved SMP domains (Synaptotagmin-like Mitochondrial and lipid-binding Proteins) within subunits of the ERMES complex, which extends to Arabidopsis proteins [52, 53]. Direct biochemical approaches are required to identify the subunits of this complex in plants. A study analyzing the outer mitochondrial proteome of mitochondria identified 42 mitochondrial outer membrane proteins from

Arabidopsis, several of which had not been previously characterized [38]. Furthermore, transient over-expression of several of these proteins linked to GFP resulted in aberrations to mitochondrial morphology [38], and thus provide useful targets for further characterization to determine if they play a role in β -barrel assembly and/or ER networks.

2.3 *The MINOS complex*

The SAM and TOM complex have also been linked to a complex of the inner membrane termed Mitochondrial Inner Organising system (MINOS). This complex is composed of six subunits, Fcj1, Mio10, Aim5, Aim13, Aim37 and Mio27 initially identified as mutants that exhibit altered cristae morphology [54-58]. The main role of the MINOS complex has been proposed to be in the maintenance of the inner membrane architecture, but specific roles in biogenesis have also been uncovered. Fcj1 is involved in the import of cysteine rich proteins located in the IMS [54]. MINOS has also been shown to interact independently with the TOM and the SAM complex and is involved in the import of β -barrel proteins [55] and SOD1 variants in the intermembrane space [59]. Whilst MINOS subunits show strong conservation between yeast, fungi and mammals [60] candidate orthologs only exhibit 27-32% identity in Arabidopsis and thus it is unclear if this complex exists in plants.

These newly discovered complexes involving TOM, SAM, ERMES and MINOS collectively involved in mitochondrial biogenesis, morphology and communication are proposed to be part of a larger network termed ERMIONE (ER-mitochondria organizing network) [61]. Spanning multiple membranes and with multiple functions, these complexes highlight the complex and dynamic nature of the mitochondrial import machinery and opens an exciting new area for research in plant mitochondria. However, it is likely that many would show embryo lethal phenotypes in plants, the use of biochemical approaches, combined with inducible genetic knock-downs or over-expression will be required to identify and characterize these complexes in plants.

3. Intermembrane Space

The mitochondrial intermembrane space (IMS) contains the “tiny Tims”, which are small soluble cysteine-rich proteins Tim 8, 9, 10 and 13 that form a hexameric ring like structure (Figure 1). Tiny Tims are involved in the import of the carrier type proteins inserted into inner membrane or β -barrel proteins, designed for insertion back into the outer membrane [9]. Import of the cysteine-rich tiny Tims requires the MIA machinery consisting of Mia40 and Erv1 (Figure 1), which are responsible for the oxidative folding and maturation of IMS proteins through the formation of disulphide bonds between cysteine residues in the disulphide relay system [62].

3.1 *The tiny TIMs*

Tim 8, 9, 10 and 13, belong to a highly conserved protein family identified in a wide range of eukaryotic lineages [5]. In yeast, both Tim 9 and 10 are essential for yeast viability and form a hexameric chaperone complex directing carrier precursor proteins from the TOM complex to the inner membrane TIM22 complex [63-65]. Tim 8 and Tim13 form a similar chaperone complex, though are not essential for yeast viability [65]. The Tiny Tim complexes in yeast appear to show differential substrate specificity, with Tim9-Tim10 being cross-linked to carrier proteins such as the ATP/ADP carrier, outer membrane translocation pore Tom40, and the inner membrane Tim17 and 22 [66-68]. Additionally, yeast Tim8-Tim13 has so far only been identified to be involved in the import of Tim23 and the aspartate-glutamate carriers [65, 69].

In plants, there are orthologs to all of the yeast tiny Tims [37]. Direct biochemical reconstitution of IMS depleted mitochondria revealed that Tim9 and 10 are associated with carrier import assembly in plants [70]. Additionally, Tim9 and Tim10 could stimulate the import and assembly of carrier type proteins containing N-terminal cleavable presequences (a plant specific feature unique to some carrier proteins) to a greater extent than those carriers with only internal targeting signals [71]. The stimulation of carrier import in plants using enriched biochemical fractions has never been reported in yeast, presumably as it cannot be achieved technically. Thus, while genetic approaches show the essential role of Tim9-Tim10 in yeast the direct biochemical complementation in plants points to a mechanistic differences of how this pathway operates [70, 72]. Taken together, the common occurrence of the N-terminal extension of plant carrier proteins that seem to enhance the efficiency of insertion into the inner membrane, and the absence of the yeast specific Tim12,

Tim18 and Tim54 components, suggests that the carrier import pathway may display diversity in higher eukaryotes.

3.2 *The Mia40-Erv1 pathway*

The disulphide relay system involves the import of proteins located in the IMS that are rich in cysteine residues with twin CX(9)C or CX(3)C motifs [73]. This pathway consists of only two proteins, Mia40 and Erv1, both of which are essential for yeast viability [74-77]. Mia40 acts to transfer precursor proteins from the outer membrane to the IMS (Figure 1), upon which the cysteine residues are oxidised by Mia40, which is subsequently oxidized by Erv1 [62]. Plants contain orthologs to the yeast Mia40 and Erv1 proteins, but important differences appear to exist between yeast and plants with respect to function. Mia40 in plants is not an essential protein, and is also located both in the mitochondria and peroxisomes [78, 79]. Whereas Erv1 is essential in plants, as in yeast [78], however, the predicted Erv1 protein differs to that of yeast Erv1 and appears more similar to yeast Erv2 and that of parasitic organisms like Trypanosomes and Plasmodium [5, 80]. Thus, while some organisms lack a gene encoding Mia40 (Trypanosomes and Plasmodium), and others have the genes (plants), it is apparent that the function differs with respect to oxidative protein folding upon import into mitochondria [81]. Furthermore, the dual-location of Mia40 has only recently been shown in higher plants, such as Arabidopsis and rice, while this was not seen in Physcomitrella [78, 79]. Moreover, this dual-targeting ability also appears to have been acquired alongside the dual-targeting of the Mia40 substrate proteins superoxide dismutase (CSD1) and copper chaperone for SOD1 (Ccs1) in Arabidopsis [79]. This suggests that in plants, Mia40 function has extended its role to peroxisomes as well and may also be involved in the fatty acid oxidation in the peroxisome [78].

4. **Inner Membrane**

In plants, analyses of the inner membrane components shows that they are more conserved with the yeast inner membrane components compared to the outer membrane components, with most showing strong orthology to their yeast counterparts [5]. While this is the case in terms of orthology, the expansion of gene families encoding inner membrane components combined with novel associations with respiratory chain complexes, means that the inner membrane translocases in

plants may have undergone both neo and sub-functionalisation resulting in several notable differences.

4.1 *The inner membrane translocases*

It has been shown in yeast that all mitochondrial precursor proteins targeted to the inner membrane or the matrix are required to pass via the channel complexes TIM17:23 or TIM22 [3]. Precursors are either directly transferred from the outer membrane TOM40 complex via a dynamic interaction with TIM17:23 complex or co-chaperoned via the tiny Tims to the TIM22 complex [82]. The TIM17:23 complex, which is also conserved in plants, is responsible for the import of the majority of the mitochondrial proteome, and exists as two functionally and structurally distinct forms [9]. The initial form termed, TIM23-SORT, contains the channel forming proteins Tim23, and Tim17, along with its associated proteins Tim50 and Tim21. All components of TIM23-SORT are fundamental subunits responsible for the direct translocation of precursor proteins from the TOM40 complex through to the matrix or for insertion into the inner membrane [82]. The second form of the TIM17:23 complex in yeast, termed TIM23-PAM, functions in the transfer of proteins through the inner membrane to the matrix [39, 83]. This complex also contains Tim17, Tim23, and Tim50 along with the Presequence translocase Associated Motor (PAM) complex, comprising of mtHSP70, Tim44 and its associated co-chaperones; Pam16, Pam17 and Pam18 [39, 83]. This complex drives the translocation through the inner membrane via ATPase activity and cyclic binding of the presequence to promote transfer towards the matrix [84]. Associated chaperones Pam16, 17 and 18 are thought to modulate mtHSP70 activity and association with the TIM17:23 channel [85, 86]. Based on the high level of orthology between these yeast inner membrane proteins their Arabidopsis counterparts, with the exception of Pam17, which is absent in plants and brown algae [5], it is very likely that these pathways are also functionally conserved in plants (Figure 1).

Interestingly, it has been shown that genes encoding these plant mitochondrial inner membrane translocases are highly expressed during seed development and germination, when mitochondrial biogenesis is actively occurring [17, 87]. It was also revealed that many of the genes showing this high expression during seed germination are essential for viability in Arabidopsis, whereby a loss-of-

function resulted in embryo lethal phenotypes [87]. Thus, the conservation and divergence of the plant inner membrane translocases are discussed below, particularly revealing their essential role for seed viability.

4.2 *The PReprotein and Amino Acid Transporter family - plant specific expansion*

Various studies, from yeast to higher eukaryotes, have shown the presence of Preprotein and Amino Acid Transporter (PRAT) domain-containing proteins [88, 89]. PRATs are thought to have evolved from a single eubacterial ancestral LivH amino acid permease, characterized by four transmembrane domains and a conserved motif of $[G/A]X_2[F/Y]X_{10}RX_3Dx_6[G/A/S]GX_3G$, where X is any amino acid [89]. In Arabidopsis, this family consists of 16 members encoded by 17 genes [88, 90]. The PRAT family of proteins in plants has clearly expanded in number, where 17 genes encode PRAT proteins in Arabidopsis, compared to just three in yeast [88] and brown algae - Ectocarpus [91]. Ten of the 17 Arabidopsis PRAT genes encode proteins that are located in mitochondria, while the other proteins are located in plastids, and have proposed roles in metabolite transport [88, 92]. For the mitochondrial PRATs; three genes encode Tim17, three genes encode Tim23 and two genes encode Tim22 [88]. Additionally, there are 4 genes encoding other PRAT proteins in Arabidopsis that are yet to be characterized [88]. One such PRAT protein termed B14.7, was initially identified as a Complex I subunit in Arabidopsis, having orthologs in bovine and *N. crassa* [93-95]. Interestingly, it was recently shown that this PRAT is also found in the TIM17:23 complex [13]. The function of the remaining mitochondrial PRAT proteins is unknown and challenges lie ahead in identifying the role of each of these specific protein isoforms in plant mitochondria. However, examination of the expression, predicted structure and experimental data on these PRAT proteins reveals a complex picture in terms of regulation (sub-functionalisation) and function (neo-functionalisation).

4.3 *TIM17*

With 17 PRAT family members in Arabidopsis, it has been proposed that a large expansion of these occurred in plants, pointing to the acquisition of new functions in higher eukaryotes and possible additional plant specific functions [6]. For example with regards to the Tim17 subfamily, both AtTim17-1 and AtTim17-2 contain

a plant specific C-terminal extension not evident in AtTim17-3 [90]. This extension, in particular, for AtTim17-2, links the inner and outer membrane and has been shown to exhibit substrate binding specificities [96]. AtTim17-2 was only able to complement a *tim17* yeast deletion strain upon removal of the C-terminal extension [96]. Another factor that may provide insight into the specialised functionalisation of these three isoforms is their transcript expression profiles. The expression profiles of AtTim17-1, AtTim17-2 and AtTim17-3 appear to vary across tissue types, development and in response to environmental stress. The expression of AtTim17-1 appears low in many tissue and developmental stages, unlike AtTim17-2, which is the most abundant isoform across development [22]. Notably, AtTim17-1 does show some expression during seed development [97] and has been linked with the mitochondrial stress response where up to 30-fold induction was seen in response to ultraviolet light, high salt and high temperature stress [98, 99]. The induction of AtTim17-1 transcript following stress may alter mitochondrial import ability, as stress treatments have also altered the import ability of several precursor proteins in isolated mitochondria [100]. AtTim17-3 on the other hand shows little expression specificity and moderate expression levels compared to AtTim17-2 [6, 22]. Furthermore, AtTim17-3 is most homologous to yeast Tim17 (50% identity) and whilst AtTim17-1 (48% identity) and AtTim17-2 (40% identity) contain a PRAT domain, AtTim17-3 contains a degenerate PRAT domain [88] and may represent a redundant isoform.

Given these differences in the Arabidopsis *Tim17* genes, we obtained knock-out lines for all 3 isoforms to gain an insight into their specificity and function (Table 1). Firstly, it was seen that *Attim17-1* knock-out plants showed faster germination rates compared to wild-type (Col-0) plants (Table 1), which supports a role for AtTim17-1 during germination and is also consistent with the increased transcript expression seen during seed development (Wang and Murcha – unpublished data). In contrast, no altered phenotype was observed in *Attim17-3* knock-out plants (Table 1), supporting the proposal that it is a redundant isoform. Lastly, it has been shown that AtTim17-2 is the most highly expressed isoform, both at the transcript and protein levels (Wang and Murcha - unpublished data), and that AtTim17-2 transcript expression peaks during seed germination [87]. Interestingly, screening of knock-out lines for *Attim17-2* (GK-561E03) failed to identify homozygous genotypes and subsequent self-fertilisation and screening of heterozygous progeny failed to identify

any homozygous lines (Figure 2). Closer inspection of silique development in the heterozygous germplasm (*AtTim17-2* (-/+)) revealed that embryos aborted early in seed development, indicating that *AtTim17-2* is an essential gene in *Arabidopsis* (Figure 2; Table 1). Ratios of aborted/viable seeds were determined using 20 siliques from three individual plants (n=20) and the percentage of defective seeds was shown to be $26.5 \pm 2\%$, consistent with an embryo lethal phenotype (Figure 2). Thus, while *AtTim17-2* is not the most orthologous isoform to its yeast counterpart, it is the only essential Tim17 isoform in plants and contains a plant specific C-terminal extension that may also contribute to this essential role.

4.4 *TIM23*

There are also three isoforms for Tim23 in *Arabidopsis*, one of which contains a PRAT domain (*AtTim23-3*) whilst *AtTim23-1* and *AtTim23-2*, contain a degenerate PRAT domain [88]. The three isoforms exhibit 70-92% sequence identity between each other [88]. Additionally, all three genes for Tim23 show different levels of orthology to the single Tim23 protein in yeast, with *AtTim23-1*, *AtTim23-2* and *AtTim23-3* exhibiting 40%, 39% and 41% amino acids sequence identity, respectively. Examination of transcript expression for each of these isoforms shows that all are most highly expressed during germination compared to other stages over development [87]. However, like *AtTim17-2*, *AtTim23-2* is the most highly expressed isoform at the transcript level compared to *AtTim23-1* and *AtTim23-3* [87]. As there is 92% amino acids sequence identity between *AtTim23-1* and *AtTim23-2*, isoform specific antibodies have not been produced to date. Thus, when Tim23 protein abundance was examined by immunodetection with isolated mitochondria, it was shown that *AtTim23-1* and/or *AtTim23-2* protein is present in isolated mitochondria from 2 week old plants, while *AtTim23-3* was not detected [13]. This study also characterized two T-DNA insertion mutants lines of *Attim23-2*, and found that one of these lines was an over-expressing line, showing a ~2-fold increase in *AtTim23-2* protein abundance compared to wild-type (Col-0) [13]. Notably, the other T-DNA insertion line in the *AtTim23-2* coding region had no effect on growth, possibly due to compensation by the other isoform(s) [13].

Thus, in order to examine this more closely, we cross fertilised homozygous

knock-out lines of *AtTim23-1* with *AtTim23-2*, *AtTim23-2* with *AtTim23-3* and *AtTim23-1* with *AtTim23-3* (Table 1). Interestingly, it was seen that these crosses failed to produce any double homozygous lines. Closer investigation of lines containing a homozygous genotype of one *AtTim23* gene and a heterozygous genotype of the other gene revealed defective seeds within the silique (Figure 2). The defective seeds appear to have been aborted in the later stages of embryo development due to the presence of an intact seed coat, indicated by the red arrows (Figure 2). The high expression of all *AtTim23* genes previously reported during early germination [87] also supports these findings suggesting a crucial role of Tim23 for embryo viability. However, unlike the single essential gene in yeast, in plants we show that the removal of any two genes encoding Tim23 results in an embryo lethal phenotype (Figure 2; Table 1), suggesting that each isoform may exhibit some specialized function in plants and at least 2 isoforms are essential for seed viability.

4.5 TIM22

The TIM22 complex is responsible for translocation of carrier type membrane proteins through and into the inner membrane containing internal targeting signals (Figure 1). In yeast, the TIM22 complex comprises of the essential translocation channel protein Tim22 and all of its accessory proteins - Tim54, Tim18 and Tim12, which are essential for yeast viability [101-104]. A recently identified Tim18-associated protein, SDH3, has also been found to be located within the respiratory Complex II in yeast and thus is involved in both biogenesis and electron transfer [11]. In plants, homologs to yeast Tim54, 18 and 12 are not identified in any plant genome database [105] and it is expected that additional plant specific proteins are likely involved to replace the essential functions of Tim54 and 18. Whilst there has been little characterisation of the TIM22 complex in plants, preliminary data suggests that Tim22 may also associate with respiratory complexes, as it has been identified to be associated with Complex I by MS analysis of the mitochondrial complex proteome [94, 106]. This is also supported by the observation that the expression pattern of *Tim22* genes in *Arabidopsis* show similarity to that seen for genes encoding respiratory chain components, i.e. increasing in expression over the course of germination and remaining high in vegetative tissues, rather than peaking in expression early during seed development and germination as seen for genes encoding proteins in TIM17:23 [87].

In Arabidopsis, two genes encode Tim22 and both are 100% identical, even up to 500 bp upstream of the ATG start codon, suggesting a very recent gene duplication event. Therefore, in order to identify T-DNA insertional knock-out lines encoding Tim22, all available lines for each gene, *AtTim22-1* (At1g18320) and *AtTim22-2* (At3g10110), were obtained and screened using primers specific for each gene at -500 bp from the start codon (Figure 3; Table 1). One homozygous line for each gene was identified, GK-848H04 for *Attim22-1* (At1g18320) and SAIL_623_F03 for *Attim22-2* (At3g10110) (Table 1). Whilst the homozygous line for At1g18320 did not exhibit any obvious deleterious phenotype (data not shown), the homozygous plant for the insertional knock-out within the At3g10110 gene resulted in a sterile plant with defects in silique maturation and seed production (Figure 3). This phenotype is also consistent with the identification of this gene as being required for female gametophyte development and function in Arabidopsis [107]. Thus, the *AtTim22-2* gene encoded by At3g10110 is also essential for seed viability in Arabidopsis.

4.6 *Tim21*

In Arabidopsis, AtTim21 is encoded by a single gene At4g00026, shown to interact with the TIM17:23 complex and respiratory complex III [13], similar to the function of yeast Tim21 as a core component of TIM17:23 and transiently interacting with the TOM and respiratory components [108, 109]. In yeast, the Tim21 is non-essential [110] yet Arabidopsis knock-outs of AtTim21 (*sh3*) has been shown to result in a dwarf phenotype resulting in seedling lethality [111]. The Arabidopsis genome also encodes two additional Tim21-like proteins (At2g40800 and At3g56430) proteins 108-165 AA longer than AtTim21 (At4g00026). The absence of these Tim21-like proteins in yeast suggests possible specialised functions and further analysis would be required to determine if they have a role in mitochondrial protein import.

4.7 *Tim50*

Tim50 recognizes of the targeting peptide within the presequence in the IMS domain, transferring it from the TOM complex to the TIM17:23 channel (Figure 1). Phylogenetic analysis of Tim50 across eukaryotic systems suggests that this protein was acquired early in eukaryotes and has evolved independently in each of the

major lineages [91]. Interestingly, AtTim50 is the only subunit of the TIM17:23 complex encoded by one gene in plants. Whilst Tim50 is essential in yeast, a recent report by Kumar et al., 2012 characterized a *Attim50* knock-out line (SALK_059376) as being viable with only minimal phenotypic abnormalities such as reduced hypocotyl length and enlarged mitochondria [112]. In contrast, another study has shown AtTim50 to be an essential gene for early embryonic development [18]. The apparent minimal phenotypic aberrations in the AtTim50 knockout characterized by Kumar et al., 2012 is surprising, given the essential nature of Tim50 in other systems such as yeast, *Drosophila* and *C.elegans* [113-115]. Thus, further characterisation of this mutant would clarify if AtTim50 is in fact an essential protein in Arabidopsis.

In order to clarify this, we examined all available AtTim50 insertional knock-out lines in Arabidopsis. Genotyping identified a homozygous line (SALK_000523) with the insertion located at position +2211 bp from the translation start site (Figure 4A). Immunodetection with an antibody raised against Arabidopsis Tim50 [13], with isolated mitochondria isolated from 2 week old homozygous seedlings detected a band, with an apparent molecular weight of 42 kDa, compared to a band with an apparent molecular weight of 43 kDa from wild-type mitochondria (Figure 4A). Considering the position of the T-DNA within the *AtTim50* gene, western blot analysis suggests that the T-DNA insert results in a truncated protein (Figure 4A). Phenotypic examination showed this plant to have a retarded growth phenotype when compared to Col-0 (Figure 4A). The previously published *Attim50* knock-out line (SALK_059376) [112], was then also screened for homozygosity and immunodetected was carried out using isolated mitochondria (Figure 4B). Immunodetection of AtTim50 showed the presence of a 43 kDa band (Figure 4B). The intensity of this AtTim50 detection suggests that the T-DNA insert within SALK_059376 results in a *AtTim50* knock-down (Figure 4B), with equal protein loading confirmed using antibodies against porin. To ensure correct genotyping was carried out, additional screening primers were used as outlined previously [112] and sequencing of the T-DNA insert confirms its location within the 4th intron. Overall, the observed retarded development seen here, when only a truncated AtTim50 protein is present (Figure 4A), supports the previous report of AtTim50 as an essential Arabidopsis protein, necessary for seed viability in Arabidopsis [18].

4.8 *Tim44*

In addition to Tim50, Tim44 also associates with the TIM17:23 complex, likely as part of the plant equivalent of the TIM17:23 PAM complex (Figure 1). In an effort to gain insight into AtTim44 function, T-DNA insertional knock-out lines of *Attim44* were also examined (Table 1). In plants, AtTim44 is encoded by two genes and it was shown that knocking out each of these individually does not affect plant development (Table 1). However, crossing these, i.e. the *Attim44-1* (-/-) homozygous knock-out line and *Attim44-2* (-/-) homozygous knock-out line failed to produce any double knock-out homozygous lines (Table 1). Analysis of the *Attim44-1* (-/-)::*Attim44-2* (+/-) plant, revealed early embryonic abortion within the silique at a ratio of 34±8% consistent with an embryo lethal phenotype (Figure 2). Thus, this suggests that both isoforms of AtTim44 can functionally replace each other and that AtTim44 is an essential protein for seed viability (Figure 2).

5. Matrix

5.1 *MPP and PrepP*

As mitochondrial proteins are imported from the inner membrane into the matrix, processing steps are necessary before the imported protein is fully functional. To do this, the mitochondrial processing peptidase (MPP) and the Presequence Peptidase (PrepP) are utilised (Figure 1) [116]. In Arabidopsis MPP is encoded by two genes, denoted α -MPP (At3g16480) and β -MPP (At3g02090). MPP functions to cleave the targeting signal prior to assembly and interestingly, although there is orthology (31 and 41% respectively) between these and their yeast counterparts, it has been shown that there are significant differences in plants. Specifically, the plant MPP has been shown to be an integral component of the respiratory cytochrome bc1 complex and thus is bi-functional, involved in both processing and electron transfer [117-119]. This unique feature is plant specific, not seen in yeast and mammals, and provides an example of links between the sharing of proteins between protein import and respiratory chain complexes [117].

In Arabidopsis PrepP is encoded by 2 genes, AtPrepP1 and AtPrepP2 exhibiting 87% identity. These encode peptidases involved in the degradation of presequences following MPP cleavage [120, 121]. Initially identified in plants, AtPrepP has the ability to complement its yeast non-essential ortholog (Mop112/Cym1) suggesting

functional conservation [122]. However it may yet have undergone neo-functionalisation as AtPrepP1 and AtPrepP2 are both co-localised in both mitochondria and chloroplast [123]. Deletion of both isoforms of AtPrepP results in minor growth aberrations suggesting additional proteases may function to degrade presequences [124].

6. Linking mitochondrial biogenesis to mitochondrial activity

In yeast, the TIM17:23 complex has been identified to form dynamic supercomplexes with both the TOM40 channel and Complex III and Complex IV of the mitochondrial respiratory chain via Tim21 [125], proposed to promote efficient translocation at contact sites by utilising the membrane potential of the inner membrane [3, 125]. Examination of the AtTim23-2 over-expressing line revealed that Complex I was reduced in abundance by ~90% [13]. Further analysis of independent Complex I knock-out lines showed that AtTim23-2 was conversely increased in abundance, thus supporting the link between the TIM17:23 complex and respiratory chain Complex I in plants [13, 126]. Further analysis of the connection between TIM17:23 and Complex I showed that another PRAT protein B14.7, previously shown by two independent groups to be a subunit of Complex I [94, 95], was also a subunit of TIM17:23 [13]. Furthermore, AtTim23-2 and AtB14.7 imported and assembled into Complex I and TIM17:23 confirming that these two complexes share both of these subunits. The overexpression of AtTim23-2 also resulted in a 2-3-fold increase in transcript abundance of mitochondrial encoded genes, organelle translation, and protein import. Transcript analysis of nuclear genes encoding mitochondrial proteins also displayed an increase in abundance, in particular for genes encoding proteins involved in mitochondrial biogenesis [13, 126]. These findings uncovered a link between TIM17:23 and Complex I, and importantly revealed that by changing the abundance of AtTim23-2, mitochondrial biogenesis could be altered. Thus, an important regulatory mechanism linking mitochondrial biogenesis and activity has been uncovered and highlights the dynamic and complex regulatory mechanisms of protein import in higher eukaryotes. A recent study in yeast has identified a novel subunit of the TIM17:23 complex, Mgr2 involved in coupling TIM17:23 to the TOM complex and the respiratory chain, is required for efficient import by stabilizing the

TOM-TIM supercomplex [127]. In Arabidopsis there appears to be one ortholog encoded by At3g07910 that exhibits a low percentage identity (32%). However, it does contain a ROMO1 (reactive mitochondrial oxygen species modulator 1) conserved domain also found in yeast Mgr2. More interestingly At3g07910 similarly contains regions of homology to several PRAT family members and thus is a likely candidate for further characterization in plant mitochondria.

5.3 *AtB14.7 (At2g42210) is essential for seed viability*

Screening of the T-DNA insertional knock-out line for AtB14.7 (At2g42210) (SAIL_434_E06) (Figure 5) failed to identify any homozygous genotypes and subsequent self fertilisation and screening of heterozygous progeny failed to identify any homozygous lines. Inspection of silique development in the heterozygous germplasms (AtB14.7 (+/-)) found defective seeds at a ratio comparable to the defective seed ratios exhibited in wild-type (Col-0). Pollen viability and total pollen counts were investigated by Alexander staining and found to be significantly lower in heterozygous plants compared to wild-type (Col-0) (Figure 5). Thus, AtB14.7 is essential for seed viability in Arabidopsis.

7. **The essential role of mitochondrial import during germination**

In this review, we have shown that several mitochondrial inner membrane import components are essential for seed viability in Arabidopsis (Figure 1-5; Table 1), consistent with their relatively high levels of transcript abundance during germination. It has been reported that genes peaking in expression during germination are enriched in mitochondrial proteins, particularly those encoding RNA processing functions such as the mitochondrial pentatripeptide repeat (PPR) containing proteins [87]. Thus, the high expression of several inner membrane import components during seed germination [87] supports the findings that a loss-of-function of these results in an embryo lethal phenotype (Table 1). Close examination of mitochondrial biogenesis during germination revealed that at transcript and protein abundance of mitochondrial import component did not correlate during germination [17].

Specifically, it was seen that the proteins encoding import components are present very early during germination, peaking in abundance before the respective transcripts in Arabidopsis [17]. This is also supported by the findings in rice, where it was shown that within 30 min of imbibition (water up-take), mitochondrial protein import begins [15]. Taken together, the recent findings linking AtTim23-2 protein abundance with altered mitochondrial biogenesis [13], as well as the observed embryo lethal phenotypes presented here after the loss-of-function of several inner membrane import components (Table 1), indicates that mitochondrial protein import plays a crucial role in early germination in plants, facilitating a move from a dormant metabolic inert state to an active metabolic state, to drive germination.

8. Concluding remarks

• *The conservation of inner membrane translocases*

In yeast, all components of the TIM17:23-sort complex are essential for viability, with the exception of Tim21 [109]. As shown above, in Arabidopsis; AtTim17, AtTim23, AtTim44 and AtTim50 are also shown to be essential subunits for seed viability (Table 1), whilst AtTim21 has acquired an essential function during vegetative growth [111] (Table 1). This shows a high level of conservation for the essential subunits of plant mitochondrial import apparatus.

• *The acquisition and divergence of function*

In plants, the presence of multi-gene families has resulted in neo-specialisation and neo-functionalisation of the mitochondrial import components. AtTim17 and AtTim23 are examples, where expansion of these gene families in plants resulted in the different isoforms acquiring new functions or showing different tissue specific expression. Of the three isoforms of AtTim17; AtTim17-2 maintains the essential function, whilst the loss-of-function of AtTim17-1 and AtTim17-3 did not result in embryo lethal phenotypes (Table 1). Interestingly, expression profiling revealed higher expression of AtTim17-1 during seed development, and further examination using AtTim17-1 knock-outs revealed a germination specific phenotype (Wang and Murcha - unpublished data), suggesting neo-specialisation, with this isoform having a unique role in seed development and germination. The C-terminal extension of

AtTim17-2 appears to contain conserved motifs predicted to bind nucleic acids, and as plant mitochondria have the ability to import tRNA, this merits an investigation of the role of AtTim17-2 in the import of tRNA [5].

- ***New levels of dynamics with the respiratory chain and supercomplex structures***

While only one gene encodes TIM23 in yeast, three genes encode for AtTim23. Interestingly, this has allowed some redundancy, where it was seen that while deletion of one gene has no effect on seed viability, the deletion of two AtTim23 encoding genes does result in an embryo lethal phenotype (Figure 2). Whilst there appears to be few predicted structural differences between the three AtTim23 isoforms, expression analysis indicates differences in abundance with *AtTim23-2* showing the highest transcript abundance [22]. Studies in Arabidopsis have shown that AtTim23-2 interacts with respiratory Complex I and III [13], which raises the possibility that different isoforms may have preference for protein-protein interactions. These differences could then facilitate the formation of dynamic and variant forms of complexes and supercomplexes within the inner mitochondrial membrane. In addition, it has been shown that AtB14.7 is also an essential inner membrane PRAT protein (Table 1). It has previously been shown that AtB14.7 has a dual-location, both within the TIM17:23 complex and the respiratory Complex I in Arabidopsis [13]. However, the specific role of AtB14.7 has yet to be elucidated. B14.7 has been characterized as a Complex I subunit in *N. crassa*, bovine and now Arabidopsis [13], suggesting an important function in higher eukaryotes.

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Table 1. Summary of T-DNA insertional knock-out lines for mitochondrial membrane import components in *Arabidopsis*. T-DNA insertional knock-out lines were screened for homozygosity and examined for growth defects. Where multiple isoforms exist for each component double knock-out lines were created to determine lethality of multiple gene inactivation. Abbreviations: Tom = Translocases of the Outer Membrane, Tim = Translocase of the Inner Membrane, NdufA11 = NADH dehydrogenase [ubiquinone] 1 subunit 11 (Human), B14.7 = NADH dehydrogenase [ubiquinone] 1 subunit 14.7 (Bovine), HM = Homozygous, Het = Heterozygous, bp = base pair, Ref = Reference, At = *Arabidopsis thaliana*.

Figure 1. Diagrammatic representation of protein import pathways and components in plant mitochondria. Targeting signals on cytosolically located precursor proteins determine the pathway of import. All precursors (containing a cleavable presequence, Twin CxC, β -barrel or carrier type proteins) pass through the Translocase of the Outer Membrane (TOM). The pathways diverge in the Intermembrane Space (IMS). Insertion of β -barrel proteins into the outer membrane is carried out via the Sorting and Assembly Machinery (SAM). Carrier import pathway proteins translocate into the inner membrane via small soluble Tim proteins through the TIM22 channel located in the inner membrane. Twin CxC proteins located within the IMS, are imported via the MIA-Erv1 pathway. For the majority of mitochondrial proteins that contain a cleavable presequence, translocation is carried out through the TIM17:23 complex with the Presequence Assisted Motor (PAM) complex or inserted directly into the inner membrane. The TIM17:23 complex can exist as 2 forms, PAM and SORT. In addition Tim23 interacts with the Complex I subunit B14.7. Following translocation, the presequence is removed via Mitochondrial Processing Peptidase (MPP), integrated into the cytochrome bc1 complex. The presequence is further processed via a matrix located Presequence Peptidase (PrepP). The essential subunits required for seed viability are circled. A membrane potential is required for translocation across the inner membrane ($\Delta\Psi$). Abbreviations: TOM = Translocase of the Outer Membrane, TIM = Translocase of the Inner membrane, IMS = Intermembrane Space, SAM = Sorting and Assembly Machinery, PrepP = Presequence Peptidase, MPP = Mitochondrial Processing

Peptidase. The subunits known to be essential for plant viability are bolded. SAM50 is bolded in gray as no experimental verification of its essential function has been reported in plants.

Figure 2. Germplasms resulting in defective embryo development. Seed sets for *Attim17-2* (+/-) heterozygous knock-out and double knock-out lines, *Attim44-1* (-/-)::*Attim44-1* (+/-), *Attim23-1* (-/-)::*Attim23-2* (+/-), *Attim23-1* (-/-)::*Attim23-3* (+/-) and *Attim23-2* (-/-)::*Attim23-3* (+/-) exhibit seed lethal phenotypes. The germplasms were screened to confirm genotype and siliques dissected to confirm the lethality of gene inactivation. Values indicate the average percentage of aborted progeny from three biological replicates $\pm$ S.D (n = 60). Scale bar, 0.1 cm. Arrow indicates defective embryo. Abbreviations: (+/-) = heterozygous knock-out, (-/-) = homozygous knock-out, Col-0 = wild-type.

Figure 3. Deletion of *AtTim22-2* results in plant sterility. The T-DNA insertional knock-out line for *AtTim22-2* (At3g10110) (SAIL_623_F03) was screened for homozygosity. *Attim22-2* homozygous plants exhibit smaller flowers and poorly developed siliques with little to no seed. Scale bar, 0.1 cm, (-/-) = homozygous knock-out.

Figure 4. Identification of *Attim50* mutants. The T-DNA insertional knock-out lines for *AtTim50* (At1g55900) were screened for homozygosity and immunodetected with *AtTim50* antibody to confirm loss of function. (A) The line, SALK_000523 (*Attim50* (-/-) KO1) with the T-DNA insert at position +2211 in the last intron, displays an aberrant growth phenotype compared to wild-type (Col-0). Immunodetection of mitochondria isolated from 2 week old seedlings indicate that the insertion results in a truncated *AtTim50* of apparent molecular weight of 42 kDa. (B) The *Attim50* (-/-) KO2 line, SALK_059376, previously identified to be a *Attim50* knock-out [59] was rescreened for homozygosity. Homozygous lines display no apparent growth aberrations and immunodetection against *AtTim50* suggests that the T-DNA insertion results in a knock-down, not a knock-out. The mitochondrial control (Porin) shows equal protein loading. Abbreviations: RP = Right border screening primer, LP

= Left border screening primer, BP = T-DNA border screening primer, kDa = kilodaltons (-/-) = homozygous knock-out.

Figure 5. Identification of a *Atb14.7* heterozygous mutant. (A) screening of the T-DNA insertional knock-out line for *AtB14.7* (SAIL_434_E06) failed to identify any homozygous lines. Dissection of *Atb14.7* (+/-) heterozygous plants show all seed sets to be viable and subsequent genotyping only identified Col-0 or heterozygous germplasm. Values indicate the average percentage of aborted progeny per silique $\pm$ S.D (n = 60) from three biological replicates. Scale bar, 0.1 cm. (B) The number of pollen grains and percentage of defective pollen from *Atb14.7* (+/-) heterozygous plants as determined by Alexander staining. Arrows indicate defective pollen grains. * indicates statistically significant difference compared with wild-type (Col-0) ($p < 0.01$). Scale bars, 20 μ m. Error bars indicate S.E, (+/-) = heterozygous knock-out.

References

- [1] M.W. Gray, G. Burger, B.F. Lang, Mitochondrial evolution, *Science*, 283 (1999) 1476-1481.
- [2] J. Dudek, P. Rehling, M. van der Laan, Mitochondrial protein import: Common principles and physiological networks, *Biochim Biophys Acta*, 1833 (2013) 274-285.
- [3] M. van der Laan, D.P. Hutu, P. Rehling, On the mechanism of preprotein import by the mitochondrial presequence translocase, *Biochim Biophys Acta*, 1803 (2010) 732-739.
- [4] C. Carrie, M.W. Murcha, E. Giraud, S. Ng, M.F. Zhang, R. Narsai, J. Whelan, How do plants make mitochondria?, *Planta*, 237 (2013) 429-439.
- [5] C. Carrie, M.W. Murcha, J. Whelan, An in silico analysis of the mitochondrial protein import apparatus of plants, *BMC Plant Biol*, 10 (2010) 249.
- [6] O. Duncan, M.W. Murcha, J. Whelan, Unique components of the plant mitochondrial protein import apparatus, *Biochim Biophys Acta*, 1833 (2013) 304-313.
- [7] T. Lithgow, A. Schneider, Evolution of macromolecular import pathways in mitochondria, hydrogenosomes and mitosomes, *Philos Trans R Soc Lond B Biol Sci*, 365 (2010) 799-817.
- [8] E. Eckers, M. Cyrklaff, L. Simpson, M. Deponte, Mitochondrial protein import pathways are functionally conserved among eukaryotes despite compositional diversity of the import machineries, *Biol Chem*, 393 (2012) 513-524.
- [9] A. Chacinska, C.M. Koehler, D. Milenkovic, T. Lithgow, N. Pfanner, Importing mitochondrial proteins: machineries and mechanisms, *Cell*, 138 (2009) 628-644.
- [10] N.V. Dudkina, R. Kouril, K. Peters, H.P. Braun, E.J. Boekema, Structure and function of mitochondrial supercomplexes, *Biochim Biophys Acta*, 1797 (2010) 664-670.
- [11] N. Gebert, M. Gebert, S. Oeljeklaus, K. von der Malsburg, D.A. Stroud, B. Kulawiak, C. Wirth, R.P. Zahedi, P. Dolezal, S. Wiese, O. Simon, A. Schulze-Specking, K.N. Truscott, A. Sickmann, P. Rehling, B. Guiard, C. Hunte, B. Warscheid, M. van der Laan, N. Pfanner, N. Wiedemann, Dual function of Sdh3 in the respiratory chain and TIM22 protein translocase of the mitochondrial inner membrane, *Mol Cell*, 44 (2011) 811-818.

- [12] B. Kulawiak, J. Hopker, M. Gebert, B. Guiard, N. Wiedemann, N. Gebert, The mitochondrial protein import machinery has multiple connections to the respiratory chain, *Biochim Biophys Acta*, (2012).
- [13] Y. Wang, C. Carrie, E. Giraud, D. Elhafez, R. Narsai, O. Duncan, J. Whelan, M.W. Murcha, Dual Location of the Mitochondrial Preprotein Transporters B14.7 and Tim23-2 in Complex I and the TIM17:23 Complex in Arabidopsis Links Mitochondrial Activity and Biogenesis, *Plant Cell*, 24 (2012) 2675-2695.
- [14] G. Giaever, A.M. Chu, L. Ni, C. Connelly, L. Riles, S. Veronneau, S. Dow, A. Lucau-Danila, K. Anderson, B. Andre, A.P. Arkin, A. Astromoff, M. El-Bakkoury, R. Bangham, R. Benito, S. Brachat, S. Campanaro, M. Curtiss, K. Davis, A. Deutschbauer, K.D. Entian, P. Flaherty, F. Foury, D.J. Garfinkel, M. Gerstein, D. Gotte, U. Guldener, J.H. Hegemann, S. Hempel, Z. Herman, D.F. Jaramillo, D.E. Kelly, S.L. Kelly, P. Kotter, D. LaBonte, D.C. Lamb, N. Lan, H. Liang, H. Liao, L. Liu, C. Luo, M. Lussier, R. Mao, P. Menard, S.L. Ooi, J.L. Revuelta, C.J. Roberts, M. Rose, P. Ross-Macdonald, B. Scherens, G. Schimmack, B. Shafer, D.D. Shoemaker, S. Sookhai-Mahadeo, R.K. Storms, J.N. Strathern, G. Valle, M. Voet, G. Volckaert, C.Y. Wang, T.R. Ward, J. Wilhelmy, E.A. Winzeler, Y. Yang, G. Yen, E. Youngman, K. Yu, H. Bussey, J.D. Boeke, M. Snyder, P. Philippsen, R.W. Davis, M. Johnston, Functional profiling of the *Saccharomyces cerevisiae* genome, *Nature*, 418 (2002) 387-391.
- [15] K.A. Howell, K. Cheng, M.W. Murcha, L.E. Jenkin, A.H. Millar, J. Whelan, Oxygen initiation of respiration and mitochondrial biogenesis in rice, *J Biol Chem*, 282 (2007) 15619-15631.
- [16] K.A. Howell, A.H. Millar, J. Whelan, Ordered assembly of mitochondria during rice germination begins with pro-mitochondrial structures rich in components of the protein import apparatus, *Plant Mol Biol*, 60 (2006) 201-223.
- [17] S.R. Law, R. Narsai, N.L. Taylor, E. Delannoy, C. Carrie, E. Giraud, A.H. Millar, I. Small, J. Whelan, Nucleotide and RNA metabolism prime translational initiation in the earliest events of mitochondrial biogenesis during Arabidopsis germination, *Plant Physiol*, 158 (2012) 1610-1627.
- [18] I. Tzafrir, A. Dickerman, O. Brazhnik, Q. Nguyen, J. McElver, C. Frye, D. Patton, D. Meinke, The Arabidopsis SeedGenes Project, *Nucleic Acids Res*, 31 (2003) 90-93.
- [19] F.N. Vogtle, S. Wortelkamp, R.P. Zahedi, D. Becker, C. Leidhold, K. Gevaert, J. Kellermann, W. Voos, A. Sickmann, N. Pfanner, C. Meisinger, Global analysis of the mitochondrial N-proteome identifies a processing peptidase critical for protein stability, *Cell*, 139 (2009) 428-439.
- [20] Y. Abe, T. Shodai, T. Muto, K. Mihara, H. Torii, S. Nishikawa, T. Endo, D. Kohda, Structural basis of presequence recognition by the mitochondrial protein import receptor Tom20, *Cell*, 100 (2000) 551-560.
- [21] J. Brix, K. Dietmeier, N. Pfanner, Differential recognition of preproteins by the purified cytosolic domains of the mitochondrial import receptors Tom20, Tom22, and Tom70, *J Biol Chem*, 272 (1997) 20730-20735.
- [22] R. Lister, O. Chew, M.N. Lee, J.L. Heazlewood, R. Clifton, K.L. Parker, A.H. Millar, J. Whelan, A transcriptomic and proteomic characterization of the Arabidopsis mitochondrial protein import apparatus and its response to mitochondrial dysfunction, *Plant Physiol*, 134 (2004) 777-789.
- [23] K.A. Rimmer, J.H. Foo, A. Ng, E.J. Petrie, P.J. Shilling, A.J. Perry, H.D. Mertens, T. Lithgow, T.D. Mulhern, P.R. Gooley, Recognition of mitochondrial targeting sequences by the import receptors Tom20 and Tom22, *J Mol Biol*, 405 (2011) 804-818.
- [24] R. Lister, C. Carrie, O. Duncan, L.H. Ho, K.A. Howell, M.W. Murcha, J. Whelan, Functional definition of outer membrane proteins involved in preprotein import into mitochondria, *Plant Cell*, 19 (2007) 3739-3759.
- [25] S. van Wilpe, M.T. Ryan, K. Hill, A.C. Maarse, C. Meisinger, J. Brix, P.J. Dekker, M. Moczko, R. Wagner, M. Meijer, B. Guiard, A. Honlinger, N. Pfanner, Tom22 is a multifunctional organizer of the mitochondrial preprotein translocase, *Nature*, 401 (1999) 485-489.
- [26] T. Lithgow, T. Junne, K. Suda, S. Gratzer, G. Schatz, The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast, *Proc Natl Acad Sci U S A*, 91 (1994) 11973-11977.

- [27] D.A. Court, F.E. Nargang, H. Steiner, R.S. Hodges, W. Neupert, R. Lill, Role of the intermembrane-space domain of the preprotein receptor Tom22 in protein import into mitochondria, *Mol Cell Biol*, 16 (1996) 4035-4042.
- [28] T. Komiya, S. Rospert, C. Koehler, R. Looser, G. Schatz, K. Mihara, Interaction of mitochondrial targeting signals with acidic receptor domains along the protein import pathway: evidence for the 'acid chain' hypothesis, *Embo J*, 17 (1998) 3886-3898.
- [29] M. Moczko, U. Bomer, M. Kubrich, N. Zufall, A. Honlinger, N. Pfanner, The intermembrane space domain of mitochondrial Tom22 functions as a trans binding site for preproteins with N-terminal targeting sequences, *Mol Cell Biol*, 17 (1997) 6574-6584.
- [30] D. Macasev, E. Newbigin, J. Whelan, T. Lithgow, How do plant mitochondria avoid importing chloroplast proteins? Components of the import apparatus Tom20 and Tom22 from Arabidopsis differ from their fungal counterparts, *Plant Physiol*, 123 (2000) 811-816.
- [31] D. Macasev, J. Whelan, E. Newbigin, M.C. Silva-Filho, T.D. Mulhern, T. Lithgow, 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 (2004) 1557-1564.
- [32] W. Werhahn, A. Niemeyer, L. Jansch, V. Kruff, U.K. Schmitz, H. Braun, Purification and characterization of the preprotein translocase of the outer mitochondrial membrane from Arabidopsis. Identification of multiple forms of TOM20, *Plant Physiol*, 125 (2001) 943-954.
- [33] K. Dietmeier, A. Honlinger, U. Bomer, P.J. Dekker, C. Eckerskorn, F. Lottspeich, M. Kubrich, N. Pfanner, Tom5 functionally links mitochondrial preprotein receptors to the general import pore, *Nature*, 388 (1997) 195-200.
- [34] H. Kato, K. Mihara, Identification of Tom5 and Tom6 in the preprotein translocase complex of human mitochondrial outer membrane, *Biochem Biophys Res Commun*, 369 (2008) 958-963.
- [35] S. Schmitt, U. Ahting, L. Eichacker, B. Granvogel, N.E. Go, F.E. Nargang, W. Neupert, S. Nussberger, Role of Tom5 in maintaining the structural stability of the TOM complex of mitochondria, *J Biol Chem*, 280 (2005) 14499-14506.
- [36] T. Becker, L.S. Wenz, N. Thornton, D. Stroud, C. Meisinger, N. Wiedemann, N. Pfanner, Biogenesis of mitochondria: dual role of Tom7 in modulating assembly of the preprotein translocase of the outer membrane, *J Mol Biol*, 405 (2011) 113-124.
- [37] R. Lister, M.W. Murcha, J. Whelan, The Mitochondrial Protein Import Machinery of Plants (MPIMP) database, *Nucleic Acids Res*, 31 (2003) 325-327.
- [38] O. Duncan, N.L. Taylor, C. Carrie, H. Eubel, S. Kubiszewski-Jakubiak, B. Zhang, R. Narsai, A.H. Millar, J. Whelan, Multiple lines of evidence localize signaling, morphology, and lipid biosynthesis machinery to the mitochondrial outer membrane of Arabidopsis, *Plant Physiol*, 157 (2011) 1093-1113.
- [39] N. Bolender, A. Sickmann, R. Wagner, C. Meisinger, N. Pfanner, Multiple pathways for sorting mitochondrial precursor proteins, *EMBO Rep*, 9 (2008) 42-49.
- [40] N.C. Chan, T. Lithgow, The peripheral membrane subunits of the SAM complex function codependently in mitochondrial outer membrane biogenesis, *Mol Biol Cell*, 19 (2008) 126-136.
- [41] L.F. Sogo, M.P. Yaffe, Regulation of mitochondrial morphology and inheritance by Mdm10p, a protein of the mitochondrial outer membrane, *J Cell Biol*, 126 (1994) 1361-1373.
- [42] K.H. Berger, L.F. Sogo, M.P. Yaffe, Mdm12p, a component required for mitochondrial inheritance that is conserved between budding and fission yeast, *J Cell Biol*, 136 (1997) 545-553.
- [43] S.M. Burgess, M. Delannoy, R.E. Jensen, Mmm1 encodes a mitochondrial outer membrane protein essential for establishing and maintaining the structure of yeast mitochondria, *J Cell Biol*, 126 (1994) 1375-1391.
- [44] M.J. Youngman, A.E. Hobbs, S.M. Burgess, M. Srinivasan, R.E. Jensen, Mmm2p, a mitochondrial outer membrane protein required for yeast mitochondrial shape and maintenance of mtDNA nucleoids, *J Cell Biol*, 164 (2004) 677-688.
- [45] K. Yamano, S. Tanaka-Yamano, T. Endo, Tom7 regulates Mdm10-mediated assembly of the mitochondrial import channel protein Tom40, *J Biol Chem*, 285 (2010) 41222-41231.

- [46] C. Meisinger, S. Pfannschmidt, M. Rissler, D. Milenkovic, T. Becker, D. Stojanovski, M.J. Youngman, R.E. Jensen, A. Chacinska, B. Guiard, N. Pfanner, N. Wiedemann, The morphology proteins Mdm12/Mmm1 function in the major beta-barrel assembly pathway of mitochondria, *EMBO J*, 26 (2007) 2229-2239.
- [47] C. Meisinger, M. Rissler, A. Chacinska, L.K. Szklarz, D. Milenkovic, V. Kozjak, B. Schonfisch, C. Lohaus, H.E. Meyer, M.P. Yaffe, B. Guiard, N. Wiedemann, N. Pfanner, The mitochondrial morphology protein Mdm10 functions in assembly of the preprotein translocase of the outer membrane, *Developmental cell*, 7 (2004) 61-71.
- [48] J.G. Wideman, S.W. Lackey, M.A. Srayko, K.A. Norton, F.E. Nargang, Analysis of Mutations in *Neurospora crassa* ERMES Components Reveals Specific Functions Related to beta-Barrel Protein Assembly and Maintenance of Mitochondrial Morphology, *PloS one*, 8 (2013) e71837.
- [49] B. Kornmann, E. Currie, S.R. Collins, M. Schuldiner, J. Nunnari, J.S. Weissman, P. Walter, An ER-mitochondria tethering complex revealed by a synthetic biology screen, *Science*, 325 (2009) 477-481.
- [50] K. Yamano, S. Tanaka-Yamano, T. Endo, Mdm10 as a dynamic constituent of the TOB/SAM complex directs coordinated assembly of Tom40, *EMBO Rep*, 11 (2010) 187-193.
- [51] A.H. Michel, B. Kornmann, The ERMES complex and ER-mitochondria connections, *Biochemical Society transactions*, 40 (2012) 445-450.
- [52] I. Lee, W. Hong, Diverse membrane-associated proteins contain a novel SMP domain, *FASEB journal : official publication of the Federation of American Societies for Experimental Biology*, 20 (2006) 202-206.
- [53] K.O. Kopeck, V. Alva, A.N. Lupas, Homology of SMP domains to the TULIP superfamily of lipid-binding proteins provides a structural basis for lipid exchange between ER and mitochondria, *Bioinformatics*, 26 (2010) 1927-1931.
- [54] K. von der Malsburg, J.M. Muller, M. Bohnert, S. Oeljeklaus, P. Kwiatkowska, T. Becker, A. Loniewska-Lwowska, S. Wiese, S. Rao, D. Milenkovic, D.P. Hutu, R.M. Zerbes, A. Schulze-Specking, H.E. Meyer, J.C. Martinou, S. Rospert, P. Rehling, C. Meisinger, M. Veenhuis, B. Warscheid, I.J. van der Klei, N. Pfanner, A. Chacinska, M. van der Laan, Dual role of mitofilin in mitochondrial membrane organization and protein biogenesis, *Developmental cell*, 21 (2011) 694-707.
- [55] M. Bohnert, L.S. Wenz, R.M. Zerbes, S.E. Horvath, D.A. Stroud, K. von der Malsburg, J.M. Muller, S. Oeljeklaus, I. Perschil, B. Warscheid, A. Chacinska, M. Veenhuis, I.J. van der Klei, G. Daum, N. Wiedemann, T. Becker, N. Pfanner, M. van der Laan, Role of mitochondrial inner membrane organizing system in protein biogenesis of the mitochondrial outer membrane, *Mol Biol Cell*, 23 (2012) 3948-3956.
- [56] M. Harner, C. Korner, D. Walther, D. Mokranjac, J. Kaesmacher, U. Welsch, J. Griffith, M. Mann, F. Reggiori, W. Neupert, The mitochondrial contact site complex, a determinant of mitochondrial architecture, *EMBO J*, 30 (2011) 4356-4370.
- [57] S. Hoppins, S.R. Collins, A. Cassidy-Stone, E. Hummel, R.M. Devay, L.L. Lackner, B. Westermann, M. Schuldiner, J.S. Weissman, J. Nunnari, A mitochondrial-focused genetic interaction map reveals a scaffold-like complex required for inner membrane organization in mitochondria, *J Cell Biol*, 195 (2011) 323-340.
- [58] A.K. Alkhaja, D.C. Jans, M. Nikolov, M. Vukotic, O. Lytovchenko, F. Ludewig, W. Schliebs, D. Riedel, H. Urlaub, S. Jakobs, M. Deckers, MINOS1 is a conserved component of mitofilin complexes and required for mitochondrial function and cristae organization, *Mol Biol Cell*, 23 (2012) 247-257.
- [59] A. Varabyova, U. Topf, P. Kwiatkowska, L. Wrobel, M. Kaus-Drobek, A. Chacinska, Mia40 and MINOS act in parallel with Ccs1 in the biogenesis of mitochondrial Sod1, *Febs J*, (2013).
- [60] R.M. Zerbes, I.J. van der Klei, M. Veenhuis, N. Pfanner, M. van der Laan, M. Bohnert, Mitofilin complexes: conserved organizers of mitochondrial membrane architecture, *Biol Chem*, 393 (2012) 1247-1261.
- [61] M. van der Laan, M. Bohnert, N. Wiedemann, N. Pfanner, Role of MINOS in mitochondrial membrane architecture and biogenesis, *Trends in cell biology*, 22 (2012) 185-192.

- [62] K. Hell, The Erv1-Mia40 disulfide relay system in the intermembrane space of mitochondria, *Biochim Biophys Acta*, 1783 (2008) 601-609.
- [63] C.M. Koehler, S. Merchant, W. Oppliger, K. Schmid, E. Jarosch, L. Dolfini, T. Junne, G. Schatz, K. Tokatlidis, Tim9p, an essential partner subunit of Tim10p for the import of mitochondrial carrier proteins, *Embo J*, 17 (1998) 6477-6486.
- [64] S. Senapin, X.J. Chen, G.D. Clark-Walker, Transcription of TIM9, a new factor required for the petite-positive phenotype of *Saccharomyces cerevisiae*, is defective in *spt7* mutants, *Current genetics*, 44 (2003) 202-210.
- [65] A.J. Davis, N.N. Alder, R.E. Jensen, A.E. Johnson, The Tim9p/10p and Tim8p/13p complexes bind to specific sites on Tim23p during mitochondrial protein import, *Mol Biol Cell*, 18 (2007) 475-486.
- [66] A. Vasiljev, U. Ahting, F.E. Nargang, N.E. Go, S.J. Habib, C. Kozany, V. Panneels, I. Sinning, H. Prokisch, W. Neupert, S. Nussberger, D. Rapaport, Reconstituted TOM core complex and Tim9/Tim10 complex of mitochondria are sufficient for translocation of the ADP/ATP carrier across membranes, *Mol Biol Cell*, 15 (2004) 1445-1458.
- [67] C.T. Webb, M.A. Gorman, M. Lazarou, M.T. Ryan, J.M. Gulbis, Crystal structure of the mitochondrial chaperone TIM9.10 reveals a six-bladed alpha-propeller, *Mol Cell*, 21 (2006) 123-133.
- [68] N. Wiedemann, K.N. Truscott, S. Pfannschmidt, B. Guiard, C. Meisinger, N. Pfanner, 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 (2004) 18188-18194.
- [69] K. Roesch, P.J. Hynds, R. Varga, L. Tranebjaerg, C.M. Koehler, The calcium-binding aspartate/glutamate carriers, citrin and aralar1, are new substrates for the DDP1/TIMM8a-TIMM13 complex, *Human molecular genetics*, 13 (2004) 2101-2111.
- [70] R. Lister, B. Mowday, J. Whelan, A.H. Millar, Zinc-dependent intermembrane space proteins stimulate import of carrier proteins into plant mitochondria, *Plant J*, 30 (2002) 555-566.
- [71] M.W. Murcha, A.H. Millar, J. Whelan, The N-terminal cleavable extension of plant carrier proteins is responsible for efficient insertion into the inner mitochondrial membrane, *J Mol Biol*, 351 (2005) 16-25.
- [72] M.W. Murcha, D. Elhafez, A.H. Millar, J. Whelan, The N-terminal extension of plant mitochondrial carrier proteins is removed by two-step processing: the first cleavage is by the mitochondrial processing peptidase, *J Mol Biol*, 344 (2004) 443-454.
- [73] K. Gabriel, D. Milenkovic, A. Chacinska, J. Muller, B. Guiard, N. Pfanner, C. Meisinger, Novel mitochondrial intermembrane space proteins as substrates of the MIA import pathway, *J Mol Biol*, 365 (2007) 612-620.
- [74] A. Chacinska, S. Pfannschmidt, N. Wiedemann, V. Kozjak, L.K. Sanjuan Szklarz, A. Schulze-Specking, K.N. Truscott, B. Guiard, C. Meisinger, N. Pfanner, Essential role of Mia40 in import and assembly of mitochondrial intermembrane space proteins, *EMBO J*, 23 (2004) 3735-3746.
- [75] N. Mesecke, N. Terziyska, C. Kozany, F. Baumann, W. Neupert, K. Hell, J.M. Herrmann, A disulfide relay system in the intermembrane space of mitochondria that mediates protein import, *Cell*, 121 (2005) 1059-1069.
- [76] N. Terziyska, T. Lutz, C. Kozany, D. Mokranjac, N. Mesecke, W. Neupert, J.M. Herrmann, K. Hell, Mia40, a novel factor for protein import into the intermembrane space of mitochondria is able to bind metal ions, *FEBS Lett*, 579 (2005) 179-184.
- [77] M. Rissler, N. Wiedemann, S. Pfannschmidt, K. Gabriel, B. Guiard, N. Pfanner, A. Chacinska, The essential mitochondrial protein Erv1 cooperates with Mia40 in biogenesis of intermembrane space proteins, *J Mol Biol*, 353 (2005) 485-492.
- [78] C. Carrie, E. Giraud, O. Duncan, L. Xu, Y. Wang, S. Huang, R. Clifton, M. Murcha, A. Filipovska, O. Rackham, A. Vrielink, J. Whelan, Conserved and novel functions for *Arabidopsis thaliana* MIA40 in assembly of proteins in mitochondria and peroxisomes, *J Biol Chem*, 285 (2010) 36138-36148.
- [79] L. Xu, C. Carrie, S.R. Law, M.W. Murcha, J. Whelan, Acquisition, conservation, and loss of dual-targeted proteins in land plants, *Plant Physiol*, 161 (2013) 644-662.

- [80] E. Eckers, C. Petrungaro, D. Gross, J. Riemer, K. Hell, M. Deponte, Divergent molecular evolution of the mitochondrial sulfhydryl:cytochrome C oxidoreductase Erv in opisthokonts and parasitic protists, *J Biol Chem*, 288 (2013) 2676-2688.
- [81] S. Basu, J.C. Leonard, N. Desai, D.A. Mavridou, K.H. Tang, A.D. Goddard, M.L. Ginger, J. Lukes, J.W. Allen, Divergence of Erv1-associated mitochondrial import and export pathways in trypanosomes and anaerobic protists, *Eukaryotic cell*, 12 (2013) 343-355.
- [82] A. Chacinska, M. van der Laan, C.S. Mehnert, B. Guiard, D.U. Mick, D.P. Hutu, K.N. Truscott, N. Wiedemann, C. Meisinger, N. Pfanner, P. Rehling, Distinct forms of mitochondrial TOM-TIM supercomplexes define signal-dependent states of preprotein sorting, *Mol Cell Biol*, 30 (2010) 307-318.
- [83] K. Wagner, D.U. Mick, P. Rehling, Protein transport machineries for precursor translocation across the inner mitochondrial membrane, *Biochim Biophys Acta*, 1793 (2009) 52-59.
- [84] A. Chacinska, M. van der Laan, C.S. Mehnert, B. Guiard, D.U. Mick, D.P. Hutu, K.N. Truscott, N. Wiedemann, C. Meisinger, N. Pfanner, P. Rehling, Distinct forms of mitochondrial TOM-TIM supercomplexes define signal-dependent states of preprotein sorting, *Mol Cell Biol*, 30 (2010) 307-318.
- [85] A.E. Frazier, J. Dudek, B. Guiard, W. Voos, Y. Li, M. Lind, C. Meisinger, A. Geissler, A. Sickmann, H.E. Meyer, V. Bilanchone, M.G. Cumsky, K.N. Truscott, N. Pfanner, P. Rehling, Pam16 has an essential role in the mitochondrial protein import motor, *Nat Struct Mol Biol*, 11 (2004) 226-233.
- [86] K.N. Truscott, K. Brandner, N. Pfanner, Mechanisms of protein import into mitochondria, *Curr Biol*, 13 (2003) R326-337.
- [87] R. Narsai, S.R. Law, C. Carrie, L. Xu, J. Whelan, In-depth temporal transcriptome profiling reveals a crucial developmental switch with roles for RNA processing and organelle metabolism that are essential for germination in Arabidopsis, *Plant Physiol*, 157 (2011) 1342-1362.
- [88] M.W. Murcha, D. Elhafez, R. Lister, J. Tonti-Filippini, M. Baumgartner, K. Philippar, C. Carrie, D. Mokranjac, J. Soll, J. Whelan, Characterization of the preprotein and amino acid transporter gene family in Arabidopsis, *Plant Physiol*, 143 (2007) 199-212.
- [89] J. Rassow, P.J. Dekker, S. van Wilpe, M. Meijer, J. Soll, The preprotein translocase of the mitochondrial inner membrane: function and evolution, *J Mol Biol*, 286 (1999) 105-120.
- [90] M.W. Murcha, R. Lister, A.Y. Ho, J. Whelan, Identification, expression, and import of components 17 and 23 of the inner mitochondrial membrane translocase from Arabidopsis, *Plant Physiol*, 131 (2003) 1737-1747.
- [91] L. Delage, C. Leblanc, P. Nyvall Collen, B. Gschloessl, M.P. Oudot, L. Sterck, J. Poulain, J.M. Aury, J.M. Cock, In silico survey of the mitochondrial protein uptake and maturation systems in the brown alga *Ectocarpus siliculosus*, *PLoS one*, 6 (2011) e19540.
- [92] B. Pudelski, A. Schock, S. Hoth, R. Radchuk, H. Weber, J. Hofmann, U. Sonnewald, J. Soll, K. Philippar, The plastid outer envelope protein OEP16 affects metabolic fluxes during ABA-controlled seed development and germination, *J Exp Bot*, 63 (2012) 1919-1936.
- [93] J. Carroll, R.J. Shannon, I.M. Fearnley, J.E. Walker, J. Hirst, Definition of the nuclear encoded protein composition of bovine heart mitochondrial complex I. Identification of two new subunits, *J Biol Chem*, 277 (2002) 50311-50317.
- [94] J. Klodmann, H.P. Braun, Proteomic approach to characterize mitochondrial complex I from plants, *Phytochemistry*, 72 (2010) 1071-1080.
- [95] E.H. Meyer, N.L. Taylor, A.H. Millar, Resolving and identifying protein components of plant mitochondrial respiratory complexes using three dimensions of gel electrophoresis, *J Proteome Res*, 7 (2008) 786-794.
- [96] M.W. Murcha, D. Elhafez, A.H. Millar, J. Whelan, The C-terminal region of TIM17 links the outer and inner mitochondrial membranes in Arabidopsis and is essential for protein import, *J Biol Chem*, 280 (2005) 16476-16483.

- [97] M. Schmid, T.S. Davison, S.R. Henz, U.J. Pape, M. Demar, M. Vingron, B. Scholkopf, D. Weigel, J.U. Lohmann, A gene expression map of *Arabidopsis thaliana* development, *Nature genetics*, 37 (2005) 501-506.
- [98] O. Van Aken, B. Zhang, C. Carrie, V. Uggalla, E. Paynter, E. Giraud, J. Whelan, Defining the mitochondrial stress response in *Arabidopsis thaliana*, *Mol Plant*, 2 (2009) 1310-1324.
- [99] E. Giraud, O. Van Aken, V. Uggalla, J. Whelan, REDOX regulation of mitochondrial function in plants, *Plant Cell Environ*, 35 (2011) 271-280.
- [100] N.L. Taylor, C. Rudhe, J.M. Hulett, T. Lithgow, E. Glaser, D.A. Day, A.H. Millar, J. Whelan, Environmental stresses inhibit and stimulate different protein import pathways in plant mitochondria, *FEBS Lett*, 547 (2003) 125-130.
- [101] C. Sirrenberg, M.F. Bauer, B. Guiard, W. Neupert, M. Brunner, Import of carrier proteins into the mitochondrial inner membrane mediated by Tim22, *Nature*, 384 (1996) 582-585.
- [102] O. Kerscher, N.B. Sepuri, R.E. Jensen, Tim18p is a new component of the Tim54p-Tim22p translocon in the mitochondrial inner membrane, *Mol Biol Cell*, 11 (2000) 103-116.
- [103] O. Kerscher, J. Holder, M. Srinivasan, R.S. Leung, R.E. Jensen, The Tim54p-Tim22p complex mediates insertion of proteins into the mitochondrial inner membrane, *J Cell Biol*, 139 (1997) 1663-1675.
- [104] C. Sirrenberg, M. Endres, H. Folsch, R.A. Stuart, W. Neupert, M. Brunner, Carrier protein import into mitochondria mediated by the intermembrane proteins Tim10/Mrs11 and Tim12/Mrs5, *Nature*, 391 (1998) 912-915.
- [105] C. Carrie, M.W. Murcha, J. Whelan, An in silico analysis of the mitochondrial protein import apparatus of plants, *BMC Plant Biol*, 10 (2010) 249.
- [106] J. Klodmann, S. Sunderhaus, M. Nimtz, L. Jansch, H.P. Braun, Internal architecture of mitochondrial complex I from *Arabidopsis thaliana*, *Plant Cell*, 22 (2010) 797-810.
- [107] G.C. Pagnussat, H.J. Yu, Q.A. Ngo, S. Rajani, S. Mayalagu, C.S. Johnson, A. Capron, L.F. Xie, D. Ye, V. Sundaresan, Genetic and molecular identification of genes required for female gametophyte development and function in *Arabidopsis*, *Development*, 132 (2005) 603-614.
- [108] M. van der Laan, N. Wiedemann, D.U. Mick, B. Guiard, P. Rehling, N. Pfanner, A role for Tim21 in membrane-potential-dependent preprotein sorting in mitochondria, *Curr Biol*, 16 (2006) 2271-2276.
- [109] D. Mokranjac, D. Popov-Celeketi, K. Hell, W. Neupert, Role of Tim21 in mitochondrial translocation contact sites, *J Biol Chem*, 280 (2005) 23437-23440.
- [110] A. Chacinska, M. Lind, A.E. Frazier, J. Dudek, C. Meisinger, A. Geissler, A. Sickmann, H.E. Meyer, K.N. Truscott, B. Guiard, N. Pfanner, P. Rehling, Mitochondrial presequence translocase: switching between TOM tethering and motor recruitment involves Tim21 and Tim17, *Cell*, 120 (2005) 817-829.
- [111] H. Hamasaki, T. Yoshizumi, N. Takahashi, M. Higuchi, T. Kuromori, Y. Imura, H. Shimada, M. Matsui, SD3, an *Arabidopsis thaliana* homolog of TIM21, affects intracellular ATP levels and seedling development, *Mol Plant*, 5 (2012) 461-471.
- [112] S. Kumar, T. Yoshizumi, H. Hongo, A. Yoneda, H. Hara, H. Hamasaki, N. Takahashi, N. Nagata, H. Shimada, M. Matsui, *Arabidopsis* mitochondrial protein TIM50 affects hypocotyl cell elongation through intracellular ATP level, *Plant science : an international journal of experimental plant biology*, 183 (2012) 212-217.
- [113] D. Mokranjac, S.A. Paschen, C. Kozany, H. Prokisch, S.C. Hoppins, F.E. Nargang, W. Neupert, K. Hell, Tim50, a novel component of the TIM23 preprotein translocase of mitochondria, *EMBO J*, 22 (2003) 816-825.
- [114] S. Sugiyama, S. Moritoh, Y. Furukawa, T. Mizuno, Y.M. Lim, L. Tsuda, Y. Nishida, Involvement of the mitochondrial protein translocator component tim50 in growth, cell proliferation and the modulation of respiration in *Drosophila*, *Genetics*, 176 (2007) 927-936.
- [115] H. Yamamoto, M. Esaki, T. Kanamori, Y. Tamura, S. Nishikawa, T. Endo, Tim50 is a subunit of the TIM23 complex that links protein translocation across the outer and inner mitochondrial membranes, *Cell*, 111 (2002) 519-528.

- [116] M. Kwasniak, L. Pogorzelec, I. Migdal, E. Smakowska, H. Janska, Proteolytic system of plant mitochondria, *Physiol Plant*, 145 (2012) 187-195.
- [117] A.C. Eriksson, S. Sjoling, E. Glaser, Characterization of the bifunctional mitochondrial processing peptidase (MPP)/bc1 complex in *Spinacia oleracea*, *J Bioenerg Biomembr*, 28 (1996) 285-292.
- [118] H.P. Braun, M. Emmermann, V. Kruft, U.K. Schmitz, The general mitochondrial processing peptidase from potato is an integral part of cytochrome c reductase of the respiratory chain, *Embo J*, 11 (1992) 3219-3227.
- [119] H.P. Braun, M. Emmermann, V. Kruft, M. Bodicker, U.K. Schmitz, The general mitochondrial processing peptidase from wheat is integrated into the cytochrome bc1-complex of the respiratory chain, *Planta*, 195 (1995) 396-402.
- [120] P. Moberg, A. Stahl, S. Bhushan, S.J. Wright, A. Eriksson, B.D. Bruce, E. Glaser, Characterization of a novel zinc metalloprotease involved in degrading targeting peptides in mitochondria and chloroplasts, *Plant J*, 36 (2003) 616-628.
- [121] A. Stahl, P. Moberg, J. Ytterberg, O. Panfilov, H. Brockenhuus Von Lowenhielm, F. Nilsson, E. Glaser, Isolation and identification of a novel mitochondrial metalloprotease (PreP) that degrades targeting presequences in plants, *J Biol Chem*, 277 (2002) 41931-41939.
- [122] N. Alikhani, A.K. Berglund, T. Engmann, E. Spanning, F.N. Vogtle, P. Pavlov, C. Meisinger, T. Langer, E. Glaser, Targeting capacity and conservation of PreP homologues localization in mitochondria of different species, *J Mol Biol*, 410 (2011) 400-410.
- [123] E. Glaser, S. Nilsson, S. Bhushan, Two novel mitochondrial and chloroplastic targeting-peptide-degrading peptidasomes in *A. thaliana*, *AtPreP1* and *AtPreP2*, *Biol Chem*, 387 (2006) 1441-1447.
- [124] S. Nilsson Cederholm, H.G. Backman, P. Pesaresi, D. Leister, E. Glaser, Deletion of an organellar peptidasome PreP affects early development in *Arabidopsis thaliana*, *Plant Mol Biol*, 71 (2009) 497-508.
- [125] R.A. Stuart, Supercomplex organization of the oxidative phosphorylation enzymes in yeast mitochondria, *J Bioenerg Biomembr*, 40 (2008) 411-417.
- [126] W.Y. Murcha MW, Whelan J., A molecular link between the mitochondrial preprotein transporters and respiratory chain complexes, *Plant Signal Behav*, 7 (2012) 1594-1597.
- [127] M. Gebert, S.G. Schrempp, C.S. Mehnert, A.K. Heisswolf, S. Oeljeklaus, R. Ieva, M. Bohnert, K. von der Malsburg, S. Wiese, T. Kleinschroth, C. Hunte, H.E. Meyer, I. Haferkamp, B. Guiard, B. Warscheid, N. Pfanner, M. van der Laan, Mgr2 promotes coupling of the mitochondrial presequence translocase to partner complexes, *J Cell Biol*, 197 (2012) 595-604.

Table 1. Summary of T-DNA insertional knock-out lines for mitochondrial membrane import components in Arabidopsis. T-DNA insertional knock-out lines were screened for homozygosity and examined for growth defects. Where multiple isoforms exist for each component double knock-out lines were created to determine lethality of multiple gene inactivation. Abbreviations: Tom = Translocases of the Outer Membrane, Tim = Translocase of the Inner Membrane, Ndufa11 = NADH dehydrogenase [ubiquinone] 1 subunit 11 (Human), B14.7 = NADH dehydrogenase [ubiquinone] 1 subunit 14.7 (Bovine), HM = Homozygous, Het = Heterozygous, bp = base pair, Ref = Reference, At = Arabidopsis thaliana.

Name	Vital in yeast	Gene name (AGI)	Line details	Insert location	Genotype	Altered Phenotype	Details	Ref
Tom20	Yes	AtTom20-2	SALK_067986	+1255 bp intron	HM	-	Non-essential gene	[24]
		(At1g27390)	SALK_134973	+126 bp intron	HM	-		[24]
		AtTom20-3	GK-554C03	+971 bp intron	HM	-	Non-essential gene	[24]
		(At3g27080)	SAIL_88_A08	+733 bp exon	HM	-		[24]
Tom20-4	Yes	AtTom20-4	SALK_147093	+579 bp exon	HM	-	Non-essential gene	[24]
		(At5g40930)	SALK_004057	+422 bp intron	HM	-		[24]
		AtTom40-1	SALK_128170	+1796 bp exon	Het	Defective embryo	essential gene	unpublished data
		(At3g20000)	AtTom40-2	SALK_004057	+422 bp intron	HM	Non-essential gene	unpublished data
OM64	NA	OM64	SALK_068772		HM	-	Non-essential gene	[24]
		(At5g09420)	SALK_089921		HM	-		[24]
Sam37/Metaxin	No	Sam37/Metaxin	SALK_107629		HM	Delayed growth	Non-essential gene	[24]
		(At2g19080)	SALK_039892		HM			[24]
Tim23	Yes	AtTim23-1	SALK_030470	+699 bp 3'utr	HM	-	Essential gene	Attim23-2: Attim23-3 not viable
		(At1g17530)	SALK_107963	+656 bp 3'utr	HM	-		
		AtTim23-2	SALK_143656	-135 bp 5'utr	HM	Delayed growth	Over-expressor	[13]
		(At1g72750)	GK-689C11	+43 bp exon	HM		Knock-out	[13]
Tim17	Yes	AtTim23-3	SAIL_1151_B01	+548 bp exon	HM	-	Essential gene	Attim23-1: Attim23-2 not viable
		(At3g04800)	SALK_129386	+415 bp exon	HM	-		
		AtTim17-1	SALK_091528	-400 bp 5'utr	HM	Faster germination	Non-essential gene	unpublished data
		(At1g20350)	SALK_092885	+360 bp exon	HM	Faster germination		
Tim17	Yes	AtTim17-2	GK-561E03	+160 bp exon	Het	Defective embryo	Essential gene	
		(At2g37410)						
		AtTim17-3	SALK_048425	+543 bp 3'utr	HM	-	Non-essential gene	
		(At5g11690)	SALK_125567	+471 bp 3'utr	HM	-		
Tim22	Yes	AtTim22-1	GK-848H04	+71 bp exon	HM	-	Non-essential gene	not expressed
		(At1g18320)						
		AtTim22-2	SAIL_623_F03	+60 bp exon	HM	Sterile plant	Essential gene	
		(At3g10110)						
Tim50	Yes	AtTim50	SALK_000523	+2211 bp intron	HM	Delayed growth	Essential gene	[112]
		(At1g55900)	SALK_059376	+1177 bp intron	HM	-		
Tim44	Yes	AtTim44-1	SALK_015319	+34 bp exon	HM	-	Essential gene	Attim44-1: Attim44-2 not viable
		(At2g20510)						
Tim44	Yes	AtTim44-2	SALK_146901	-227 bp 5'utr	HM	-	Essential gene	Attim44-1: Attim44-2 not viable
		(At2g36070)						
Tim21	No	AtTim21	sd3-2	intron	HM	seedling lethal	Essential gene	[111]
(At4g00026)								
NdufsA11/B14.7	NA	AtB14.7	SAIL_434_E06	+619 bp intron	Het	Defective pollen	Essential gene	
(At2g42210)

Fig. 1. Diagrammatic representation of protein import pathways and components in plant mitochondria. Targeting signals on cytosolically located precursor proteins determine the pathway of import. All precursors (containing a cleavable presequence, Twin Cx/C, β -barrel or carrier type proteins) pass through the Translocase of the Outer Membrane (TOM). The pathways diverge in the InterMembrane Space (IMS). Insertion of β -barrel proteins into the outer membrane is carried out via the Sorting and Assembly Machinery (SAM). Carrier import pathway proteins translocate into the inner membrane via small soluble Tim proteins through the TIM22 channel located in the inner membrane. Twin Cx/C proteins located within the IMS, are imported via the MIA-Erv1 pathway. For the majority of mitochondrial proteins that contain a cleavable presequence, translocation is carried out through the TIM17:23 complex with the Presequence Assisted Motor (PAM) complex or inserted directly into the inner membrane. The TIM17:23 complex can exist as 2 forms, PAM and SORT. In addition Tim23 interacts with the Complex I subunit B14.7. Following translocation, the presequence is removed via Mitochondrial Processing Peptidase (MPP), integrated into the cytochrome bc1 complex. The presequence is further processed via a matrix located Presequence Peptidase (PrepP). The essential subunits required for seed viability are circled. A membrane potential is required for translocation across the inner membrane ($\Delta\Psi$). Abbreviations: TOM = Translocase of the Outer Membrane, TIM = Translocase of the Inner membrane, IMS = Intermembrane Space, SAM = Sorting and Assembly Machinery, PrepP = Presequence Peptidase, MPP = Mitochondrial Processing Peptidase. The subunits known to be essential for plant viability are bolded. SAM50 is bolded in grey as it is not yet known if it is essential.

germplasm		% defective seeds
Col-0		1.3 ± 0.02
<i>Attim17-2</i> (+/-)		26.5 ± 0.08
<i>Attim44-1</i> (-/-) :: <i>Attim44-2</i> (+/-)		34.5 ± 0.08
<i>Attim23-1</i> (-/-) :: <i>Attim23-2</i> (+/-)		24.8 ± 0.08
<i>Attim23-2</i> (-/-) :: <i>Attim23-3</i> (+/-)		23.9 ± 0.07
<i>Attim23-1</i> (-/-) :: <i>Attim23-3</i> (+/-)		29.3 ± 0.08

Fig. 2. Germplasms resulting in defective embryo development. Seed sets for *Attim17-2* (+/-) heterozygous knock-out and double knock-out lines, *Attim44-1* (-/-)::*Attim44-2* (+/-), *Attim23-1* (-/-)::*Attim23-2* (+/-), *Attim23-1* (-/-)::*Attim23-3* (+/-) and *Attim23-2* (-/-)::*Attim23-3* (+/-) exhibit seed lethal phenotypes. The germplasms were screened to confirm genotype and siliques dissected to confirm the lethality of gene inactivation. Values indicate the average percentage of aborted progeny from three biological replicates ± S.D (n = 60). Scale bar, 0.1 cm. Arrow indicates defective embryo. Abbreviations: (+/-) = heterozygous knock-out, (-/-) = homozygous knock-out, Col-0 = wild-type.

Fig. 3. Deletion of *AtTim22-2* results in plant sterility. The T-DNA insertional knock-out line for *AtTim22-2* (At3g10110) (SAIL_623_F03) was screened for homozygosity. *Attim22-2* homozygous plants exhibit smaller flowers and poorly developed siliques with little to no seed. Scale bar, 0.1 cm, (-/-) = homozygous knock-out.

Fig. 4. Identification of *Attim50* mutants. The T-DNA insertional knock-out lines for *AtTim50* (At1g55900) were screened for homozygosity and immunodetected with *AtTim50* antibody to confirm loss of function. (A) The line, SALK_000523 (*Attim50* (-/-) KO1) with the T-DNA insert at position +2211 in the last intron, displays an aberrant growth phenotype compared to wild-type (Col-0). Immunodetection of mitochondria isolated from 2 week old seedlings indicate that the insertion results in a truncated *AtTim50* of apparent molecular weight of 42 kDa. (B) The *Attim50* (-/-) KO2 line, SALK_059376, previously identified to be a *Attim50* knock-out [59] was rescreened for homozygosity. Homozygous lines display no apparent growth aberrations and immunodetection against *AtTim50* suggests that the T-DNA insertion results in a knock-down, not a knock-out. The mitochondrial control (Porin) shows equal protein loading. Abbreviations: RP = Right border screening primer, LP = Left border screening primer, BP = T-DNA border screening primer, kDa = kilodaltons (-/-) = homozygous knock-out.

Fig. 5. Identification of a *Atb14.7* heterozygous mutant. (A) screening of the T-DNA insertional knock-out line for *AtB14.7* (SAIL_434_E06) failed to identify any homozygous lines. Dissection of *Atb14.7 (+/-)* heterozygous plants show all seed sets to be viable and subsequent genotyping only identified Col-0 or heterozygous germplasm. Values indicate the average percentage of aborted progeny per silique $\pm$ S.D (n = 60) from three biological replicates. Scale bar, 0.1 cm. (B) The number of pollen grains and percentage of defective pollen from *Atb14.7 (+/-)* heterozygous plants as determined by Alexander staining. Arrows indicate defective pollen grains. * indicates statistically significant difference compared with wild-type (Col-0) ($p < 0.01$). Scale bars, 20 μ m. Error bars indicate S.E, (+/-) = heterozygous knock-out.

Graphical Abstract

Highlights

- Up to two billion years of evolution separate mitochondria from various groups
- Small gene families encode mitochondrial protein import components in plants
- Components of the import apparatus play role(s) in signalling and development