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REVIEW

The TIM23 mitochondrial protein import complex: function and dysfunction

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Abstract Mitochondria acquire the majority of their proteins from the cytosol in a process that is mediated by intricate multimeric machineries designed to allow proteins to cross and/or to insert themselves into the two mitochondrial membranes. Ongoing studies carried out in yeast over the past few decades have led to the discovery of numerous protein components that constitute several mitochondrial translocases. One of these complexes, the mitochondrial TIM23, is the major translocase for matrix proteins and is the focus of this review. The components of the TIM23 complex are categorized into four functional types. The first type plays the role of receptor for preproteins in the intermembrane space. The second type forms the actual channel that allows proteins to cross the inner mitochondrial membrane. The third species functions as part of the motor that mediates the final steps of import across the inner membrane. Additional components play regulatory roles orchestrating the action of this myriad of subunits. Recent studies provide new insights into the function of the mammalian TIM23 complex and the role that it plays under pathological conditions.

Keywords Mitochondrial protein import · TOM complex · TIM23 complex · Import motor · Pam complex

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Introduction

The odyssey of nuclear-encoded but mitochondrially localized proteins to their final destination begins upon their translation in the cytosol. Most mitochondrial proteins are translocated posttranslationally across the mitochondrial outer membrane, but some also undergo cotranslational import (Knox et al. 1998; Lesnik et al. 2014; Yogev et al. 2007). Proteins that localize to the matrix need to cross both the outer and inner mitochondrial membranes. This process is mediated by two machineries acting in a consecutive concerted manner, the TOM (translocase of the outer mitochondrial membrane) and the TIM23 (translocase of the inner mitochondrial membrane) complexes.

The TOM complex is the main entry pore for most of the proteins that are destined for the mitochondria (Dukanovic and Rapaport 2011). Once a precursor protein is synthesized in the cytosol, it is escorted to the mitochondrial outer membrane by cytosolic chaperones. The translocation event commences upon interaction with the receptor subunits of the outer-membrane TOM complex. These receptor subunits also recognize the escorting chaperones thereby forming a receptor-precursor-chaperone complex. Further binding of ATP to this complex causes the dissociation of the precursor protein from the chaperone and its insertion into the outer-membrane import channel (Komiya et al. 1997; Young et al. 2003). Once the precursor protein has crossed the outer membrane, it binds components of the TOM complex located on the *trans* side of the membrane. This facilitates passage of the precursor proteins across the outer membrane and into the inter-membrane space (IMS; Bolliger et al. 1995; Mayer et al. 1995) in which additional translocases assist the polypeptide in reaching its final destination.

Precursor proteins that utilize the TIM23 complex cross the inner membrane and eventually reach their correct destination.

These include proteins that are translocated into the matrix, a number of inner membrane proteins, and a few IMS proteins, and even some outer membrane proteins have recently been shown to be processed by this translocase (Sinzel et al. 2016; Song et al. 2014; Wenz et al. 2014). In this review, we elaborate on our present knowledge about the function of the TIM23 complex and the specific roles of its constituent components and cofactors.

The TIM23 complex

TIM23 is a hetero-oligomeric complex that is anchored to the mitochondrial inner membrane. It consists of a membrane-embedded core complex and an import motor, which is largely exposed to the matrix side of the inner membrane. The integral membrane protein sub-complex constitutes the import channel and consists of three essential subunits: Tim23 (Dekker et al. 1993), Tim17 (Maarse et al. 1994), and Tim50 (Geissler et al. 2002; Mokranjac et al. 2003a; Yamamoto et al. 2002), plus the non-essential Mgr2 (Gebert et al. 2012) and Tim21 (Chacinska et al. 2005; Mokranjac et al. 2005). The import motor (also called the PAM or presequence translocase-associated motor complex) is responsible for completing protein translocation across the inner membrane in a process that derives energy from ATP hydrolysis. It consists of several components: Tim44 (Horst et al. 1993; Maarse et al. 1992), the 70-kDa mitochondrial heat shock protein (mtHsp70; Kang et al. 1990; Schneider et al. 1994), Tim16 (also called Pam16; Frazier et al. 2004; Kozany et al. 2004), Tim14 (also called Pam18; Li et al. 2004; Mokranjac et al. 2003b; Truscott et al. 2003), Pam17 (van der Laan et al. 2005), and Mge1 (Laloraya et al. 1994; Voos et al. 1994; Fig. 1a).

The TIM23 complex is extremely complex and dynamic, reflecting the elaborate pathway that the precursor protein must go through once it enters the inter-membrane space. As a first step, the precursor is recognized by the receptor subunits of TIM23 in the IMS. Then, it is transferred to the import channel found in the inner membrane. Once in the import pore, the precursor is sorted either to the inner membrane or the matrix in a process that is not fully understood. For matrix-destined proteins, the import motor takes over and actively translocates the protein into the matrix. Finally, regardless of the precursor protein's final destination, the targeting signal is cleaved in the matrix, and the precursor reaches its mature form.

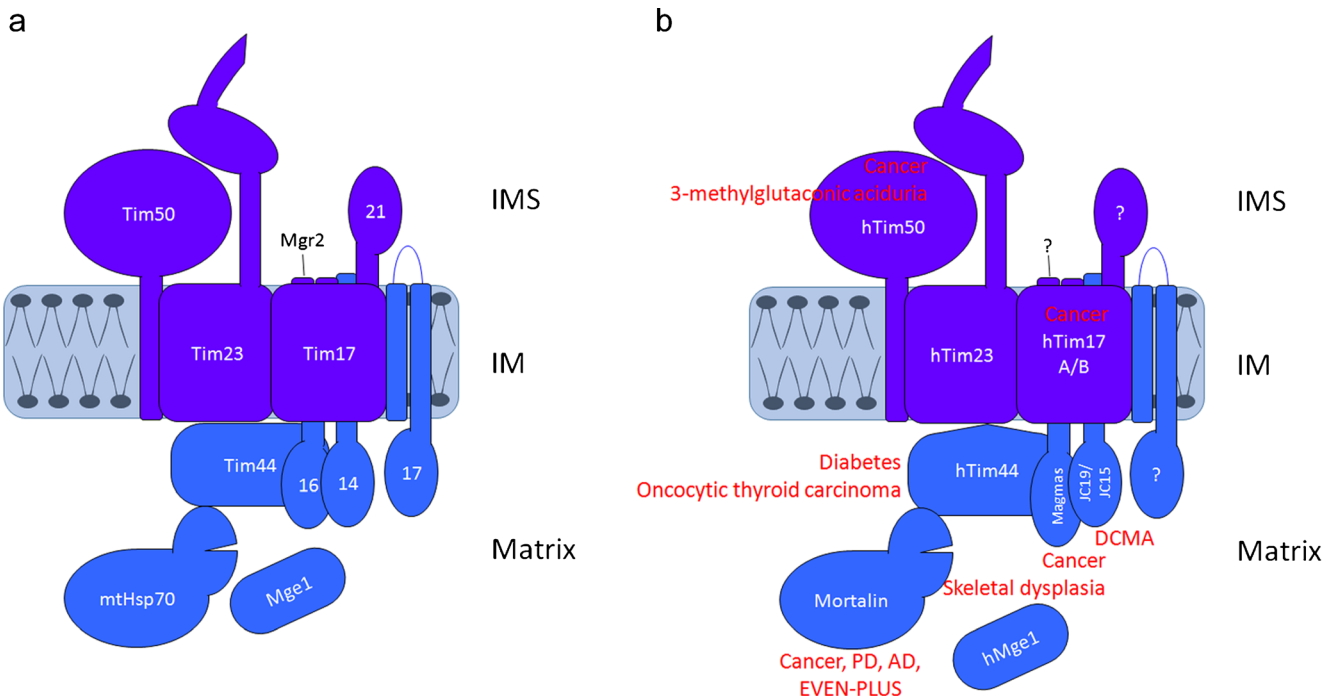

Fig. 1 TIM23 (translocase of the inner mitochondrial membrane) complex. **a** Various members of the TIM23 complex have been identified in yeast. The TIM23 complex consists of integral membrane proteins (*purple*) that participate in the initial import from the TOM (translocase of the outer mitochondrial membrane) complex to the TIM23 complex and partial import across the inner membrane (*IMS* inter-membrane space, *IM* inner membrane, *Mge1* cochaperone). Complete import across the inner membrane and into the matrix requires the associated import motor (*blue*) and additional energy, which is supplied by ATP and utilized by mtHsp70 (70-kDa mitochondrial heat shock protein). **b** Human TIM23 complex. Human homologs of various complex members have been identified and studied. Almost all members are associated with diseases (*red*). Members of the complex that have not been studied in humans are indicated by *question marks* (*PD* Parkinson's disease, *AD* Alzheimer's disease, *DCMA* dilated cardio myopathy with ataxia, *EVEN-PLUS* an autosomal recessive syndrome characterized by epiphysis and vertebral dysplasia and numerous other abnormalities)

Over the past few years, a large amount of information has been accumulated regarding the structural organization of the complex, in yeast and its homologs in mammals. Here, we review the latest discoveries regarding the structure and function of the TIM23 complex and the way that various components of the system are involved in disease and pathological conditions.

Tim50 and Tim23 act as receptors for incoming precursor proteins

Tim23 and Tim50 are both essential proteins of the TIM23 complex in yeast. Tim50 exposes, to the IMS, a large hydrophilic domain that is anchored in the membrane by one transmembrane helix (Geissler et al. 2002; Mokranjac et al. 2003a; Yamamoto et al. 2002). Tim23 consists of two main domains: its N-terminal half is hydrophilic and is exposed to the IMS, whereas its C-terminal half is predicted to span the inner membrane with four trans-membrane segments (TM1-4; Dekker et al. 1993). Tim50 has been found to keep the translocation channel closed in a presequence-regulated mode (Meinecke et al. 2006). In the IMS, Tim23 and Tim50 bind each other, and this interaction has been determined to be essential for protein translocation by the TIM23 complex (Gevorkyan-Airapetov et al. 2009; Tamura et al. 2009). Both proteins also bind the incoming precursor protein, and together, they act as receptors for the imported presequence (Bauer et al. 1996; Geissler et al. 2002; Marom et al. 2011; Mokranjac et al. 2009; Schulz et al. 2011). Tim50 has been shown to bind precursor proteins while still passing through the TOM complex. Hence, Tim50 is the first component of the TIM23 complex that is expected to interact with the precursor protein and mediates the transfer of the incoming protein from the TOM complex to the TIM23 complex (Chacinska et al. 2003; Geissler et al. 2002; Mokranjac et al. 2003a, 2009; Waegemann et al. 2015; Yamamoto et al. 2002). Tim50 binds the precursor at two different binding sites that communicate with each other. The C-terminal binding site seems to transfer the precursor protein to the Tim50 core domain, and from there, the precursor protein is transferred to the inner membrane channel (Rahman et al. 2014).

The hydrophilic N-terminal half of Tim23 is intrinsically unstructured (de la Cruz et al. 2010; Gevorkyan-Airapetov et al. 2009), implying that it is extremely flexible and should be able to bind various partner proteins. Indeed, in addition to Tim50 and the incoming precursor protein, the N-terminal domain also forms dimers (Alder et al. 2008b; Bauer et al. 1996) and binds Tom22 and Tim21 at several contact sites in its soluble domain (Bajaj et al. 2014; Tamura et al. 2009). Furthermore, its extreme N-terminus has been shown to span the outer membrane once a precursor protein is in transit (Popov-Celeketic et al. 2008), thereby enabling a tight connection between the two mitochondrial membranes. Tim50 also interacts with various partner proteins, among them Tom22 and Tim21 (Chacinska et al. 2003; Lytovchenko et al. 2013; Shiota et al. 2011; Tamura et al. 2009; Waegemann et al. 2015). This demonstrates that the hand-over of the precursor protein to the Tim23 channel is complicated and involves cooperation of several different components of both the TOM and TIM23 complexes.

In short, the IMS domains of both Tim23 and Tim50 serve as active components of the complex in the IMS side. Tim50 is the first component of the complex to interact with the incoming precursor protein and, together with Tim23, directs the precursor to the import channel. Tim23 serves as a hub that binds various subunits of the complexes thereby keeping the components in close proximity to each other, while enabling the flexibility that is needed for the transfer of the protein from the TOM complex to the import channel.

Crossing the inner membrane

Once the precursor has reached the import channel, it next has to cross the inner membrane. This process is driven by three main forces. The first is the membrane potential across the inner membrane. A second diving force is the increasing affinities of the precursor proteins to the components on the *trans* side (i.e., Tim44) of the complex compared with its affinities to the components in the *cis* side (i.e., Tim23 and Tim50; Marom et al. 2011). Finally, the third driving force is the motor function that is carried out by the ATP hydrolyzing mtHsp70 chaperone and its associated subunits.

The precursor crosses the inner membrane via the voltage-gated channel of TIM23. The pore has been found to be ~13 Å wide and can therefore accommodate only one α-helix in transit (Schwartz and Matouschek 1999; Truscott et al. 2001). Early on, Tim23 was shown to constitute an integral part of the import channel itself. Tim17 is an additional essential core protein of the TIM23 complex, and its membrane domain is homologous to Tim23 at the primary sequence level (Kubrich et al. 1994; Sirrenberg et al. 1996). It is predicted to span the inner membrane of the mitochondrion with four trans-membrane helices and exposes short N-terminal and C-terminal segments to the IMS (Kubrich et al. 1994). Tim17 was initially thought to constitute part of the import channel. However, the Tim23 C-terminal membrane domain was eventually shown to be sufficient to form a pore, which lacked the pre-sequence sensitivity (Truscott et al. 2003). In mitoplasts prepared from wild-type mitochondria, the TIM23 complex possesses a twin-pore structure, whereas in mitoplasts prepared from Tim17-depleted mitochondria, the twin-pore collapses into a single pore. Hence, Tim17 seems to regulate the formation of the correct twin-pore structure, and this function is attributed to its C-terminal segment (Martinez-Caballero et al. 2007). The N-terminal segment of

Tim17 has been found to regulate voltage gating of the channel (Martinez-Caballero et al. 2007).

Recent work has revealed that TMs 1 and 2 of Tim23 are found in close proximity to the substrate during its import suggesting that they comprise part of the import channel (Alder et al. 2008a; Pareek et al. 2013). TMs 1 and 2 have also been found to mediate the interaction of Tim23 with Tim17 and the dimerization of Tim23 through the GxxxG motifs present throughout these helices (Alder et al. 2008b; Demishtein-Zohary et al. 2015; Pareek et al. 2013). Furthermore, we have determined that the destabilization of GxxxG interactions in these helices causes Tim44 to dissociate from the TIM23 complex (Demishtein-Zohary et al. 2015).

Taken together, the data suggest the following sequence of events. Once the precursor has reached the import channel, it is bound by the channel-forming Tim23 subunit, while Tim17 regulates the formation of the correct channel structure and presequence sensitivity. The membrane potential and the increasing affinities of the precursor protein to Tim44 in *trans* are the main forces that drive the precursor across the inner membrane and to emerge in the matrix.

Final steps of translocation into the matrix

Final translocation across the inner membrane into the matrix is facilitated by the translocation motor and fueled by ATP hydrolysis. Tim44 acts as an anchor that recruits mtHsp70 and other members of the motor to the TIM23 complex (Kronidou et al. 1994). MtHsp70 contains an ATPase domain and a peptide-binding pocket. Upon binding ATP, the peptide-binding pocket of mtHsp70 opens thus allowing substrate binding. ATP hydrolysis to ADP causes the binding pocket to lock on the bound substrate, concomitant with the release of mtHsp70 from Tim44 and the further insertion of the precursor into the matrix (Liu et al. 2003; Slutsky-Leiderman et al. 2007). Release of the peptide from mtHsp70 is enabled once ADP is removed and exchanged with ATP (Bukau et al. 2006). The membrane-associated J-protein complex, Tim14/Tim16, and the cochaperone, Mge1, modulate the function of mtHsp70. Tim14 stimulates ATP hydrolysis to ADP thereby causing the binding pocket to close (Mokranjac et al. 2003b; Truscott et al. 2003). Tim16 strongly interacts with Tim14 and seems to regulate the ability of the latter to stimulate the ATPase function of mtHsp70 (Li et al. 2004). Mge1 is a nucleotide exchange factor that replaces ADP with ATP, enabling the release of the bound peptide and the recycling of mtHsp70 (Miao et al. 1997).

The interface between the import motor and the membrane-embedded channel complex has been mapped to a number of different interaction points. First, Tim44 has been found to bind Tim23 (Ting et al. 2014) and Tim17 (Banerjee et al. 2015). A tertiary interaction seems to occur between Tim23, Tim17, and Tim44 and enables the emerging precursor protein to immediately bind mtHsp70 for further translocation into the matrix. A second interface is found between Tim44 and Tim16 (D'Silva et al. 2008; Schilke et al. 2012). An additional interaction site has been shown to exist between Tim17 and Tim14 (Chacinska et al. 2005). Tim14 and Tim16 form a tight complex, the destabilization of which causes Tim14 to dissociate from the TIM23 supercomplex (D'Silva et al. 2008). Hence, Tim16 also recruits Tim14 to the membrane-embedded complex. Pam17 has also been shown to bind Tim17, further stabilizing the membrane-embedded complex (Ting et al. 2014). In addition, the first loop of Tim23 has also been determined to be important for the recruitment of PAM members (Pareek et al. 2013).

Thus, the import motor is bound to the membrane-embedded complex via a number of different subunit interactions to ensure its presence upon emergence of the precursor from the import channel and to allow non-interrupted import across the inner membrane into the matrix.

Regulation of the function of the complex

Once the precursor enters the inner membrane channel, it is then either sorted to the inner membrane or fully transferred to the import motor for its final translocation to the matrix. The way that this decision is made remains to be elucidated. Several non-essential components including Tim21, Mgr2, and Pam17 have been suggested to regulate this process.

The N-terminal domain of Tim21 spans the inner membrane of the mitochondria with one predicted transmembrane helix, and its C-terminal domain exposes a hydrophilic domain to the IMS (Chacinska et al. 2005; Mokranjac et al. 2005). Tim21 has been shown to connect the TIM23 complex to the TOM complex via interaction with Tom22 (Albrecht et al. 2006; Chacinska et al. 2005, 2010; Mokranjac et al. 2005; Tamura et al. 2009). It has been found to play a role in forming the TOM-TIM23-precursor complex; however, it can be exchanged by a new Tim21 molecule, once this complex is formed (Ieva et al. 2014), demonstrating the dynamic nature of its interaction with the core of the TIM23 complex. Tim21 has also been shown to couple the respiratory-chain super-complexes (complex III and IV) to the TIM23 complex (van der Laan et al. 2006; Wiedemann et al. 2007). Indeed, it has been found to bind both complexes independently, again, highlighting its highly dynamic character.

Mgr2, a second regulator of the complex, is predicted to consist of two transmembrane helices. It has been proposed to connect Tim21 to the core TIM23 complex because, in its absence, Tim21 binds only the respiratory complexes (Gebert et al. 2012). Recent work has shown Mgr2 to regulate and control the lateral release of inner membrane proteins from the TIM23 complex to the inner membrane (Ieva et al. 2014). In its absence, the lateral release of the protein to the

inner membrane is enhanced, whereas over-expression of Mgr2 delays the lateral release of the protein to the inner membrane.

Pam17, a third regulatory protein of the complex, is considered to be a part of the import motor and to participate in the initial transfer across the inner membrane (Schiller 2009; van der Laan et al. 2005). Data suggest that this protein modulates the activity of the complex in a manner antagonistic to that of Tim21 (Popov-Celeketic et al. 2008); however, the exact mode of action has yet to be clarified.

Human TIM23 under pathological conditions

In recent years, the human TIM23 complex has attracted considerable attention. Several human orthologs of yeast TIM23 subunits have been studied, mostly in the context of their involvement in a variety of pathological conditions, as summarized in Table 1 (see also Fig. 1b).

An indication that defects in import machinery components can lead to pathological situations was first demonstrated with the human deafness dystonia syndrome, which was shown to be caused by mutations in human Tim8 (DDP1). Tim8, together with Tim13, is involved in the import of Tim23 into mitochondria, providing indirect evidence that import defects can cause mitochondrial diseases (Roesch et al. 2002; Rothbauer et al. 2001). Surprisingly, the biogenesis of Tim23 is not affected by reduced levels of DDP1; however, alterations in mitochondrial morphology have been observed (Engl et al. 2012).

Although no specific disease has been directly linked to Tim23, homozygous knockout mice are not viable, indicating that, similar to the situation in yeast, Tim23 is also essential in mice (Ahting et al. 2009). Moreover, heterozygous mice, with a 50 % reduction of the protein levels, exhibit a reduced lifespan, neurological phenotypes, and signs of premature aging.

Table 1 Human orthologs of yeast TIM23 (translocase of the inner mitochondrial membrane) subunits and their involvement in a variety of pathological conditions (*DCMA* dilated cardio myopathy with ataxia, *EVEN-PLUS* a syndrome characterized by epiphyseal, vertebral, and ear dysplasia and numerous other abnormalities)

Human orthologs of yeast TIM23 subunits	Associated pathologies
hTim17	Breast cancer
hTim50	Breast cancer 3-Methylglutaconic aciduria
hTim44	Diabetes Oncocytic thyroid carcinoma
hTim16 (Magmas)	Cancer Skeletal dysplasia
hTim14	DCMA syndrome
hmtHsp70 (mortalin)	Alzheimer's disease Parkinson's disease Cancer EVEN-PLUS

Despite the structural conservation between the yeast and human TIM23 complexes, a number of interesting differences have been observed between these translocases. Whereas in yeast, only one gene of Tim17 exists, two paralogs have been described in humans, namely Tim17a and Tim17b, the latter of which exists as two isoforms, namely $Tim17b_1$ and $Tim17b_2$ (Bauer et al. 1999). A recent study of the human TIM23 complex has demonstrated that the three Tim17 variants (Tim17a, $Tim17b_1$, and $Tim17b_2$) are present in three distinct forms of the human TIM23 complex: human translocase B1, human translocase B2, and human translocase A, in which $Tim17b_1$, $Tim17b_2$, and Tim17a are integrated, respectively (Sinha et al. 2014). Tim17a has been found to serve as a suppressor of mitochondrial DNA instability in human cells and, in this way, may prevent disorders connected with mitochondrial DNA loss (Iacovino et al. 2009). Furthermore, high expression of Tim17a has been observed to be associated with breast cancer (Salhab et al. 2010; Xu et al. 2010; Yang et al. 2016).

Another study demonstrating differences between the human and yeast complexes was reported for Tim50. Human Tim50 was shown to possess phosphatase activity in vivo, the role of which is unknown (Guo et al. 2004). However, in yeast, the active site residues of the motif are not conserved, and no evidence for phosphatase activity has been found (Geissler et al. 2002). In cells, the loss of Tim50 causes mitochondrial permeabilization and dysfunction followed by cell death (Guo et al. 2004). Furthermore, like human Tim17a, the expression of Tim50 levels are increased in breast cancer, and the suppression of its expression inhibits the ability of cancer cells to proliferate (Gao et al. 2015). Recently, a homozygous point mutation in hTim50 (G372S) has been linked to 3-methylglutaconic aciduria disorder, characterized by epilepsy, microcephaly, developmental delay, and visual deficit spastic quadriplegia (Serajee and Huq 2015).

Comparison of the human import motor with that of yeast has shown hTim44 to be loosely associated with the inner membrane. A single proline to glutamine point mutation in hTim44 has been found to be correlated with oncocytic thyroid carcinoma (Bonora et al. 2006). In addition, Tim44 has been revealed to be upregulated in hyperglycemic states in mice with diabetes (Wada and Kanwar 1998). Indeed, a transgenic mouse overexpressing Tim44 exhibits improved insulin sensitivity and is protected from type 2 diabetes and obesity (Wang et al. 2015).

Human Tim16 is termed Magmas, and its expression has been demonstrated to increase in prostate cancer cells (Jubinsky et al. 2005). Additionally, a homozygous missense mutation (N76D) in MAGMAS correlates with

severe skeletal dysplasia (Mehawej et al. 2014). Human mitochondria contain two different Tim14 homologs: DnaJC19 (JC19) and DnaJC15 (JC15). Both human B translocases interact with the JC19 DnaJ protein, whereas translocase A interacts with the JC15 DnaJ protein (Sinha et al. 2014). A point mutation in JC19 resulting in defective splicing and loss of the full-length DNAJC19 transcript has been found to be associated with dilated cardiomyopathy with ataxia (DCMA) syndrome, an inherited condition represented by heart problems, movement difficulties, and impairment in multiple body systems (Davey et al. 2006).

Finally, mortalin, the human mitochondrial Hsp70 protein, is involved in numerous cellular processes and has been demonstrated to be involved in a large number of pathological conditions. In yeast, three homologs of mtHsp70 are present in the mitochondria. The most important is the product of the Ssc1 gene, which serves as a motor component and as a major protein-folding machine (Horst et al. 1997). In human mitochondria, only one homolog exists. In addition to playing an active role in the import of proteins into the mitochondria and in the folding of proteins in the matrix, mortalin has been detected in the cytosol and the nucleus. In the cytosol, mortalin has been shown to bind and inactivate p53, a tumor suppressor protein, leading to the uncontrolled proliferation of the cells. Indeed, the upregulation of mortalin has been shown to be linked to tumorogenesis in several studies (Ando et al. 2014; Lu et al. 2011a, 2011b; Wadhwa et al. 2006). Altered expression of mortalin has been found to be connected with Parkinson's (Burbulla et al. 2014; De Mena et al. 2009; Jin et al. 2006) and Alzheimer's (Park et al. 2014) diseases. Notably, three mutations, namely R126W, A476T, and P509S, have been associated with Parkinson's disease (Burbulla et al. 2010; De Mena et al. 2009); these point mutations have been identified in heterozygous patients and are absent among the control group. However, the contribution of these mutations in mortalin for the development of early onset Pakinsons' disease is not clear (Freimann et al. 2013). Recently, two separate single homozygous point mutations in mortalin have also been found to be associated with the EVEN-PLUS syndrome, an autosomal recessive syndrome characterized by epiphysis and vertebral dysplasia and numerous other abnormalities (Royer-Bertrand et al. 2015). In these patients, the mutations in mortalin are homozygous. Interestingly, one of these mutations, R126W maps to the same amino acid whose mutation has been associated with Parkinson's disease. This highlights the great importance of the fine tuning of the protein level in vivo: if one normal copy of the protein exists, its function is partly impaired, resulting in early onset Parkinson's disease, whereas two dysfunctional copies cause severe congenital malformations and skeletal dysplasia.

Concluding remarks

Intense research over the past few decades has led to a general understanding of the way that proteins undergo translocation into mitochondria. Most of the subunits of the large TIM23 translocation supercomplex have been identified, and their specific functions are slowly being elucidated. Linkage of many diseases to mutations in the various components of the TIM23 complex highlights the great importance of each individual component and the necessity for the correct cooperation and balance between the subunits. With the exception of Tim23, all the essential Tim components in yeast have been directly linked to human diseases (Table 1). Point mutations have been associated with diseases with regard to all the essential Tim components, except for Tim23 and Tim17. Since these proteins are essential, their mutation probably leads to embryonic lethality. However, we cannot exclude the possibility that point mutations in these proteins resulting in their partial function might be connected with diseases in the future.

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