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In vivo studies of *Saccharomyces cerevisiae* Hmi1 helicase mitochondrial transport

Magistritöö

Dmitri Lubenets

Juhendaja: Prof. Juhan Sedman

Opponent: PhD Tiina Alamäe

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ABBREVIATIONS

ADH	alcohol dehydrogenase
ADP	adenosine diphosphate
ATP	adenosine triphosphate
CIAP	calf intestine alkaline phosphatase
DTT	dithiothreitol
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
FOA	5'-fluoroorotic acid
HS	hypersuppressiveness
IM	inner membrane
IMS	inter membrane space
MIP	mitochondrial intermediate peptidase
MPP	matrix processing peptidase
MSF	mitochondrial import stimulating factor
mtDNA	mitochondrial deoxyribonucleic acid
MTS	mitochondrial targeting sequence
OM	outer membrane
ORF	open reading frame
PEG	polyethylene glycol
SD	selective drop out
ssDNA	single stranded deoxyribonucleic acid
TIM	inner membrane translocase
TOM	outer membrane translocase
Tris	Tris(Hydroxymethyl)aminomethane
WT	wild type

INTRODUCTION

Mitochondria are organelles in eukaryotic cells that participate in many different metabolic processes. These processes include energy production via degradation of fatty acids, citric acid cycle, and respiration. Mitochondria are surrounded by double membrane and have its own genome, mitochondrial DNA (mtDNA). It has been shown that topology of mtDNA is dependent on many factors, such as chemical environment surrounding cell, and the proteome of the host cell. It has been reported that many different proteins are essential for mtDNA replication and stability. These enzymes encoded by nuclear genes and after synthesis in cytoplasm are transported into mitochondria using special targeting or, also called, signal sequences.

After synthesis on cytoplasmic ribosomes mitochondrial matrix proteins bind special class of cytosolic polypeptides. These polypeptides, called chaperones, direct preproteins to the surface of the organelle. On the exterior of the mitochondria preproteins make contact with translocase complex, and are then transported through the outer membrane. The inner mitochondrial membrane contains another translocase complex, which interacts with preproteins and directs them into the matrix. Inside of the matrix targeting sequences of preproteins are proteolytically cleaved off and processed peptides obtain their proper tertiary structure with the help of mitochondrial chaperones.

In the present study Hmi1p mitochondrial transport has been studied. Hmi1p utilizes C-terminal α-helical signal sequence, which is both necessary and sufficient for helicase translocation through outer and inner membranes into mitochondrial matrix. During mitochondrial import Hmi1p interacts with components of the outer and inner membrane translocases via its C-terminal targeting sequence, and signal peptide is proteolytically removed upon arrival into the matrix. Using recombinant DNA technology Hmi1p mutants were obtained and transport of enzyme with shortened C-terminal signal sequences was studied. In addition, the mitochondrial transport of Hmi1p containing different N-terminal targeting sequences was studied. Finally, using oligonucleotide

directed mutagenesis we substituted original amino acids S665, G674, N686 and G688 with prolines, and examined import efficiency of these mutant forms of Hmi1 helicase.

LITERATURE REVIEW

Yeast mitochondrial genome

General features

Mitochondria are intracellular organelles that are surrounded by double membrane and have its own genome. The outer membrane is fairly smooth. But the inner mitochondrial membrane is highly convoluted, and form folds called cristae. Mitochondria proliferate by the growth and division of preexisting mitochondria, and major components of mitochondrial growth are the synthesis of proteins encoded by mtDNA and the import of nuclear-encoded polypeptides (Attardi *et. al.,* 1988). Mitochondria play essential roles in cellular energy production and function in numerous vital metabolic pathways. For example citric acid cycle, urea cycle, fatty acids oxidation and respiratory chain. Many different proteins support the metabolic role of mitochondria. Nuclear genes encode the major part of these proteins. However, some polypeptides that are encoded by mitochondrial DNA are also involved.

For many years mitochondrial DNA was thought to be a circular molecule, supporting an idea that mitochondria had evolved from bacteria. Although some pictures of circular mtDNA were obtained (Fig. 1), the majority of electron microscopic images of mtDNA showed it as a linear molecule. For over 30 years these linear molecules mostly observed

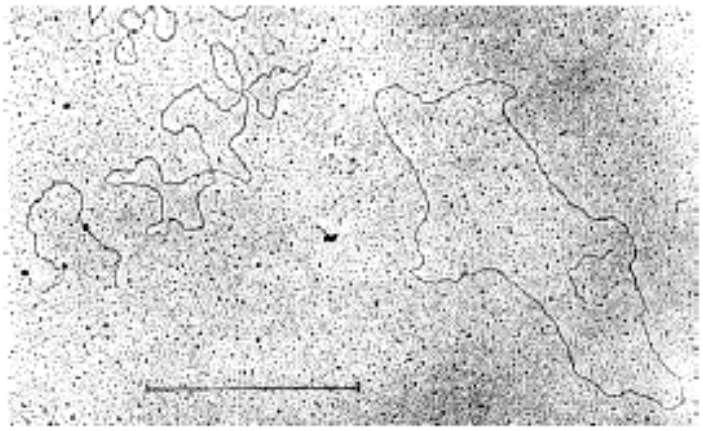

Fig 1. Electron microscopic image of circular mitochondrial DNA. Visualized mtDNA was isolated from mouse mitochondria. Scale bar, 1μm. (Sinclair *et. al*., 1966).

in yeast mitochondria were regarded as broken circles, until in 1991 Clark-Walker and his colleagues finally revealed the truth. Using pulsed-field gel technology they found that *S. cerevisiae* mitochondrial genome consists of linear arrays approximately 75kb long and that the lengths of some mtDNA molecules ranged up to sizes greater than 75kb (Maleszka *et. al.,* 1991).

In yeast cells, the mitochondrial genomes are organized in 10 – 20 distinct nucleoids (protein – DNA complexes), which are spherical or ovoid, measuring 0.3 – 0.6 μm in diameter. Nucleoids contain 3 – 4 mitochondrial genome copies and as many as 20 different polypeptides. Mitochondrial DNA molecules are synthesized throughout the cell cycle and the normal level of mtDNA per cell is about 20% of the nuclear content (Moraes, 2001). In general, the mtDNA is very AT – rich. Most genes have a composition of about 60 – 70% A+T some are 85 – 90% AT - rich. Genes are separated by long regions of approximately 95% A+T and these spacer regions are punctuated by small regions of high GC – content called GC clusters (Kang *et. al.,* 1989).

Yeast mitochondrial genome contains several open reading frames (ORFs). Genes encoding three subunits of cytochrome c oxidase and ATP synthase subunits 6, 8 and 9 are among them. In addition, the mitochondrial genome encodes 15S and 21S ribosomal RNAs, 24 tRNAs that can recognize all codons, and the 9S RNA component of RNase P (de Zamaroczy *et. al.,* 1986 and Foury *et. al.,* 1998). For replication initiation the genome has eight replication origin – like elements, each approximately 270 base pairs long (Dujon *et. al.,* 1980).

Petite yeast and hypersuppressiveness

Spontaneously or under certain environmental conditions, segments of the wild type (rho^+) mitochondrial DNA of yeast cells are excised and tandemly amplified to form a defective genome. Excisions take place by internal recombination processes between direct nucleotide repeats present in the abundant noncoding sequences of the wild type genome. The normal mtDNA fragments are reiterated in a head-to-tail fashion or in a

head-to-head mode to form longer molecules in amount comparable to rho^+ genome (de Zamaroczy *et. al.,* 1983). The mutant mitochondrial DNA may contain a section of rho^+ DNA as short as 35 base pairs (Fangman *et. al.,* 1989).

There are four different types of defective mitochondrial genome. Yeast cells with no mitochondrial DNA are called rho^0, and cells with mutated mtDNA are called rho^-. Both types of cells grow slower on fermentable carbon source and are thus called *petite*. Petite cells are defective in respiration and can not grow on non – fermentable carbon sources, such as glycerol. The frequency of spontaneous appearance of petite mutants is quite high, about 1% per generation in most laboratory strains (de Zamaroczy *et. al.,* 1983). Mating different rho^- mutants with normal cells (also called grandes) revealed three types of respiration – deficient yeast. The first type cells are yeast with neutral rho^- genome. When these are genetically crossed with grandes only the wild type progeny appear. The second are moderately suppressive rho^- cells. Mating these with rho^+ cells give moderate proportion of petites. The last type is hypersuppressive (HS) rho^- cells. Mating rho^+ with HS cells generates nearly 100% of petite descendants (Blanc *et. al.,* 1980 and Williamson, 2002).

Using HS yeast strain KL14 – 4A/I21/HS416 Dujon and Blanc showed that after crosses with rho^+ strains, the mitochondrial genome of the progeny is indistinguishable from that of the HS parent (Blanc *et. al.,* 1980). This brings an idea that a defective genome has an advantage in replication or segregation over the wild type mtDNA during heteroplasmic yeast stage. Sequencing of the mutated HS rho^- genome revealed that it contains numerous repeats of the replication origin – like sequence. Thus, in petite x grande crosses it would easily out-replicate the wild type mtDNA molecules of the zygotic partner and dominate the zygotic progeny (Blanc *et. al.,* 1980).

Not only spontaneous mutations lead to petite yeast appearance. Some chemicals function as inductors and increase the wild type mitochondrial genome instability. Among them are ethidium bromide and nitrosoguanidine, which act directly on mtDNA, and promote rho^- formation. Other petite yeast inductors were shown to affect mitochondrial

membranes, leaving genetic material intact. These are ethanol, isopropanol, *tert*-butanol and sodium dodecyl sulfate (Jimenez *et. al.,* 1988). In addition, some proteins encoded by nuclear genes are absolutely necessary for rho^+ genome maintenance.

Proteins that affect the stability of mitochondrial genome

Since the discovery of the petite mutants, it has been shown that more than 100 nuclear genes directly or indirectly influence the fate of the rho^+ mitochondrial DNA. Several examples of these proteins that belong to different functional classes are given below.

It is not surprising that mutations in enzymes involved in transport of preproteins through mitochondrial membranes and mtDNA metabolism can cause a complete loss of mitochondrial DNA and/or lead to truncated forms (rho^-) of this genome. Preproteins processing peptidases also play important role in mtDNA stability, as many proteins obtain their active form only after proteolytic digestion.

Two DNA helicases found in yeast mitochondrial matrix, Pif1p and Hmi1p, participate in mtDNA maintenance. Pif1 helicase unwinds duplex DNA in 5'-3' direction and participates in mtDNA maintenance at high temperatures and specific type of mtDNA recombination (Lahaye *et. al.,* 1991 and 1993). Pif1p is not the major replicative helicase in mitochondria, as it is not required for mtDNA maintenance under optimal growth conditions. In contrast, Hmi1 helicase is necessary for mitochondrial genome stability under regular growth conditions. Yeast strains with deleted HMI1 gene were unable to grow on non – fermentable carbon sources and lost rho^+ mitochondrial DNA at 30^oC when grown on glucose-containing medium (Sedman *et. al.,* 2000).

The mitochondrial nucleoid is a complex nucleoprotein structure that functions in packaging, storing and organization of metabolic and segregational activities associated with mtDNA maintenance and inheritance. One component of mitochondrial nucleoid, an Mgm101 protein has been shown to be involved in mtDNA maintenance. Studies of Mgm101p revealed that this protein does not participate in mtDNA packaging,

segregation, or partitioning, as *mgm101* mutants did not show changes in nucleoid morphology. It is also unlikely that Mgm101p is involved in mtDNA replication. It has been found that Mgm101p is able to bind mtDNA directly, and that it participates in repair of oxidatively damaged mitochondrial DNA. Comparative analysis of the wild type and *mgm101* cells showed that mtDNA of mutant yeast was more sensitive to UV irradiation and was hypersensitive to damage induced by gamma rays and H_2O_2 treatment. Thus, by maintaining the repairing processes Mgm101p is directly involved in mtDNA maintenance (Chen *et. al.,* 1993 and Meeusen *et. al.,* 1999).

Protein of mitochondrial nucleoid, a catalytic subunit of mitochondrial RNA polymerase encoded by *RPO41* gene also plays an important role in wild type mtDNA stability (Greenleaf *et. al.,* 1986 and Fangman *et. al.,* 1990). Yeast mitochondrial RNA polymerase probably consists of two or more separate components, one of which can catalyze the synthesis of RNA chains and others participate in promoter recognition. The 145 kDa subunit encoded by the RPO41 gene represents the catalytic core of RNA polymerase. The mtDNA transcription efficiency in yeast with disrupted RPO41 gene was 400 times lower than in wild type cells. Mutant cells were unable to synthesize proteins encoded by mitochondrial genome, and thus were respiration deficient and lacked mitochondrial DNA (Wang *et. al.,* 1999).

Another mitochondrial protein that is involved in maintenance of the mitochondrial genome via protein synthesis is Mmf1p. Although the precise function of this polypeptide is unknown, there is data indicating that Mmf1p is involved in mitochondrial protein translation. Strains with MMF1 replaced with a gene that confers resistance to kanamycin lose their mitochondrial DNA and grow slower. Interestingly, mitochondrial genome can still be maintained in strains with deleted MMF1 gene (*Δmmf1*), but only if the dissection of mutant yeast obtained by sporulation was performed on plates containing a non – fermentable carbon source. The *Δmmf1* cells lose their rho^+ mitochondrial DNA when grown on glucose-containing medium. Moreover, on glycerol medium *Δmmf1* yeast show a temperature-sensitive defect, being unable to grow at 37^oC (Oxelmark *et. al.,* 2000).

Not only DNA or RNA interacting enzymes are involved in mitochondrial DNA maintenance. Additionally, some matrix proteases can indirectly participate in this process. For instance, the dynamin-related GTPase, Mgm1p (note, not previously described Mgm101p) is critical for the formation of normal inner membrane structures, inheritance of mitochondria, and mtDNA stability. This enzyme exists in two forms with molecular weight of 100 and 90 kDa, where smaller form is a product of proteolytic cleavage of the larger one. The processing of Mgm1p is maintained by a serine protease Pcp1p localized in mitochondrial matrix. Pcp1p is a yeast homolog of *Drosophila* rhomboid-1, an intramembrane serine protease that cleaves the TGFα-like growth factor, Spitz, in the ER membrane (Urban *et. al.,* 2001). Cells lacking Pcp1p produce only the larger form of Mgm1p (100 kDa), which is unable to fulfill its functions. In yeast with deleted PCP1 gene mitochondrial morphology is very similar to that found in cells with deleted MGM1 gene. Both types of cells contain partially fragmented mitochondria and rapidly lose their mtDNA (Sesaki *et. al.,* 2003). Thus, by producing the functional form of Mgm1p serine protease Pcp1 also takes part in yeast mitochondrial DNA maintenance.

All proteins described above encoded by nuclear genes and after synthesis on cytoplasmic ribosomes are transported into mitochondria. We will next point our attention on mitochondrial import machinery and general processes that accompany mitochondrial membrane transport of proteins.

Transport of proteins across the mitochondrial membranes

General features

During cell growth and proliferation many different proteins encoded by nuclear genes are synthesized in cytoplasm and are then transported into mitochondria. After translocation, these polypeptides find themselves in all submitochondrial compartments including outer membrane (OM), intermembrane space (IMS), inner membrane (IM) and the matrix. Separate translocases in the mitochondrial outer membrane (TOM complex) and in the inner membrane (TIM complex) facilitate recognition of proteins and transport

across two membranes. In addition, special factors in cytosol and the mitochondrial matrix assist in targeting of polypeptides and their proper folding (Daum *et. al.,* 1982).

A protein that functions in mitochondrial matrix is generally synthesized with N-terminal extension, the matrix targeting sequence (MTS). Signal sequences commonly form amphipathic α-helix that has positively charged residues on one side of the helix and hydrophobic amino acids on the other side. The targeting sequence is proteolytically cleaved upon arrival into the matrix. The length of the MTS varies from 15 to 70 amino acids with the average number of 30 residues. Protein that must be transported into intermembrane space has two targeting sequences the matrix and IMS targeting sequences. Such a protein is first transported into the matrix, premier presequence is removed and via the second one polypeptide is directed into intermembrane space. The inner membrane proteins possess only matrix targeting sequence. There is no exact data how IM proteins get into the proper location. However, structural studies of inner membrane translocases show that some of them could open laterally releasing the IM protein into lipid bilayer. The outer membrane proteins possess the matrix targeting sequence and a stop-transfer sequence just after it. This stop-transfer sequence forms a long α-helix, which is both essential and sufficient for suspending the translocation of protein through the mitochondrial OM (Lodish *et. al.,* 2000). Again, it is thought that outer membrane translocase opens laterally and the protein diffuses into membrane.

The TOM and TIM complexes

Each of the two mitochondrial membranes has translocation machinery for preproteins. These machineries work cooperatively in the translocation of preproteins, but each translocation complex can act independently. *In vitro* studies showed that translocation of polypeptides across OM can occur separately of translocation across the inner membrane (Hwang *et. al.,* 1989).

The yeast outer membrane translocation complex is composed of at least eight proteins. These components mediate recognition of preproteins, transfer of polypeptides through

the outer membrane, and insertion of resident OM proteins. The schematic representation of TOM complex is presented in Figure 2.

Two receptor complexes needed for recognition of preprotein signal sequence have been discovered in outer membrane of mitochondria, Tom20-Tom22 and Tom37 – Tom70-Tom71. The cytosolic domain of Tom22 has an abundance of negatively charged residues (Nakai *et. al.,* 1995), and can directly interact with positively charged targeting sequences of preproteins. Tom20 is exposed to cytosol and is thought to assist Tom22 in presequences recognition.

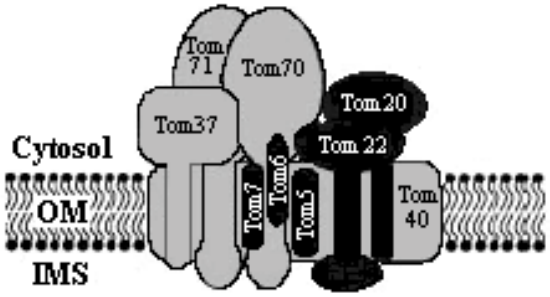

Fig 2. The schematic representation of mitochondrial outer membrane translocase complex components.

The sub complex formed by Tom22 and Tom20 was defined as the main structural element required for binding of signal sequences at the surface of the mitochondrial outer membrane (Mayer *et. al.,* 1995). It is thought that negatively charged cytosolic domain of Tom22 provides the docking site for positively charged targeting sequences, and after interaction with this site preproteins are guided into the outer membrane translocation pore. The second outer membrane receptor complex consists of three components. Tom70 and Tom71 are the largest components of TOM complex. They closely related structurally (70% of similarity), and both proteins are anchored to the mitochondrial OM via their N-terminus, thereby exposing a large C-terminal domain to the cytosol. Tom70 is the central component of Tom37 – Tom70-Tom71 receptor complex, as *in vitro* studies showed that most proteins can use Tom70 as an entry site (Hines, *et. al.* 1993). Tom 37 and Tom71 are thought to support Tom70 function. Tom37 is exposed at the cytosolic surface of the mitochondria and needed for preproteins binding only at high temperatures. The function of Tom71 is largely unknown, as deletion of TOM71 gene minimally affected the import of Tom70-dependent preproteins (Schlossmann, *et. al.,* 1996).

The major component of general insertion pore of the TOM complex is TOM40. It was shown that TOM40 makes contacts with preproteins as they traverse the outer membrane. Smaller TOM components seem to be a part of this pore, but their precise function is poorly understood. Tom7 and Tom6 seem to modulate the dynamics of the general insertion pore complex while Tom5 is involved in preprotein transfer from receptors to Tom40 (Ryan *et. al.,* 2000).

There are only three major components of the TIM complex Tim17, Tim23 and Tim44 (Fig 3).

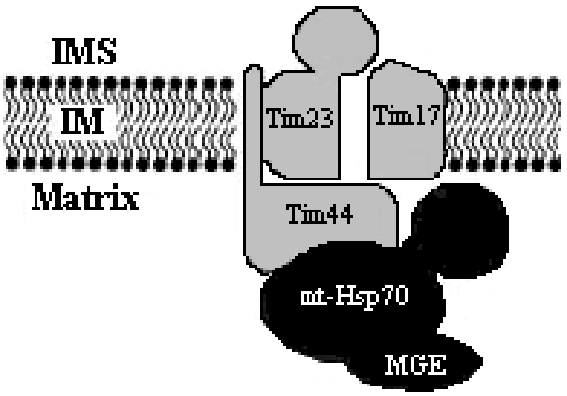

Fig 3. The schematic representation of mitochondrial inner membrane translocase complex components.

Tim23 is an integral protein of the yeast inner mitochondrial membrane. One part of Tim23 is hydrophilic and is exposed to the intermembrane space, while the hydrophobic portion is likely to span IM three or four times (Kübrich *et. al.,* 1994). Tim 17 is also resident inner membrane protein and it shares sequence similarity with hydrophobic part of Tim23. In contrast, Tim44 is hydrophilic protein that associates with IM. Tim44 could be easily extracted from membranes at alkaline pH, suggesting that it is not an integral membrane protein (Blom *et. al.,* 1993). The translocation pore of the inner mitochondrial membrane is composed of Tim17 and Tim23 proteins. Tim44 binds this complex from the matrix side of the inner membrane and plays a role in protein transduction by recruiting matrix chaperon mtHsp70 close to the preprotein import site.

The cytosolic factors assisting the preprotein transport

During or after synthesis the preproteins exist in a loosely folded state and could aggregate or be degraded by cellular proteases. There are cytosolic factors known as chaperones that prevent these reactions. These chaperones include members of the 70kDa heat shock protein family (Hsp70), which bind proteins targeted to different subcellular compartments in many places of polypeptide chain. However, there is one chaperone dedicated to only mitochondrial protein import: the heterodimeric protein called mitochondrial import stimulating factor (MSF) consisting of 30 and 32kDa subunits. MSF has two major activities. It recognizes and forms stable complexes with signal sequences and main part of mitochondrial precursors, and promotes unfolding of preproteins. After binding, MSF delivers preproteins to the receptor components of the TOM complex (Hachiya *et. al.,* 1994).

The involvement of Hsp70 or MSF in targeting of preproteins to the mitochondrial surface depends on the preprotein, and it is not clear how proteins choose one or the other chaperone. Although no ATP dependence was observed with Hsp70, the targeting of both MSF-preprotein complexes and MSF-Hsp70-preprotein complexes to mitochondria required ATP hydrolysis. It was found that MSF is able to hydrolyze ATP. Its ATPase activity is induced by binding of presequences and positively charged residues in signal sequences appear to play an important role in this stimulation. In the presence of non hydrolysable analog of ATP, import of the MSF-Hsp70-preprotein ternary complex ceased and the MSF-preprotein complex was found at the mitochondrial surface. In addition, the MSF binding to the outer membrane occurred only in the presence of a functional matrix-targeting sequence (Neupert, 1997).

There are two pathways described for preprotein targeting into mitochondria. One pathway depends on Hsp70 and is ATP independent, while the other needs MSF and is stimulated by ATP hydrolysis (Fig.4). It was proposed that Hsp70-preprotein complex interact directly with TOM complex subunits Tom20-Tom22 without ATP requirement. In contrast, MSF-preprotein complexes first bind to Tom37-Tom70 subunits, from where

the preprotein is transferred upon ATP-dependent release of MSF to the Tom20-Tom22 subunits and enters the translocation channel (Schatz, 1996).

Preprotein translocation mechanism

Although several protein components of the outer mitochondrial membrane preprotein translocation machinery have been identified, little is known about the translocation mechanism at the molecular level. As soon as preprotein is directed to the mitochondrial surface, it interacts with receptor components of the TOM complex and enters the translocation pore. The translocation of protein through the outer mitochondrial membrane requires no membrane potential on the OM or any additional energy except that needed for MSF releasing. In contrast, two kinds of energy are required for preprotein translocation across the IM. First one is the electrical membrane potential $\Delta\psi$ on the inner mitochondrial membrane.

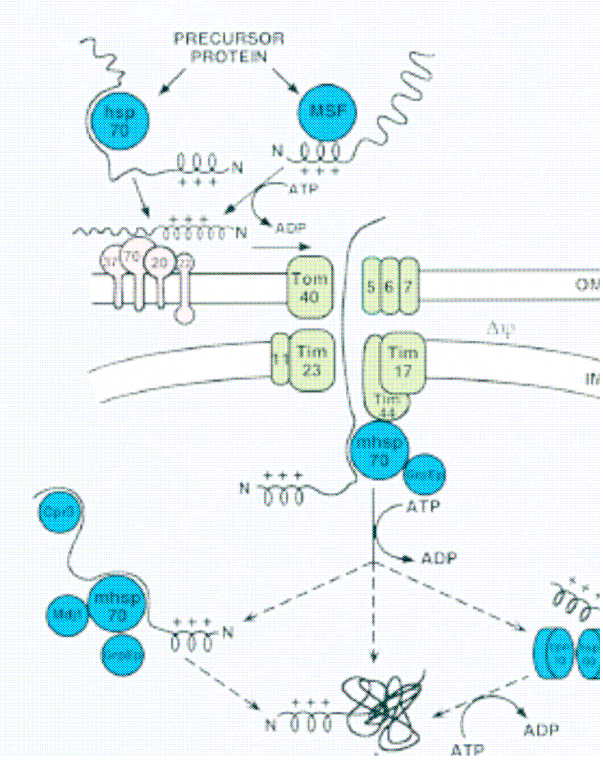

Fig 4. The schematic representation of the mitochondrial protein import machinery. Signal sequence of the preprotein is represented as a spiral with + signs. Tom and Tim proteins are medium-shaded. TOM complex receptor components are light-shaded. Chaperones and other folding helpers are dark-shaded.

It was shown that Δψ is necessary for insertion and transport of MTS across the Tim23 channel, but not for the translocation of the mature part of the polypeptide chain (Herrmann *et. al.,* 2000). It is also known that the membrane potential can promote unfolding of folded domains by an unknown mechanism (Huang *et. al.,* 2002).

The second energy source is also required for inward movement of precursor proteins. An import motor mediates this process, and utilizes ATP hydrolysis energy. The motor represented by "hand-over-hand" model has minimum three components: Tim44, the mitochondrial heat-shock protein mtHsp70 and Mge1. This model implies that Tim44 sits at the matrix face of the inner mitochondrial membrane bound to Tim23-Tim17 complex. MtHsp70 is attached to Tim44, so the mitochondrial chaperon is situated closely to the import site. At this stage mtHsp70 exists in an ATP-bound form and its peptide-binding site is open. As preprotein signal sequence enters the matrix mtHsp70 binds to it. Subsequently, ATP is hydrolyzed and peptide-binding site closes on a short stretch of amino acid residues. At the same time the chaperon is released from Tim44 and the polypeptide chain moves further inward. Next, the mtHsp70 in a complex with ADP is released from the chain. This reaction necessitates the nucleotide exchanging factor Mge1, which eliminates bound ADP and allows regeneration of the ATP-bound form and opening of the peptide-binding site (Fig. 5). After that the mtHsp70 molecule is ready for the second round of protein import (Neupert *et. al.,* 2002).

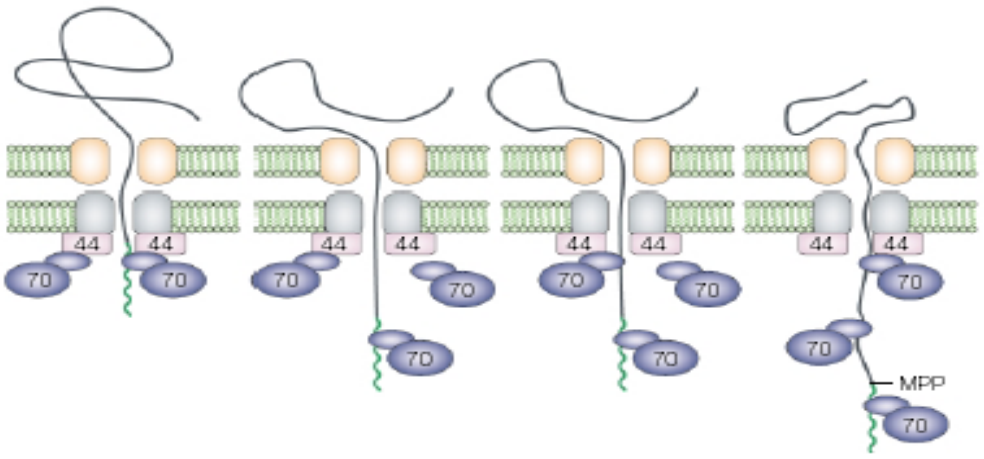

Fig 5. The model of mitochondrial import machinery. Protein is represented as a black line. MPP is mitochondrial processing peptidase. The Tim23-Tim17-Tim44 complex presumably acts as a dimmer or higher oligomer. In this way more than one mtHsp70 can be recruited to the import site.

Preprotein processing peptidases and the matrix components assisting the proper folding of the imported proteins

As soon as preprotein enters the mitochondrial matrix its signal sequence is cleaved off by specific peptidase, and matrix chaperones or chaperonins assist in the proper folding of the protein. All matrix-processing peptidases (MPP) identified so far consist of two components termed α-MPP and β-MPP. Their molecular masses are 55 and 52 kDa respectively. In yeast two MPP subunits tightly interact with each other, so that mature enzyme exists as a heterodimer. In contrast in *Neurospora crassa* α-MPP and β-MPP work separately. In addition, the β-MPP was shown to be a part of cytochrome bc1 complex. Studies of recombinant α and β forms of the MPP revealed that only β-MPP has the catalytic activity (Neupert, 1997). Unfortunately, the precise mechanism of cooperation of the two MPP components and the preprotein cleavage remains unknown.

In addition to the cleavage by MPP some matrix proteins are further digested by mitochondrial intermediate peptidase (MIP). In yeast this enzyme consists of 772 amino acid residues and cleaves polypeptides in conserved motif RX(↓)(F/L/I)XX(T/S/G)XXXX(↓) releasing the corresponding octapeptide (Branda *et. al.,* 1995). A search through known yeast mitochondrial preproteins revealed many substrates for mitochondrial intermediate peptidase. These are components of mitochondrial electron transport chain, citric acid cycle and mitochondrial genetic machinery. Deletion of MIP gene in yeast leads to respiratory-deficient phenotype, as the unprocessed preproteins do not fold properly (Isaya *et. al.,* 1994).

Because preproteins are imported into the mitochondrial matrix in the elongated form they must obtain a native conformation during or after the entering process. The system consisting of mtHsp70 and its cochaperones Mdj1 and Mge1 has the capacity to fold some transferred proteins (Rospert *et. al.,* 1996). Mge1 is the nucleotide exchanging factor that removes ADP from nucleotide binding pocket of mtHsp70 allowing the regeneration of ATP-bound form. Mdj1 is a heat shock protein that acts as an essential cofactor of the mtHsp70 (Fig. 4). The disruption of MDJ1 gene resulted in a respiratory-deficient phenotype, loss of mitochondrial DNA and nonviability at 37^{o}C (Rowley *et. al.,*

1994). In addition, the proper folding of many imported proteins requires the chaperonin system consisting of Hsp60 and Hsp10. In yeast, Hsp10 is a protein with molecular weight of 11.4kDa that structurally forms a heptameric ring. It is required for mediation of the Hsp60-dependent folding, which makes both proteins essential for the final step of mitochondrial transport.

The role of mitochondrial chaperonins in the assembly of oligomeric proteins appears to rely on folding of separate proteins. When subunits of one multimeric enzyme are released from chaperonin the multimeric protein would assemble spontaneously (Zheng *et. al.,* 1993).

Despite the fact that two protein folding complexes may function independently, there is a hypothesis that both systems in the mitochondrial matrix work sequentially. This hypothesis implies that the imported preprotein interacts first with mtHsp70 followed by binding to Hsp60 and no complex alone can facilitate the proper protein folding (Endo, 1991).

Yeast mitochondrial Hmi1 helicase

Yeast mitochondrial helicase Hmi1p ("Helicase in Mitochondria 1") is 706 amino acids long protein with molecular weight of 80.5kDa. It is encoded by nuclear gene YOL095c, which is located on the left arm of chromosome XV (nucleotides: 141346 – 139226).

Hmi1p is a single stranded DNA dependent NTPase that is able to unwind distributively DNA duplexes in 3'-5' direction in the presence of ATP and magnesium ions. For initiation of DNA unwinding reaction Hmi1p requires 9 to 16 base pairs long ssDNA region at 3' end of the duplex depending on the topology of DNA substrate (Kuusk *et. al.,* 2005). Hmi1 helicase has seven conserved motifs that characterize the superfamily 1 of DNA helicases (Sedman *et. al.,* 2000). Hmi1p is homologous to PcrA from *S. aureus* and *E. coli* Rep and UvrD helicases. Alignment of these four enzymes revealed that HMI1 gene has small distinctive differences compared to the bacterial helicases. These

are present as short insertions and deletions in the nucleic acid sequence of yeast helicase. However, it is also true that all these differences are mostly located within or between α-helices, strictly beyond the conserved "helicase" motifs and structural β-sheets.

Hmi1 helicase is not essential for yeast survival. However, it is required for the stability of the functional rho^+ mitochondrial DNA on fermentable and non – fermentable carbon sources. In the near past Hmi1p was thought to be dispensable for maintenance of the defective rho^- mitochondrial genome (Sedman *et. al.,* 2000), because yeast with deleted HMI1 gene were still able to maintain their rho^- mtDNA. However, recently it has been shown that Hmi1p stimulates the synthesis of long tandemly repeated (concatemeric) rho^- mitochondrial DNA molecules. In addition, it has been found that ATPase activity of helicase is not necessary for rho^+ mtDNA maintenance and is not required for the synthesis of long concatemeric rho^- molecules (Sedman *et. al.,* 2005).

Because Hmi1p is distributive enzyme, it can not be the major helicase in mtDNA replication machinery. Still, it can participate in replication initiation, recombination or reparation processes. In addition, by analogy to PcrA helicase, whose activity is stimulated by RepD protein, Hmi1p processivity could be maintained by adaptor protein yielding helicase processive enough for unwinding DNA duplex during replication process.

As Hmi1p is synthesized in cytoplasm it must be transported into mitochondria. Generally mitochondrial matrix enzymes encoded by nuclear genes possess presequences at their N-terminus. Yeast mitochondrial DNA helicase Pif1 is transported into organelle via 45 amino acids long mitochondrial targeting sequence at its N-terminus. This presequence is cleaved off in mitochondrial matrix, so the mature Pif1p helicase starts at the 46^{th} residue (Lahaye *et. al.,* 1991 and 1993). No such sequence was found at Hmi1p N-terminus. However, analysis of Hmi1p coding sequence revealed that carboxyl-terminal region contains a stretch of approximately 30 amino acids resembling mitochondrial targeting presequences. Like Pif1p presequence Hmi1p signal sequence forms an amphipathic α-helix with positively charged amino acids on one side and non-

polar residues on the other side of the helix. When this sequence was removed by the recombinant DNA technology, no translocation of Hmi1p across mitochondrial membranes occurred. Although classical protein import into mitochondria occurs in N- to C-terminal direction, the Hmi1p helicase was shown to enter organelle in opposite direction (C- to N-terminal). This process requires membrane potential, the Tim17-Tim23 translocase and mitochondrial Hsp70 (Lee *et. al.,* 1999).

Previous studies of Hmi1p mitochondrial transport revealed some interesting aspects in this process. Up to date, Hmi1 helicase is the only enzyme possessing C-terminal mitochondrial targeting sequence. It is transported in C- to N-terminal direction, and signal sequence is proteolytically cleaved off inside of the matrix. However, further investigations of Hmi1p mitochondrial transport are needed in order to understand how does the C-terminal signal sequence function, and to determine if the unusual C-terminal targeting sequence has a specific biological role.

AIMS OF THIS STUDY

The aims of this study were to find the minimum deletion from Hmi1p C-terminus required for termination of mitochondrial transport of the helicase, to understand if Hmi1p can be transported efficiently with a N-terminal signal. And to examine the mitochondrial transport of Hmi1p containing C-terminal signal peptide that is mutated to make it less flexible.

MATERIALS AND METHODS

Bacterial and yeast strains and growth conditions

The parental yeast strain was **W303** *MATa/α, leu2-3, 112, trp1-1, ura3-1, can1-100, ade2-1, his3-11, 15 RAD5*. In the present study, as a starting material the parental strain with disrupted YOL095c ORF (Sedman *et. al.,* 2000), and a copy of HMI1 with native promoter and ADH terminator cloned into a shuttle vector pRS316 (Sikorski *et. al.* 1989) was used. For intermediate cloning procedures *E. coli* strains **DH5α** *F¯φ80dlacZΔM15 Δ(lacZYA-argF) U169 deoR, recA1, endA1, hsdR17 (r_k^- m_k^+) phoA, supE44 λ^- thi-1 gyrA96, relA1* and **TOP10** *F- mcrA D (mrr-hsdRMS-mcrBC) f80lacZDM15, DlacX74, deoR, recA1, araD139, D (ara-leu) 7697, galU, galK, rpsL, (Str^R) endA1, nupG* were used. For single stranded DNA preparation *Escherichia coli* strain **BW313** *ung-1, relA1, dut-1, spoT1, thi-1, l- F+* was used.

All yeast strains were grown aerobically at 30^{o}C on selective drop out (SD) medium supplemented with different amino acids (2% glucose, 0.67% yeast nitrogen base without amino acids, 0.2% drop out mix, 0.01% of histidine, 0.02% of adenine, 0.01% of lysine, 0.005% of uracil, 0.01% of tryptophan, 0.025% of leucine, 1.8% of agar for plates). At different stages of experiments SD medium containing distinct amino acid composition was used: without tryptophan and uracil (-TU), without tryptophan and leucine (-TL), without tryptophan, uracil and leucine (-TUL). Bacteria were grown aerobically at 37^{o}C on LB plates (1.5% of bacto-agar, 1% of bacto-tryptone, 0.5% of bacto-yeast extract, 1% of NaCl) or in liquid 2YT medium (1.6% of bacto-tryptone, 1% of bacto-yeast extract, 0.5% of NaCl).

Restriction and ligation procedures

In the present study all digestions of DNA with restriction endonucleases were performed according to standard Fermentas protocol.

Different DNA fragments were ligated at room temperature for 2 hours in reaction mixture containing 1µl of 10X Fermentas ligation buffer (400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP pH 7.8), appropriate amount of DNA fragments and 1 unit of T4 DNA ligase (Fermentas).

C-terminal deletion mutants of Hmi1p helicase

Deletion mutants D6Ser, D11Leu, D15Ala, D18Phe, D24Leu, and D33Gly were made by replacing the wild type C-terminus of the HMI1 gene with native promoter and ADH terminator cloned in pRS315 vector with a PCR fragment carrying the corresponding deletion, and a stop codon after the last amino acid residue. Oligonucleotides used for PCR reactions were as following. Upstream primer Rep427, 5'-CCTCTAGAATGAGCGCTCTCAAAGG-3' and reverse primers D6Ser, 5'-CCGGATCCATGCTCTTACAACCTCTTAGTG-3'; D11Leu, 5'-CCGGATCCTATAG TGAAGAATATGATCT-3'; D15Ala, 5'-CCGGATCCTATGCTCTATAAAATCCAA A-3'; D18Phe, 5'-CCGGATCCTAAAATCCAAAATTTTTACGTAAC-3'; D24Leu, 5'-CCGGATCCTATAACTGATTGTATCGCTGCA-3'; D33Gly, 5'-CCGGATCCTAACC ATGTGTCACCTTAAC-3' (Fig. 6). All reverse primers contained BamHI restriction site near their 5' end. The fragments were amplified from yeast genomic DNA using 5 units of Pfu DNA polymerase possessing the proofreading activity. For one PCR reaction 30 pmoles of each primer and approximately 0.3 micrograms of genomic DNA template were used.

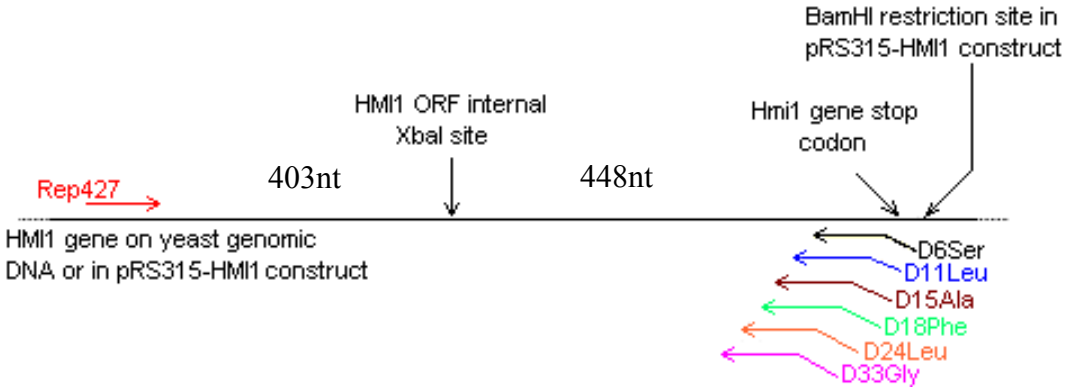

Fig 6. Schematic diagram representing primers used for construction of deletion mutants. HMI1 gene internal XbaI restriction site, position of the stop codon and BamHI restriction site in pRS315-HMI1 construct are indicated. Colored arrows represent different primers. The lengths of Rep427-XbaI and XbaI-stop codon fragments are correspondingly 403 and 448 base pairs.

After preheating at 95°C for 2 minutes, 25 PCR cycles were as following, 95°C – 30sec, 53°C – 30sec, 72°C – 2min. The amplified fragments were analyzed on 1.0% agarose gel. About 2μg of each type of DNA segments and pRS315 vector containing HMI1 ORF were digested with XbaI restrictase. After phenol/chloroform treatment DNA was precipitated with ethanol and dissolved in 10μl of H_2O. Subsequently, DNA fragments were digested with BamHI restrictase. Digested material was loaded onto 1.0% agarose gel and fragments of appropriate size were isolated. After that DNA was extracted from gel using gel extraction kit (UltraClean™) accordingly to standard purification protocol. After purification, amplified fragments were ligated with XbaI/BamHI digested pRS315-HMI1 and transformed into DH5α cells. Selection of plasmid containing bacteria was performed on LB plates containing 100μg/ml of ampicillin. Single colony of overnight bacteria was inoculated into 2ml of 2YT medium supplemented with 100μg/ml of ampicillin. After 6 hours of incubation at 37°C bacteria were harvested by centrifugation and plasmids were extracted. Extracted plasmids containing HMI1 gene with altered C-terminuses were analyzed on 0.8% agarose gel, and controlled by sequencing.

Transformation into yeast

Yeast strain W303α hmi1::TRP1, pRS316-HMI1 was grown for 2 days on synthetic dropout medium (-TU) plates. Fresh yeast colony was inoculated into 30ml of liquid SD (–TU) medium and grown at 30°C overnight. Logarithmic phase cells were harvested by centrifugation at 2000rpm for 5min (Hettich Zentrifugen 1617 rotor), resuspended in 1 volume of 0.1M Li-acetate – TE buffer (0.1M LiAc, 10mM Tris pH 7.5, 1mM EDTA) (LiAc-TE) and centrifuged again, as previously. Pelleted cells were resuspended in appropriate amount of LiAc-TE buffer in order to get 50μl of yeast solution per one transformation reaction. Subsequently, 100μg of denatured carrier DNA and app. 1μg of pRS315 vector containing wild type or mutated HMI1 gene were added to 50μl of yeast solution. Cells were briefly mixed and 0.5ml of FTS (40% polyethylene glycol, 0.1M LiAc, 10mM Tris pH 7.5, 1mM EDTA) was added. After that yeast cells were incubated at 30°C for 15 minutes. Next, heat shock was performed at 42°C for 15min. Yeast cells were pelleted in an Eppendorf miniSpin centrifuge for 1min at 5000rpm and FTS was

removed. Cells were centrifuged again and all traces of FTS were removed with a narrow pipette tip. Pellet was dissolved in 30µl of water and spread on Petri dish containing SD (–TUL) medium. Subsequently, cells were grown for 2 days.

Plasmid-shuffling assay

W303 (hmi1::TRP1, pRS316-HMI1) transformed with any kind of pRS315-HMI1 was grown for 48 hours at 30°C. About 10 colonies were collected and resuspended in 100µl of H_2O. Solution was diluted 40 times with water and cells were counted. Approximately 1.5 million of cells were inoculated into 2ml of SD (–TL) medium and grown for 4.5 hours. During this time yeast divided approximately two times. Subsequently, about $1.5x10^5$ cells were spread on SD (–TL) agar plates supplemented with 1mg/ml of 5'-fluoroorotic acid (FOA). After 3 days of incubation at 30°C and 3 days growth at room temperature, FOA resistant cells, the ones having lost the pRS316-HMI1 plasmid containing the URA3 gene were analyzed according to their color (see results and discussion part).

Preparation of single stranded DNA

For N-terminal mutagenesis of Hmi1 helicase the region of pRS315-HMI1 between KpnI and XbaI restriction sites were cloned into pGEM-7zf(+) vector. For C-terminal mutagenesis the XbaI/BamHI fragment was cloned into pUC119 vector (fig. 7).

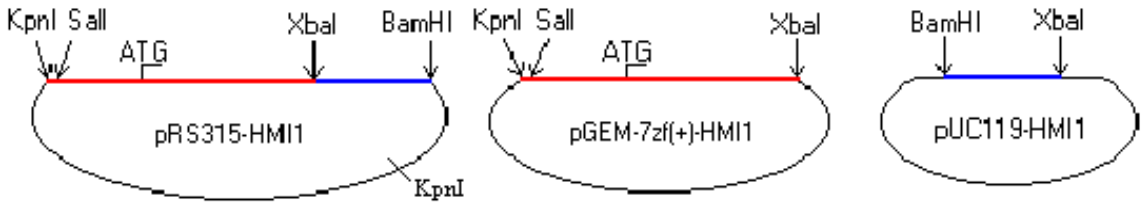

Fig 7. Schematic representation of cloning procedures. HMI1 gene N-terminal mutagenesis was performed in pGEM7zf(+) vector. C-terminal mutagenesis was performed in pUC119 vector. The restriction sites of KpnI, SalI, XbaI and BamHI are shown by arrows. Cloned KpnI – XbaI fragment is in red, XbaI – BamHI is in blue.

Plasmids pGEM-7zf(+)-HMI1 or pUC119-HMI1 were transformed into *E.coli* strain BW313 and grown overnight on LB plates supplemented with 100μg/ml of ampicillin. Next day single colony was inoculated into 10ml of 2YT medium and grown until OD_{600} 0.2. Subsequently, approximately $1.2x10^9$ plaque forming units of VCS M13 helper phage were added. Cells were then grown for 1.5 hours at 37°C. After that 50μg/ml of kanamycin were added and BW313 were grown for 12 – 14 hours more at 37 degrees. Grown overnight bacteria were harvested by centrifugation in Eppendorf miniSpin centrifuge at +4°C, 13400rpm for 5min. The supernatant was put into new plastic tube and centrifuged once more. Again, 1ml of supernatant was put into new tube and 200ml of 20% PEG6000, 2.5N NaCl solution was added. Mixture was centrifuged as previously and supernatant was removed. Pellet was resuspended in 100μl of TE pH 8.0 and 50μl of phenol/chloroform was added. After vigorous mixing solution was centrifuged as previously for 1min at +4°C and upper aqueous phase (app. 90μl) was put into new tube. Subsequently, 300μl of 96% ethanol and 16μl of 3M Na-acetate pH 5.2 were added. Mixture was incubated at room temperature for 15min and ssDNA was precipitated by centrifugation in miniSpin centrifuge at 13400rpm for 10min at +4°C. After washing with 80% ethanol ssDNA was dissolved in TE buffer (10mM Tris pH 7.5, 1mM EDTA) and stored at –20°C. The single stranded DNA was analyzed by gel electrophoresis.

HMI1 gene N-terminal and C-terminal mutagenesis

To clone different mitochondrial targeting sequences at the N-terminus of Hmi1p helicase we made an NcoI restriction site (5'-CCATGG-3') near the 5' end of HMI1 ORF. We substituted AG nucleotides in the original HMI1 sequence 5'-AGATGG-3' containing start codon with CC nucleotides. An oligonucleotide 5'-GCTTGTCCAT**GG**GTCTTCAACG-3' was perfectly complementary to pGEM-7zf(+)-HMI1 ssDNA except for two GG nucleotides in the middle of oligonucleotide. For mutagenesis reaction app. 100ng of ssDNA and 5ng of oligonucleotide were used. 5μl of reaction mixture containing template DNA, oligonucleotide and 0.5μl of buffer A (0.2M Tris, 0.1M $MgCl_2$, 0.5M NaCl, 10mM DTT, pH 7.5) were incubated at 55°C for 5 min and slowly cooled down to room temperature. Then, 0.5μl of buffer B (0.2M Tris, 0.1M

$MgCl_2$, 0.1M DTT, pH7.5), 0.5µl of 2mM dNTPs, 0.5µl of 10mM ATP, 6 units of T4 DNA ligase and 2 units of T7 DNA polymerase were added. Final volume of reaction mixture was adjusted to 10µl with water. Subsequently, solution was incubated for 5min at room temperature and for 60min at 37 degrees. After incubation, *E.coli* strain DH5α was transformed with 1µl of mutagenesis reaction mixture. Selection of cells containing plasmid was performed on ampicillin containing LB plates. Next day, some colonies were separately inoculated into 2ml of 2YT medium and grown at 37^{o}C for 6 hours. Plasmids were evolved and pGEM-7zf(+) vectors containing HMI1 gene with NcoI site were selected by digestion with NcoI restrictase. Obtained plasmid was called pGEM-7zf(+)-NcoHMI1. The pGEM-7zf(+)-NcoHMI1 was digested with SalI and XbaI restrictases (fig. 7). The HMI1 fragment was cloned between these two sites into pRS315 vector carrying HMI1 gene with wild type, D15 and D35 C-terminal signal sequences. The accuracy of obtained constructs was controlled by digestion with NcoI and sequencing.

At Hmi1p C-terminus we wanted to disrupt the α-helical structure of mitochondrial targeting sequence substituting amino acids S665, G674, N686 and G688 for proline residues. For this purpose the XbaI/BamHI fragment of wild type HMI1 gene was cloned into pUC119 vector (fig. 7) and ssDNA was extracted as previously described. Mutagenesis reactions were performed essentially as described above using four different oligonucleotides. S665P, 5'-GTCACATACAGGTCGTTTCAAA-3'; G674P, 5'-CACATTATAAGGATGTGTCACC-3'; N686P, 5'-AAATCCAAAAGGTTTACGT AAC-3' and G688P, 5'-TCTATAAAATGGAAAATTTTTA-3'. Altered HMI1 C-terminal sequences were cloned from pUC119-HMI1 to pRS315-HMI1 using the same restrictases. New pRS315-HMI1 constructs were transformed into *E.coli* TOP10 strain. Plasmids were extracted and the presence of mutations was controlled by sequencing.

Cloning of MTS at Hmi1p N-terminus

DNA sequences encoding 15 and 35 amino acids from C-terminus of Hmi1p helicase and 63 amino acids from N-terminus of Pif1p helicase were amplified from yeast genomic

DNA using 2.5 units of Pwo DNA polymerase. Oligonucleotides used were as following. SIGN15, 5'-TGAACCCATGGCTAGAGCATATTCTTCA-3'; SIGN35, 5'-TGAACCCATGGCTACACATGGTTATAAT-3'; 3PAG, 5'-GCGGCTCATGACTATACGTCTGAAAAC-3'; PPFNCO, 5'-TGAACCCATGGGTAAGTGGATAAGATCAACC-3'; PIFR1189, 5'-GCCGCCATGGATTTTGAAAGTATGGA-3' (fig. 8).

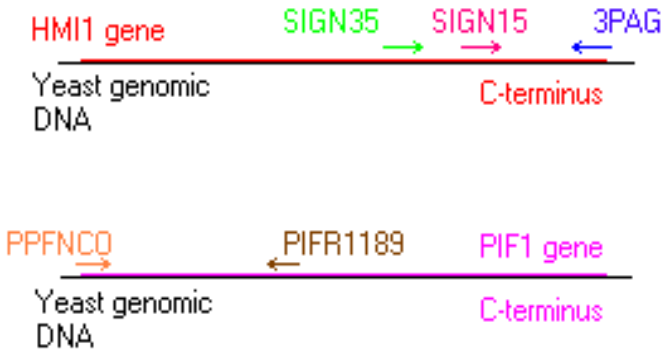

Fig 8. Schematic diagram representing oligonucleotides used for Hmi1 and Pif1 signal sequences amplification. Black line represents the genomic DNA. Long red and magenta lines correspondingly represent HMI1 and PIF1 genes. Colored arrows represent approximate position of primers. SIGN35, green; SIGN15, pink; 3PAG, blue; PPFNCO, orange; PIFR1189, brown.

After preheating for 2min at 95°C, 25 PCR cycles were as following 95°C – 30sec, 55°C – 30sec, 72°C – 20sec for longer fragments and 15sec for the shorter one. Single PCR reaction contained 30pmol of each primer and about 0.3µg of genomic DNA template. Oligonucleotides SIGN15, SIGN35, PPFNCO and PIFR1189 had NcoI restriction site near their 5' ends. PAG3 had PagI restriction site at its 5' end. PRS315-NcoHMI1 construct containing wild type, D15 and D33 C-terminal signal sequences, as well as amplified DNA fragments were digested with NcoI and PagI, using approximately 2µg of amplified DNA. After digestion with NcoI and PagI restriction endonucleases the same sticky ends were formed. Restriction material was loaded onto 2% agarose gel and bands of appropriate size were isolated. Subsequently, DNA was extracted using gel extraction kit (UltraClean™). Digested plasmid was treated with Calf intestine alkaline phosphatase (CIAP) according to standard Fermentas protocol. Fragments amplified by PCR were ligated with CIAP treated pRS315-NcoHMI1. Ligated material was transformed into *E.coli* DH5α cells. Selection of bacteria was performed of LB plates containing 100µg/ml of ampicillin. Next day single colony from each plate was inoculated into 2ml of 2YT medium with 100µg/ml of ampicillin and grown at 37 degrees for 6 hours. Plasmids were extracted, and controlled by sequencing. Thus, totally 9 constructs

containing N-terminal Hmi15, Hmi35 and Pif1, and C-terminal wild type, D15 and D33 signal sequences were obtained.

RESULTS

According to standard process of preprotein mitochondrial transport, after the synthesis on cytoplasmic ribosomes Hmi1p must interact with cytosolic chaperons Hsp70 or MSF via its C-terminal mitochondrial targeting sequence. Amino acids of α-helical MTS would make contact with components of TOM and TIM complexes and after translocation whole signal sequence would be removed by proteolytic cleavage. The full-length carboxyl-terminal targeting sequence is absolutely necessary for successful maintaining of these three major steps of Hmi1p mitochondrial transport. However, as we show in this thesis, some mutant proteins with slightly shortened MTS were also able to penetrate mitochondria, but with reduced efficiency.

Basic principles of experimental procedures

After cloning procedures the pRS315 vector containing different HMI1 deletion mutants were transformed into yeast strain W303α, hmi1::TRP1, pRS316-HMI1 and cells were grown on SD (-TL, +FOA) agar plates. 5'-fluoroorotic acid is a powerful agent for the selection of Ura- cells amid a population of Ura+ cells. FOA is highly toxic to cells with a functioning URA3 gene encoding orotidine 5'-monophosphate decarboxylase. This enzyme facilitates the final step in the *de novo* biosynthesis of uridine monophosphate and incorporation of 5'-fluoroorotic acid results in the formation of 5'-fluorouridylate, which is harmful to cell propagation (Boeke *et al.*, 1984). As pRS316 vector has URA3 gene, yeasts containing it were not viable in the presence of FOA. This allowed the use of plasmid shuffle and cells containing pRS315-HMI1 with LEU marker were the only ones that could grow on the selective drop out medium. Thus, survivors contained only one plasmid carrying HMI1 gene with C-terminal deletions.

Because W303 yeast strain has ade2-1 phenotype, respiratory deficient cells could be easily detected by analyzing color of the colonies (Fig. 9).

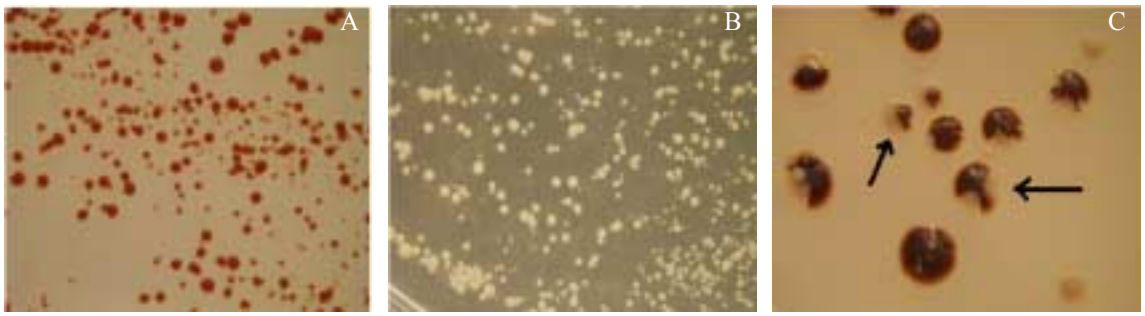

Fig 9. Yeast colonies of different color. A) Red yeast colonies. Appear on FOA plates if Hmi1 helicase can freely pass into mitochondria. B) White yeast colonies appear on FOA plates if Hmi1p can not penetrate mitochondria. C) Sectored colonies are indicated by black arrows. Show up when mitochondrial import of the helicase is partially impeded.

ADE2 gene product functions in adenine biosynthesis. In respiration-competent yeast cells mutations in this gene cause the accumulation of intermediate molecule P-ribosylaminoimidazole (AIR), which is converted to form the red pigment. However, if yeast strain loses the ability to carry out respiration (due to loss or damage of mitochondrial genome) cells will turn white. This provides the simple method for Hmi1p mitochondrial transport detection. If the helicase freely gets into the matrix then mtDNA is stably maintained and yeast forms red colonies. On the contrary, when Hmi1p translocation is impeded the mitochondrial genome becomes unstable and white colonies appear. Occasionally, yeast colonies appear as sectored, which means that both red and white cells are present in one colony. When Hmi1p mitochondrial transport is not fully abolished and proceeds with low efficiency the number of Hmi1 helicase molecules inside the matrix will be smaller. Under this condition mitochondrial genome is only partially maintained and, as a result, only few red colonies will appear. The majority of cells will exhibit the mixed phenotype or will be white indicating the lack of Hmi1p helicase in the mitochondrial matrix. However, a few sectored yeast cells could appear even if wild type HMI1 gene is expressed. Production of red pigment is unfavorable to yeast. Red strains will always grow slower than isogenic white strains. This results in a selection for any mutants that may prevent the red pigment from being produced. Since there are at least six genes functioning before ADE2 the spontaneous mutation rate could be quite high.

C-terminal deletion mutants of Hmi1p helicase

In the present study C-terminal deletion mutants of Hmi1p with MTS shortened by 5 to 32 amino acids were made (Fig. 10 and 15), and their ability to translocate into mitochondria was examined.

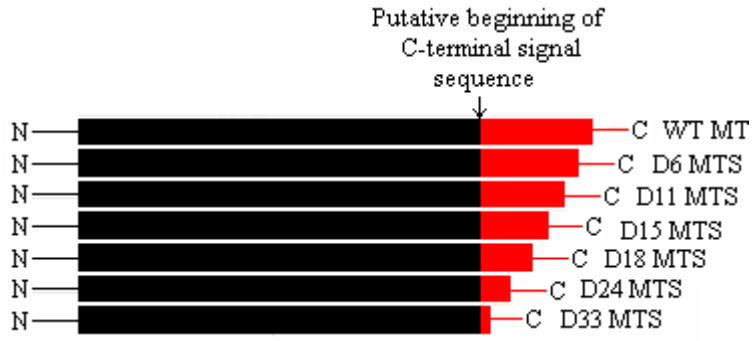

Fig 10. Hmi1p C-terminal deletion mutants. Black bar represents the catalytic part of the helicase. The red bar represents mitochondrial targeting sequence. Shortened signal sequences are shown, beginning with full-length wt MTS and ending with targeting sequence shortened by 32 amino acids (D33 MTS).

The results of mitochondrial transport of Hmi1p C-terminal deletion mutants are summarized in Figure 11. As we can see from this chart even the wild type helicase used for positive control does not provide 100% of red colonies. This result appears because mitochondrial genome may spontaneously transform into rho^0 or rho^- state. In fact, yeast population containing the wild type HMI1 gene numbered only about 80% of the red cells and about 15% of the white ones. The average percentage of sectored colonies was quite low, about 5%.

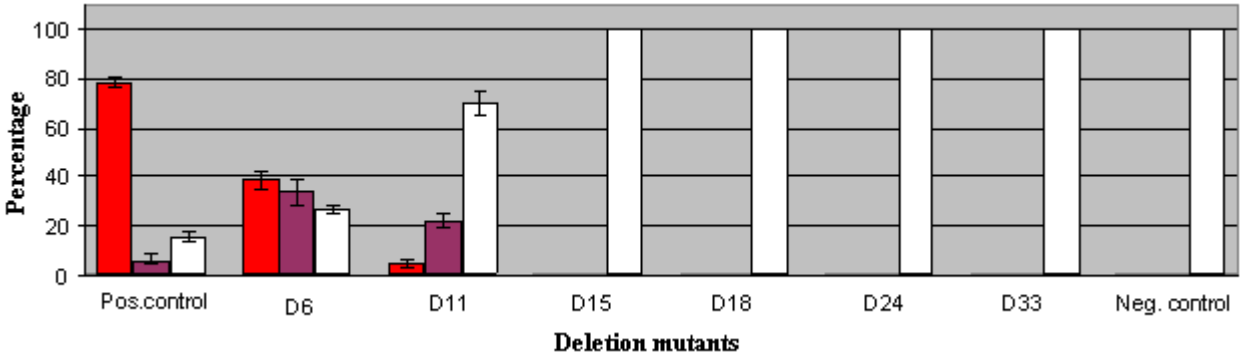

Fig 11. Average percentage of yeast colonies of different color. The red bars represent percentage of red colonies; the white and magenta bars represent numbers of white and sectored colonies correspondingly.

Deletion of just 5 amino acids from C-terminus of Hmi1p helicase resulted in a serious drop in Hmi1p import efficiency and, as a result, in mtDNA stability. In this population red colonies accounted for about 40%, while the white and sectored ones comprised

correspondingly about 25 and 35% of all colonies in this culture. This decrease in efficiency of Hmi1p mitochondrial transport could be explained by the absence of two positively charged arginines (Arg704 and Arg705). These residues may be important for interactions with components of the TOM complex. Without them weaker contacts are formed, which may result in a less efficient protein translocation.

Deletion of 10 amino acids (D11) almost fully abolished the Hmi1p import into mitochondria. Only 5% of colonies exhibited red phenotype, while the majority of cells were white (about 70%). Sectored cells comprised approximately 25% of all colonies. In signal sequence of Hmi1p with 10 C-terminal amino acids deleted two additional positively charged residues were absent. These are Arg697 and Lys700. If we look at the structure of Hmi1p signal sequence (Fig. 12), all four positively charged residues (Arg697, Lys700, Arg704 and Arg705) will appear on one side of this α-helix. This means that all four residues could be essential for interactions with TOM complex components and for overall Hmi1p mitochondrial transport.

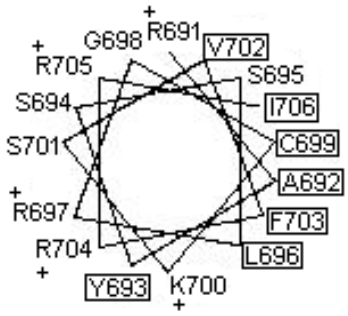

Fig 12. Representation of α-helix of the Hmi1 18 C-terminal amino acids starting from Arg691. Hydrophobic residues are indicated with boxes, and a positively charged residues are indicated with "+".

The last four deletion mutants of Hmi1p (D15 to D33) were not able to penetrate mitochondria and resulted in 100% of white colonies in every culture. This result indicates that Hmi1p with D15 signal sequence is a minimal deletion mutant required for termination of helicase mitochondrial transport.

Mitochondrial transport of Hmi1p with N-terminal signal sequences

The presence of N-terminal mitochondrial targeting sequence is favorable to cells. After synthesis on cytosolic ribosomes preproteins with N-terminal MTS could be cotranslationally transported into mitochondria and no additional energy for preprotein unfolding is needed. Despite this, Hmi1 helicase possesses MTS at its carboxyl-terminus. In addition, some polypeptides like mitochondrial inner membrane protein Bcs1p have internal signal sequences. Previous studies showed that both Hmi1p and Bcs1p were able to efficiently penetrate mitochondria in abnormal C- to N-terminal direction (Fölsch *et. al.*, 1996 and Lee *et. al.*, 1999). This raised question whether Hmi1p signal peptide must be C-terminal or the enzyme with N-terminal MTS will be imported as well.

To solve this problem we made pRS315-NcoHMI1 construct containing NcoI endonuclease restriction site just at HMI1 gene start codon region. DNA sequences encoding amino acids Thr672 – Ile706 and Arg691 – Ile706 of Hmi1 helicase, and amino acids Met1 – Lys63 of Pif1 helicase were cloned into pRS315-NcoHMI1 containing wild type, D15 and D33 C-terminal signal sequences. Thus, totally 9 constructs namely (C-terminus/N-terminus) WT/H15, WT/H35, WT/Pif1, D15/H15, D15/H35, D15/Pif1, D33/H15, D33/H35 and D33/Pif1 were obtained (Fig. 13).

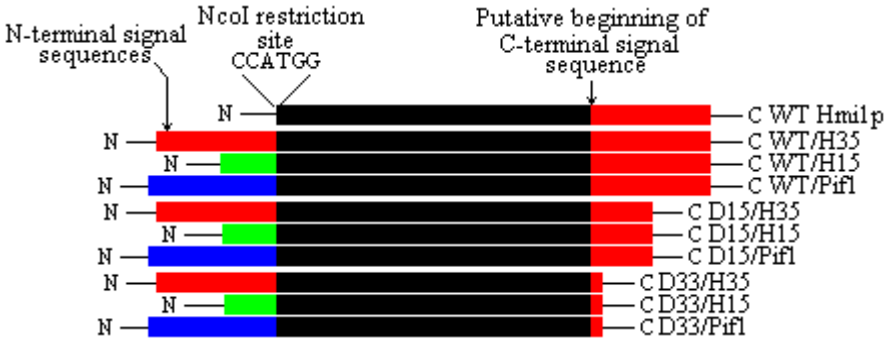

Fig 13. Hmi1 helicase with different N-terminal signal sequences. Black bars represent the Hmi1 helicase. Long red bars represent Hmi1 full-length signal sequences. Short red bars at C-terminus represent D15 or D33 deletion mutants. Green bars represent 15 amino acids from carboxyl end transported to the N-terminus. Blue bars represent N-terminal signal sequences from Pif1 helicase.

The results of mitochondrial transport of Hmi1p with different N- and C-terminal mitochondrial targeting sequences are summarized in Figure 14. Two positive controls were used in this experiment. The first one contained wild type HMI1 gene (assigned in chart as Pos. control) and the other contained HMI1 gene with NcoI restriction site at the start codon region (assigned in chart as NcoHMI1). Expression of the wt HMI1 gene resulted in appearance of approximately 75% of red colonies. Sectored and white cells comprised correspondingly 12 and 13% of all colonies.

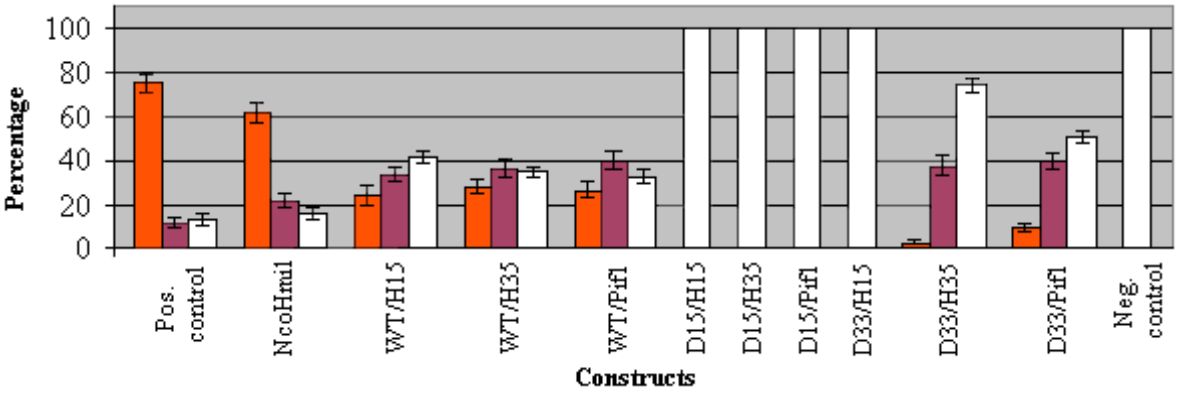

Fig 14. Average percentage of yeast colonies on FOA plates. Different constructs expressing Hmi1p helicase with distinct C-terminal/N-terminal signal sequences are named. H15 and H35 are stated respectively for 15 and 35 amino acids from Hmi1p carboxyl end put to the N-terminus. Pif1 is stated for 63 amino acids from N-terminus of Pif1 helicase put to the N-terminus of Hmi1 helicase. Red bars represent percentage of red colonies; white bars represent white colonies and magenta bars, sectored colonies.

These results are in good correlation with previously described for Hmi1p deletion mutants where percents were 80, 5 and 15 for red, sectored and white colonies respectively. Similar outcome was obtained expressing NcoHMI1 gene. Here, percentage of red colonies was a little bit lower than for the wild type helicase comprising about 65% of all cells. However, the number of sectored colonies increased to 20%, while percentage of white ones remained stable at about 15%.

Because C- to N-terminal transport proceeds with similar efficiencies as N- to C-terminal transport we expected Hmi1 helicase with N-terminal signal sequences to be effectively translocated across outer and inner mitochondrial membranes. However, helicase containing wild type C-terminal signal sequence and 15 amino acids from carboxyl end at N-terminus showed reduced efficiency in mitochondrial transport. Approximately three

times less red colonies (25%) appeared comparing to positive control and NcoHMI1. About 40% of colonies were of white color or sectored (35%), indicating decrease in the ability of Hmi1p to penetrate mitochondria. Similar results were obtained for Hmi1p containing wild type C-terminal MTS and full length Hmi1 or Pif1 targeting sequences at N-terminus. Only about 25% of all colonies were red. Sectored cells comprised approximately 40% and white took about 35% in both WT/H35 and WT/Pif1.

Out of nine constructs four resulted in 100% of white colonies. Two of them (D15/H15 and D33/H15) were actually predicted to give white cells, as signal sequences on both sides of the protein were clearly too short to provide mitochondrial transport. However, other two variants of Hmi1 helicase (D15/H35 and D15/Pif1) were expected to penetrate mitochondria and give at least some red colonies. Surprisingly, even original N-terminal signal sequence of Pif1 helicase was not able to support protein import. Despite this, both types of Hmi1 helicases containing full-length N-terminal targeting sequences of Hmi1p or Pif1p, and no MTS at C-terminus (D33) were capable to enter mitochondria. Although the number of red colonies was very low, less than 2% for D33/H35 and nearly 9% for D33/Pif1, percentage of sectored cells in both cases was about 40%, close to that of WT/H15, WT/H35 and WT/Pif1. White cells comprised 75% and 50% correspondingly for D33/H35 and D33/Pif1. In summary, it is possible to say that the import of Hmi1p with one MTS in all cases was more successful than with two signal sequences. Helicases possessing wild type MTS and any N-terminal targeting sequence were imported less effectively than helicases with only one original MTS at C-terminus.

Mitochondrial transport of Hmi1p containing full-length C-terminal MTS with altered amino acids

The C-terminus of Hmi1 helicase does not contain any negatively charged residues and can be predicted by *in silico* analysis to form an amphiphilic α-helix (Fig. 15). In the present study we wanted to investigate whether only strict α-helical structure of signal sequence is able to mediate mitochondrial transport of Hmi1p or MTS with abnormal secondary structure can substitute. For this purpose we performed oligonucleotide

directed mutagenesis and made four different mutants of Hmi1 helicase. In these mutants original amino acids Ser665, Gly674, Asn686 and Gly688 were replaced with proline residues (Fig. 15). Introducing these replacements we wanted to destabilize the original structure of mitochondrial targeting sequence in different positions and analyze the effect of these changes to protein import.

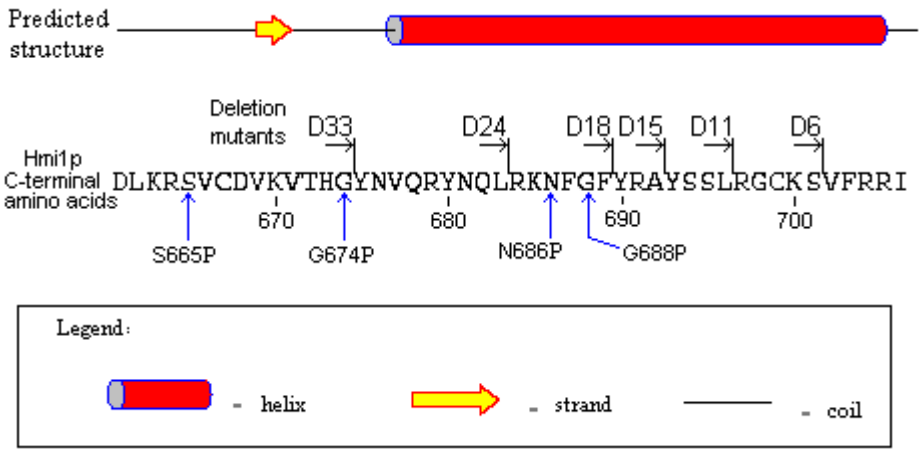

Fig 15. Putative structure of Hmi1p signal sequence. Putative secondary structure of 46 carboxyl-terminal amino acids region from Hmi1 helicase predicted using PSIPRED program. Blue arrows indicate the original residues substituted for prolines.

Like in previous experiments we used the wild type Hmi1p as a positive control and empty pRS315 vector as a negative control (Fig. 16). Similarly to our previous results about 70% of red colonies appeared on FOA plates with yeast cells expressing wild type Hmi1 helicase. 20% of colonies were of white color, and sectored ones comprised about 10%. Surprisingly, the biggest negative effect on Hmi1p mitochondrial transport had mutation at position S665P, situated before the beginning of predicted α-helix. Only 40% of all colonies were of red color and 25% of sectored ones. About one third of colonies were of white color indicating the decrease in Hmi1p mitochondrial import efficiency. The least negative effect gave the mutation at position N686P, which is located near the center of α-helix. Here, the number of all types of colonies was very close to those from positive control 60, 25 and 15% for correspondingly red, white and sectored cells. Mutations at positions G674P situated near the beginning of α-helix and G688P, which is located near the center of Hmi1p mitochondrial targeting sequence resulted in analogous effect to Hmi1p mitochondrial transport. In both cases helicase mitochondrial transport

was a little bit less efficient than in the case of wt Hmi1p or N686P. The red colonies comprised about 55%, white 25% and mixed colonies about 20%.

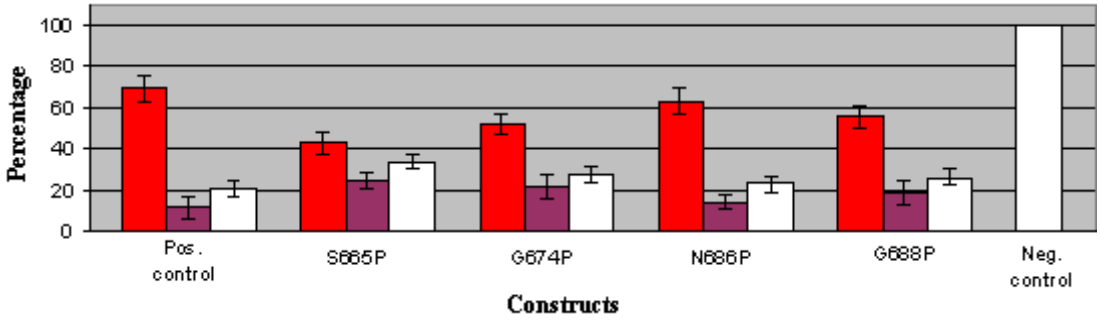

Fig 16. Average percentage of yeast colonies containing pRS315-HMI1 with mutated signal sequences. Constructs are named accordingly point mutation they contain. Red, white and magenta bars represent red, white and sectored colonies respectively.

In summary, the mitochondrial transport of Hmi1 helicase with G674, N686 and G688 amino acids substituted for proline residues was only slightly affected. Import efficiency of these constructs and wild type Hmi1p was very similar and quite high. The highest negative effect on mitochondrial transport has mutation at position S665. Substitution of this residue for proline resulted in approximately two times less efficient mitochondrial transport than in the case of wt Hmi1p.

DISCUSSION

The recently identified DNA helicase, Hmi1p, is located in the mitochondrial matrix. This enzyme is directed and imported into mitochondria via unusual C-terminal cleavable targeting sequence. Approximately 30 amino acids long MTS structurally forms amphipathic α-helix with positively charged residues on one side of the helix and hydrophobic residues on the other (Fig. 12). As demonstrated here, deletion of just 14 C-terminal residues totally prevents helicase from being transported into the matrix. Deleted sequence (Y693 – I706) contained 7 hydrophobic and 4 positively charged amino acids. Two docking sites for targeting sequences of preproteins, the Tom22 and Tom70 have many negatively charged residues on their cytosolic surfaces. Thus, Hmi1p amino acids Arg697, Lys700, Arg704 and Arg705 could be very important for interaction with TOM complex receptor components Tom20-Tom22 and Tom37-Tom70. The absence of four positively charged residues may result in a weaker interaction with Tom22 or Tom70, so that the mutant helicase would dissociate before it could be guided into the outer membrane translocation pore. In addition, the inability of D15 Hmi1 helicase to penetrate mitochondria could be explained by a poor binding to cytosolic chaperone. There are two different chaperons that may participate in preprotein targeting to mitochondrial surface. Hsp70 binds the whole protein preventing it from folding, and thus it is unlikely that Hsp70 – Hmi1p interaction could be destabilized by deletion of just 14 C-terminal residues. However, the other chaperone, MSF interacts directly with signal sequence and guides preproteins to mitochondrial surface (Fig. 4). It has been shown that this preprotein – MSF reciprocal action is largely depends on positively charged residues of mitochondrial targeting sequence (Komiya *et. al.,* 1994). Furthermore, the MSF binding to the outer membrane occurs only in the presence of functional MTS. Thus, deletion of about half of signal sequence could result in an inefficient binding of MSF to the OM and, as a result, inability of D15 Hmi1p to penetrate mitochondria. Unfortunately, there is no data about what pathway is used for Hmi1p direction to mitochondrial surface and further studies are needed to reveal the way of helicase targeting to TOM receptor components.

Nuclear-encoded mitochondrial matrix proteins generally contain N-terminal targeting signals and are imported in a linear N- to C-terminal fashion. However, it has been shown that mitochondrial import machinery displays no preference for the direction of the import process (Fölsch *et. al.,* 1998), and preproteins could be efficiently transported in C- to N-terminal way. Supporting these statement studies of mitochondrial transport of dehydrofolate reductase (DHFR) with signal sequences of Hmi1p and Bcs1p attached to DHFR C-terminus showed that chimeric protein accumulates in the mitochondrial matrix (Lee *et. al.,* 1999 and Fölsch *et. al.,* 1998).

In the present study we wanted to compare the efficiency of mitochondrial transport of Hmi1p with different N- and C-terminal MTS. We put 63 N-terminal amino acids of Pif1 helicase and 35 and 15 C-terminal amino acids of Hmi1p to the N-terminus of Hmi1 helicase and examined the mitochondrial transport of these chimeric polypeptides. Surprisingly, Hmi1p containing wt C-terminal signal sequence and full length Pif1 and Hmi1 targeting sequences, as well as WT/H15 construct were imported less efficiently than wild type helicase without N-terminal MTS. This brings the idea that two signal sequences in one protein may compete with each other for cellular resources needed for mitochondrial import. Indeed, both N- and C-terminal presequences require for cytosolic chaperones binding and could contend for subsequent interaction with TOM complex components. Supporting this theory, D33/H35 and D33/Pif1 proteins were imported poorly, but still more effectively than D15/H35 and D15/Pif1. About half of the wild type MTS is present on the C-terminus of D15/H35 and D15/Pif1 constructs. This remaining part of the carboxyl-terminal α-helix could interact with cytosolic chaperones and Tom20-Tom22 or Tom37-Tom70 complexes competing with N-terminal signal sequences. On the contrary, Hmi1p with 32 C-terminal amino acids deleted can not interact with MSF and TOM recognition complexes, and thus no competition between N- and C-terminal signal sequences can occur. However, there might be another reason explaining the decreased appearance of red yeast cells in population expressing Hmi1p with N-terminal signal sequences. The N-terminal peptide could interfere with normal activity of Hmi1p inside of the mitochondrial matrix. It is still unknown at what position Hmi1 signal sequences are exactly processed in mitochondrial matrix. Lee and coworkers

proposed that Arg691, which is followed by alanine and tyrosine, conforms to a classical mitochondrial processing peptidase cleavage motif. This means that after digestion of N-terminal H35 targeting sequence 14 amino acids are still present at the N-terminus of Hmi1 helicase. The H15 signal sequence would not be processed at all, as this peptide contains no residue corresponding to Arg691. Signal sequence of Pif1 helicase is digested at 45th residue, so there could be additionally 18 residues at the N-terminus of Hmi1p after processing of the full length Pif1 MTS. It is possible that these short leftovers of targeting sequences may somehow inhibit helicase activity, which results in a less stable mtDNA and higher percent of white or sectored colonies appearance.

Finally, the inability of Hmi1p to penetrate mitochondria with N-terminal signal sequence can be explained by cytoplasmic function of the helicase. When diploid yeast cells starve for nitrogen they differentiate into a filamentous growth form, and Hmi1 is one of several proteins regulating the appearance of this phenotype (Lorenz *et. al.,* 2000). In addition, it was found that Hmi1p make contacts with yeast Ste5 protein that participates in signal transduction pathways in cytoplasm (unpublished data). Clearly, Hmi1p with N-terminal MTS can not fulfill its cytoplasmic role, as it is cotranslationally transported into mitochondria. In contrast, helicase containing C-terminal signal sequence may fold and function for some time in cytoplasm before its mitochondrial import occurs. To test this possibility further investigation must be performed, and the mitochondrial transport of different Hmi1p mutants must be studied.

In α-helical structure of the MTS the polypeptide backbone is wound around the axis of the molecule and side groups of amino acid residues protrude outward from the helical backbone. A single turn of the helix extends for about 0.56nm. It contains 3.6 amino acid residues, and the whole structure is largely stabilized by hydrogen bonding. Angles of N – C_α and C_α – C bonds are very strict and comprise correspondingly -60^o and -45^o to -50^o. For these reasons proline residues are absolutely incompatible with α-helical protein structure. The nitrogen atom of proline is a part of a rigid ring, which prevents any rotations around the N – C_α bond. In addition, the nitrogen atom of a proline residue can not form any hydrogen bonds in peptide linkage. In fact, prolines are very rarely found

within α-helical structures. As it was shown above, substitution of the amino acid S665 for proline had the highest negative effect on Hmi1 mitochondrial import. The overall efficiency of Hmi1p mitochondrial transport was close to that of D6 Hmi1 helicase. However, serine 665 is situated beyond the α-helix predicted for MTS. It is possible to say that this point mutation can affect the activity of Hmi1p, which result in a less stable mtDNA. Substitution of other three residues in Hmi1p targeting sequence for proline had only minor effect on helicase mitochondrial transport. Indeed, incorporation of single proline can not seriously preclude the import of Hmi1p. The overall effect of one proline on tertiary structure of MTS would be negligible and additional mutations must be introduced into targeting sequence in order to obtain constructs containing two or three proline residues at the same time. This will allow more detailed investigation of influence of MTS topology to mitochondrial transport.

In summary, the mitochondrial transport of Hmi1 helicase is strongly depends on the presence of full length C-terminal targeting sequence. Studies of mitochondrial import of Hmi1p with different N-terminal signal sequences showed that even original N-terminal MTS of Pif1 helicase can not provide efficient mitochondrial transport of Hmi1p. Moreover, deletion of just 5 amino acids, or substitution of only one original residue for proline results in a less efficient transport of Him1 helicase comparing to Hmi1p with wt MTS.

SUMMARY

Mitochondria are organelles in eukaryotic cell. They have their own genome, whose stability is strongly depends on proteins encoded by nuclear DNA. Those proteins are synthesized in cytoplasm and directed into mitochondria via their mitochondrial targeting sequences. Two molecular chaperones play important role in mitochondrial transport, maintaining preproteins in the unfolded state and delivering them to the surface of the organelle. On the exterior of the mitochondria the outer membrane translocase complex interacts with signal sequence of preprotein and transports it through the outer membrane. In intermembrane space preprotein is recognized by inner membrane translocase complex components and directed into the matrix. In the mitochondrial matrix signal sequence of protein is removed and mitochondrial chaperonins maintain the proper folding of matrix resident protein.

Hmi1p is the helicase that functions in the mitochondrial matrix. It has unusual C-terminal MTS and transported into mitochondria in uncommon C- to N-terminal direction. In the present study we investigated the mitochondrial import of Hmi1p containing C-terminal signal sequences of different length and found that removal of 14 amino acids from C-terminus results in the inability of helicase to penetrate mitochondria. Studies of mitochondrial transport of mutant Hmi1p showed that mitochondrial transport of helicase containing different N-terminal signal sequences proceeds with minor efficiency, and only when C-terminal MTS is removed. Substitution of original amino acids S665, G674, N686 and G688 in wt targeting sequence of Hmi1p for prolines showed that destabilization of α-helical structure of MTS only slightly affect the mitochondrial transport of the helicase. Mutant proteins are successfully imported with efficiency close to that of the wild type protein.

KOKKUVÕTE

Mitokondrid on eukarüootsete rakkude organellid. Mitokondrid omavad oma genoomi, mille stabiilsus sõltub tuuma genoomi poolt kodeeritud valkudest, mis sünteesitakse tsütoplasmas ja suunatakse mitokondrisse spetsiaalsete N-terminaalsete signaaljärjestuste abil. Mitokondriaalses transpordis omavad olulist rolli kaks molekulaarset tšaperooni, mis takistavad prevalgu argregeerumist ja viivad selle mitokondri välispinnale. Välismembraani translokaasi komponendid interakteeruvad prevalguga ja transpordivad selle mitokondri intermembraansesse ruumi, kus preproteiin tuntakse ära sisemembraani translokaasi komponentide poolt ja suunatakse maatriksisse. Mitokondriaalses maatriksis preproteiini signaaljärjestus lõigatakse maha ja valk saavutab oma õige tertsiaarstruktuuri mitokondriaalsete tšaperoniinide abil.

Hmi1 on tuuma poolt kodeeritud mitokondri maatriksi helikaas. Selle helikaasi import mitokondrisse toimub ebatavalise C-terminaalse signaaljärjestuse abil C – N suunas. Antud töös uuriti Hmi1 mitokondriaalset transporti ja leiti, et nimetatud valku ei transpordita mitokondrisse, kui temalt on deleteeritud 14 C-terminaalset aminohapet.
Mutantsete Hmi1 helikaaside abil näidati, et Hmi1 mitokondriaalne transport N-terminaalse signaaljärjestuse abil on ebaefektiivne ja võimalik ainult siis, kui C-terminaalne signaaljärjestus on elimineeritud. Aminohapete S665, G674, N686 ja G688 asendamine proliiniga Hmi1 C-terminaalses signaaljärjestuses näitas, et α-helikaase struktuuri destabiliseerimine ei takista mitokondriaalset transporti. Mutantseid helikaase, milledes üks signaalpeptiidi aminohape oli asendatud proliiniga, transporditi mitokondrisse sarnase efektiivsusega kui metsiktüüpi Hmi1p.

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REFERENCES

Attardi, G. and G. Schatz (1988). "Biogenesis of mitochondria." Annu Rev Cell Biol **4**: 289-333.

Blanc, H. and B. Dujon (1980). "Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness." Proc Natl Acad Sci U S A **77**(7): 3942-6.

Blom, J., M. Kubrich, et al. (1993). "The essential yeast protein MIM44 (encoded by MPI1) is involved in an early step of preprotein translocation across the mitochondrial inner membrane." Mol Cell Biol **13**(12): 7364-71.

Boeke, J. D., F. LaCroute, et al. (1984). "A positive selection for mutants lacking orotidine-5'-phosphate decarboxylase activity in yeast: 5-fluoro-orotic acid resistance." Mol Gen Genet **197**(2): 345-6.

Branda, S. S. and G. Isaya (1995). "Prediction and identification of new natural substrates of the yeast mitochondrial intermediate peptidase." J Biol Chem **270**(45): 27366-73.

Chen, X. J., M. X. Guan, et al. (1993). "MGM101, a nuclear gene involved in maintenance of the mitochondrial genome in Saccharomyces cerevisiae." Nucleic Acids Res **21**(15): 3473-7.

Daum, G., P. C. Bohni, et al. (1982). "Import of proteins into mitochondria. Cytochrome b2 and cytochrome c peroxidase are located in the intermembrane space of yeast mitochondria." J Biol Chem **257**(21): 13028-33.

de Zamaroczy, M. and G. Bernardi (1986). "The GC clusters of the mitochondrial genome of yeast and their evolutionary origin." Gene **41**(1): 1-22.

de Zamaroczy, M., G. Faugeron-Fonty, et al. (1983). "Excision sequences in the mitochondrial genome of yeast." Gene **21**(3): 193-202.

Dujon, B. (1980). "Sequence of the intron and flanking exons of the mitochondrial 21S rRNA gene of yeast strains having different alleles at the omega and rib-1 loci." Cell **20**(1): 185-97.

Endo, T. (1991). "Co-operative binding of hsp60 may promote transfer from hsp70 and correct folding of imported proteins in mitochondria [corrected]." FEBS Lett **293**(1-2): 1-3.

Fangman, W. L., J. W. Henly, et al. (1990). "RPO41-independent maintenance of [rho-] mitochondrial DNA in Saccharomyces cerevisiae." Mol Cell Biol **10**(1): 10-5.

Folsch, H., B. Gaume, et al. (1998). "C- to N-terminal translocation of preproteins into mitochondria." Embo J **17**(22): 6508-15.

Folsch, H., B. Guiard, et al. (1996). "Internal targeting signal of the BCS1 protein: a novel mechanism of import into mitochondria." Embo J **15**(3): 479-87.

Foury, F., T. Roganti, et al. (1998). "The complete sequence of the mitochondrial genome of Saccharomyces cerevisiae." FEBS Lett **440**(3): 325-31.

Greenleaf, A. L., J. L. Kelly, et al. (1986). "Yeast RPO41 gene product is required for transcription and maintenance of the mitochondrial genome." Proc Natl Acad Sci U S A **83**(10): 3391-4.

Hachiya, N., T. Komiya, et al. (1994). "MSF, a novel cytoplasmic chaperone which functions in precursor targeting to mitochondria." Embo J **13**(21): 5146-54.

Herrmann, J. M. and W. Neupert (2000). "Protein transport into mitochondria." Curr Opin Microbiol **3**(2): 210-4.

Hines, V. and G. Schatz (1993). "Precursor binding to yeast mitochondria. A general role for the outer membrane protein Mas70p." J Biol Chem **268**(1): 449-54.

Huang, S., K. S. Ratliff, et al. (2002). "Protein unfolding by the mitochondrial membrane potential." Nat Struct Biol **9**(4): 301-7.

Hwang, S., T. Jascur, et al. (1989). "Disrupted yeast mitochondria can import precursor proteins directly through their inner membrane." J Cell Biol **109**(2): 487-93.

Isaya, G., D. Miklos, et al. (1994). "MIP1, a new yeast gene homologous to the rat mitochondrial intermediate peptidase gene, is required for oxidative metabolism in Saccharomyces cerevisiae." Mol Cell Biol **14**(8): 5603-16.

Jimenez, J., E. Longo, et al. (1988). "Induction of petite yeast mutants by membrane-active agents." Appl Environ Microbiol **54**(12): 3126-32.

Kang, Y. W. and D. L. Miller (1989). "Structure and transcription of the mitochondrial genome in heteroplasmic strains of Saccharomyces cerevisiae." Nucleic Acids Res **17**(21): 8595-609.

Komiya, T., N. Hachiya, et al. (1994). "Recognition of mitochondria-targeting signals by a cytosolic import stimulation factor, MSF." J Biol Chem **269**(49): 30893-7.

Kubrich, M., P. Keil, et al. (1994). "The polytopic mitochondrial inner membrane proteins MIM17 and MIM23 operate at the same preprotein import site." FEBS Lett **349**(2): 222-8.

Kuusk, S., T. Sedman, et al. (2005). "Hmi1p from Saccharomyces cerevisiae mitochondria is a structure specific DNA helicase." J Biol Chem.

Lahaye, A., H. Stahl, et al. (1991). "PIF1: a DNA helicase in yeast mitochondria." Embo J **10**(4): 997-1007.

Lahaye, A., S. Leterme, et al. (1993). "PIF1 DNA helicase from Saccharomyces cerevisiae. Biochemical characterization of the enzyme." J Biol Chem **268**(35): 26155-61.

Lee, C. M., J. Sedman, et al. (1999). "The DNA helicase, Hmi1p, is transported into mitochondria by a C-terminal cleavable targeting signal." J Biol Chem **274**(30): 20937-42.

Lodish, H., Berk, A, et al. (2002). “Molecular cell biology”

Lorenz, M. C., N. S. Cutler, et al. (2000). "Characterization of alcohol-induced filamentous growth in Saccharomyces cerevisiae." Mol Biol Cell **11**(1): 183-99.

Maleszka, R., P. J. Skelly, et al. (1991). "Rolling circle replication of DNA in yeast mitochondria." Embo J **10**(12): 3923-9.

Mayer, A., F. E. Nargang, et al. (1995). "MOM22 is a receptor for mitochondrial targeting sequences and cooperates with MOM19." Embo J **14**(17): 4204-11.

Meeusen, S., Q. Tieu, et al. (1999). "Mgm101p is a novel component of the mitochondrial nucleoid that binds DNA and is required for the repair of oxidatively damaged mitochondrial DNA." J Cell Biol **145**(2): 291-304.

Moraes, C. T. (2001). "What regulates mitochondrial DNA copy number in animal cells?" Trends Genet **17**(4): 199-205.

Nakai, M. and T. Endo (1995). "Identification of yeast MAS17 encoding the functional counterpart of the mitochondrial receptor complex protein MOM22 of Neurospora crassa." FEBS Lett **357**(2): 202-6.

Neupert, W. and M. Brunner (2002). "The protein import motor of mitochondria." Nat Rev Mol Cell Biol **3**(8): 555-65.

Neupert, W. (1997). "Protein import into mitochondria." Annu Rev Biochem **66**: 863-917.

Oxelmark, E., A. Marchini, et al. (2000). "Mmf1p, a novel yeast mitochondrial protein conserved throughout evolution and involved in maintenance of the mitochondrial genome." Mol Cell Biol **20**(20): 7784-97.

Rospert, S., R. Looser, et al. (1996). "Hsp60-independent protein folding in the matrix of yeast mitochondria." Embo J **15**(4): 764-74.

Rowley, N., C. Prip-Buus, et al. (1994). "Mdj1p, a novel chaperone of the DnaJ family, is involved in mitochondrial biogenesis and protein folding." Cell **77**(2): 249-59.

Ryan, M. T., R. Wagner, et al. (2000). "The transport machinery for the import of preproteins across the outer mitochondrial membrane." Int J Biochem Cell Biol **32**(1): 13-21.

Schatz, G. (1996). "The protein import system of mitochondria." J Biol Chem **271**(50): 31763-6.

Schlossmann, J., R. Lill, et al. (1996). "Tom71, a novel homologue of the mitochondrial preprotein receptor Tom70." J Biol Chem **271**(30): 17890-5.

Sedman, T., P. Joers, et al. (2005). "Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial DNA molecules in yeast Saccharomyces cerevisiae." Curr Genet **47**(4): 213-22.

Sedman, T., S. Kuusk, et al. (2000). "A DNA helicase required for maintenance of the functional mitochondrial genome in Saccharomyces cerevisiae." Mol Cell Biol **20**(5): 1816-24.

Sesaki, H., S. M. Southard, et al. (2003). "Mgm1p, a dynamin-related GTPase, is essential for fusion of the mitochondrial outer membrane." Mol Biol Cell **14**(6): 2342-56.

Sikorski, R. S. and P. Hieter (1989). "A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in Saccharomyces cerevisiae." Genetics **122**(1): 19-27.

Sinclair, J. H. and B. J. Stevens (1966). "Circular DNA filaments from mouse mitochondria." Proc Natl Acad Sci U S A **56**(2): 508-14.

Urban, S., J. R. Lee, et al. (2001). "Drosophila rhomboid-1 defines a family of putative intramembrane serine proteases." Cell **107**(2): 173-82.

Wang, Y. and G. S. Shadel (1999). "Stability of the mitochondrial genome requires an amino-terminal domain of yeast mitochondrial RNA polymerase." Proc Natl Acad Sci U S A **96**(14): 8046-51.

Williamson, D. (2002). "The curious history of yeast mitochondrial DNA." Nat Rev Genet **3**(6): 475-81.

Zheng, X., L. E. Rosenberg, et al. (1993). "GroEL, GroES, and ATP-dependent folding and spontaneous assembly of ornithine transcarbamylase." J Biol Chem **268**(10): 7489-93.