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Functional characterization of MCJ in the protein import into human mitochondria

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Erklärung

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Für meine Familie.

Im Andenken an Dr. rer. nat. Erich Klier,
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1 Introduction

1.1 Biogenesis of mitochondria requires import of nuclear encoded proteins

Mitochondria are essential organelles of all eukaryotic cells. They harbor many metabolic enzymes such as the pyruvate dehydrogenase, the enzymes of the citrate cycle and the respiratory chain complexes and are required for ATP generation by oxidative phosphorylation. Therefore, they are called the “powerhouses” of the cell. Mitochondria make other major contributions to the metabolism of the cell by ß-oxidation of activated fatty acids, by providing reduction equivalents generated in the citrate cycle, and by FeS-cluster biogenesis, to name only a few. Additionally, they exhibit a strong calcium buffering capacity within the cell and, furthermore, mitochondria play a very important role in the intrinsic pathway of apoptosis.

Having evolved from endosymbiontic bacteria mitochondria represent organelles which are enclosed by two membranes, but form a dynamic network in the cell which constantly grows (by fusion) and shrinks (by fission).

Despite mitochondria contain their own DNA, the majority of the genes coding for mitochondrial proteins has been transferred to the nucleus of the cell during evolution. This means, that only 13 proteins are encoded in the human mitochondrial genome, whereas the majority of mitochondrial proteins is synthesized in the cytosol as precursor proteins (herein after also named preproteins) and has to be imported into the mitochondria. This process is vital for the biogenesis of functional mitochondria and therefore for the many important processes mitochondria fulfill in the cell.

1.2 Import of mitochondrial proteins into the different compartments of the organelle

All mitochondrial proteins which are synthesized in the cytosol need to be transported to their specific compartment within the mitochondria where they obtain their mature fold and functionality. While low molecular weight molecules can diffuse through porins in the outer mitochondrial membrane, the mitochondrial proteins are transported by specialized translocases. Dependent on specific localization / targeting signals in their amino acid sequences the mitochondrial proteins are sorted to their target compartment within mitochondria. These compartments comprise:

- the outer membrane,

- the intermembrane space which is enclosed by the outer and inner membrane,
- the inner membrane which is folded into invaginations, the so called cristae, and which is tightly packed with proteins, and
- the matrix space.

A short overview of the translocases involved in the biogenesis of the proteins of these different compartments as it has been studied mainly in *S. cerevisiae* is given in figure 1 and is described in the following chapters.

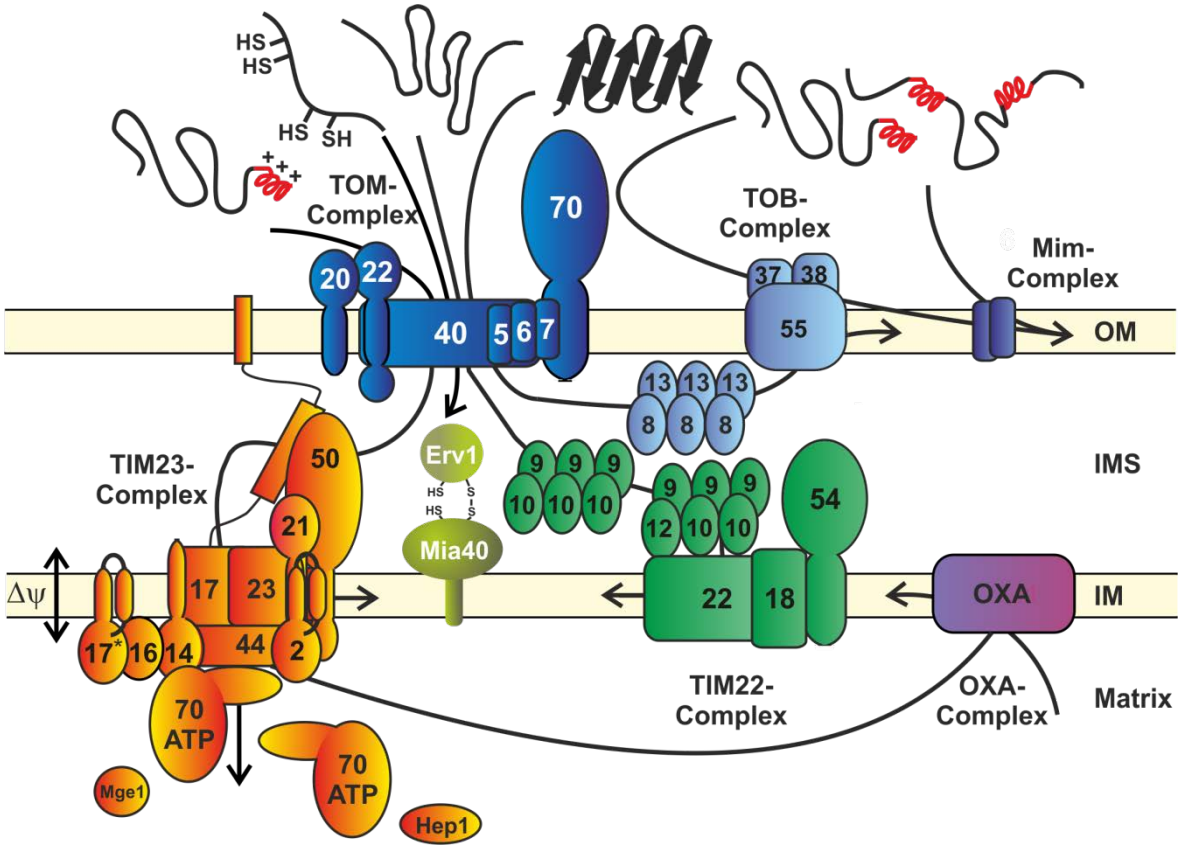

Figure 1 Model of the different translocases present in the mitochondrial membranes adapted from Mokranjac and Neupert 2010

Mitochondrial preproteins contain various specific targeting information depicted by the motifs in the precursor proteins. Upon recognition of the targeting information the preproteins are sorted to their target compartment by different translocases.

1.2.1 Sorting of proteins into and across the outer membrane

Proteins destined for the outer membrane are discussed to be inserted by two different major pathways. ß-Barrel proteins were shown to use the TOM (Translocase of the outer membrane) and the TOB (Topogenesis of the mitochondrial outer membrane ß-barrel proteins) complex, whereas α-helical proteins were demonstrated to be transported by the Mim (mitochondrial import) complex with the help of components of the TOM and TOB complex. An overview about these complexes and the pathways is given here:

1.2.1.1 The TOM complex

The TOM (Translocase of the outer membrane) complex acts as the entry gate from the cytosolic side for the vast majority of mitochondrial proteins independent of their target compartment. The TOM complex accepts the preproteins by direct interaction with them or by interaction with their cytosolic chaperone shuttles (Zara et al. 2009, Fan and Young 2011, Rimmer et al. 2011). It consists of the receptor components Tom20, Tom22, Tom70 and Tom71 (with the latter playing a minor role) and a peptide conducting pore formed by Tom40 and the small Toms, Tom5, Tom6 and Tom7 (Endo and Yamano 2010). The TOM complex provides the docking platform for a variety of preproteins by having various receptor domains. Tom20 for example is specialized to recognize the matrix targeting signal (MTS) of many matrix proteins, whereas Tom70 binds not only different specific stretches of MTS-less carrier proteins but also EEVD motifs of cytosolic chaperones. Once accepted by the receptor components, the mitochondrial preproteins are transported through the peptide conducting pore, which is mainly formed by Tom40. The exact mechanism of translocation has been discussed for a long time. Preferred interaction of Tom40 with unfolded peptide stretches may play a role as well as a chaperone-like stabilization of the pore spanning state of the imported preprotein until it is accepted on the IMS side of the outer membrane (Becker et al. 2008, Walther and Rapaport 2009, Endo and Yamano 2010).

1.2.1.2 Sorting of outer membrane ß-barrel proteins

Tom20 and Tom70 are crucial for the recognition of ß-barrel proteins at the mitochondrial surface (Keil et al. 1993, Rapaport and Neupert 1999). After translocation across the TOM complex, outer membrane ß-barrel proteins are recognized by a chaperone system, called the small Tim proteins. Five different small Tim proteins exist: Tim9, Tim10, Tim12, Tim8 and Tim13 (see 1.2.2.2 intermembrane space). The heterohexameric Tim8-Tim13 complex shuttles the ß-barrel proteins through the intermembrane space (Hoppins and Nargang 2004, Webb et al. 2006, Beverly et al. 2008, Baker et al. 2009). It shields the unfolded or partially folded proteins from aggregation in the IMS. Next, the ß-barrel proteins are delivered to the TOB/SAM complex (Topogenesis of the mitochondrial outer membrane ß-barrel proteins / Sorting and assembly machinery) of the outer membrane for their membrane insertion (Kozjak et al. 2003, Paschen et al. 2003, Wiedemann et al. 2003, Thornton et al. 2010, Zeth 2010, Wenz et al. 2014). The TOB complex consists of the essential membrane-embedded subunit Tob55 (Sam50), the essential membrane attached subunit Tob38 (Sam35) and a non-essential membrane attached subunit Tob37 (Mas37/Sam37) (Walther and Rapaport 2009). At the IMS side of the TOB complex, a POTRA (polypeptide-transport-associated) domain recognizes the arriving preproteins (Habib et al. 2007). It has been discussed that ß-barrel proteins are recognized by a C-terminal ß-signal (Kutik et al. 2008), but it also seems that targeting information is located throughout the protein (Paschen et al. 2005). The

interior of the TOB complex allows for ß-barrel formation and maturation of the preproteins. The subsequent release of a ß-barrel protein from the TOB complex into the outer membrane is then facilitated by Mdm10 (Meisinger et al. 2004). Lately, it was shown that a Tom22 mediated TOM/TOB supercomplex assembly facilitates the ß-barrel insertion (Qiu et al. 2013).

1.2.1.3 Sorting of outer membrane α-helical proteins

In contrast to outer membrane ß-barrel proteins, outer membrane spanning α-helical proteins are inserted into the membrane in different ways. α-Helical proteins can be divided into proteins containing a single transmembrane segment and proteins containing multiple transmembrane segments. Signal anchored proteins containing a single N-terminal transmembrane segment, like Tom20, were reported to be inserted into the membrane independently of the TOM complex and with the help of the Mim complex. The oligomeric Mim complex consists of Mim1 and Mim2, both spanning the outer membrane once with their C-termini facing the IMS (Dimmer and Rapaport 2010, Becker et al. 2011, Papic et al. 2011, Dimmer et al. 2012). Tail anchored proteins containing a single C-terminal transmembrane segment, like the small Toms, are discussed to be inserted into the outer membrane with the help of TOB components, Mdm10 and also Mim1 (Becker et al. 2008). For the biogenesis of the tail anchored protein Tom22 the recognition by Tom20 and Tom70 has been also found crucial (Keil and Pfanner 1993, Nakamura et al. 2004). For biogenesis of multispanning outer membrane proteins initial interaction with the receptor Tom70 and subsequent insertion via Mim1 was reported (Becker et al. 2011). Recently, it was found that also Mim2 plays a role in the insertion of membrane proteins containing multiple transmembrane segments (Dimmer et al. 2012).

Interestingly, as another possibility for the biogenesis of outer membrane proteins it was also suggested, that the TOM complex itself may release transmembrane segments of proteins laterally into the outer membrane (Harner et al. 2011). However, no physiological substrate for this pathway has been identified yet. The release of transmembrane segments into the outer membrane directly from the TOM complex has been only observed for a Tim23 preprotein artificially arrested in the TOM complex.

1.2.2 Import of proteins into the mitochondrial IMS

The intermembrane space of mitochondria harbors important proteins not only for respiration but also for transport of proteins, lipids, metabolites or metal ions and for apoptosis. Proteins which are located in the intermembrane space can be divided in three major classes dependent on their import route. In addition, some IMS proteins have not been classified yet and it is still under debate how they are imported.

1.2.2.1 Class I - Import of proteins with a matrix targeting signal

The precursors of class I IMS proteins contain a bipartite sorting signal which comprises an N-terminal matrix targeting signal followed by a transmembrane segment.

The matrix targeting signal (MTS) is necessary and sufficient to direct the N-terminus of the preproteins to the mitochondrial matrix. The MTS sequence usually counts 15 to 55 amino acid residues, among them several net positive charges, and has the ability to form an amphipathic helix with one hydrophobic and one positively charged side (von Heijne 1986, Roise and Schatz 1988, Vogtle et al. 2009). Sequential binding of this helix at various interaction sites of receptor components in the TOM and TIM23 complexes has been suggested to lead to directed transport of the proteins into the matrix (Moczko et al. 1997, Komiya et al. 1998, Marom et al. 2011). The membrane potential is necessary and sufficient to direct the MTS to the negatively charged matrix side of the inner membrane (Schleyer et al. 1982, Stuart et al. 1996, Geissler et al. 2000), where it is cleaved off the preprotein mainly by the matrix processing peptidase MPP (Taylor et al. 2001).

The transmembrane segment (TMS) following the MTS functions as a “stop-transfer”-signal and determines the lateral release of the preprotein from the TIM23 translocase into the inner membrane (Glick et al. 1992). How the “stop-transfer”-signal is exactly recognized, is still under discussion (see 1.3.3 different sorting mechanisms of the TIM23 translocase). To obtain a soluble IMS protein, the TMS is cleaved off either by the IMP complex in the IMS (for example for cytochrome b_2) or by Pcp1 in the inner membrane (for example for Mgm1) and the soluble protein is released into the IMS (Glick et al. 1992, Nunnari et al. 1993, Urban et al. 2001).

1.2.2.2 Class II - Import of proteins by the folding trap mechanism

Many soluble IMS proteins do not contain matrix targeting signals but are trapped in the intermembrane space by transient interaction with a partner protein that helps their folding and / or incorporation of a cofactor and subsequent release as a mature protein. Since passage through the TOM complex requires an at least partially unfolded state, these proteins are trapped in the IMS and cannot slide back through the TOM complex once they are folded (Herrmann and Hell 2005).

The best understood pathway in the folding trap mechanism is the Mia40-Erv1-disulfide relay system for oxidative folding. Mia40 and Erv1 (ALR1 in humans (Hagiya et al. 1994)) are ubiquitously expressed and highly conserved proteins in the IMS. Mia40 (mitochondrial intermembrane space transport and assembly) contains a structural twin Cx_9C motif, a hydrophobic substrate binding groove and a redox active CPC motif (Grumbt et al. 2007, Banci et al. 2009, Kawano et al. 2009). Substrates of Mia40 are often small (<20kDa) proteins and contain

conserved cysteine residues which are in a reduced state for passage of the TOM complex, probably stabilized by Zinc binding (Morgan et al. 2009). They also contain a MISS (mitochondrial IMS-sorting signal) / ITS (IMS-targeting signal) consensus sequence involving hydrophobic residues and a cysteine residue in an α-helical conformation. This sequence directs the preprotein most likely to the binding groove of Mia40 (Milenkovic et al. 2009, Sideris et al. 2009). The preprotein interacts with Mia40 covalently by formation of a mixed disulfide and is released in an oxidized state with an intramolecular disulfide bond (Chacinska et al. 2004, Terziyska et al. 2005, Morgan and Lu 2008, Banci et al. 2009, Kawano et al. 2009). Re-oxidation of Mia40 requires Erv1 (Lee et al. 2000, Mesecke et al. 2005, Coppock and Thorpe 2006, Fass 2008, Terziyska et al. 2009), which in turn transfers electrons to FAD which leads in the end to oxidation of cytochrome c of the respiratory chain (Allen et al. 2005, Farrell and Thorpe 2005, Bihlmaier et al. 2007, Dabir et al. 2007). Hot13 is a small cysteine rich protein that likely promotes the function of the disulfide-relay system (Curran et al. 2004, Mesecke et al. 2008).

The substrates oxidized by the Mia40-Erv1-disufide relay system fall into two major groups, namely proteins containing a twin Cx_3C motif or a Cx_9C motif. Proteins of both groups form two intramolecular disulfide bonds required for their proper function (Curran et al. 2002, Lu et al. 2004, Mesecke et al. 2005, Webb et al. 2006). *In vitro,* it has been shown that Mia40 can introduce both disulfide bonds consecutively (Bien et al. 2010). All proteins with a twin Cx_3C motif are the small Tim proteins, Tim9, Tim10, Tim8, Tim13 and Tim12, and function in the transport of mitochondrial membrane proteins (see ß-barrel proteins and TIM22 translocase). Mutation of one cysteine residue of DDP1, the human Tim8 homologue, was reported to cause the Mohr-Tranebjaerg syndrome, a neurodegenerative disorder with deafness and dystonia (Hofmann et al. 2002, Roesch et al. 2002). Proteins with a Cx_9C motif are functionally a more heterogeneous group of proteins. Many of them, such as Cox17, are involved in assembly of the cytochrome *c* oxidase (Glerum et al. 1996, Carr and Winge 2003, Gabriel et al. 2007, Longen et al. 2009), but others, such as Mdm35, contribute for example to the lipid homeostasis of mitochondria (Tamura et al. 2009, Potting et al. 2010). Recently, additional substrates of the Mia40-Erv1-disulfide relay system not containing twin Cx_3C or twin Cx_9C motifs have been described, indicating a broader substrate specificity of the system than thought (Reddehase et al. 2009).

Further proteins containing cysteine motifs but not twin Cx_3C or twin Cx_9C motifs also reside in the IMS and are imported in a folding trap manner, not directly dependent on the Mia40-Erv1-disulfide relay system. Import and maturation of Sod1 for example is facilitated by its copper chaperone, Ccs1, a Mia40 substrate, that introduces a disulfide bond and the cofactor copper into Sod1 (Culotta et al. 1997, Casareno et al. 1998, Sturtz et al. 2001, Field et al. 2003).

The best studied example for maturation by introduction of a cofactor is apocytochrome *c*. It interacts tightly with its heme lyase CCHL in the IMS which introduces its heme and thereby renders holocytochrome *c*. This interaction drives the import reaction (Mayer et al. 1995).

1.2.2.3 Class III - Import of proteins by stable attachment to the membrane

The third class of proteins is directed to the IMS by an internal targeting signal comprising specifically arranged hydrophilic amino acids. These residues are thought to be required for stable interaction with binding sites at the inner or outer membrane of the IMS. The best characterized example of such class III IMS proteins are the heme lyases (Steiner et al. 1995, Diekert et al. 1999).

1.2.3 Sorting of proteins into the inner mitochondrial membrane

Mitochondrial inner membrane proteins are transported dependent on their structure either by the TIM22 translocase or by the TIM23 translocase.

1.2.3.1 Substrates of the TIM22 translocase

Multispanning inner membrane proteins like members of the mitochondrial carrier protein family with six transmembrane segments (for example AAC) and subunits of inner membrane translocases itself with four transmembrane segments (for example Tim23) do not contain a matrix targeting signal. They contain targeting information within their hydrophobic segments and the mostly positively charged matrix exposed loops in between them (Davis et al. 1998, Wiedemann et al. 2001, Brandner et al. 2005). These proteins are delivered to the mitochondrial surface by ATP dependent chaperones, pass the TOM complex in a loop-like conformation and are accepted in the IMS by the small Tim proteins. The small Tim proteins (mainly Tim9-Tim10) shield the inner membrane preprotein from aggregation in the aqueous environment (Koehler et al. 1998, Koehler et al. 1998, Sirrenberg et al. 1998, Endres et al. 1999, Curran et al. 2002, Curran et al. 2002, Baker et al. 2009). With the help of Tim12 the preprotein is directed to the TIM22 translocase (Sirrenberg et al. 1996, Sirrenberg et al. 1998, Endres et al. 1999). The TIM22 translocase consists of four integral membrane proteins: Tim22 forms a channel in the inner membrane (Rehling et al. 2003) and Tim54 possesses a binding site for the small Tim proteins (Gebert et al. 2008, Wagner et al. 2008). Tim18 is involved in the assembly of the TIM22 translocase (Kerscher et al. 2000) and interacts with Sdh3 being the fourth TIM22 protein and at the same time a subunit of Complex II (Gebert et al. 2011). The TIM22 complex mediates the loop-like insertion of the preprotein into its channel and subsequent integration into the inner membrane in a membrane potential dependent manner (Wiedemann et al. 2001, Rehling et al. 2003, Gebert et al. 2008). Apart from the TIM22 translocase, cardiolipin was found to be important for generation of stable and functional carrier proteins. After membrane insertion the carrier proteins dimerize, thereby obtaining their mature structure (Jiang et al. 2000, Dyall et al.

2003, Claypool 2009). Lately, it was suggested that Oxa1 (see 1.2.3.2 “conservative sorting”) might also be necessary for carrier protein import (Hildenbeutel et al. 2012).

1.2.3.2 Substrates of the TIM23 translocase

Membrane proteins containing one or multiple transmembrane segments downstream of an N-terminal matrix targeting signal are inserted into the inner membrane by the “stop-transfer”-pathway or the “conservative sorting”-pathway. These preproteins do not cross the IMS in a soluble form but exist in a TOM-TIM spanning intermediate connecting the TOM and TIM23 translocases to form a supercomplex (Dekker et al. 1997, Chacinska et al. 2003).

Import of preproteins that are arrested in the TIM23 translocase by a “stop-transfer”-signal and subsequently laterally released into the inner membrane, occurs in a membrane potential dependent manner (Glick et al. 1992). The N-terminal MTS is proteolytically cleaved off in the matrix and renders the mature inner membrane protein with its C-terminus in the IMS (Taylor et al. 2001, Gakh et al. 2002, Vogtle et al. 2009).

In case of less hydrophobicity or a proline-rich, “broken” helix in one of its transmembrane segments the preprotein is fully translocated to the matrix in an ATP dependent manner (see below for TIM23 translocase and import motor) (Meier et al. 2005) and re-exported to the inner membrane via Oxa1. This pathway is called “conservative sorting”-pathway (Hartl et al. 1986). Oxa1 contains five transmembrane segments in the inner membrane and a C-terminal binding domain for mitochondrial ribosomes in the matrix (Jia et al. 2003, Szyrach et al. 2003). Oxa1 is necessary for insertion of mitochondrially encoded proteins which are mostly very hydrophobic and contain multiple transmembrane segments. It was also shown to be responsible for insertion of transmembrane segments of nuclear encoded mitochondrial proteins in the “conservative sorting”-pathway (He and Fox 1997, Hell et al. 1998).

It has been shown that these two pathways, both dependent on the TIM23 translocase, can closely cooperate. For example, the import of the ABC protein Mdl1, which spans the inner membrane multiple times, involves both pathways for its different transmembrane segments. It was reported that the first two TMS are laterally released from the TIM23 translocase, whereas the “conservative sorting”-pathway is necessary for correct insertion of the full length Mdl1 protein (Bohnert et al. 2010).

Some proteins reside in the inner mitochondrial membrane with one TMS and do not contain an N-terminal matrix targeting signal (for example Bcs1 or Tim14). They are not imported by either of these pathways. They harbor a putative targeting signal downstream of their transmembrane

segment. It is speculated that they are imported in a loop-like conformation through the TIM23 translocase bringing their C-terminus to the matrix (Fölsch et al. 1996).

1.2.4 Import of proteins into the mitochondrial matrix

Proteins, which need to be imported into the mitochondrial matrix, use the TOM and TIM23 translocases pathway. As mentioned earlier, they usually contain a cleavable N-terminal MTS which directs the N-terminus of the preprotein in presence of the membrane potential directly from the TOM to the TIM23 translocase and into the matrix. However, complete translocation of the full-length preprotein across the inner membrane into the matrix requires not only the membrane potential, but also energy derived from the hydrolysis of ATP. The mechanism discussed for the TIM23 mediated translocation of preproteins into the matrix is explained in chapter 1.3.

1.3 The TIM23 translocase

The TIM23 translocase is the major translocase of the inner membrane transporting nuclear encoded mitochondrial proteins with an MTS. It is required for integration of proteins into the inner membrane employing the “stop-transfer”-pathway or the “conservative sorting”-pathway and for import of proteins into the matrix. The TIM23 translocase consists of eight essential subunits forming a membrane embedded translocation channel and a membrane associated motor which hydrolyses ATP.

1.3.1 The membrane embedded part of the TIM23 translocase

Tim23 is the central component of the translocase and the one its name is derived from. It is anchored in the inner membrane by four transmembrane segments present in its C-terminus (Emtage and Jensen 1993). This membrane embedded part of Tim23 contributes largely to the aqueous peptide conducting channel of the translocase (Kubrich et al. 1994, Berthold et al. 1995, Milisav et al. 2001) which is selective for cations and voltage gated (Truscott et al. 2001). Moreover, the C-terminus of Tim23 interacts with Tim17 (Berthold et al. 1995, Dekker et al. 1997, Ryan et al. 1998). The N-terminus of Tim23 fulfills additional functions: The approximately first 20 amino acid residues can insert into the outer membrane resulting in the Tim23 protein spanning two membranes at the same time (Donzeau et al. 2000). The function of this second membrane insertion is still under discussion. The second segment of the Tim23 N-terminus is responsible for the interaction with Tim50 (Geissler et al. 2002, Yamamoto et al. 2002, Tamura et al. 2009) as well as with the presequences of incoming preproteins (Truscott et al. 2001) and was shown to be required for the dimerization of Tim23 which is dependent on the membrane potential (Bauer et al. 1996).

Tim17 spans the inner membrane with four transmembrane segments in a similar way as Tim23 (Ryan et al. 1994). In contrast to Tim23, Tim17 exposes only a few amino acid residues into the IMS. Tim17 is involved in the formation and the voltage gating of the channel (Milisav et al. 2001, Chacinska et al. 2005, Meier et al. 2005, Martinez-Caballero et al. 2007).

Tim50 is the first component of the Tim23 translocase to interact with the incoming preprotein as soon as it appears on the IMS side of the TOM complex (Yamamoto et al. 2002, Mokranjac et al. 2003, Mokranjac et al. 2009, Tamura et al. 2009, Schulz et al. 2011, Shiota et al. 2011, Lytovchenko et al. 2013). Tim50 is anchored in the inner membrane and exposes its large C-terminal domain into the IMS (Geissler et al. 2002, Yamamoto et al. 2002, Mokranjac et al. 2003). Moreover, Tim50 interacts not only with the preproteins, but also with Tim21, when preproteins are absent (Lytovchenko et al. 2013), and with Tim23, in order to transfer the preprotein from the TOM to the TIM23 complex and to translocate it across the inner membrane (Yamamoto et al. 2002, Gevorkyan-Airapetov et al. 2009). In addition, Tim50 has been suggested to play a role in the gating of the channel of the translocase (Meinecke et al. 2006).

Additionally, three non-essential components are found in the translocase.

Tim21, which is anchored in the inner membrane by a single transmembrane segment, exposes its C-terminus into the IMS and was reported to bind to the TOM complex *in vitro* (Chacinska et al. 2005, Mokranjac et al. 2005, Albrecht et al. 2006). Tim21 binds to Tim50 in the absence of preproteins and is released from the TIM23 translocase upon presequence binding and connection of the translocation channel with the import motor components (Chacinska et al. 2005, van der Laan et al. 2005, Popov-Celeketic et al. 2008, Lytovchenko et al. 2013). Moreover, Tim21 was also discussed to be involved in the interaction of the TIM23 translocase with respiratory chain complexes (van der Laan et al. 2006, Wiedemann et al. 2007).

Pam17, which is also anchored in the inner membrane but exposed to the matrix (van der Laan et al. 2005), was shown to decrease the interaction of the Tim14-Tim16 sub-complex with the Tim17-Tim23 core (van der Laan et al. 2005, Hutu et al. 2008). It might also influence the Tim14-Tim16 interaction itself negatively and was suggested to have an antagonistic effect with Tim21 on the TIM23 translocation activity (Chacinska et al. 2005, van der Laan et al. 2005, Popov-Celeketic et al. 2008).

Mgr2, which also resides in the inner membrane, is required for interaction of Tim21 with the core components of the translocase and for coupling of the TOM with the TIM23 complex during precursor protein import (Gebert et al. 2012). Mgr2 itself lacks the typical N-terminal MTS, but is

processed by IMP in the IMS. It has been shown that this processing is required for complete assembly of the TIM23 translocase (Ieva et al. 2013).

1.3.2 The import motor of the TIM23 translocase

1.3.2.1 Components of the import motor

The components of the import motor are conserved and represent a specialized chaperone system. They bind to the membrane embedded part of the translocase via Tim44.

The peripheral membrane protein Tim44 is attached to the matrix side of the Tim23 translocase (Maarse et al. 1992, Scherer et al. 1992, Moro et al. 1999). Tim44 interacts at its N-terminus with the core of the translocase (Berthold et al. 1995, Schiller et al. 2008) and at its C-terminus directly with the lipid phase of the cardiolipin containing inner membrane (Handa et al. 2007, Marom et al. 2009). Tim44 is seen as the coordinating component of the translocase, since it interacts not only with the core of the translocase but also with the mitochondrial Hsp70 (mtHsp70) chaperone and with the Tim14-Tim16 co-chaperone sub-complex, thereby positioning these constituents of the import motor at the channel exit of the TIM23 translocase in the mitochondrial matrix (Rassow et al. 1994, Schneider et al. 1994, Merlin et al. 1999, Krimmer et al. 2000, Moro et al. 2002, Mokranjac et al. 2003, Kozany et al. 2004, Slutsky-Leiderman et al. 2007, Hutu et al. 2008, Schiller et al. 2008).

The central import motor component is the mtHsp70 (Ssc1 in yeast). MtHsp70 is a chaperone (Hartl 1998) homologous to the bacterial DnaK (Genevaux et al. 2007). MtHsp70 consists of an N-terminal ATPase / nucleotide binding domain (NBD) and a C-terminal peptide binding domain (PBD) which are connected via a short linker segment. The PBD comprises a ß-stranded binding pocket and an α-helical lid-domain (Kang et al. 1990, Bukau et al. 2006, Hartl and Hayer-Hartl 2009). Within the mitochondrial matrix mtHsp70 is not only involved in import, but also in protein folding with the help of Mdj1 (Craig et al. 1987, Rowley et al. 1994). MtHsp70 mediates import by repeated ATP hydrolysis driven cycles of binding and release of a preprotein (Scherer et al. 1990, Stuart et al. 1994, Ungermann et al. 1994). It is regulated by Mge1, Tim14, Tim16 and the presence of a preprotein.

The soluble matrix protein and GrpE homologue Mge1 acts as a nucleotide exchange factor for mtHsp70 (Bolliger et al. 1994, Westermann et al. 1995). It was first proposed to accelerate the release of ADP from mtHsp70, thereby helping to release the polypeptide from the PBD of mtHsp70 (Schneider et al. 1996, Dekker and Pfanner 1997, Miao et al. 1997). Newer measurements, however, revealed that Mge1 rather facilitates the binding of ATP from a nucleotide-free state of mtHsp70 than the release of ADP (Sikor et al. 2013). Mge1 forms a dimer

(Schneider et al. 1996, Harrison et al. 1997, Oliveira et al. 2006) and was suggested to be sensitive to thermal (Grimshaw et al. 2001, Moro and Muga 2006) and oxidative stress (Marada et al. 2013).

Additionally, J co-chaperones increase the rate of ATP hydrolysis at the mtHsp70 ATPase domain, thereby triggering high affinity binding of the polypeptide by the peptide binding domain. J proteins are named after the founding member, *E. coli* DnaJ and are also called Hsp40 proteins (Genevaux et al. 2007, Kampinga and Craig 2010, Hartl et al. 2011). DnaJ interacts via its J domain with the NBD of DnaK (bacterial Hsp70) and stimulates the ATPase activity of DnaK (Ahmad et al. 2011). In yeast, two such J co-chaperones are found: Tim14 (also termed Pam18) and Mdj2. Whereas Mdj2 is not required for yeast cell viability, Tim14 is an essential subunit of the TIM23 translocase (Mokranjac et al. 2003, Mokranjac et al. 2005). Tim14 is an integral membrane protein spanning the inner membrane once. Its N-terminus is located in the IMS (D'Silva et al. 2003, Mokranjac et al. 2003, Truscott et al. 2003) and was shown to interact with Tim17 (Chacinska et al. 2005, D'Silva et al. 2008). Tim14 contains a J domain (Kelley 1998) with a characteristic HPD motif which is essential for stimulation of the mtHsp70 ATPase activity and thereby for the function of the TIM23 translocase (D'Silva et al. 2003, Truscott et al. 2003, Mokranjac et al. 2005).

Tim16 (also termed Pam16), an essential J-like protein, is located in the matrix attached to the inner membrane and interacts at its N-terminus with the N-terminal part of Tim44 (Frazier et al. 2004, Kozany et al. 2004, Mokranjac et al. 2007, D'Silva et al. 2008). Tim16 binds to Tim14 (Kozany et al. 2004, Mokranjac et al. 2006), thereby inhibiting the stimulatory activity of Tim14 (Li et al. 2004).

1.3.2.2 Mode of operation of the import motor

The complex and coordinated interplay between the components of the import motor leads to the translocation of a preprotein into the mitochondrial matrix. Whereas the MTS mediated insertion of the preprotein into the TIM23 translocation channel requires only the presence of membrane potential (Bauer et al. 1996, Truscott et al. 2001), the complete translocation into the matrix requires ATP hydrolysis (Neupert and Brunner 2002).

The mtHsp70 is recruited to the membrane attached Tim44 in an ATP bound state (Schneider et al. 1994, Schneider et al. 1996), although the interaction of mtHsp70 and Tim44 is found to be most stable in presence of ADP (Kronidou et al. 1994, Rassow et al. 1994, Schneider et al. 1994). The interaction with Tim44 was localized to the NBD (Krimmer et al. 2000), the PBD (Moro et al. 2002), or to both of the domains (D'Silva et al. 2004) of mtHsp70 and is still under debate. However, in this ATP bound state, the PBD of mtHsp70 is in an open conformation (Gragerov et al.

1994, Bukau and Horwich 1998). As soon as the translocating preprotein appears at the matrix side of the TIM23 translocase, it interacts with Tim44 and is bound by the PBD of mtHsp70. At the same time, binding of the preprotein at the PBD leads to ATP hydrolysis at the NBD of mtHsp70 inducing a conformational change which allows tight binding of the preprotein at the PBD (Gragerov et al. 1994, Rudiger et al. 1997, Rudiger et al. 1997, Bukau and Horwich 1998, Mayer et al. 2000, Mayer et al. 2000). The binding leads together with the presence of Mge1 to a release of mtHsp70 from Tim44 (Liu et al. 2003, Slutsky-Leiderman et al. 2007) which allows by Brownian motion an inward movement of the preprotein together with mtHsp70 into the matrix and recruitment of another mtHsp70 molecule to Tim44 (Strub et al. 2002, D'Silva et al. 2004). The ATP hydrolysis at the NBD is stimulated by the J co-chaperone Tim14 since the basal ATPase rate of mtHsp70 is low. Tim14 is stabilized in a non-stimulating conformation by Tim16 to prevent futile cycles of stimulation in absence of a preprotein (Mokranjac et al. 2006). Tim16 does not mask the HPD motif of Tim14, but rather keeps Tim14 in a certain conformation that prevents it from its stimulatory activity on mtHsp70 (Mokranjac et al. 2006). Upon a conformational change of the Tim14-Tim16 sub-complex Tim14 interacts with mtHsp70 and stimulates its ATPase activity. The exchange of ADP to new ATP at the NBD of mtHsp70 is accelerated with the help of Mge1 and allows for a new cycle of preprotein binding (Schneider et al. 1996). The repetition of these cycles in a “hand-over-hand” model is thought to lead to the vectorial movement of the preprotein into the mitochondrial matrix (Ungermann et al. 1994). Since the translocase is probably present as an oligomer (Berthold et al. 1995, Bauer et al. 1996, Moro et al. 1999), at least two Tim44 proteins provide a binding platform for more than one single mtHsp70 enabling efficient recruitment and release of mtHsp70 proteins.

The preprotein has to cross the membrane in an unfolded or at least partially unfolded state since the channel can only accommodate the diameter of max. two helices (Rassow et al. 1990, Schwartz et al. 1999, Truscott et al. 2001). Although the membrane potential can contribute to the preprotein unfolding (Huang et al. 2002, Krayl et al. 2007), it is necessary that the mitochondria employ a preprotein unfolding mechanism (Eilers and Schatz 1986, Pfanner and Neupert 1986, Eilers et al. 1987, Matouschek et al. 1997).

Different models for the exact mechanism of translocation and the involved unfolding process have been discussed during the past years.

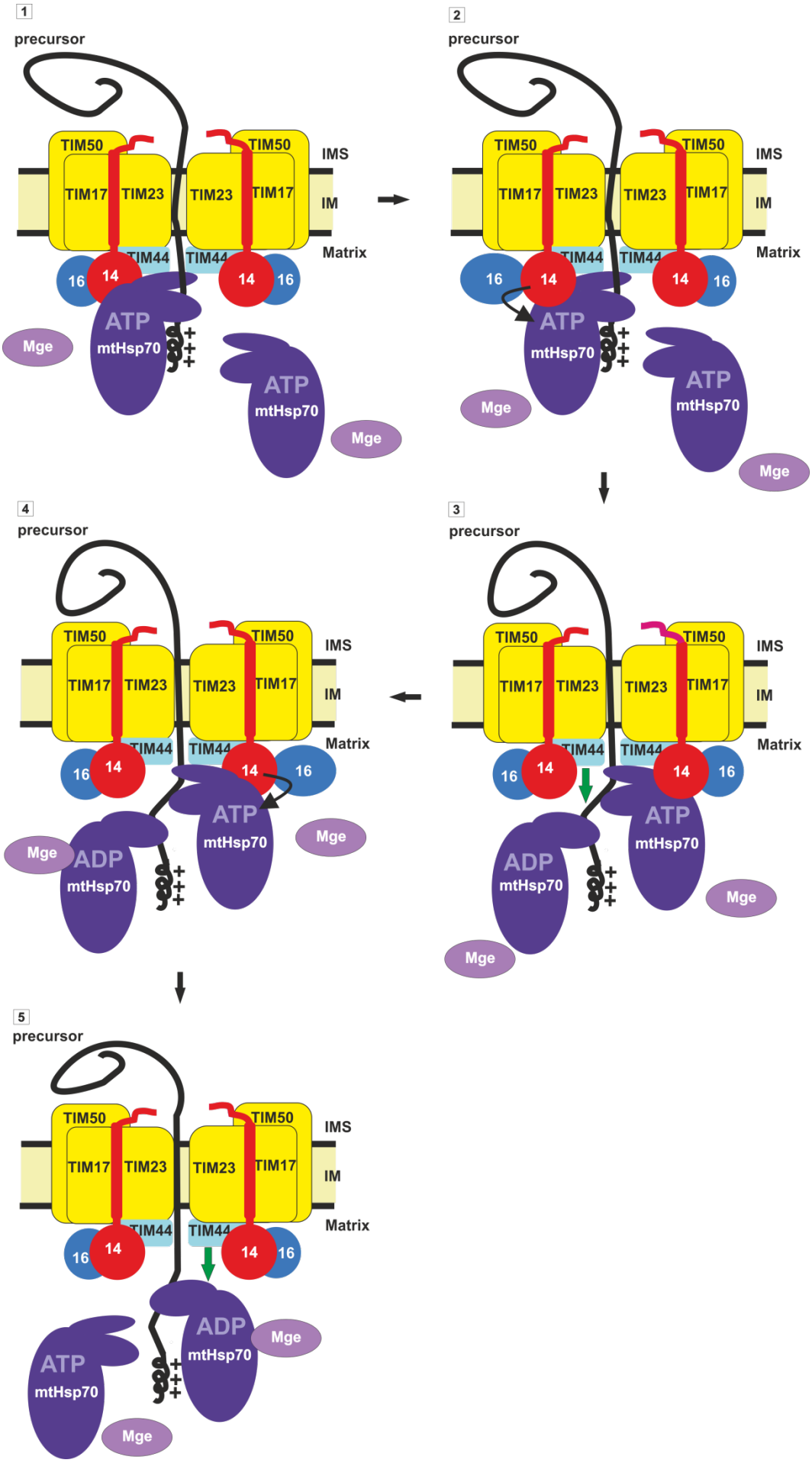
1
precursor
TIM50
TIM17
TIM23
TIM23
TIM17
TIM50
IMS
IM
Matrix
TIM44
TIM44
16
14
14
16
ATP
mtHsp70
Mge
ATP
mtHsp70
Mge
2
precursor
TIM50
TIM17
TIM23
TIM23
TIM17
TIM50
IMS
IM
Matrix
TIM44
TIM44
16
14
14
16
ATP
mtHsp70
Mge
ATP
mtHsp70
Mge
3
precursor
TIM50
TIM17
TIM23
TIM23
TIM17
TIM50
IMS
IM
Matrix
TIM44
TIM44
16
14
14
16
ADP
mtHsp70
Mge
ATP
mtHsp70
Mge
4
precursor
TIM50
TIM17
TIM23
TIM23
TIM17
TIM50
IMS
IM
Matrix
TIM44
TIM44
16
14
14
16
Mge
ADP
mtHsp70
ATP
mtHsp70
Mge
5
precursor
TIM50
TIM17
TIM23
TIM23
TIM17
TIM50
IMS
IM
Matrix
TIM44
TIM44
16
14
14
16
ADP
mtHsp70
Mge
ATP
mtHsp70
Mge

Figure 2 Model of the mechanism of the ATP dependent operation of the import motor at the matrix side of the TIM23 translocase

1: ATP bound mtHsp70 is attached to the translocase via its interaction with Tim44. The translocating preprotein interacts with the PBD of mtHsp70 at the exit of the Tim17-Tim23 channel.

2: Upon a conformational change of the Tim14-Tim16 complex Tim14 stimulates the ATP hydrolysis of mtHsp70 (arrow).

3: The mtHsp70 binds the preprotein tightly and is released from Tim44. The the preprotein with mtHsp70 is allowed to move inwards (green arrow). Another mtHsp70 bound at the second Tim44 at the channel exit interacts with the preprotein.

4: The ATPase activity of the second mtHsp70 is stimulated by Tim14 leading to tight binding of the preprotein. Mge facilitates the nucleotide exchange from ADP to a new ATP at the NBD of mtHsp70 in the matrix, thereby triggering the release of the preprotein from the ATP bound mtHsp70 in the matrix.

5: The ADP bound mtHsp70 binds the preprotein tightly and is released from Tim44.

It has been found that the preprotein in the translocase can undergo random backward and forward Brownian movement (Yamano et al. 2008). In the "Brownian ratchet" model the mtHsp70 is seen as a component which traps every forward movement by repeated cycles of preprotein binding and subsequent release from Tim44, thereby preventing backsliding of the preprotein (Neupert et al. 1990, Simon et al. 1992, Simon and Blobel 1993, Schneider et al. 1994, Liu et al. 2003, Yamano et al. 2008). Since the "breathing" – the dynamic local unfolding – of proteins can be trapped by an interacting chaperone, it is conceivable that the import motor "makes use" of these thermal fluctuations of the preprotein in every cycle of mtHsp70 binding and collects all these small movements to a complete unidirectional transport in total (Huang et al. 1999, Lim et al. 2001). Experimental evidence was for example obtained by the observation of paused import of folded domains in preproteins (Gaume et al. 1998, Okamoto et al. 2002). The relatively new idea of "entropic pulling" brings the entropy gain into play which is achieved by the freedom gain of the preprotein leaving the translocation channel and the PBD of mtHsp70 (De Los Rios et al. 2006, Goloubinoff and De Los Rios 2007).

In the "power stroke model" the mtHsp70 is seen as a machine actively pulling the preprotein into the matrix triggered by the ATP hydrolysis. The conformational change of mtHsp70 results in a power stroke in which one domain of mtHsp70 is still fixed at the channel exit and the other domain (seen as a "lever arm") moves into the matrix pulling the preprotein with it (Glick 1995, Krimmer et al. 2000). The pulling would have to lead to a complete unfolding of the cytosolic portion of the preprotein and thereby enable translocation (Glick et al. 1993, Glick 1995, Pfanner and Meijer 1995, Horst et al. 1997, Jensen and Johnson 1999, Voisine et al. 1999, Pfanner and Geissler 2001, Krayl et al. 2007). Additionally, interactions between the preprotein and the translocation channel could stabilize the translocation intermediate after inward movement of one mtHsp70 molecule. During this time, before the next mtHsp70 molecule binds the preprotein

at the channel exit, backsliding of the unfolded preprotein would be allowed (Dekker et al. 1997, Chauwin et al. 1998, Marom et al. 2011).

1.3.3 Proposed mechanisms of action of the TIM23 translocase in different sorting pathways

Apart from the two models proposed for the mode of operation of the import motor, different models have also been proposed for the whole TIM23 translocase to distinguish between matrix import and lateral release of inner membrane / IMS proteins.

The “single entity model” suggests that the essential components of the TIM23 translocase act as one assembled unit with the import motor always connected to the translocation channel irrespective of the presence and type of a preprotein (Mokranjac et al. 2005). Several conformational changes are necessary to perform one or the other import route. One of them leads to the insertion of the N-terminus of Tim23 into the outer membrane in presence of a translocating preprotein. Moreover, the presence of a matrix targeted preprotein or of a laterally sorted preprotein changes the interactions of Tim23 with the other translocase components in different ways compared to the empty translocase. The non-essential subunit Pam17 was found in close proximity to Tim23 in absence of translocating preproteins and in presence of a laterally sorted preprotein. In contrast, the other non-essential subunit Tim21 is in complex with the translocase preferentially in presence of a matrix targeted preprotein. Pam17 and Tim21 do not interact with each other. These data and data obtained from interaction analysis of translocase components in presence of over-expressed Pam17 or Tim21 suggest that these two subunits exert an antagonistic effect on the translocase in terms of discrimination between different types of preproteins (Mokranjac et al. 2005, Popov-Celeketic et al. 2008, Tamura et al. 2009).

The “modular model” suggests that the translocase contains distinct modules dependent on the need of an import motor for matrix import. For the lateral release of a preprotein, the translocase consists only of the subunits Tim17, Tim23, Tim50 and Tim21 and is called $TIM23^{SORT}$. Tim21 is required for interaction with the Tom complex and for mediation of the lateral insertion of the preprotein. When a matrix targeted preprotein is present, Tim21 dissociates from the complex and the import motor gets attached to the core. Tim17 is required to mediate this “switch” between the two forms of the translocase (Chacinska et al. 2005, van der Laan et al. 2005, Chacinska et al. 2009).

Which of these models holds true, is still under discussion and further studies are necessary to support one or the other.

1.4 Human homologues of the TIM23 translocase components

Despite having a good understanding of how the translocation of preproteins into mitochondria takes place and how the TIM23 translocase functions in yeast (Mokranjac and Neupert 2009, Mokranjac and Neupert 2010), it is still puzzling how these processes are mediated in higher eukaryotes such as humans. First insights into the biogenesis of human mitochondria were provided by the identification of mammalian homologues of yeast translocase components (Wada and Kanwar 1998, Bauer et al. 1999, Guo et al. 2004, Davey et al. 2006, Sinha et al. 2010). Moreover, pathologic phenotypes were reported for lack, overexpression or mutation of some of the human homologues. However, the mechanisms underlying the mitochondrial transport in humans are poorly understood.

1.4.1 Identification of mammalian homologues

Table 1 gives an overview of the identified mammalian components of the TIM23 translocase, their yeast homologues and the phenotypes observed in mammals. The presence of homologous proteins in mammals suggests a conserved mechanism for the mitochondrial import via the TIM23 pathway. Several observations are in agreement with this suggestion: TIM23 (Ishihara and Mihara 1998) was reported to interact with TIM17A and TIM17B, the two human homologues of Tim17 (Bauer et al. 1999). However, more and more evidence is provided that the two TIM17 proteins fulfill additional and separate functions: TIM17B is seen as the “housekeeping” mitochondrial translocase component (Rainbolt et al. 2013), whereas TIM17A was reported to be up-regulated in breast cancer (Xu et al. 2010, Salhab et al. 2012), to be a stress sensor involved in the mitochondrial unfolded protein response (Aldridge et al. 2007, Rainbolt et al. 2013) as well as to be involved in mtDNA maintenance (Iacovino et al. 2009). TIM17A and TIM17B were not observed to reside in the same translocase (Bauer et al. 1999). The human TIM50 was first described in 2004 and later found to interact with mitochondrial targeting sequences (Guo et al. 2004, Zhang et al. 2012). Lately, the human TIM21 was reported to be involved not only in preprotein import via the TIM23 translocase but also in mediation of the assembly of respiratory chain complexes and therefore to link preprotein import with complex assembly (Mick et al. 2012). The mitochondrial localization of the mammalian TIM44 was confirmed in the late 1990s and its up-regulation was associated with hyperglycemia (Wada and Kanwar 1998). Mortalin, the human mtHsp70, was found to be differentially expressed in cancer cells compared to normal cells (Kaul et al. 2007). Also in Alzheimer’s and Parkinson’s disease expression patterns of mortalin were altered. Furthermore, it was reported to change its subcellular localization from mitochondria in normal cells to the cytosol in cancer cells where it interacts with a variety of cytosolic proteins. These findings suggest that mortalin can fulfill additional functions besides its role in the preprotein import into mitochondria (Webster et al. 1994, Kaul et al. 2007).

Additionally, the presence of a stimulating co-chaperone, DNAJC19, was demonstrated as well as its inhibition by a J-like protein termed MAGMAS, both homologues of the yeast Tim14 and Tim16, respectively (Taylor et al. 2003, Sinha et al. 2010). DNAJC19 was found to be mutated in cases of DCMA syndrome (Davey et al. 2006, Ojala et al. 2012), a severe heart disease, and altered expression levels of DNAJC19 were detected in autistic brain tissue (Anitha et al. 2012). MAGMAS was first identified as a gene to be switched on upon stimulation of a murine myeloid cell line by GM-CSF (granulocyte macrophage colony stimulating factor) and was therefore termed mitochondria-associated granulocyte macrophage CSF signaling molecule (MAGMAS) (Jubinsky et al. 2001).

For two of the components (Mdj2 and Pam17), no human homologues have been identified. Several studies have been published for Romo1, the human homologue of the relatively newly identified Mgr2, but until today, there is no evidence that this protein is a component of the TIM23 translocase in human (Chung et al. 2009, Lee et al. 2010, Lee et al. 2011, Lee et al. 2011, Chung et al. 2012, Shin et al. 2013, Kim et al. 2014, Norton et al. 2014).

yeast	**human**	**Phenotype**
Tim23	**TIM23**	reduced lifespan of heterozygous knockout mouse (Ahting et al. 2009)
Tim17	**TIM17A**	overexpression prevents mtDNA loss, upregulated in breast cancer on RNA and protein level (Iacovino et al. 2009, Xu et al. 2010, Salhab et al. 2012)
	TIM17B	
Tim50	**TIM50**	loss causes mitochondrial membrane permeabilization (Guo et al. 2004)
Tim21	**TIM21**	loss disturbs early-assembly of respiratory chain intermediates (Mick et al. 2012)
Mgr2	**Romo1**	overexpression promotes ROS production (Chung et al. 2012)
Tim44	**TIM44**	upregulated in diabetic kidney (Wada and Kanwar 1998)
Ssc1	**Mortalin**	altered expression in cancer, AD, PD (Kaul et al. 2007)
Mge1	**Mge**	
Tim14	**DNAJC19**	mutation causes dilated cardiomyopathy with ataxia, reduced expression in autism (Davey et al. 2006, Anitha et al. 2012)
	MCJ	methylation controlled loss of expression causes chemoresistance of tumor cells (Shridhar et al. 2001)
Tim16	**MAGMAS**	upregulated upon GM-CSF treatment; upregulated in neoplastic prostate; overexpressed in mouse and human ACTH-secreting pituitary adenomas (Tagliati et al. 2010)
Mdj2	not found	
Pam17	not found	

Table 1 *S. c.* TIM23 translocase components and their human homologues

For all characterized translocase components of yeast with exception of Mdj2 and Pam17, human homologues have been identified. For Tim17 and Tim14 two human homologues have been found.

For some of the translocase components, like Tim17, more than one human homologue was identified, which might indicate a more complex composition and / or regulation of the TIM23 translocase in higher eukaryotes compared to the unicellular organism *S. cerevisiae*. DNAJC19 and MCJ are sequence homologues of the yeast Tim14 protein.

1.4.2 MCJ – Methylation controlled J protein

Shridar *et al*. first described that loss of expression of MCJ confers chemoresistance to ovarian cancer cells. This loss of expression resulted from methylation of the MCJ gene and led to the name methylation controlled J protein (Shridhar et al. 2001). Later it was described in more detail: The expression of MCJ is tissue specific and depends on the methylation state of a CpG island in the first intron and exon of the gene (Strathdee et al. 2004). Loss of MCJ expression was observed in different cancers (Ehrlich et al. 2002, Lindsey et al. 2006, Muthusamy et al. 2006) and correlated with increased chemoresistance of tumor cell lines (Strathdee et al. 2005, Hatle et al. 2007). MCJ was suggested to be located in the Golgi apparatus in a study using HA-tagged MCJ protein (Hatle et al. 2007).

MCJ belongs to the family of J proteins. In human, 41 J proteins are identified today. Three classes of J proteins can be distinguished by their domain structure: DNAJA, DNAJB and DNAJC (Kampinga et al. 2009). J proteins belonging to the DNAJA class are to a large extent homologous to DnaJ and are characterized by an N-terminal J domain, a G/F rich region, a C rich region and their C-terminus is variable. J proteins belonging to the DNAJB class contain the N-terminal J domain and the G/F rich region. DNAJC class J proteins are characterized by containing a J domain which does not necessarily have to be located at the N-terminus of the protein. MCJ contains a J domain at the C-terminus and lacks the G/F rich region and lacks also the C rich region. It is therefore classified as a C-type DNAJ-protein and also named DNAJC15 in the latest consensus nomenclature (Kampinga et al. 2009). Also DNAJC19, the second human Tim14 homologue, is a C-type J protein.

1.5 Aims of the present study

Most of our knowledge about the import of mitochondrial proteins has been obtained from studies in *S. cerevisiae*. At the beginning of this study, almost nothing was known about this process in human cells. The aim of this study was to identify components of the human TIM23 translocase and to determine how they act in the translocation of preproteins across the mitochondrial inner membrane. Despite its previous localization to the Golgi compartment (Hatle et al. 2007), the J protein MCJ should be characterized, because it shows high sequence homology

to the yeast Tim14 protein and therefore might play a role in the preprotein import into human mitochondria.

First, the subcellular and submitochondrial localization of MCJ should be addressed. Next, it was the aim to identify interaction partners of MCJ and to answer the question, whether MCJ might be a component of the human TIM23 translocase. Moreover, the function of MCJ in the TIM23 translocase should be investigated. It should be tested, how the absence of MCJ affects the import of preproteins into mitochondria. Employing recombinant proteins, it should be analyzed, whether MCJ functions as a co-chaperone of mortalin. Additionally, it should be tested, whether MCJ and DNAJC19, the second human homologue of yeast Tim14, are able to functionally replace Tim14 in yeast cells. Finally, the aim was to elucidate whether the two homologues, MCJ and DNAJC19, might be present in distinct TIM23 translocases with different functions or together in one single TIM23 translocase.

2 Materials and Methods

2.1 Material

2.1.1 Instruments

Table 2 gives an overview about the instruments used for this study and from which company they were purchased.

product	company
AEKTA	GE Healthcare
Balance max = 2200g; d = 0,01g	Sartorius
Balance max = 81g; d = 0,01mg	Mettler Toledo
Centrifuge 5417R	Eppendorf
Centrifuge 5424	Eppendorf
Densitometer	GE Healthcare
Developing machine Curix 60	AGFA
Freezer -20°c	Liebherr
Freezer -80°C	GFL
Refrigerator	Liebherr
GelDoc It	UVP
Heating Block	Eppendorf
Incubator 30°C/37°C	Memmert
Incubator 30°C/37°C shaking	Infors HAT multitron
Incubator 37°C, 5 %CO2	Binder
Incubator 68°C	Memmert
Laminar Air Flow Hera Safe	Thermo electron corporation
Light microscope Axiovert 40 CFL	Zeiss
pH-Meter Lab850	Schott
Photometer Bio	Eppendorf
Pipets Pipetman 1-10, 2-20, 10-100, 20-200, 200-1000µl	Gilson
Power supply EPS 601	GE Healthcare
Sonifier 250	Branson Ultrasonics
Thermal Cycler Primus	MWG Biotech
Thermomixer comfort	Eppendorf
Transilluminator	UVP
Ultracentrifuge Optima LE-80K	Beckmann Coulter
UV-Spectrophotometer V-600	Jasco
Vortex	Scientific Industries Inc.
Waterbath	GFL

Table 2 List of instruments

2.1.2 Laboratory consumables

Table 3 provides an overview over the laboratory consumables used in this study.

product	company
^{35}S-Methionine	MP Biomedicals
Amicon Ultracel 3k	Millipore
Blot dishes	Stricker
Cell culture dishes 10 cm, 6-well plates	TPP
Cell culture dishes 24.5 x 24.5 cm	Nunc
Cell scraper	TPP
Cuvettes, semi-micro 10 x 10 x 45 mm	Greiner
Electroporation cuvettes 1 ml	Biozym
Falcons 15, 50 ml	Omnilab
Films Fuji Super RX 13 x 18	PW Medizintechnik
Gel extraction kit miGel	Metabion
Glass plates 14 x 16.5 cm	Glaserei Peter Wenzel
Glass plates with ears 14 x 16.5 cm	Glaserei Peter Wenzel
Glutathionsepharose 4B	GE Healthcare
Hamilton 50 µl	VWR
IgG sepharose	ICN
Midiprep Kit	Promega
Minisart 0.2 µm Filter	Sartorius
Ni-NTA agarose	Quiagen
Nitrocellulose membrane	Whatman
Omnifix 2 ml, 5 ml	Braun
Pasteur Pipets 150 mm	Stricker
PCR purification kit miPCR	Metabion
PCR tubes	Sarstedt
PD10 columns	GE/VWR
Pipet tips	Sarstedt
Protein A-Sepharose CL-4B	GE Healthcare
Reaction tubes 1.5 ml	Sarstedt
Sensitive films Z350397	Kodak
Serological pipettes 2, 5, 10, 25 ml	TPP
Sterican needles 0.45 x 25 mm	Braun
Sulfolink gel	Pall
Whatman paper	Schleicher & Schüll

Table 3 List of laboratory consumables

2.1.3 Reagents

Table 4 provides an overview about the reagents used in this study.

product	Company	product number
5'FOA	Thermofischer	R0812
Acetic acid	Applichem	A0820
Acrylamide/Bisacrylamide 37,5 : 1	Serva	10688
Adenine sulfate	Sigma	A9126
αFlag affinity gel	Sigma	A2220

product	Company	product number
Agar agar	Serva	11396
Agarose	Sigma	A9539
αHis antibody	Quiagen	34660
Ampicillin sodium salt	Sigma	A9518
ATP	Roche	10127531
ß-Mercaptoethanol	Sigma	M6250
Bactotryptone	BD	211701
Blasticidin S-HCl	Life Technologies	R210-01
Bradford reagent	BioRad	
Bromphenolblue	Sigma	114405
BSA, fatty-acid free	Serva	11932
Citrate	Merck	1064329051
CNBr activated sepharose	Life Technologies	17043001
Complete EDTA free protease inhibitor	Roche	04693132N9M13
Developing Solution G153	PW Medizintechnik	1100002
Digitonin	Merck	300410
DMEM high glucose	Sigma	D5671
DMSO	Sigma	D2438
dNTPs	NEB	
Doxycycline	Sigma	D9891
DTT	Gerbu	1008
Dulbecco's PBS	Sigma	D8537
EDTA	Merck	108418
Ethidiumbromide 1 %	Sigma	E1510
Fetal Bovine Serum	Life Technologies	10270106
Fixation solution G354	PW Medizintechnik	1200002
Freund's Incomplete Adjuvans	Sigma	F5506
Galactose	Applichem	G0625
Glycerol	Sigma	G7757
Glycin	Sigma	G8790
Goat anti-mouse IgG	BioRad	1706516
Goat anti-rabbit IgG	BioRad	170-6515
H_2O_2 30 %	Applichem	A0626
HEPES	Serva	25245.05
Histidin	Sigma	H8000
Hydrochloric acid 37.5 %	Applichem	A0659
Hygromycin B	Life Technologies	10687
Imidazole	Merck	104716
IPTG	Gerbu	1043
KCl	Sigma	104936
KH_2PO_4	Merck	1048719
KOH	Merck	105033
Lactic acid	Merck	1003669
LDH	Sigma	L2500

product	Company	product number
Leucin	Fluka	61820
L-Glutamine 200x	Sigma	G7513
L-glutathione reduced	Roth	63822
L-Methionine	Fluka	64319
Luminol	Sigma	A8511
Lysine	Fluka	62840
Lysozyme	Serva	28262
MEM media	Sigma	M1018
Milk powder	Pasteurapotheke	-
Na_2HPO_4	Merck	106586
NaCl	Merck	1064009029
NADH	Gerbu	1051
Non-essential amino acids 100x	Sigma	M7145
p-Coumaric acid	Sigma	G9008
PEG3350	Sigma	P3640
Penicillin/streptomycin	Sigma	P4458
PEP	Sigma	860077
PMSF	Serva	32395
Ponceau S	Sigma	P7170
Protein marker, unstained	Fermentas	SM0431
Proteinase K	Roche	3115801001
Restriction endonucleases and corresponding buffers	NEB	various
Sucrose	Roth	4661.3
Titer Max Gold Adjuvant	Sigma	T2684
Tris-HCl	Applichem	A1379
TritonX-100	Merck	108603
Trypsin	Sigma	T1763
Trypsin-EDTA	Sigma	T3924
Tryptophan	Sigma	T0254
Uracil	Sigma	U1128
Western Blocking Reagent	Roche	1096176
X-fect reagent	Clonetech/Takara	631318
Yeast extract	Serva	2454003
Yeast nitrogen base	Life Technologies	25685033
Zymolyase	Wak	120491

Table 4 List of reagents

2.1.4 Plasmids

Table 5 provides an overview over the plasmids used in this study. For those plasmids which were generated during this study, the primers used for cloning are listed with the restriction sites highlighted in green; tags encoded in the primers are highlighted in purple.

Name in this study	Protein encoded [tag]	Vector backbone	Primers used for cloning 5' -> 3'	Restriction site – gene insertion (aa residues encoded)- Restriction site
DNAJC19(s)	***H. s.* DNAJC19 aa 24-116 [GST (N-term)]**	pGEX6p1	CCCGAATTCCAAGCCATGAAGCATATGG	**EcoRI - DNAJC19 (24-116) - Xho**
			CCCTCGAGTCATTTTTTAGCTTGACCTTCTAGT	
MCJ(s)	***H. s.* MCJ aa 57-150 [GST (N-term)]**	pGEX6p1	CCCGAATTCGCATTTCGGATATGGAAACC	**EcoRI - MCJ (57-150) - Xho**
			CCCCTCGAGTCAATGTTTGGTGGTTGTTTC	
MAGMAS(s)/MCJ(s)	***H. s.* MAGMAS aa 25-125 [his (N-term)]**	pETduet1	CCGGATCCCTCCAGGAGGCACAGCAG	**BamHI - MAGMAS (25-125) - NotI**
			CCGCGGCCGCTCACTGCCCTTTTTCTCT	
	***H. s.* MCJ aa 57-150 [-]**		CCCCATATGGCATTTCGGATCTGG	**NdeI - ATG - MCJ (57-150) - XhoI**
			CCCCTCGAGTCAATGTTTGGTGGTTGTTTC	
MAGMAS(s)	***H. s.* MAGMAS aa 25-121 [his (N-term)]**	pQE30	CCGGATCCCTCCAGGAGGCACAGCAG	**BamHI - MAGMAS (25-125) - Xho -**
			CCCCTCGAGTCACGTATGGGGCATCTGCCCTTTTTC	
MAGMAS	***H. s.* MAGMAS aa 2-125 [his (N-term)]**	pQE30	CCCGGATCCGCCAAGTACCTGGCCCAGATC	**BamHI - MAGMAS (2-125) - XhoI**
			CCCCTCGAGTCACGTATGGGGCATCTGCCCTTTTTC	
TIM50	**H. s. TIM50 aa 2-457 [his (N-term)]**	pQE30	CCGGATCCGCCTCAGCTTTATCTCTGGGCAATA	**BamHI - TIM50 (2-457) - HindIII**
			CCAAGCTTTCAGGGCTGTTTGGAGCGAGGCCACAAGCG	
MCJ	***H. s.* MCJ aa 1-150 [-]**	pYX142	CCCGGATCCATGGCTGCCCGTGGTGTCATCGCTCCAG	**BamHI - MCJ (1-150) - TGA - Xho**
			CCCCTCGAGTCAATGTTTGGTGGTTGTTTC	
DNAJC19	***H. s.* DNAJC19 aa 1-116 [-]**	pYX142	CCCGGATCCATGGCCAGTACAGTGGTAGCAGTTGGAC	**BamHI - DNAJC19 (1-116)- Xho**
			CCCTCGAGTCATTTTTTAGCTTGACCTTCTAGT	
MCJ	***H. s.* MCJ aa 1-150 [-]**	pRS314-yTim14 promoter (Mokranjac et al. 2005)	CCCGGATCCATGGCTGCCCGTGGTGTCATCGCTCCAG	**EcoRI – yTim14PROM -BamHI – MCJ (1-150) - PstI – yTim14TERM - XhoI**
			CCCCTGCAGTCAATGTTTGGTGGTTGTTTCTAGC	
DNAJC19	***H. s.* DNAJC19 aa 1-116 [-]**	pRS314-yTim14 promoter	CCCGGATCCATGGCCAGTACAGTGGTAGCAGTTGGAC	**EcoRI – yTim14PROM -BamHI - DNAJC19 (1-116) - NotI - yTim14TERM - XhoI**
			CCGCGGCCGCTCATTTTTTAGCTTGACCTTCTAGT	

Name in this study	Protein encoded [tag]	Vector backbone	Primers used for cloning 5' -> 3'	Restriction site – gene insertion (aa residues encoded)- Restriction site
MCJ-FLAG	***H. s.* MCJ aa 1-150 [flag (C-term)]**	pCDNA5 FRT TO	CCGGTACCATGGCTGCCCGTGGTGTC CCCTCGAGTCACTTATCGTCGTCATCCTTGTAATCATGTTTG GTGGTTGTTTC	**Kpn – ATG –MCJ (2-150) – FLAG –TGA – Xho**
DNAJC19-FLAG	***H. s.* DNAJC19 aa 1-116 [flag (C-term)]**	pCDNA5 FRT TO	CCGGTACCATGGCCAGTACAGTGGTAGC CCCTCGAGTCACTTATCGTCGTCATCCTTGTAATCATCTTTT TTAGCTTGACCTTC	**Kpn – ATG –DNAJC19 (2-116) – FLAG – TGA – Xho**
yTim14	**S. c. Tim14 aa 1-168 [-]**	pRS314-yTim14 promoter (Mokranjac et al. 2005)	(Mokranjac et al. 2005)	
Su9-DHFR$_{mut}$	***N. c. Su9 aa 1-69 - GIHRGI - M. m.* DHFR aa 1-187 (C7S/S42C/ D49C) [-]**	pGEM4	(Teichmann et al. 1996)	
F1ß	***N. c.* F1ß aa 1-519 [-]**	pJLA503	(Rassow et al. 1990, Sirrenberg et al. 1997)	
Flp Recombinase	**Flp Recombinase [-]**	pOGG44	commercially available from Invitrogen	
ANT3	***H. s.* ANT3 aa 1-297 [-]**	pCMV-SPORT6	purchased from Source Bioscience (SourceBioScience) no. IRATp970C0314D	

Table 5 List of plasmids used in this study

2.1.5 Antibodies

Table 6 provides an overview over the antibodies used in this study.

antibody	generated by	antigen
αMCJ	Commercial, Pineda (rabbit)	ESLRYAEYLQPSAKRPDADC
αTIM17A	Commercial, Pineda (rabbit)	CQLPSSPFGDYRQYQ
αTIM17B	Commercial, Pineda (rabbit)	CKDGTPAPGYPSYQQYH
αDNAJC19	rabbit for this study	DNAJC19(s) native
αMAGMAS	rabbit for this study	MAGMAS on NC
αTIM50	rabbit for this study	TIM50 on NC
αTIM44	Commercial, Protein Tech (mouse)	gene ID 10469
αHsp60	Commercial, BD (mouse)	*H.s.* Hsp60 aa 1-200
αTIM23	Commercial, BD (mouse)	*R. n.* Tim23 aa 5-126
αGM130	Commercial, BD (mouse)	*R. n.* GM130 aa 869-982
αCalnexin	Commercial, BD (mouse)	*H. s.* Calnexin aa 116-301
αEEA1	Commercial, BD (mouse)	*H. s.* EEA1 aa 3-281
αMTCO1	Commercial, abcam (mouse)	*H. s.* complex IV su1
αNDUFA9	Commercial, abcam (mouse)	*B. t.* purified mt complex I

Table 6 List of antibodies

2.1.6 Yeast strains

Table 7 provides a list of the yeast strains used and generated in this study.

yeast strain	genotype	reference
YPH499 wt	MATα ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1	(Johnston and Davis 1984, Sikorski and Hieter 1989)
YPH499Δ*TIM14* + pVTUyTim14	YPH499 with *tim14::HIS3* pVTUyTim14 [*TIM14*, *URA3*, 2µ]	(Mokranjac et al. 2005)
YPH499Δ*TIM14* + pRS314-yTim14	YPH499 with *tim14::HIS3* pRS314-yTim14 [*TIM14*, *TRP1*, cen]	(Mokranjac et al. 2005)
YPH499Δ*TIM14* + pRS314-MCJ	YPH499 with *tim14::HIS3* pRS314-yTim14 [*MCJ*, *TRP1*, cen]	this study
YPH499Δ*TIM14* + pRS314-DNAJC19	YPH499 with *tim14::HIS3* pRS314-yTim14 [*DNAJC19*, *TRP1*, cen]	this study
YPH499Δ*TIM14* + pYX142-DNAJC19	YPH499 with *tim14::HIS3* pYX142-DNAJC19 [*DNAJC19*, *LEU2*, 2µ]	this study
YPH499Δ*TIM14* + pYX142-MCJ	YPH499 with *tim14::HIS3* pYX142-DNAJC19 [*MCJ*, *LEU2*, 2µ]	this study

Table 7 List of yeast strains

2.1.7 Cell lines

The cell lines used in this study are listed in table 8. HeLa cells were purchased from CilBiotech.

Cell line	media	serum	antibiotics			Ref.	additional remarks
MCF7	MEM	10% FBS	Pen/ Strep			(Hatle et al. 2007)	
MCF7/ siMCJ	MEM	10% FBS	Pen/ Strep			(Hatle et al. 2007)	
HEK293 FlpIn TRex	DMEM	10% FBS w/o Tetracycline	Pen/ Strep	Blasticidin	Zeocin	this study	
HEK293 FlpIn MCJ-FLAG	DMEM	10% FBS w/o Tetracycline	Pen/ Strep	Blasticidin	Hygro-mycin	this study	inducible with Doxycycline
HEK293 FlpIn DNAJC19-FLAG	DMEM	10% FBS w/o Tetracycline	Pen/ Strep	Blasticidin	Hygro-mycin	this study	inducible with Doxycycline

Table 8 List of cell lines used in this study

All cell lines were cultivated in the indicated media containing FBS and antibiotics as listed. HeLa cells were used from a frozen cell pellet purchased from CilBiotech.

2.2 Methods

2.2.1 Molecular Biology Methods

2.2.1.1 Agarose gel electrophoresis of DNA

The separation of DNA fragments was performed using horizontal agarose gel electrophoresis in TAE buffer (40 mM Tris-$C_2H_3O_2$ pH 7.5, 20 mM $NaC_2H_3O_2$, 1 mM EDTA). 1.0 % or 1.5 % (w/v) agarose (dependent of the size of the DNA fragments to be observed) was dissolved in TAE buffer by heating it up in a microwave. When cooled to about 60°C, 17 µl of a 0.25 % (w/v) ethidiumbromide solution was added per 100 ml of agarose and gels (5 x 10 cm or 10 x 20 cm) were casted. Dissolved agarose was stored at 68°C until further use. DNA samples were supplemented with DNA loading buffer (4 % (v/v) glycerol, 0.025 % (w/v) bromphenol-blue, 0.025 % (w/v) xylecyanol) and loaded onto the gel. Gels were run at 90-150 V depending on the size of the gel. DNA bands were visualized with UV light at 302 nm. To isolate DNA from agarose gels, the Wizard SV Gel and PCR Clean Up System® kits were used according to the manufacturer's protocol.

2.2.1.2 Amplification of DNA by Polymerase Chain Reaction (PCR)

Amplification of specific DNA sequences was carried out by PCR (Mullis et al. 1986) using specifically designed primers (Metabion) and Taq polymerase. Table 9 shows the reaction mixture; in table 10 a typical PCR program is listed. Annealing temperatures were adjusted for each set of primers individually.

Component	Amount
Taq standard buffer 10x	10 µl
dNTPs	0.2 µM
Primers 5' -> 3' and 3' -> 5'	0.5 µM each
Template DNA	100 ng
Taq polymerase	0.25 units
ddH_2O	ad 100 µl

Table 9 Composition of a standard PCR preparation

Step of PCR program	Temperature	Time
First denaturation	94°C	5 min
Cycle (repeated 30x)		
denaturation	94°C	30 s
Primer annealing	55-70°C	30 s
Primer extension	72°C	1 min / kb
Cycle (repeated 30 x)		
Final extension	72°C	10 min

Table 10 PCR program

2.2.1.3 Restriction digest of DNA

Plasmid DNA and DNA material which was obtained by PCR or by isolation from an agarose gel were digested with 2-5 U restriction endonucleases / 1 µg DNA in a reaction buffer specific for the restriction enzymes at 37°C for 1-2 h.

2.2.1.4 Ligation of DNA

To complete cloning of a plasmid of interest, the target DNA insert was ligated into the vector backbone both being digested with the same restriction enzymes (2.2.1.3). 2 U T4 ligase per 150 ng of linearized vector backbone and about 1 µg insert DNA dependent on the molar ratio between vector and insert were used. The reaction was carried out in 10 µl of ligation buffer for 20 min at room temperature. One mix containing ddH_2O instead of insert DNA served as a negative control.

2.2.1.5 Preparation of electrocompetent **E. coli** *cells*

500 ml of LB media were inoculated with a pre-culture of *E. coli* cells (see 2.2.2.1) and incubated at 37°C rotating at 130 rpm. As soon as an OD_{600} of 0.5 was reached, cells were cooled on ice for 30 min. The cells were subsequently centrifuged at 2 700 g for 20 min at 4°C. Pelleted cells were gently resuspended in 200 ml of ice cold 10 % (w/v) glycerol and centrifuged again. The cells were resuspended in 1 ml 10 % (w/v) glycerol, aliquoted à 40 µl and stored at -80°C.

2.2.1.6 Transformation of electrocompetent E. coli *cells*

40 µl of electrocompetent *E. coli* cells were thawed on ice and mixed either with 100 ng of plasmid DNA or 1 µl of a ligation mix (see 2.2.1.4) in an electroporation cuvette and incubated for 2 min on ice. The cuvette was placed in the electroporator and the settings were adjusted to 1.5 kV, 400 Ω, 25 µF and the cells were treated with a short electrical pulse. The time constant was 8-9 ms. Subsequently, cells were transferred into 1 ml of ice-cold SOC media and shaken at 37°C (or 30°C for ΔDnaK cells) for 30 min. Afterwards, the *E. coli* cells were transferred to LB Amp media to select for transformed cells.

2.2.1.7 Isolation of DNA from E. coli

Plasmid DNA was purified from *E. coli* cells either as a "mini-prep" or as a "midi-prep". For a "mini-prep" 2-3 ml of *E. coli* culture was used and DNA was isolated using the Nucleospin Plasmid QuickPure® kit according to the manufacturer's protocol. For a "midi-prep" 40-50 ml of *E. coli* culture was used and DNA was isolated using the PureYield Plasmid Midiprep System® kit according to the manufacturer's protocol. "Mini-preps" resulted in the isolation of 50 µl of DNA sample containing around 80 ng/µl DNA and were used for qualitative control of cloning. "Midi-preps" yielded 600 µl of DNA sample containing around 300 ng/µl DNA and were used to prepare plasmid stocks. All DNA concentrations were measured with a NanoDrop® photometer at 260 nm using 1 µl of DNA sample against ddH_2O. DNA was sequenced by Eurofins MWG Operon® or GATC Biotech®. Analysis of the results was performed by sequence alignment using the computer program ApE.

2.2.1.8 Transformation of yeast cells

S. cerevisiae cells were transformed using the lithium acetate method as described in the "Quick and Easy TRAFO Protocol" (Woods and Gietz 2001). All solutions were autoclaved before use or filtered through a 0.2 µm filter. 50 µl of yeast cells were washed in ddH_2O and resuspended in 1 ml 100 mM $LiC_2H_3O_2$. After 5 min incubation at 30°C, cells were re-isolated by centrifugation and overlaid with 240 µl 50 % (w/v) PEG, 36 µl 1 M $LiC_2H_3O_2$, 25 µl 2 mg/ml ss-DNA, 5 µl plasmid DNA (containing 1 mg DNA) and 45 µl ddH_2O. Cells were resuspended in this transformation mix by vortexing and incubated at 42°C for 20 min. Afterwards cells were pelleted, resuspended in 200 µl ddH_2O and plated on selective media. Plates were incubated at 30°C for 3 to 8 days until colonies were visible.

2.2.2 Cell biology and *in organello* methods

2.2.2.1 Cultivation of E. coli *cells*

E. coli cells were cultivated in liquid media (usually LB-Amp, or LB in the case of cultivation of electro competent cells) shaking at 130 rpm after inoculation with a single *E. coli* colony from a

plate grown overnight at 37°C in the case of BL21(DE3) strains or at 30°C in the case of BL21(DE3) ΔDnaK strains. This pre-culture was diluted and grown further as described for expression of recombinant proteins or directly used for plasmid isolations or for preparation of electro competent cells.

Table 11 lists the media used for *E. coli* cells in this study. All media were prepared in double distilled water and autoclaved before the addition of antibiotics. For preparation of solid media, 1.7 % (w/v) of separately autoclaved bacto agar was added to the liquid media.

LB media	**LB Amp**	**SOC**
0.5 % (w/v) yeast extract	0.5 % (w/v) yeast extract	0.5 % (w/v) yeast extract
1 % (w/v) bacto-tryptone	1 % (w/v) bacto-tryptone	2 % (w/v) bacto-tryptone
1 % (w/v) NaCl	1 % (w/v) NaCl	8.56 mM NaCl
	100 µg/ml ampicillin	2.5 mM KCl
		10 mM $MgCl_2$
		20 mM glucose

Table 11 Media for *E. coli* cultivation

2.2.2.2 Cultivation of human cell lines

Cell lines were cultivated at 37°C and 5 % CO_2, in suitable media indicated in table 8. All tissue culture handling was carried out under aseptique conditions in a laminar air flow hood. Cell lines were passaged at about 80 % confluence. To this end, adherent cells were washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) to remove residual media and subsequently incubated with Trypsin-EDTA 1x solution for several minutes until they started to detach from the dish. Trypsin activity was stopped by addition of 2 fold excess of media. Cells were detached completely from the dish by gentle pipetting. Cells were collected by centrifugation at 700 g for 3 min and resuspended in fresh media. Cells were seeded into fresh dishes, thereby diluting them 1:3 – 1:10 depending on the confluence.

2.2.2.3 Generation of stable FlpIn 293 T-REx cell lines coding for MCJ-FLAG and DNAJC19-FLAG

For generation of stable cell lines the Flp-In™ 293 T-REx cell line was used. The cells were cultured in media containing 100 µg/ml Zeocin. Flp-In™ 293 T-REx cells harbor one single stably integrated FRT site at a locus which is controlled by tetracycline dependent repressor (TET-OFF system) (Invitrogen). Upon co-transfection of a pCDNA5 FRT TO plasmid encoding a gene of interest together with the pOGG44 plasmid coding for the Flp recombinase the nucleotide sequence of the gene of interest is stably integrated into that locus. Here, a pCDNA5 FRT TO plasmid encoding either MCJ-FLAG or DNAJC19-FLAG downstream of a CMV promoter was used and integration of

MCJ-FLAG or DNAJC19-FLAG, respectively, rendered stable cell lines expressing MCJ-FLAG or DNAJC19-FLAG. The targeted integration of the pCDNA5 FRT TO construct was controlled by the resulting resistance of the transfected cells against Hygromycin B and loss of resistance against Zeocin. The empty pCDNA5 FRT TO vector served as a negative control for the integration.

Transfection of a 9 : 1 ratio of pOGG44 : pCDNA5 FRT TO was performed in 6-well plates using the X-fect reagent according to the manufacturer's protocol. After 24 h the cells were washed with PBS and fresh media was added to the cells. After 48 h the cells were split to about 30 % confluence into fresh media containing 150 µg/ml Hygromycin B. Media was replaced to fresh Hygromycin B containing media every three days. After 10 to 14 days foci of dividing cells were identified. Three individual foci per cell line were picked and transferred to a separate culture dish. The cells were expanded in Hygromycin B containing media and tested for Zeocin sensitivity by monitoring their growth in Hygromycin B and Zeocin containing media, respectively, in parallel. Hygromycin resistant clones which were sensitive for Zeocin were used for this study and kept in Hygromycin B containing media throughout the study.

2.2.2.4 Induction of protein expression in stably transfected FlpIn 293 T-REx cell lines

Expression of the flag-tagged constructs in the stably transfected FlpIn 293 T-Rex cell lines was induced using 1.0 or 0.1 µg/ml Doxycycline as indicated for 24 h.

2.2.2.5 Isolation of mitochondria from human cells

Cells were detached from the culture dish using a cell scraper and collected by centrifugation at 700 g. Cells were washed once with PBS and subsequently with PBS containing 5 mM EDTA. Approximately 200 µl of cells were resuspended in 2 ml of standard isolation buffer (SB: 70 mM sucrose, 220 mM mannitol, 5 mM EDTA, 20 mM Hepes pH 7.2) containing 1 x complete EDTA free protease inhibitor. The cells were disrupted by pulling them three times though a 26 G needle. Centrifugation at 2 000 g, 4°C for 10 minutes removed intact cells and cell debris (pellet). The supernatant was collected and separated by a second centrifugation at 16 500 g, 4°C for 10 min into the mitochondrial pellet and the post-mitochondrial supernatant containing cytoplasm and other organelles like Golgi vesicles and lysosomes. This procedure was repeated twice with the cell debris pellet fraction to isolate mitochondria from remaining intact cells. The mitochondrial fraction was gently resuspended in SB and adjusted to a protein concentration of 10 mg/ml. Mitochondria were flash frozen in liquid nitrogen and stored at -80°C.

2.2.2.6 Ultrapurification of mitochondria

Crude mitochondria as obtained by the isolation method described in 2.2.2.5 were further purified by sucrose step gradient ultracentrifugation at 134 000 g for 1 h at 2°C. The gradient

consisted of four sucrose layers (15, 23, 32 and 60 %). Mitochondria at the interface of the 32 and 60 % sucrose layer were harvested, pelleted and resuspended in SB.

2.2.2.7 Cultivation of yeast cells

Following yeast transformation or inoculation from glycerol stocks (yeast cells suspended in 15 % (v/v) glycerol, stored at -80°C) yeast cultures were grown on agar plates at 30°C. For all plasmid shuffling experiments single colonies were used.

Table 12 provides a list of media used for cultivation of yeast cells in this study. All media were prepared in double distilled water and autoclaved before use. Glucose, galactose and the auxotrophy selection markers were autoclaved separately and added afterwards. For preparation of the corresponding solid media, liquid media were supplemented with 2 % (w/v) separately autoclaved bacto-agar.

YP (non-selective)	**YPD (non-selective)**	**SD (selective)**
1 % (w/v) bacto-peptone	1 % (w/v) bacto-peptone	0.17 % yeast nitrogen base
1 % (w/v) yeast extract	1 % (w/v) yeast extract	0.5 % $(NH_4)_2SO_4$
pH adjusted to 5.5	2 % glucose	2 % glucose
	pH adjusted to 5.5	appropriate amount of auxotrophic markers
		ad 1 l H_2O

Auxotrophic markers used for selective media
20 mg/l adenine sulfate
20 mg/l uracil
20 mg/l L-tryptophan
30 mg/l L-leucine
20 mg/l L-histidine
30 mg/l L-lysine

Table 12 Media for yeast cultivation

2.2.2.8 Plasmid shuffling

A haploid *TIM14* deletion yeast strain harboring a copy of wild-type yeast Tim14 on the URA3 containing plasmid pVTU (Mokranjac et al. 2003) was transformed with plasmids expressing either MCJ or DNAJC19. As a positive control, a plasmid expressing yeast Tim14 was used, as a negative control the corresponding empty vector was used. Cells were grown on selective media until colonies were visible. The cells of these colonies carried the transformed plasmid with the marker

for which the media selected and the pVTU plasmid with the endogenous yeast Tim14. Single colonies were transferred to non-selective media for 48 h to allow for loss of plasmids. The same colonies were subsequently transferred to media selecting for the transformed plasmid but additionally containing 1 mg/ml 5-fluoroorotic acid (5'-FOA). Cells still carrying the pVTU plasmid with the URA3 gene did not grow under these conditions. URA3 codes for the enzyme orotine-5-monophosphate decarboxylase which converts 5'-FOA into the toxic compound 5'-fluorouracil. Cells that have lost the URA3 plasmid do not convert 5'-FOA into the toxin and grow in the presence of 5'-FOA.

2.2.2.9 Growth test of S. cerevisiae *cells*

To test for growth phenotypes, drop dilution assays were performed. The yeast strains were grown overnight in 20 ml of the corresponding media and in the morning diluted to an OD_{600} of 0.1. The cultures were further incubated at 30°C on a rotating plate. When sufficient density was reached, each strain was diluted to an OD_{600} of ca. 0.5 which served as a stock for serial dilutions (five times 1 : 10) in dd water. 3 µl of each dilution were pipetted onto a plate with media allowing for growth and incubated at 30°C for 5 days.

2.2.2.10 Preparation of yeast cell extracts

Yeast cells were grown to the logarithmic phase and 2.5 OD were harvested by centrifugation. The pelleted cells were resuspended in 100 µl ddH_2O and 100 µl 0.2 M NaOH were added. The mix was incubated for 5 min at room temperature and centrifuged at 18 000 g for 5 min at room temperature. The pellet was resuspended in 50 µl Laemmli buffer (10 % glycerol, 2 % SDS, 10 mM Tris pH 6.8, 0.1 g/l bromphenol-blue, 5 % ß-mercaptoehtanol) and proteins were denatured at 95°C for 3 minutes. Undissolved material was removed by centrifugation at 18 000 g for 5 minutes at room temperature. The supernatant was analyzed by SDS-PAGE and immunoblotting.

2.2.2.11 Subfractionation of human mitochondria

To assess the localization and topology of proteins in the different mitochondrial sub-compartments, gradual opening of mitochondria was combined with proteinase K treatment. In presence of an intact outer membrane, only outer membrane proteins are accessible to externally added protease whereas in mitoplasts, which do not contain an intact outer membrane IMS proteins and IMS facing inner membrane proteins are accessible. Mitoplasts are generated by disrupting the outer membrane by hypotonic swelling. To this end, mitochondria were diluted 10-fold in 20 mM HEPES-KOH, pH 7.4. Mitoplasts and mitochondria were incubated with or without 50 mg/ml proteinase K for 20 min on ice. Mitochondria and mitoplasts were re-isolated, washed and precipitated with 12 % (w/v) TCA. The fractions were analyzed by SDS–PAGE and immunoblotting.

2.2.2.12 Carbonate extraction of membrane proteins from mitochondria

Carbonate extraction was performed to distinguish integral membrane proteins from membrane attached and soluble proteins. Isolated human mitochondria were incubated in 0.1 M sodium carbonate buffer at pH 11.5 for 20 min on ice. Soluble and membrane attached proteins were separated from integral membrane proteins by centrifugation at 124 000 g for 30 min at 4°C. The supernatant containing the soluble and membrane attached proteins was precipitated with 12 % TCA. Samples were analyzed by SDS–PAGE and immunodecoration with antibodies against the indicated proteins.

2.2.2.13 Import of radiolabelled precursor proteins into isolated mitochondria

Import reactions were carried out at 34°C in 100 µl import buffer (0.6 M sorbitol, 80 mM KCl, 10 mM Mg-acetate, 2 mM K-phosphate, 2.5 mM EDTA, 2.5 mM $MnCl_2$ and 50 mM HEPES, pH 7.2) supplemented with 10 mM ATP, 10 mM GTP, 5 mM NADH, 20 mM citrate, 10 mM phosphocreatine (PC) and 100 µg/ml creatine kinase (CK). When indicated, the mitochondrial membrane potential was dissipated by addition of 2 µM valinomycin in absence of nucleotides, NADH, citrate, PC and CK. 50 µg of mitochondria and 3–6 % (v/v) of reticulocyte lysate containing the precursor protein were used per reaction. Following incubation, the samples were treated with 100 µg/ml proteinase K for 15 min on ice to remove non-imported preprotein. The protease was stopped by addition of 2 mM PMSF. Mitochondria were re-isolated, washed with SH-KCl buffer (0.6 M sorbitol, 50 mM Hepes pH 7.4, 80 mM KCl) and subjected to SDS–PAGE and autoradiography.

2.2.3 Protein chemistry methods

2.2.3.1 SDS-PAGE

To separate proteins by their molecular weight under denaturing conditions a discontinuous vertical one dimensional SDS gel electrophoresis was used (Laemmli 1970). 14 cm x 10 cm gels containing a 1 cm bottom gel below a 7 cm separating gel below a 2 cm stacking gel were prepared according to table 13.

The amount of acrylamide varied dependent on the size of proteins run on the gel. Samples were loaded after supplementation with Laemmli buffer (60 mM Tris pH 6.8, 2 % (w/v) SDS, 10 % glycerol, 5 % (v/v) ß-mercaptoethanol, 0.05 % (w/v) bromphenol-blue). Separation was carried out at 35 mA for 1.5-2 h in electrophoresis buffer (25 mM Tris, 62 mM glycine, 0.1 % SDS).

bottom gel	separating gel	stacking gel
20 % (w/v) acrylamide	14 or 16 % (w/v) acrylamide,	5 % (w/v) acrylamide,
0.4 % (w/v) bis-acrylamide	0.28 or 0.33 % (w/v) bis-acrylamide	0.1 % (w/v) bis-acrylamide
375 mM Tris pH 8.8	375 mM Tris pH 8.8	60 mM Tris pH 6.8
0.1 % (w/v) SDS	0.1 % (w/v) SDS	0.1 % (w/v) SDS
0.05 % (w/v) APS	0.05 % (w/v) APS	0.05 % (w/v) APS
0.05 % (v/v) TEMED	0.05 % (v/v) TEMED	0.05 % (v/v) TEMED

Table 13 Components of SDS gels used in this study

2.2.3.2 Coomassie Blue Staining of SDS-gels

To visualize proteins on SDS-gels, gels were rinsed in ddH_2O and incubated in Coomassie Blue staining solution (30 % (v/v) methanol, 10 % (v/v) acetic acid, 0.1 % (w/v) Serva Blue R-250) for 1-3 h at room temperature. Next, gels were transferred to a destaining solution (30 % (v/v) methanol, 10 % (v/v) acetic acid) for 1 h. The destaining solution was exchanged to a fresh destaining solution several times every 2-12 h until blue protein bands were visible on transparent background. The used destaining solution was destained by activated charcoal for reuse.

2.2.3.3 Western Blotting

To transfer proteins which were separated by SDS-PAGE onto a nitrocellulose membrane semi-dry blotting was performed (Kyhse-Andersen 1984) using 17 cm x 12 cm blotting paper and 15 cm x 10 cm nitrocellulose membranes. All materials including the blotting chamber and the gel were soaked in blotting buffer (20 mM Tris, 150 mM glycine, 0.02 % (w/v) SDS, 20 % (v/v) methanol) prior to assembly. The blot was assembled as follows: cathode – blotting paper – polyacrylamide gel – NC membrane – blotting paper – anode. Transfer of the proteins was carried out at 250 mA for 1 h. To visualize proteins on the membrane, they were stained with Ponceau S solution (0.2 % (w/v) Ponceau S, 3 % (w/v) TCA).

2.2.3.4 Autoradiography

Radiolabelled proteins on nitrocellulose membranes were visualized using highly sensitive films (Kodak) which were developed automatically with the developing machine and corresponding solutions by AGFA. For quantification of the bands, the films were scanned with a densitometer and analyzed using the computer software Labscan® and ImageMaster®.

2.2.3.5 TCA precipitation

Samples were mixed with TCA to a final concentration of 12 % (v/v) and incubated at least 30 min at -20°C. After centrifugation at 20 000 g, 4°C for 20 min, the pelleted protein samples were washed with 1 ml ice-cold acetone and vortexed thoroughly. After another centrifugation step the

supernatant was removed, samples were dried at room temperature for 3-5 min and resuspended in Laemmli buffer.

2.2.3.6 Expression of recombinant proteins in E. coli

E. coli cells carrying the expression plasmid were grown in a shaking incubator at 30°C or 37°C in 2 to 6 l LB Amp media until they reached an OD_{600} of about 1.0. Protein expression was induced by addition of 1 mM IPTG and carried out for 3 h at 37°C or overnight at lower temperature (16°C). Cells were harvested by centrifugation at 4 400 g for 15 min and washed in ddH_2O before they were stored at -20°C until further use.

2.2.3.7 Purification of recombinant proteins

2.2.3.7.1 Purification of native his-tagged proteins

The bacterial pellet was thawed on ice, resuspended in 20-40 ml (dependent on the amount of *E. coli* cells harvested) lysis buffer (50 mM Tris pH 8.0, 200 mM NaCl, 20 mM imidazole, 5 % (w/v) glycerol, 1 mM PMSF) containing 1 mg/ml lysozyme and incubated with agitation for 45 minutes at 4°C. Sonication was performed at level 4, 80 % duty cycle for 10 x 12 s on ice to break the cells. Cell debris was pelleted at 27 500 g for 15 minutes and the supernatant was loaded onto a preequilibrated Ni-NTA column. The bed volume was 1-4 ml dependent on the amount of *E. coli* cells harvested and the expression level. Binding was allowed at a flow-rate of 1 ml/min. The column was washed with 50-100 ml washbuffer (50 mM Tris pH 8.0, 200 mM NaCl, 50 mM imidazole, 5 % (w/v) glycerol, 1 mM PMSF). The his-tagged protein was eluted in 1 ml fractions with 10 ml of elution buffer (50 mM Tris pH 8.0, 200 mM NaCl, 500 mM imidazole, 5 % (w/v) glycerol, 1 mM PMSF). Small aliquots of all fractions were analyzed by SDS-PAGE and subsequent Coomassie staining for purity of the protein. Protein containing elution fractions were pooled and concentrated over a suitable Amicon Spin Column if necessary. The elution buffer was exchanged to storage buffer (20 mM Hepes pH 7.4, 100 mM KCl, 5 % glycerol, 5 mM $MgCl_2$) via a PD10 column according to the manufacturer's protocol. Aliquots were flash frozen in liquid nitrogen and stored at -80°C.

2.2.3.7.2 Purification of denatured his-tagged proteins

The bacterial pellet was thawed on ice, resuspended in 20 ml lysis buffer (100 mM NaH_2PO_4, 6 M guanidinium-HCl, 10 mM Tris pH 8.0) and incubated with agitation for 45 minutes at room temperature. Cell debris was pelleted at 20 000 g for 15 minutes and the supernatant was loaded onto a preequilibrated Ni-NTA column. The bed volume was 1-4 ml dependent on the amount of *E. coli* cells harvested and the expression level. Binding was allowed at a flow-rate of 1 ml/min. The column was washed with 15 ml lysis buffer and 15 ml washbuffer (100 mM NaH_2PO_4, 6 M

guanidinium-HCl, 10 mM Tris pH 6.3). The his-tagged protein was eluted in 1 ml fractions with 10 ml of elution buffer (100 mM NaH_2PO_4, 6 M guanidinium-HCl, 10 mM Tris pH 4.5). Small samples of all fractions were diluted 4 fold in water and supplemented with Laemmli buffer without SDS to avoid precipitation of guanidinium. SDS-PAGE and subsequent Coomassie staining were applied for control of purity of the protein. Protein containing elution fractions were pooled, aliquoted and stored at -80°C.

2.2.3.7.3 Purification of GST-tagged proteins

The bacterial pellet was thawed on ice, resuspended in 20-40 ml (dependent on the amount of *E. coli* cells harvested) PBS containing 1 mM PMSF and 1 mg/ml lysozyme and incubated with agitation for 45 minutes at 4°C. Sonication was performed at level 4, 80 % duty cycle for 10 x 12 s on ice to break the cells. Cell debris was pelleted at 27 500 g for 15 minutes and the supernatant was loaded onto a pre-equilibrated GSH sepharose column. The bed volume was 1-4 ml dependent on the amount of *E. coli* cells harvested and the expression level. Binding was allowed at a flow-rate of 1 ml/min. The column was washed with 50-100 ml PBS containing 1 mM PMSF. Either the GST-tagged protein was eluted in 1 ml fractions with 10 ml of PBS containing 20 mM GSH (reduced) and 1 mM PMSF or the protein was cleaved off the tag by incubating the sepharose beads with bound protein overnight at 4°C with an appropriate amount of PreScission protease® and collecting the flow-through. Small aliquots of all fractions were analyzed by SDS-PAGE and subsequent Coomassie staining for purity of the protein. Protein containing elution fractions or the flow-through containing the cleaved protein were concentrated over a suitable Amicon® Spin Column if necessary. The buffer was exchanged to storage buffer (20 mM Hepes pH 7.4, 100 mM KCl, 5 % glycerol, 5 mM $MgCl_2$) via a PD10 column according to the manufacturer's protocol. Aliquots were flash frozen in liquid nitrogen and stored at -80°C.

2.2.3.8 In vitro *synthesis of radiolabelled preproteins*

For preparation of radiolabelled proteins the TNT® Coupled Reticulocyte Lysate System (Promega) was used according to the manufacturer's protocol using ^{35}S-methionine.

2.2.3.9 Determination of protein concentration

The concentration of proteins was determined with the Bradford assay (Bradford 1976). A calibration curve was prepared from five differently concentrated bovine IgG standards in 5 fold diluted Bradford reagent. Extinction was measured in an Eppendorf BIO spectrophotometer at 595 nm. Three technical replicates of the sample were measured and the average protein concentration was calculated.

2.2.3.10 Enzyme-coupled spectrophotometric ATPase activity assay

The ATPase activity of recombinant mortalin in presence and absence of J co-chaperones was determined using a spectrophotometric assay (Norby 1988). In this assay, ATP hydrolysis was enzymatically linked to the oxidation of NADH to NAD^+ which could be photometrically monitored at 360 nm. The following equations describe the mechanism of the experiment.

$$ATP \xrightarrow{\text{Mortalin}} ADP + P_i$$

$$ADP + PEP \xrightarrow{\text{PK}} ATP + pyruvate$$

$$pyruvate + NADH/H^+ \xrightarrow{\text{LDH}} lactate + NAD^+$$

From recording the decrease of $NADH/H^+$ concentration over time, the rate of ATP hydrolysis can be calculated per mol mortalin per time.

The assay was carried out at 25°C in buffer containing 20 mM HEPES pH 7.4, 80 mM KCl, 5 mM $MgCl_2$, 2 mM ATP, 3 mM PEP, 0.12 mM NADH, 39 U/ml PK, 37 U/ml LDH and 2 µM of human Mge. The reaction was started by addition of 1 µM mortalin. The rates of ATP hydrolysis and standard deviations were calculated from the changes in absorbance measured in time ($n \geq 5$, $t \geq 300$ s). When indicated, 7.5 µM of purified MCJ(s) or MAGMAS(s) or 10 µM of purified DNAJC19(s) was added to the reaction. To test for the inhibition by MAGMAS, either the purified 7.5 µM MCJ(s) was pre-incubated for 5 min at 25°C in an 1 : 1 molar ratio with purified MAGMAS(s) and then added to the assay or 7.5 µM purified MCJ(s)–MAGMAS(s) complex was added to the assay.

2.2.4 Immunology methods

2.2.4.1 Generation of antibodies

Antibodies against MAGMAS, TIM50 and TIM21 were generated using the full length proteins, as indicated in table 6. These proteins were recombinantly expressed and purified via affinity chromatography (see 2.2.2.3.7). 100-200 µg of the protein per lane were run in an SDS-PAGE and transferred onto a NC membrane, where they were visualized by Ponceau S staining. The bands referring to the size of the recombinant protein were cut out of the membrane, dissolved in DMSO and mixed in a 1:1 ratio of *Freund's Adjuvant Incomplete*. For generation of antibodies against DNAJC19, 250 µl (1 mg/ml) of the soluble domain of the protein in PBS was added to 250 µl of *Freund's Adjuvant Incomplete* directly after the purification. 100 – 500 µg antigen mixed with *Freund's Adjuvant Incomplete* were injected into rabbits. Injections were repeated every 6 weeks. 10 days after the injection 20 ml blood were collected.

2.2.4.2 Generation of serum from blood

The agglutinated rabbit blood was centrifuged at 3 200 g for 10 min at room temperature to remove the agglutinated material. The supernatant was centrifuged at 13 000 g for 15 min at 4°C to remove the remaining insoluble material. This second supernatant was incubated in a waterbath at 56°C for 30 min to inactivate factors of the complement system. Serum was stored at -20°C.

2.2.4.3 Purification of antibodies

To enrich the specifically generated antibodies from rabbit serum, the serum was affinity purified over an antigen containing column. Therefore, two different methods were used dependent on whether the antigen was (1) a small peptide or (2) a complete protein or domain of a protein.

(1) To immobilize the HPLC purified and lyophilized peptide (delivered by Pineda), SulfoLink® coupling gel was used. The gel (stored at 4°C) was brought to room temperature and 1 ml of the gel was transferred into a column and washed with 6 ml of coupling buffer I (50 mM Tris pH 8.5, 5 mM EDTA). 1 mg of the peptide of interest was dissolved in 1 ml of the coupling buffer I and incubated for 15 min under rotation and subsequently for 30 min without agitation with the washed coupling gel at room temperature. After removal of the buffer, the gel was washed with 3 ml of coupling buffer I. To block nonspecific binding sites on the gel, the gel was incubated with 1 ml of blocking buffer (50 mM cysteine, 5 mM EDTA, 50 mM Tris pH 8.5) in the same manner as described for the coupling buffer I containing the peptide. Blocking buffer was removed and the gel was washed first with 16 ml of 1 M NaCl and second with 4 ml 10 mM Tris pH 7.4. The column containing the immobilized peptide on the SulfoLink® gel was stored in 10 mM Tris pH 7.4 containing 0.05 % NaN_3 at 4°C until use.

(2) To immobilize recombinantly expressed and purified proteins or domains of proteins on a column of CNBr-activated sepharose, ca 4 mg of the protein were transferred to coupling buffer II (0.1 M $NaHCO_3$, 0.5 M NaCl pH 8.3) via a PD10 column according to the manufacturer's protocol. 500 mg CNBr-activated sepharose 4B was swollen in 10 ml of 1 mM HCl for 20 min at room temperature and subsequently washed with 200 ml of 1 mM HCl. 1 ml of the washed sepharose was transferred into a column and incubated with the antigen contained in 3.5 ml of coupling buffer II for 1 h at room temperature under rotation. After removal of the buffer, the sepharose beads were washed with 6 ml of coupling buffer II and nonspecific binding sites on the beads were blocked by incubation with 2 ml of 1 M ethanolamine pH 8.0 at room temperature for 2 h. The beads were washed three times alternating with 6 ml of washbuffer I (0.1 M $NaC_2H_3O_2$, 0.5 M NaCl pH 4.0) and 6 ml of washbuffer II (0.5 M NaCl, 0.1 M Tris pH 8.0). The column containing the

immobilized protein on the sepharose beads was stored in 10 mM Tris pH 7.4 containing 0.05 % NaN_3 at 4°C until use.

For affinity purification of the antibodies, the column containing the immobilized antigen was equilibrated with 10 ml 10 mM Tris pH 7.4. 10 ml of rabbit serum were added to 20 ml of loading buffer (1 mM EDTA, 1 mM PMSF, 10 mM Tris pH 7.4) and loaded onto the gel / beads. Unbound material was collected in the flow-through. The gel / beads was washed with 10 ml 10 mM Tris pH 7.4, with 10 ml of 10 mM Tris pH 7.4 containing 0.5 M NaCl and with 10 ml of 100 mM sodium citrate pH 4.0. Bound antibodies were eluted in 1 ml fractions with 10 ml of 100 mM glycine pH 2.5 and neutralized with 200 µl of 1 M Tris pH 8.8 per fraction. The gel / beads was washed with 10 ml 10 mM Tris pH 7.4 and stored in 10 mM Tris pH 7.4 containing 0.05 % NaN_3 at 4°C until the next use. The eluted fractions were tested in immunodecoration experiments and antibody-rich fractions were pooled, aliquoted and stored at -20°C.

2.2.4.4 Immunoblotting of proteins on nitrocellulose membranes

To prevent unspecific binding of antibodies to the membrane, the membrane was rinsed with TBS (150 mM NaCl, 10 mM Tris pH 7.5) and incubated in 5 % (w/v) milkpowder / TBS or 1 % WBR for 30 minutes at room temperature depending on the reagent in which the antibody was diluted (see table 6). Concentration of the antibodies was determined individually for each antibody. The membrane was incubated in the antibody solution either for 1.5 h at room temperature or overnight at 4°C. The antibody solution was collected and stored at -20°C, the membrane was washed 5 min in TBS, 5 min in TBS containing 0.05 % (v/v) Triton X-100 and 5 min in TBS at room temperature. The washed membrane was incubated for 1 h at room temperature in milk containing 1:10 000 diluted secondary mouse or rabbit HRP coupled antibody, respectively, depending on the first antibody's origin. The membrane was washed again three times as described above and covered with freshly prepared ECL reagent (1:1 mixture of ECL1 (0.44 mg/mL Luminol, 66.5 µg/ml p-coumaric acid, 100 mM Tris pH 8.5) and ECL2 (0.03 % (v/v) H_2O_2, 100 mM Tris pH 8.5)). Luminescent signals were visualized on X-ray films, which were developed automatically with an AGFA developing machine and the corresponding solutions by AGFA.

2.2.4.5 Immunoprecipitation

Mitochondria isolated from HeLa cells were lysed in lysisbuffer (20 mM TrisHCl pH 7.4, 80 mM KCl, 1 mM PMSF with complete EDTA-free protease-inhibitor cocktail (Roche)) containing either 2.5 % or 1 % digitonin as indicated or 1 % Triton X-100 for 20 min at 4°C. For lysis in SDS, mitochondria were incubated for 10 min at room temperature in 1 % SDS and subsequently diluted 10 times in 0.05 % Triton X-100 to the final volume. After a clarifying spin at 45 000 g, 4°C for 25 min, the lysate was incubated for 90 min at 4°C with antibodies or pre-immune IgGs prebound to protein-A

sepharose. For αflag pull-down the commercially available αflag beads were used after three washing steps in lysis buffer. The supernatant was removed and the beads were washed twice with 1 ml of buffer containing 0.1 % digitonin or 0.05 % Triton X-100. Bound proteins were eluted with Laemmli buffer. Proteins of total cell lysates, of the supernatant after the immunoprecipitation and of the eluate were analyzed by SDS–PAGE and immunoblotting with antibodies against the indicated proteins.

3 Results

Many components of the human TIM23 translocase have homologues in yeast. The presence of more than one human homologue for some of the components suggests a more complex composition and / or function of the human translocase compared to yeast. DNAJC19, one of the Tim14 homologues, has been shown to interact with MAGMAS and to stimulate the ATPase activity of mtHsp70 (Sinha et al. 2010) implying that it functions in the TIM23 translocase. However, MCJ, the other Tim14 homologue, was poorly characterized as a Golgi protein involved in chemoresistance of tumor cells (Hatle et al. 2007). Thus, it was analyzed in this study, whether MCJ is also a mitochondrial protein and potentially plays a functional role in the TIM23 translocase.

3.1 MCJ is a sequence homologue of yeast Tim14

MCJ is a 150 amino acid residue protein with a molecular mass of 16.39 kDa. In figure 3, a sequence alignment of MCJ with various Tim14 proteins is shown. The sequence of the J domain is conserved throughout evolution and between the two human Tim14 homologues, whereas the N-terminus is not conserved and is not present in DNAJC19, the second human Tim14 homologue. Identical residues are highlighted in yellow.

```
S.c. Tim14                MSSQSNTGNSIEAPQLPIPGQTNGSANVTVDGAGVNV  38
C.e. Tim14                                                        0
H.s. MCJ                                              MAARGVIAP   9
H.s. DNAJC19                                                      0

S.c. Tim14    GIQNGSQGQKTGMDLYFDQALNYMGEHPVITGFGAFLTLYFTAGAYKS   85
C.e. Tim14                                MTGGLIVAGLGLAAVGFGARYV   22
H.s. MCJ      VGESLRYAEYLQPSAKRPDADVDQQRLVRSLIAVGLGVAALAFAGRYA   57
H.s. DNAJC19                              MASTVVAVGLTIAAAGFAGRYV   22

S.c. Tim14    ISKGLNGGKSTT.............AFLKGGFDPKMNSKEALQILNLT  120
C.e. Tim14    LRNQALIKKGMEAIPVAGGA...FSNYYRGGFDQKMSRAEAAKILGVA   67
H.s. MCJ      FRIWKPLE.QVITETAKKISTPSFSSYYKGGFEQKMSRREAGLILGVS  104
H.s. DNAJC19  LQAMKHMEPQVKQVFQSLPKSAFSGGYYRGGFEPKMTKREAALILGVS   70

S.c. Tim14    ENTLTKKKLKEVHRKIMLANHPDKGGSPFLATKINEAKDFLEKRGISK  168
C.e. Tim14    PSAKPA.KIKEAHKKVMIVNHPDRGGSPYLAAKINEAKDLMESSKS    112
H.s. MCJ      PSAGKA.KIRTAHRRVMILNHPDKGGSPYVAAKINEAKDLLETTTKH   150
H.s. DNAJC19  PTANKG.KIRDAHRRIMLLNHPDKGGSPYIAAKINEAKDLLEGQAKK   116
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Figure 3 Alignment of MCJ with various Tim14 proteins

MCJ shows high sequence homology with Tim14 proteins. Identical residues are highlighted. The J domain is underlined. S.c.: *Saccharomyces cerevisiae*; C.e.: *Caenorhabditis elegans*; H.s.: *Homo sapiens*.

3.2 MCJ is a J domain containing protein with a putative TMS

To further characterize the structure of MCJ, its amino acid sequence was subjected to a Kyte-Doolittle-hydropathy plot (Artimo et al. 2012). The result is shown in figure 4. Score values above zero indicate hydrophobicity of the protein at the underlying residue positions. Three highly hydrophobic stretches were observed from amino acids 3 to 8, 38 to 57 and 99 to 105.

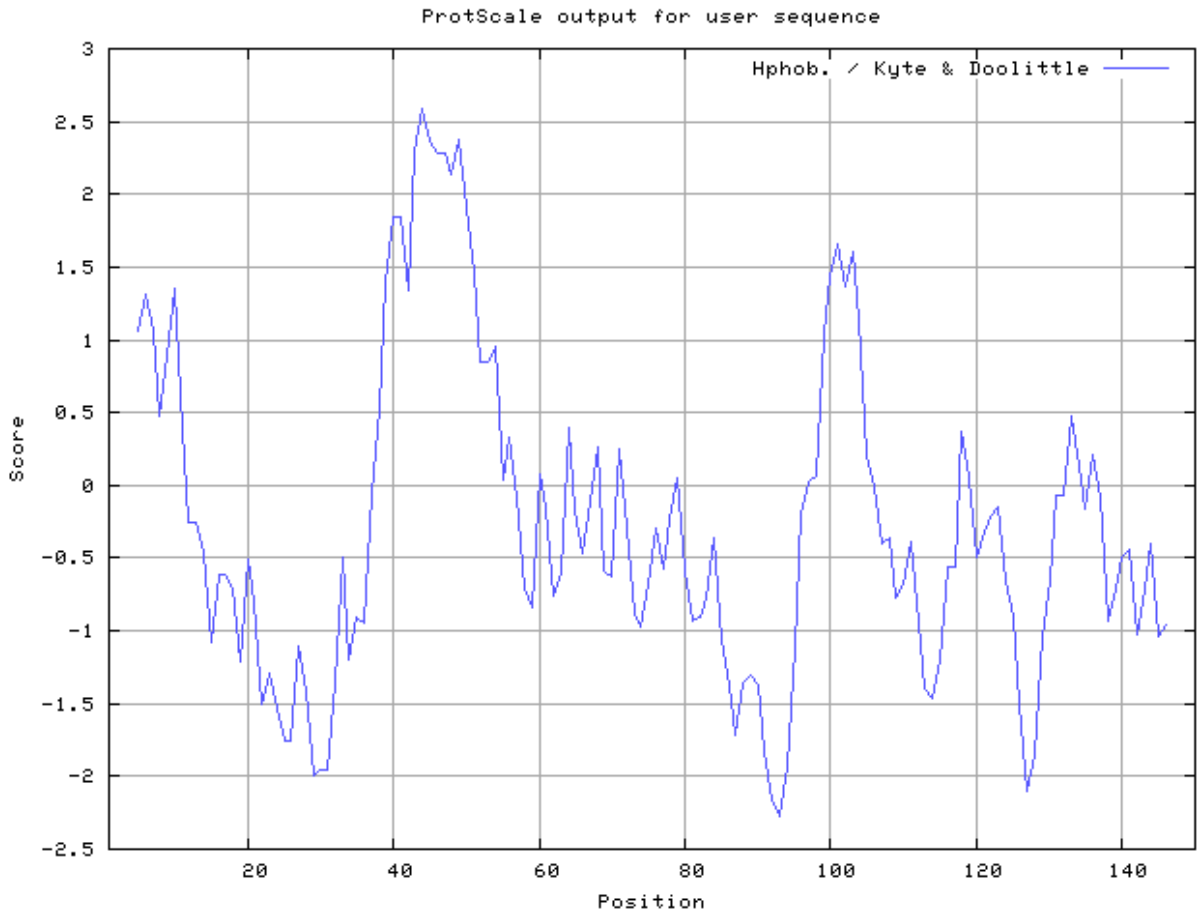

Figure 4 Hydropathy plot of the MCJ amino acid sequence

Residues in the region of amino acids 3 to 8, 38 to 57 and 98 to 105 exhibit hydrophobicity.

Transmembrane segments (TMS) are not only characterized by hydrophobicity of their residues but they also need to have a certain length to span a lipid bilayer. In order to analyze more precisely, whether the region around the residues 38 to 57 which is long enough to span a membrane can be predicted as a transmembrane segment, the amino acid sequence of MCJ was subjected to a transmembrane prediction software ("TMpred" (Hofmann and Stoffel 1993)). This software does not only take the hydrophobicity of residues into account, but also compares it to known transmembrane segments and returns a probability of each residue to be part of a TMS. Therefore, the TMS prediction is more specific and reliable. The result for the MCJ sequence is shown in figure 5. Residues 36 to 56 are predicted as a transmembrane segment of MCJ.

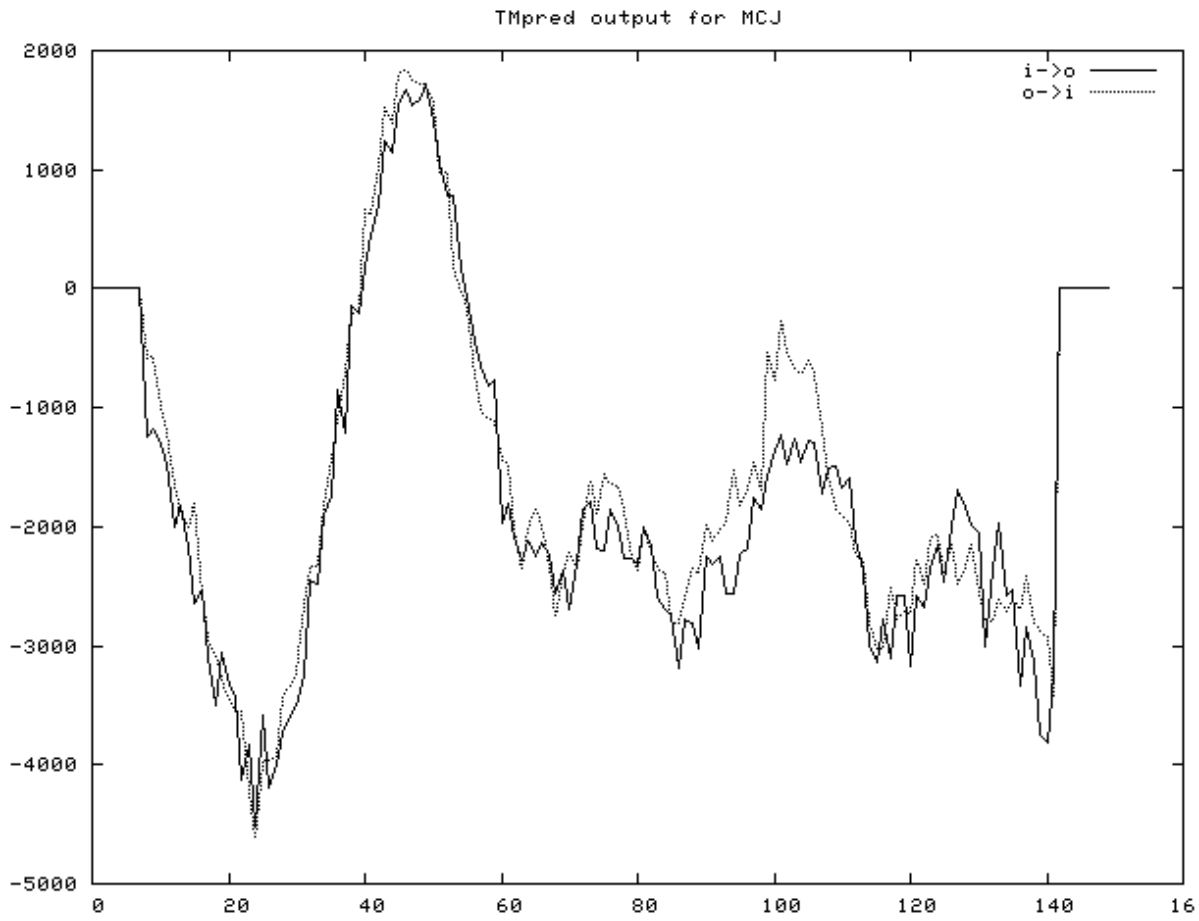

Figure 5 Result of the TMS prediction of the amino acid sequence of MCJ

The peak above zero indicates a predicted transmembrane segment. The black line represents the inside-to-outside (i->o) orientation, the dotted line the outside-to-inside (o->i) orientation. The o->i model is strongly preferred (http://embnet.vital-it.ch/cgi-bin/TMPRED_form_parser).

In summary, MCJ consists of a non-conserved N-terminal tail, a predicted trans-membrane segment and a highly conserved C-terminal J domain.

3.3 Antibodies against the N-terminus of MCJ recognize MCJ but not DNAJC19

Since the N-terminal tail of MCJ is not conserved, it is the part that is highly specific for the protein. In order to obtain specific polyclonal antibodies against MCJ that do not cross-react with the other Tim14 homologue, antibodies against a peptide in the MCJ N-terminus were raised and affinity purified. The antibodies were tested in an immunoblot with isolated mitochondria from HeLa cells and different amounts of recombinant soluble domain of DNAJC19 (DNAJC19(sol)). The antibodies against MCJ recognized only MCJ but not DNAJC19 in the HeLa mitochondria. Even in the presence of high amounts of recombinant DNAJC19(sol) protein on the same NC-membrane, antibodies against MCJ did not recognize DNAJC19(sol), as shown in figure 6.

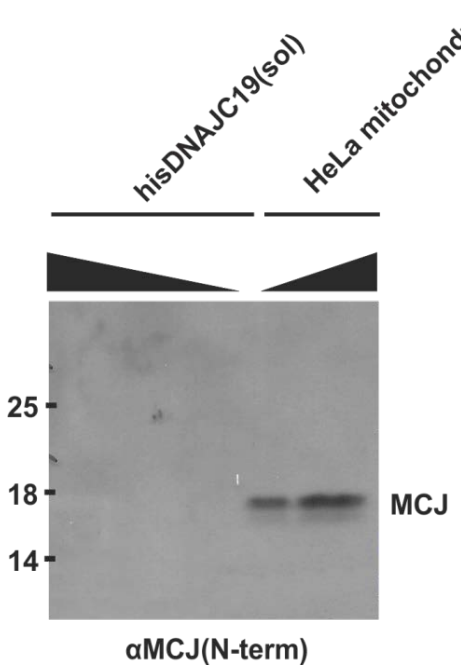

Figure 6 Antibodies against the N-terminus of MCJ do not recognize DNAJC19, but specifically recognize MCJ.

250, 125 and 60 ng recombinant histagged soluble domain of DNAJC19 (DNAJC19(sol)) and 25 and 50 µg isolated HeLa mitochondria were subjected to SDS-PAGE and immunoblotting with antibodies directed against the N-terminus of MCJ (αMCJ(N-term)). A band at 18 kDa corresponding to endogenous MCJ in HeLa mitochondria was detected. No band corresponding to recombinant (about 12 kDa) or endogenous (14 kDa) DNAJC19 was detected.

3.4 MCJ is located in the mitochondrial inner membrane

3.4.1 MCJ is located in mitochondria

To analyze the cellular location of endogenous MCJ, subfractionation of HeLa cells was performed. The cells were disrupted and the post-nuclear supernatant was separated from cell debris and nuclei by a low speed centrifugation step. Additional centrifugation of the post-nuclear supernatant at higher speed yielded a crude mitochondrial pellet and the post-mitochondrial supernatant. The mitochondrial fraction was further purified by a sucrose gradient ultracentrifugation. Equal amounts of all fractions were subjected to SDS-PAGE and immunoblotting with antibodies directed against proteins specific for distinct cellular compartments.

As shown in figure 7, MCJ behaves like the mitochondrial marker proteins TIM44, TIM23 and MAGMAS. They are all found in the cell extracts (Total) and in the mitochondrial fraction, but are depleted from the post-mitochondrial fraction. After the sucrose gradient centrifugation, the mitochondrial marker proteins are further enriched in the purified mitochondrial fraction. In contrast, EEA1, a marker for endosomal proteins, and GM130, a marker for Golgi proteins, are present in the post-mitochondrial fraction and absent from the mitochondrial fraction. Calnexin,

the marker for ER proteins, is present in the crude mitochondrial fraction. Upon purification of the mitochondria the amount of co-purified ER protein decreases. Co-purification of ER vesicles together with mitochondria is commonly observed as these organelles are in close contact (English and Voeltz 2013, Kornmann 2013).

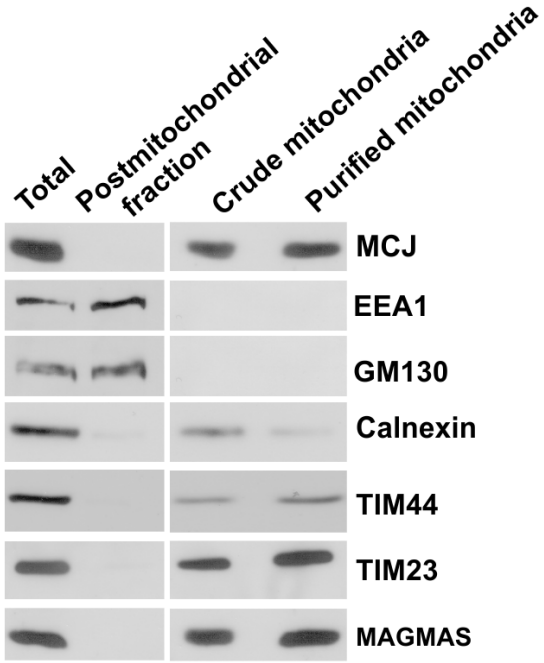

Figure 7 MCJ is enriched in purified mitochondria.

Mitochondria were isolated from HeLa cells as described in 2.2.2.5 and purified by a sucrose gradient centrifugation as described in 2.2.2.6. Equal amounts of all fractions were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins. Different subcellular compartments are represented by organellar marker proteins: MAGMAS, TIM23, TIM44: mitochondria; EEA1: endosomes; GM130: Golgi; Calnexin: ER. MCJ was detected in the crude and purified mitochondrial fractions.

From these results it is concluded that MCJ is a mitochondrial protein and is not present in the Golgi apparatus.

3.4.2 MCJ is located in the inner mitochondrial membrane with the N-terminus in the intermembrane space (IMS)

To assess the location of MCJ within mitochondria, alkaline carbonate extraction of isolated mitochondria was performed. This method separates integral membrane proteins from peripheral membrane proteins and soluble proteins. In good agreement with the prediction of a transmembrane segment, MCJ was found in the pellet fraction, as was the known inner membrane protein TIM23. In contrast, TIM44, an inner membrane associated matrix protein, was recovered in the supernatant.

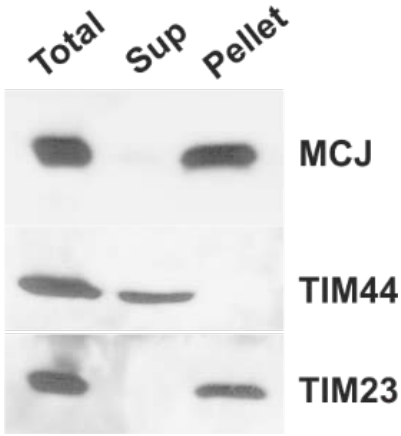

Figure 8 MCJ is present in the pellet fraction of an alkaline carbonate extraction of HeLa mitochondria.

Mitochondria were incubated in 0.1 M sodium carbonate buffer pH 11.5 on ice. Soluble and membrane fractions were separated by high-speed centrifugation. Soluble and peripheral membrane proteins were recovered in the supernatant (Sup) and precipitated with TCA. Integral membrane proteins were pelleted and resuspended in Laemmli buffer. Equal amounts of all fractions were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins. TIM44 represents a matrix protein peripherally attached to the inner membrane; TIM23 represents an integral membrane protein.

To address the questions in which of the mitochondrial membranes MCJ is located and which topology it has, isolated HeLa mitochondria were subjected to submitochondrial fractionation. In presence of proteinase K, proteins facing the outside of intact mitochondria are digested. Upon addition of hypotonic buffer, mitochondria osmotically swell and the outer membrane is disrupted, generating mitoplasts. Thereby, IMS proteins and proteins facing the IMS side of the inner membrane become accessible for externally added proteinase K, whereas matrix proteins stay shielded from the protease by the inner mitochondrial membrane.

When intact mitochondria were treated with PK, full length MCJ was still detected by immunoblotting, indicating that MCJ is not located in the outer membrane of mitochondria. Mitochondria incubated in absence of PK served as a control. In contrast, upon generation of mitoplasts prior to PK addition, MCJ was not longer detected in the immunoblot. Also the mitochondrial inner membrane proteins TIM23 and TIM17B which expose amino acid residues into the IMS were not detected under these conditions. TIM44 served as a control, that the matrix of mitochondria was protected from protease treatment under all tested conditions.

Since the MCJ antibody is specific for the N-terminal tail of MCJ and did not detect any smaller fragment, this result demonstrates that MCJ is located in the inner mitochondrial membrane with the N-terminus facing the intermembrane space.

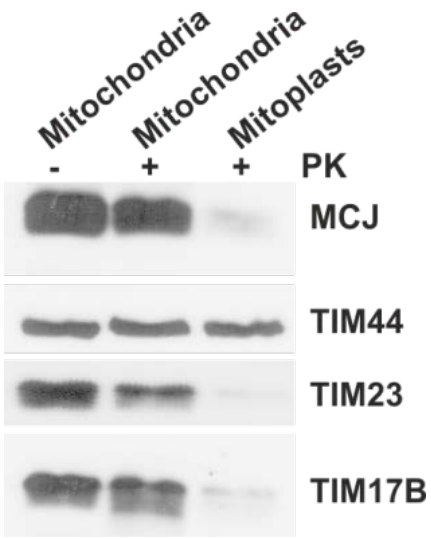

Figure 9 Subfractionation of isolated HeLa mitochondria showing that the N-terminus of MCJ faces the IMS

50 µg of mitochondria were incubated either in standard isolation buffer in absence (lane 1) or presence (lane 2) of PK or in hypotonic swelling buffer containing 20 mM Hepes pH 7.4 in presence of PK (lane 3). PK was inhibited after 10 minutes by addition of PMSF as described in 2.2.2.11. Mitochondria and mitoplasts, respectively, were re-isolated, washed and subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins. TIM23 and TIM17B expose amino acid residues into the IMS which becomes accessible for PK upon generation of mitoplasts. TIM44 is located in the matrix and remained protected under all conditions tested indicating that the inner membrane was still intact after the hypotonic swelling treatment.

In summary, these findings suggest that MCJ is an inner mitochondrial membrane protein whose N-terminus resides in the IMS, as depicted in the scheme shown in figure 10.

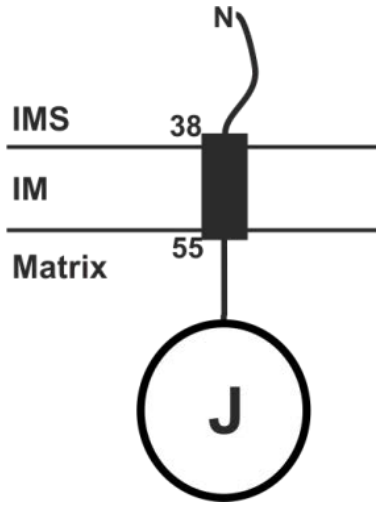

Figure 10 Topology model of the MCJ protein located in the inner mitochondrial membrane

MCJ is located in mitochondria with one transmembrane segment spanning the inner mebrane. Its N-terminus faces the IMS and the J domain localizes to the matrix.

3.5 MCJ is a component of the TIM23 translocase

3.5.1 MCJ co-purifies with TIM17B

To obtain information about the function of the MCJ protein, a search for interaction partners was performed. Since MCJ was found to be a mitochondrial inner membrane protein with high sequence homology to Tim14, the question was addressed, whether MCJ interacts with components of the TIM23 translocase. To this end, mitochondria were lysed in buffer containing digitonin, a detergent gently solubilizing the mitochondrial membranes but not disrupting the TIM23 translocase complex (Bauer et al. 1999, Moro et al. 1999, Kozany et al. 2004). After a clarifying spin, the mitochondrial lysate was subjected to co-immunoprecipitation with antibodies against TIM17B, an integral part of the TIM23 translocase. Under these conditions, the antibodies against TIM17B precipitated TIM17B together with TIM23. Fractions of MCJ, MAGMAS and TIM50 were also found in the bound material. Hsp60 served as a control for unspecific binding and was not detected in the precipitated fraction. These results show that MCJ is co-isolated with and thus interacts with components of the TIM23 translocase.

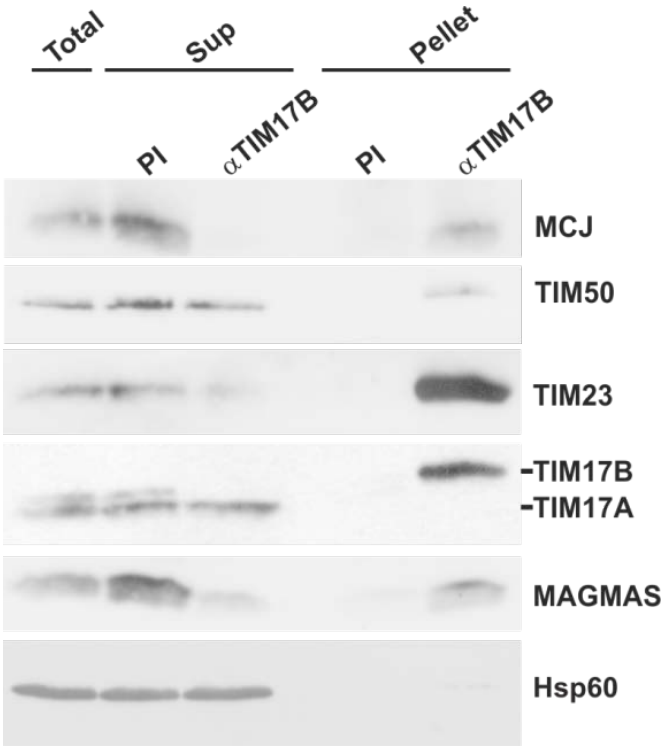

Figure 11 Co-immunoprecipitation of MCJ with TIM17B

HeLa mitochondria were solubilized in buffer containing 2.5 % digitonin and co-immunoprecipitated with antibodies against TIM17B pre-bound to Protein-A-sepharose beads as described in 2.2.4.5. Pre-immune IgGs (PI) served as a control. Bound proteins were eluted with Laemmli buffer. 20 % of total and non-bound (Sup) fractions and 100 % of the bound fraction (Pellet) were subjected to SDS–PAGE and immunoblotting with antibodies against the indicated proteins.

To test whether MCJ interacts specifically with TIM17B or with both TIM17 homologues, mitochondria were lysed in buffer containing digitonin and subjected in parallel to co-immunoprecipitation with antibodies against TIM17A and TIM17B, respectively. Aliquots of the total mitochondrial lysate, of the supernatant and the eluate were analyzed by SDS-PAGE and immunoblotting. MCJ was found in the eluate fractions of the TIM17A and the TIM17B co-immunoprecipitations together with the translocase components TIM23 and MAGMAS.

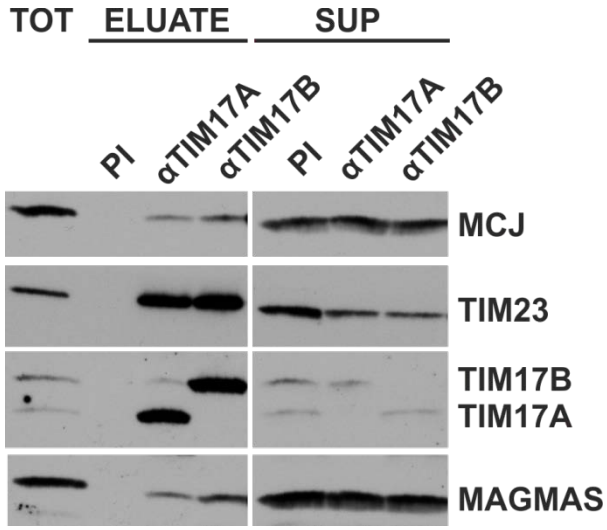

Figure 12 Co-immunoprecipitation of MCJ with TIM17A and TIM17B

HeLa mitochondria were solubilized in buffer containing 2.5 % digitonin and co-immunoprecipitated with antibodies against TIM17A or TIM17B pre-bound to Protein-A-sepharose beads as described in 2.2.4.5. Pre-immune IgGs (PI) served as a control. Bound proteins were eluted with Laemmli buffer. 10 % of total (TOT) and non-bound (SUP) fractions and 100 % of the bound fraction (ELUATE) were subjected to SDS–PAGE and immunoblotting with antibodies against the indicated proteins.

This result indicates that MCJ interacts with TIM23 translocases containing TIM17A as well as with translocases containing TIM17B.

3.5.2 MAGMAS and the TIM17 homologues can be co-purified with MCJ

When the co-immunoprecipitation was performed with antibodies against MCJ, MAGMAS and also a fraction of TIM17A and TIM17B were precipitated together with MCJ from digitonin solubilized mitochondria. *Vice versa*, antibodies against MAGMAS co-precipitated MAGMAS and MCJ, as well as to a low extent TIM17A. The negative control Hsp60 was not found in the precipitated fraction, confirming specificity of the results.

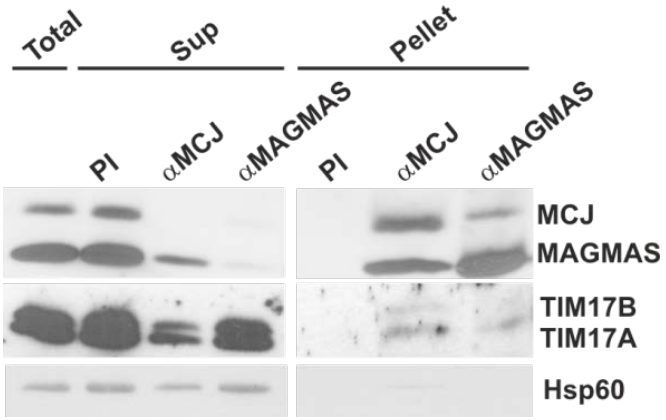

Figure 13 Co-immunoprecipitation of MAGMAS, TIM17A and TIM17B with MCJ

HeLa mitochondria were solubilized in buffer containing 2.5 % digitonin and co-immunoprecipitated with antibodies against MCJ and MAGMAS, respectively. Pre-immune IgGs (PI) served as a control. Bound proteins were eluted with Laemmli buffer. 20 % of total and non-bound (Sup) fractions and 100 % of the bound fraction (Pellet) were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated protein.

These findings confirm that MCJ is a component of the human TIM23 translocase.

3.6 MCJ forms a stable sub-complex with MAGMAS

3.6.1 MCJ and MAGMAS can be co-precipitated from TritonX-100 solubilized mitochondria

In order to characterize the interaction of MCJ with TIM23 translocase components in more detail, it was tested, whether the interaction is still observed under more stringent lysis conditions. To this end, mitochondria were lysed with buffer containing TritonX-100, a detergent in which the Tim17-Tim23 complex of yeast is not stable. Immunoprecipitation with antibodies against MCJ precipitated MCJ and a fraction of MAGMAS, but not TIM17A. This finding suggests a stable interaction between MCJ and MAGMAS under conditions that abolish interactions between other translocase components.

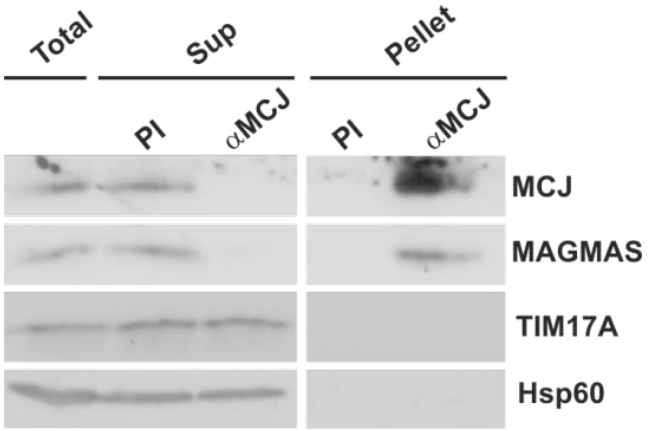

Figure 14 MCJ interacts with MAGMAS in presence of Triton X-100.

HeLa mitochondria were solubilized in buffer containing 1.0 % Triton X-100 and co-immunoprecipitated with antibodies against MCJ. Pre-immune IgGs (PI) served as a control. Bound proteins were eluted with Laemmli buffer. 20 % of total and non-bound (Sup) fractions and 100 % of the bound fraction (Pellet) were subjected to SDS–PAGE and immunoblotting with antibodies against the indicated proteins.

3.6.2 The soluble domains of MCJ and MAGMAS interact in *E. coli* upon co-expression

Next, the formation of a sub-complex of MCJ and MAGMAS was analyzed in *E. coli* cells, a heterologous expression system. The soluble domains of MCJ and MAGMAS (MCJ(s) and MAGMAS(s)) were co-expressed from a pETDuet-plasmid in *E. coli*, with MAGMAS(s) containing a his-tag. After lysis of the bacteria, his-MAGMAS(s) was purified via Ni-NTA agarose chromatography from the bacterial extract. Indeed, MCJ(s) was co-isolated with his-tagged MAGMAS(s).

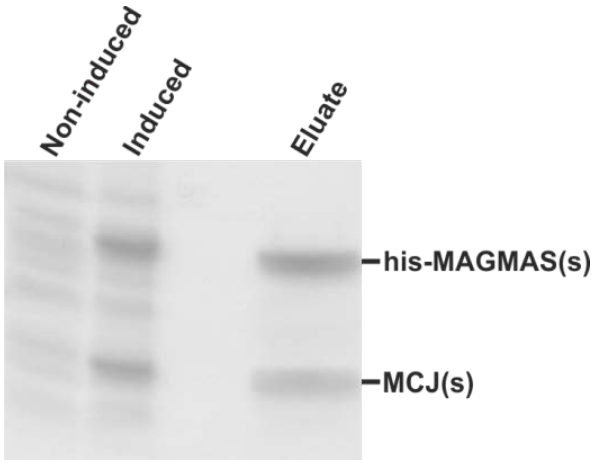

Figure 15 Co-expression and purification of the soluble domains of MAGMAS and MCJ from *E. coli*

Expression of the soluble domains of MAGMAS and MCJ was induced with 1 mM IPTG in *E. coli* cells containing a pETDuet-plasmid coding for his-MAGMAS(s) and MCJ(s). Samples were taken before (non-induced) and after 16 h of induction (induced). Protein purification via Ni-NTA was performed as described in 2.2.3.7.1. The samples and the eluate were analyzed by SDS-PAGE and Coomassie Blue staining.

The co-purification of recombinant MCJ(s) with MAGMAS(s) from the *E. coli* lysate supports the previous observation that MCJ and MAGMAS form a stable sub-complex under physiological conditions and reveals that the soluble domains are sufficient for the interaction of MCJ and MAGMAS.

3.6.3 The soluble domains of MCJ and MAGMAS interact *in vitro*

To test whether MAGMAS and MCJ are capable to interact *in vitro* upon mixing of their soluble domains, a pull-down experiment was performed with the recombinant soluble domains of both proteins. First, the soluble domains were separately expressed and purified. Next, his-MAGMAS(s) was bound to Ni-NTA beads and used as bait. MCJ(s) purified as a GST fusion construct was incubated with the MAGMAS(s) on the beads. GST alone served as a negative control. After two hours of incubation the beads were washed and the bound proteins were eluted with buffer containing 300 mM imidazole. Bands corresponding to MAGMAS(s) and GST-MCJ(s) were observed in the lane of the bound fraction in the Coomassie stain, whereas the band corresponding to GST without MCJ(s) was detected in the lane representing the supernatant. This result shows that the soluble domains of MAGMAS and MCJ not only form a stable complex *in vivo*, but also upon mixing of the single domains in the test tube.

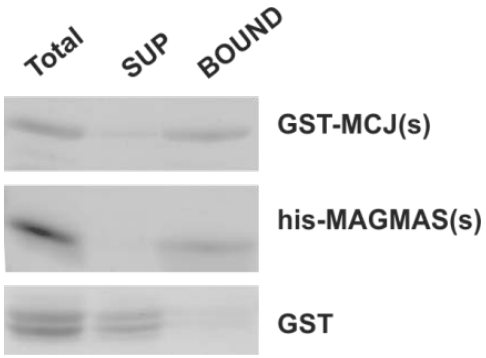

Figure 16 *In vitro* interaction of the soluble domains of MCJ and MAGMAS

Purified his-MAGMAS(s) was pre-bound to Ni-NTA. Purified GST and GST-MCJ(s), respectively, were incubated with the beads for 2 h at 4°C. The supernatant (SUP) was removed and the beads were washed. Bound proteins (BOUND) were eluted with buffer containing 300 mM imidazole and samples of all fractions were analyzed by SDS-PAGE and Coomassie Blue staining.

Thus, the interaction between MCJ and MAGMAS is mediated by their soluble domains. This interaction is highly stable and independent of the help of any other proteinaceous factors.

3.7 The absence of MCJ specifically affects the preprotein import via the TIM23 translocase

To test whether MCJ plays a functional role in the import of preproteins into the mitochondrial matrix via the TIM23 translocase, mitochondria lacking MCJ were investigated regarding their import capability.

3.7.1 Protein amounts in mitochondria lacking MCJ

To obtain mitochondria lacking MCJ, MCF7 cells stably expressing shRNA against MCJ (MCF7/siMCJ) were used (Hatle et al. 2007). The corresponding wild-type MCF7 cells served as a control. Mitochondria were isolated from both cell lines and equal amounts of mitochondria were subjected to SDS-PAGE and immunoblotting. The MCJ protein was not detectable in mitochondria from MCF7/siMCJ cells compared to mitochondria from MCF7 wild-type cells, as shown in figure 17. Other mitochondrial proteins were present in equal amounts in both cell lines. These data demonstrate efficient knock-down of the MCJ protein in MCF7/siMCJ cells without affecting overall protein levels in mitochondria.

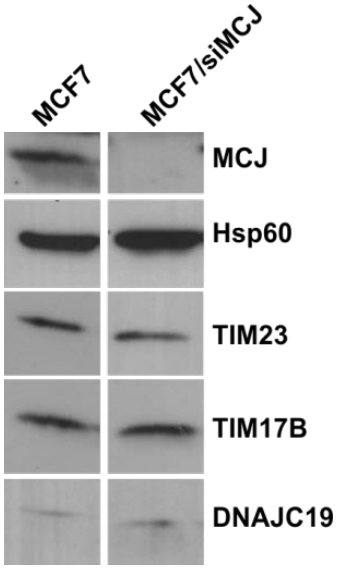

Figure 17 Protein levels of MCF7 and MCF7/siMCJ cells

Equal amounts of mitochondria isolated from MCF7 and MCF7/siMCJ cells were analyzed by SDS-PAGE and immunoblotting with antibodies against the indicated proteins. MCJ was not detected in MCF7/siMCJ mitochondria. Comparable amounts of the analyzed control proteins were detected in mitochondria isolated from both cell lines.

3.7.2 Import of the precursor proteins Su9-DHFR$_{mut}$ and F1ß is reduced in mitochondria lacking MCJ

To address whether MCJ plays a functional role in the TIM23 translocase mediated import of precursor proteins, an *in vitro* import assay was performed to compare import of the matrix targeted precursor proteins Su9-DHFR$_{mut}$ and F1ß into mitochondria of wild-type MCF7 and

MCF7/siMCJ cells. Su9-DHFR$_{mut}$ represents a well-established model preprotein for import mediated by the TIM23 translocase. It is a fusion protein composed of the presequence (MTS) of the subunit 9 of the *Neurospora crassa* ATPase and the mouse dihydrofolate reductase and carries mutations in the DHFR part which prevent it from folding. F1ß is the ß-subunit of the F1-ATPase of *Neurospora crassa*. Both precursors were translated *in vitro* in presence of radioactive ^{35}S methionine.

Mitochondria were incubated in import buffer together with the radiolabelled precursor protein Su9-DHFR$_{mut}$ for 30 minutes. One sample was incubated in buffer containing valinomycin to dissipate the mitochondrial membrane potential and therefore served as a negative control. Aliquots were taken at different time points and treated with proteinase K to digest non-imported precursor protein. Mitochondria were re-isolated, washed and subjected to SDS-PAGE. Imported and processed Su9-DHFR$_{mut}$ was visualized by autoradiography. The amount of imported preprotein increased over a time course of 30 minutes, whereas no imported material was visible in the sample without membrane potential. The signals of imported and processed Su9-DHFR$_{mut}$ observed in the mitochondria from MCF7/siMCJ cells were reduced compared to the signals in mitochondria from MCF7 cells.

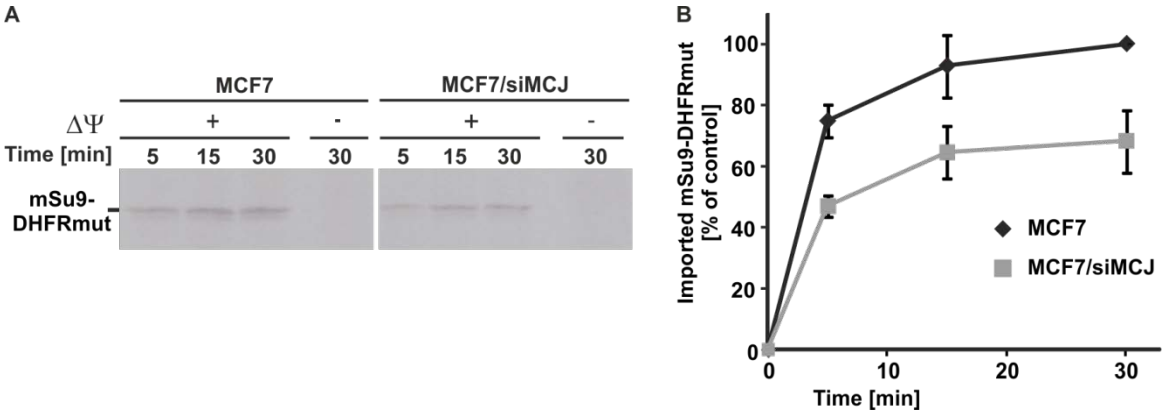

Figure 18 The import efficiency of Su9-DHFR$_{mut}$ into mitochondria is reduced to about 60 % in absence of MCJ.

A. Import of radiolabeled preprotein Su9-DHFR$_{mut}$ was performed as described in 2.2.2.13. Samples were analyzed by SDS-PAGE and autoradiography. Reduced import was observed in mitochondria isolated from MCF7/siMCJ cells. B. For quantification of the results densitometry of four independent import experiments was performed. The amount of material imported into mitochondria from wild-type cells after 30 minutes was set to 100 %.

The import efficiency of the preprotein Su9-DHFR$_{mut}$ into mitochondria lacking MCJ was decreased to around 60 % compared to the import efficiency into wild-type mitochondria. This result shows that MCJ indeed plays a functional role in the preprotein import via the TIM23 translocase.

In order to confirm this result, the import of the precursor protein F1ß into mitochondria isolated from MCF7 and MCF7/siMCJ cells was tested as described above. In accordance with the result for Su9-DHFR$_{mut}$, the levels of imported and processed F1ß (mF1ß, mature F1ß) were diminished in MCF7/siMCJ mitochondria compared to the levels in MCF7 mitochondria.

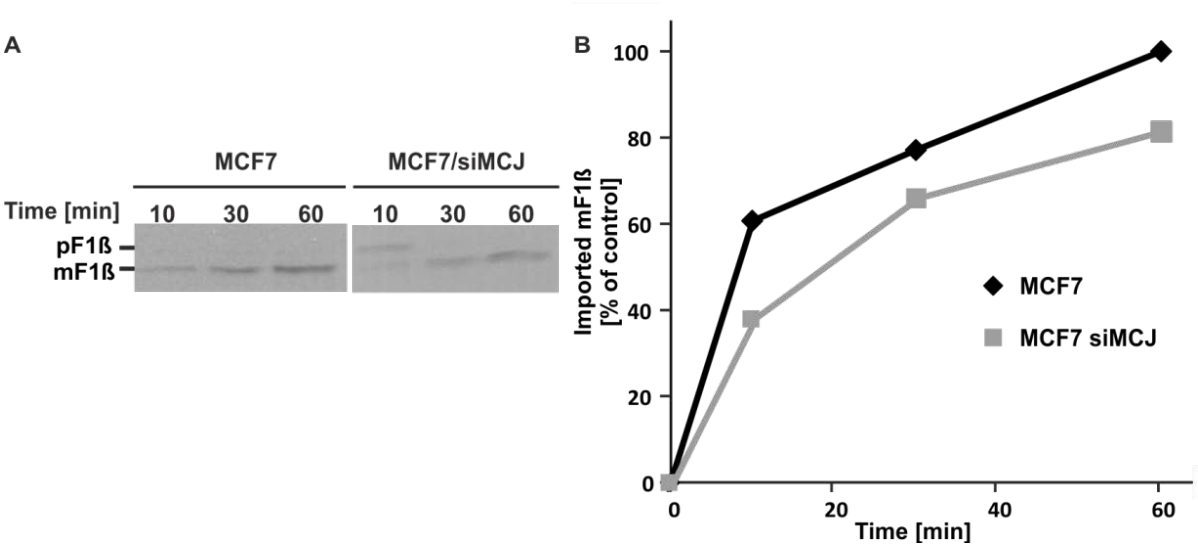

Figure 19 The import efficiency of F1ß is reduced in absence of MCJ.

A. Import of radiolabeled preprotein F1ß was performed as described in 2.2.2.13. Samples were analyzed by SDS-PAGE and autoradiography. Reduced import was observed in mitochondria isolated from MCF7/siMCJ cells. B. For quantification of the results densitometry was performed. The amount of material imported into mitochondria from wild-type cells after 60 minutes was set to 100 %. pF1ß: precursor F1ß.

Taken together, these results show that the absence of MCJ from mitochondria results in a defect of the preprotein import activity via the TIM23 translocase. The residual import rate observed in MCF7/siMCJ mitochondria was most likely due to the presence of the MCJ homologue, DNAJC19.

3.7.3 Absence of MCJ does not affect the import via the TIM22 pathway

To test whether the import defect of mitochondria lacking MCJ is specific for the TIM23 translocase, the import via the TIM22 pathway was investigated. As described in the introduction most carrier proteins of the inner membrane are transported by the TIM22 complex in a membrane potential dependent manner. If the lack of MCJ affected mitochondrial function in general or disturbed the mitochondrial membrane potential, the import via the TIM22 translocase would also be affected.

To address this question, the human ANT3 carrier protein was transcribed and translated as a radiolabelled preprotein *in vitro* as described before. *In vitro* import of the ANT3 preprotein was performed with mitochondria isolated from MCF7 and MCF7/siMCJ cells. The amounts of ANT3 imported into the mitochondria were not reduced in the absence of MCJ indicating that the

TIM22 import pathway was still intact in absence of MCJ. This clearly demonstrates that the absence of MCJ specifically affects the import of the TIM23 pathway.

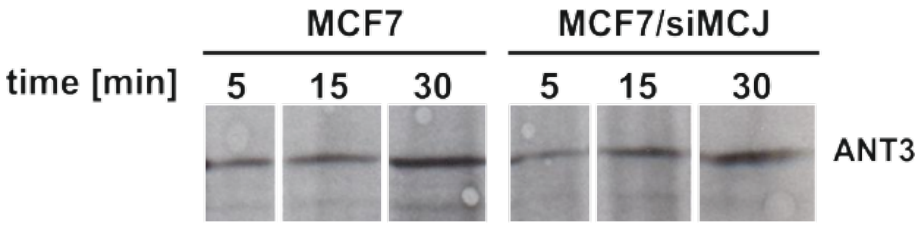

Figure 20 The import efficiency of ANT3 is not reduced in absence of MCJ.

Import of radiolabeled preprotein ANT3 was performed as described in 2.2.2.13. Samples were analyzed by SDS-PAGE and autoradiography. Equal amounts of imported protein were observed in mitochondria isolated from MCF7 and MCF7/siMCJ cells.

3.8 MCJ stimulates the ATPase activity of Mortalin *in vitro*

Since MCJ acts in the preprotein import via the TIM23 translocase and is a J protein, the question was addressed, whether MCJ interacts with mortalin, the human mtHsp70, in a J protein like manner. To test this, the ATPase activity of recombinantly expressed and purified mortalin was measured in an enzyme coupled photometric assay in presence or absence of MCJ. The stimulation of the ATPase activity of mortalin by DNAJC19, the second human Tim14 homologue, served as a control.

In absence of J proteins the basal ATPase activity of mortalin was determined to be 0.033 mol ATP / mol mortalin / minute. In presence of 10 fold molar excess of the soluble domain of DNAJC19, DNAJC19(s), the ATPase activity of mortalin was stimulated about three fold compared to its basal ATPase activity. This stimulation by DNAJC19(s) is consistent with reported measurements (Sinha et al. 2010). In presence of 7.5 fold molar excess of the soluble domain of MCJ, MCJ(s), the ATPase activity of mortalin was stimulated about 3.5 fold.

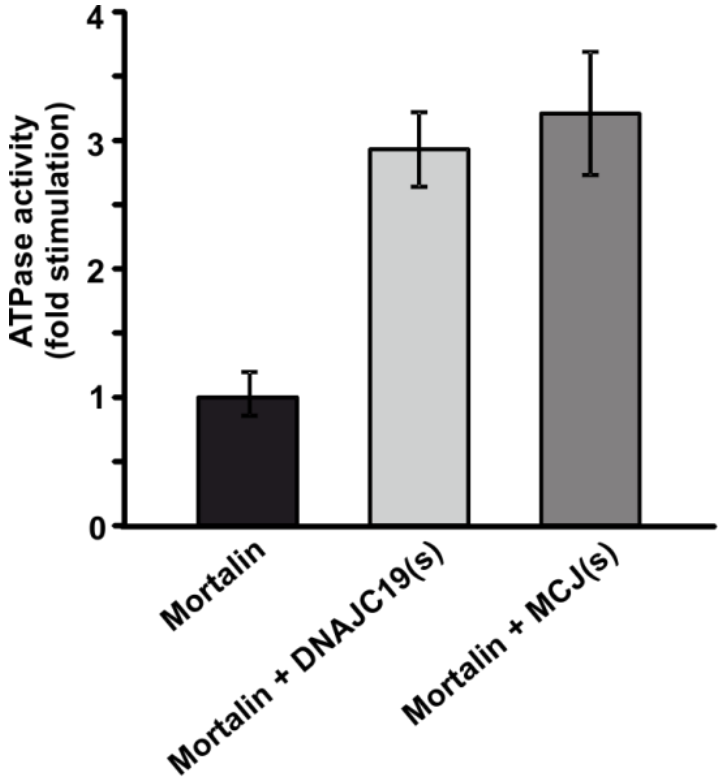

Figure 21 ATPase activity of mortalin in absence or presence of its J co-chaperones

The ATPase activity of 1 µM mortalin was measured in presence of stoichiometric amounts of its nucleotide exchange factor Mge1 and excess of phosphoenolpyruvate (PEP), NADH/H^+, PK and LDH as described in 2.2.3.10. The soluble domain of either MCJ or DNAJC19 was added as indicated. The reaction was started by the addition of excess ATP. Each reaction was monitored at 360 nm for at least 300 seconds in, at least, five technical replicates (n≥5). The basal ATPase activity of mortalin was determined to be 0.033 mol ATP / mol mortalin / minute and set to 1.

This result confirms that MCJ contains a functional J domain which is able to stimulate the ATPase activity of mortalin *in vitro*.

3.9 The stimulatory effect of MCJ on mortalin is counteracted by MAGMAS

Next, the influence of MAGMAS on the stimulatory effect of MCJ was tested, since the two proteins interact very stably via their soluble domains. In yeast, the MAGMAS homologue Tim16 inhibits the stimulatory activity of Tim14 (Kozany et al. 2004, Mokranjac et al. 2006, Li et al. 2004).

The ATPase activity of mortalin in presence of MCJ(s) was compared to its ATPase activity in presence of MCJ(s) in complex with MAGMAS(s). Either a 7.5 fold molar excess of purified MCJ(s)/MAGMAS(s) complex was used in the reaction or equimolar amounts of MCJ(s) and MAGMAS(s) were pre-incubated and added to the reaction.

In both cases, the stimulatory effect of MCJ on the ATPase activity of mortalin was abolished. MAGMAS(s) alone had no effect on the ATPase activity of mortalin, as shown in figure 22. This

result shows that the stimulatory effect of MCJ on mortalin is counteracted by the interaction of MCJ with MAGMAS.

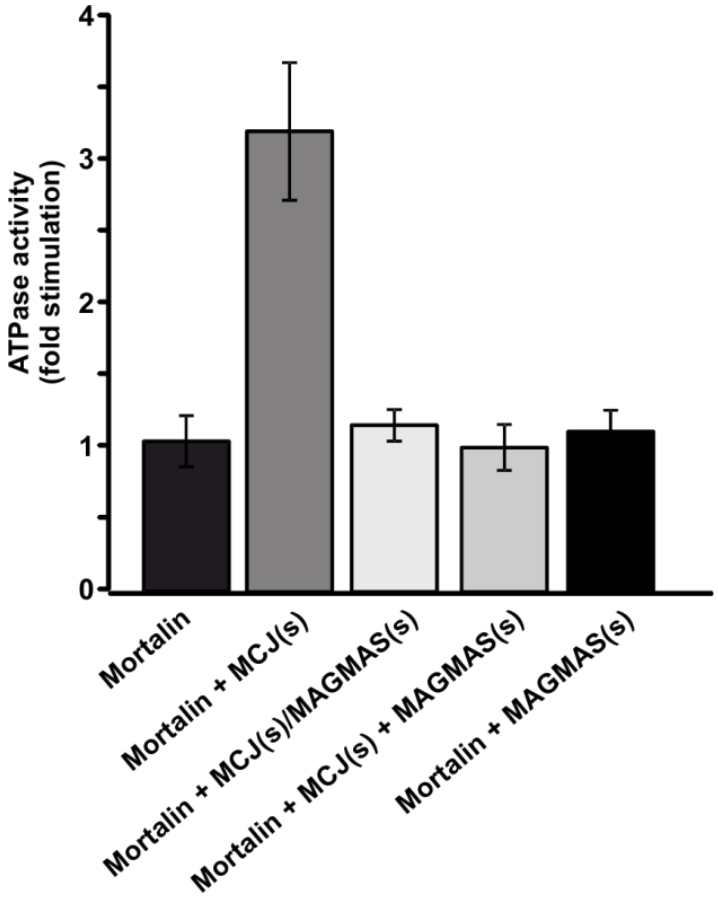

Figure 22 Stimulation of the ATPase activity of mortalin by MCJ in absence or presence of MAGMAS

The ATPase activity of 1 µM Mortalin was measured as described in 2.2.3.10. The simulation by the soluble domain of 7.5 µM MCJ was compared to the stimulation observed in presence of 7.5 µM of the purified MCJ(s)/MAGMAS(s) complex or of pre-incubated MCJ(s) + MAGMAS(s). Addition of MAGMAS(s) alone served as a control.

3.10 MCJ rescues the lethal *TIM14* deletion in *S. cerevisiae*

The deletion of *TIM14* in *S. cerevisiae* is lethal (Winzeler et al. 1999). To test whether MCJ or DNAJC19, the human homologues of the protein, can rescue this phenotype, centromeric plasmids were generated containing the open reading frame of either DNAJC19 or MCJ, respectively, each under either the strong constitutive TPI promoter or the endogenous yeast *TIM14* promoter. Each of these plasmids was introduced into a separate YPH499Δ*TIM14* yeast strain via plasmid shuffling. A plasmid encoding the endogenous yeast Tim14 and an empty plasmid served as a positive and negative control, respectively. Under expression of the yeast *TIM14* promoter, both human J proteins, MCJ and DNAJC19, were able to rescue the lethal *TIM14* deletion in yeast. Interestingly, only MCJ, but not DNAJC19, was able to complement the growth of the yeast cells upon overexpression.

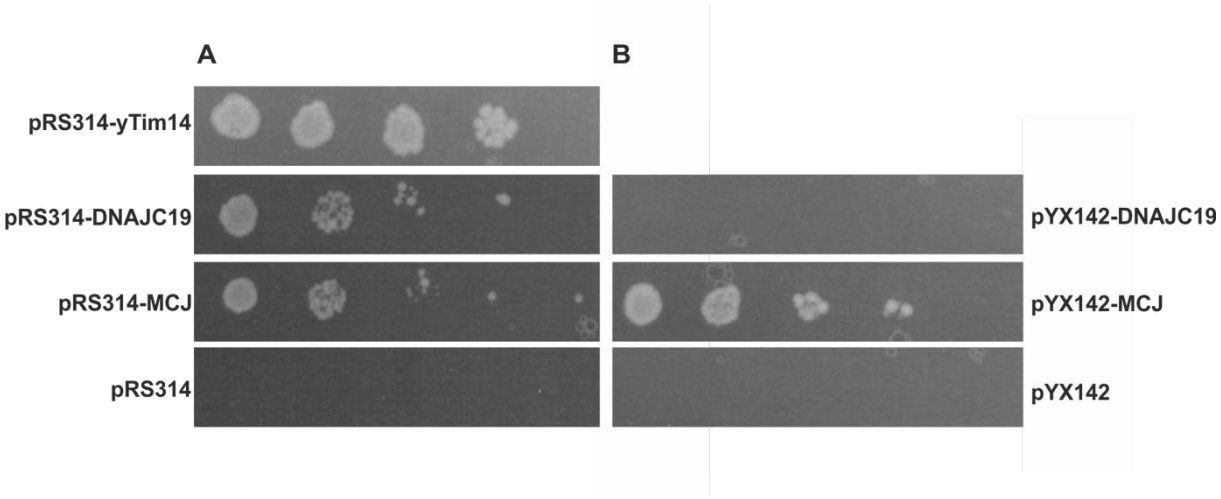

Figure 23 Complementation assay with MCJ and DNAJC19 in YPH499Δ*TIM14* yeast cells

YPH499Δ*TIM14* yeast cells harboring either yeast Tim14, MCJ or DNALC19 expressed either from a pRS314 plasmid containing the endogenous yeast Tim14 promoter (pRS314-) (A) or from a pYX142 plasmid containing the TPI promoter (pYX142-) (B) were tested for growth in a drop dilution assay on media containing 5'-FOA (see 2.2.2.9). The empty plasmid served as a negative control.

These results confirm that MCJ is a functional homologue of yeast Tim14 and is able to act as a J co-chaperone of the import motor of the TIM23 translocase independent of its expression levels. DNAJC19 is also a functional homologue of yeast Tim14. However, it rescues the lethal *TIM14* deletion only upon expression under the endogenous Tim14 promoter.

3.11 DNAJC19 is a component of the human TIM23 translocase

DNAJC19 rescues the *TIM14* deletion in yeast and stimulates the ATPase activity of mortalin. Thus, it should be analyzed whether DNAJC19 is also present in the human TIM23 translocase.

3.11.1 Components of the TIM23 translocase co-purify with DNAJC19-FLAG

To this end, a stable HEK 293 T-REx cell line was generated expressing DNAJC19-FLAG from a tetracycline receptor repressed promoter. The cells were induced with 1 µg/ml doxycycline for 24 h, harvested, lysed and tested for DNAJC19-FLAG expression by SDS-PAGE and immunoblotting. Indeed, in presence of doxycycline DNAJC19-FLAG was present in the cells after 24 h compared to non-induced cells.

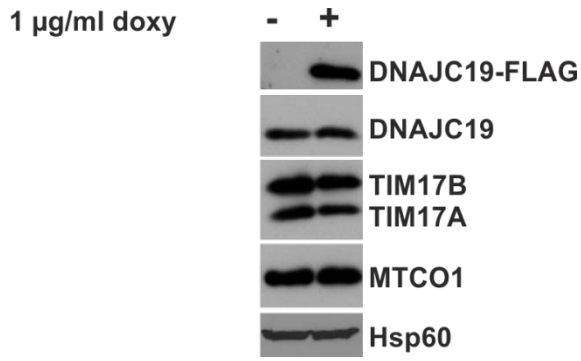

Figure 24 Induction of DNAJC19-FLAG in HEK 293 T-REx cells

HEK 293 T-REx cells expressing DNAJC19-FLAG in presence of 1 µg/ml doxycycline for 24 h were harvested, lysed in Laemmli buffer and subjected to SDS-PAGE and immunoblotting against the indicated proteins.

Then, mitochondria were isolated from the induced DNAJC19-FLAG expressing cells by differential centrifugation as described in 2.2.2.5. Fractions of the total cell lysate, the post-mitochondrial supernatant and the mitochondrial fraction were analyzed by SDS-PAGE and immunoblotting. DNAJC19-FLAG was recovered in the mitochondrial fraction and therefore behaves like the endogenous mitochondrial proteins DNAJC19, MAGMAS, TIM17A and TIM17B. The Golgi protein GM130 served as a control. This result demonstrates that DNAJC19-FLAG localizes correctly to the mitochondria like the endogenous DNAJC19 protein and other mitochondrial proteins.

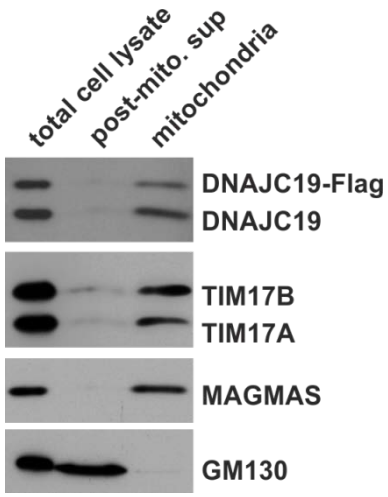

Figure 25 DNAJC19-FLAG is located in mitochondria.

Mitochondria were isolated as described in 2.2.2.5 from cells expressing DNAJC19-FLAG. Equal amounts of all fractions were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins.

To test for interaction partners of DNAJC19-FLAG, the isolated mitochondria containing DNAJC19-FLAG were lysed in buffer containing 1 % digitonin. After a clarifying spin, the mitochondrial lysate was subjected to a pull-down with anti-flag affinity beads (commercially available from Sigma). Pull-down from mitochondria lysed in buffer containing 1 % SDS served as a control. The pull-

down with the anti-flag affinity beads depleted DNAJC19-FLAG from both mitochondrial lysates. Under digitonin conditions the translocase components MAGMAS, TIM17A, TIM17B and TIM23 were specifically co-isolated with DNAJC19-FLAG. This result indicates that DNAJC19 is a component of the human TIM23 translocase.

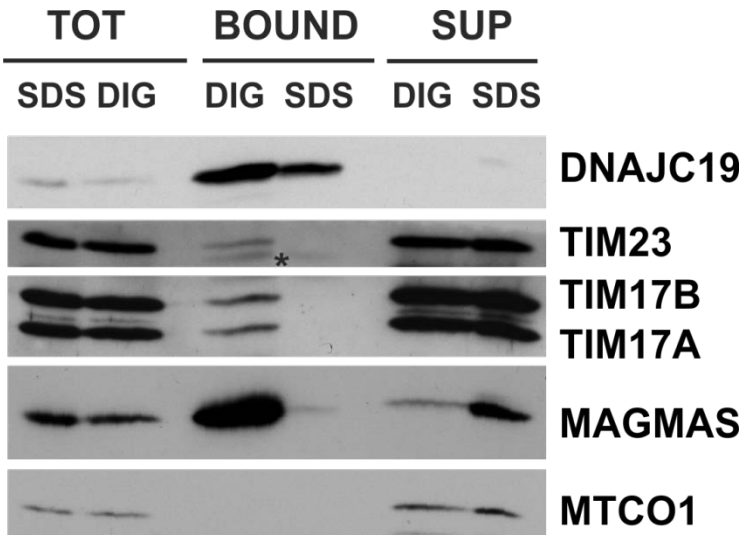

Figure 26 Co-isolation of MAGMAS, TIM17A, TIM17B and TIM23 with DNAJC19-FLAG

Mitochondria were isolated from DNAJC19-FLAG expressing cells, solubilized in buffer containing 1 % digitonin and co-immunoprecipitated with antibodies against the FLAG-tag. Mitochondria lysed in 1 % SDS served as a control and were diluted 10 x before the pull-down. Bound proteins were eluted with Laemmli buffer. 10 % of total and non-bound (SUP) fractions and 100 % of the bound fraction (BOUND) were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins. The asterisk indicates the light chains of the antibodies against the flag peptide.

3.11.2 DNAJC19 co-purifies with components of the TIM23 translocase

It should be also tested *vice versa* whether DNAJC19 can be co-purified with TIM23 translocase components. To this end, HeLa mitochondria were lysed in buffer containing digitonin. After a clarifying spin, the mitochondrial lysate was subjected to co-immunoprecipitation with antibodies against TIM17A and TIM17B. Aliquots of the total mitochondrial lysate, of the supernatant and the eluate were analyzed by SDS-PAGE and immunoblotting. Under these conditions, the antibodies against TIM17B and to a lower extent also the antibodies against TIM17A co-precipitated DNAJC19 together with MAGMAS and TIM23. The pre-immune serum served as a negative control.

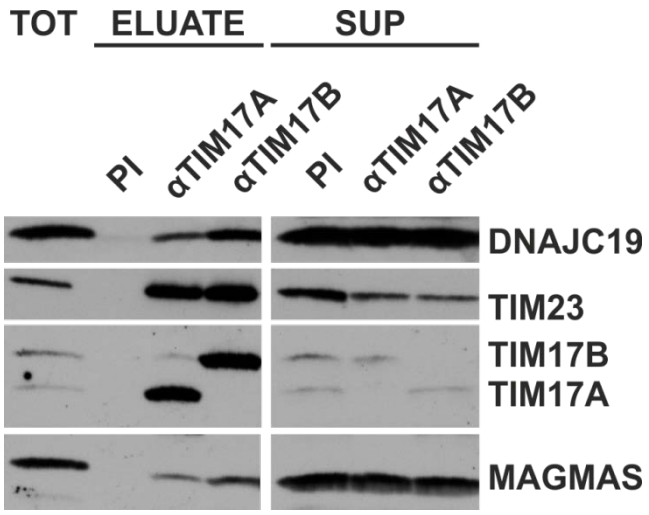

Figure 27 Co-immunoprecipitation of DNAJC19 with TIM17A and TIM17B

HeLa mitochondria were solubilized in buffer containing 1.0 % digitonin and co-immunoprecipitated with antibodies against TIM17A and TIM17B pre-bound to Protein-A-sepharose beads as described in 2.2.4.5. Pre-immune IgGs (PI) served as a control. Bound proteins were eluted with Laemmli buffer. 10 % of total and non-bound (SUP) fractions and 100 % of the bound fraction (BOUND) were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins.

These results confirm that DNAJC19 interacts with components of the TIM23 translocase.

3.12 Co-isolation of DNAJC19 and MCJ

The previous results show that both J proteins, MCJ and DNAJC19, are part of the TIM23 translocase. Therefore, the question arose, whether they could function in one TIM23 translocase or might be present in two distinct translocases. To address this, it should be tested whether DNAJC19 can be co-isolated with MCJ and MCJ-FLAG, respectively.

3.12.1 Expression of MCJ-FLAG

A stable HEK 293 T-REx cell line expressing MCJ-FLAG upon induction with doxycycline was generated. The recommended concentration by the manufacturer of 1 µg/ml doxycycline led to strong over-expression of MCJ-FLAG. Therefore, 0.1 µg/ml doxycycline for 24 h was used for induction of MCJ-FLAG expression. Only a slight overexpression of MCJ-FLAG compared to non-induced cells was observed under these conditions. Other mitochondrial proteins were virtually not affected by the presence of doxycycline, shown by TIM17A, TIM17B and NDUFA9 in figure 28. Thus, all MCJ-FLAG inductions were carried out with 0.1 µg/ml doxycycline for 24 h.

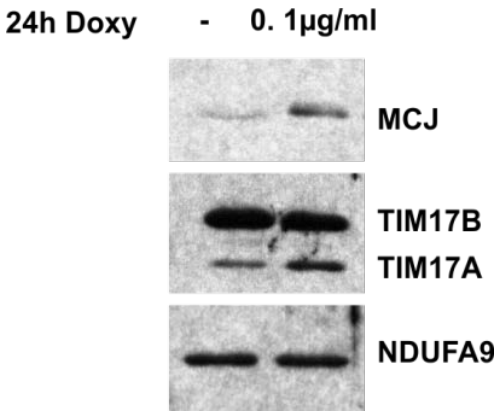

Figure 28 Expression of MCJ-FLAG in HEK 293 T-REx cells

Expression of MCJ-FLAG was induced in HEK 293 T-REx cells by addition of 0.1 µg/ml doxycycline for 24 h. Mitochondria isolated from cells grown in presence (0.1 µg/ml) or absence (-) of doxycycline (Doxy) were analyzed by SDS-PAGE and immunoblotting against the indicated proteins.

To test for the correct location of MCJ-FLAG in the cells, mitochondria were isolated as described in 2.2.2.5 and fractions of the total cell lysate, the post-mitochondrial supernatant and the mitochondria were analyzed by SDS-PAGE and immunoblotting. MCJ-FLAG was found in the mitochondrial fraction together with MAGMAS and TIM17B. The ER protein Calnexin served as a control.

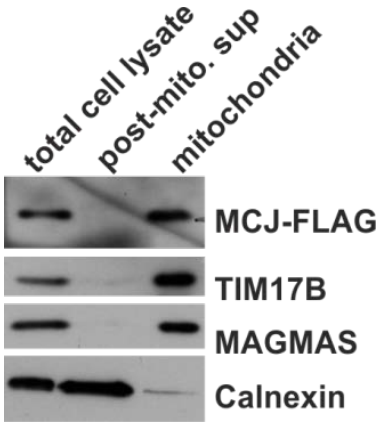

Figure 29 MCJ-FLAG is located in mitochondria.

Mitochondria were isolated as described in 2.2.2.5 from cells expressing MCJ-FLAG. Equal amounts of all fractions were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins.

These data show that the FLAG-tagged MCJ protein is sorted correctly to the mitochondrial compartment within the cell.

3.12.2 Co-purification of DNAJC19 with MCJ-FLAG

Mitochondria were isolated from MCJ-FLAG expressing cells and lysed in buffer containing 1 % digitonin. After a clarifying spin, the mitochondrial lysate was subjected to pull-down with anti-

flag affinity beads. A pull-down from mitochondria lysed in buffer containing 1 % SDS served as a control. The pull-down from digitonin lysed mitochondria isolated the translocase components MAGMAS, TIM17A, TIM17B and TIM23 together with MCJ-FLAG. In addition, DNAJC19 was detected in the bound fraction. The pull-down from mitochondria lysed in SDS did not co-purify any of the translocase components together with MCJ, demonstrating specificity of the co-isolation.

This result indicates that MCJ and DNAJC19 are located in one complex.

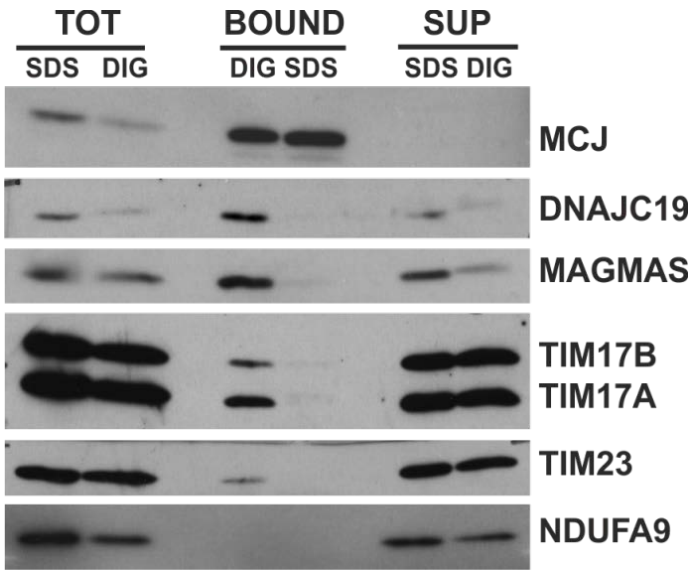

Figure 30 Pull-down of DNAJC19 with MCJ-FLAG

Mitochondria isolated from MCJ-FLAG expressing cells were solubilized in buffer containing 1 % digitonin or 1 % SDS. The SDS containing sample was diluted 10 x before the pull-down. The lysates were incubated with anti-flag affinity beads for 2 h at 4°C. Bound proteins were eluted with Laemmli buffer. 10 % of total and non-bound (SUP) fractions and 100 % of the bound fraction (BOUND) were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins.

3.12.3 DNAJC19 is co-precipitated with endogenous MCJ

Next, it was tested, whether endogenous MCJ and DNAJC19 are present together in a complex. HeLa mitochondria containing endogenous levels of untagged MCJ and DNAJC19 were lysed in buffer containing 1 % digitonin and subjected to co-immunoprecipitation with antibodies against the N-terminus of MCJ. These antibodies isolate specifically MCJ and do not recognize DNAJC19 (see 3.3). In the immunoblot of the bound fraction, bands corresponding to MCJ and its interaction partners MAGMAS, TIM17A, TIM17B and TIM23 were observed. Also DNAJC19, but not MTCO1, a subunit of the cytochrome oxidase, was co-precipitated with MCJ. The pre-immune serum served as a negative control.

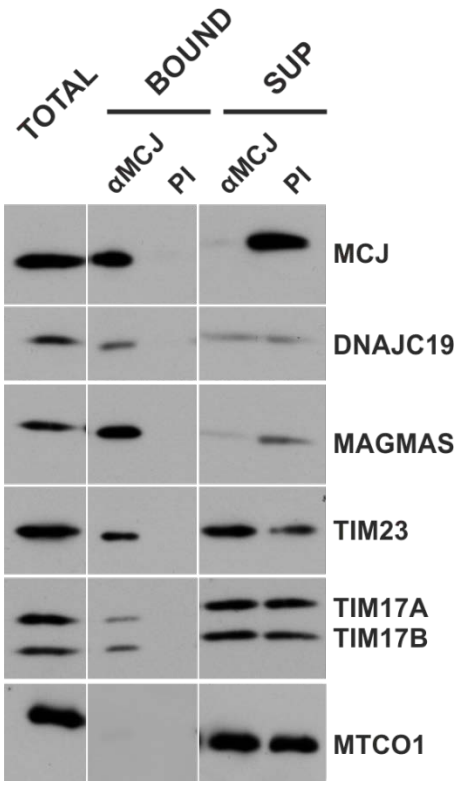

Figure 31 Co-isolation of DNAJC19 with MCJ

HeLa mitochondria were solubilized in buffer containing 1 % digitonin and co-immunoprecipitated with antibodies against DNAJC19. Pre-immune IgGs (PI) served as a control. Bound proteins were eluted with Laemmli buffer. 10 % of total and non-bound (SUP) fractions and 100 % of the bound fraction (BOUND) were subjected to SDS-PAGE and immunoblotting with antibodies against the indicated proteins.

This result confirms that MCJ and DNAJC19 are, at least partially, present together in a complex in human mitochondria. Both J proteins interact with components of the TIM23 translocase suggesting that there are translocases in human mitochondria which contain both co-chaperones, MCJ and DNAJC19.

4 Discussion

At the beginning of this study, MCJ was known to be expressed from a methylation controlled gene. MCJ was suggested to reside in the Golgi apparatus being involved in chemoresistance of different cancer cell lines (Hatle et al. 2007). However, the physiological role of the MCJ protein was poorly characterized. This study identified MCJ as a functional J co-chaperone of the human mitochondrial TIM23 translocase.

It was clearly demonstrated in this study that MCJ resides in the inner mitochondrial membrane with its N-terminal tail facing the intermembrane space. It interacts with components of the TIM23 translocase, namely TIM17A, TIM17B, TIM23 and MAGMAS (3.5 and 3.12). It is found in complexes that TIM17A and TIM17B form with TIM23 and TIM50. The J domain of MCJ stimulates the ATPase activity of mortalin, the central component of the import motor of the TIM23 translocase. This activity is counteracted by MAGMAS upon formation of a stable complex between MCJ and MAGMAS. Reduced *in vitro* import of model substrates into isolated mitochondria lacking MCJ (as shown in 3.7) demonstrates the importance of MCJ in the TIM23 import pathway. MCJ is also able to complement the lethal *TIM14* deletion in *S. cerevisiae*, implying that MCJ is not only a sequence homologue of yeast Tim14, but a functional co-chaperone of the TIM23 translocase. The characterization of the second human Tim14 homologue, DNAJC19, revealed that it is also part of the TIM23 translocase as a J co-chaperone, as shown by its ability to stimulate the ATPase activity of mortalin and by its complementation of the *TIM14* deletion in yeast. Finally, the question was addressed whether MCJ and the second human Tim14 homologue, DNAJC19, are both present in one single translocase. In pull-down experiments with flag-tagged MCJ as well as with endogenous MCJ, it was shown that MCJ and DNAJC19 are found in the same TIM23 translocase. Thus, the results shown in this study suggest that both J co-chaperones are not mutually exclusively present in the TIM23 translocase.

4.1 MCJ is a mitochondrial protein

As described in 3.1, MCJ harbors a transmembrane segment and a conserved C-terminal J domain. The residues between the TMS and the C-terminal domain are not conserved. This stretch of the protein may form an amphipathic helix, as it was reported for Bcs1, another mitochondrial inner membrane protein (Fölsch et al. 1996). This amphipathic helix can serve as a mitochondrial targeting signal and can cooperate with the TMS to form a loop-like structure which acts as a "thickened" amphipathic helix required for preprotein import into mitochondria (Fölsch et al. 1996). The identification of MCJ herein as a mitochondrial protein is in good agreement with the predictions of such a mitochondrial targeting signal, as was found in Bcs1.

The localization of MCJ to the Golgi, which was previously suggested (Hatle et al. 2007), was based on studies using over-expressed MCJ protein, a method which can lead to mislocalization of proteins or changes in organellar dynamics. In this study, the endogenous MCJ protein was used for localization experiments and clearly found in the mitochondrial fraction. The complementation of the *TIM14* deletion in yeast as well as the interaction with TIM23 translocase components in human mitochondria confirm the localization to the mitochondrial compartment. The mitochondrial location of MCJ was later conceded by Hatle and colleagues (Hatle et al. 2013).

4.2 MCJ and DNAJC19 differ in their N-termini

The two human homologues of Tim14, MCJ and DNAJC19, share sequence homology within their transmembrane segments and their J domains, and exhibit the same topology. The major structural difference between the two human Tim14 homologues is present in the N-terminal parts of the proteins. MCJ contains a 39 amino acid N-terminal segment in the intermembrane space, which is lacking in DNAJC19. MCJ shares this feature with the yeast Tim14, which also contains an extended N-terminus followed by the transmembrane segment. However, this N-terminal part of the protein is not conserved on the amino acid level and previous studies with yeast cells expressing a Tim14 variant, which lacks the N-terminal extension, have shown that it is not essential (Mokranjac et al. 2006, Mokranjac et al. 2007). Nonetheless, these yeast cells exhibited a growth defect at higher temperatures on lactate media, indicating an adverse effect on respiration (D'Silva et al. 2008). Moreover, the N-terminal stretch of Tim14 has been shown to interact with the Tim17 protein, suggesting a stabilizing role (Chacinska et al. 2005, D'Silva et al. 2008). Later, it was suggested that the N-terminus of Tim14 regulates the import of membrane spanning inner membrane proteins via the TIM23 translocase, since Popov-Celeketic and colleagues found that lateral release of inner membrane precursor proteins from the TIM23 translocase is increased in the absence of the N-terminus of Tim14 (Popov-Celeketic et al. 2011). Thus, it can be hypothesized that in human, the N-terminus of MCJ might also play a role in mediating the import of matrix targeted precursor proteins by decreasing the efficiency or the probability of the lateral release. How this could occur still remains unclear. Furthermore, MCJ was found in this study to be able to rescue the *TIM14* deletion in yeast, both when overexpressed and when expressed under the endogenous *TIM14* promoter. In contrast, DNAJC19 was only able to rescue the deletion when expressed under the endogenous promoter. This finding points to a functional role of MCJ which cannot be fulfilled by DNAJC19. It can be hypothesized that the N-terminal part of MCJ is involved in this additional function since DNAJC19 lacks this stretch. To better understand such a potential mechanism and whether distinct roles of

MCJ and DNAJC19 based on their different N-terminal structures exist in human mitochondria, further studies on MCJ and its IMS segment in comparison to DNAJC19 will have to be conducted.

4.3 Two Tim14 homologues are present in yeast mitochondria

Not only in humans, but also in yeast, a second homologue of Tim14 is found. Mdj2 is a J domain containing inner membrane protein of yeast mitochondria (Westermann and Neupert 1997). Although it interacts with components of the TIM23 translocase (Mokranjac et al. 2005), its absence does not affect the import of preproteins via the TIM23 translocase. Moreover, Mdj2 is not essential for cell viability and was reported to only rescue the lethal *TIM14* deletion upon overexpression (Mokranjac et al. 2005). The physiological function of the Mdj2 protein remains unclear. Since MCJ and DNAJC19 show clearly more sequence homology to Tim14 than to Mdj2, the human proteins do not resemble a Tim14/Mdj2 pair, but rather represent two homologues of the Tim14 protein.

4.4 Functions of MCJ and DNAJC19

Why are two homologues of the Tim14 protein found in human cells? As shown in 3.8, both J proteins, MCJ and DNAJC19, stimulate the ATPase activity of mortalin. Both of them are inhibited by MAGMAS ((Sinha et al. 2010), this study) and therefore show a similar behavior as their yeast homologue, since Tim14 is inhibited by the yeast homologue of MAGMAS, Tim16 (Frazier et al. 2004, Kozany et al. 2004). Thus, both human J proteins most likely share an overlapping function. Such redundancy can explain why cells and tissues survive in the absence of either J protein.

4.4.1 Redundant functions of MCJ and DNAJC19?

Since the presence of a J protein in the yeast TIM23 translocase is essential for yeast cell viability, it is highly probable that also in human at least one of the J proteins is required for correct mitochondrial and cellular functions. The presence of two homologues compared to yeast might represent a “back-up” system of higher eukaryotes to ensure survival of cells also in the absence of one or the other J protein. Since ovarian cancer cells and MCF7 cells are able to survive in the absence of MCJ expression and / or even thrive under chemotherapeutic treatment (Shridhar et al. 2001, Strathdee et al. 2005, Lindsey et al. 2006, Hatle et al. 2007), it seems that expression of DNAJC19 is sufficient for viability of these cells. The presence of DNAJC19 also explains the observation that import of precursor proteins in the *in vitro* import assay is not completely abolished upon silencing the expression of MCJ (compare 3.7.2). On the other hand, DNAJC19 can most likely not be completely replaced by MCJ, since a stop mutation in the gene coding for DNAJC19 leads to a severe cardiac phenotype (Davey et al. 2006, Ojala et al. 2012).

The fact that individual phenotypes were reported for loss (of function) of DNAJC19 and MCJ strengthens the idea that the J proteins fulfill distinct additional functions which might also be dependent on the cell-type or other influences like the expression level of one or the other protein.

4.4.2 The role of DNAJC19 in mitochondrial biogenesis is important for normal function of heart and brain tissue

A splice site mutation in DNAJC19 was first reported to correlate with dilated cardiomyopathy with cerebellar ataxia and 3-methylglutaconic aciduria (Davey et al. 2006) and later another mutation was found to confirm this correlation (Ojala et al. 2012). The mutations lead to loss or incomplete expression of the J domain of DNAJC19. Since the J domain is essential for the function of DNAJC19, these mutations are fatal for the stimulatory effect of DNAJC19 on the mtHsp70 activity. Thus, it can be concluded that the loss of the DNAJC19 stimulated import motor activity of the TIM23 translocase leads to severe defects in mitochondrial biogenesis and therefore causes defects especially in tissues that rely on high oxidative metabolism. Another disease in which mitochondrial import is impaired is the Mohr-Tranebjaerg syndrome (Tranebjaerg et al. 1995), a progressive neurodegenerative disorder, in which the biogenesis of the TIM23 translocase itself amongst others seems to be affected (Rothbauer et al. 2001). Therefore, the loss of functional DNAJC19 is in good agreement with a severe cardiac and neuronal phenotype. Moreover, it has been reported before that the three other classified 3-methylglutaconic acidurias are also caused by dysfunctions of known or predicted mitochondrial proteins (Anikster et al. 2001, Ly et al. 2003, Gunay-Aygun 2005). The identification of altered expression patterns of DNAJC19 in at least two brain regions of autism patients also speaks for the importance of functional mitochondrial biogenesis in normal brain function (Anitha et al. 2012).

4.4.3 The role of MCJ in chemoresistance and mitochondrial import may link mitochondrial biogenesis and cancer

Interestingly, MCJ has been reported to be absent from tumor cells and cancer cell lines which are more resistant to chemotherapeutics as the corresponding tissue expressing MCJ (Shridhar et al. 2001, Strathdee et al. 2005, Hatle et al. 2007). Thus, mitochondrial import and the process of chemoresistance could be linked. At the moment, it can only be speculated how these processes could be connected. Chemoresistance, as it is understood up to now, can be mediated by various cellular processes, such as decreased drug uptake by the cell, increased metabolism of the drug, modification or modified expression of the drug target or increased drug export out of the cell (Soengas and Lowe 2003, Ali et al. 2012). Another important mechanism not only playing a role in

tumorigenesis but also in increasing loss of chemosensitivity of cancer cells is apoptosis. Since MCJ deficient ovarian cancer cells have been shown to be less sensitive to apoptosis (Shridhar et al. 2001) the link might lie in this process. Apoptosis is the process of programmed cell death and is very tightly regulated and occurs upon certain stimuli (Munoz-Pinedo 2012). It is activated by either the extrinsic or the intrinsic signaling pathway and leads in the end to the controlled degradation of the cells by proteolysis. This process is vital for development of tissues and to ensure controlled clearance of unwanted cells by an organism. However, apoptosis has to be tightly regulated and several mechanisms in the signaling cascades leading to the final degradation are accurately balanced between the reaction to pro-survival and pro-death stimuli. Malignant cells have been found to have developed mechanisms unbalancing these signaling cascades and to avoid pro-death processes in favor of uncontrolled pro-survival mechanisms. Additionally, many chemotherapeutic approaches make use of the cell's apoptotic pathway (Khan et al. 2013). After reacting of the drug with its target, the subsequent cellular processes lead to death of the tumor cell. However, in evading apoptotic mechanisms, tumor cells can avoid drug effects.

Since mitochondria play an essential role in the intrinsic pathway of apoptosis activation (Tait and Green 2010, Martinou and Youle 2011), altered mitochondrial biogenesis might lead to altered apoptotic processes. It might be possible that MCJ is especially required for the mitochondrial import of pro-apoptotic proteins which fulfill their function within mitochondria. In case of loss of the MCJ protein, the apoptotic process could be disturbed, since such a factor would not be correctly located in mitochondria. It is also conceivable that MCJ might be required for the mitochondrial import and therefore sequestration of an anti-apoptotic factor of the cell. In this case, the anti-apoptotic factor would exert its effect for example in the cytosol or elsewhere in the cell like in the nucleus. For an apoptotic process, this factor would have to be transported into mitochondria to prevent its anti-apoptotic action. Upon loss of MCJ such a mechanism might be diminished. Another possibility, how MCJ could be involved in apoptosis, could be an additional function of MCJ elsewhere in mitochondria apart from the translocase. Very recently, MCJ was suggested to be involved in the recruitment of cyclophilin D to the mitochondrial permeability transition pore and to promote the opening of its channel under chemotherapeutic treatment (Sinha and D'Silva 2014). However, this role of the MCJ protein was only observed upon over-expression of MCJ, which might cause excess MCJ protein to interact with additional proteins apart from the translocase. How MCJ is involved in a potential pro-apoptotic mechanism under physiological conditions remains to be elucidated.

4.5 Presence of two homologues of other translocase components

Tim14 is not the only protein which is present in two homologues in human. Also the homologues of Tim17, TIM17A and TIM17B, are both found in the human TIM23 translocase (Bauer et al. 1999). This result raises the possibility that differently composed translocases might exist in human cells. Bauer and colleagues found that TIM17A and TIM17B both interact with TIM23 but do not interact with each other (Bauer et al. 1999). However, it remained unclear whether these two complexes fulfill distinct functions in terms of precursor protein import or whether they can replace each other. Later, TIM17A was shown to be a stress-responsive gene (Aldridge et al. 2007) and was suggested to be involved in adaption to stress and in the associated regulations in import behavior of the TIM23 translocase (Rainbolt et al. 2013). Additionally, the TIM17A protein has been reported to be involved in breast cancer progression (Salhab et al. 2012). None of these publications finds TIM17B involved in the same processes. Rainbolt and colleagues actually report that in absence of TIM17A, a complex corresponding to the size of a TIM23-TIM17B pair can still be detected (Rainbolt et al. 2013). Despite both TIM17 homologues are ubiquitously expressed (Bauer et al. 1999), tissue specificities were suggested: One of the homologues, TIM17B, might be especially involved in house-keeping functions, whereas the other homologue, TIM17A, could be a regulatory element in tissues, where fast adaption to changing conditions is needed, like in response to stress (Rainbolt et al. 2013).

This example illustrates that the duplication of a homologue during evolution might lead to a more precise regulation of the complex it is involved in. Also the J proteins of the human TIM23 translocase might fulfill this type of additional different functions, which lead to their distinct phenotypes and potencies in the complementation of the yeast homologue (see 3.10).

4.6 DNAJC19 and MCJ are present in the same translocase

Considering the behavior of TIM17A and TIM17B, it was surprising that MCJ and DNAJC19 are located in the same translocase and can be co-isolated. The presence of two J proteins in one TIM23 translocase may be explained by a dimeric nature of the TIM23 translocase. Since it was shown in yeast that Tim23 can dimerize in a membrane potential dependent manner (Bauer et al. 1996), the translocase can most probably also interact with two molecules of Tim44, which also exists as a dimer (Moro et al. 1999). Thereby, more than one mtHsp70 can bind to the core of the translocase during matrix import. In a “hand-over-hand” model, several mtHsp70 molecules act subsequently of each other in binding to the Tim44 proteins and being released into the matrix with the preprotein bound (see 1.3.2.2 and figure 2). The movement into the matrix occurs upon a conformational change dependent on the hydrolysis of ATP at the nucleotide biding domain of mtHsp70. In yeast, Tim14-Tim16 sub-complexes are located in close proximity to Tim44 and to the

translocase channel exit. Upon a conformational change of the sub-complex, Tim14 stimulates the ATPase activity of the mtHsp70 at the channel exit, thereby allowing tight binding of the preprotein and release from Tim44.

In human, it is conceivable that instead of two Tim14-Tim16 sub-complexes, one MAGMAS-MCJ sub-complex and one MAGMAS-DNAJC19 sub-complex are present at the channel exit of a dimeric TIM23 translocase, as depicted in the model in figure 32.

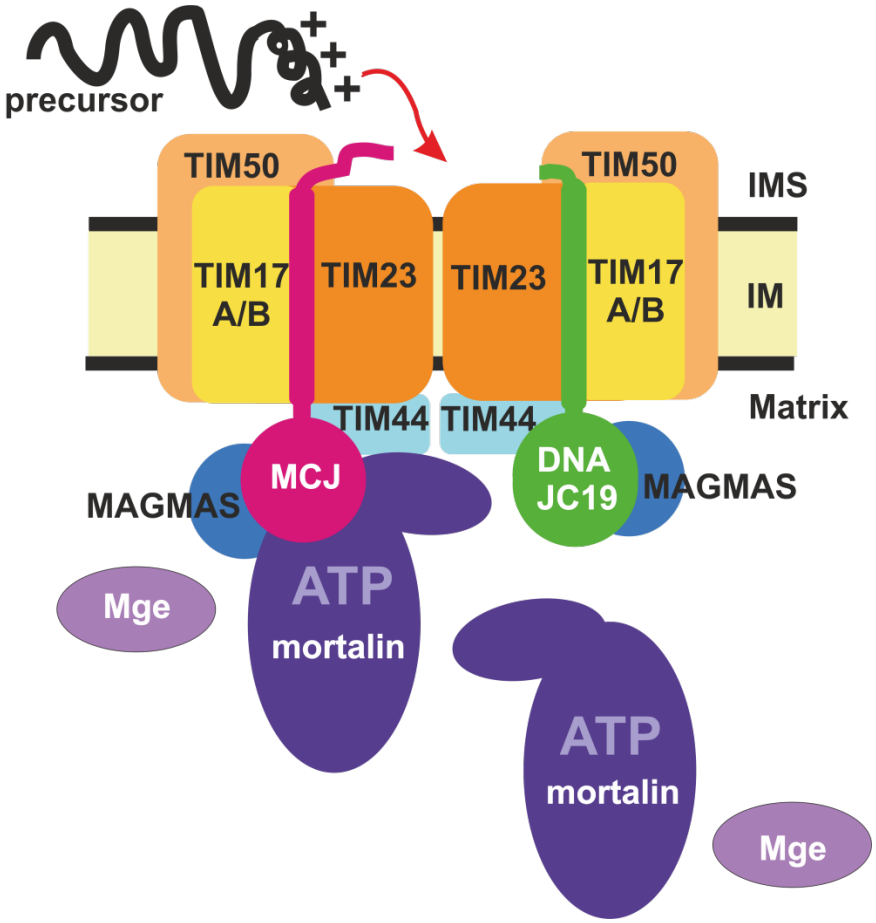

Figure 32 Model of MCJ and DNAJC19 in the human TIM23 translocase

MCJ (pink) and DNAJC19 (green) reside in the inner mitochondrial membrane with their N-termini facing the IMS. MCJ and DNAJC19 interact with components of the TIM23 translocase. The J domains of MCJ and DNAJC19 are able to stimulate the ATPase activity of mortalin, the human mtHsp70 chaperone. MAGMAS (blue) counteracts this stimulatory activity of MCJ and DNAJC19 upon formation of a stable sub-complex with either of them. MCJ and DNAJC19 play a functional role in the import of preproteins (precursor) via the TIM23 translocase.

Thereby, it is possible that MCJ interacts with one “half” / part of the translocase dimer and DNAJC19 with the other. One of the J proteins, for example MCJ, might be “released” from its stabilizing complex with MAGMAS and stimulate mtHsp70 at a time when DNAJC19 is in a stable complex with MAGMAS on the other TIM44 molecule at the translocase exit. It is possible that the J proteins take turns in stimulating different mortalin molecules present at the translocase exit after each other. Moreover, MCJ and DNAJC19 might exhibit different efficiencies of stimulation

dependent on the preprotein that is present in the translocation channel and could thereby be responsible for the import of different preproteins via the same translocase. However, it cannot be excluded that additional translocases exist which harbor only one of the J proteins. Such translocases could also possess different substrate specificities. The existence of translocases with distinct J co-chaperones might also explain the different phenotypes observed for MCJ and DNAJC19.

A major challenge of future studies will be to dissect what the exact differences between MCJ and DNAJC19 are, regarding their presence in distinct translocases, their different roles when present in the same translocase and their potential functions in other mitochondrial processes.

5 Summary

The majority of the mitochondrial proteome is encoded in the nucleus of the cell, translated at cytosolic ribosomes as precursor proteins and has to be imported into the organelle. In yeast, several translocases have been identified which transport the precursor proteins across and into the two membranes of mitochondria. The TIM23 translocase is necessary for translocation of precursor proteins into the inner membrane and into the mitochondrial matrix. Although several homologues of the yeast translocase components are present in humans, little is known about the structure and the function of the human TIM23 translocase. In order to identify components of the human TIM23 translocase, the human J protein MCJ was characterized and compared to DNAJC19. Both proteins, MCJ and DNAJC19, are homologous to Tim14 of the yeast translocase. MCJ has been reported to play a role in chemosensitivity of cancer cells (Shridhar et al. 2001, Strathdee et al. 2005, Hatle et al. 2007), but its physiological role was completely unclear at the beginning of this study.

Subcellular and submitochondrial fractionation revealed that MCJ is a mitochondrial protein, spanning the inner membrane once with its N-terminus facing the IMS. This result corrected the reported localization of MCJ in the Golgi compartment (Hatle et al. 2007). In line with the mitochondrial location, co-immunoprecipitation experiments demonstrated that MCJ interacts with components of the TIM23 translocase. It forms a highly stable sub-complex with MAGMAS, a component of the import motor of the translocase, mediated by the soluble domains of both proteins. MCJ is important for the mitochondrial import of precursor proteins translocated by the TIM23 translocase. Employing an *in vitro* import assay, this study shows that the import of the matrix targeted precursor proteins Su9-DHFR$_{mut}$ and F1ß was impaired in mitochondria lacking MCJ. This is a specific defect of the TIM23 translocase pathway, since the import of ANT3, a control substrate which uses a different import pathway, was not reduced in mitochondria lacking MCJ. *In vitro*, MCJ stimulates the ATPase activity of mortalin, the mtHsp70 chaperone of the human TIM23 translocase, suggesting that MCJ acts as a J co-chaperone of the translocase. Consistent with these results, MCJ was able to rescue the lethal *TIM14* deletion in yeast when overexpressed and when expressed under the endogenous yeast Tim14 promoter. The other Tim14 homologue, DNAJC19, was also able to rescue this deletion, but only when expressed under the endogenous promoter. As confirmed in this study, DNAJC19 is indeed a component of the human TIM23 translocase. Interestingly, DNAJC19 was co-isolated from mitochondria together with MCJ, suggesting that both J proteins are present together in the TIM23 translocase.

Taken together, the results of this study identified MCJ as a novel functional component of the human TIM23 translocase. MCJ contributes to the import of mitochondrial precursor proteins

being a stimulating co-chaperone of the TIM23 translocase import motor. Moreover, this study shows that DNAJC19, beside MCJ, also resides in the human TIM23 translocase as a stimulating J protein.

6 Literature

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7 Table of figures

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9 Abbreviations

µF	Micro Farad
aa	Amino acid
AD	Alzheimer's Disease
ADP	Adenosindiphosphate
Amp	Ampicillin
ANT3	Adenine nucleotide translocator 3
APS	Ammoniumpersulfate
ATP	Adenosintriphosphate
BSA	Bovine serum albumin
C. elegans	*Caenorhabditis elegans*
cDNA	Complementary desoxyribonucleic acid
Co-IP	Co-immunoprecipitation
Da	Dalton
dd	Double distilled
DHFR	Dihydrofolate reductase
DNA	Desoxyribonucleic acid
DTT	Dithiothreitol
E. coli	*Eschericchia coli*
ECL	Electrochemical luminescence
EDTA	Ethylenedinitrilotetraacetic acid
et al.	*Et alii*
F1ß	ß-subunit of ATP synthase
Fe/S cluster	Iron-sulfur cluster
Gal	Galactose
h	Hour
HCl	Hydrochloric acid
Hepes	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
His	Histidine
HPD	His-pro-asp / Histidin-proline-aspartic acid
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IM	Inner membrane
IMS	Intermembrane space
IP	Immunoprecipitation
IPTG	Isopropyl-β-D-thiogalactopyranosid
kb	Kilobases
kDa	Kilodalton
kV	Kilo volts
LB	Luria-bertani
LMU	Ludwig-Maximilians-Universität München
MCJ	Methylation controlled J protein
Mia	Mitochondrial intermembrane space import and assembly (machinery)
Mim	Mitochondrial import (protein)
min	Minute
MPP	Matrix processing peptidase
mRNA	Messenger ribonucleic acid

ms	Milliseconds
mt	Mitochondrial
N. c.	*Neurospora crassa*
NAD	Nicotinamide adenin dinucleotide
NEB	New England Biolabs
Ni-NTA	Nickel nitrilotriacetic acid
OM	Outer membrane
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PD	Parkinson's Disease
PEG	Polyethyleneglycol
Pel	Pellet
Pen/Strep	Penicillin/streptomycin
PK	Proteinase K
PMSF	Phenylmethylsunfonylfluoride
S. cerevisiae	*Saccharomyces cerevisiae*
S.c.	*Saccharomyces cerevisiae*
SAP	Shrimp alkaline phosphatase
SDS	Sodium dodecylsulfate
SDS	Sodiumdodecylsulfate
ss-DNA	Single stranded DNA
Su9	Subunit 9 of Fo-atpase from Neurospora crassa
Sup	Supernatant
TBS	Tris buffered Saline
TCA	Trichloracetic acid
TEMED	N, n, n‟, n‟-tetramethylethylendiamine
TIM	Translocase of the mitochondrial inner membrane
TMS	Transmembrane segment
TOB	Topogenesis of the mitochondrial outer membrane ß-barrel proteins
TOM	Translocase of the mitochondrial outer membrane
TPI	Triose phosphate isomerase
WB	Western blot
WBR	Western blocking reagent
wt	Wild type
Δψ	Membrane potential
Ω	Ohm

10 Publication resulting from this thesis

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