Review

Sylvie Callegari, Luis Daniel Cruz-Zaragoza and Peter Rehling*

From TOM to the TIM23 complex – handing over of a precursor

<https://doi.org/10.1515/hsz-2020-0101>

Received January 3, 2020; accepted February 13, 2020

Abstract: Mitochondrial precursor proteins with amino-terminal presequences are imported via the presequence pathway, utilizing the TIM23 complex for inner membrane translocation. Initially, the precursors pass the outer membrane through the TOM complex and are handed over to the TIM23 complex where they are sorted into the inner membrane or translocated into the matrix. This handover process depends on the receptor proteins at the inner membrane, Tim50 and Tim23, which are critical for efficient import. In this review, we summarize key findings that shaped the current concepts of protein translocation along the presequence import pathway, with a particular focus on the precursor handover process from TOM to the TIM23 complex. In addition, we discuss functions of the human TIM23 pathway and the recently uncovered pathogenic mutations in TIM50.

Keywords: membrane translocation; mitochondria; presequence pathway; protein translocation; TIM23 complex; TIM50.

Introduction

Mitochondria are integral to cell function, primarily as the site of ATP production, but they also play key roles in metabolic and signaling processes. Over 1000 nuclear-encoded proteins are required for mitochondria to fulfill their functions, all of which are translated on cytosolic ribosomes as mitochondrial precursor proteins and

imported into their appropriate mitochondrial subcompartment (Morgenstern et al., 2017; Vogtle et al., 2017). The import process is highly selective and requires specialized machinery that can recognize and sort the different categories of mitochondrial precursor proteins. Approximately two thirds of mitochondrial precursor proteins have an N-terminal, positively charged, presequence that is approximately 15–50 amino acids long (Chacinska et al., 2009). The presequence targets precursor proteins to the translocase of the outer membrane (TOM complex), which is the main entry gate across the outer mitochondrial membrane. From there, presequence-containing precursors are passed onto the translocase of the inner membrane 23 (TIM23 complex) and the presequence is cleaved by the mitochondrial presequence peptidase. Precursors are then directed into either the inner membrane or the matrix. This pathway is known as the presequence pathway, or more commonly, the TIM23 pathway.

The TIM23 complex is the designated inner membrane translocase for translocation of presequence-containing precursors. It cooperates with the TOM complex to coordinate precursor passage across the two mitochondrial membranes. The dynamic nature of the complex enables it to switch composition to enable a precursor to be released laterally into the inner membrane, or driven into the matrix (Chacinska et al., 2005). Unraveling the mechanisms of the TIM23 pathway has spanned several decades, which is testament to the complexity and intricacies of the pathway. In this review, we outline the structural components of the presequence pathway from a chronological perspective and describe the latest model for precursor translocation, with a special focus on precursor handover between the TOM complex and TIM23 complex.

Most of the seminal work on this pathway has been performed using yeast. However, as more information is starting to emerge about the human presequence pathway, it is becoming clear that there are functional differences. An overview of the human TIM23 pathway will be given, with respect to some of the newly acquired functions of translocase components. Mutations in components of the TIM23 machinery have been linked to mitochondrial disease (Kang et al., 2018; Pfanner et al., 2019). Several of

*Corresponding author: Peter Rehling, Department of Cellular Biochemistry, University Medical Center Göttingen, D-37073 Göttingen, Germany; and Max Planck Institute for Biophysical Chemistry, D-37077 Göttingen, Germany, e-mail: peter.rehling@medizin.uni-goettingen.de. <https://orcid.org/0000-0001-5661-5272>
Sylvie Callegari and Luis Daniel Cruz-Zaragoza: Department of Cellular Biochemistry, University Medical Center Göttingen, D-37073 Göttingen, Germany

these have been within the intermembrane space (IMS)-filling component of the TIM23 complex, TIM50, which is critical for precursor handover (Shahrour et al., 2017; Reyes et al., 2018; Tort et al., 2019). The pathogenic effects of these mutations underlie the importance of efficient precursor handover for human health.

A short history of mitochondrial presequence translocase components

The earliest accounts of mitochondria at the beginning of the 20th century, described them as intracellular structures that contain proteins and lipids (Regaud, 1908). Several decades later, once mitochondrial isolation methods had been established, it became apparent that mitochondria had the ability to synthesize their own proteins (McLean et al., 1958), a phenomenon that was explained shortly thereafter by the discovery of mitochondrial DNA (Nass and Nass, 1963; Luck and Reich, 1964; Schatz et al., 1964) and mitochondrial ribosomes (Kuntzel and Noll, 1967). However, the complexity of mitochondrial biogenesis was not fully realized until the late 1960s by a series of experiments that used radiolabeling of isolated rat mitochondria to track the fate of mitochondrial translated products. After radiolabeling, mitochondrial membranes were fractionated and remarkably, labeled translation products could only be observed in the inner mitochondrial membrane (Beattie et al., 1967; Neupert et al., 1967; Werner and Neupert, 1972). This implied that the remainder of the mitochondrial proteome originates from nuclear genes, is translated on cytosolic ribosomes and is then imported into mitochondria. These paradigm-shifting findings gave birth to the field of mitochondrial protein import (Figure 1).

The establishment of an *in vitro* transport system that enabled the monitoring of mitochondrial protein uptake, for example, of *in vitro* translated proteins, provided a means to assess the mechanism of protein translocation (Hallermayer et al., 1977; Harmey et al., 1977; Maccacchini et al., 1979a,b; Nelson and Schatz, 1979; Gasser et al., 1982). Early import experiments led to the identification of cytosolic precursors for mitochondrial proteins that are imported into mitochondria in a kinetically resolvable manner. Remarkably some of these precursors underwent proteolytic processing. An amphiphatic N-terminal cleavable signal was later described to be the mitochondrial targeting signal which directs proteins of the presequence pathway to specific receptors on the outer mitochondrial

Figure 1: The presequence pathway – a timeline of discovery.

membrane (Riezman et al., 1983; Hurt et al., 1984; Roise et al., 1988).

The translocase of the outer membrane

The discovery, that mitochondria selectively take up proteins from the cytosol, initiated a long quest to identify the pathways and machinery that enable protein import. The first component identified was the pore-forming protein in the outer mitochondrial membrane, Tom40 (Vestweber et al., 1989; Baker et al., 1990; Kiebler et al., 1990; Hill et al., 1998). This was closely followed by the purification of a receptor complex from the outer mitochondrial membrane, which resulted in the identification of the presequence receptor subunit, Tom20 (Sollner et al., 1989; Moczko et al., 1992) and a receptor specializing in the recognition of precursors with internal targeting signals, Tom70 (Hines et al., 1990; Sollner et al., 1990; Steger et al., 1990). Another receptor component, Tom22, was later characterized and found to mediate the transfer of precursors from Tom20 and Tom70 to the Tom40 import pore (Lithgow et al., 1994). In addition to the import pore and the main receptors, the TOM complex is made up of

several small molecular weight constituents, Tom5, Tom6 and Tom7 (Kassenbrock et al., 1993; Alconada et al., 1995; Honlinger et al., 1996; Dietmeier et al., 1997). These aid in the assembly and stability of the TOM complex. The TOM complex is broadly regarded as the general entry site for mitochondrial proteins and a detailed review on this translocase is included in this issue (Bausewein et al., 2020).

The presequence translocase

These early studies indicated that different pathways exist for presequence-containing precursors and precursors with internal targeting signals (Zimmerman et al., 1979; Riezman et al., 1983). The subset of mitochondrial proteins that contain presequences are directed to the mitochondrial matrix or the inner membrane – but how do they get there? It was believed that, like their passage through the outer membrane, this process is facilitated by a proteinaceous import channel within the inner membrane. To decipher this translocation machinery, researchers exploited the power of yeast genetics. In one approach, a screen was devised to obtain mutants of *Saccharomyces cerevisiae* that were unable to import a mitochondrial targeted reporter enzyme (Maarse et al., 1992). Concomitantly, in an alternative approach, another group of researchers analyzed temperature sensitive mutants of *S. cerevisiae* that accumulate cytosolic precursors at the non-permissive temperature of 37°C (Emtage and Jensen, 1993). Analysis of these mutants uncovered the first translocase components of the inner mitochondrial membrane, Tim44, Tim23 and Tim17. All of these components were found to be not only required for import, but also essential for cell viability (Maarse et al., 1992, 1994; Scherer et al., 1992; Blom et al., 1993; Dekker et al., 1993; Emtage and Jensen, 1993; Ryan et al., 1994; Martinez-Caballero et al., 2007).

The Tim23 and Tim17 proteins display sequence similarity, although they cannot functionally compensate for each other (Maarse et al., 1994; Ryan et al., 1994). Both proteins have four transmembrane spans and possess a topology in which the N- and C-termini face the IMS. Although Tim23 alone has channel activity, it is natively found in complex with Tim17 (Dekker et al., 1997; Ryan et al., 1998; Truscott et al., 2001) and requires Tim17 to maintain pore structure (Meier et al., 2005; Martinez-Caballero et al., 2007). Therefore, it is believed that, together, these two subunits make up the channel unit of the translocase. A unique feature of Tim23 is its hydrophilic N-terminal segment, which was later shown to bind incoming presequences (Bauer et al., 1996; Truscott et al., 2001; de la Cruz et al., 2010).

Tim44 is peripherally associated with the channel at the inner mitochondrial membrane and has a large matrix facing domain. The primary function of Tim44 is to act as a scaffold for the binding of the chaperone mtHsp70 (Kronidou et al., 1994; Schneider et al., 1994). mtHsp70 is located in the mitochondrial matrix and had already been identified several years earlier in an analysis of temperature-sensitive import-deficient yeast mutants. At that time it was shown to have a role in the import and folding of the mature precursor within the matrix (Kang et al., 1990). Some years later, the ATP-activated mtHsp70 was discovered to provide the driving force that moves precursors into the matrix (Ungermann et al., 1994; Berthold et al., 1995).

Despite the identification of translocase constituents, it remained unclear how the TIM23 complex coordinates with the TOM complex to receive cargo, or how it is capable of sorting precursors into their diverse compartments – the inner membrane or the matrix. In 2002, three separate groups identified a sixth core component of the TIM23 machinery (Geissler et al., 2002; Yamamoto et al., 2002; Mokranjac et al., 2003a,b). This 50 kDa protein with a large IMS spanning domain was termed Tim50. Like the Tim23 IMS domain, Tim50 is also capable of binding precursors, but with a greater affinity (de la Cruz et al., 2010; Marom et al., 2011). The Tim50 IMS region is composed of two domains; a C-terminal presequence binding domain (PBD) and a core domain. Both domains are capable of interacting with presequences, but the core domain can additionally associate with Tim23, Tim21 and the PBD region of Tim50 itself (Schulz et al., 2011; Qian et al., 2011; Lytovchenko et al., 2013; Rahman et al., 2014). Therefore, Tim50 represents the main inner membrane receptor for matrix-targeted precursors, guiding them from the TOM complex to the channel insertion site (Geissler et al., 2002; Yamamoto et al., 2002; Mokranjac et al., 2003a,b).

Subsequently, two DnaJ-homologs of 14 and 16 kDa were identified (D'Silva et al., 2003; Mokranjac et al., 2003a,b; Truscott et al., 2003; Kozany et al., 2004). These proteins can both dynamically associate with mtHsp70 and Tim44, and together, they make up the presequence translocase-associated motor (PAM). The 14 and 16 kDa proteins were therefore termed Pam18 (Tim14) and Pam16 (Tim16), respectively. While the membrane anchored Pam18 stimulates the ATPase activity of mtHsp70 at the channel exit, the membrane associated Pam16 inhibits the stimulatory effect of Pam18 when in complex together. In this way, by working in concert, Pam18 and Pam16 control the activity of the import motor. Another new translocase component, termed Pam17, was found some years later (van der Laan et al., 2005). Pam17 is anchored to the membrane and is recruited to the translocase by Tim50 where

it has a specific role in promoting the translocation of precursors that are hypersensitive to membrane potential, possibly by regulating the gating activity of Tim23 (Schendzielorz et al., 2017).

Precursors that are destined for the inner membrane contain a hydrophobic stop transfer (sorting) signal adjacent to the presequence and are imported independently of the mtHsp70 motor in a process driven solely by the membrane potential (Gartner et al., 1995; Gruhler et al., 1997). However, the mechanics by which the translocase discriminates between matrix transport and membrane insertion remained unaddressed. The identification of the inner membrane protein, Tim21 (Chacinska et al., 2005; Mokranjac et al., 2005) led to the discovery of a state of the TIM23 complex that lacks the import motor and is dedicated to lateral release. The purified complex is capable of inserting precursors with sorting signal into the lipid phase of a membrane in a reconstituted system (van der Laan et al., 2007). Accordingly, the TIM23 complex exists in two different modular states; the TIM23^{SORT}, which contains Tim21 but lacks the PAM complex, and the TIM23^{MOTOR}, in which the motor is associated with the translocase but Tim21 is absent (Chacinska et al., 2005). Interestingly, Tim21 also interacts with the IMS domain of Tom22, which has been suggested to act as a *trans* binding site for presequences at the TOM complex. Binding of Tim21 to Tom22 is likely to facilitate the release of precursors from the TOM complex and direct them to Tim50 (Mokranjac et al., 2005; Albrecht et al., 2006).

The latest component of the TIM23 complex to be discovered was Mgr2, a small hydrophobic protein with two transmembrane spans (Gebert et al., 2012). Mgr2 was suggested to support binding of Tim21 to the TIM23^{SORT} complex and regulates the lateral release of precursors into the inner membrane (Ieva et al., 2014). As Mgr2 can be crosslinked to precursors in transit, it suggests that it may be a component of the channel (Gebert et al., 2012).

The discovery of the components of the presequence machinery spanned over two decades and had provided an architectural map of a complex but fascinating machinery. In the research that follows, many mechanistic aspects of the presequence translocase continue to be dissected. Below we outline the mechanisms of precursor translocation by TIM23 upon handover from the TOM complex.

From TOM to TIM23: the precursor handover

The transport of proteins into mitochondria and across the inner membrane requires the transfer of precursors

from the TOM complex to the inner membrane translocation machineries. Upon their exit from the Tom40 pore, pre-sequence containing precursors are passed on to the TIM23 complex in a process that relies on an intricate relay of interactions between the TOM complex and constituents of the TIM23 complex. The precursor exit site on the IMS side of the TOM complex is a composite site, made up of the IMS domain of Tom22, Tom7 and Tom40, all of which are able to bind presequences (Bolliger et al., 1995; Moczko et al., 1997; Rapaport et al., 1997; Komiyama et al., 1998; Kanamori et al., 1999; Araiso et al., 2019; Tucker and Park, 2019). Once positioned at this exit site, the precursor is then transferred to Tim50, the primary receptor of the TIM23 complex (Figure 2).

To facilitate the handover process, the two translocases draw into close proximity, thereby forming a TOM-TIM23 supercomplex (Dekker et al., 1997; Chacinska et al., 2010; Gold et al., 2014). The formation of the TOM-TIM23 supercomplex is triggered by the precursor protein. In fact, the supercomplex can only be stabilized for detection using *in-vitro* mitochondrial import of an artificial precursor with a folded C-terminal domain that cannot pass the Tom40 pore, thereby trapping both complexes together (Rassow et al., 1990; Dekker et al., 1997).

The generation and dynamics of the TOM-TIM23 supercomplex are critical to our understanding of precursor transfer between the translocases. The C-terminal IMS domain of Tom22 is critical for the stabilization of the TOM-TIM23 supercomplex. Ablation of the IMS domain of Tom22 dramatically affected supercomplex stability, resulting in a reduction in presequence import efficiency (Chacinska et al., 2010). As the main presequence receptor, Tim50 also supports supercomplex formation, but once formed, Tim50 is dispensable for maintaining the stability of the supercomplex (Chacinska et al., 2010). Crosslinking studies have shown that the IMS domain of Tim50 can form a direct link with the IMS domain of Tom22, which not only facilitates the formation of the supercomplex, but also positions Tim50 in a primed state to receive the precursor from the Tom22 IMS (Shiota et al., 2011). In the presence of precursor protein, the crosslinks between Tom22 and Tim50 are diminished, suggesting that the precursor competes for the binding site on the Tim50 IMS, which then causes the two subunits to dissociate (Shiota et al., 2011). Another contact between the TOM and TIM23 translocases occurs through the interaction between Tim21 and the IMS domain of Tom22 (Mokranjac et al., 2005; Albrecht et al., 2006; van der Laan et al., 2006). However, the role of Tim21 in supercomplex formation is less clear. The interaction between the Tom22 IMS and Tim21 is transient and once the precursor has been

Figure 2: Mechanism of precursor handover and translocation via TIM23.

(A) After translocation through the TOM complex, the presequence-containing precursor is positioned at the TOM complex exit site. The TOM and TIM23 complexes associate via interactions between the Tom22^{IMS} and Tim21, Tim23^{IMS} and Tim50. The presequence is then recognized and bound by Tim50^{PBD}. (B) After the handover process, Tim50 then shifts the precursor protein from the Tim50^{PBD} to the Tim50^{CORE}. From the Tim50^{CORE}, the precursor is passed onto the Tim23^{IMS}, from where it has access to the channel. The channel is activated by Tim50 and the precursor is then driven through the channel, along the electrochemical gradient ($\Delta\psi$) across the inner membrane and assisted by cation-selective residues lining the pore of the channel. (C) The TIM23 complex exists in two states. TIM23^{MOTOR} is the motor associated form for matrix-destined precursor proteins. ATP-powered mtHsp70 associates with the translocase and, upon hydrolysis of ATP by Pam18 (18), binds to the emerging precursor and dissociates from the translocase. Subsequent mtHsp70 molecules bind to the precursor in a similar manner to draw it further into the matrix in a ratchet-like motion. TIM23^{SORT} is specialized for the lateral release of inner membrane bound precursor proteins that have a hydrophobic “stop-transfer” signal after the presequence. TIM23^{SORT} is defined by the presence of Tim21 and the lateral gatekeeper Mgr2. Both are released from the complex, allowing the “stop-transfer” signal lateral migration into the inner membrane.

transferred to the TIM23 complex, Tim21 dissociates from the translocase (Lytovchenko et al., 2013).

At the inner membrane, Tim50 is set to receive the incoming precursor protein from the TOM complex. The IMS domain of Tim50 has two presequence binding regions; one within its C-terminal presequence binding domain, Tim50^{PBD}, and a second region, which has an even higher presequence affinity, within its core domain, Tim50^{CORE} (Schulz et al., 2011; Qian et al., 2011; Lytovchenko et al., 2013; Rahman et al., 2014). Notably, the binding region within Tim50^{CORE} is also capable of binding the Tim50^{PBD}, suggesting that upon receiving the presequence at the PBD domain, Tim50 can then transfer it to its higher affinity core domain (Rahman et al., 2014). Crystallization of the core domain of Tim50 enabled visualization of the large presequence binding groove and identified a nearby extended beta loop, which not only assists in the recognition and binding of presequences, but is also required for interaction with the IMS domain of Tim23 (Geissler et al., 2002; Yamamoto et al., 2002; Tamura et al., 2009; Qian et al., 2011; Li and Sha, 2015; Dayan et al., 2019). As the IMS region of Tim23 is also capable of binding presequences, this arrangement would conveniently position Tim23^{IMS} in a primed position to receive the incoming precursor from Tim50^{CORE}.

While Tim50 remains bound to Tim23, the gating activity of the channel remains closed, thereby preventing ion leakage across the inner membrane (Meinecke et al., 2006; Alder et al., 2008). At this point, Tim23 exists as a dimer in a state that is dependent on the electrochemical proton gradient across the inner membrane (Bauer et al., 1996). However, once the precursor has been transferred to the Tim23^{IMS}, Tim50 separates from the translocase, the Tim23 dimer dissociates and the channel is activated (Bauer et al., 1996; Meinecke et al., 2006). Upon opening of the channel, the precursor protein, with its positively charged presequence is drawn through the channel by the membrane potential (Martin et al., 1991). Furthermore, the channel is lined with cation selective amino acids that recognize the positively charged presequence and act to further drive it through the channel (Denkert et al., 2017). Once inside the channel, the precursor can be laterally released or translocated further into the matrix.

Full matrix translocation of a precursor requires additional driving forces. In this case, the emerging precursor binds to Tim44 and is then transported to the matrix, by the ATP-powered presequence translocase-associated motor (PAM) (Marom et al., 2011). The mtHsp70 protein is the central component of the PAM, which is recruited to the translocase by Tim44 and regulated by the Pam18/Pam16 complex (Kronidou et al., 1994; Schneider et al.,

1994; Hutu et al., 2008). The function of the import motor in precursor translocation is reviewed in detail elsewhere in this issue (Mokranjac, 2020). The association of Pam18 to the TIM23 complex favors matrix transport of precursors by stimulating mtHsp70. Additionally, Pam18 spans the inner membrane with a single transmembrane span that, when associated with TIM23, physically obstructs the lateral gate (D'Silva et al., 2003; Mokranjac et al., 2003a,b; Truscott et al., 2003). Therefore, in the case of precursors with a stop transfer (sorting) signal, which are destined for the inner membrane, Pam18 must dissociate from the translocase to enable precursor access to the lateral gate (Schendzielorz et al., 2018).

In contrast to matrix-translocated precursors, laterally released proteins do not require any additional forces other than membrane potential. The hydrophobic sorting signal, located downstream of the presequence, stalls the precursor in the channel. The release of the precursors into the lipid bilayer is thought to occur through a lateral gate of the translocase. Mgr2 has been proposed to act as a gatekeeper, partitioning proteins depending on the hydrophobicity of their sorting signals (Ieva et al., 2014; Lee et al., 2019). Mgr2 or Pam18 are mutually exclusively positioned at the lateral gate (Schendzielorz et al., 2018). Tim21 has been shown to be exclusively present in the translocase in the absence of the import motor. However, the role of Tim21 in lateral sorting is still unclear. Interestingly, Tim21 interacts with proton pumping respiratory chain complexes and may therefore position the translocase in regions of higher membrane potential to facilitate sorting (van der Laan et al., 2006).

Conservation of the presequence pathway in mammals and its role in disease

The TIM23 complex is essential for life and as such, the yeast complex remains largely conserved in mammals. In human, the five membrane integrated subunits of the translocase are; the receptor TIM50, the channel forming units TIM23 and TIM17A/B, as well as the TIM23^{SORT} components TIM21 and ROMO1 (human homolog of yeast Mgr2) (Bauer et al., 1999; Guo et al., 2004; Mick et al., 2012; Sinha et al., 2014; Wrobel et al., 2016; Richter et al., 2019). Additionally, the majority of motor subunits also have human counterparts. These include; TIM44, DNAJC15 and DNAJC19 (both homologs of Pam18), MAGMAS (PAM16), Mortalin (mtHSP70) and GrpE (human homolog of yeast Mge1) (Bhattacharyya et al., 1995; Bauer et al., 1999;

Borges et al., 2003; Sinha et al., 2010) (Figure 3). The only yeast TIM23 constituent without a human homolog is Pam17. The lack of Pam17 in mammals possibly reflects a greater resilience to fluctuations in bioenergetic availability, whereby Pam17 is no longer required to support translocation of membrane potential-hypersensitive precursors (Schendzielorz et al., 2017).

Although these components are all conserved, most have undergone at least some degree of functional divergence and this is strongly evidenced by differences in the interaction networks of presequence pathway components between yeast and human (Pfanner et al., 2019). One well-described example is TIM21, which in mammals, has acquired the additional function where it shuttles newly imported components of respiratory complexes I

and IV from the TIM23 complex to early assembly intermediates, known as mitochondrial translation regulation assembly intermediate of cytochrome *c* oxidase, MITRAC (Mick et al., 2012; Richter-Dennerlein et al., 2016). In doing so, TIM21 coordinates the import of nuclear encoded respiratory complex subunits with the synthesis of mitochondrial encoded subunits. ROMO1 is important for the association of TIM21 with the translocase and consequently also affects the biogenesis of respiratory chain complexes (Richter et al., 2019). ROMO1 has also functionally diverged from its yeast counterpart Mgr2, as it is not essential for the lateral sorting of precursor proteins, but instead has a specific role in the import of the protease YME1L1 and itself displays a very short half-life (Richter et al., 2019). YME1L1 was recently found to mediate the

Figure 3: The human TIM23 complex – TIM50 in disease.

(A) The human TIM23 complex is formed by the core components TIM50 (50), TIM23 (23) and TIM17A/B (17A/B). The sorting elements are TIM21 (21) and ROMO1, and the motor elements include TIM44 (44), PAM18 (18; DNAJC15 and DNAJC19), PAM16 (16; MAGMAS), mtHSP70 (Mortalin) and GrpE. During precursor sorting into the inner membrane, TIM21 can associate with an emerging nuclear-encoded polypeptide for a respiratory complex component. It then delivers the polypeptide to the actively translating mitochondrial-encoded subunit, still associated with the mitoribosome, in a process facilitated by respiratory complex intermediates known as MITRAC and the insertase OXA1L. This forms part of sequential OXPHOS complex assembly. (B) The four characterized patient mutations on TIM50 are indicated.

stress-induced proteolysis of proteins essential for mitochondrial biogenesis; such as translocase components, lipid transfer proteins and metabolic enzymes. Proteolysis by YME1L1 enables rapid adaptation to stress conditions and the limiting of mitochondrial biogenesis supports cell growth (MacVicar et al., 2019). Therefore, as a regulator of YME1L1 levels, ROMO1 is vital for controlling mitochondrial proteostasis and quality control.

A substrate of YME1L1 is TIM17A (Rainbolt et al., 2013). TIM17A and TIM17B exist as translocase components in a mutually exclusive manner (Bauer et al., 1999). Under stress conditions, TIM17A is rapidly degraded and consequently, protein import is reduced (Rainbolt et al., 2013). It therefore appears that in mammals, entry via the presequence pathway is under proteolytic control, which would enable a rapid mitochondrial response during stress conditions. Import motor components also appear to have acquired new functions in mammals. Although DNAJC15 and DNAJC19 can stimulate the activity of mtHSP70 (Sinha et al., 2010; Schusdziarra et al., 2013), only DNAJC15 can complement the activity of yeast Pam18 (Schusdziarra et al., 2013). In fact, DNAJC19 forms a complex with prohibitins and is involved in cardiolipin remodeling, so its role in mitochondrial import is still unclear (Richter-Dennerlein et al., 2014). The activity of the PAM relies on the regeneration of mtHSP70. In human, this is expected to occur via the Mge1 homolog, GrpE, but although GrpE was shown to bind to mtHSP70 *in vitro*, its function, including its exchange factor activity, has not yet been verified (Choglay et al., 2001).

Human TIM50 is also likely to have functionally diversified. Although both the yeast and human TIM50 possess a C-terminal CTD-like phosphatase domain, human TIM50 lacks the C-terminal PBD binding domain that in yeast is required for precursor handover (Qian et al., 2011), suggesting that the mechanism of precursor handover in humans may differ from that in yeast.

Several TIM23 components have been implicated in various diseases and these have been described in several recent reviews (Kang et al., 2018; Pfanner et al., 2019). Intriguingly, three separate reports have recently described mutations in TIM50, which is critical for precursor handover. These studies report on four unrelated patients, all of which experienced severe lactic acidosis and seizures (Shahrour et al., 2017; Reyes et al., 2018; Tort et al., 2019). Two patients had homozygous missense mutations (p.Arg217Trp and p.Thr252Met) in conserved residues within the TIM50 IMS domain and both patients also exhibited intellectual disability and 3-methylglutaconic aciduria (Shahrour et al., 2017). Another subject, with compound heterozygous mutations

within the transmembrane region of TIM50 (p.Ser112* and p.Gly190Ala) had strongly reduced levels of TIM50 and compromised mitochondrial import. Consequently, biochemical analyses revealed defects in the steady state levels of complex I, II and IV and a general respiratory deficiency (Reyes et al., 2018). Finally, a fourth subject with compound heterozygous mutations in highly conserved residues within the IMS domain (p.Arg114Gln and p.Gly269Ser) also exhibited 3-methylglutaconic aciduria, as well as vision loss, symptoms of Leigh syndrome and dilated hypercardiomyopathy (Tort et al., 2019). Like the previous patient, this subject had reduced levels of TIM50 protein and compromised respiratory capacity. Furthermore, the patient exhibited a general reduction in the activity of complexes I–V and defective respiratory complex assembly (Tort et al., 2019).

Interestingly, loss of TIM50 in both zebra fish and mice also resulted in increased heart and cardiomyocyte size (Guo et al., 2004; Tang et al., 2017), which appears to be linked to increased ROS production in the absence of TIM50 (Tang et al., 2017). TIM50 expression has additionally been implicated in various cancers (Gao et al., 2016; Zhang et al., 2019). Overall, these studies provide an insight into the function of human TIM50 but further studies are needed to explain the diversity in the physiological and biochemical phenotypes.

Conclusions and future perspectives

Mitochondrial function depends on the efficient and regulated import of nuclear-encoded proteins, most of which are directed through the presequence pathway. In work that has spanned decades, researchers have developed specialized tools to unravel the machinery and intricate details of this highly sophisticated import system. In this review we focused on the handover of the precursor protein from the TOM complex to the TIM23 complex; a process that requires a juxtapositioning of the inner and outer membranes, precursor recognition by the TIM23 complex, precursor translocation through the channel and its subsequent release into the inner membrane or the matrix.

Despite the current model, several key aspects of precursor handover remain to be resolved; (1) How do the two membranes come into close contact? Are there other protein complexes that facilitate this? (2) How do the TOM complex and TIM23 cooperate during precursor handover and how is the TOM-TIM23 supercomplex generated? (3) How does precursor transfer occur in human

mitochondria? These questions require high resolution analysis of the dynamics and proteomic landscape of the inner mitochondrial membrane, as well as structural studies of the TIM23 complex, alone and in complex with TOM. Furthermore, although components of the TIM23 complex are well conserved in mammals, the major functional variations indicate that there are evolutionary differences in the translocation process. Already, the lack of an extended presequence binding domain in the IMS of human TIM50 implies a different precursor handover mechanism. Therefore, detailed mechanistic and structural studies are warranted to elucidate the translocation mechanism of the human presequence translocase. This would not only enable a greater understanding of the role of pathogenic mutations in TIM23 components, but may pave the way for therapeutic modulation of the pathway.

Acknowledgments: This work was supported by the Deutsche Forschungsgemeinschaft: SFB1190, Funder Id: <http://dx.doi.org/10.13039/501100001659>, Project P13 (P.R.); SFB860, Funder Id: <http://dx.doi.org/10.13039/501100001659>, Project B01 (P.R.) and the Max Planck Society (P.R.).

References

- Albrecht, R., Rehling, P., Chacinska, A., Brix, J., Cadamuro, S.A., Volkmer, R., Guiard, B., Pfanner, N., and Zeth, K. (2006). The Tim21 binding domain connects the preprotein translocases of both mitochondrial membranes. *EMBO Rep.* 7, 1233–1238.
- Alconada, A., Kubrich, M., Moczek, M., Honlinger, A., and Pfanner, N. (1995). The mitochondrial receptor complex: the small subunit Mom8b/Isp6 supports association of receptors with the general insertion pore and transfer of preproteins. *Mol. Cell. Biol.* 15, 6196–6205.
- Alder, N.N., Jensen, R.E., and Johnson, A.E. (2008). Fluorescence mapping of mitochondrial TIM23 complex reveals a water-facing, substrate-interacting helix surface. *Cell* 134, 439–450.
- Araiso, Y., Tsutsumi, A., Qiu, J., Imai, K., Shiota, T., Song, J., Lindau, C., Wenz, L.S., Sakaue, H., Yunoki, K., et al. (2019). Structure of the mitochondrial import gate reveals distinct preprotein paths. *Nature* 575, 395–401.
- Baker, K.P., Schaniel, A., Vestweber, D., and Schatz, G. (1990). A yeast mitochondrial outer membrane protein essential for protein import and cell viability. *Nature* 348, 605–609.
- Bauer, M.F., Gempel, K., Reichert, A.S., Rappold, G.A., Lichtner, P., Gerbitz, K.D., Neupert, W., Brunner, M., and Hofmann, S. (1999). Genetic and structural characterization of the human mitochondrial inner membrane translocase. *J. Mol. Biol.* 289, 69–82.
- Bauer, M.F., Sirrenberg, C., Neupert, W., and Brunner, M. (1996). Role of Tim23 as voltage sensor and presequence receptor in protein import into mitochondria. *Cell* 87, 33–41.
- Bausewein, T., Naveed, H., Liang, J., and Nussberger, S. (2020), submitted for publication [Epub ahead of print].
- Beattie, D.S., Basford, R.E., and Koritz, S.B. (1967). The turnover of the protein components of mitochondria from rat liver, kidney, and brain. *J. Biol. Chem.* 242, 4584–4586.
- Berthold, J., Bauer, M.F., Schneider, H.C., Klaus, C., Dietmeier, K., Neupert, W., and Brunner, M. (1995). The MIM complex mediates preprotein translocation across the mitochondrial inner membrane and couples it to the mt-Hsp70/ATP driving system. *Cell* 81, 1085–1093.
- Bhattacharyya, T., Karnezis, A.N., Murphy, S.P., Hoang, T., Freeman, B.C., Phillips, B., and Morimoto, R.I. (1995). Cloning and subcellular localization of human mitochondrial hsp70. *J. Biol. Chem.* 270, 1705–1710.
- Blom, J., Kubrich, M., Rassow, J., Voos, W., Dekker, P.J., Maarse, A.C., Meijer, M., and Pfanner, N. (1993). The essential yeast protein MIM44 (encoded by MPI1) is involved in an early step of preprotein translocation across the mitochondrial inner membrane. *Mol. Cell. Biol.* 13, 7364–7371.
- Bolliger, L., Junne, T., Schatz, G., and Lithgow, T. (1995). Acidic receptor domains on both sides of the outer membrane mediate translocation of precursor proteins into yeast mitochondria. *EMBO J.* 14, 6318–6326.
- Borges, J.C., Fischer, H., Craievich, A.F., Hansen, L.D., and Ramos, C.H. (2003). Free human mitochondrial GrpE is a symmetric dimer in solution. *J. Biol. Chem.* 278, 35337–35344.
- Chacinska, A., Lind, M., Frazier, A.E., Dudek, J., Meisinger, C., Geissler, A., Sickmann, A., Meyer, H.E., Truscott, K.N., Guiard, B., et al. (2005). Mitochondrial presequence translocase: switching between TOM tethering and motor recruitment involves Tim21 and Tim17. *Cell* 120, 817–829.
- Chacinska, A., Koehler, C.M., Milenkovic, D., Lithgow, T., and Pfanner, N. (2009). Importing mitochondrial proteins: machineries and mechanisms. *Cell* 138, 628–644.
- Chacinska, A., van der Laan, M., Mehnert, C.S., Guiard, B., Mick, D.U., Hutu, D.P., Truscott, K.N., Wiedemann, N., Meisinger, C., Pfanner, N., et al. (2010). Distinct forms of mitochondrial TOM-TIM supercomplexes define signal-dependent states of preprotein sorting. *Mol. Cell. Biol.* 30, 307–318.
- Choglay, A.A., Chapple, J.P., Blatch, G.L., and Cheetham, M.E. (2001). Identification and characterization of a human mitochondrial homologue of the bacterial co-chaperone GrpE. *Gene* 267, 125–134.
- D'Silva, P.D., Schilke, B., Walter, W., Andrew, A., and Craig, E.A. (2003). J protein cochaperone of the mitochondrial inner membrane required for protein import into the mitochondrial matrix. *Proc. Natl. Acad. Sci. U. S. A.* 100, 13839–13844.
- Dayan, D., Bandel, M., Gunsell, U., Nussbaum, I., Prag, G., Mokranjac, D., Neupert, W., and Azem, A. (2019). A mutagenesis analysis of Tim50, the major receptor of the TIM23 complex, identifies regions that affect its interaction with Tim23. *Sci. Rep.* 9, 2012.
- de la Cruz, L., Bajaj, R., Becker, S., and Zweckstetter, M. (2010). The intermembrane space domain of Tim23 is intrinsically disordered with a distinct binding region for presequences. *Protein Sci.* 19, 2045–2054.
- Dekker, P.J., Keil, P., Rassow, J., Maarse, A.C., Pfanner, N., and Meijer, M. (1993). Identification of MIM23, a putative component of the protein import machinery of the mitochondrial inner membrane. *FEBS Lett.* 330, 66–70.
- Dekker, P.J., Martin, F., Maarse, A.C., Bomer, U., Muller, H., Guiard, B., Meijer, M., Rassow, J., and Pfanner, N. (1997). The Tim core

- complex defines the number of mitochondrial translocation contact sites and can hold arrested preproteins in the absence of matrix Hsp70-Tim44. *EMBO J.* 16, 5408–5419.
- Denkert, N., Schendzielorz, A.B., Barbot, M., Versemann, L., Richter, F., Rehling, P., and Meinecke, M. (2017). Cation selectivity of the presequence translocase channel Tim23 is crucial for efficient protein import. *eLife* 6, e28324.
- Dietmeier, K., Honlinger, A., Bomer, U., Dekker, P.J., Eckerskorn, C., Lottspeich, F., Kubrich, M., and Pfanner, N. (1997). Tom5 functionally links mitochondrial preprotein receptors to the general import pore. *Nature* 388, 195–200.
- Emtage, J.L. and Jensen, R.E. (1993). MAS6 encodes an essential inner membrane component of the yeast mitochondrial protein import pathway. *J. Cell. Biol.* 122, 1003–1012.
- Gao, S.P., Sun, H.F., Jiang, H.L., Li, L.D., Hu, X., Xu, X.E., and Jin, W. (2016). Loss of TIM50 suppresses proliferation and induces apoptosis in breast cancer. *Tumour Biol.* 37, 1279–1287.
- Gartner, F., Voos, W., Querol, A., Miller, B.R., Craig, E.A., Cumsy, M.G., and Pfanner, N. (1995). Mitochondrial import of subunit Va of cytochrome c oxidase characterized with yeast mutants. *J. Biol. Chem.* 270, 3788–3795.
- Gasser, S.M., Daum, G., and Schatz, G. (1982). Import of proteins into mitochondria. Energy-dependent uptake of precursors by isolated mitochondria. *J. Biol. Chem.* 257, 13034–13041.
- Gebert, M., Schrempp, S.G., Mehnert, C.S., Heisswolf, A.K., Oeljeklaus, S., Ieva, R., Bohnert, M., von der Malsburg, K., Wiese, S., Kleinschroth, T., et al. (2012). Mgr2 promotes coupling of the mitochondrial presequence translocase to partner complexes. *J. Cell. Biol.* 197, 595–604.
- Geissler, A., Chacinska, A., Truscott, K.N., Wiedemann, N., Brandner, K., Sickmann, A., Meyer, H.E., Meisinger, C., Pfanner, N., and Rehling, P. (2002). The mitochondrial presequence translocase: an essential role of Tim50 in directing preproteins to the import channel. *Cell* 111, 507–518.
- Gold, V.A., Ieva, R., Walter, A., Pfanner, N., van der Laan, M., and Kuhlbrandt, W. (2014). Visualizing active membrane protein complexes by electron cryotomography. *Nat. Commun.* 5, 4129.
- Gruhler, A., Arnold, I., Seytter, T., Guiard, B., Schwarz, E., Neupert, W., and Stuart, R.A. (1997). N-terminal hydrophobic sorting signals of preproteins confer mitochondrial hsp70 independence for import into mitochondria. *J. Biol. Chem.* 272, 17410–17415.
- Guo, Y., Cheong, N., Zhang, Z., De Rose, R., Deng, Y., Farber, S.A., Fernandes-Alnemri, T., Alnemri, E.S. (2004). Tim50, a component of the mitochondrial translocator, regulates mitochondrial integrity and cell death. *J. Biol. Chem.* 279, 24813–24825.
- Hallermayer, G., Zimmermann, R., and Neupert, W. (1977). Kinetic studies on the transport of cytoplasmically synthesized proteins into the mitochondria in intact cells of *Neurospora crassa*. *Eur. J. Biochem.* 81, 523–532.
- Harmey, M.A., Hallermayer, G., Korb, H., and Neupert, W. (1977). Transport of cytoplasmically synthesized proteins into the mitochondria in a cell free system from *Neurospora crassa*. *Eur. J. Biochem.* 81, 533–544.
- Hill, K., Model, K., Ryan, M.T., Dietmeier, K., Martin, F., Wagner, R., and Pfanner, N. (1998). Tom40 forms the hydrophilic channel of the mitochondrial import pore for preproteins. *Nature* 395, 516–521.
- Hines, V., Brandt, A., Griffiths, G., Horstmann, H., Brutsch, H., and Schatz, G. (1990). Protein import into yeast mitochondria is accelerated by the outer membrane protein MAS70. *EMBO J.* 9, 3191–3200.
- Honlinger, A., Bomer, U., Alconada, A., Eckerskorn, C., Lottspeich, F., Dietmeier, K., and Pfanner, N. (1996). Tom7 modulates the dynamics of the mitochondrial outer membrane translocase and plays a pathway-related role in protein import. *EMBO J.* 15, 2125–2137.
- Hurt, E.C., Pesold-Hurt, B., and Schatz, G. (1984). The amino-terminal region of an imported mitochondrial precursor polypeptide can direct cytoplasmic dihydrofolate reductase into the mitochondrial matrix. *EMBO J.* 3, 3149–3156.
- Hutu, D.P., Guiard, B., Chacinska, A., Becker, D., Pfanner, N., Rehling, P., and van der Laan, M. (2008). Mitochondrial protein import motor: differential role of Tim44 in the recruitment of Pam17 and J-complex to the presequence translocase. *Mol. Biol. Cell.* 19, 2642–2649.
- Ieva, R., Schrempp, S.G., Opalinski, L., Wollweber, F., Hoss, P., Heisswolf, A.K., Gebert, M., Zhang, Y., Guiard, B., Rospert, S., et al. (2014). Mgr2 functions as lateral gatekeeper for preprotein sorting in the mitochondrial inner membrane. *Mol. Cell.* 56, 641–652.
- Kanamori, T., Nishikawa, S., Nakai, M., Shin, I., Schultz, P.G., and Endo, T. (1999). Uncoupling of transfer of the presequence and unfolding of the mature domain in precursor translocation across the mitochondrial outer membrane. *Proc. Natl. Acad. Sci. U. S. A.* 96, 3634–3639.
- Kang, P.J., Ostermann, J., Shilling, J., Neupert, W., Craig, E.A., and Pfanner, N. (1990). Requirement for hsp70 in the mitochondrial matrix for translocation and folding of precursor proteins. *Nature* 348, 137–143.
- Kang, Y., Fielden, L.F., and Stojanovski, D. (2018). Mitochondrial protein transport in health and disease. *Semin. Cell Dev. Biol.* 76, 142–153.
- Kassenbrock, C.K., Cao, W., and Douglas, M.G. (1993). Genetic and biochemical characterization of ISP6, a small mitochondrial outer membrane protein associated with the protein translocation complex. *EMBO J.* 12, 3023–3034.
- Kiebler, M., Pfaller, R., Sollner, T., Griffiths, G., Horstmann, H., Pfanner, N., and Neupert, W. (1990). Identification of a mitochondrial receptor complex required for recognition and membrane insertion of precursor proteins. *Nature* 348, 610–616.
- Komiya, T., Rospert, S., Koehler, C., Looser, R., Schatz, G., and Mihara, K. (1998). Interaction of mitochondrial targeting signals with acidic receptor domains along the protein import pathway: evidence for the ‘acid chain’ hypothesis. *EMBO J.* 17, 3886–3898.
- Kozany, C., Mokranjac, D., Sichting, M., Neupert, W., and Hell, K. (2004). The J domain-related cochaperone Tim16 is a constituent of the mitochondrial TIM23 preprotein translocase. *Nat. Struct. Mol. Biol.* 11, 234–241.
- Kronidou, N.G., Oppliger, W., Bolliger, L., Hannavy, K., Glick, B.S., Schatz, G., and Horst, M. (1994). Dynamic interaction between Isp45 and mitochondrial hsp70 in the protein import system of the yeast mitochondrial inner membrane. *Proc. Natl. Acad. Sci. U. S. A.* 91, 12818–12822.
- Kuntzel, E. and Noll, H. (1967). Mitochondrial and cytoplasmic polysomes from *Neurospora crassa*. *Nature* 215, 1340–1345.
- Lee, S., Lee, H., Yoo, S., Ieva, R., van der Laan, M., von Heijne, G., and Kim, H. (2019). The Mgr2 subunit of the TIM23 complex

- regulates membrane insertion of marginal stop-transfer signals in the mitochondrial inner membrane. *FEBS Lett.* DOI: 10.1002/1873-3468.13692.
- Li, J. and Sha, B. (2015). The structure of Tim50(164–361) suggests the mechanism by which Tim50 receives mitochondrial presequences. *Acta. Crystallogr. F: Struct. Biol. Commun.* **71**, 1146–1151.
- Lithgow, T., Junne, T., Suda, K., Gratzer, S., and Schatz, G. (1994). The mitochondrial outer membrane protein Mas22p is essential for protein import and viability of yeast. *Proc. Natl. Acad. Sci. U. S. A.* **91**, 11973–11977.
- Luck, D.J. and Reich, E. (1964). DNA in mitochondria of *Neurospora crassa*. *Proc. Natl. Acad. Sci. U. S. A.* **52**, 931–938.
- Lytovchenko, O., Melin, J., Schulz, C., Kilisch, M., Hutu, D.P., and Rehling, P. (2013). Signal recognition initiates reorganization of the presequence translocase during protein import. *EMBO J.* **32**, 886–898.
- Maarse, A.C., Blom, J., Grivell, L.A., and Meijer, M. (1992). MPI1, an essential gene encoding a mitochondrial membrane protein, is possibly involved in protein import into yeast mitochondria. *EMBO J.* **11**, 3619–3628.
- Maarse, A.C., Blom, J., Keil, P., Pfanner, N., and Meijer, M. (1994). Identification of the essential yeast protein MIM17, an integral mitochondrial inner membrane protein involved in protein import. *FEBS Lett.* **349**, 215–221.
- Maccacchini, M.L., Rudin, Y., Blobel, G., and Schatz, G. (1979a). Import of proteins into mitochondria: precursor forms of the extramitochondrially made F1-ATPase subunits in yeast. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 343–347.
- Maccacchini, M.L., Rudin, Y., and Schatz, G. (1979b). Transport of proteins across the mitochondrial outer membrane. A precursor form of the cytoplasmically made intermembrane enzyme cytochrome c peroxidase. *J. Biol. Chem.* **254**, 7468–7471.
- MacVicar, T., Ohba, Y., Nolte, H., Mayer, F.C., Tatsuta, T., Sprenger, H.G., Lindner, B., Zhao, Y., Li, J., Bruns, C., et al. (2019). Lipid signalling drives proteolytic rewiring of mitochondria by YME1L. *Nature* **575**, 361–365.
- Marom, M., Dayan, D., Demishtein-Zohary, K., Mokranjac, D., Neupert, W., and Azem, A. (2011). Direct interaction of mitochondrial targeting presequences with purified components of the TIM23 protein complex. *J. Biol. Chem.* **286**, 43809–43815.
- Martin, J., Mahlke, K., and Pfanner, N. (1991). Role of an energized inner membrane in mitochondrial protein import. Delta psi drives the movement of presequences. *J. Biol. Chem.* **266**, 18051–18057.
- Martinez-Caballero, S., Grigoriev, S.M., Herrmann, J.M., Campo, M.L., and Kinnally, K.W. (2007). Tim17p regulates the twin pore structure and voltage gating of the mitochondrial protein import complex TIM23. *J. Biol. Chem.* **282**, 3584–3593.
- McLean, J.R., Cohn, G.L., Brandt, I.K., and Simpson, M.V. (1958). Incorporation of labeled amino acids into the protein of muscle and liver mitochondria. *J. Biol. Chem.* **233**, 657–663.
- Meier, S., Neupert, W., and Herrmann, J.M. (2005). Conserved N-terminal negative charges in the Tim17 subunit of the TIM23 translocase play a critical role in the import of preproteins into mitochondria. *J. Biol. Chem.* **280**, 7777–7785.
- Meinecke, M., Wagner, R., Kovermann, P., Guiard, B., Mick, D.U., Hutu, D.P., Voos, W., Truscott, K.N., Chacinska, A., Pfanner, N., et al. (2006). Tim50 maintains the permeability barrier of the mitochondrial inner membrane. *Science* **312**, 1523–1526.
- Mick, D.U., Dennerlein, S., Wiese, H., Reinhold, R., Pacheu-Grau, D., Lorenzi, I., Sasarman, F., Weraarpachai, W., Shoubbridge, E.A., Warscheid, B., et al. (2012). MITRAC links mitochondrial protein translocation to respiratory-chain assembly and translational regulation. *Cell* **151**, 1528–1541.
- Moczko, M., Dietmeier, K., Sollner, T., Segui, B., Steger, H.F., Neupert, W., and Pfanner, N. (1992). Identification of the mitochondrial receptor complex in *Saccharomyces cerevisiae*. *FEBS Lett.* **310**, 265–268.
- Moczko, M., Bomer, U., Kubrich, M., Zufall, N., Honlinger, A., and Pfanner, N. (1997). The intermembrane space domain of mitochondrial Tom22 functions as a trans binding site for preproteins with N-terminal targeting sequences. *Mol. Cell. Biol.* **17**, 6574–6584.
- Mokranjac, D., Paschen, S.A., Kozany, C., Prokisch, H., Hoppins, S.C., Nargang, F.E., Neupert, W., and Hell, K. (2003a). Tim50, a novel component of the TIM23 preprotein translocase of mitochondria. *EMBO J.* **22**, 816–825.
- Mokranjac, D., Sichting, M., Neupert, W., and Hell, K. (2003b). Tim14, a novel key component of the import motor of the TIM23 protein translocase of mitochondria. *EMBO J.* **22**, 4945–4956.
- Mokranjac, D., Popov-Celeketić, D., Hell, K., and Neupert, W. (2005). Role of Tim21 in mitochondrial translocation contact sites. *J. Biol. Chem.* **280**, 23437–23440.
- Mokranjac, D. (2020), submitted for publication [Epub ahead of print].
- Morgenstern, M., Stiller, S.B., Lubbert, P., Peikert, C.D., Dannenmaier, S., Drepper, F., Weill, U., Hoss, P., Feuerstein, R., Gebert, M., et al. (2017). Definition of a high-confidence mitochondrial proteome at quantitative scale. *Cell Rep.* **19**, 2836–2852.
- Nass, S. and Nass, M.M. (1963). Intramitochondrial fibers with DNA characteristics. II. enzymatic and other hydrolytic treatments. *J. Cell. Biol.* **19**, 613–629.
- Nelson, N. and Schatz, G. (1979). Energy-dependent processing of cytoplasmically made precursors to mitochondrial proteins. *Proc. Natl. Acad. Sci. U. S. A.* **76**, 4365–4369.
- Neupert, W., Brdiczka, D., and Bucher, T. (1967). Incorporation of amino acids into the outer and inner membrane of isolated rat liver mitochondria. *Biochem. Biophys. Res. Commun.* **27**, 488–493.
- Pfanner, N., Warscheid, B., and Wiedemann, N. (2019). Mitochondrial proteins: from biogenesis to functional networks. *Nat. Rev. Mol. Cell. Biol.* **20**, 267–284.
- Qian, X., Gebert, M., Hopker, J., Yan, M., Li, J., Wiedemann, N., van der Laan, M., Pfanner, N., and Sha, B. (2011). Structural basis for the function of Tim50 in the mitochondrial presequence translocase. *J. Mol. Biol.* **411**, 513–519.
- Rahman, B., Kawano, S., Yunoki-Esaki, K., Anzai, T., and Endo, T. (2014). NMR analyses on the interactions of the yeast Tim50 C-terminal region with the presequence and Tim50 core domain. *FEBS Lett.* **588**, 678–684.
- Rainbolt, T.K., Atanassova, N., Genereux, J.C., and Wiseman, R.L. (2013). Stress-regulated translational attenuation adapts mitochondrial protein import through Tim17A degradation. *Cell Metab.* **18**, 908–919.
- Rapaport, D., Neupert, W., and Lill, R. (1997). Mitochondrial protein import. Tom40 plays a major role in targeting and translocation of preproteins by forming a specific binding site for the presequence. *J. Biol. Chem.* **272**, 18725–18731.
- Rassow, J., Hartl, F.U., Guiard, B., Pfanner, N., and Neupert, W. (1990). Polypeptides traverse the mitochondrial envelope in an extended state. *FEBS Lett.* **275**, 190–194.

- Regaud, C. (1908). Regarding the mitochondrion of the seminal epithelium. IV. Facts and hypotheses relative to their constitution. *Comptes Rendus Des Seances De La Societe De Biologie Et De Ses Filiales* 65, 718–720.
- Reyes, A., Melchionda, L., Burlina, A., Robinson, A.J., Ghezzi, D., and Zeviani, M. (2018). Mutations in TIMM50 compromise cell survival in OxPhos-dependent metabolic conditions. *EMBO Mol. Med.* 10, e8698.
- Richter, F., Dennerlein, S., Nikolov, M., Jans, D.C., Naumenko, N., Aich, A., MacVicar, T., Linden, A., Jakobs, S., Urlaub, H., et al. (2019). ROMO1 is a constituent of the human presequence translocase required for YME1L protease import. *J. Cell. Biol.* 218, 598–614.
- Richter-Dennerlein, R., Korwitz, A., Haag, M., Tatsuta, T., Dargazanli, S., Baker, M., Decker, T., Lamkemeyer, T., Rugarli, E.I., and Langer, T. (2014). DNAJC19, a mitochondrial cochaperone associated with cardiomyopathy, forms a complex with prohibitins to regulate cardiolipin remodeling. *Cell Metab.* 20, 158–171.
- Richter-Dennerlein, R., Oeljeklaus, S., Lorenzi, I., Ronsor, C., Bareth, B., Schendzielorz, A.B., Wang, C., Warscheid, B., Rehling, P., and Dennerlein, S. (2016). Mitochondrial protein synthesis adapts to influx of nuclear-encoded protein. *Cell* 167, 471–483.
- Riezman, H., Hay, R., Witte, C., Nelson, N., and Schatz, G. (1983). Yeast mitochondrial outer membrane specifically binds cytoplasmically-synthesized precursors of mitochondrial proteins. *EMBO J.* 2, 1113–1118.
- Roise, D., Theiler, F., Horvath, S.J., Tomich, J.M., Richards, J.H., Allison, D.S., and Schatz, G. (1988). Amphiphilicity is essential for mitochondrial presequence function. *EMBO J.* 7, 649–653.
- Ryan, K.R., Menold, M.M., Garrett, S., and Jensen, R.E. (1994). SMS1, a high-copy suppressor of the yeast mas6 mutant, encodes an essential inner membrane protein required for mitochondrial protein import. *Mol. Biol. Cell.* 5, 529–538.
- Ryan, K.R., Leung, R.S., and Jensen, R.E. (1998). Characterization of the mitochondrial inner membrane translocase complex: the Tim23p hydrophobic domain interacts with Tim17p but not with other Tim23p molecules. *Mol. Cell. Biol.* 18, 178–187.
- Schatz, G., Haslbrunner, E., and Tuppy, H. (1964). Deoxyribonucleic acid associated with yeast mitochondria. *Biochem. Biophys. Res. Commun.* 15, 127–132.
- Schendzielorz, A.B., Schulz, C., Lytovchenko, O., Clancy, A., Guiard, B., Ieva, R., van der Laan, M., and Rehling, P. (2017). Two distinct membrane potential-dependent steps drive mitochondrial matrix protein translocation. *J. Cell. Biol.* 216, 83–92.
- Schendzielorz, A.B., Bragoszewski, P., Naumenko, N., Gomkale, R., Schulz, C., Guiard, B., Chacinska, A., and Rehling, P. (2018). Motor recruitment to the TIM23 channel's lateral gate restricts polypeptide release into the inner membrane. *Nat. Commun.* 9, 4028.
- Scherer, P.E., Manning-Krieg, U.C., Jenö, P., Schatz, G., and Horst, M. (1992). Identification of a 45-kDa protein at the protein import site of the yeast mitochondrial inner membrane. *Proc. Natl. Acad. Sci. U. S. A.* 89, 11930–11934.
- Schneider, H.C., Berthold, J., Bauer, M.F., Dietmeier, K., Guiard, B., Brunner, M., and Neupert, W. (1994). Mitochondrial Hsp70/MIM44 complex facilitates protein import. *Nature* 371, 768–774.
- Schulz, C., Lytovchenko, O., Melin, J., Chacinska, A., Guiard, B., Neumann, P., Ficner, R., Jahn, O., Schmidt, B., and Rehling, P. (2011). Tim50's presequence receptor domain is essential for signal driven transport across the TIM23 complex. *J. Cell. Biol.* 195, 643–656.
- Schusdziarra, C., Blamowska, M., Azem, A., and Hell, K. (2013). Methylation-controlled J-protein MCJ acts in the import of proteins into human mitochondria. *Hum. Mol. Genet.* 22, 1348–1357.
- Shahrour, M.A., Staretz-Chacham, O., Dayan, D., Stephen, J., Weech, A., Damseh, N., Pri Chen, H., Edvardson, S., Mazaheri, S., Saada, A., et al. (2017). Mitochondrial epileptic encephalopathy, 3-methylglutaconic aciduria and variable complex V deficiency associated with TIMM50 mutations. *Clin. Genet.* 91, 690–696.
- Shiota, T., Mabuchi, H., Tanaka-Yamano, S., Yamano, K., and Endo, T. (2011). In vivo protein-interaction mapping of a mitochondrial translocator protein Tom22 at work. *Proc. Natl. Acad. Sci. U. S. A.* 108, 15179–15183.
- Sinha, D., Joshi, N., Chittoor, B., Samji, P., and D'Silva, P. (2010). Role of Magmas in protein transport and human mitochondria biogenesis. *Hum. Mol. Genet.* 19, 1248–1262.
- Sinha, D., Srivastava, S., Krishna, L., and D'Silva, P. (2014). Unraveling the intricate organization of mammalian mitochondrial presequence translocases: existence of multiple translocases for maintenance of mitochondrial function. *Mol. Cell. Biol.* 34, 1757–1775.
- Sollner, T., Griffiths, G., Pfaller, R., Pfanner, N., and Neupert, W. (1989). MOM19, an import receptor for mitochondrial precursor proteins. *Cell* 59, 1061–1070.
- Sollner, T., Pfaller, R., Griffiths, G., Pfanner, N., and Neupert, W. (1990). A mitochondrial import receptor for the ADP/ATP carrier. *Cell* 62, 107–115.
- Steger, H.F., Sollner, T., Kiebler, M., Dietmeier, K.A., Pfaller, R., Trulzsch, K.S., Tropschug, M., Neupert, W., and Pfanner, N. (1990). Import of ADP/ATP carrier into mitochondria: two receptors act in parallel. *J. Cell. Biol.* 111, 2353–2363.
- Tamura, Y., Harada, Y., Shiota, T., Yamano, K., Watanabe, K., Yokota, M., Yamamoto, H., Sesaki, H., and Endo, T. (2009). Tim23-Tim50 pair coordinates functions of translocators and motor proteins in mitochondrial protein import. *J. Cell. Biol.* 184, 129–141.
- Tang, K., Zhao, Y., Li, H., Zhu, M., Li, W., Liu, W., Zhu, G., Xu, D., Peng, W., and Xu, Y.W. (2017). Translocase of inner membrane 50 functions as a novel protective regulator of pathological cardiac hypertrophy. *J. Am. Heart Assoc.* 6, e004346.
- Tort, F., Ugarteburu, O., Texido, L., Gea-Sorli, S., Garcia-Villoria, J., Ferrer-Cortes, X., Arias, A., Matalonga, L., Gort, L., Ferrer, I., et al. (2019). Mutations in TIMM50 cause severe mitochondrial dysfunction by targeting key aspects of mitochondrial physiology. *Hum. Mutat.* 40, 1700–1712.
- Truscott, K.N., Kovermann, P., Geissler, A., Merlin, A., Meijer, M., Driessen, A.J., Rassow, J., Pfanner, N., and Wagner, R. (2001). A presequence- and voltage-sensitive channel of the mitochondrial preprotein translocase formed by Tim23. *Nat. Struct. Biol.* 8, 1074–1082.
- Truscott, K.N., Voos, W., Frazier, A.E., Lind, M., Li, Y., Geissler, A., Dudek, J., Muller, H., Sickmann, A., Meyer, H.E., et al. (2003). A J-protein is an essential subunit of the presequence translocase-associated protein import motor of mitochondria. *J. Cell Biol.* 163, 707–713.
- Tucker, K. and Park, E. (2019). Cryo-EM structure of the mitochondrial protein-import channel TOM complex at near-atomic resolution. *Nat. Struct. Mol. Biol.* 26, 1158–1166.

- Ungermann, C., Neupert, W., and Cyr, D.M. (1994). The role of Hsp70 in conferring unidirectionality on protein translocation into mitochondria. *Science* 266, 1250–1253.
- van der Laan, M., Chacinska, A., Lind, M., Perschil, I., Sickmann, A., Meyer, H.E., Guiard, B., Meisinger, C., Pfanner, N., and Rehling, P. (2005). Pam17 is required for architecture and translocation activity of the mitochondrial protein import motor. *Mol. Cell. Biol.* 25, 7449–7458.
- van der Laan, M., Meinecke, M., Dudek, J., Hutu, D.P., Lind, M., Perschil, I., Guiard, B., Wagner, R., Pfanner, N., and Rehling, P. (2007). Motor-free mitochondrial presequence translocase drives membrane integration of preproteins. *Nat. Cell. Biol.* 9, 1152–1159.
- van der Laan, M., Wiedemann, N., Mick, D.U., Guiard, B., Rehling, P., and Pfanner, N. (2006). A role for Tim21 in membrane-potential-dependent preprotein sorting in mitochondria. *Curr. Biol.* 16, 2271–2276.
- Vestweber, D., Brunner, J., Baker, A., and Schatz, G. (1989). A 42K outer-membrane protein is a component of the yeast mitochondrial protein import site. *Nature* 341, 205–209.
- Vogtle, F.N., Burkhart, J.M., Gonczarowska-Jorge, H., Kucukose, C., Taskin, A.A., Kopczynski, D., Ahrends, R., Mossmann, D., Sickmann, A., Zahedi, R.P., et al. (2017). Landscape of submitochondrial protein distribution. *Nat. Commun.* 8, 290.
- Werner, S. and Neupert, W. (1972). Functional and biogenetical heterogeneity of the inner membrane of rat-liver mitochondria. *Eur. J. Biochem.* 25, 379–396.
- Wrobel, L., Sokol, A.M., Chojnacka, M., and Chacinska, A. (2016). The presence of disulfide bonds reveals an evolutionarily conserved mechanism involved in mitochondrial protein translocase assembly. *Sci. Rep.* 6, 27484.
- Yamamoto, H., Esaki, M., Kanamori, T., Tamura, Y., Nishikawa, S., and Endo, T. (2002). Tim50 is a subunit of the TIM23 complex that links protein translocation across the outer and inner mitochondrial membranes. *Cell* 111, 519–528.
- Zhang, X., Han, S., Zhou, H., Cai, L., Li, J., Liu, N., Liu, Y., Wang, L., Fan, C., Li, A., et al. (2019). TIMM50 promotes tumor progression via ERK signaling and predicts poor prognosis of non-small cell lung cancer patients. *Mol. Carcinog.* 58, 767–776.
- Zimmerman, R., Paluch, U., Sprinzl, M., and Neupert, W. (1979). Cell-free synthesis of the mitochondrial ADP/ATP carrier protein of *Neurospora crassa*. *Eur. J. Biochem.* 99, 247–252.