

Review

A journey along the TIM23 complex, the major protein translocase of the mitochondrial inner membrane

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Summary. Proper structure and function of mitochondria depends on translocation of over a thousand different mitochondrial proteins from the cytosol. Mitochondrial proteins carry a number of different targeting signals and are translocated into the mitochondria by a number of different protein translocases. Here, we provide an overview of mitochondrial protein translocation pathways, and focus on the TIM23 complex, the major protein translocase of the mitochondrial inner membrane.

Keywords: mitochondria, protein translocation, protein sorting, targeting signal, presequence, TIM23 complex.

INTRODUCTION

The most fascinating feature of eukaryotic cells is their intracellular compartmentalization. During evolution, membrane-enclosed structures, or organelles, were formed to allow for more efficient metabolic processes to occur. Organelles contain their own specific sets of enzymes, metabolites, structural elements and, in some cases, even their own genetic material, distinct from the nuclear genome. Mitochondria are involved in various vital cellular processes, including energy metabolism, the synthesis of a number of different biomolecules and various signaling events. Thus, it is not surprising that dysfunction of mitochondria is associated with a myriad of human diseases (Nunnari and Suomalainen 2012). Mitochondria are double membrane-surrounded organelles, resulting in the formation of four different sub-

compartments: from outside to inside, the outer membrane (OM), intermembrane space (IMS), inner membrane (IM) and the innermost matrix. Even though they contain their own genetic material and complete transcription and translation systems, only 8 out of ca. 1000 proteins in yeast and 13 out of ca. 1500 proteins in humans are encoded by mitochondrial DNA (mtDNA). All other mitochondrial proteins are expressed from nuclear DNA (ncDNA), translated on cytosolic ribosomes and require specialized translocation machineries for their correct targeting, translocation and sorting within mitochondria (Neupert 2015; Wiedemann and Pfanner 2017).

In this review, we first give a brief overview of mitochondrial protein translocation pathways and then focus on the TIM23 complex, the major protein translocase of the mitochondrial IM.

“Know the ropes”: Protein translocation into mitochondria

Proteins destined to mitochondria are synthesized in the cytosol as precursor proteins with specific cleavable or internal targeting signals. These different mitochondrial targeting signals will guide the precursor proteins to their final destination within the mitochondria (Fig. 1). Essentially all mitochondrial proteins use the TOM (translocase of the outer membrane) complex as a general gate for passage through the OM. The TOM complex is the most abundant protein translocase of mitochondria and coordinates import of mitochondrial proteins with the downstream translocases. Below, the various mitochondrial protein translocation pathways are briefly described.

β -barrel proteins of the OM, such as porin, Tom40, Sam50/Tob55 and Mdm10, are initially translocated completely across the OM using the TOM complex. In the IMS, they are handled by the small TIM chaperones, which guide them to the TOB/SAM complex (topogenesis of mitochondrial OM β -barrel proteins/sorting and assembly machinery) for insertion into the OM from the IMS side. The single- and multi-spanning α -helical OM proteins use the TOM and MIM complexes for insertion into the OM. Many small IMS proteins, including small TIMs, contain conserved

cysteine motifs and undergo oxidative folding after they traverse the TOM complex. This process is mediated by the disulfide relay system, also called the MIA (mitochondrial IMS assembly) pathway. Inner membrane proteins with multiple α -helical transmembrane segments (TMs), such as metabolite carriers and the core components of mitochondrial IM translocases, Tim23, Tim17 and Tim22, contain internal targeting signals and use the TIM22 complex (translocase of the inner membrane 22) for insertion into the IM in a membrane potential ($\Delta\Psi$) dependent manner. Like the clients of the TOB/SAM complex, the hydrophobic TIM22 substrates require small TIM proteins to chaperone them through the aqueous IMS from the TOM complex in the OM to the TIM22 complex in the IM. Hydrophobic IM proteins that are encoded in the mtDNA and translated on mitochondrial ribosomes are inserted into the IM from the matrix side with the help of the OXA (oxidase assembly) complex. The vast majority of mitochondrial proteins, however, follow the so-called presequence pathway. They contain N-terminal, cleavable presequences (also called matrix targeting signals) and are translocated by the TOM and TIM23 complexes across the OM and IM, respectively. The TIM23 complex utilizes the $\Delta\Psi$ energy across the IM and ATP in the matrix to trans-

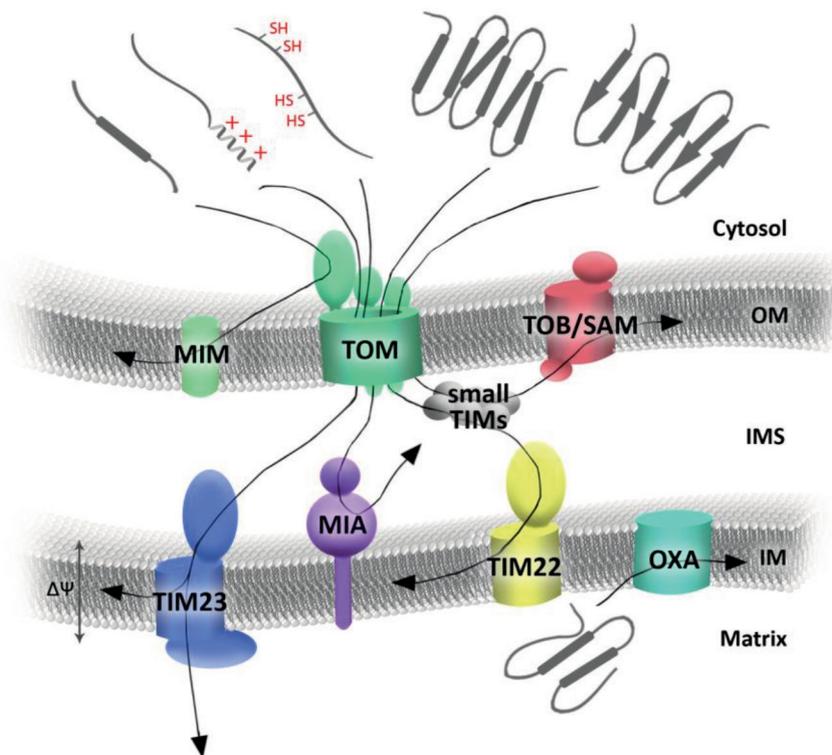


Fig. 1. An overview of mitochondrial protein translocation pathways. See text for details. OM, outer membrane; IMS, intermembrane space; IM, inner membrane.

locate proteins across and insert them into the IM. In the next sections, we describe in more detail the TIM23 complex.

THE TIM23 COMPLEX

The TIM23 complex tightly cooperates with the TOM complex during import of ncDNA-encoded mitochondrial proteins that have N-terminal presequences. Almost 70% of proteins targeted to mitochondria have this type of targeting signal. Presequences are usually 8 to 80 amino acid residues long, and are typically found at the N-terminal end of the protein. They are characterized by the ability to form an amphipathic helix with a net positive charge (+3 to +6) on one side and a hydrophobic surface on the opposite side. Once in the matrix, presequences are usually cleaved by mitochondrial processing peptidase and, in some cases, peptidases such as mitochondrial intermediate peptidase and Icp55 can further process incoming proteins (Mossmann et al. 2012).

By default, presequences target precursor proteins to the matrix. However, the TIM23 complex can also open laterally and release precursor proteins into the IM, if an additional downstream, hydrophobic lateral sorting signal is present (also known as 'stop-transfer'). Some of the laterally sorted precursor proteins are further processed by the inner membrane peptidase on the IMS side of the IM, releasing soluble proteins into the IMS. In case of presequence-con-

taining precursor proteins with multiple TMs, some TMs can be laterally sorted and some completely translocated by the TIM23 complex. The TIM23 complex can also import IM proteins with an N-out, C-in topology, such as Bsc1 and Tim14. Here the amphipathic signal sequence is proposed to be formed by a tight hairpin structure between the TM and a positively charged patch directly behind it. Besides import into matrix, IM and IMS, the TIM23 complex was recently reported to be involved in an unusual import pathway of some OM proteins (Song et al. 2014; Wenz et al. 2014; Sinzel et al. 2016).

“Running a tight ship”: Functional organization of the TIM23 complex

The TIM23 complex can be functionally divided into IMS-exposed receptors that recognize precursor proteins, the IM-integrated translocation channel through which the precursor proteins cross the IM in a $\Delta\Psi$ -dependent manner, and a matrix-exposed import motor that uses the energy of ATP hydrolysis to provide unidirectional transport into the matrix (Fig. 2).

Genetic and biochemical studies, performed mainly with baker's yeast, *Saccharomyces cerevisiae*, showed that at least eleven, highly evolutionary conserved subunits form the TIM23 complex (Fig. 2). Eight of them, Tim23, Tim17,

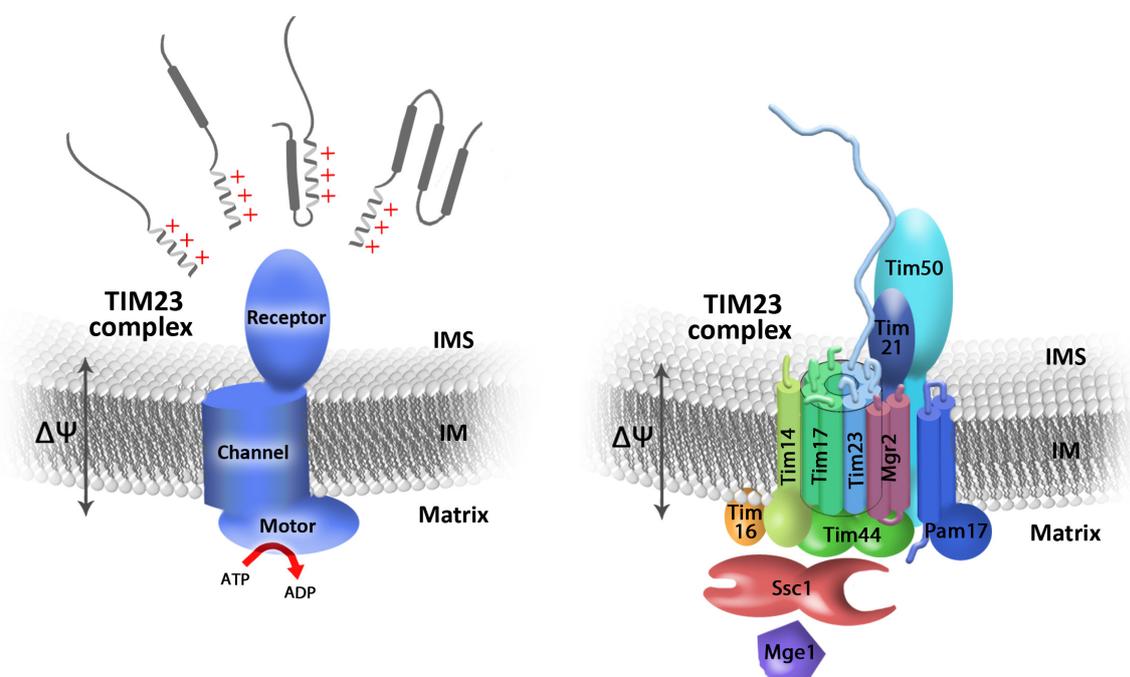


Fig. 2. Schematic representation of the TIM23 complex. The TIM23 complex can be functionally divided into three functional units - receptors, translocation channel and import motor (left panel). Eleven subunits of the TIM23 complex were identified to date. These are Tim23, Tim17, Tim50, Tim44, Tim14, Tim16, mtHsp70 (Ssc1), Mge1, Tim21, Mgr2 and Pam17 (right panel). See text for details. IMS, intermembrane space; IM, inner membrane.

Tim50, Tim44, Tim16 (Pam16), Tim14 (Pam18), mtHsp70 (Ssc1) and Mge1, are essential for yeast cell viability, demonstrating their essential roles in mitochondrial biogenesis. Deletions of the remaining three subunits, Tim21, Pam17 and Mgr2, which appear to modulate the import process, can be tolerated by yeast cells.

“Hoist the anchor”: Receptors of the TIM23 complex receive incoming precursor proteins

The subunit of the TIM23 complex that first recognizes presequences from the TOM complex is the main receptor Tim50. It has a large C-terminal globular domain in the IMS, a single transmembrane segment in the IM and a short segment in the matrix (Geissler et al. 2002; Yamamoto et al. 2002; Mokranjac et al. 2003a). The C-terminal IMS domain of Tim50, that is sufficient to support the function of the full-length protein (Mokranjac et al. 2009), can be divided in two subdomains - a highly conserved core domain and a presequence binding domain (PBD) at the very C-terminus that appears to be fungi-specific. Tim50 is in close proximity of the TOM complex and recognizes presequences as soon as they appear at the outlet of the TOM complex (Yamamoto et al. 2002; Mokranjac et al. 2003a; Mokranjac et al. 2009). Both the core domain of Tim50 and the PBD were shown to bind to presequences (Lytovchenko et al. 2013; Rahman et al. 2014). However, how the two cooperate during translocation of proteins into mitochondria remains unknown.

Tim50 cooperates with the IMS-exposed domain of Tim23 during recognition of presequences. Mutations that destabilize interactions between Tim50 and Tim23 impair Tim50 receptor function, reducing the efficiency of protein import along the presequence pathway and impairing the growth of yeast cells (Gevorkyan-Airapetov et al. 2009; Mokranjac et al. 2009; Tamura et al. 2009). Intriguingly, mutations of Tim50 that impair binding to Tim23 map to two distinct patches on Tim50 (Dayan et al. 2019). Whether two patches are directly involved in Tim23 binding remains unclear. Interaction between Tim50 and Tim23 is important for maintaining the IM permeability barrier (Meinecke et al. 2006) and is also modulated by the lipid composition of the membrane (Malhotra et al. 2017).

The IMS-exposed domain of Tim23 appears to be a central hub in the IMS that regulates transfer of precursor proteins between the translocation channels in the outer and inner membranes. This intrinsically disordered domain was shown to interact, in a likely highly dynamic manner, with a number of the components of both TOM and TIM23 complexes, with incoming precursor proteins and with the mitochondrial membranes (Gevorkyan-Airapetov et al. 2009; Tamura et al. 2009; de la Cruz et al. 2010; Marom et al. 2011; Lytovchenko et al. 2013; Bajaj et al. 2014a, 2014b). It can

also dimerize in response to $\Delta\Psi$ and the dimers dissociate in response to presequence binding (Bauer et al. 1996). Protease accessibility experiments with intact mitochondria demonstrated that ~20 residues at the very N-terminus of Tim23 can even extend outside of the mitochondria. The extent of exposure of Tim23 on the mitochondrial surface is regulated by Tim50, the TOM complex and the translocation activity of the TIM23 complex (Popov-Celeketic et al. 2008; Gevorkyan-Airapetov et al. 2009; Tamura et al. 2009; Waegemann et al. 2015). Molecular details of all of these interactions and their dynamics during transfer of precursor proteins from the translocation channel in the OM to the translocation channel in the IM remain to be examined. However, it is clear that a $\Delta\Psi$ dependent step is required to transfer the precursor protein to the translocation channel and initiate translocation across the IM.

“Go with the flow”: Aqueous channel allows precursors to pass the IM

The molecular nature of the translocation channel of the TIM23 complex is still unclear. Tim17 and the C-terminal domain of Tim23 belong to same protein family and have four predicted transmembrane segments, which embed the proteins in the IM. Electrophysiological measurements with recombinant Tim23 have shown that Tim23 alone can form an aqueous channel (Truscott et al. 2001). Whether Tim17 contributes to the formation of the translocation channel is still not clear. A more regulatory role was proposed for this protein in stabilizing the twin pore structure of the TIM23 channel and regulating its voltage gating (Martinez-Caballero et al. 2007). Unlike Tim23, Tim17 does not have a large N-terminal extension in the IMS. However, a ca. 10 amino acid long N-terminal extension of Tim17, which contains a conserved negatively charged motif, was reported to be important for gating of the TIM23 channel, and thus for protein import (Meier et al. 2005a). Gating of the TIM23 channel is also dependent on two conserved cysteine residues in Tim17 that form a disulfide bond between TM 1 and TM2 (Ramesh et al. 2016). Both Tim17 and Tim23 contain multiple GxxxG motifs in their TMs. These motifs play important roles in maintaining the structural integrity of the TIM23 complex and are likely involved in TM packing (Demishtein-Zohary et al. 2015, 2017). TM1 and TM2 of Tim23 are in direct contact with translocating proteins, TM2 and likely also TM1 face an aqueous environment, and are hence likely directly involved in forming the protein-conducting channel (Alder et al. 2008a). TM1 is in close proximity to Tim17 and Tim50 and, together with TM2, responds dynamically to the presence of $\Delta\Psi$ by undergoing conformational changes that likely lead to channel opening (Alder et al. 2008b; Malhotra et al. 2013). Whereas TM3 and TM4 of Tim23 are dispensable

for yeast cell viability (Pareek et al. 2013), all four TMs of Tim17 are essential (Demishtein-Zohary et al. 2017). TM1 and TM2 of Tim17 are important for the interaction with Tim23 and TM3 and TM4, with the short loop in between, play an essential role in recruiting the import motor (Demishtein-Zohary et al. 2017).

Membrane potential is required to activate the translocation channel of the TIM23 complex. Even though the true nature of the voltage sensor of TIM23 complex remains unclear, this role has been attributed to the IMS domain of Tim23, which can dimerize in response to $\Delta\Psi$ (Bauer et al. 1996), and the conserved negatively charged residues in the N-terminal stretch of Tim17 (Meier et al. 2005a). Membrane potential also generates an electrophoretic force that was suggested to pull positively charged presequences towards the matrix. Matrix exposed loops of Tim17 and Tim23 recruit Tim44, the organizer of the import motor, to the matrix face of the translocation channel (Ting et al. 2014, 2017; Demishtein-Zohary et al. 2017). Thus, presequences can be handed-over to the import motor of the TIM23 complex.

“Land ahoy!”: Import motor provides unidirectionality to the import process

Complete translocation into the matrix requires the ATP-dependent action of the import motor of the TIM23 complex. Tim44 is a peripheral membrane protein that couples the import motor to the translocation channel. It is comprised of an N-terminal intrinsically disordered domain and a C-terminal globular domain. The C-terminal domain engages Tim17 and Tim23 and the N-terminal domain recruits the import motor components (Schiller et al. 2008; Banerjee et al. 2015; Ting et al. 2017). Tim44 not only acts as a platform for recruitment of the import motor to the exit of the translocation channel, but it also interacts with presequences (Marom et al. 2011; Ting et al. 2017).

The import motor components are mitochondrial Hsp70 (mtHsp70, also known as Ssc1 in yeast) and its co-chaperones Tim14 (Pam18), Tim16 (Pam16) and Mge1. The energy of ATP is used by mtHsp70 for further import of precursor proteins into the matrix and the other subunits regulate this process. mtHsp70 is a member of the conserved family of Hsp70 chaperones (Rosenzweig et al. 2019). They are comprised of an N-terminal nucleotide-binding domain (NBD) which binds ATP and a C-terminal substrate-binding domain (SBD) which binds aggregation-prone stretches of hydrophobic amino acid residues that are exposed in unfolded proteins. When ATP is bound to the NBD, the SBD is in an open conformation, characterized by low substrate affinity. When ATPase activity is triggered, NBD hydrolyzes ATP to ADP and the SBD closes, trapping the bound substrate. Upon ADP release and binding of a new molecule

of ATP to NBD, the SBD opens, releasing the bound substrate. Two types of co-chaperones, J-proteins and nucleotide exchange factors (NEFs), enable this cycle to progress in a regulated and efficient manner. J-proteins stimulate the ATPase activity of NBD and NEFs help the release of ADP, resetting the cycle by allowing a new ATP molecule to bind. In the TIM23 complex, Tim14 is the J-protein that stimulates ATP hydrolysis by mtHsp70, and thus enables tight binding of incoming proteins to mtHsp70 (D’Silva et al. 2003; Mokranjac et al. 2003b; Truscott et al. 2003). J-like protein Tim16 recruits Tim14 to the TIM23 complex, regulating the stimulatory activity of Tim14 (Frazier et al. 2004; Kozany et al. 2004; Li et al. 2004; D’Silva et al. 2005; Mokranjac et al. 2006). The molecular understanding of the function of the import motor is still missing. It is likely that, once the translocating protein is stably captured by mtHsp70 upon ATP hydrolysis, mtHsp70 is released from the channel into the matrix, taking along a segment of the translocating protein and leaving space for another molecule of mtHsp70 to bind to the next incoming segment of the translocating protein. In the matrix, the nucleotide exchange factor Mge1 exchanges an ADP molecule with ATP, releasing the captured protein from mtHsp70 and resetting the cycle. mtHsp70 not only has a role during the import process, but is also important for folding of precursor proteins, which arrive in an unfolded state in the matrix.

“Trim the sails”: Lateral insertion by the TIM23 complex and regulatory subunits

The TIM23 complex can import precursor proteins into the matrix and sort them laterally into the IM. The unique feature of the TIM23 complex among protein translocases in the cell is that it can discriminate between *bona fide* transmembrane segments. It can open laterally to release ‘stop-transfer’ signals into the IM but it can also translocate some transmembrane segments completely into the matrix. The latter ones are then inserted into the IM from the matrix side by the OXA complex. In comparison to the TMs that are translocated by the TIM23 complex, the TMs that are laterally sorted are slightly more hydrophobic, lack proline residues and are flanked by charged residues (Meier et al. 2005b).

Which subunits of the TIM23 complex recognize lateral sorting signals and form the lateral gate and how TMs are laterally inserted into the IM remains unclear. Certain mutations of Tim17 specifically impair lateral insertion and have no effect on translocation of proteins into the matrix (Chacinska et al. 2005). In addition to stimulating the ATPase activity of Ssc1, Tim14 seems to have an additional active role during lateral insertion (Popov-Celeketić et al. 2011; Schendzielorz et al. 2018). The role of the three non-essential subunits of the TIM23 complex, Tim21, Pam17 and Mgr2,

also appears to be in differential sorting of proteins. Deletion of Mgr2 promotes lateral insertion, whereas its upregulation delays it (Ieva et al. 2014). One model of function of the TIM23 complex proposed that lateral insertion is mediated by a Tim21-containing, but motor-free TIM23 complex (Chacinska et al. 2005). However, subsequent experiments revealed that the import motor associates with the channel irrespective of the translocation activity of the TIM23 complex (Tamura et al. 2006; Popov-Celeketic et al. 2008), suggesting that the TIM23 complex functions as a single entity that is actively remodeled to translocate proteins across or insert them into the IM membrane (Popov-Celeketic et al. 2008). The latter model proposed that the conformational changes that underly differential sorting of proteins are driven by recognition of targeting signals in the translocating proteins, and modulated by the antagonistic behavior of Tim21 and Pam17. Tim21 was also found to bind very efficiently to the TOM complex *in vitro* (Chacinska et al. 2005; Mokranjac et al. 2005), however, the *in vivo* significance of this finding appears to be limited (Waegemann et al. 2015). The IMS-exposed domain of Tim21 seems to modulate interactions among the receptors of the TIM23 complex and their interplay with the presequences (Lytovchenko et al. 2013). Tim21 may also improve the energetics of the TIM23 complex, by coupling it to the respiratory chain complexes (van der Laan et al. 2006). Pam17 was recently reported to promote translocation of hypersensitive precursor proteins into the matrix (Schendzielorz et al. 2017). These proteins appear to require $\Delta\Psi$ not only for translocation of their presequences but also of their mature parts.

“Loose cannon”: TIM23 complex and human disease

Inspection of eukaryotic genome sequences has shown that components of the TIM23 complex are highly conserved over the course of evolution, suggesting that the structure and function of the complex are equally conserved. Direct analyses of the human TIM23 complex are still in their very early stages. The data available so far revealed only very few differences between yeast and human TIM23 complexes (Demishtein-Zohary and Azem 2017; Kang et al. 2018). Whereas in yeast all components are encoded by single genes (except for Tim14 that has a paralog Mdj2), the human genome encodes for three different Tim17 (hTim17A, hTim17B1 and hTim17B2) and two different Tim14 (DnaJC15 and DnaJC19) proteins. All other subunits have one corresponding homolog, though a homolog of Pam17 has not yet been identified. In addition, unlike its yeast counterpart, hTim50 was reported to possess phosphatase activity, the role of which still needs to be resolved. So far, three distinct TIM23 complexes in human mitochondria have been identified. Tim17A appears to associate only with DnaJC15

and Tim17B1 and B2 with DnaJC19. Tim17B-containing complexes are essential for protein import into the matrix and, thus, cell viability, whereas the TIM23 complex containing Tim17A appears dispensable for cell growth (Sinha et al. 2014).

Concerning the essential nature of the majority of the TIM23 components for biogenesis of mitochondria and the viability of yeast cells, it has been assumed that loss of function mutations in TIM23 components would be embryonically lethal in humans. Indeed, homozygous Tim23 knockout is lethal in mice and heterozygous mice have neurological phenotypes and reduced life span (Ahting et al. 2009). It has therefore come as a surprise when point mutations and alteration in expression of different essential subunits of the TIM23 complex were identified in patients with different disorders (Demishtein-Zohary and Azem 2017; Kang et al. 2018). Mutations in hTim50 are associated with microcephaly, encephalopathy, epilepsy, delayed growth, vision loss, breast cancer and 3-methylglutaconic aciduria. hTim44 is linked to oncocytic thyroid carcinoma and diabetes. hTim16 (Magma) and hmtHsp70 (Mortalin) are found mutated in skeletal dysplasia. Mortalin is also linked to EVEN-PLUS syndrome and Parkinson's disease. Loss of DnaJC19 has been found in patients with DCMA syndrome. Certain cancer types showed changes in the expression levels of Tim17A, Tim50, Magma, DnaJC15 and Mortalin. Recently, it has been shown that mutant huntingtin protein, which causes Huntington's disease, clogs the TIM23 channel, preventing import of endogenous matrix proteins and thus likely contributing to the pathology of the disease (Yablonska et al. 2019). In general, it is likely that any problem in the function of the TIM23 complex will cause problems in biogenesis of mitochondria and will thus result in malfunctioning mitochondria. Why mutations identified so far display such specific phenotypes and tissue specificity remains to be determined.

CONCLUDING REMARKS

Our understanding of the TIM23 complex has considerably improved over the years. Still, the increasing number of reports demonstrating the potential involvement of the TIM23 complex in various human diseases reminds us that the way ahead is still a very long one. Molecular understanding of the structure and function of the TIM23 complex under physiological conditions is necessary to understand the pathologies that result from its malfunction. How are presequences recognized and, what is the degree of flexibility in recognition? What kind of conformational changes enable movement of presequences from their initial recognition in the IMS till their cleavage in the matrix? When

and how do the presequences sense and harvest the $\Delta\Psi$ electrophoretic force? How does the translocation channel look like and how does it open laterally? How is the energy of ATP hydrolysis converted into unidirectional transport across the IM? These are just some of the questions that need to be answered in the future.

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