Involvement of Deafness Dystonia Protein 1 in the Pathomechanism of Deafness-Dystonia-Optic Neuronopathy Syndrome

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SUMMARY

Deafness-Dystonia-Optic Neuronopathy (DDON) syndrome is a rare X-linked progressive neurodegenerative disorder resulting from mutations in the *TIMM8A* gene encoding for the deafness dystonia protein 1 (DDP1). Despite important progress in identifying and characterizing novel mutations in this gene, little is known about the underlying pathomechanism. Deficiencies in the biogenesis of hTim23 and consecutive alterations in biogenesis of inner membrane and matrix proteins have been proposed to serve as one possible mechanistic explanation. To shed new light on the role of DDP1 in the biogenesis of mammalian mitochondria, we investigated the effects of reduced or elevated DDP1 levels on mitochondrial dynamics and function. Our results show a reduction in the import of β-barrel proteins into mitochondria from cells overexpressing DDP1. This effect was not observed when the DDON-related mutant form DDP1-C66W was overexpressed. Live cell microscopy of primary fibroblasts derived from DDON patients and of DDP1 downregulated HeLa cells displayed alterations of mitochondrial morphology with notable extensions in the length of mitochondrial tubules, whereas overexpression of DDP1 induced the formation of hollow spherical mitochondria. Interestingly, overexpression of TIMM13 alone, or both TIMM13 and DDP1, revealed a wild-type phenotype morphology. Blue native-PAGE analysis of cells with elevated amounts of DDP1 and TIMM13 showed a reduction of unassembled DDP1 with concomitant formation of high molecular mass complexes containing both proteins. These results support the notion that the unassembled DDP1 molecules might cause the phenotype of the DDP1 overexpressing cells.

Of note, knockdown of the *TIMM8A* gene by RNA interference did not show an influence on the oxygen respiration rate and the mitochondrial membrane potential. Taken together, these results suggest that alterations in the levels of DDP1 can affect the morphology of mitochondria and thus shed new light on the pathogenetic mechanisms of DDON.

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1 INTRODUCTION

1.1 Mitochondrial structure, function and genetics

Mitochondria play a central role in eukaryotic cells by fulfilling not only an important function in the cell's energy production but also in other metabolic pathways such as amino acid synthesis, tricarboxylic acid cycle, beta-oxidation as well as in calcium homeostasis and free radical scavenging. These organelles contain two membranes and two aqueous spaces and harbor a complete genome in the matrix (Schmidt et al., 2010). However, the majority of mitochondrial proteins are nuclear encoded and need to be imported into the organelle after being synthesized as precursor molecules on cytosolic ribosomes.

The mitochondrial genome in humans is comprised of 16596 nucleotide base pairs forming a closed circular loop of double stranded DNA, present in hundreds or thousands of copies in each cell (polyplasmy). The mtDNA is coding for two ribosomal RNA genes, 22 tRNA genes and 13 polypeptides that serve as subunits of the respiratory chain and are necessary for the assembly of the oxidative phosphorylation machinery (Anderson et al., 1981). Moreover, the mtDNA has a remarkably concise genome, does not harbor introns and contains only a small 1.1 kb non-coding region, known as the D-loop. Due to the absence of protective histones and poor repair mechanisms, alongside with high replication rates, mtDNA accumulates mutations faster than nDNA. Consequentially, a replicative mutant mtDNA is occasionally formed (Yu-Wai-Man et al., 2011). Thus, a dual population of mutant mtDNA and normal mtDNA coexist within one cell, a condition termed heteroplasmy (Chinnery, 2002; Lightowlers et al., 1997). This situation is in most cases inconsequential.

Selective pressure, such as replicative advantage of the mutant mtDNA, might shift the mutational load towards the tissue-specific "threshold" level and thus lead to occurrence of clinical signs and symptoms. The specific threshold though, is highly variable and strongly depends on energy demands of the tissue with symptom manifestation in highly energy consuming tissues already at a lower mutational burden as compared to

less metabolically active tissues (Chinnery et al., 2000; Chinnery et al., 1999; Corral-Debrinski et al., 1992; Lightowlers et al., 1997; Nekhaeva et al., 2002; Shoubridge et al., 1990; Taylor and Turnbull, 2005; Wang et al., 2001).

Differences in mutant mtDNA levels of certain tissues may also be a consequence of mitotic segregation resulting from differential distribution of these mitochondria in the oocyte to either of the two daughter cells. This would promote the inheritance of significantly more mutated mtDNA in one daughter cell and be amplified by recurrence of this process during organogenesis (Lightowlers et al., 1997).

Considering the important role of mitochondria in a eukaryotic cell, it is not surprising that mitochondrial dysfunction manifests in patients with severe clinical symptoms (Harman, 1957; Raha and Robinson, 2000).

1.2 Mitochondrial import

Mitochondrial function relies on a well-organized and efficient transport of roughly 1500 different mitochondrial proteins to their corresponding mitochondrial subcompartments. Nuclear encoded preproteins that are synthesized in the cytosol on free ribosomes utilize cytosolic factors and the import machineries for their translocation (Dolezal et al., 2006; Neupert and Herrmann, 2007b). Currently, five classes of precursor proteins are known, each following a specific import route. The vast majority uses as a common entry gate the translocase of the outer membrane (TOM) complex and then various pathways to the different mitochondrial subcompartments are employed. Four main pathways are currently well described whereas the insertion of α-helical proteins into the outer membrane is still unclear and a matter of intense research.

I) Presequence protein translocation to the matrix and inner mitochondrial membrane
II) Carrier pathway to the inner mitochondrial membrane
III) Oxidative folding pathway in the mitochondrial intermembrane space

IV) Insertion pathways of the outer mitochondrial membrane

1.2.1 The TIM23 pathway

Presequence protein translocation to the matrix and inner mitochondrial membrane.

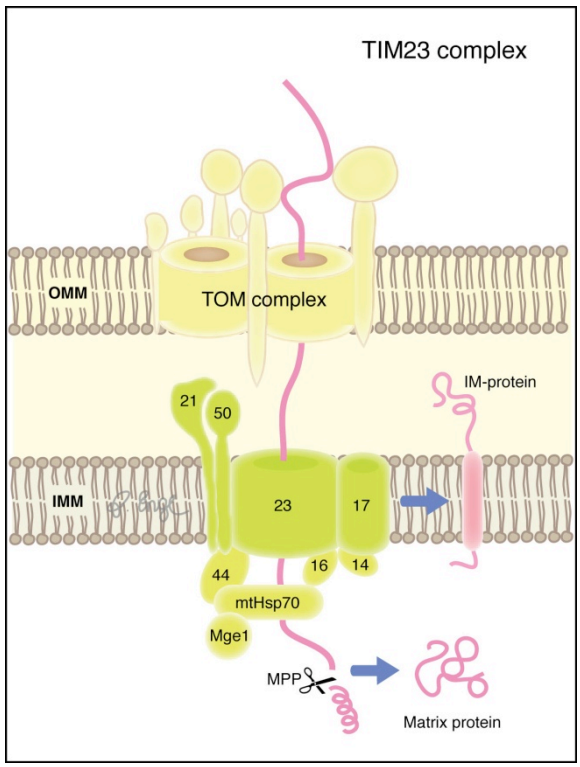


Fig. 1.1 *TIM23 complex. The translocase of the inner mitochondrial membrane transfers preproteins that were translocated through the TOM complex further to the matrix or releases them laterally into the IMM. This process is driven by an electrochemical membrane potential and the chaperone mtHsp70.*

The majority of matrix proteins and some inner membrane proteins utilize the presequence translocation pathway for import. Cleavable precursors are transferred from the TOM complex to the presequence translocase of the inner membrane (TIM23) complex to be further relayed to the matrix or released laterally into the inner membrane. The positively charged presequences (usually consisting of 15-50 amino acids) at the N-termini of preproteins form amphipathic α-helices that are recognized by TOM and

TIM23 complexes in a sequential manner (Chacinska et al., 2009; Dolezal et al., 2006; Neupert and Herrmann, 2007b; Saitoh et al., 2007). Translocation is energetically driven by the electrical membrane potential across the inner membrane and ATP hydrolysis.

The TIM23 complex can be divided into two mechanistic groups which are suggested to act in a sequential and cooperative manner: 1) the membrane sector which forms the conducting channel and 2) the import motor which actively drives the matrix translocation processes (Neupert and Herrmann, 2007a).

Three essential inner mitochondrial membrane proteins constitute the membrane sector: the channel forming proteins Tim23 and Tim17, and Tim50 that exposes a large domain to the IMS (Geissler et al., 2002; Yamamoto et al., 2002), presumably functioning as a passive import receptor and regulator of the import channel's permeability (Fig. 1.1) (Chacinska et al., 2005; Mokranjac et al., 2003; Mokranjac et al., 2009; Tamura et al., 2009). The fourth subunit Tim21 is like Tim50 in transient interaction with the TOM complex (Chacinska et al., 2005; Mokranjac et al., 2005) and additionally promotes a dynamic remodeling of the TIM23 complex by coupling it to the respiratory chain complexes III and IV (Dienhart and Stuart, 2008; van der Laan et al., 2006; Wiedemann et al., 2007).

Translocating polypeptides that contain a hydrophobic sorting signal C-terminally to the presequence are arrested in the inner membrane and laterally released into the inner mitochondrial membrane (the so-called stop-transfer-mechanism).

If complete translocation across the inner membrane into the matrix is required, the import motor has to take over (Neupert and Herrmann, 2007b). Tim44, Tim14 (Pam18), Tim16 (Pam 16), mtHsp70 and Mge1 mediate vectorial movement of the matrix-destined precursor proteins through cycles of ATP binding and hydrolysis. The motor subunits interact with several sites on the TIM23 complex and ensure complete translocation of the matrix precursor proteins. Then these proteins are

ultimately liberated into the matrix and proteolytically processed by the mitochondrial processing peptidase (MPP) to remove the presequence (Fig. 1.1) (Schmidt et al., 2010).

1.2.2 The TIM22 pathway

The carrier pathway to the inner membrane.

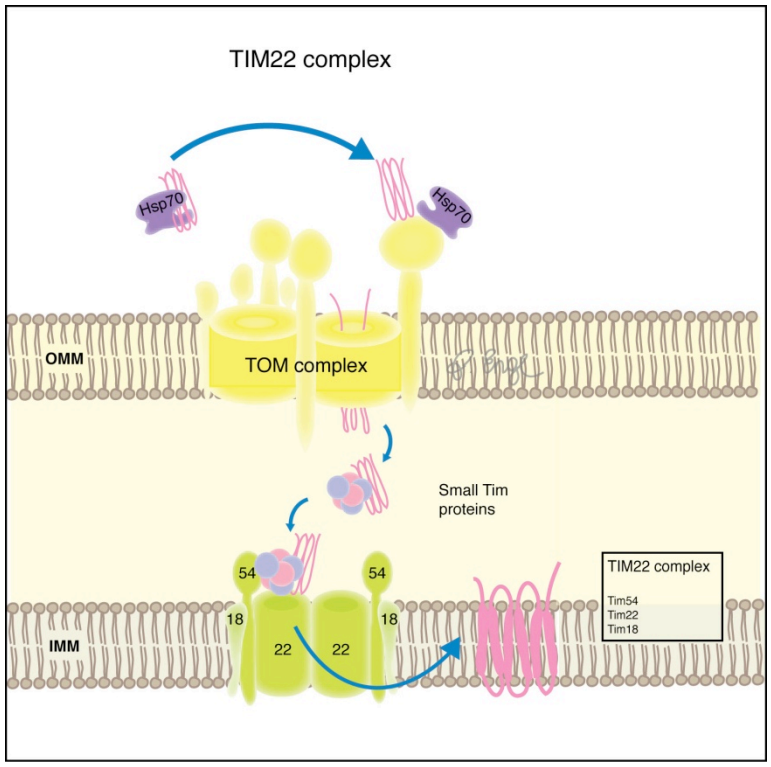


Fig 1.2 *TIM22 complex. Metabolite carrier proteins with internal targeting signals are prevented from aggregation by cytosolic chaperones such as Hsp70. They cross the TOM complex to reach the IMS where small Tim proteins shuttle them to the TIM22 complex for their final insertion into the IMM.*

A large family of metabolite carrier proteins such as the ADP/ATP carrier (AAC) as well as the multispan proteins Tim23, Tim17 and Tim22 take an alternative route for their integration into the inner membrane (Palmieri et al., 2006).

Such largely hydrophobic precursors without cleavable presequences are shuttled by the help of cytosolic chaperones from the Hsp70 and Hsp90

families to the TOM complex (Young et al., 2003). On this complex they interact with Tom70 that exhibits a binding site for the precursor protein as well as for the chaperone itself (Wiedemann et al., 2001; Young et al., 2003; Zara et al., 2009). Release and transfer to the core portion of the TOM complex is followed by threading the precursor through the pore in a loop conformation (Wiedemann et al., 2001).

To avoid aggregation upon contact with the aqueous intermembrane space, Tim9 and Tim10 and the homologous Tim8-Tim13, bind the precursor when still residing in the pore and subsequently guide the precursor protein to the mitochondrial inner membrane (Baker et al., 2009; Curran et al., 2002; Paschen et al., 2000; Roesch et al., 2002; Rothbauer et al., 2001; Vasiljev et al., 2004; Webb et al., 2006).

Next, the precursor protein is relayed to the carrier translocase, or TIM22 complex, in the inner membrane that provides a dedicated machinery for membrane insertion of this family of precursor proteins. The TIM22 complex consists of Tim22, Tim54, Tim18 and Tim12 along with a sub-population of Tim9 and Tim10 (Fig. 1.2) (Kerscher et al., 1997; Kerscher et al., 2000; Koehler et al., 1998; Koehler et al., 2000; Rehling et al., 2003; Sirrenberg et al., 1996).

Tim54 is thought to provide a binding site for the Tim9-Tim10-Tim12 complex. The precursor protein is finally inserted in a loop-like conformation into the twin pore formed by the major subunit Tim22 and is then released into the lipid core of the membrane (Gebert et al., 2008; Kerscher et al., 1997; Rehling et al., 2004; Wagner et al., 2008; Wiedemann et al., 2001).

1.2.3 Oxidative folding pathway in the mitochondrial intermembrane space

The identification of the MIA (Mitochondrial Intermembrane space Assembly) machinery provides new insights into the import of proteins into the intermembrane space. IMS proteins are predominantly synthesized without cleavable presequences but contain Cys motifs (Gabriel et al.,

2007; Longen et al., 2009) which are recognized along with a hydrophobic residue by Mia40 (Milenkovic et al., 2009; Sideris et al., 2009).

Mia40 is an essential protein for cell viability and is the core component of the MIA machinery. After formation of a transient disulphide bond (Banci et al., 2009; Kawano et al., 2009), Mia40, acting as a protein disulphide carrier, transfers disulphide bonds to the imported proteins leading to their oxidation and formation of the mature form. Thus, many IMS proteins are in an oxidized state. This finding is in contrast to the classic belief that the IMS possesses a reducing environment (Chacinska et al., 2004; Naoe et al., 2004; Terziyska et al., 2005).

Disulphide bonds are not formed *de novo* by Mia40 but are rather the result of a complex interplay between Mia40, which functions as an oxidoreductase that is part of a disulphide-transferring reaction chain (disulphide relay) (Allen et al., 2005; Bien et al., 2010; Mesecke et al., 2005; Rissler et al., 2005), and Erv1 (sulphhydryl oxidase essential for cell viability and respiration).

Erv1 generates disulphide bonds that are transferred to Mia40 by forming transient intermolecular disulphide bonds and these disulphides are ultimately transferred onto the substrate. This process is coupled to electron removal and thus an electron flow from imported substrates via Mia40 to Erv1 and finally via cytochrome c to the respiratory chain is anticipated (Allen et al., 2005; Bihlmaier et al., 2007; Dabir et al., 2007).

1.2.4 Insertion pathways of the outer mitochondrial membrane

1.2.4.1 The TOM complex of the outer mitochondrial membrane

The TOM complex is a heteromolecular protein complex consisting of receptor proteins such as Tom70, Tom20 and Tom22 and the main translocation pore formed by Tom40 (Rapaport, 2005b). The small Tom proteins, Tom5, 6 and 7 function as regulatory factors in stabilization and dissociation processes (Alconada et al., 1995; Dembowski et al., 2001; Honlinger et al., 1995; Schmitt et al., 2005; Sherman et al., 2005).

Substrate proteins that are synthesized in the cytosol are recognized by the complex and transferred across the outer membrane.

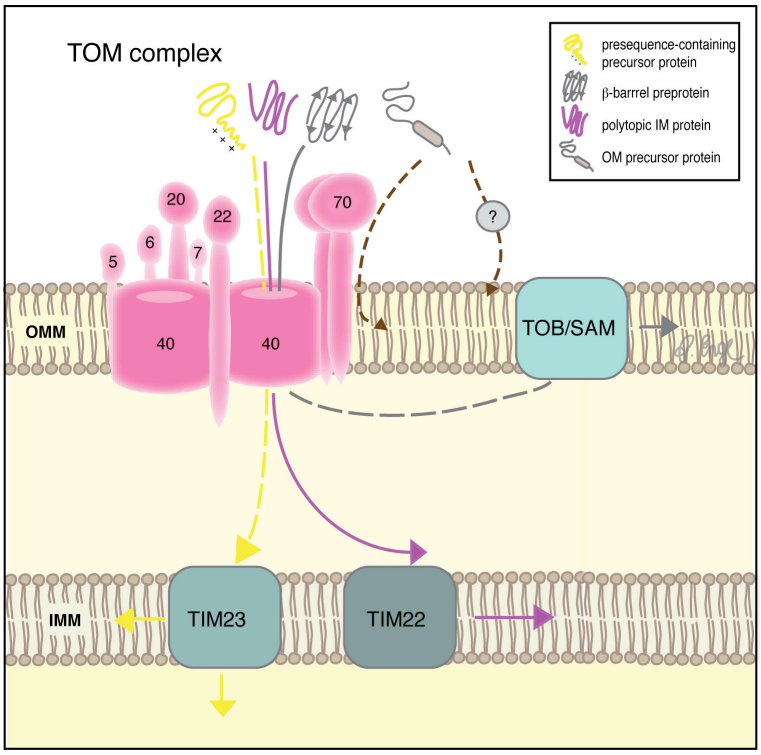


Fig. 1.3 *Translocase of the outer membrane. The TOM complex mediates the translocation of virtually all mitochondrial preproteins and serves as an entry site for polytopic inner membrane proteins, presequence containing precursor proteins and β-barrel precursors. Unknown additional factors involved in the insertion of α-helical OM proteins are indicated by question marks.*

Tom70 and Tom20, who constitute the two major receptors of the translocase, differ in substrate specificity but are able to substitute for the other due to overlapping functions (Fig. 1.3). Hydrophobic precursor proteins containing internal targeting sequences are preferentially recognized by Tom70 whereas Tom20 mainly functions as a receptor for preproteins harboring N-terminal presequences.

The protein conducting pore is formed by Tom40 and together with the other components forming the TOM complex in the outer membrane efficient translocation of proteins destined for the various

submitochondrial compartments can be achieved (Neupert and Herrmann, 2007b).
Proteins destined for the mitochondrial outer membrane are synthesized on cytosolic ribosomes and utilize various sorting pathways. Two types of integral membrane proteins can be distinguished: α-helical proteins and β-barrel proteins.

1.2.4.2 Outer membrane insertion of α-helical proteins

Single span outer membrane proteins comprise a group of proteins which differ in the location of their transmembrane segments (Schmidt et al., 2010). N-terminally anchored proteins, or signal anchored proteins, such as Tom20 and Tom70 do not use receptors, but seemingly insert via assistance of Mim1 (Becker et al., 2008; Hulett et al., 2008; Popov-Celeketic et al., 2008) without using the general import pore of the TOM complex (Ahting et al., 2005). Nonetheless, the TOM complex is required for insertion probably by facilitating insertion at its protein-lipid interface (Rapaport, 2005a).
Tail anchored precursors such as the small TOM proteins depend on Mim1 (Thornton et al., 2010) whereas other tail-anchored proteins seem to require none of the known translocation machineries while the lipid composition comprises a major factor in their insertion process (Kemper et al., 2008; Setoguchi et al., 2006). It is currently unclear whether this insertion process is driven by an unknown insertase or occurs as an unassisted process.
Precursors with multiple transmembrane segments were found to rely on Tom70 and Mim1 for insertion but on no other TOM components (Becker et al., 2011; Otera et al., 2007; Papic et al., 2011).

1.2.4.3 The β-barrel pathway

β-barrel proteins comprise an interesting class of outer mitochondrial membrane proteins that are also found in outer membranes of Gram-

negative bacteria and chloroplasts (Neupert and Herrmann, 2007a; Voulhoux et al., 2003; Wu et al., 2005).

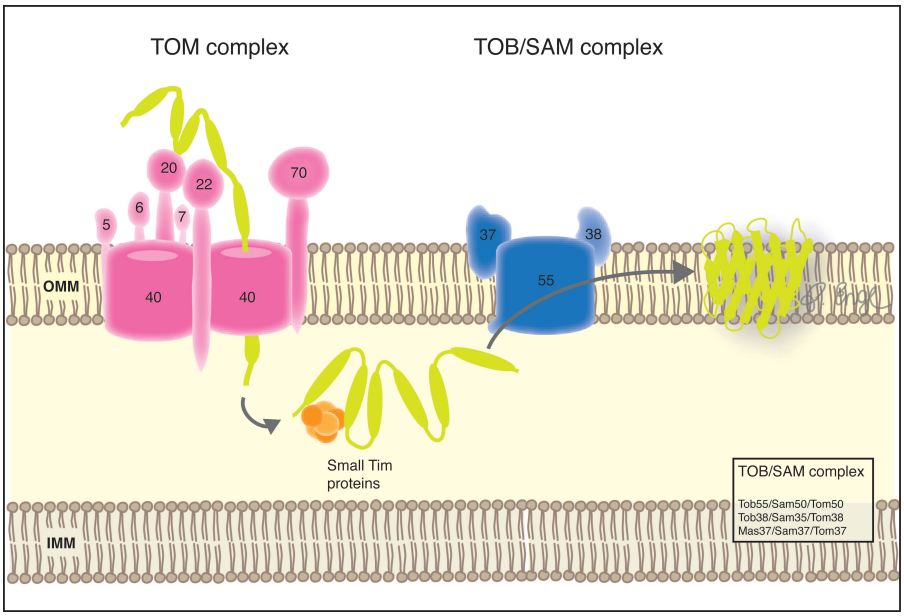


Fig. 1.4 *β-barrel pathway. Precursors of β-barrel proteins are recognized by receptors of the TOM complex and subsequently translocated through the TOM import pore. Upon translocation the precursor proteins are relayed to the small Tim proteins and further transferred to the TOB complex that facilitates their insertion into the outer membrane. Figure illustrated by the author based on the original model by W. Neupert and J. M. Herrmann, Annual Review of Biochemistry, 2007.*

Precursors of β-barrel proteins cross the outer membrane via the TOM complex and are transferred to the TOB complex with the assistance of the intermembrane space chaperones Tim9-Tim10 and Tim8-Tim13 (Fig. 1.4) (Neupert and Herrmann, 2007a).

The final release into the lipid phase of the outer mitochondrial membrane is promoted by Mas37, the third subunit of the core TOB complex (Chan and Lithgow, 2008; Dukanovic et al., 2009). The exact mechanism, however, how release and insertion are accomplished remains to be elucidated.

1.3 Mitochondrial morphology and neurodegeneration

Mitochondria are extremely dynamic organelles, constantly undergoing fusion and fission events. These processes are essential for cell fate as they are tightly linked to apoptosis and maintenance of cellular homeostasis. An increasing number of proteins participating in these processes have been discovered in recent years with mitochondrial morphology deregulation proposed as one emerging concept of neurodegenerative diseases (Chan, 2006; Frank, 2006; Han et al., 2011; Otera and Mihara, 2011).

1.3.1 Mitochondrial fission

Drp1 in mammalian cells and its yeast homologue Dnm1 are key players in mitochondrial fission processes (Table 1.1 and Fig. 1.5) and were reported to promote their action via GTP hydrolysis and assembly of higher order structures (Ingerman et al., 2005; Pitts et al., 2004; Praefcke and McMahon, 2004; Smirnova et al., 1998).
The soluble protein contains a GTPase domain at the C-terminus and an N-terminal GTPase effector domain (GED). The latter domain is associated with self-assembly and membrane constriction that ultimately leads to membrane scission (Smirnova et al., 1998). Lack of DRP1 thus promotes a shift towards fusion with formation of a highly interconnected mitochondrial network (Otsuga et al., 1998; Smirnova et al., 1998). The cellular function of this protein was intensively studied in yeast and the current models propose a dynamic interplay between Dnm1, Fis1 and the adapter molecules Mdv1 and Caf4 (Griffin et al., 2005; Mozdy et al., 2000; Tieu and Nunnari, 2000).

Fis1, an outer membrane protein, serves as an interaction interface for the adaptor molecule Mdv1. By presenting a six-helices bundle with tandem TPR motifs of Fis1 binding to an N-terminal Mdv1 structure can be established (Lackner and Nunnari, 2009; Yoon et al., 2003; Zhang and Chan, 2007). Subsequently membrane-associated Mdv1 acts as a

nucleator for Dnm1 oligomers and stabilizes advantageous conformations (Lackner et al., 2009). Ultimately these oligomeric structures that form spirals around the mitochondria separate the membranes after GTP-hydrolysis.

Caf4, an Mdv1 paralog, is also able to form contacts with Fis1 and to recruit Dnm1. However, the physiological importance of this particular complex is currently unclear (Griffin et al., 2005). Similarly unresolved is the mechanism by which cytosolic Dnm1 is recruited to discrete spots on the mitochondrial outer membrane (Westermann, 2010).

Mammalian FIS1 was also reported to participate in fission processes since induced fragmentation was observed in cells overexpressing the protein and its depletion resulted in a hyperfused and elongated network (James et al., 2003b; Stojanovski et al., 2004; Yoon et al., 2003). However, the distinct morphological function of FIS1 was recently challenged as another protein, Mff was suggested as the anchor for DRP1 on mitochondria (Otera and Mihara, 2011; Otera et al., 2010). Mff was found recently in a comprehensive RNAi study in *Drosophila* to be a novel component of the fission process (Gandre-Babbe and van der Bliek, 2008).

A number of posttranslational modifiers have been implicated in the regulation of fission events (Fig. 1.5)(Cho et al., 2010; Otera and Mihara, 2011; Westermann, 2010).
DRP1 function was reported to be modified by phosphorylation (Cereghetti et al., 2008; Chang and Blackstone, 2007; Cribbs and Strack, 2007; Han et al., 2008; Taguchi et al., 2007), sumoylation (Braschi et al., 2009), and ubiquitination (Karbowski et al., 2007; Nakamura et al., 2006; Park et al., 2010; Yonashiro et al., 2006).

Table 1.1 Mitochondrial fission factors *(Table adapted from B. Westermann, Nature Reviews Molecular Cell Biology, 2010)*

Mammalian protein	Yeast protein	Proposed function	Reference
DRP1 (DLP1)	Dnm1	mitochondrial division	Ingerman et al., 2005; Legesse-Miller et al., 2003; Otsuga et al., 1998; Smirnova et al., 1998
hFIS1	Fis1	OM, recruits DNM1/DRP1	Jakobs et al., 2003; James et al., 2003a; Mozdy et al., 2000; Yoon et al., 2003; Zhang and Chan, 2007
MFF	---	DRP1 receptor	Gandre-Babbe and van der Bliek, 2008; Otera et al., 2010
---	Mdv1, Caf4	Dnm1 and Fis1 adaptor	Griffin et al., 2005; Lackner et al., 2009; Schauss et al., 2006; Tieu and Nunnari, 2000; Tieu et al., 2002

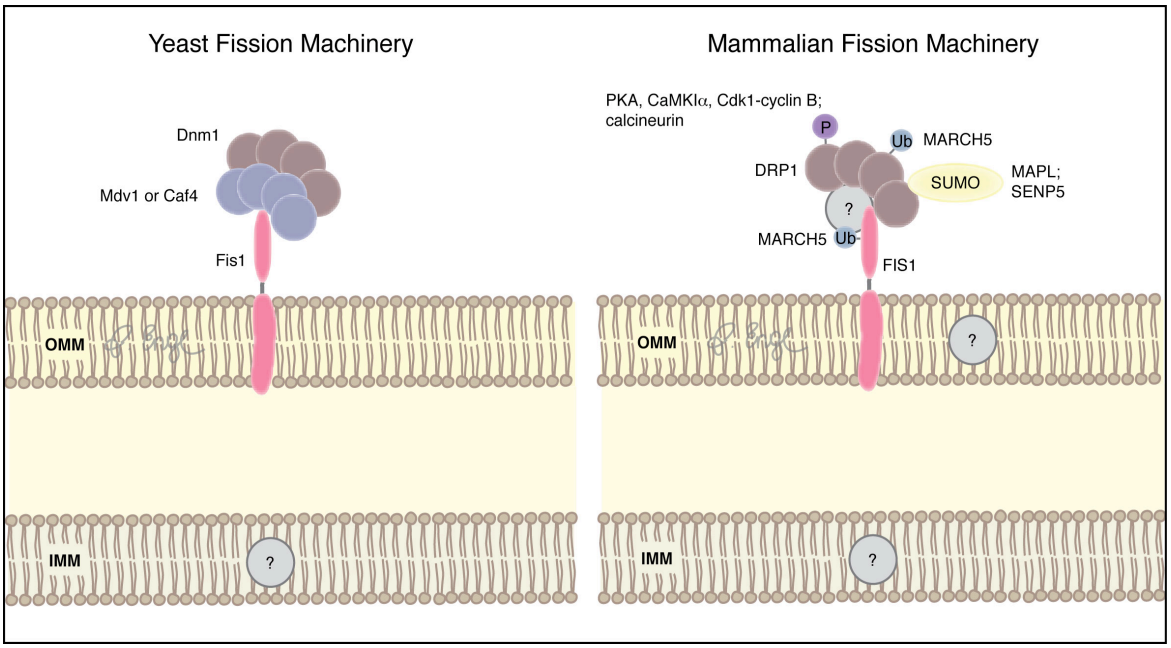


Fig. 1.5 *Fission machinery in yeast and in mammalians. Components of the fission machinery assemble in higher molecular weight complexes in a yet to be resolved stoichiometry. Several post-translational modifications of these proteins modify the fission events. Coordination of fission events might be influenced by still unknown additional factors (indicated by question marks). Figure illustrated by the author based on the original model by B. Westermann, Nature Reviews Molecular Cell Biology, 2010.*

1.3.2 Mitochondrial fusion

Mitochondrial fusion enables cells to respond to different conditions thus maintaining respiratory function and mtDNA exchange. A balance between fusion and fission events is essential for a series of cellular processes such as neuronal plasticity, calcium signaling, embryonic development and apoptosis (Chan, 2006).

Content mixing of essential mitochondrial components and mtDNA in murine models with fusion defects underlines the crucial role of fusion events. This mechanism prevents respiratory dysfunction and eventually impaired outgrowth of cellular processes and neurodegeneration due to loss of mtDNA and other critical mitochondrial contents amongst a

mitochondrial population (Chen and Chan, 2010). Coordinated fusion of four membranes requires a complex interplay between various fusogenic elements (Fig. 1.6 and Table 1.2).

To date, 3 large GTPase proteins MFN1, MFN2 and OPA1 are the known key mediators of fusion in mammalian cells (Table 1.2) (Alexander et al., 2000; Chen et al., 2003; Delettre et al., 2000; Santel and Fuller, 2001). MFN1 and MFN2 are highly homologous proteins in the outer mitochondrial membrane that are able to form in trans hetero- or homo-oligomers (Chen et al., 2003). These contacts lead to initial tethering of the two opposing membranes and subsequent fusion although the exact mechanism following the initial tethering step is not evident yet (Koshiba et al., 2004).

OPA1, the other key player in mitochondrial fusion processes, can localize to different mitochondrial sub-compartments depending on its isoform. Eight mRNA splice forms and posttranslational cleavage result in "long" isoforms with the transmembrane domain of the protein anchored in the inner mitochondrial membrane and the "short" isoforms that reside in the intermembrane space. Both forms are essential for proper fusion events (Griparic et al., 2007; Song et al., 2007).

Different proteases were proposed to be involved in the processing of OPA1 (Fig. 1.6). Candidates include i) i-AAA protease Yme1L, ii) the m-AAA proteases Afg3L1, Afg3L2 or Paraplegin in mice mitochondria (Afg3L2 and Paraplegin in humans) (Cipolat et al., 2006; Duvezin-Caubet et al., 2007; Guillery et al., 2008; McQuibban et al., 2003; Song et al., 2007), and iii) Oma1 (Ehses et al., 2009; Head et al., 2009; McBride and Soubannier, 2010), or iv) PARL (Ehses et al., 2009).

OPA1 promotes inner membrane fusion whereas MFN1 and MFN2 are required for outer membrane fusion. Although the fusion process of both membranes is usually coordinated and synchronized, this process can be

functionally uncoupled under certain conditions (Malka et al., 2005; Meeusen et al., 2004; Song et al., 2009).

Table 1.2 Mitochondrial fusion factors *(Table adapted from B. Westermann, Nature Reviews Molecular Cell Biology, 2010)*

Mammalian protein	Yeast protein	Proposed function	Reference
MFN1, MFN2	Fzo1	OM fusion	Chen et al., 2003; Fritz et al., 2001; Hermann et al., 1998; Koshiba et al., 2004; Meeusen et al., 2004; Rapaport et al., 1998; Rojo et al., 2002; Santel and Fuller, 2001
OPA1	Mgm1	IM fusion	Cipolat et al., 2004; DeVay et al., 2009; Meeusen et al., 2006; Olichon et al., 2003; Wong et al., 2000
---	Ugo1	OM fusion and linker between Fzo1 and Mgm1	Hoppins et al., 2009; Sesaki and Jensen, 2001, 2004

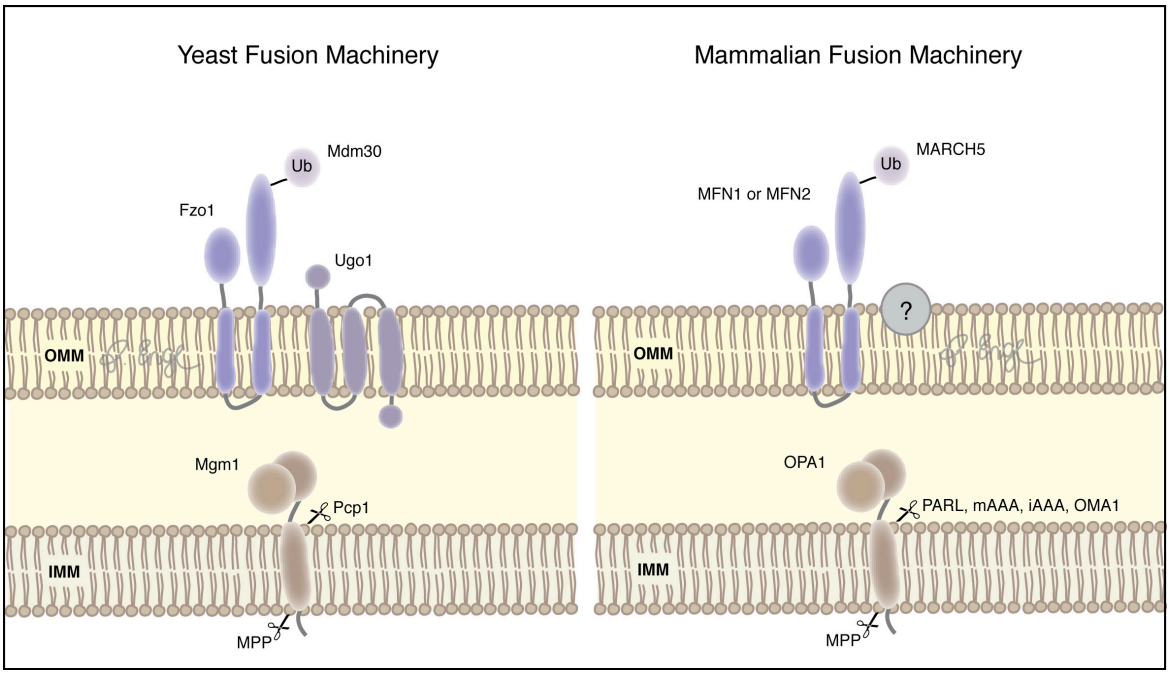


Fig. 1.6 *Fusion machinery in yeast and in mammalian cells. The components of the fusion machinery Fzo1 (in yeast) and MFN1/MFN2 (in mammals) are post-translationally ubiquitinated by Mdm30 or MARCH5, respectively. The inner membrane proteins Mgm1 and OPA1 are processed after import to remove the mitochondrial targeting sequence. Figure illustrated by the author based on the original model by B. Westermann, Nature Reviews Molecular Cell Biology, 2010.*

1.3.3 Function and dysfunction of mitochondrial dynamics

Mitochondrial function is required in regions of high energy demand. Therefore proper regulation of mitochondrial dynamics is a key factor in processes as rapid axonal transport of mitochondria along microtubules.

This transport towards dendritic spines is required for synapse-formation and ultimately for neuronal survival and is mediated by kinesin (anterograde transport), the adaptor molecules Milton1/2 (Brickley et al., 2005; Stowers et al., 2002) and Miro (Guo et al., 2005).

Retrograde transport to the cell soma by dynein-mediated processes ensures restoration of activity of energetically compromised mitochondria by fusion with respiratory active mitochondria, thereby allowing complementation of mtDNA products. Alternatively, the damaged or surplus mitochondria can be eliminated by mitophagy (Chang and

Reynolds, 2006; Hollenbeck and Saxton, 2005; Li et al., 2004; Westermann, 2010). Disruption of fusion or fission in mammalian cells, as seen for mitofusins or DRP1 knockout mice, leads to severe consequences. Lack of these proteins seems to interfere with mammalian embryonic development, thus resulting in embryonic death (Chen et al., 2003; Ishihara et al., 2009; Wakabayashi et al., 2009).

Highly interconnected mitochondria act as electrically united systems that secure ATP production by dissipating the generated membrane potential in the cell periphery over large distances towards remote parts of the network. This mechanism was reported to be a major factor in metabolic energy pathways of large cells as in the case of muscle cells (Amchenkova et al., 1988; Skulachev, 2001).

Another indicator for mitochondrial fusion connected to energy dissipation is the occurrence of markedly increased fusion events under stress conditions involving mitochondrial ATP production (Tondera et al., 2009) and in the cell cycle during the G_1 to S transition (Mitra et al., 2009) or under nutrition starvation (Gomes et al., 2011).

Hence, mitochondrial dynamics, defined by a concerted interplay between various elements and processes, seem to enable the cell to respond and adapt to the specific requirements by altering the shape of the mitochondrial network.

1.3.4 Mitochondrial dynamics and neurodegeneration

Defects of mitochondrial dynamics seemingly show a tropism for neuronal tissue. These cells have a high energy demand and are strictly relying on mitochondrial energy production. Thus they are predominantly sensitive and vulnerable regarding perturbations of mitochondrial distribution and function (Knott et al., 2008). Neuronal defects were reported in association with mutations in *OPA1*, *MFN2* and *DRP1*, all causing mitochondrial morphology alterations. Mutations in *MFN2* give rise to Charcot-Marie-Tooth disease type 2a (CMT2A), a neurodegenerative disease characterized by degeneration of long sensory and motor nerves (Zuchner et al., 2004).

Impairment of mitochondrial fusion and resulting changes in movement and/or distribution might hinder recruitment to synapses and over time culminate in severe physiological conditions (Chan, 2006).
OPA1 mutations are causative for the major form of autosomal dominant optic atrophy (ADOA), the most common variant of inherited blindness in infants, which is due to progressive degeneration of retinal ganglion cells (Alexander et al., 2000; Delettre et al., 2000). In some clinical studies reduced oxidative phosphorylation in myoctes and a reduced copy number of mtDNA was revealed (Kim et al., 2005; Lodi et al., 2004). These defects might be correlated to a critical reduction of membrane potential (Chen et al., 2005). Additionally, loss of OPA1 promotes cristae remodeling and apoptosis (Griparic et al., 2004; Olichon et al., 2003).

A direct link between fission components and neurodegenerative diseases was not reported to date although a recent case report described a dominant-negative mutation in *DRP1* that is associated with neurological defects including visual impairment, microencephaly and irregular brain development in a newborn (Waterham et al., 2007).
Extensive studies will be necessary to extend our knowledge of neurodegeneration and mitochondrial dynamics to unravel the still unresolved conundrums of fusion and fission.

1.4 Mitochondrial diseases

Since mitochondria play a crucial role in many metabolic pathways it is not surprising that mitochondrial dysfunction manifests in patients with severe clinical symptoms (Harman, 1957; Raha and Robinson, 2000). Mitochondrial diseases comprise a vast spectrum of clinical syndromes and genetic features thereby complicating initial diagnosis and management. Mutations in mitochondrial DNA and in nuclear genes encoding mitochondrial proteins have been associated with pathological manifestations in a variety of systems like the central nervous system (CNS), leading to seizures and stroke like episodes, the auditory system (symptoms ranging from prelingual deafness to progressive hearing loss

over decades), the optic system and also in tissues with high metabolic energy demand such as nerves and muscles (Cacace and Pinheiro, 2011; DiMauro, 2007; Simon and Johns, 1999; Yu-Wai-Man et al., 2011).
Although great efforts and considerable progress have been made in the past decades, a common pathogenetic mechanism of mitochondrial mutations and dysfunction ultimately causing cell death is still undisclosed.
A potential "lowest common denominator" linking these diseases seems to be mitochondrial dysfunction, affecting in the long run the function of the respiratory chain and oxidative phosphorylation and culminating in reduced ATP levels in the cell, and alteration in regulation of mitochondrial morphology processes or mitochondrial protein import (Campello and Scorrano, 2010; Chan, 2006; DiMauro, 2007; Kwong et al., 2006).
In addition, also other neurodegenerative diseases like Alzheimer's and Huntington's disease, not primarily thought of as mitochondrial, have been widely reasoned to have a "secondary" mitochondrial involvement during disease progression (Kwong et al., 2006).

1.4.1 Epidemiology and diagnosis

Mitochondrial diseases can present at any time from the neonatal period to onset in the late years whereas onsets in infancy, in general, tend to be more severe than those with their onset in adulthood. High clinical heterogeneity does not only have an immense impact on individual diagnosis but also impedes epidemiological studies of disease prevalence.

Initial diagnosis might lack detection of a multi-organ involvement, the hallmark of mitochondrial diseases, and tissue specific manifestation can progress very slowly (McFarland et al., 2002; McFarland and Turnbull, 2009; Taylor and Turnbull, 2005).
A plethora of symptoms and high phenotypic expression variability of the same mutation, as seen for example in the m.3243 mutation that can lead to three different syndromes (MELAS-mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes; MIDD-maternally inherited

diabetes and deafness; CPEO-chronic progressive external ophthalmoplegia), hampers clinical diagnosis (McFarland and Turnbull, 2009).

A mitochondrial pathogenetic origin is often considered only if the classical neurological manifestations such as seizures, neuropathy and dystonia coexist with other conditions like diabetes, or visual and auditory impairment. Differential diagnosis requires extensive clinical assessment with specific emphasis on issues addressing maternal health, family history including childhood deaths, obstetric history and laboratory evaluation.

Attention should be also paid to endocrine malfunction, the gastrointestinal system, cardiac abnormalities and developmental progress. Examination of the auditory and visual system might give additional information about their possible involvement although probably initially not causing symptomatic problems (Cacace and Pinheiro, 2011; DiMauro et al., 2006; McFarland and Turnbull, 2009; Taylor and Turnbull, 2005; Yu-Wai-Man et al., 2011).

1.4.2 Mitochondrial involvement in neurodegenerative diseases

1.4.2.1 Neurodegeneration arising from mutations in mtDNA

MtDNA mutations tend to affect multiple organs, preferentially tissues with high energy metabolism, causing a multisystemic clinical picture. Nevertheless, the detrimental effects of certain mutations seem to have a tropism for the nervous system (Kwong et al., 2006).

1.4.2.1.1 Leber's hereditary optic neuropathy (LHON)

LHON is a maternally inherited degeneration of retinal ganglion cells and nerve fiber layers. The majority of patients carries one of the three primary mutations in mtDNA genes encoding subunits of complex1: (i) m.3460G>A (Howell et al., 1991; Huoponen et al., 1991), (ii) m.11778G>A (Wallace et al., 1988), or (iii) m.14484T>C (Johns et al., 1992; Mackey and Howell, 1992). The predominantly male carriers suffer from acute or subacute

painless vision loss with variability in disease onset as well as in progression. This variability is indicative of modulation by epigenetic and environmental factors (Yu-Wai-Man et al., 2011).

1.4.2.1.2 Neuropathy ataxia and retinitis pigmentosa (NARP)

The most common mutation in NARP is a T-G transition at position m.8993 in the mtDNA gene coding for ATPase6, an essential subunit of the ATP synthase. This disease is characterized by proximal muscle weakness, seizures, ataxia, retinitis pigmentosa and developmental delay at a modest mutational burden of about 70 % (Holt et al., 1990). A shift of this heteroplasmic condition towards higher percentages of mutated mtDNA molecules results in a far more severe condition, termed maternally inherited Leigh syndrome. This latter syndrome is a fatal encephalopathy with onset within the first year of life (Leigh, 1951; Tatuch and Robinson, 1993).

1.4.2.2 Neurodegeneration associated with nDNA encoded mitochondrial proteins

1.4.2.2.1 Friedreich ataxia (FA)

The most common inherited form of ataxia is the FA that is associated with degeneration of large-caliber neurons in the spinocerebellar, sensory, and motor pathways. The disease is manifesting with progressive gait and limb ataxia, spasticity and axonal sensory neuropathy (Durr et al., 1996; Harding, 1981). Genetically, a GAA nucleotide repeat extension in the first intron of the FA gene leads to decreased levels of the protein frataxin (Campuzano et al., 1996; Grabczyk and Usdin, 2000; Sakamoto et al., 1999). This protein was reported to regulate mitochondrial iron levels by participating in transport, iron-sulfur cluster biogenesis and detoxification of surplus iron.

Accumulation of high iron amounts in mitochondria might thus result in an increase of oxygen-based free radicals promoting mitochondrial damage and therefore in the long run neuronal cell death (Babcock et al., 1997;

Bulteau et al., 2004; Foury and Cazzalini, 1997; Gakh et al., 2006; Puccio et al., 2001; Ristow et al., 2003; Simon et al., 2004; Thierbach et al., 2005; Wong et al., 1999).

1.4.2.2.2 Hereditary spastic paraplegia

More than 20 genetic loci have been linked with hereditary spastic paraplegia, a heterogeneous group of neurodegenerative disorders characterized by muscle weakness and spasticity (Kwong et al., 2006). Mutations identified to be of mitochondrial origin were found in the *SPG13* gene (encoding the mitochondrial chaperone Hsp60) and *SPG7* gene (encoding for paraplegin, a mitochondrial metalloprotease subunit) (Casari et al., 1998; Hansen et al., 2002). These mutations lead to respiratory chain dysfunction and increased oxidative stress sensitivity (Atorino et al., 2003).

1.4.2.3 Mitochondrial neurodegenerative diseases deriving from mutant proteins with multiple intracellular localizations

1.4.2.3.1 Parkinson's disease (PD)

The hereditary form of PD emanates from mutations in any of these six different genes: *α-synuclein* (Polymeropoulos et al., 1997), *DJ1* (Bonifati et al., 2003), *PINK1* (Valente et al., 2004), *parkin* (Kitada 1998), *OMI/HTRA2* (Strauss et al., 2005), and *LRKK2* (Zimprich et al., 2004). These mutations cause a substantial loss of dopaminergic neurons in the substantia nigra.

Clinically the disease manifests with the symptom triad bradykinesia, rigor and resting tremor resulting in postural instability. Accumulating evidence suggests that a strong relation between oxidative damage, mitochondrial dysfunction and protein degradation contributes to the pathomechanism of PD (Kwong et al., 2006).

1.4.2.3.2 Amyotrophic lateral sclerosis (ALS)

ALS is in 90 % of the cases of unknown origin, the remaining 10 % can be tracked back to genetic causes, therefore termed Famytrophic lateral sclerosis. A subset of the latter cases is derived from mutations in the superoxide dismutase 1 (*SOD1)* gene (Rosen et al., 1993). SOD1 containing aggregates that were postulated to contribute to mitochondrial dysfunction were found in the IMS and matrix of the organelle (Vijayvergiya et al., 2005). Neuronal degeneration, due to the selectivity for spinal and cortical motor neurons, results in a fatal condition marked by progressive skeletal muscle atrophy, paralysis and demise (Mulder and Kurland, 1987).

"Secondary" mitochondrial involvement was also postulated in diseases such as Alzheimer's disease (although there are reports on the presence of Aβ peptide in mitochondria) and Huntington's disease.

1.4.2.4 Deafness-Dystonia-Optic Neuronopathy (DDON) syndrome

DDON or previously termed DFN-1, Deafness Dystonia Syndrome or Mohr-Tranebjaerg syndrome (MTS; MIM 304700) belongs to the family of mitochondrial diseases and emerges upon mutations in the *TIMM8A* gene which encodes for the deafness dystonia protein 1 (DDP1). Mutations in the *DDP1* coding region itself as well as mutations affecting additional genes up- and downstream of the *DDP1* gene locus, resulting in the deletion of the entire *TIMM8A* gene, have been described.
DDON therefore comprises a single gene disorder with frameshift, nonsense, missense and stop mutations or deletions and splice site mutations affecting the *TIMM8A* gene locus (Aguirre et al., 2006; Aguirre et al., 2008; Binder et al., 2003; Ezquerra et al., 2005; Jin et al., 1996; Kim et al., 2007; Swerdlow and Wooten, 2001; Tranebjaerg et al., 2000a; Tranebjaerg et al., 2001; Ujike et al., 2001), as well as a contiguous gene deletion syndrome disrupting coding regions for *BTK, TAF7L* and *DRP2* (Jyonouchi et al., 2008; Pizzuti et al., 2004; Richter et al., 2001; Sediva et al., 2007; Tranebjaerg et al., 2000b). Deletion of the entire T*IMM8A* gene

locus or mutations within, causing a frameshift, result in an absent, truncated or malfunctioning gene product (Tranebjaerg, 1993).
DDON was first described in 1960 in a Norwegian family with progressive sensorineural hearing impairment. Initially, isolated deafness was suggested until an extensive clinical restudy of this family in 1995 by Tranebjaerg et al. demonstrated the underlying syndromic character with an X-linked inheritance pattern (Tranebjaerg et al., 1995). Further characterization revealed the causative single base pair deletion in exon 1 in an affected male from the original Norwegian DFN-1/MTS family (Jin et al., 1996; Mohr and Mageroy, 1960).

1.4.2.4.1 Symptomatic presentation and prevalence of DDON

DDP1 mutations manifest with a variable onset of slowly progressive ataxia or dystonia and progressive postlingual sensorineural hearing impairment in early childhood. Progressive degeneration of the brain stem, corticospinal tract and the basal ganglia was reported in patients developing the movement disorder (Wallace and Murdock, 1999). In addition, other clinical characteristics as mental deterioration, paranoia, behavioral abnormalities and cortical blindness were described, although not in all cases and also not in all individuals of the same affected family (Aguirre et al., 2006; Binder et al., 2003; Ezquerra et al., 2005; Jin et al., 1996; Kim et al., 2007; Swerdlow and Wooten, 2001; Tranebjaerg et al., 2000a; Tranebjaerg et al., 2001; Tranebjaerg et al., 1995; Ujike et al., 2001).

A vast clinical phenotypic variability, both interfamilial and intrafamilial, can thus not solely be explained by different mutations of the *DDP1* gene but might rather indicate an environmental and genetic modulation of the clinical symptoms (Aguirre et al., 2006).

Prelingual or postlingual sensorineural hearing impairment is always the primary presenting symptom emerging in early childhood, typically followed by slowly progressive dystonia or ataxia in the teens, a gradual impairment in visual acuity in the 20ties and dementia from approximately

40 years of age on. The time of onset of hearing impairment seems to be more consistent than the neurologic, neuropsychiatric and ophthalmologic signs (Tranebjaerg, 1993).

Sensorineural hearing impairment, as the least varying symptom, starts generally at an age of 18 months, although reports also described affected individuals with congenital prelingual hearing impairment (Swerdlow and Wooten, 2001; Ujike et al., 2001). Profound hearing loss before the age of ten years due to a rapid progression of cochlear and vestibular neuronal cell death results in an auditory neuropathy associated with absent auditory brain stem responses (Bahmad et al., 2007).

The onset of neurological symptoms which might either appear as dystonia or ataxia in early childhood or later results in a progressive movement disorder characterized by walking difficulties, brisk tendon reflexes, ankle cloni and extensor plantar responses (Hayes et al., 1998; Jensen, 1981; Jensen et al., 1987; Scribanu and Kennedy, 1976; Tranebjaerg et al., 1995; Ujike et al., 2001).

Destruction of cell bodies of neurons, termed as neuronopathy, leading to optical neuropathy might be subclinical for years (Ujike et al., 2001), as in childhood vision and vision fields are normal (Ponjavic et al., 1996; Tranebjaerg et al., 2001). Decline in visual acuity and subsequent blindness occur usually around age 30 to 40 years (Ponjavic et al., 1996; Tranebjaerg et al., 2000a; Tranebjaerg et al., 2000b; Tranebjaerg et al., 2001; Tranebjaerg et al., 1995), and might present initially with photopobia, defect in color vision and central scotomas in the late teens (Ponjavic et al., 1996).

Until recently a total number of 67 affected individuals in at least 31 families worldwide were reported thus occurring with a prevalence between 70 and 329 per million (Tranebjaerg, 1993).

1.4.2.4.2 Pathomechanism underlying DDON

The pathomechanism of DDON is not yet completely elucidated. Studies in

yeast indicate that the yeast homologue of DDP1, Tim8, resides in the intermembrane space (IMS) of mitochondria and forms there a hetero-oligomeric 70 kDa complex with Tim13. This complex was initially suggested to assist the import process of Tim23 precursor protein by stabilizing it as the newly synthesized protein is transferred through the IMS (Davis et al., 2000; Koehler et al., 1999; Paschen et al., 2000; Rothbauer et al., 2001). Truncation or absence of DDP1 as well as the missense mutation C66W in the conserved "twin CX_3C" motif (Tranebjaerg et al., 2000a) cause a defect in assembly of the DDP1/TIMM13 complex (Hofmann et al., 2002; Roesch et al., 2002). Hence, DDP1 mutations were suggested to result in impaired hTim23 biogenesis in the affected tissue (Roesch et al., 2002; Rothbauer et al., 2001). Additionally reported substrates of DDP1/TIMM13 in mammalian mitochondria are the aspartate/glutamate carriers, citrin and aralar1, participating in the aspartate-malate NADH shuttle. Due to shared tissue expression patterns of aralar1, DDP1 and TIMM13 and high metabolic demands in large cerebellar neurons, decreased NADH levels in these mitochondria together with a decrease in hTim23 biogenesis and the consecutive alterations in matrix import of precursor proteins were proposed as molecular mechanism of this neurodegenerative disease (Roesch et al., 2004).

However, studies in *Saccharomyces cerevisiae* and *Neurospora crassa* point out an involvement of the Tim8-Tim13 complex also in the import pathway of outer membrane β-barrel precursor proteins. Lack of Tim8 and Tim13 partially impairs the assembly pathway Tom40 and Tob55/Sam50 (Habib et al., 2005; Hoppins and Nargang, 2004; Wiedemann et al., 2004). Considering the important functions of these β-barrels proteins, their reduced import in DDP1 mutated cells can also contribute to the pathomechanism of DDON.

1.5 Aims of this study

Deafness-Dystonia-Optic Neuronopathy (DDON) syndrome is a rare X-linked progressive neurodegenerative disorder resulting from mutations in

the *TIMM8A* gene encoding for the deafness dystonia protein 1 (DDP1). Although a lot of progress has been made in recent years little is known about the underlying pathomechanism.

The aim of this study was to elucidate in detail the function of DDP1 especially in a mammalian cell culture system.

One particular point was to investigate the impact of altered DDP1 levels on the import of β-barrel proteins such as Tom40 in comparison to import in mitochondria derived from cells overexpressing the mutant form of DDP1.

Complex formation of the wild type protein compared to the mutant form of DDP1 constituted another main point with specific emphasis on the effects of co-overexpression of the partner protein TIMM13.

A special interest was to conduct morphological studies of primary cells derived from DDON patients and cells with altered DDP1 levels that would provide further information about possible consequences of these conditions.

To assess potential variations regarding bioenergetic features, it was imperative to analyze DDP1 downregulated or lacking cells in terms of growth behavior, membrane potential and respiratory functions.

Addressing these issues might help to expand the information on DDON and in the long run maybe contribute to a more precise concept of the pathomechanism underlying this syndrome.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1. Growth Media for *E. coli*

2.1.1.1 LB medium

LB medium contained 1 % (w/v) bacto tryptone, 0.5 % (w/v) yeast extract and 0.5 % (w/v) NaCl in ddH_20, pH 7.0, and was autoclaved.

For LB solid media 1.5 % (w/v) agar was added to liquid LB and autoclaved. When required the medium was supplemented with antibiotics (100 µg/ml ampicillin or 50 µg/ml kanamycin).

For purification of proteins with MBP-tag, LB medium prepared as above was supplemented with 0.3 M saccharose and 0.2 % (w/v) glucose.

2.1.1.2 Super optimal broth with catabolite repression (SOC) medium

SOC medium contained 2 % (w/v) bacto tryptone, 0.5 % (w/v) yeast extract, 0.05 % (w/v) NaCl and 2.5 mM KCL in water, pH 7.0. The medium was autoclaved and subsequently supplemented with 10 mM $MgCl_2$ and 20 mM glucose.

2.1.2 Bacterial strains

Bacterial strains used in this work are listed in Table 2.1.

Table 2.1: Bacterial Strains

Organism	Strain	Source/Reference
E. Coli	BL-21 (DE3)	kind gift of Dr. T. Stehle
E. Coli	XL1 Blue	Stratagene

2.1.3 Buffers and solutions

Table 2.2: Buffers for plasmid isolation from *E. coli*

E1	50 mM Tris-HCl, 10 mM EDTA in purified water, 100 µg/ml RNAse
E2	200 mM NAOH, 1 % (w/v) SDS in purified water
E3	3 M potassium acetate in purified water, pH adjusted to 5.5 with acetic acid

Table 2.3: Buffers for preparation of competent *E. coli*

TfbI	30 mM potassium acetate, 100 mM RbCl, 10mM $CaCl_2$, 50 mM $MnCl_2$, 15 % (v/v) glycerol, pH 5.8
TfbII	10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15 % (v/v) glycerol, adjust to pH 6.5 with NaOH

Table 2.4: Buffers for purification of MBP-tagged proteins

Column buffer	20 mM HEPES, pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol, 2 mM EDTA, 1 mM PMSF
Elution buffer	20 mM HEPES, pH 7.4, 200 mM NaCl, 10 mM β-mercaptoethanol, 2 mM EDTA, 1 mM PMSF, 10 mM maltose

Table 2.5: Buffers for SDS-PAGE

2x Lämmli SDS-gel loading buffer	60 mM Tris-HCl, 1 % (w/v) SDS, 10 % (v/v) glycerol, 5 % β-mercaptoethanol, 0.01 % (w/v) bromphenol-blue in purified water
1.0 M Tris-HCl, pH 8.8	
1.0 M Tris-HCl, pH 6.8	
SDS running buffer	386 mM glycine, 49.5 mM Tris, 0.1 % (w/v) SDS

Table 2.6: Whole cell lysis buffer

Ripa buffer	25 mM Tris, pH 7.6, 150 mM NaCl, 1 % NP-40, 1 % sodium deoxycholate, 0.1 % (w/v) SDS, Complete, EDTA-free; Protease Inhibitor Cocktail Tablets (Roche)

Table 2.7: Buffers and solutions for immunoblotting and immunodetection

Blotting buffer	20 mM Tris, 150 mM glycine, 0.02 % SDS, 20 % (v/v) methanol in ddH_20
Tris buffered saline (TBS)	5 mM Tris, 150 mM NaCl in purified water, pH 7.5
Blocking buffer	TBS supplemented with 5 % (w/v) skim dry milk, or 3 % (w/v) BSA
Stripping buffer	62.5 mM Tris, pH 6.8, 2 % (w/v) SDS, 50 mM DTT
ECL	0.2 mM p-coumaric acid, 1.25 mM Luminol, 100 mM Tris, pH adjusted to 8.5 with HCl. Shortly before use it was mixed 1: 1000 with 30 % H_2O_2.

Table 2.8: Buffers for isolation of mitochondria

HBB	20 mM HEPES, pH 7.6, 220 mM Mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF
HBA	20 mM HEPES, pH 7.6, 220 mM mannitol, 70 mM sucrose, 1 mM EDTA, 0.5 mM PMSF, 2 mg/ml BSA
PBS	137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2mM KH2PO4

Table. 2.9: Buffer for in vitro import of proteins into isolated mitochondria

HBB-import buffer	HBB supplemented with 0.5 mM magnesium acetate, 20 mM sodium succinate, 5 mM NADH and 1 mM ATP

Table 2.10: Buffers for BN-PAGE

10x Cathode buffer	500 mM Tricine, 150 mM Bis-Tris pH 7.0, 0.2 % (w/v) CBB-G250
10x Anode buffer	500 mM Bis-Tris adjusted to pH 7.0
3x Gel buffer	150 mM Bis-Tris, 1.5 M 6-Aminocaproic acid, pH 7.0
10x BN Sample buffer	5 % (w/v) Coomassie BB-G250 (ServaG), 100 mM Bis-Tris pH 7.0, 500 mM 6-Aminocaproic acid
Lysis buffer	20 mM HEPES adjusted to pH 7.5, 100 mM NaCl, 10 % (w/v) glycerol
Transfer buffer	100 mM Tris, 200 mM glycine, 0.01 % (w/v) SDS, 5 % (v/v) methanol

Table 2.11: Buffers for agarose gel electrophoresis

TAE	4.84 g/l Tris-Base, 1.14 ml/l acetic acid, 1 mM EDTA, pH 8.0
6x DNA Loading buffer	6 % (v/v) glycerol, 0.05 % (w/v) bromphenol-blue, 0.05 % (w/v) xylencyanol
DNA Loading buffer OrangeG	15 % (w/v) FicollTM PM400, 5 % (v/v) glycerol, 0.05 % (w/v) OrangeG powder

Table 2.12: Buffers for PCR reaction

10x Pfu buffer with $MgSO_4$	100 mM $(NH4)_2SO_4$, 100 mM KCl, 1 % Triton X-100, 1 mg/ml BSA, 20 mM $MgSO_4$, 200 mM Tris, pH adjusted to 8.8 with HCl
10x Taq buffer with $(NH_4)_2SO_4$	200 mM $(NH_4)_2SO_4$, 0.1 % Tween-20, 750 mM Tris, pH adjusted to 8.8 with HCl

Table 2.13: T4 DNA Ligase buffer

T4 DNA Ligase buffer	100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP, 400 mM Tris, pH adjusted to 7.8 with HCl

2.1.4 Primers and constructs

Table 2.14: Constructs used in this study

Construct	Vector	Selection marker	PCR amplified or subcloned
MBP-DDP1	pMal- c2X	Amp	PCR amplification
pCS2-DDP1	pCS2	Amp	subcloned
6xmyc-DDP1	6xmyc pCS2	Amp	subcloned
pcDNA5-DDP1	pcDNA/FRT	Hyg	subcloned
pcDNA5-C66W	pcDNA/FRT	Hyg	subcloned
6xpCS2-C66W	pCS2	Amp	subcloned
pCS2-TIMM13	pCS2	Amp	subcloned
6xmyc-TIMM13	6xmyc pCS2	Amp	PCR amplification
pGEM4Z-hTim23	pGEM4Z	Amp	gift of Dr. S. Hofmann
pGEM4Z-hTom40	pGEM4Z	Amp	gift of Dr. S. Hofmann
pGEM4-VDAC1	pGEM4	Amp	PCR amplification
pGEM4-AAC	pGEM4	Amp	(Pfanner and Neupert, 1987)
pGEM4-pSU9-DHFR	pGEM4	Amp	(Pfanner et al., 1987)

Vectors pCS2, 6xmyc pCS2, pcDNA5/FRT, pMal-c2X (generous gifts of Dr. T. U. Mayer) are modified version of the original plasmids with an extended multiple cloning site (MCS). The construct pDS56/RBSII-VDAC1-6xHis (kind gift of C. Zeth) served as a template for pGEM4-hVDAC1. Constructs encoding DDP1 (NCBI acc. no. NG_011734) and TIMM13 (NCBI acc. no. NM_012458.2) were generated by PCR amplification of the corresponding cDNA using a human cDNA library as a template.

The plasmids containing the C66W mutation were generated by site-directed mutagenesis using the QuikChange Site Directed Mutagenesis Kit (Stratagene) and the MBP-DDP1 construct as a template. The following primers, designed according to the manufacturer´s guidelines, were used. Forward primer: 5`-GAG GCC TGT TTT GTG AAC TGG GTT GAG CGC TTC ATT GAT AC-3` and reverse primer (reverted and complemented): 5‘-GTA TCA ATG AAG CGC TCA ACC CAG TTC ACA AAA CAG GCC TC-3‘. The resulting insert encoding the mutated C66W version was subcloned into the respective vectors. All constructs were verified by DNA sequencing.

Table 2.15: Oligonucleotide primers

Construct	5’ primer	3’ Primer (sequence reverted and complemented)
MBP-DDP1	5’-CCC GGC CGG CCG ATG GAT TCC TCC TCC TCT TC-3’	5’-CAG TTT TCT CAG AAA GCC TTT CTG ACT GAG GCG CGC CGG G -3’
6xmyc-TIMM13	5’-AA GGC CGG CCA ATG GAG GGC GGC TTC G-3’	5’-TTG GCG CGC CTT CAC ATG TTG GCT CGT TCC-3’
pGEM4-VDAC1	5’-CCC GAG CTC ATG GCT GTG CCA CCC ACG TAT G-3’	5’-CCC GGT ACC TTA TGC TTG AAA TTC CAG TCC TAG ACC AAG C-3’

2.1.5 Antibodies

Table 2.16: Antibodies used in this study

Antibody	Reference
α-alpha-tubulin	Sigma

α-c-Myc	Santa Cruz
α-DDP1	Pineda
α-GAPDH	Assay Designs
α-Hsp60	kind gift of Dr. A. Azem
α-MBP	anti Tob55 cross-reactivity
α-Mia40	kind gift of Dr A. Reichert
α-MnSOD	Assay Designs, Enzo Life Sciences
α-Tim23	BD Transduction Laboratories
α-Tom20	Santa Cruz
α-Tom40	Santa Cruz
α-Tom70	kind gift of Dr. K. Mihara
α-VDAC1	Calbiochem, Abcam

Antibodies were diluted for the specific applications as recommended by the manufacturer.

2.2 Molecular Biology Methods

2.2.1 Transformation of *E. coli*

Plasmid DNA was incubated on ice for 30 min with 100 µl of chemically competent XL-1 blue *E. coli* cells and subsequently a heat shock of 42°C was applied for 45 sec. After cooling on ice for another 2 min, 1 ml SOC medium was added and incubated for 30 min at 37°C under constant agitation. A portion (100 µl) of this cell suspension was plated on LB agar plates supplemented with the respective antibiotics.
In case of transforming a ligation reaction, the reaction mixture was pelleted at this point, the growth medium was discarded and the cell pellet was resuspended and plated on an LB agar plate supplemented with the required antibiotic. Agar plates were incubated over night at 37°C for a maximum of 16 hours followed by examination of the colony growth.

2.2.2 Plasmid preparation

Plasmid DNA was isolated for control digestions from transformed *E. coli* cells by alkaline lysis (Birnboim and Doly, 1979). Small scale plasmid preparation with NucleoSpin™Plasmid QuickPure kit (Macherey & Nagel) following the supplier's instructions was used for PCR cloned constructs destined for sequencing.
For cell culture transfection plasmids were isolated by using the Pure Yield Plasmid Midiprep System (Promega).

2.2.3 Agarose gel electrophoresis

DNA fragments were separated according to their molecular mass on 1-3 % agarose gels containing 0.5 µg/ml ethidium bromide. Loading buffer was added to the samples and electrophoresis was performed in TAE buffer. Bands were visualized by UV-illumination.

2.2.4 Isolation of DNA from agarose gels

Gel bands corresponding to fragments of interest were excised and purified using the "peqGold Gel Extraction Kit" (PeqLab, Germany) following the manufacturer's instructions.

2.2.5 Enzymatic manipulation of plasmids

2.2.5.1 Restriction digest of DNA

Restriction digest of DNA (plasmids and PCR fragments) was carried out under optimized conditions according to the manufacturer's instructions. For cloning and subcloning experiments, 1-2 µg of plasmid-DNA and 50µl of PCR reactions were usually digested with the corresponding amount of the appropriate enzymes.

2.2.5.2 Dephosphorylation of DNA

Dephosphorylation of 1-2 µg restriction-digested cloning vector was routinely performed with 1 -1.5 µl of shrimp alkaline phosphatase (SAP) in a total reaction volume of 30 µl according to the manufacturer's instructions.

2.2.5.3 Ligation with T4 DNA ligase

Routine ligation of DNA fragments was performed with 1.5 µl T4 DNA ligase in a reaction volume of 10 µl. Different molar ratios of vector:insert ranging from 1:10 to 1:3 were tested. T4 DNA ligase was inactivated prior to the transformation into competent *E. coli* cells.

Restriction enzymes, SAP, T4 DNA Ligase, *Pfu*- and *Taq*- DNA Polymerases were purchased from Fermentas. Restriction enzymes *FseI* and *AscI* were ordered from New England Biolabs. Proteinase K was acquired from Roche. All enzymes were used in assays with optimized conditions according to the manufacturer's instructions.

2.2.6 RNA isolation

Total RNA from induced and non-induced HeLa cells was extracted using PrepEase RNA Spin Kit (Affymetrix). First-strand cDNA was synthesized from 500 ng RNA using iScript cDNA Synthesis kit (Biorad) according to the manufacturer's recommendations.

2.2.7 Quantitative Real Time-PCR

RT-PCR amplification was performed on first-strand cDNA using TIMM13-specific primers (QT00203266, Qiagen), and GAPDH-specific primers (QT01192646, Qiagen) as a control. The reaction was performed with the

Maxima SYBR Green/ROX qPCR Master Mix (Fermentas) kit and an AppliedBiosystems StepOne real time PCR system according to the Fermentas Maxima SYBR Green/ROX qPCR Master Mix manual.

2.2.8 Polymerase chain reaction

PCR was employed to amplify DNA fragments. A standard 50 µl PCR-reaction mix contained 1-1.5 µl thermostable DNA polymerase (*Pfu*-polymerase for cloning purposes or *Taq*-polymerase for control reactions), 5 µl appropriate polymerase reaction buffer (10x *Pfu*- or 10x *Taq*-buffer), 2 µl of 10 mM dNTPs, 12.5 pmol of 5' and 3' oligonucleotide primers, 100 ng plasmid DNA template and ddH_2O_2.
For amplification in a Thermal Cycler (Biometra) the following standard program (see Table 2.17) was employed and modified according to the melting temperatures of the primers used and length of the desired DNA product.

Table 2.17: Standard PCR program

Step		Condition (time/temperature)
Initial denaturation of template DNA	#1x	5 min/95°C
Denaturation	#5x	1 min/95°C
Annealing of oligonucleotide primers		1 min/ TM primer- ~10-15°C
Elongation		~1 min /1 kbp/ 72°C
Denaturation	#20-25x	1 min/95°C
Annealing of oligonucleotide primers		1 min/ TM primer - ~5-10°C
Elongation		~1 min /1 kbp/ 72°C
Final elongation step	#1x	4 min/72°C

2.3 Cell Biology Methods

2.3.1 Cell lines, cell culture growth conditions and media

2.3.1.1 Cell lines

HEK 293, SHSY-5Y (kind gifts of Drs. T. Stehle and P. Kahle, respectively), and HeLa cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, PAA, Germany), low glucose (PAA, Germany) supplemented with 10 % (v/v) FBS-Gold (PAA, Germany), 2 mM glutamine, and 5 mg/l gentamycin. The cells were kept at 37°C under 5 % CO_2 atmosphere.

The HeLa cell lines stably expressing DDP1 or the C66W variant in a tetracycline-inducible manner are based on a HeLa cell line that was generated using the FlpIn system (Invitrogen) (Florian and Mayer, 2011). The coding sequences of DDP1 or its C66W variant were cloned into a modified version of the pcDNA5/FRT vector. Each construct was co-transfected with pOG44 (for Flp recombinase expression) into the tet-inducible, FRT site-containing HeLa clone.
Positive clones were selected with hygromycin B and maintained in Dulbecco's Modified Eagle's Medium, low glucose (DMEM, PAA, Germany), supplemented with 10 % (v/v) tetracycline-negative FBS (PAA, Germany), 2 mM glutamine and 200 mg/500 ml hygromycin B. DDP1 or DDP1-C66W expression was induced immediately after seeding with 1 µg/ml tetracycline for the indicated period of time or 2 days, if not stated otherwise. Expression levels of the respective proteins were analyzed by immunodetection with a DDP1 specific antibody.

Cells were maintained in culture by growing them until ca. 90 % confluency and passaged in 1:8 to 1:3 dilutions (for HeLA and HEK293 cells) or in 1:3 dilutions for primary fibroblasts and SHSY-5Y cells.

2.3.1.2 Primary fibroblasts

For culturing of primary fibroblasts derived from MTS patients harboring the c. 116delT mutation (Tranebjaerg et al., 1995) and from a healthy donor (kind gift of Dr. M. Berneburg), DMEM high glucose (PAA, Germany) with stable glutamine was supplemented with 20 % (v/v) FCS-Gold and 5 mg/l gentamycin. The cells were cultivated at 37°C under a 5 % CO_2 atmosphere.
For growth analysis, cells were maintained in DMEM without glucose (PAA, Germany), and glutamine was supplemented with 15 % (v/v) FCS-Gold, 10 mM HEPES, 2.25 mM uridine, 2 mM sodium pyruvate, 5 mg/l gentamycin and 10 mM galactose, 200 mg/l glucose or 4.5 g/l glucose.

Oxygen consumption analysis was performed on cells grown in DMEM medium without glucose (PAA, Germany), supplemented with 10 % (v/v) FCS-Gold, 10 mM HEPES, 2.25 mM uridine, 2 mM sodium pyruvate, 5 mg/l gentamycin and either 10 mM galactose or 4.5 g/l glucose. Respiration analysis was performed with Dulbecco's Modified Eagle's Medium, containing either 10 mM galactose or 4.5 g/l glucose, supplemented with 1 g/100 ml sodium pyruvate and 1 g/100 ml glutamine during measurement recordings.

Antibiotics for cell culture were purchased from PAA, Invivogen, Invitrogen and Sigma Aldrich, whereas additives and supplements were from Sigma-Aldrich.

2.3.2 Transfection

Cells destined for transfection were cultivated in the respective growth medium without antibiotics and seeded one day prior to transfection.

Transfection was performed according to the HiPerFect transfection handbook guidelines for traditional transfection of adherent cells. RNAi-

mediated gene knockdown in HeLa cells was done with HiPerFect transfection reagent and siRNA oligos against *DDP1* or control oligos against *Luciferase Gl2*. Cell densities, transfection time points and final concentration of the oligonucteotides applied, were individually assessed for each experiment and are being discussed elsewhere.

Routinely, RNA interference studies were conducted as following: HeLa cells were transfected using HiPerFect transfection reagent with 5 nM siRNA oligonucleotides 1 (Hs_TIMM8A_1) and 2 (Hs_TIMM8A_6) for specific gene knockdown of *DDP1* or with control siRNA oligonucleotide directed against *Luciferase GL2*. Oligonucleotides for specific downregulation of *DDP1* were purchased from Qiagen as well as the control siRNA duplexes against *Luciferase GL2* (see Table 2.18).

Table 2.18: Target sequences of employed siRNA duplexes

Product name	target sequence
Luciferase GL2 siRNA	AAC GTA CGC GGA ATA CTT CGA
Hs_TIMM8A_5	ATG TGC CTA AAT AAG TTA TAA
Hs_TIMM8A_1	CAG GTA GAG GTG CAT GCC TAA
Hs_TIMM8A_3	TAG TGG GAC CAC ATA CGT AAA
Hs_TIMM8A_6	CAG AAG CTA ACA GCA GTT TCA

For transient transfection with plasmid DNA solutions of adherent cells using FuGENE6 (Roche), cells were plated one day prior to transfection to yield a 50-70 % confluency on transfection day. For co-transfection experiments, HeLa cells were transfected with a total of 120 ng DNA in a total volume of 250 µl growth medium for microscopy experiments or a total of 26 µg DNA in a total volume of 40 ml growth medium for BN-PAGE experiments. Transfection was performed according to the FuGENE6 transfection reagent manual.

Opti-MEM serum-reduced medium (Gibco) was used for plasmid DNA

transfection and in siRNA transfection procedures as a serum free culture medium without additives.

2.3.3 High resolution live cell imaging

For fluorescence microscopy, cells were incubated 1.5 days after transfection for 30 min with medium containing 40-100 nM MitoTracker Red CMXRos (Molecular Probes) which was subsequently replaced by CO_2-independent Live cell medium (Invitrogen). Cells were visualized on a DeltaVision microscope and deconvolved using softWoRx 4.0 (both Applied Precision) and image 3D segmentation and rendering was performed with Imaris software (Bitplane).

For morphological quantification cells were classified into three categories based on the appearance of the mitochondrial network structure: elongated structures presenting as extended cables, hollow-spheres or regular mitochondrial tubules. For live cell imaging we used either HeLa cells or HeLa cells stably expressing GFP-tagged histone H2B (kind gift of Dr. T. U. Mayer).

2.3.4 Bioenergetic analysis of cells with altered DDP1 levels

2.3.4.1 Membrane potential measurements by fluorescence-activated cell sorting (FACS)

Mitochondrial membrane potential ($\Delta\Psi$) of Hela cells after RNAi mediated gene knockdown (cells were transfected twice within 48 hours) was assessed via FACS analysis. Cells were harvested and resuspended followed by a subsequent incubation for 15-30 min at 37°C in 2 % FCS-PBS in the presence of 100 nM tetramethylrhodamine ethyl ester (TMRE). A cell population treated for 2-3 hours with 1 µM staurosporine (STS) in order to dissipate the membrane potential, served as a negative control. After incubation, cells were washed and resuspended in 1x PBS. Analysis

of TMRE-fluorescence was examined on a BD LSR II system equipped with FACSDiva software (Becton Dickinson).

2.3.4.2 Respiration analysis

For oxyometry studies HeLa cells were transfected twice with 25 nM siRNA and cultivated in Dulbecco's Modified Eagle's Medium. The medium, containing either 10 mM galactose or 4.5 g/l glucose, was supplemented during measurement recordings with 1 g/100 ml sodium pyruvate and 1 g/100 ml glutamine. Respiration of intact cells at 37°C was measured with a Clark type electrode oxygraph, in a water-jacketed chamber connected to a circulating water bath. Cells were trypsinized and resuspended in 1.1 ml respiration medium and 1 ml of the cell suspension was used for respiration recordings, whereas 100 µl were utilized for cell counting. The instrument was calibrated prior to recording and oxygen consumption was documented until respiration ratios dropped below the threshold.

2.3.4.3 Growth analysis of primary patient fibroblasts

For growth analysis, fibroblasts (matched as closely as possible for similar passage numbers and age) were cultivated for 12 days in Dulbecco's Modified Eagle's Medium containing 4.5 g/l glucose, 200 mg/l glucose or 10 mM galactose according to previously described procedures (Bonnet et al., 2007). Subsequently the number of viable cells was monitored by Trypan blue staining and cell counting.

2.4 Biochemical Methods

2.4.1 Preparation of protein extracts

Adherent cells were thoroughly washed once with 1x PBS, subsequently incubated with RIPA buffer at 4°C for 5 min under constant agitation and finally harvested with a cell scraper. Lysates were centrifuged for 15 min at

14000 x g to remove unsolubilized cell debris.

2.4.2 Determination of protein concentration

Protein concentrations were determined according to the method described by M. M. Bradford (Bradford, 1976). Accordingly, Bradford reagent (Roti-Quant, Roth) was used for determination of protein concentrations of the samples. Samples with 1 to 15 µg BSA content were used as internal standards. Quantification was performed by measuring OD_{595}, using the standard curve feature of an Eppendorf BioPhotometer.

2.4.3 SDS-PAGE and Western blotting

Sodium-dodecyl-sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and semi-dry western blotting were essentially carried out according to standard protocols. Buffers and reagents used are listed in Tables 2.5 and 2.7.
Proteins were transferred onto nitrocellulose membranes for 1 h at room temperature with a current of approximately 1.5 mA/cm^2.

2.4.4 Blue-native gel electrophoresis (BN-PAGE)

Blue-native gel electrophoresis was employed to investigate mitochondrial membrane proteins and complexes under native (non-denaturing) conditions (Schagger et al., 1994).

Isolated mitochondria (100 µg) were solubilized for 30 min on ice with lysis buffer (Table 2.10) supplemented with the indicated detergent. Subsequently samples were sedimented by a centrifugation step (17500 x g, 15 min, 2°C) and the supernatant was mixed with BN sample buffer (Table 2.10) and loaded, together with a molecular weight markers mix (NativeMark, Invitrogen), on a 5-16 % native gradient gel.
Electrophoresis and Western blotting was essentially carried out as

previously described (Kyhse-Andersen, 1984; Schagger et al., 1994; Towbin et al., 1979).

2.4.5 Isolation of mitochondria

2.4.5.1 Isolation of mitochondria from adherent cells

Mitochondria were isolated from mammalian cells according to published procedures (Johnston et al., 2002). Cells were grown to confluency, washed with PBS, and harvested. Cells were then pelleted (800 × g, 5 min, 2°C) and resuspended in HBB buffer supplemented with 2 mg/ml BSA (=HBA buffer) followed by homogenization with a needle and subsequent differential centrifugation steps (Johnston et al., 2002). Protein content of isolated mitochondria was determined according to procedures described by Clarke at al. (Clarke, 1976).

2.4.5.2 Isolation of mitochondria from murine liver

Mitochondria were isolated from mouse liver of C57BL/6N mice (kindly provided by Dr. R. Feil and M. Thunemann) by mincing the liver and subsequent homogenization with a potter. The crude suspension was subjected to differential centrifugation essentially as described previously (Frezza et al., 2007). Isolated mitochondria were resuspended in chilled HBB buffer and protein concentration was determined using the Bradford protein assay (Roti-Quant, Roth).

2.4.6 *In vitro* import of proteins into isolated mitochondria

Precursor proteins were synthesized by coupled transcription/translation in reticulocyte lysate (TNT Coupled Reticulocyte Lysate System, Promega) in the presence of [^{35}S]methionine using SP6 RNA polymerase. For *in vitro* import, isolated mitochondria (25-100 µg) were incubated with

radiolabeled precursor proteins at 25°C for different time periods in import buffer (Tables 2.8 and 2.9). Subsequently, samples were transferred on ice and treated with proteinase K (100 µg/ml) to remove non-imported material. The protease was inactivated by addition of 2 mM phenylmethylsulphonyl fluoride (PMSF) and then mitochondria were re-isolated and washed with import buffer.

To specifically rupture the mitochondrial outer membrane, isolated mitochondria were subjected to osmotic shock treatment for 30 min on ice with 10 mM HEPES, pH 7.5. Swollen mitochondria (mitoplasts) were reisolated and resuspended in fresh import buffer that was supplemented with 100 µg/ml creatine kinase and 5 mM creatine phosphate before the import reaction.

For alkaline extraction mitochondria or mitoplasts were isolated and suspended on ice for 30 min in 100 mM sodium carbonate, pH 11.5. The pellet resulting from a centrifugation step (100000 x g, 30 min, 2°C) was processed for SDS-PAGE.
Precipitation of proteins from aqueous supernatant was achieved by addition of 12 % trichloroacetic acid. The samples were vortexed and incubated for 20-30 min on ice followed by a centrifugation step (36000 x g, 20 min, 2°C). The resulting protein pellet was washed with chilled acetone and re-centrifuged (36000 x g, 10 min, 2°C). The protein pellet was dried at RT and finally dissolved in 2x sample buffer.

Isolated mitochondria were resuspended in SDS sample buffer and subjected to SDS-PAGE. Endogenous proteins and protein complexes were analyzed by Western blotting and detection via specific antibodies, whereas radiolabeled proteins were detected by autoradiography. Quantification of electrophoretic bands was done using AIDA Image Analyzer software (Raytest).

2.4.7 Purification of maltose binding protein (MBP)-fusion proteins

Purification of recombinant maltose binding protein (MBP, MW = 42 kDa) fused to DDP1 (MBP-DDP1) from *E. coli* was performed as following. Constructs encoding MBP-DDP1 and MBP alone were transformed into BL21-(DE3) cells and LB-medium for MBP-purification was inoculated with a single colony and grown at 37°C to an OD_{600nm} of 0.5. Subsequently the culture was supplemented with 0.3 mM IPTG for ~2 hours and cells were pelleted. Next, cells were resuspended in column buffer (Table 2.4), lysozyme (0.5 mg/ml) was added and the resulting solution was subjected to sonification followed by a clarifying centrifugation step. The supernatant was incubated with amylose resin for 2 hours at 4°C, the resin was washed twice and bound proteins were eluted with elution buffer (Table 2.4).

3 RESULTS

DDP1 is a soluble 11 kDa intermembrane space protein that forms and functions as a complex together with TIMM13 in a 1:1 stoichiometry (Rothbauer et al., 2001).

A specific antibody was required to detect the protein in Western blot analysis and to estimate the expression levels after up- and downregulation of the DDP1 variants as well as to investigate complex formation in BN-PAGE. To that goal we generated a polyclonal antibody in rabbits. To assess the specificity of the generated antibodies we tested various human cell lines for expression of DDP1. As a control, preimmune serum of the corresponding rabbit was tested for possible cross-reactivity bands.

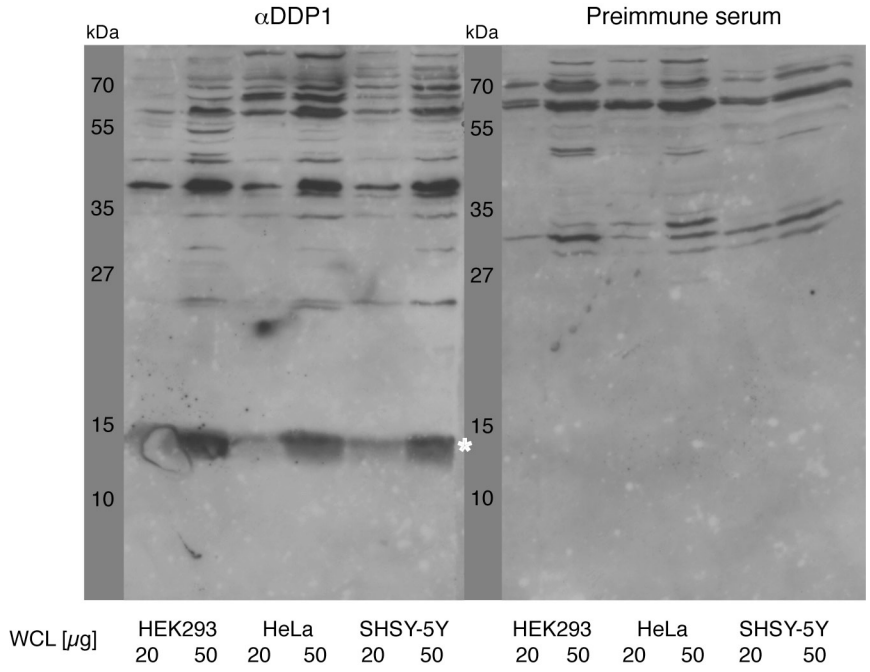


Figure 3.1: *Antibody specificity and expression pattern of DDP1 in different mammalian cell lines. Different amounts of whole cell lysates (WCL) derived from three different cell lines were tested for protein expression levels of DDP1 and specificity of the generated antibody. The DDP1 band is indicated with an asterisk.*

All cell lines showed a specific band that migrates with an apparent Mw of 14 kDa. when immunodecorated with the DDP1 specific, affinity purified antibody (Fig. 3.1). This band showed an increased intensity when higher amounts of protein were loaded. Of note, no cross-reactivity or unspecific band at the range of 10-27 kDa (where a band corresponding to DDP1 could be expected) was observed. Interestingly, all cell lines, despite their different tissue origin, ranging from cervix tissue to dopaminergic neuronal tissue, express DDP1 to a detectable level.

3.1 Generation of two novel cell lines inducibly overexpressing either DDP1 or its C66W variant

The complex of the small chaperones DDP1 and TIMM13 resides in the mitochondrial IMS. This complex plays a multifaceted and not yet completely understood role in the import of multispan inner membrane precursor proteins like the aspartate/glutamate carriers, and Tim23 (Paschen et al., 2000; Roesch et al., 2004; Rothbauer et al., 2001). Several reports suggested that the homologous complex in yeast mitochondria also facilitates the transfer of precursors of β-barrel proteins through the mitochondrial IMS (Habib et al., 2005; Hoppins and Nargang, 2004; Wiedemann et al., 2004).

To elucidate the potential function of DDP1 in mammalian cells we monitored the capacity of mitochondria isolated from cells overexpressing DDP1 to import *in vitro* various precursor proteins. To efficiently and reproducibly upregulate DDP1 levels as well as the mutant form C66W, two cell lines were generated based on a FlpIn system to ensure stable integration and uniform expression levels. Stable integration of different tet-inducible genes occurs at the same genomic locus and only once, thereby ensuring homogenous expression in all cells of a HeLa cell clone expressing DDP1 or C66W under a tetracycline inducible promoter. By employing this technique, comparisons of expression levels of the WT and

the mutant forms (MT) are valid due to expression regulation by the same promoter and insertion of both genes at the same gene locus. This enabled us to study the effects of upregulated DDP1 or DDP1-C66W levels in detail.

To monitor DDP1 expression, various cell clones were picked individually to guarantee a homogenous cell population deriving from a single cell clone and were passaged individually until the first induction tests with tetracycline. All cell clones showed a similar DDP1 expression pattern after the same induction period and did not exhibit clone specific variances (data not shown). To determine the optimal induction time with tetracycline, cell lines expressing either native DDP1 or its C66W variant were induced for 48 and 72 hours (Figure 3.2, A).

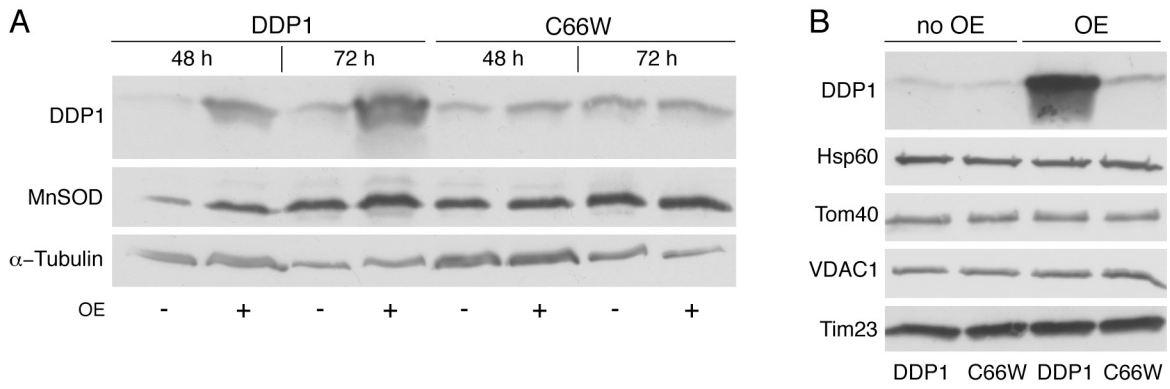


Figure 3.2: *DDP1-C66W, the mutant form of DDP1, cannot be expressed to a similar extent as the native protein. Whole cell lysate (100 µg) of cells induced for 48 and 72 hours to overexpress (OE, +) DDP1 or its C66W variant were analyzed by SDS-PAGE and immunodecoration with the indicated antibodies. Non-induced cells (OE, -) served as a control. (B) Mitochondria (20 µg) isolated from the aforementioned cell lines were analyzed by SDS-PAGE and immunodecoration with antibodies directed against the indicated mitochondrial proteins. VDAC1 and Tom40, outer membrane proteins; DDP1, soluble IMS protein; Tim23, inner membrane protein; Hsp60, soluble matrix protein.*

The DDP1 overexpressing cell lines showed elevated amounts of DDP1 already after 48 hours of induction whereas markedly lower DDP1 levels were observed in the cells expressing the mutant protein. The same effect could be observed after 72 hours. Mitochondria derived from control and induced cells were additionally analyzed for variances in various mitochondrial protein steady-state levels. The results of Figure 3.2, B demonstrate that protein levels in control mitochondria were similar to those in mitochondria from induced cells.

We next wanted to verify that these findings also hold true for cells transiently transfected with the respective plasmids and thus exclude the possibility that this phenomenon results from possible artifacts of the generated cell lines. To that end we transfected HeLa cells with pCS2-DDP1, pCS2-C66W or N-terminally Myc-tagged versions of DDP1 and C66W (Figure 3.3).

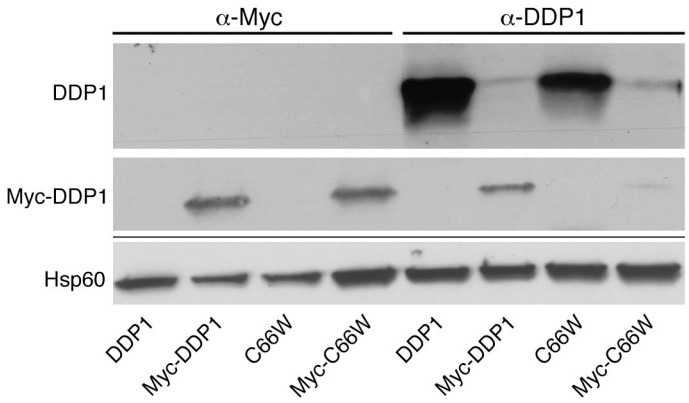


Figure 3.3: *The mutant form of DDP1 cannot be expressed to such an extent as the wild type form. Hela cells were transiently transfected with plasmids encoding for native DDP1 or its C66W mutant form, in either case with or without a N-terminal Myc-tag. Mitochondria (50 µg) isolated from these cells were subjected to SDS-PAGE and subsequent immunodecoration with the indicated antibodies directed against DDP1, the Myc-epitope or Hsp60.*

Untagged and tagged versions of C66W could not be expressed to a similar extent as compared to the respective native protein. In both cases less myc-C66W and C66W was detectable (compare lane two to lane four after correcting for loading).

To control for proper localization of the DDP1 molecules under these overexpression conditions, mitochondria were isolated from induced and control cells and subjected to proteinase K treatment to remove non-imported protein molecules that should be exposed on the mitochondrial surface. Additionally, mitochondria were incubated in hypotonic swelling buffer for 30 min to selectively open the outer mitochondrial membrane to generate mitoplasts and thereby releasing components of the intermembrane space. After these treatments intact and swollen mitochondria were reisolated by a centrifugation step and analyzed by SDS-PAGE and immunodecoration (Figure 3.4).

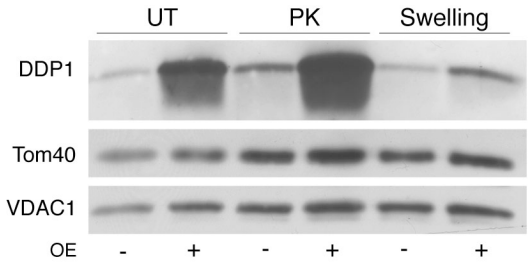


Figure 3.4: *Overexpression of DDP1 does not lead to submitochondrial mislocaliazation. Mitochondria derived from cells with elevated amounts of DDP1 and from control cells were left untreated (UT, 25 µg), subjected to proteinase K treatment (PK, 50 µg), or incubated in hypotonic buffer for osmotic swelling (50 µg). The outer membrane proteins Tom40 and VDAC1 as well as DDP1 as a soluble intermembrane space protein were used as controls for validation of the treatments.*

Proteinase K treatment did not result in a change in signal intensity of DDP1, whereas a pronounced decrease was evident after release of intermembrane proteins by hypotonic swelling. Of note, control proteins Tom40 and VDAC1, both located in the outer membrane were not affected

by these procedures. Thus, it can be concluded that the elevated amounts of DDP1 are correctly localized in the mitochondrial IMS.

3.2 Mitochondria with elevated levels of DDP1 are compromised in their capacity to import β-barrel proteins *in vitro*

We next wanted to test whether elevated levels of DDP1 affect the capacity of mitochondria to import precursor proteins *in vitro*. For this purpose radiolabeled precursor proteins of the inner membrane protein hTim23, the inner membrane ADP-ATP carrier (AAC), the outer membrane β-barrel proteins hTom40 and VDAC1, and the matrix-destined protein pSu9-DHFR were utilized. The latter one was used to control for the general import capacity of the employed organelles.

The import assay was performed with mitochondria derived from either HeLa cells overexpressing DDP1 in an inducible manner or from control cells.
Our results suggest that overexpression of DDP1 does not affect the ability of mitochondria to import hTim23, AAC and the control protein pSu9-DHFR. In contrast, import levels of hTom40 and VDAC1 into the organelles from the DDP1 overexpressing cells were compromised (Figures 3.5, 3.6 and 3.7). Western blot analysis using antibodies against DDP1 confirmed the high expression levels of DDP1 in mitochondria derived from the overexpression cell line (Figure 3.5).
We also observed that elevated levels of DDP1 did not cause alterations in the steady state levels of the matrix proteins MnSOD and Hsp60, as well as the β-barrel proteins Tom40 and VDAC1 (Figure 3.5, A, lower panels).

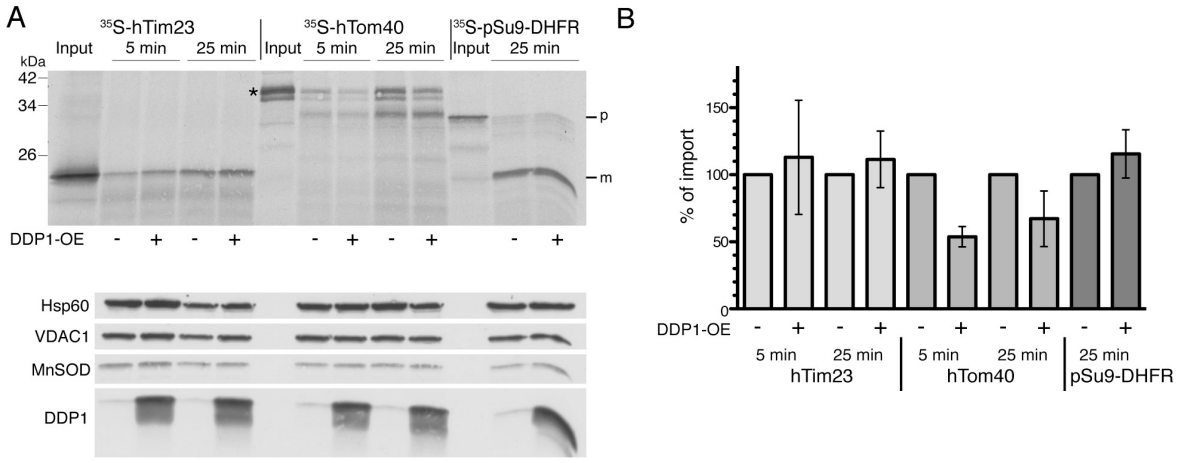


Figure 3.5. *Mitochondria isolated from DDP1 overexpressing HeLa cells have a reduced capacity to import hTom40. (A) Mitochondria isolated from HeLa cells overexpressing DDP1 were incubated at 25°C for the indicated time periods with radiolabeled precursors of hTim23, hTom40 or pSu9-DHFR. The import reactions were stopped by addition of proteinase K to digest non-imported molecules and mitochondria were subsequently reisolated and analyzed by SDS-PAGE followed by autoradiography (upper panel) and then immunodecoration (lower panels). Immunodecoration was performed with antibodies against the indicated mitochondrial proteins. The precursor and mature forms of pSu9-DHFR are indicated as p and m, respectively. (B) The bands representing the PK-protected hTom40 (indicated with an asterisk) and hTim23 as well as the mature form of pSu9-DHFR were quantified. The intensity of the bands corresponding to material imported into control mitochondria was set as 100 %. The average values for three independent experiments are presented together with error bars that represent standard deviations.*

Notably, the import of AAC, which was found in yeast cells to be a substrate of the Tim9-Tim10 complex but not of the Tim8-Tim13 complex (Leuenberger et al., 1999) was also not affected (Figure 3.7).

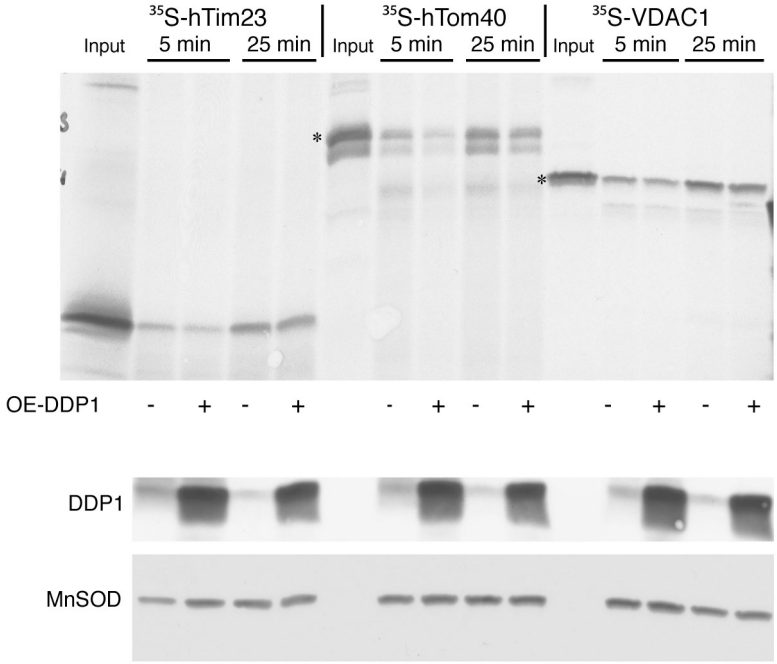


Figure 3.6 *Reduced import efficiencies of two tested β-barrels in mitochondria derived from DDP1 overexpressing cells. Isolated mitochondria from an inducible DDP1-overexpressing cell line were incubated with radiolabeled precursor proteins of hTom40, VDAC1 and hTim23 for 25 min at 25°C. Further treatment and analysis were as described in the legend to Figure 3.5. Asterisks indicate the PK protected precursor forms.*

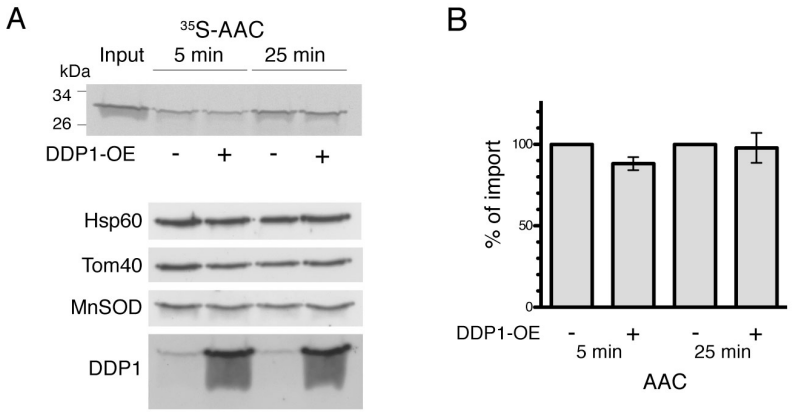


Figure 3.7 *Import of AAC into mitochondria isolated from DDP1 overexpressing and control cells does not exhibit differences in import efficiencies of AAC. Experimental procedure was performed as stated in the legend to Figure.3.5.*

The DDON-related C66W replacement in DDP1 results in a full-length protein that is efficiently imported *in vitro* into mitochondria but degraded more rapidly than the native counterpart (Hofmann et al., 2002). In order to understand whether this variant could possibly interfere with the biogenesis of mitochondrial precursor proteins, we checked the import properties of mitochondria isolated from cells inducibly overexpressing this mutant form.

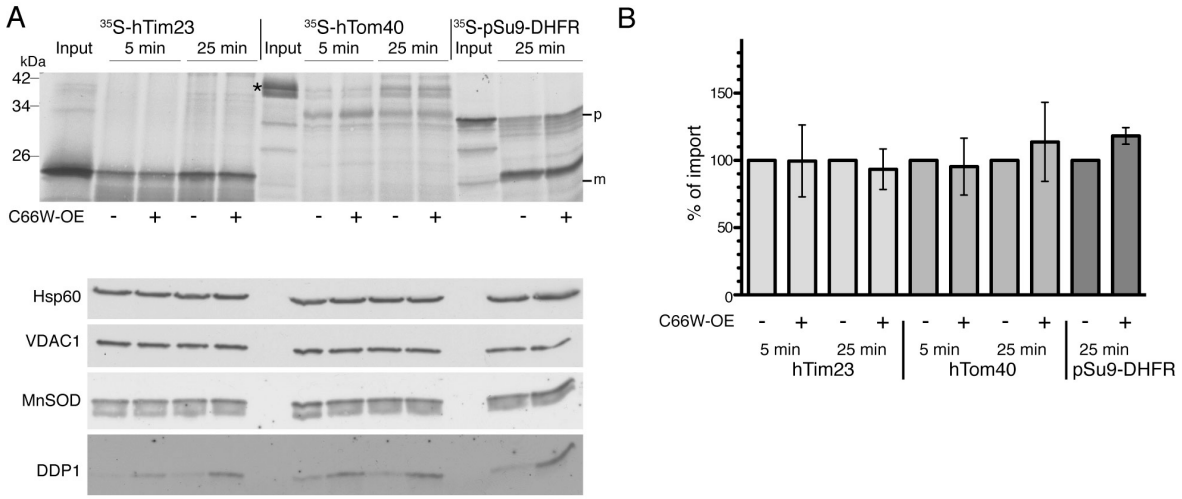


Figure 3.8 *Mitochondria isolated from cells overexpressing the DDP1-C66W variant exhibit a normal import capacity. (A) Mitochondria isolated from HeLa cells overexpressing DDP1-C66W were incubated with radiolabeled proteins and analyzed as described in the legend to Fig. 3.5, A. (B) The bands representing the imported protein molecules were quantified as described in the legend to Fig. 3.5, B. The average values for three independent experiments are presented together with error bars that represent standard deviations.*

Import efficiencies of all tested precursor proteins into organelles isolated from cells overexpressing the mutant form of DDP1 were similar to those into control mitochondria (Figure 3.8, A and B). Similarly, the steady-state levels of the monitored mitochondrial proteins were unaffected by the overexpression of the C66W mutant (Figure 3.8, A lower panels). Of

note, detected levels of the C66W variant were remarkably lower than those of the native DDP1 (compare Figs. 3.7, A; 3.8, A; 3.2 and 3.3).

These observations are in agreement with the previous reports regarding the reduced stability of the mutant protein (Hofmann et al., 2002). We conclude that overexpression of DDP1, but not of its C66W mutant, specifically reduces the capacity of mitochondria to import *in vitro* precursor molecules of β-barrel proteins.

The small Tim proteins, Tim8 and Tim13, form a hetero-oligomeric 70 kDa complex in the IMS. Thus we asked whether the observed reduced β-barrel import upon overexpression of DDP1 is due to an assembly defect of this complex. To address this question, mitochondria overexpressing either the native form of DDP1 or the mutant form were solubilized in buffer containing the detergents digitonin or Triton X-100 and subjected to blue native-PAGE and subsequent Western blot analysis (Figure 3.9).

DDP1 overexpression resulted in a massive formation of a complex with an apparent molecular mass of ~70 kDa that migrates similarly to the complex observed in the wild type organelle. However, in mitochondria overexpressing DDP1 we also observed a band with lower apparent MW that might represent unassembled molecules. In contrast, overexpression of the C66W variant did not lead to accumulation of unassembled molecules and significantly less of the ~70 kDa complex was observed (Figure 3.9). Of note, the complex containing the C66W mutation of DDP1 migrated somewhat higher in the native gel system probably due to an altered conformation of the hetero-oligomeric complex.

A similar behavior of the complex was observed when mitochondria were lysed with another detergent, Triton X-100 (Figure 3.9). The levels of the translocase of the mitochondrial outer membrane (TOM complex) were not altered in the various samples and served as a loading control (Figure 3.9).

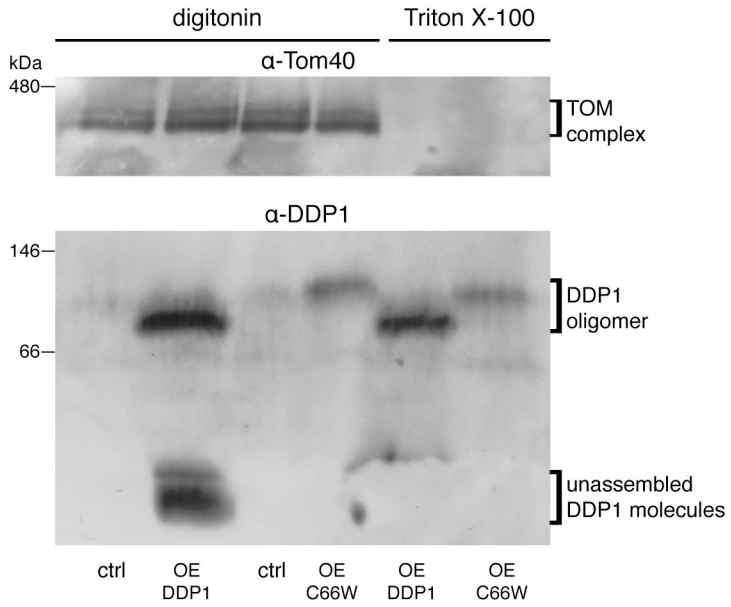


Figure 3.9 *Complex assembly of DDP1 and its C66W variant. Mitochondria (50 µg) were solubilized for 30 min on ice in buffer containing 1.6 % of either digitonin or Triton X-100. Samples were subjected to BN-PAGE and immunodecoration with antibodies against hTom40 and DDP1. The assembled TOM complex as well as the unassembled and oligomeric forms of DDP1 are indicated. Notably, the TOM complex is not stable in the presence of Triton X-100.*

Taken together, these results suggest that unassembled DDP1 molecules might interfere with the import of β-barrel proteins and further demonstrate that the mutant form is still able to assemble into oligomeric structures.

Next we asked if upregulation of DDP1 could in turn also lead to an increase in the expression levels of its partner protein TIMM13. Unfortunately, in the absence of a functional TIMM13 antibody we could not address this question by Western blotting and this absence also prevented us from testing whether the increased levels of the 70 kDa complex represent homo-oligomers of DDP1 or additional hetero-hexamers harboring both DDP1 and TIMM13. However, we did perform qRT-PCR on these cells and observed similar mRNA levels of TIMM13 in DDP1 overexpressing cells compared to those in control cells (data not shown).

3.3 Reduced levels of DDP1 do not affect biogenesis of hTim23 and hTom40

The small Tim proteins residing in the IMS facilitate in yeast the import of β-barrels and multispan inner membrane proteins (Davis et al., 2000; Habib et al., 2005; Hoppins and Nargang, 2004; Leuenberger et al., 1999; Paschen et al., 2000; Roesch et al., 2004; Rothbauer et al., 2001; Wiedemann et al., 2004).

To imitate a condition comparable to DDON in patients we aimed to downregulate DDP1 levels and then to evaluate the possible consequences in terms of import capacity, morphology and bioenergetic properties. To that end, four different oligonucleotides, all targeted against untranslated regions of *DDP1*, were employed to specifically reduce DDP1 levels in HeLa cells. As a negative control siRNA directed against *Luciferase Gl2* was utilized. HeLa cells, transfected with 5 nM siRNA, were harvested and checked for their knockdown efficiency. In all four cases a reduction of DDP1 expression levels was apparent in comparison to the *Luciferase Gl2* control cells and untreated cells. A marked reduction in the case of oligo 1 and oligo 6 (termed from this point on siRNA1 and siRNA2, respectively) was evident, thus leading to the decision to use these two most potent oligonucleotides in follow-up experiments.

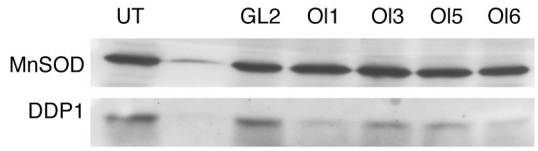


Figure 3.10: *Downregulation of DDP1 with 4 different siRNA dupexes showed a marked reduction in DDP1 protein levels. Whole cell lysates of HeLa cells treated with 5 nM siRNA oligonucleotides (Ol) directed against DDP1 or Luciferase (Gl2) and of untreated (UT) cells were analyzed by SDS-PAGE followed by immunodecoration with the indicated antibodies. MnSOD levels served as a loading control.*

To specifically assess the effects on import of the reported substrate Tim23 and β-barrel proteins we compared the import efficiency of mitochondria isolated from HeLa cells with downregulated DDP1 levels to that of organelles isolated from control cells. Isolated organelles were incubated with radiolabeled precursor proteins and analyzed by SDS-PAGE followed by autoradiography. Reduced DDP1 levels did not affect the amounts of radiolabeled precursor proteins of VDAC1 and hTim23 imported into mitochondria (Figure 3.11). A significant reduction of import efficiencies was observed in each case at an import temperature of 4°C which, as expected, prevented an efficient import. Of note, pSu9-DHFR, a fungal derived presequence of an ATPase subunit coupled to dihydrofolate reductase, could not be processed to the mature form in DDP1 downregulated mitochondria. Due to technical difficulties this experiment could only be performed once with this specific set-up.

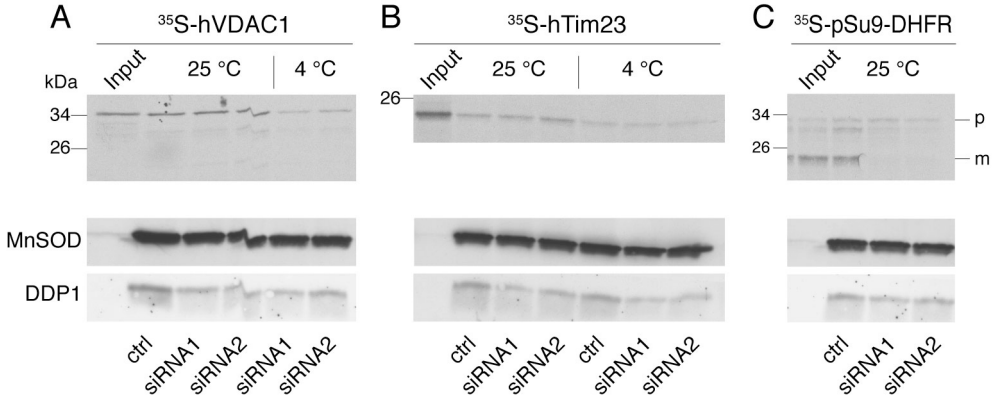


Figure 3.11 *Cells with reduced levels of DDP1 do not show a reduction in import efficiency. Cells transfected with two different siRNAs directed against DDP1 or with control siRNA were isolated and incubated with radiolabeled precursor proteins of VDAC1 (A), Tim23 (B) or the control protein pSu9-DHFR (C) for 25 min at the indicated temperature. After import, mitochondria were subjected to proteinase K treatment and reisolated. Autoradiography and immunodecoation was performed after SDS-PAGE analysis.*

To completely deplete the IMS of DDP1 and other potential import factors, we isolated mitochondria from mouse liver and subjected them to osmotic swelling. This treatment results in rupturing of the outer membrane and release of IMS elements like DDP1. Next, radiolabeled proteins were added to either intact or swollen mitochondria (mitoplasts) and import capacities of both types of organelles were monitored.

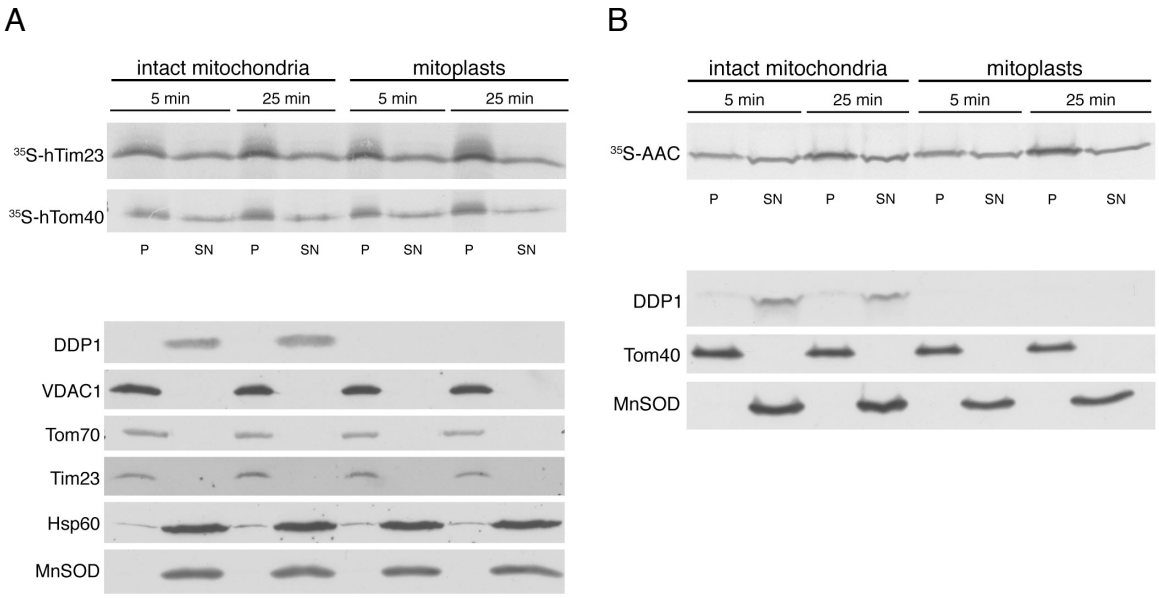


Figure 3.12. *Rupturing of the mitochondrial outer membrane does not compromise the import of hTim23, hTom40 and AAC. (A) Isolated mouse liver mitochondria were preincubated in either isotonic or hypotonic buffer resulting in intact organelles or mitochondria with a ruptured outer membrane (mitoplasts), respectively. Mitochondria and mitoplasts were then reisolated and incubated for 5 or 25 min at 25°C with radiolabeled precursors of hTim23 or hTom40 (A) or with radiolabeled precursor of AAC (B). Samples were subjected to carbonate extraction and the resulting pellet (P) and supernatant (SN) fractions were analyzed by SDS-PAGE followed by either autoradiography (upper panels) or immunodecoration with antibodies against mitochondrial proteins (lower panels). VDAC1, Tom70 and Tom40, outer membrane proteins; DDP1, soluble IMS protein; Tim23, inner membrane protein; Hsp60 and MnSOD, soluble matrix proteins.*

Intriguingly, osmotic swelling did not affect hTom40 or hTim23 import as analyzed by resistance to alkaline extraction of membrane-embedded proteins (Figure 3.12, A). Similarly, this treatment also did not compromise the import efficiency of the ADP-ATP carrier AAC (Figure 3.12, B). Effective swelling, the intactness of the mitochondrial inner membrane, and the fidelity of the alkaline treatment were confirmed by immunoblot analysis.

Membrane embedded proteins such as hTom70, VDAC1, Tom40 and hTim23 were detected in the pellet fractions of both intact and swollen mitochondria. As expected, the soluble matrix proteins Hsp60 and MnSOD were found in the supernatant fractions. Their presence in both mitochondria and mitoplasts indicates that the inner membrane was intact in these samples. The osmotic swelling effectively ruptured the outer mitochondrial membrane since the DDP1 signal was completely lost in the supernatant fraction of mitoplasts in contrast to intact mitochondria where it behaved like other soluble proteins (Figure 3.12, A and B).

Thus, it seems that DDP1 does not play a crucial role in the *in vitro* import of the investigated precursor proteins. Notably, very minor amounts of the yeast Tim9-Tim10 complex are usually associated with the mitochondrial inner membrane and are therefore not released upon osmotic swelling (Papic et al., 2011). A similar situation might also occur in mammalian mitochondria, where these residual amounts of Tim9-Tim10 molecules are able to sufficiently promote the *in vitro* import of small quantities of radiolabeled substrates.

Previous studies in *Neurospora crassa* and *Saccharomyces cerevisiae* demonstrate that the TIMM13-DDP1 complex is involved in the import pathway of β-barrel precursors (Habib et al., 2005; Hoppins and Nargang, 2004; Wiedemann et al., 2004).

We designed an experiment which would allow us to investigate a possible interaction between radioactively labeled precursor proteins of hTom40 as a model β-barrel precursor protein and recombinantly expressed and purified DDP1 (MBP-DDP1). Equivalent amounts of MBP-DDP1 and MBP (Figure 3.13, B) were preincubated with radiolabeled precursor proteins to facilitate an interaction prior to the import reaction.

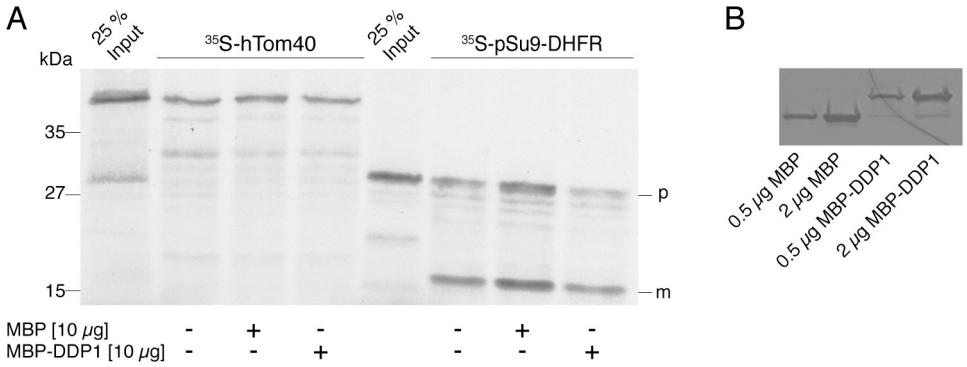


Figure 3.13: *Preincubation of radiolabeled hTom40 precursor protein with recombinant MBP-DDP1 does not alter import efficiency. Radioactively labeled precursor proteins of hTom40 and pSu9-DHFR were incubated with 10 µg of recombinant MBP or MBP-DDP1 prior to import into isolated mitochondria (15-20 µg) for 25 min at 25°C. After import mitochondria were subjected to proteinase K treatment to remove non-imported material and mitochondria were subsequently reisolated and analyzed by SDS-PAGE followed by autoradiography (A). The precursor and mature forms of pSu9-DHFR are indicated as p and m, respectively. (B) To control for equivalent amounts of the recombinant proteins MBP and MBP-DDP1, different amounts of the purified proteins were loaded onto a SDS-gel and analyzed by Coomassie staining.*

Autoradiography results showed almost identical import levels when either MBP-DDP1 or MBP served as a potential interaction partner whilst the control protein pSu9-DHFR displayed higher import levels when preincubated with MBP. Hence, this assay does not provide further evidence for the involvement of DDP1 in the import of β-barrel precursors.

To further analyze the role of DDP1 in the biogenesis of mitochondrial proteins we tested the steady state levels of mitochondrial proteins in cells where DDP1 was downregulated or completely absent. Whole cell lysates of HeLa cells specifically downregulated for DDP1 with the two most potent siRNAs were analyzed for abundance of the mitochondrial outer membrane proteins hTom40 and VDAC1, as well as for the inner membrane protein hTim23, a suggested putative substrate of the DDP1/TIMM13 complex. Although the levels of DDP1 were markedly decreased in these cells, we did not observe any reduction in the steady-state levels of the investigated proteins (Fig. 3.14, A).

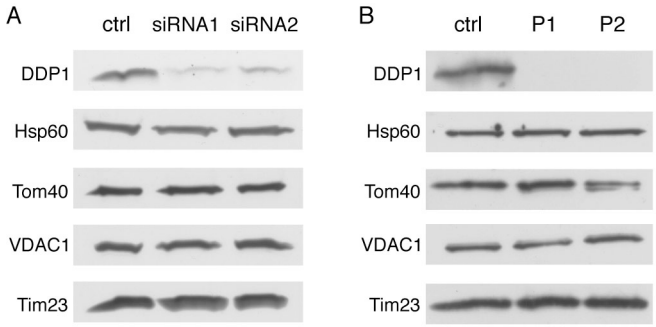


Figure 3.14. *Downregulation or absence of DDP1 does not affect the steady-state levels of mitochondrial proteins. (A) Whole cell lysates (50 µg) from HeLa cells transfected with oligonucleotides directed against either Luciferase Gl2 (as a control, ctrl) or TIMM8A (siRNA1 and 2) were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins. (B) Mitochondria (50 µg) isolated from primary fibroblasts of a control person (ctrl) or of two DDON patients diagnosed with the c.116delT mutation (P1 and P2) were analyzed by SDS-PAGE and immunodecoration with antibodies against the indicated mitochondrial proteins.*

These results are in accordance with our observation that mitochondria isolated from DDP1-depleted cells were not compromised in their capacity to import hTom40 and hTim23 *in vitro* (Fig. 3.12). A possibility that we cannot exclude is that the observed residual DDP1

molecules in the DDP1 downregulated cells are capable of supporting normal biogenesis of hTim23, VDAC1 and hTom40.

To address this point in an unrelated way, we isolated mitochondria from patients' fibroblasts, harboring the c.116delT (also known as Q38fsX64) mutation in the *TIMM8A* gene (Jin et al., 1996; Tranebjaerg et al., 1995). We observed the complete absence of DDP1 in these patient cells (Fig. 3.14, B). Nevertheless, levels of the mitochondrial marker proteins in healthy control fibroblasts were similar to those in fibroblasts derived from the DDON patients (Figure 3.14, B).

Considering these results, we conclude that in the tested cell types neither lack nor downregulation of DDP1 has a major effect on the expression levels of the tested proteins, hTom40, VDAC1 or hTim23.

3.4 Depletion of DDP1 does not affect the bioenergetic properties of mitochondria

Mutations in mitochondrial genes are often accompanied by impairment of primary mitochondrial functions like respiration and maintenance of membrane potential across the inner membrane. Due to the restricted availability of primary fibroblasts and their limited proliferation ability we could not monitor mitochondrial functions in these cells and used siRNA treated DDP1-downreguated HeLa cells.

Cells were incubated with the membrane potential sensitive dye tetramethylrhodamine ethyl ester (TMRE) and then subjected to fluorescence activated cell sorting (FACS). No decline in the TMRE signal in DDP1-downregulated cells in comparison to control cells was detected, indicating an uncompromised membrane potential in these cells (Fig. 3.15).

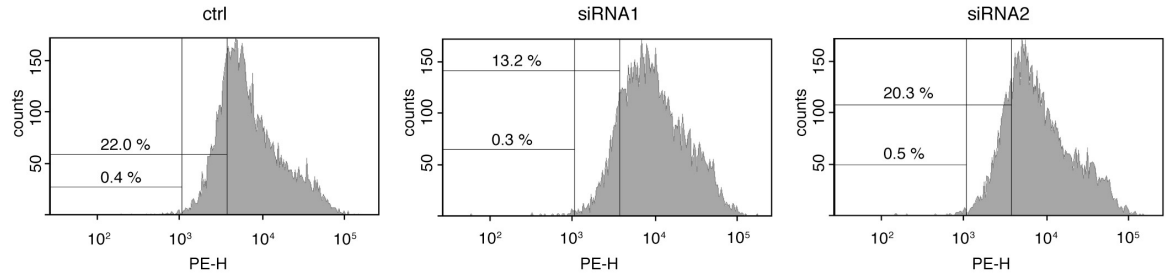


Figure 3.15 *Uncompromised membrane potential in DDP1 downregulated cells. HeLa cells described in the legend to Fig. 3.14 were harvested, incubated for 25 min with the membrane potential sensitive dye TMRE and then analyzed by FACS. Data are representative for at least three independent experiments.*

Next, respiration was monitored under normal growth conditions (4.5 g/l glucose medium) and in growth medium where glucose was substituted for galactose. This substitution can aggravate a potential respiratory dysfunction by forcing the cells to rely on oxidative phosphorylation for energy production

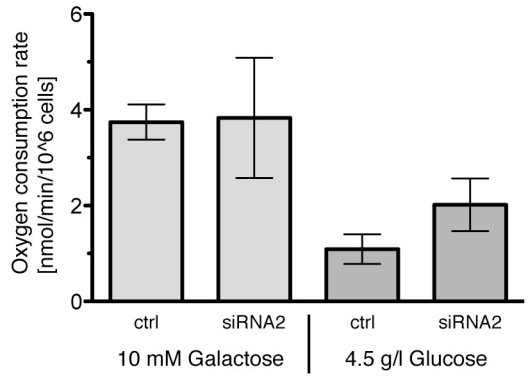


Figure 3.16 Similar *respiration levels in DDP1 downregulated cells and control cells. Oxygen consumption rates were assessed in HeLa cells transfected with oligonucleotides directed against either Luciferase Gl2 (ctrl) or TIMM8A (siRNA2). Cells were grown in a medium containing either 10 mM galactose or 4.5 g/l glucose and respiration was measured at 37°C with a Clark-type electrode oxygraph.*

As expected, cells grown in the presence of 10 mM galactose containing medium showed a higher oxygen consumption rate as compared to that of cells grown in glucose containing medium. Of note, statistically significant differences between DDP1 downregulated and control cells could not be seen in either case (Figure 3.16).
These findings are in agreement with previous studies where no major alterations in energy-generating systems were found in primary cells derived from DDON patients (Binder et al., 2003; Roesch et al., 2002).

Interference with the growth behavior could also be a consequence of a latent mitochondrial dysfunction. Primary fibroblasts from two DDON patients with the c.116delT mutation in the *TIMM8A* gene and fibroblasts from a healthy control were grown in medium with either low (200 mg/l) or high (4.5 g/l) glucose concentrations as well as in galactose (10 mM) containing medium. After 12 days, cells were harvested and viable cells were counted.

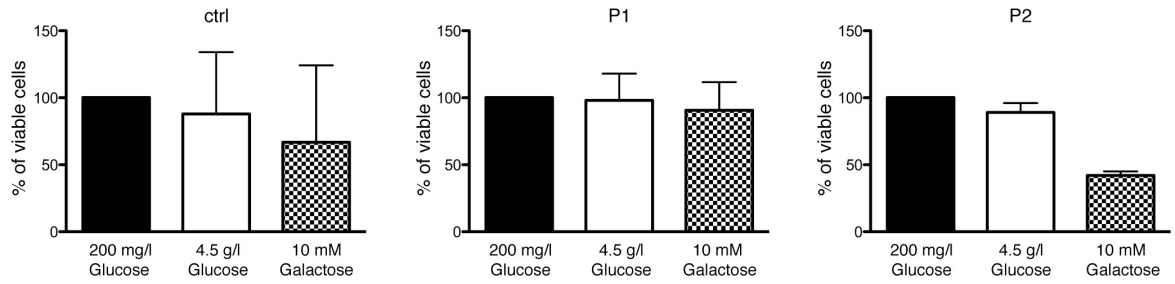


Figure 3.17 *No significant differences in the viability of patient and control cells. Fibroblasts as described in the legend to Fig. 3.14 were seeded and grown in media containing 200 mg/l glucose, 4.5 g/l glucose or 10 mM galactose. After 12 days cells were harvested and the number of viable cells was counted. The percentage of viable cells in medium containing 200 mg/l glucose was set as 100%. The average values for three independent experiments are presented together with error bars representing standard deviations.*

Fibroblasts from patient P1 and control fibroblasts from a healthy donor did not show marked differences in cell counts under either growth condition, whereas the cell number of fibroblasts from patient P2 grown in galactose-containing medium was reduced to half in comparison to the cell count in glucose containing media (Figure 3.17). Despite this difference it becomes apparent that the bioenergetic properties of cells with a reduced or absent DDP1 protein expression are not drastically altered.

3.5 Changes in the levels of DDP1 influence mitochondrial morphology

As a further step to understand the pathomechanism of DDON we investigated whether alteration in the levels of DDP1 can cause changes in mitochondrial morphology. To that goal we performed live cell microscopy of primary fibroblasts with the aforementioned mutation and of DDP1-downregulated HeLa cells.

To facilitate the visual assessment of the mitochondrial morphology, mitochondria were segmented and depicted in 3D using Imaris software. Cells harboring the mutation in the *TIMM8A* gene displayed an altered mitochondrial morphology in both DDON-patients' fibroblasts compared to control fibroblasts from a healthy donor. In particular, we noticed that the majority (~90 %) of the primary fibroblasts lacking DDP1 contained long continuous mitochondrial extensions projecting towards the cell periphery (Figure 3.18).

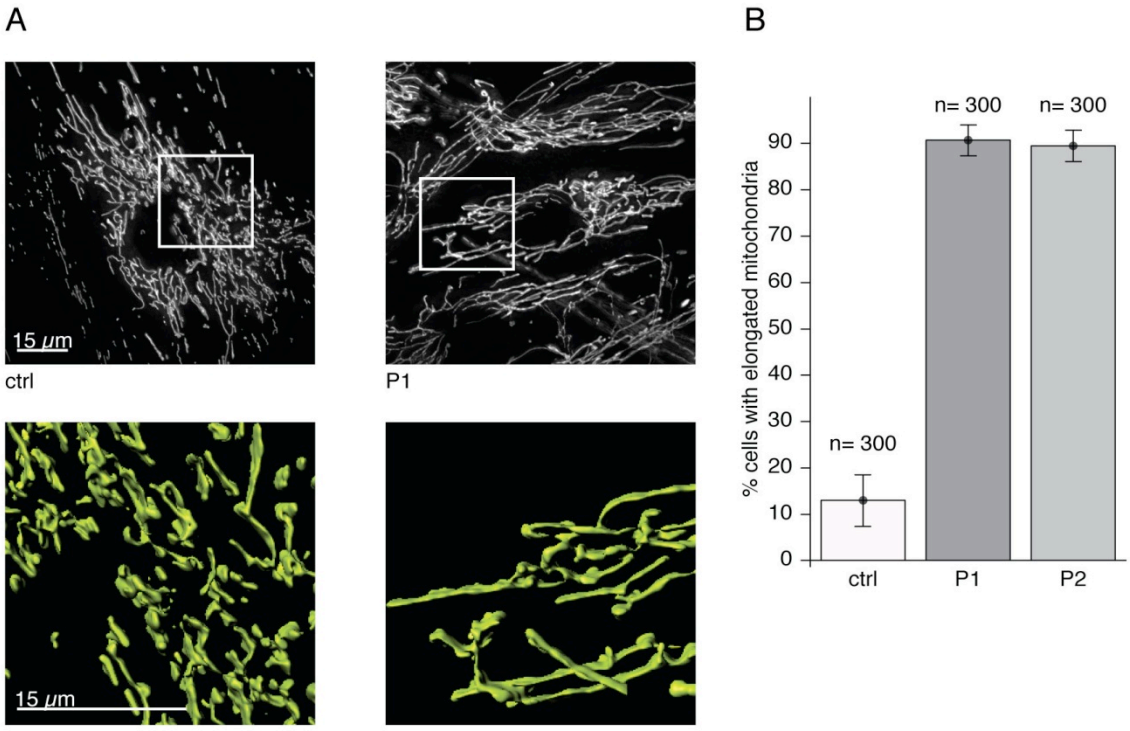


Figure 3.18 *Complete lack of DDP1 induces elongated, thread like mitochondria. (A) Primary fibroblasts as described in the legend to Figure 3.14 were incubated with MitoTracker Red for 30 min. Subsequently the medium was replaced by CO_2-independent Live cell medium and the cells were visualized by live cell microscopy. Images constitute deconvolved projections of z-stacks (upper panel). Scale bars represent 15 µm. The lower panel shows magnified insets. Mitochondria were segmented and rendered in 3D using Imaris software. (B) Cells analyzed as described in part A were categorized into normal or elongated phenotype and bar graphs showing the percentage of cells with elongated mitochondria are presented. Data represent the mean of three independent experiments with SEM.*

A similar picture could be observed in DDP1 downregulated cells, although not to the same extent as in the primary fibroblasts. Transfection with siRNA and subsequent live cell microscopy revealed extended tubular structures stretching toward the cell borders as the predominant pattern in 65-80 % of the analyzed cells (Figure 3.19).

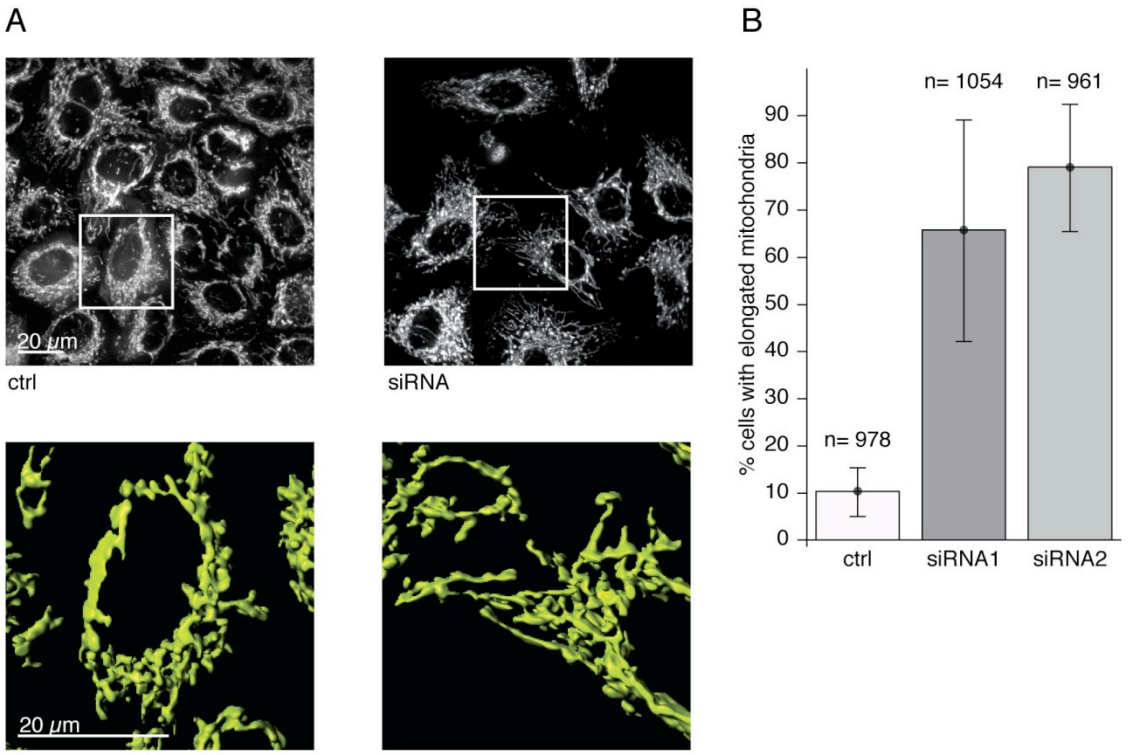
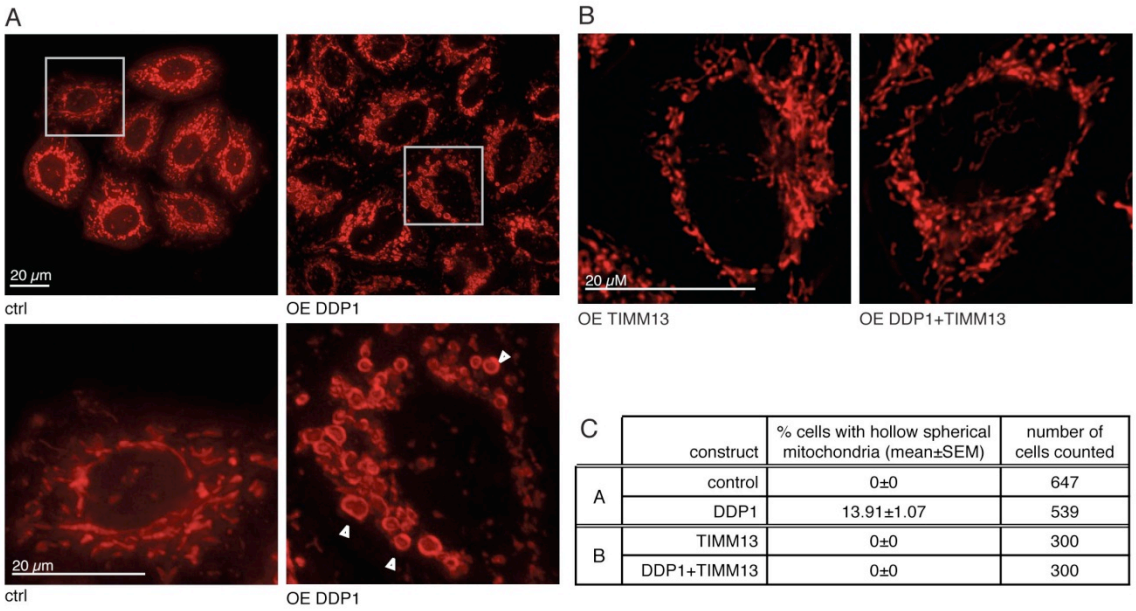


Figure 3.19 *Downregulation of DDP1 induces elongated, thread like mitochondria* (*A) HeLa cells were transfected with control siRNA or siRNA targeting TIMM8A and incubated for 36 h. Then mitochondrial morphology was assessed by live cell microscopy. Representative images for both conditions are shown. Insets display enlarged versions of segmented and 3D rendered mitochondria. Scale bars represent 20 µm. (B) Cells analyzed as described in part A were categorized into normal or elongated phenotype and bar graphs showing the percentage of cells with elongated mitochondria are presented. Data represent the mean of 3 independent experiments with SEM.*

Next, we asked if DDP1 overexpression affects mitochondrial morphology. To that end we transfected HeLa cells transiently with pCS2-DDP1 and the empty control vector and after ~36 h incubated these cells with the mitochondria specific dye MitoTracker Red CMXRos. Our images revealed that overexpression of DDP1 resulted in fragmentation of

mitochondria and appearance of a fraction of the organelle as hollow, spherical tubes (Figure 3.20, A).

This phenotype was observed in ca. 14 % (± 1 % SEM) of more than 500 analyzed cells, whereas no cells with the same phenotype were found in more than 600 control cells (Figure 3.20, C).



C

	construct	% cells with hollow spherical mitochondria (mean±SEM)	number of cells counted
A	control	0±0	647
	DDP1	13.91±1.07	539
B	TIMM13	0±0	300
	DDP1+TIMM13	0±0	300

Figure 3.20 *DDP1 overexpression induces hollow spherical mitochondria. (A) HeLa cells were transfected with a vector encoding DDP1 or with the empty vector. After 36 h, mitochondria were stained with MitoTracker Red and living cells were imaged. Deconvolved images are shown. Left upper panel: control transfected cells with regular mitochondrial morphology. Right upper panel: DDP1 overexpressing cells. The lower panels show magnified insets from the upper ones. Note the hollow spherical mitochondrial morphology in DDP1 overexpressing cells (white arrowheads). Scale bars represent 20 µm. (B) HeLa cells were transfected with a vector encoding TIMM13 (pCS2-TIMM13) alone (left) or cotransfected with pCS2-TIMM13 and pCS2-DDP1 (right). Subsequent procedures were performed as described in part A. (C) The transfected cells were analyzed by fluorescence microscopy as described in parts A and B. The percentage of cells that harbor hollow spherical mitochondria under the different transfection conditions is presented as the mean with SEM of three independent experiments.*

The hollow spheres, indicated with arrow heads (Figure 3.20, A), appeared not to be connected amongst each other but were single mitochondrial entities formed around the nucleus. This was further confirmed by segmentation of mitochondria from z-stacks and rendering of a 3D-model (movie not shown). Notably, similar morphological phenotypes were not observed when TIMM13 was overexpressed alone or in combination with DDP1 (Figure 3.20, B).

Hence, it seems that DDP1 does not behave identically to its partner protein, TIMM13. Importantly, overexpression of TIMM13 did not affect the expression levels of DDP1 (Figure 3.21).

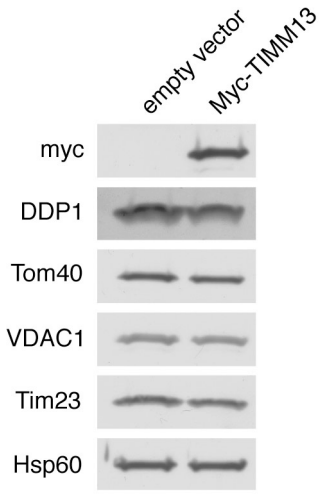


Figure 3.21: *Overexpression of Myc-tagged TIMM13 does not influence the steady state levels of the tested mitochondrial proteins. Mitochondria (50 µg) isolated from HeLa cells transfected with the plasmid coding for Myc-tagged TIMM13 or with the corresponding empty vector were analyzed by SDS-PAGE and immunodecoration. Antibodies directed against various mitochondrial proteins and the Myc-tag were employed to test for expression of Myc-TIMM13 and to assess the steady state levels of other mitochondrial proteins. VDAC1 and Tom40, outer membrane proteins; DDP1, soluble IMS protein; Tim23, inner membrane protein; Hsp60 soluble matrix protein.*

We further observed that when in addition to DDP1 also TIMM13 was overexpressed, the amounts of unassembled DDP1 were reduced concomitantly with the formation of high molecular mass complexes that contain both proteins (Figure 3.22).

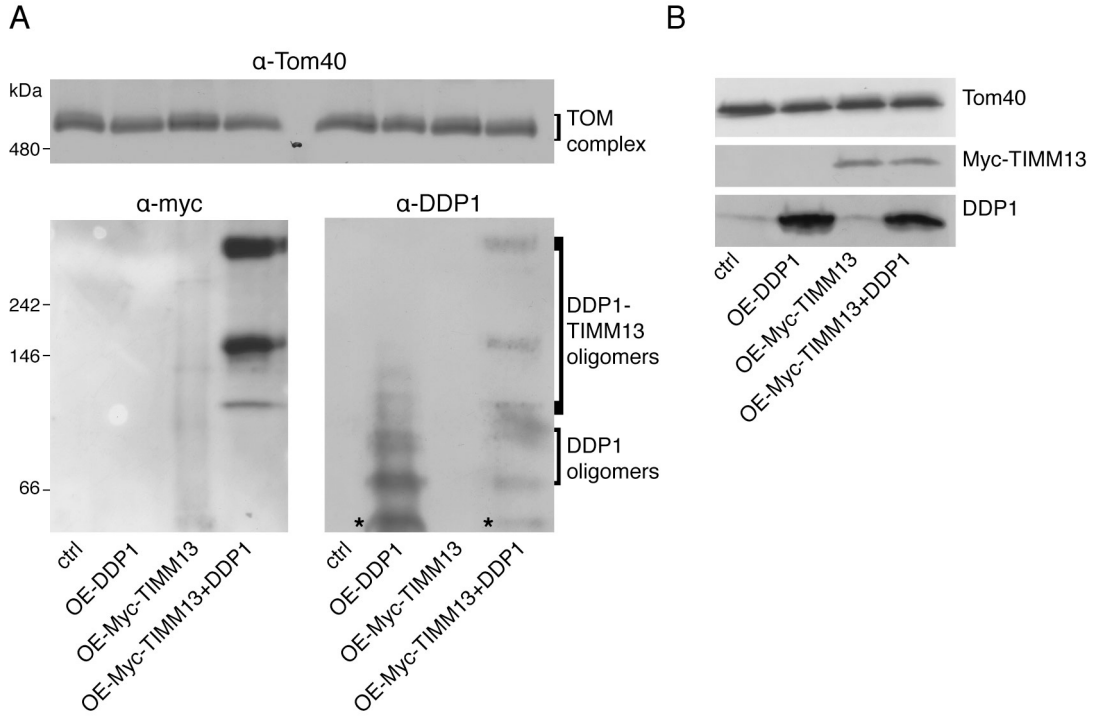


Figure 3.22 *Overexpression of both DDP1 and Myc-TIMM13 results in the formation of higher molecular weight hetero-oligomers. Mitochondria were isolated from control cells, cells overexpressing DDP1 alone, or co-overexpressing DDP1 and Myc-TIMM13. (A) Mitochondria (50 µg) were solubilized for 30 min on ice in buffer containing 1.6 % digitonin. Samples were subjected to BN-PAGE and immunodecoration with antibodies against DDP1, Myc or Tom40 (as a control). The assembled TOM complex as well as the various oligomeric forms of DDP1 are indicated. The unassembled form of DDP1 is indicated with an asterisk. (B) Mitochondria were subjected to SDS-PAGE and immunodecoration with antibodies against the indicated proteins.*

These results support the notion that the unassembled DDP1 molecules might cause the phenotypes of the DDP1 overexpressing cells. Taken

together, enhanced or reduced levels of DDP1 result in distinct and apparent mitochondrial morphology phenotypes.

Fluorescence activated cell sorting experiments and their data evaluation were carried out with the assistance of Dr. F. Essmann. Oxyometry respiration studies were performed under guidance of Dr. Y. Kamenisch. Live cell microscopy experiments and 3D-Imaris reconstructions were done in cooperation with Dr. S. Florian. Microscopy pictures for Figures 3.18, A and 3.20, B were taken by S. Florian. Experiments were designed and conducted by myself if not stated otherwise. Cells utilized for the various experiments were in each case cultivated and set up by myself according to the procedures described in the respective "Materials and Methods"-section.

4 DISCUSSION

4.1 Distinctive features of DDP1 and C66W overexpression

Deafness dystonia optic neuronopathy syndrome is a rare neurodegenerative disease with multiple clinical phenotypes. As complex as the clinical presentation is also the elucidation of the pathomechanism underlying this disease. A lot of research was primarily conducted in *Saccharomyces cerevisiae* or in primary cells derived from DDON patients. Due to restricted access and availability of primary patient tissue samples on the one hand and the possibility to transfer certain scientific questions to the mammalian system on the other, a mixed approach using primary cells and immortalized human cells was employed in this study.

A lack of commercially available antibodies to detect DDP1 necessitated us to generate a polyclonal antibody. Testing of whole cell lysates of HEK293, HeLa and SH-SY5Y cells showed a DDP1 specific band at the estimated protein size in all samples, indicating that cells originating from different tissues express DDP1 to a similar level.

After assuring the specificity of the generated antibody, further steps were taken to generate two novel cell lines. Based on a FlpIn system the generated cell line inducibly overexpressing the protein of interest allowed us to investigate, and even more importantly, to compare two different proteins. Since both genes are under the control of the same promoter and at a defined gene locus, present only once in a cell, we were able to assess the impact of overexpressing DDP1 and the mutant form C66W.

Various cell clones were tested from both the C66W and the DDP1 cell lines and in both cases similar expression levels were found amongst the different clones derived from the respective cell line. Expression of the mutant form and of the wild type form was tested under the same conditions and time course experiments showed elevated amounts of DDP1 with increasing time whereas the mutant form could only be slightly

upregulated with no increase even after 72h. Thus the wild type protein was upregulated to a high extent in comparison to the endogenous wild type levels of non-induced control cells in contrast to the mutant protein.

Previous studies with radioactively labeled precursors of DDP1 and DDP1-C66W in *in vitro* import experiments in yeast indicated that both forms were imported into isolated mitochondria with a similar efficiency but the imported mutant form was degraded faster than the wild type (Hofmann et al., 2002).
Considering that in our case whole cell lysates were compared, the possibility that the mutant protein is less stable and might be prone to degradation in the cytosol even before translocating to the IMS could explain the observed differences. Overexpression of either of the two proteins did not alter the steady state levels of mitochondrial proteins like Tom40 and Tim23 that were suggested previously as possible substrates of DDP1 (Davis et al., 2000; Hoppins and Nargang, 2004; Koehler et al., 1999; Paschen et al., 2000; Rothbauer et al., 2001; Wiedemann et al., 2004).

To exclude possible mislocalization due to the high expression levels, HeLa cells were also transiently transfected with tagged and untagged forms of DDP1 and C66W. Isolated mitochondria were analyzed and the results disclosed that in both cases additional DDP1 or C66W molecules were correctly located to mitochondria. Thus the overexpression and the Myc-tag did not influence the localization of the native or mutant protein.
In contrast N-terminally GFP-tagged DDP1 displayed a predominantly cytosolic distribution pattern in live cell microscopy experiments (data not shown). Probably the size of the GFP-tag of around 27 kDa in comparison to the 6xMyc-tag (ca. 8 kDa) prevented proper folding or translocation of the fusion protein.
A similar difference in the expression pattern of DDP1 and its C66W

variant was detected in the inducible cell lines as well as in the transiently transfected cells. These results reinforce the notion that the mutant form is less stable. Reports of DDON patient fibroblasts with a non-detectable C66W-TIMM13 complex (Roesch et al., 2002) would argue in favor of our hypothesis.
Upon confirming the proper mitochondrial localization of the overexpressed DDP1 molecules, it was essential to determine their submitochondrial localization. Mitochondria isolated from DDP1 overexpressing cells were treated with externally added protease or treated with hypotonic buffer to rupture their outer membrane. Both treatments confirmed that, as expected, DDP1 is located in the IMS.

Taken together, DDP1 molecules derived from the cell line overexpressing this protein locate to mitochondria and revealed the typical behavior of soluble IMS proteins.

4.2 Protein import studies

4.2.1 Protein import into mitochondria of DDP1 or C66W overexpressing cells

In the current study we investigated the function of DDP1 in human cells by applying *in vitro* protein import experiments as well as *ex-vivo* experiments using cells with altered DDP1 levels. Import experiments showed that upregulated amounts of DDP1, but not of the mutant form C66W, reduce the import efficiency of the β-barrel outer membrane protein hTom40 and VDAC1. Of note, such an effect was not observed in the import of the inner membrane protein hTim23. The precursor form of the latter protein was previously reported to interact with the Tim8/Tim13 complex (Davis et al., 2000; Leuenberger et al., 1999) and to rely on this complex for its efficient delivery to the inner membrane (Paschen et al., 2000; Rothbauer et al., 2001).

We propose that the compromised import of the β-barrel precursors is due to accumulation of unassembled DDP1 molecules in the IMS and the unproductive interactions of this monomeric form with either the precursor molecules or other components in the import pathway.

The mutant form was shown in previous studies to be degraded more rapidly than the wild type form (Hofmann et al., 2002). Accordingly, reduced amounts of DDP1-C66W were observed upon its overexpression and no monomeric form was detected in BN-PAGE experiments. In agreement with this, the amounts of the oligomeric form of DDP1-C66W were lower when the mutant form was upregulated as compared to the situation upon overexpression of native DDP1.
Considering the findings by Hofmann and colleagues (Hofmann et al., 2005) that this mutant form is not able to form homo-oligomers *in vitro*, our data would argue in favor of a hetero-oligomeric complex of DDP1-C66W with TIMM13. This complex formation would thereby be prone to a more rapid degradation and destabilization as also seen previously in fibroblasts derived from patients with the C66W mutation, where the complex could not be detected at all (Roesch et al., 2002).

Higher DDP1 levels did not in turn lead to an upregulation of the transcripts of the partner protein TIMM13. This observation might suggest the existence of a free pool of TIMM13 molecules in the IMS that can be recruited for complex formation upon DDP1 upregulation. Of note, upregulation of TIMM13 did not alter the steady state levels of DDP1, Tim23 or the β-barrel proteins Tom40 and VDAC1.

4.2.2 Mitochondrial protein import in cells downregulated for DDP1 or lacking DDP1

To create a situation which would allow us to test for the consequences of reduced DDP1 levels in mammalian cells we employed a siRNA approach. Four different oligonucleotides against untranslated regions of the *DDP1* gene were tested and demonstrated a decrease in all four cases, thereby strongly reducing the possibility of an unspecific secondary effect. Moreover the effects of the downregualtion with all four siRNAs were tested also in live cell microscopy experiments (data not shown) and revealed a direct correlation between the morphological phenotype of mitochondria and the knockdown efficiency of the respective oligonucleotide.
This would suggest that the protein level of DDP1 is connected to the manifestation of a mitochondrial network morphology, with cells downregulated for DDP1 displaying a phenotype of hyperelongated, thread like mitochondria. A stronger decrease in DDP1 would thereby increase the severity of mitochondrial elongation.

The significance of low DDP1 levels in terms of protein import was tested in *in vitro* experiments with cells downregulated for DDP1. Import efficiencies of the tested precursor proteins hVDAC1 and hTim23 were not affected by the reduced levels of DDP1 in the isolated mitochondria. Due to the fact that residual DDP1 molecules in the IMS were detectable, the possibility that these minor amounts were sufficient for proper insertion cannot be ruled out.

Unexpectedly, removal of soluble IMS proteins upon rupturing of the outer membrane did not show any effect on the import of hTom40, AAC or hTim23 into isolated mitochondria. This treatment results in the release of the DDP1-containing complex from the ruptured organelles but we cannot exclude the possibility that minor amounts of the TIMM9-TIMM10 complex, which are associated with the inner membrane, are still present.

Thus, it appears that although the DDP1/TIMM13 complex can interact with hTim23 precursor molecules in the IMS while they are relayed from the TOM complex to the inner membrane, such an interaction does not seem to be absolutely required for their efficient translocation and proper membrane insertion.
This is in contrast to the situation in fungi where rupture of the outer membrane or depletion of the Tim8/Tim13 complex results in a severely compromised capacity to import Tim23 or outer membrane β-barrel proteins (Habib et al., 2005; Smith et al., 1994; Wiedemann et al., 2004). This difference might indicate the existence of alternative import pathways in higher eukaryotes as compared to fungi.

Preincubation of recombinantly purified DDP1 and the radioactively labeled precursor protein hTom40 before the import reaction did not affect the import efficiency. Tom40 was imported to the same extent if preincubated with either MBP or MBP-DDP1. From this finding it can be deduced that the two proteins do not interact with each other under these experimental conditions. It is possible that the MBP-tag masked a potential interaction site or that the interaction itself is very rapid and unstable and therefore cannot to be tracked by this method.

Interestingly, neither downregulation nor absence of DDP1 resulted in changes in the steady state levels of proteins from various intra-mitochondrial compartments. Complete lack of DDP1 in the patient fibroblasts harboring the c.116delT mutation revealed a similar picture as in the downregulated cells. Thus, either altered protein levels are unlikely to contribute to the basic pathomechanism of the disease or the analyzed cells do not reflect the homeostasis of important proteins in the affected neuronal cells. Alternatively, the absence of DDP1 may compromise the steady-state levels of a yet to be identified specific substrate that fulfils an important role in mitochondrial function.

4.3 Bioenergetic properties of cells with reduced levels of DDP1

Mitochondrial diseases are often accompanied by changes in the organelles´ bioenergetic properties such as respiration rates and maintenance of membrane potential. Nevertheless, reduced DDP1 levels in HeLa cells neither resulted in alterations of the mitochondrial membrane potential in the examined cells nor compromised oxygen consumption rates even under aggravating metabolic stress conditions.
These results argue against an energetic defect as a major cause for the disease and are also in accordance with findings in primary cells from patients with other mutations in the *TIMM8A* gene (Binder et al., 2003; Roesch et al., 2002).

Our proliferation studies of primary fibroblasts with the c.116delT mutation showed no differences in the growth behavior of the control fibroblasts and P1 fibroblasts, although a reduced number of cells were counted in the case of P2 fibroblasts in galactose supplemented growth medium. Considering the large variability of clinical symptoms amongst family members with the same genetic mutation, epigenetic factors might be partly responsible for this effect.
Comprehensive studies of a large patient cohort would be essential to investigate possible epigenetic factors in detail. In summary, inadequate energy production does not provide a universal explanation for the clinical picture of DDON.

4.4 Morphological impact of DDP1 protein level alterations

Mitochondrial morphology and the factors regulating the organelles fusion and fission processes are a matter of intense research (Campello and Scorrano, 2010; Chen and Chan, 2010; Hoppins et al., 2007; Otera and

Mihara, 2011; Westermann, 2010; Youle and Narendra, 2011). It is appreciated that the cell's varying requirements result in adaptation in shape by fusion and fission events of the mitochondrial network.
Therefore we examined the morphological implications of reduced or elevated DDP1 levels. Extensive analysis and assessment was performed on more than 900 cells of each siRNA treated cell population and indistinguishable morphological changes were found upon DDP1 depletion with both short interfering oligonucleotides targeting DDP1 mRNA.

Elongated, thread like mitochondria were apparent in the majority of these cells and primary fibroblasts derived from DDON patients with the c.116delT mutation displayed a similar phenotype.

Overexpression of DDP1 on the other hand resulted in hollow spherical mitochondria of varying diameter that were not interconnected. This specific grain like structure was previously found in *C. elegans,* where overexpressing DRP-1 cells exhibited severe mitochondrial fragmentation (Labrousse et al., 1999).
Interestingly, overexpression of TIMM13 revealed an unaltered morphological phenotype and also seemed to rescue the phenotype when co-overexpressed with DDP1. This might suggest that unassembled DDP1 molecules are responsible for the phenotypic modifications and also findings of the respective BN-PAGE experiments hint at a similar explanation. Co-overexpression of TIMM13 and DDP1 resulted in a shift towards formation of higher molecular complexes and less unassembled DDP1 molecules.

Currently, the link between alterations in the DDP1 expression levels and the observed morphological changes is not clear. It was reported that in apoptotic cells DDP1 is released into the cytoplasm where it binds Drp1, a mediator of mitochondrial fission, and promotes its mitochondrial redistribution (Arnoult et al., 2005). Despite this report, we favor a scenario where the levels of DDP1 could indirectly affect the import of

additional, yet not identified, regulatory substrates, which in turn promote morphological changes in the mitochondrial network.

Although the morphology of the organelle is tightly connected to mitochondrial regulation pathways, these mitochondrial changes might not result in a direct metabolic deficit in HeLa cells and fibroblasts, but could possibly interfere with mitochondrial transport and/or function in neurons. Since the central nervous system has a high energy demand and is strongly dependent on mitochondrial ATP production (Silver and Erecinska, 1998) alterations would severely affect the cellular metabolism and in the long run lead to neurodegeneration. Fusion events are essential for exchange of contents between dysfunctional and functional mitochondria thereby also ensuring mtDNA integrity and respiration (Chen and Chan, 2010).

It is very tempting to speculate, considering the morphological changes we found in DDP1 depleted HeLa cells and primary fibroblasts of DDON patients, that this elongated tubules could reflect an intense effort to exchange mitochondrial contents to sustain the basic metabolic functions. Indeed, recent reports suggest that elongated mitochondria are less prone to removal by mitophagy (Gomes et al., 2011; Rambold et al., 2011). Although our study provides initial hints regarding the complex pathomechanism of DDON, extensive further studies employing primarily affected cells and/or a mouse model are required for obtaining a comprehensive understanding of this process and to gain a better knowledge about the correlation of mitochondrial morphology and function in different cell types.

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6 LIST OF ABBREVATIONS

$\Delta\psi$	membrane potential
AAA	ATPases associated with a variety of cellular activities
AAC	ADP/ATP carrier
ADOA	autosomal dominant optic atrophy
ADP	adenosine diphosphate
ALS	Amyotrophic lateral sclerosis
Amp	ampicillin
APP	amyloid precursor protein
APS	ammonium peroxo-disulfate
ATP	adenosine triphosphate
BN-PAGE	blue native polyacrylamide gel electrophoresis
bp	base pairs
BSA	bovine serum albumin
BTK	Bruton agammaglobulinemia tyrosine kinase
C-	carboxyl-
CCCP	carbonyl cyanide m-chlorophenylhydrazone
cDNA	complementary DNA
CMT2A	Charcot-Marie-Tooth type 2A
CNS	central nervous system
CPEO	chronic progressive external ophthalmoplegia
Cys	Cysteine
DDON	Deafness Dystonia Optic Neuronopathy Syndrome
DDP	Deafness Dystonia Protein 1
DFN	deafness
DHFR	dihydrofolate reductase
DJ1	parkinson protein 7
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
Dnm1	dynamin 1
dNTP	deoxyribonucleoside triphosphate
Drp	dynamin related protein
DTT	dithiotreitol
E. coli	*Escherichia coli*
ECL	enhanced chemiluminescence
EDTA	ethylendiamine tetraacetate
Erv	essential for respiration and viability
FA	Friedreich ataxia
FCS	fetal calf serum
g	standard gravity
G_1	Gap 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GED	GTPase effector domain
GFP	green fluorescent protein
GIP	general import pore
GTP	guanosine triphosphate

HEPES	N-2 hydroxyl piperazine-N´-2-ethane sulphonic acid
Hsp	heat shock protein
HSP	hereditary spastic paraplegia
Hyg	Hygromycin
IM	inner membrane
IMM	inner mitochondrial membrane
IMS	intermembrane space
kbp	kilobase pairs
kDa	kilodalton
LB	Luria Bertani
LRKK2	leucine-rich repeat kinase2
Mas	mitochondrial assembly
MBP	maltose binding protein
MELAS	mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes
MFN	Mitofusin
MIA	Mitochondrial Intermembrane space Assembly
MIDD	maternally inherited diabetes and deafness
MIM	mitochondrial inner membrane
Mim1	mitochondrial import 1
MnSOD	Manganese superoxide dismutase
MOM	mitochondrial outer membrane
MOPS	N-morpholinopropane sulphonic acid
MPP	mitochondrial processing peptidase
mRNA	messenger RNA
mt	mitochondrial
MT	mutant form
mtDNA	mitochondrial DNA
mtHsp	mitochondrial heat shock protein
MTS	Mohr-Tranebjaerg Syndrome
MW	molecular weight
N-	amino-
N. crassa	*Neurospora crassa*
NADH	nicotine amide adenine dinucleotide
NaOH	sodium hydroxide
NARP	Neuropathy ataxia and retinitis pigmentosa
nDNA	nuclear encoded DNA
NP-40	Nonidet P-40
ODx	optical density at x nm
Ol	oligonucleotide
OM	outer membrane
Oma1	Overlapping activity with M-AAA protease (OMA1) homolog, zinc metallopeptidase
OMI/HTRA2	HtrA serine peptidase 2
OMM	outer mitochondrial membrane
OPA1	optic atrophy 1

OXPHOS	oxidative phosphorylation
PAGE	polyacrylamide gel electrophoresis
Pam	presequence translocase-associated motor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	Parkinson's disease
PEG	polyethylene glycol
Pfu	*Pyrococcus furiosus*
PHB	prohibitin
PINK1	PTEN induced putative kinase 1
PIS	preimmune serum
PK	proteinase K
PMSF	phenylmethylsulfonyfluoride
PVDF	polyvinylidene difluoride
qRT-PCR	quantitative Real Time-Polymerase chain reaction
RNA	ribonucleic acid
RNAi	RNA interference
RNasin	ribonuclease inhibitor
ROS	reactive oxygen species
RT	room temperature
S. cerevisiae	*Saccharomyces cerevisiae*
SAM	sorting and assembly machinery
SAP	shrimp alkaline phosphatase
SD	Standard deviation
SDS	sodium dodecyl sulfate
SEM	Standard error of the mean
SOC	Super optimal broth with catabolite repression
SOD	superoxide dismutase 1
SPG	spastic paraplegia gene
STS	Staurosporine
TAF7L	TAF7-like RNA polymerase II, TATA box binding protein (TBP)-associated factor
Taq	*Thermophilus aquaticus*
TBS	TRIS buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethylene diamine
TIM	translocase of the inner mitochondrial membrane
TMRE	tetramethylrhodamine ethyl ester
TOB	topogenesis of outer membrane β-barrel proteins
TOM	translocase of the outer mitochondrial membrane
TPR	tetratricopeptide repeat
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiole
TX-100	Triton X-100
U	unit(s)
v/v	volume per volume
VDAC	Voltage dependent anion selective channel
w/v	weight per volume

WCL	whole cell lysate
wt/WT	wild type

7 APPENDIX

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Anja: Although being the youngest of the female Musketeers you are in no way standing back in terms of crazy ideas or adventurous actions. Your enthusiasm is contagious and made a lot of us to become a bit more outgoing. Thanks for lovely food tastings, “Threadless” ordering excesses, a good humor and your uncomplicated and open way in dealing with other people.

Katharina: Being in the lab together from the very beginning we had plenty of incidents and accidents to look back on. After demonstrating that working with me might end at the emergency unit with a syringe stuck in your finger, I still hope that it was mostly pleasant, at least when no mechanical shearing of cells was necessary… Thanks for your efforts when anything had to be ordered really quickly (as usual) or was already in our stock when I did not even know that I might need it yet.

Desiree, Elisa, Snow: You completed the women guard in our lab and mixed up lab life in a surprising and sometimes unexpected way. Thanks for your delicious cakes/tarts/sweets, imported or self-made, they were a treat!

Dražen: Spending a whole lot of time next to you on a bench, I think it is just fair to say that we both adopted or enforced the most doubtful character traits of each other, which finally turned our part of the lab to “the dark side”. I enjoyed very much working next to you, not only because we shared the obsessive need to clean the bench spaces regularly and to tidy up, but also because there was no need to explain why one would at least return twice to the lab after leaving (1st time to check if the thermo block is really switched off; 2nd time if

the water bath is disconnected…). It made life easier to know somebody next to you who would smile consent or start laughing when stories were being told about Balkan/family idiosyncrasies. Thanks for bringing back an atmosphere of paprika, eggplants, Vegeta and stuffed wine leaves with minced meat!

Thomas: Even though we did not spend too much time in the small lab together, I realized that working next to you is a real charm. I did not need to climb up benches anymore to reach kits (that's the downside of being really tall…) and even if we happened to be all busy at the same time, your "No problem, we can switch on x and y, I will use that" attitude was really helpful.

Tao: Although we were sometimes "Lost in Translation" I had a really good time with you and I hope I could give you proper medical advice in exchange of the awesome dumplings you were cooking for us every once in a while. Knowing that you are in the lab at any time made it quite comfortable for me since I hated to work late when nobody was around or if I just needed to call and make sure I did not leave my computer on my desk, samples on the bench…..

Kai: I would like to say thanks for your continuous efforts to entertain ("Rocket Man….") and update us on the newest video games/TV-sets/FC Bayern victories. Even though a lot changed during the past years (Italian music was partly replaced by Pop music-thank you!, the caffeine consume seemed to skyrocket with time as well as your need to work out (any connection?)) the most striking quality remained: your willingness to participate in rather dubious and questionable undertakings planned from a very adventurous part of the lab!

Hiroki: Imagine me making a 45-degree bow, Dr. Kato-san, while thanking you for your help in technical and scientific issues. Beside your extensive scientific knowledge, which I profited a lot of, it was a pleasure to get to know you as a person with an incredible humor spiced up with a great portion of sarcasm. As I am sure that I crossed several social/politeness-boundaries during your stay here (ranging from sneezing really loudly to forcing you to bring me sewing magazines from Japan from your trips home…), I hereby ask you to register them under: Momonga-just a bit weird, but not in general bad manners/intentions… maybe ok?

Denise, Martin, Frank, York, Wolf and Ulrike: You are unbelievably reliable and supportive colleagues who helped without any hesitation whenever I needed your support or time at one of your lab devices. I am very aware that this is truly outstanding. Working with you was always easy and made me recall why we do research and what I like most about it. Talking to you about experiments and experimental set-ups, as well as working with you or next to you was always a pleasure and fruitful and made 10h non-stop experiments less exhausting.

Stefan: Even though I meticulously tried to avoid the "spouses lab co-work" on the one hand because of our completely different working styles and on the other to separate private life from work, I am very thankful in retrospect. You made me question my scrupulous requests and expectations that made me often miserable (because experiments sometimes just wouldn't turn out the way planned) and thereby set things again into a more reasonable perspective. Thanks for your understanding and patience, late night microscopy shifts, junk food deliveries shortly before a hypoglycemic shock and in particular for enduring my special way…