STUDIES OF THE MITOCHONDRIAL HELICASE Hmi1p IN *CANDIDA ALBICANS* AND *SACCHAROMYCES CEREVISIAE*

PRIIT JÕERS

TARTU 2006

Department of Biochemistry, Institute of Molecular and Cell Biology, University of Tartu, Estonia

Dissertation was accepted for the commencement of the degree of Doctor of Philosophy (in Biochemistry) on November 23rd, 2006 by the council of the Institute of Molecular and Cell Biology, University of Tartu.

Opponent: Lubomir Tomaska, Associate Professor, Head of the Department of Genetics, PhD (Comenius University, Bratislava, Slovakia).

Commencement: January 15, 2007, Room 217, Riia 23, Tartu

The publication of this dissertation is granted by the University of Tartu

ISSN 1024–6479
ISBN 9949–11–510–8 (trükis)
ISBN 9949–11–511–6 (PDF)

Tartu Ülikooli Kirjastus
www.tyk.ee
Tellimus nr 648

CONTENTS

LIST OF ORIGINAL PUBLICATIONS

The current thesis is based on the following original publications referred to in the text by their Roman numerals:

I. **Joers, P**., Gerhold, J.M., Sedman, T., Kuusk, S., Sedman J. (2006) The helicase CaHmi1p is required for wild-type mitochondrial DNA organization in *Candida albicans*, *FEMS Yeast Res*, accepted.

II. Kuusk, S., Sedman, T., **Joers, P**., Sedman, J. (2005) Hmi1p from *Saccharomyces cerevisiae* mitochondria is a structure-specific DNA helicase. *J Biol Chem*, **280**, 24322–24329.

III. Sedman, T., **Joers, P**., Kuusk, S., Sedman, J. (2005) Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial DNA molecules in yeast *Saccharomyces cerevisiae*. *Curr Genet*, **47**, 213–222.

My contribution to the articles referred to in current the thesis is as follows:

Ref. I – designed and performed the experiments, analysed the experimental data and participated in writing of the paper.

Ref. II – participated in data analysis and writing of the paper.

Ref III – designed and performed the experiments, analysed the experimental data and participated in writing of the paper.

ABBREVIATIONS

5'-FOA – 5'-fluoro-orotic acid

ARS/CEN – autonomous replicating sequence/centromeric element

DAPI – 4',6-diamidino-2-phenylindole

EM – electron microscopy

ETC – electron transport chain

GCN – general amino-acid control pathway

HMG – high-mobility group

hs – hypersuppressive

mt – mitochondrial

MR – MitoTracker Red

MTS – mitochondrial targeting sequence

PFGE – pulsed field gel electrophoresis

PH – pseudohyphal

RFB – replication fork block

RFLM – restriction fragment length mapping

SF1 – helicase superfamily 1

SHAM – salicyl-hydroxamic acid

1. INTRODUCTION

The mitochondrion is the only organelle in a non-photosynthesising eukaryotic cell that possesses its own genome. Mitochondrial DNA (mtDNA) is believed to be a heavily modified remnant of the mitochondrion's free-living ancestor genome. In the course of evolution most genes were either transferred to the nucleus or lost altogether. Nevertheless, the mt genome still codes for some factors that are vital for oxidative phosphorylation. Faithful replication and maintenance of mtDNA is therefore a prerequisite for viability of multicellular organisms. Defects in these processes inflicting alterations in mtDNA sequences can cause serious disorders in humans (reviewed in Schapira et al., 2006). Studies of mtDNA maintenance in mammals are hampered by complex genetics, tissue-specific density variations and uniparental inheritance of mitochondria. The species of the taxonomic group *Ascomycota* combine the simplicity and powerful genetical approaches of unicellular organisms with basic mechanisms similar to higher eukaryotes. These properties have made the best-studied yeast *Saccharomyces cerevisiae* the prime target for mtDNA studies. The ability of this organism to survive without functional mtDNA distinguishes *S. cerevisiae* from higher eukaryotes. Another yeast, the petite-negative *Candida albicans* shares the stringent requirement for intact mt genome and might therefore prove to be a valuable model in mtDNA maintenance studies. This yeast is also a major fungal pathogen in humans, causing life-threatening infections in immunocompromised patients.

Several nucleic acid transactions like recombination, replication and transcription require separation of double-stranded DNA. Therefore mtDNA maintenance and gene expression require enzymes called helicases that catalyse the separation of DNA strands. Up to date there are two DNA helicases whose presence in mitochondria is proven: Pif1p and Hmi1p. Although the latter of these two enzymes was recently characterised by our group and shown not to be involved in transcription (Sedman et al., 2000), its precise biological function has remained an open question. This thesis literature overview describes the data available on topology, organisation, synthesis and inheritance of yeast mtDNA with focus on *S. cerevisiae*. It also covers the petite-positive phenomenon, which is the major difference between mitochondrial systems of *S. cerevisiae* and *C. albicans*. The role of known proteins involved in mtDNA maintenance and inheritance are discussed. My research has been focused on further characterisation of Hmi1p-s role in mtDNA maintenance in an effort to unravel its function. This includes the characterisation of its homologue in *C. albicans*. Although no exhaustive proof is given for the function of Hmi1p, evidence presented here allows to propose its function in mtDNA synthesis. The effects of Hmi1p deletion on *C. albicans* mtDNA also demonstrate the value of this model organism in further mtDNA studies.

2. REVIEW OF LITERATURE

2.1. Ascomycotic mtDNA organisation

2.1.1. Size and coding capacity of mtDNA

The discovery of mitochondrial DNA (mtDNA) triggered the endosymbiosis theory, according to which the mitochondrion is a monophyletic remnant of α-proteobacteria, embedded by an ancestor of modern eukaryotes (reviewed in Gray et al., 1999). Few existing amitochondrial eukaryotes are considered to have lost the mitochondrion or modified it to another related organelle referred to as hydrogenosome, rather than being diverged from the main phylogenetic tree before the endosymbiosis event (Horner et al., 1996, Bui et al., 1996). By retaining the membrane of the engulfed organism required for build-up of a proton gradient for oxidative phosphorylation, the ancestral eukaryote was able to free its own plasma membrane for other tasks, thus opening a way to the formation of multicellular organisms. Gradual reduction of the mitochondrial genome (mt genome) followed as most of its genes were lost or transferred into the nucleus and the electron transport chain (ETC) was tailored to suit the host organism's requirements.

Table 1. Size and coding capacity comparison of mt genomes between different yeast species. Var1p codes for a ribosome-associated protein and 9S RNA is a RNA component of RNase P. ETC ORF-s are either a selection or complete sets of the genes *cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *atp6*, *atp8*, *atp9*.

Species	Size (bp)	ETC ORF-s	rRNA/ tRNA	Other ORF-s	Reference
S. cerevisiae	85779	7	2/24	Var1p, 9S RNA	Foury et al., 1998
S. castellii	25753	7	2/24	Var1p, 9S RNA	Petersen et al., 2001
S. pombe	19431	7	2/25	9S RNA	Lang et al., 1983
C. albicans	40420	14	2/30	–	Anderson et al., 2001
C. parapsilosis	32745	14	2/24	–	Nosek et al., 2004
Y. lipolytica	47916	14	2/27	–	Kerscher et al., 2001
K. lactis	40291	7	2/21	Var1p	Zivanovic et al., 2005
P. canadiensis	27694	14	2/25	Var1p	Sekito et al., 1995
P. anserina	100314	13	2/27	Var1p	Cummings et al., 1990
C. glabrata	20063	7	2/23	Var1p, 9S RNA	Koszul et al., 2002

Abbreviations: *S. castellii – Saccharomyces castellii*, *S. pombe – Schizosaccharomyces pombe*, *C. parapsilosis – Candida parapsilosis*, *Y. lipolytica – Yarrowia lipolytica*, *K. lactis – Kluyveromyces lactis*, *P.canadiensis – Pichia canadiensis*, *P. anserina – Podospora anserina*, *C. glabrata – Candida glabrata*.

Ascomycota or lower fungi conjoin eukaryotic intracellular processes with several ecological and genetical benefits that makes them good model organisms. Members of this taxonomic group are mostly unicellular, occupying several ecological niches as free-living organisms or parasites. Mitochondrial genomes of this group display a great variability in size, ranging from ~20 kbp to ~100 kbp (Table 1). The size of a mt genome does not seem to have any direct link to the phylogenetical lineage, as quite big differences can be observed even within a relatively small taxonomic group. This is exemplified by comparison of two closely related yeast species *Saccharomyces cerevisiae* and *Saccharomyces castellii*, that have mtDNA with a length of 85 kbp and 25 kbp respectively (Foury et al., 1998, Petersen et al., 2002). Plasticity of mtDNA size is in contrast to the conservation of the coding potential since triplication in size is not accompanied by changes in gene content, which is identical between these two yeasts. Even species with large mt genomes like *Podospora anserina* have approximately similar numbers of genes than yeasts with substantially smaller mt genomes (Table 1). This indicates that the overall number of genes is far less amenable to changes than the length of mtDNA.

The coding potential of mt genomes is usually limited to subunits of ETC complexes and mitochondrial protein expression machinery (Table 1). Mitochondrial components of the latter comprise usually structural RNA-s required for translation (2 ribosomal RNA-s with the complete set of tRNA-s) and sometimes one or few proteins associated with mitochondrial ribosomes. The number of ETC subunits coded by mtDNA varies between different species, but usually does not exceed the set of 14 proteins. This collection of genes (*cob*, *cox1*, *cox2*, *cox3*, *nad1*, *nad2*, *nad3*, *nad4*, *nad4L*, *nad5*, *nad6*, *atp6*, *atp8*, *atp9*) codes for subunits of cytochrome c oxidase, cytochrome bc_1, NADH dehydrogenase and ATP synthetase multienzyme complexes.

2.1.2. Topology of mtDNA

Circular-mapping mt genomes

According to restriction fragment length mapping (RFLM) mitochondrial genomes can be divided into linear- or circular-mapping groups. The latter group comprises *S. cerevisiae*, the best studied member of ascomycotic division. A circular map in itself is not an exhaustive proof of circularity of DNA molecules *in vivo*, since it can be caused by linear head-to-tail concatemers and circularly permuted molecules. Early electron microscopy (EM) studies demonstrated the presence of circular mtDNA molecules with the size of mt genome monomers in *S. cerevisiae* alongside with a large number of linear DNA molecules (Hollenberg et al., 1969). At first these linear molecules were disregarded as a result of mechanical or enzymatical disruption of circular DNA molecules (reviewed in Williamson 2002). However, mounting evidence from pulsed-field

gel electrophoresis (PFGE) experiments suggest that the initial "broken circle" theory was incorrect. PFGE and moving-picture analysis of circular-mapping mt genomes from species like *Candida glabrata*, *Schizosaccharomyces pombe*, *Neurospora crassa* and *S. cerevisiae* have revealed several populations of mtDNA molecules with different topology (Skelly & Maleszka, 1989, Bendich & Smith, 1990, Maleszka et al., 1991, Bendich, 1996).

The majority of mtDNA molecules entering the gel in PFGE are polydisperse and linear, ranging between 50–100 Kbp. Surprisingly the heterogeneity of genome sizes between these species and even between different *S. cerevisiae* strains had no effect on the mobility of this population (Bendich, 1996). This may indicate that the length of these linear molecules is determined by the physical length of DNA rather than by the number of genome units.

A minor fraction of mtDNA can be observed in some experiments as two bands migrating between the compression zone (cz) and the well (Maleszka et al., 1991, Bendich, 1996). Since this area is restricted to linear molecules and introduction of single-stranded nicks in *C. glabrata* mtDNA preparations has been shown to reduce one of the bands and strengthen the other (Maleszka et al., 1991), they are believed to represent supercoiled and open forms of circular molecules. In any case, this fraction accounts only for a few percent of total mtDNA. This is in accordance with EM experiments, where circular DNA molecules were greatly outnumbered by linear molecules.

A substantial amount of mtDNA is formed by well-bound molecules that do not enter the gel. Moving-picture analysis of individual molecules from the well-bound fraction demonstrated the presence of condensated DNA structures with the shape of clots, comets and starbursts alongside with linear molecules with sizes from 25 kbp to several hundred kbp (Bendich, 1996, Jacobs et al., 1996). Behaviour of these complex structures in response to electric current and UV-irradiation suggests that they are comprised of condensed DNA regions along with double-strand (ds) and single-strand (ss) stretches and loops. In accordance with this, dsDNA molecules with ssDNA stretches were also observed in EM analysis of *C. glabrata* mtDNA (Maleszka et al., 1991). The shorter linear fragments are probably products of shearing of large immobile DNA complexes during sample preparation, since many of them are small enough to enter the gel. In conclusion, the majority of mtDNA from species with circular RFLM is present in larger molecules than mt genome unit length. Circular molecules are a small minority and *in vivo* topology is predominantly either linear or complex.

Linear-mapping mt genomes

In addition to circular-mapping genomes there is an increasing number of fungi where linear mt genomes have been reported (reviewed in Nosek et al., 1998). As demonstrated above, even in species with circular-mapping genomes the real *in vivo* conformation of mtDNA is predominantly linear. "True" linear mt

genomes are characterised by a linear RFLM and a distinct band in the PFGE analysis of mt genome unit length (Fukuhara et al. 1993, Jacobs et al., 1996, Nosek et al., 1998). Linear genophores are widespread and do not cluster into specific taxonomic groups. Indeed, linear mt genomes do not seem to be of monophyletic origin but rather have risen throughout the evolution in several occasions (Figure 1). Variability in mt genome organisation is encountered even between different strains of *Williopsis suaveolens* and *C. parapsilosis* species (Jacobs et al., 1996, Rycovska et al., 2004). This in turn indicates that the maintenance of linear and circular mt genomes probably does not require strikingly different mechanisms. However, if linear molecules are used as replication templates, organisms have to solve the end-replication problem of the lagging strand. Due to the antiparallel nature of DNA replication, the 3'-end becomes gradually shortened if no compensatory mechanism is involved. To date two different approaches for this problem have been characterised in yeast mitochondria, covalently closed ends and mitochondrial telomeres. The former solution is used by species from *Williopsis* and *Pichia* genera, where at the proximity of inverted terminal repeats ssDNA loops covalently join the ends of linear molecules (Dinouel et al., 1993). A different approach is used by *C. parapsilosis*, *Candida salmanticensis* and *Pichia philodendra*, where inverted repeats occupy the physical ends of mt genomes and a direct covalent link between two strands is absent (Nosek et al., 1995). These mitochondrial telomeric sequences consist of tandemly arranged repeat units of various lengths. In addition to mt genome ends, telomeres are present as extragenomic DNA circles termed t-circles (Tomaska et al., 2000). Telomeres are thought to be replicated from t-circles by rolling-circle replication to generate long tandem repeats which are added to the ends of linear genomes probably through a recombination-driven mechanism (Nosek et al., 2005). A telomeric 5'-overhang may direct this recombination event as there is a specific telomere-binding protein (mtTBP) that binds to and protects this 5'-overhang in ssDNA form (Tomaska et al., 1997, Nosek et al., 1999, Tomaska et al., 2001).

Wide distribution of linear genophores and different solutions for the end-replication problem suggests a polyphyletic origin of linear mt genomes. This means that yeasts with circular- and linear-mapping genomes use essentially the same maintenance machinery, although some modifications might have occurred as it is probably the case with mtTBP that shares several features with non-specific prokaryotic and mitochondrial DNA-binding proteins (Nosek et al., 1999). Although the cause for this widespread occurrence of linear mt genomes is unclear, insertion of selfish mobile elements that function as telomeric/resolution elements have been proposed (Nosek & Tomaska, 2003).

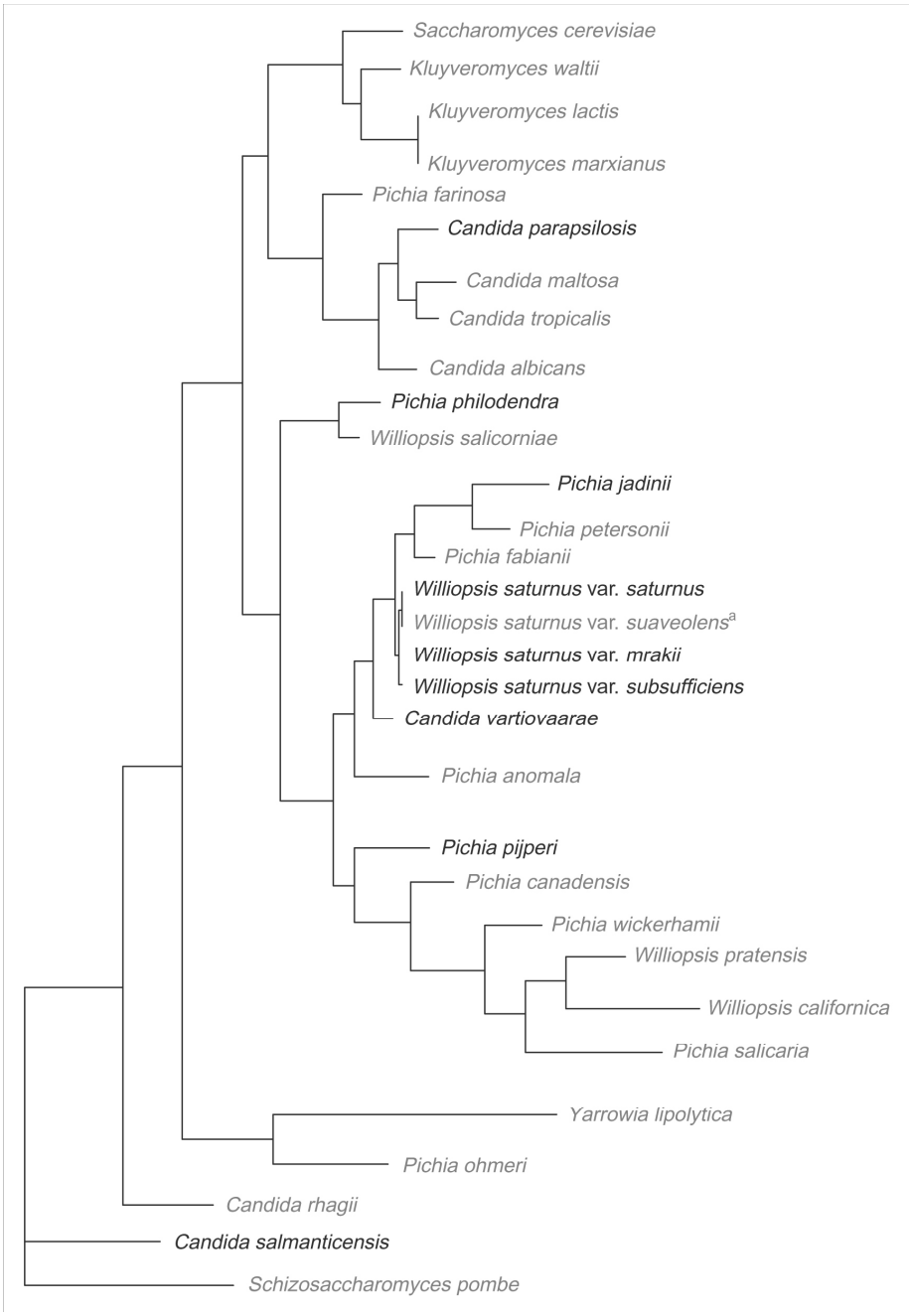


Figure 1. Distribution of linear mt genomes (reprinted from "Linear mitochondrial genomes: 30 years down the line", vol. 14, Nosek J., Tomaska, L., Fukuhara, H., Suyama, Y., Kovac, L., 184–188, copyright 1998, with permission from Elsevier). Yeasts with linear-mapping mt genomes are in black and species with circular-mapping genomes in gray.

2.2. Petite-mutation phenomenon

It has been established that yeasts can be divided into two groups according to their susceptibility to lose their wt mt genome (Bulder 1964). Yeasts that can survive and produce small colonies termed *petites* on a fermentable carbon source without functional mt genome are called petite-positive species. These species can have either wt mt genomes (ρ^+), mutated forms of mt genomes (ρ^-) or be completely devoid of mtDNA (ρ^0). In other yeast groups loss of mt genome proved to be lethal and these species were therefore termed petite-negative yeasts. The frequency of occurence is heavily in favour of petite-negative species that form the bulk of ascomycetes (Chen and Clark-Walker 1999). The petite-positive phenotypes are common only within two genera, *Saccharomyces* and *Brettanomyces/Dekkera*, plus in a few individual species that are dispersed between different monophyletic taxonomic groups (e.g. *C. glabrata*) (Bulder 1964, Hoeben *et al*. 1993, Defontaine *et al*. 1999). The distance between *Saccharomyces* and *Brettanomyces* genera in phylogenetic trees strongly suggests the polyphyletic origin of the petite-positive phenomenon. This also indicates that being petite-negative or ρ^0-lethal represents the original state of ascomycetes.

The ability for anaerobic growth and the petite-positive feature are two distinct phenomena. Genetic data propose that most petite-negative species have no biochemical restrictions for anaerobic growth. Indeed, respiration-deficient mutants can be isolated from a number of petite-negative species like *K. lactis*, *S. klyuveri*, *S. pombe* and *C. albicans* (Herman & Griffin 1968, Wolf *et al*. 1971, Moeller *et al*. 2001, Aoki & Ito-Kuwa 1987). However, as demonstrated for *S. klyuveri*, respiration-deficiency is not necessarily coupled with major alterations or loss of mtDNA (Moeller et al., 2001). Therefore susceptibility to petite-mutations is not caused by fermentative incapacity but is rather linked to the ability to tolerate the loss of some factor(s) encoded by the mtDNA. The essentiality of mtDNA in petite-negative yeasts was demonstrated by lethality of a mutant that disrupts mitochondrial protein synthesis in *K. lactis* (Pel *et al.*, 1996). An exception of this rule is *Debaryomyces occidentalis*, which can lose mt genome and still utilize non-fermentable substrates by salicyl-hydroxamic acid (SHAM)-sensitive pathway (Fernet *et al*., 2002). As mentioned above, protein coding capacity of mt genome is limited mainly to components of electron transport chain (ETC) and F_0 subunits of F_0F_1 ATP synthetase complex (complex V). Deletions of nuclear genes encoding either ETC or F_0F_1 ATP synthetase subunits can be tolerated in petite-negative yeasts on fermentable carbon source (Chen & Clark-Walker 1993, Chen *et al*. 1998). But as demonstrated for *K. lactis*, petite-negative species cannot tolerate the simultaneous loss of ETC and complex V (Clark-Walker & Chen 2001). An unifying feature of ETC and F_0F_1 ATP synthetase is the ability to uphold an electrochemical potential ($\Delta\Psi$) across the inner membrane, either by creating a proton gradient

or by hydrolysing ATP in the matrix (Giraud & Velours 1997). $\Delta\Psi$ is essential for protein transport into mitochondria, which in turn is vital for every mitochondrial processes, Fe-S cluster synthesis amongst them. One of the proteins containing Fe-S cluster is Rli1p, which is vital for cellular translation by participating in rRNA processing and ribosome subunit transport (Yarunin *et al*. 2005, Kispal *et al*. 2005). Thus the basic necessity of mitochondria in yeast is linked to cellular protein synthesis through Fe-S cluster assembly. Loss of $\Delta\Psi$ would therefore lead to failure of translation and cell death. *S. cerevisiae* is believed to be able to compensate for the loss of $\Delta\Psi$ generated by ETC by hydrolysing ATP with the F_1 part of F_0F_1 ATPase and reversing the flow of ATP/ADP exchange between matrix and cytosol (Chen & Clark-Walker 1999). This hypothesis is supported by the fact that the petite-positive state of *S. cerevisiae* can be altered by mutations in an ADP/ATP translocase and in F_0F_1 ATP synthetase α and β subunits (Lawson *et al.* 1990, Chen & Clark-Walker 1999). Likewise, mutants that convert petite-negative *K. lactis* into petite-positive, affect either α, β or γ alleles of F_1 ATPase subunits (Chen & Clark-Walker 1995, Chen & Clark-Walker 1996, Clark-Walker et al., 2000). As deletions of these genes do not affect the petite-negative state of *K. lactis*, they must be gain-of-function mutations. Since loss of the F_0 sector, that has three subunits encoded by the mt genome, does not affect the suppression of ρ^0-lethality, this function must be dependent on some activity of the F_1 subunit (Clark-Walker *et al*. 1997, Chen *et al*. 1998). As mutated F_1 subunits with decreased K_m for ATP are able to suppress the lethality of simultaneously disrupted ATPase and ETC in *K. lactis*, the increased hydrolysis of ATP by F_1 might be sufficient to uphold $\Delta\Psi$ and rescue ρ^0-lethality (Clark-Walker & Chen 2001). The difference between petite-positive and petite-negative species might therefore be determined by the ability of the F_1 part of F_0F_1 ATP synthetase to hydrolyse ATP at a sufficient level.

2.3. Inheritance of *S. cerevisiae* mtDNA

2.3.1. Replication of mtDNA

Unlike nuclear DNA, mtDNA is replicated independently of the cell cycle. According to the contemporary view, replication is thought to be initiated at specific conserved sequences termed *rep/ori* sequences. These are approximately 300 bp long sequences with three specific GC-rich blocks (A, B and C) separated by AT-rich regions (Figure 2) (de Zamaroczy *et al.* 1981). Depending on the strain considered, the number of *rep/ori* sequences in the mt genome of *S. cerevisiae* varies between 7 and 8 (Faugeron-Fonty 1984, Foury et al., 1998, de Zamaroczy *et al.* 1984). GC-blocks A and B have the ability to form a DNA hairpin, although its role and requirement for replication is unclear. A subset of

rep/oris contain a canonical mitochondrial promoter ATATAAGTA just outside of the C-block (Osinga *et al.* 1984, Baldacci & Bernardi 1982). This association with mitochondrial promoter is thought to be essential for initiation of replication, as *rep/oris* without promoters do not display supressiveness when incorporated into ρ^- genomes (MacAlpine et al., 2001). Transcription in mitochondria is carried out by a bacteriophage-like RNA polymerase Rpo41p and its specificity factor Mtf1p (Masters *et al*., 1984, Schinkel *et al*., 1988). Both of these proteins are vital for wt mt genome maintenance (Greenleaf *et al.*, 1986, Lisowsky *et al.*, 1984). As stable RNA-DNA hybrids have been observed during *in vitro* transcription from a *rep/ori* promoter (Xu & Clayton 1995), it is likely that a Rpo41-generated transcript is used as a primer for promoter strand DNA synthesis. This primer could be generated from a longer transcript through the action of MRP, a yeast RNA processing enzyme, which cuts RNA transcripts in site-specific manner (Stohl & Clayton 1992). Indeed, RNA-DNA junction molecules have been isolated from yeast that map to the *rep/ori* regions. However, exact length of RNA primer and position of RNA-DNA junction have remained a matter of debate (Baldacci *et al.* 1984, Graves *et al.* 1998), since it has been reported to occur either within or outside of C-block (Figure 2). The mechanism of non-promoter strand priming has remained unclear, although primase activity has been described in *S. cerevisiae* mitochondria (Desai *et al*. 1989). Once initiated, replication is thought to proceed on wt mtDNA in a strand-coupled manner, as DNA structures resembling a progressive replication fork have been observed (Lockshon *et al.* 1995, MacAlpine *et al.* 1998).

As mentioned earlier, *S. cerevisiae* is a petite-positive yeast with the ability to lose it's wt mt genome. After spontaneous or induced mutagenesis mtDNA might either be lost altogether (ρ^0) or exist in form of mutated mt genomes (ρ^- genomes). These ρ^- genomes consist of tandemly arranged short sequences, which are amplified to equal DNA levels of strains with wt mt genomes. ρ^- genomes can be characterised by the ability to out-compete wt mtDNA in progeny of zygotes formed after mating. The percentage of cells in zygote progeny that contain ρ^- genomes is the measure of supressiveness. Some ρ^- genomes display extreme cases of supressiveness over wt and neutral ρ^- genomes which is called hypersupressivity (hs). In that case >95% cells in zygote progeny contain supressive ρ^- genomes. This hypersupressivity requires the presence of *rep/oris* with intact promoters in amplified fragments (Blanc & Dujon, 1980). Supressivity is not caused by preferential transmission of ρ^- mtDNA during mating, as both ρ^+ and ρ^- genomes move into the medial bud (MacAlpine *et al.* 2001). Therefore it is more likely that supressivity is linked to replicational and/or segregational advantage of hs genomes. *ori4*, *ori6*, *ori7* and *ori8* contain a short insertion in their promoters and have not been found in hypersupressive ρ^- genomes (de Zamaroczy *et al.* 1981). This endorses the role of transcription from promoters adjacent to *rep/oris* in replication. However, ρ^-

genomes have been reported to be stably maintained without Rpo41p, whose absence abolishes mitochondrial RNA transcription (Fangman *et al*. 1990, Lorimer *et al*. 1995, Van Dyck & Clayton 1998). Hypersuppressive strains with disrupted *rep/ori* promoters display biased inheritance when mated with neutral ρ^-, but not with ρ^+ or hs ρ^- strains with intact promoters (MacAlpine *et al*. 2001). As ρ^- genomes devoid of *rep/ori* sequences are stably maintained and in some cases also display a moderate suppressivity, alternative systems must exist for ρ^- genome propagation that do not require transcription (Fangman *et al*. 1989). This data indicates that although various types of ρ^- genomes can be propagated in absence of promoter and/or Rpo41p-dependant activity, transcription seems to be vital for biased inheritance if a competitor genome also possesses a *rep/ori* with an intact promoter.

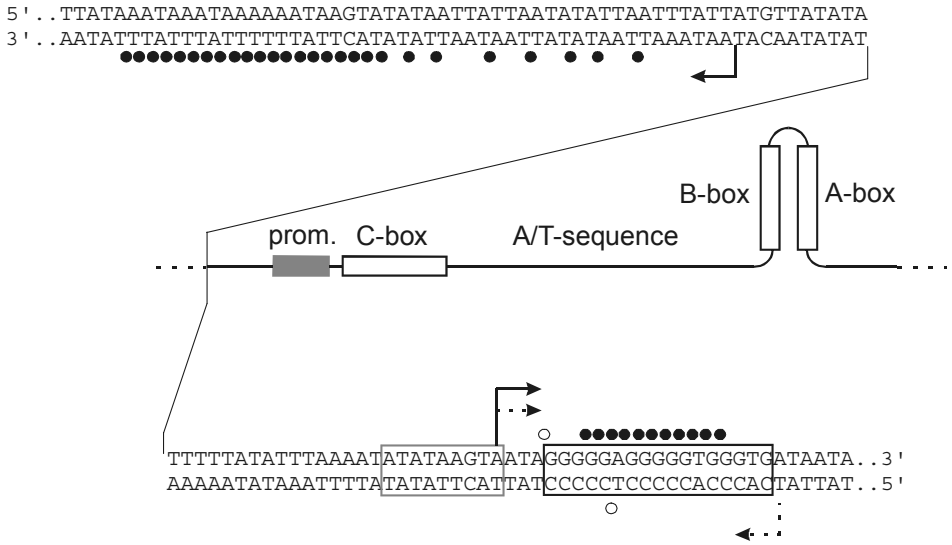


Figure 2. Structure of *S. cerevisiae* active mt replication origin *ori5*. GC-rich boxes A and B have the ability to form a hairpin. C-box is separated from them by AT-rich sequence and is in the vicinity of active mt promoter (gray). The 5'-end of a RNA transcript is depicted by broken (Baldacci et al., 1984) or solid arrows (Graves et al., 1998). Transition from RNA to DNA are marked by open (Baldacci et al., 1984) or solid circles (Graves et al., 1998).

2.3.3. Nucleoid organisation and mtDNA transmission

The copy number of the mt genome per cell varies between 25–50 in *S. cerevisiae*, depending on ploidity and enviromental conditions (Williamson, 1976). When yeast cells are stained with DAPI, mtDNA is visualised as spherical structures called chondriolites or nucleoids (Williamson and Fennell,

1979). In addition to DNA, nucleoids also contain proteins (Rickwood et al., 1981, Miyakawa & Sando, 1987). The number of nucleoids is smaller than the estimate of individual mt genomes, therefore every individual nucleoid contains several copies of the mt genome (Williamson, 1977). In progeny of cells that contain a mix of genetically different mtDNA-s (heteroplasmy), a homogenous state or homoplasmy is established after fewer cell divisions than it would be expected if every individual mtDNA molecule is propagated randomly (Dujon 1981). This data with more recent observations suggests that nucleoids are the segregational units of mtDNA (Lockshon et al., 1995, Nunnari et al., 1997, Okamoto et al., 1998, MacAlpine et al., 2000).

Fusion of two different populations of mitochondria takes place when two haploid cells form a zygote in mating (Nunnari et al., 1997). Soon after mating mitochondrial proteins from both parents are distributed throughout a novel mitochondrial reticulum. Integral membrane proteins are sorted with slightly slower kinetics than soluble matrix proteins (Azpiroz & Butow 1993, Okamoto et al. 1998). This indicates that mitochondrial constituents are mixed rapidly after cellular fusion. However, this is not the case with mtDNA nucleoids. These structures dislocate within zygote mitochondria slower than and independent of protein mixing (Azpiroz & Butow 1993). The place of the emerging bud also determines the mitochondrial genotype for following progeny. Buds emerging from either end of zygote are homoplasmic for parental mtDNA, whereas cells displaying biparental and/or recombinational mtDNA inheritance originate from a medial bud that emerges from the central zone in the zygote (Zinn et al. 1987). In crosses with ρ^+ and ρ^0 strains mtDNA is preferentially sorted into the medial bud and the ρ^0 end of the zygote usually contains no mtDNA (Nunnari et al. 1997, Okamoto et al. 1998). This indicates that a) a specific mechanism exists that ensures the segregation of mitochondrial DNA and b) mixing of parental mtDNA is limited in zygotes.

The transmission of mtDNA is influenced by modulating the amount and topology of mtDNA in nucleoids. For example, in the absence of Cce1p (Mgt1p), a cruciform cutting endonuclease required for resolving Holliday junctions, the preferential transmission of ρ^- genomes in matings is severly compromised (Zweifel & Fangman, 1991). Similar results were obtained with respiratory-competent mutants of mtDNA (Piskur 1997). Upon *CCE1* deletion nucleoids become fewer and larger (Lockshon et al. 1995). This effect is accompanied by an increase in mtDNA molecule size and in the number of Holliday junctions (Lockshon et al., 1995). Thus, the impaired transmission of mt genomes in *Δcce1* background is caused by the formation of large structures of mtDNA that are probably less efficiently segregated than wt nucleoids. Another factor controlling segregation is Mhr1p, a protein involved in homologous recombination and in repair of mtDNA (Ling et al., 1995, Ling et al., 2000). Deletion of *MHR1* leads to mtDNA instability under stress conditions (Ling et al., 1995). Elevated levels of Mhr1p lead to increase in

mtDNA concatemers and accelerates the formation of homoplasmic cells (Ling & Shibata, 2004). The topology of mtDNA changes during transmission. In buds the majority of it is present as monomers in contrast to mother cells, where the prevailing form is concatemer (Ling & Shibata, 2002). Furthermore, radioactive labeling has demonstrated that these concatemers are the immediate products of replication (Ling & Shibata, 2004). Hypothesis has therefore been put forward that these concatemers are generated by rolling-circle mechanism and are therefore genetically homogenous (Ling and Shibata, 2004). Transmission of such concatemers into nascent buds and subsequent breakdown into monomers is one possible explanation for the quick establishment of homoplasmy described by Dujon et al., 1981.

2.3.4. Nucleoid segregation

An active segregation mechanism requires tethering of nucleoids to some component of the cytosceleton that directs movement into nascent bud. In contrast to animals and *S. pombe*, baker's yeast utilises actin cables instead of microtubules to transport mitochondria to emerging buds (Simon et al., 1995, Simon et al., 1997, Lazzarino et al., 1994). Mmm1p protein that spans outer and inner membranes is localised adjacent to mt nucleoids (Burgess et al., 1994, Hobbs et al., 2001). This protein is associated only with a subset of nucleoids. The same subset exclusively co-aligned with the polymerase Mip1p and was found to be actively synthesising DNA (Meeusen & Nunnari 2003). Another protein with a similar localisation pattern, Mmm2p, is synthetically lethal with Mmm1p (Youngman et al. 2004). Deletion of either one of these proteins causes a similar phenotype – abberant mitochondrial structures and aggregated nucleoids that are associated with segregation failure (Hobbs et al. 2001, Youngman et al. 2004). The outer membrane proteins Mdm10 and Mdm12 form a complex with Mmm1p and connect mitochondria to actin cables, although their localisation is independent of actin (Boldogh et al. 2003). Putative inner membrane components of this tethering complex termed mitochore are proteins Mdm31p and Mdm32p. These proteins are required for motility and normal structure of mitochondria and also for co-localisation of Mmm1p to mtDNA (Dimmer et al. 2005). Thus the mitochore complex consisting of Mmm1p, Mmm2p, Mdm10p, Mdm12p, Mdm31 and Mdm32 links mtDNA nucleoids, mitochondria and actin cables. Although a mitochore-dependent system seemingly links mtDNA and organelle segregation, the fact that nucleoids remain in one end of the zygote after mitochondrial constituents have distributed evenly throughout novel mitochondrial space (Azpiroz & Butow 1993), suggests disparate mechanisms for transmission of mitochondrial structure and nucleoids.

2.3.5. Proteins associated with mtDNA maintenance

As mentioned before, mtDNA of *S. cerevisiae* codes only for a few proteins that, with the exception of Var1p, are components of ETC and ATP synthetase complexes. Therefore most of the proteins involved in processes inside mitochondria are encoded by the nucleus and transported to mitochondria after translation in the cytosol. The 750 currently identified mitochondrial proteins are believed to represent approximately 90% of the total mitochondrial proteome (Sickmann et al., 2003). There are approximately 30 protein-coding genes whose deletion is known to cause the loss of wt mtDNA. After excluding proteins that have either indirect (e.g. through nucleoid segregation or mitochondrial dynamics) or obscure influence on mt genome maintenance, the remaining proteins are described below. Some of these proteins have quite well established functions in mtDNA synthesis (Mip1p, Rim1p, Cdc9p, Rpo41p). Other factors like Abf2p and Mgm101p do not directly participate in mtDNA synthesis but are rather involved in various other processes in mtDNA maintenance. In addition there is also an intriguing set of bifunctional proteins (Ilv5p, Aco1p, Hsp60p) which have a dual role in metabolism and mtDNA maintenance. The functions of these proteins probably link the metabolic state of the cell to nucleoid dynamics and therefore to transmission of mtDNA. Most of these proteins are also found in nucleoids (Kaufman et al., 2000). Two known mitochondrial helicases Pif1p and Hmi1p will be described in detail in a separate subchapter.

Mip1p

The DNA polymerase from yeast mitochondria, Mip1p, has been described both in *S. cerevisiae* and *S. pombe* (Foury 1989, Ropp & Copeland, 1995). It displays a limited homology to *E.coli* Pol I-type polymerases (Blanco et al., 1991, Ito & Braithwaite 1990). Besides possessing a 5'–3' polymerase activity, Mip1p also has a 3'–5' proofreading activity, which is important for faithful synthesis of mtDNA (Foury & Vanderstraeten, 1992). As loss of the *MIP1* gene results in ρ^0 phenotype (Genga, 1986, Foury, 1989), this polymerase is probably the major replicative polymerase required both for ρ^+ and ρ^- genomes. An interesting feature is that in diploids that harbour only one copy of *MIP1*, formation of petite mutation is increased (Lecrenier & Foury, 1995). This suggests dosage-dependency of Mip1p function.

Rpo41p and Mtf1p

The DNA-dependent RNA polymerase Rpo41p is required for transcription and maintenance of the mt genome (Greenleaf et al., 1986), highlighting its involvement in both processes. It is homologous to RNA polymerases from bacteriophages T3 and T7 (Masters et al., 1987) and has a specificity factor, Mtf1p

(Schinkel et al., 1987). Although Mtf1p resembles RNA methyltransferases (Schubot et al., 2001), it acts as a bacterial-type sigma factor. It associates with the core RNA polymerase prior to DNA binding and dissociates after the initiation step (Mangus et al., 1994). Highly purified Rpo41p has been reported to recognise promoter sequence by itself, but only if the target sequence is partially opened (Matsunaga & Jaehning, 2004). Therefore Mtf1p is required for promoter opening and not for sequence recognition.

Rpo41p participates in replication by synthesising RNA transcripts from promoters associated with active origins. This transcript is probably processed to yield a RNA primer for replication initiation. Loss of Rpo41p leads to generation of cells devoid of wt mt genome. Since neutral and hypersuppressive ρ^- genomes can be maintained without Rpo41p activity (Fangman *et al*. 1990, Lorimer *et al*. 1995, Van Dyck & Clayton 1998), there are clearly other possibilities for replication priming. Nevertheless, Rpo41p is crucial for biased transmission when hs ρ^- genomes are mated with each other (MacAlpine et al., 2001).

Cdc9p

Cdc9p is an essential ligase involved in replication and repair of nuclear DNA. Two ligase isoforms are synthesised from *CDC9* mRNA from different initiation codons (Willer et al., 1999). The longer polypeptide is transported to mitochondria by a cleavable N-terminal mitochondrial targeting sequence (MTS) (Willer et al., 1999). Cdc9p is probably the sole ligase in mitochondria and is vital for propagation of both replicating and non-replicating mtDNA (Donahue et al. 2001).

Rim1p

This protein was identified as a high-copy suppressor of *Δpif1* temperature-sensitive phenotype (Van Dyck et al. 1992). Rim1p is a mitochondrially located protein and displays significant homology with ssDNA binding proteins (SSB) from *Xenopus laevis* and *E. coli*. Upon Rim1p deletion mtDNA is lost altogether (Van Dyck et al., 1992), predicting a vital function in maintenance of various mtDNA forms similarly to Mip1p.

Abf2p

The 20 kDa protein Abf2p is an abundant nucleoid component with homology to high-mobility group (HMG) proteins (Kaufman et al., 2000, Diffley & Stillman, 1991). Abf2p function requires a HMG-box domain and its deletion phenotype can be complemented by other HMG-box proteins (Kao et al., 1993, Parisi et al., 1993). HMG-box proteins bind DNA in a non-specific manner and function in DNA packaging into nucleoprotein complexes (Thomas and Travers, 2001). Since Abf2p has the ability to bind DNA non-specifically and

can also bend and wrap DNA, it probably acts as a mitochondrial histone analogue in mtDNA packaging (Diffley and Stillman, 1993, Friddle et al., 2004). This hypothesis is augmented by the fact that Abf2p deletion can be rescued by *Escherichia coli* histone-like protein HU, that participates in packaging of prokaryotic DNA (Megraw and Chae, 1993). Deletion of *ABF2* leads to changes in nucleoid morphology and petite-mutation formation on fermentable, but not on non-fermentable medium (Diffley and Stillman, 1991, Newman et al., 1996). Furthermore, in accordance with its proposed role as a mitochondrial histone, mtDNA from *Δabf2* strain is also more sensitive to DNase treatment (Newman et al., 1996). In the absence of Abf2p nucleoid segregation is delayed and recombination is decreased both during vegetative growth and matings (Okamoto et al., 1998, MacAlpine et al., 1998, Zelenaya-Troitskaya et al., 1998). Although moderate increase in Abf2p levels results in the rise of mtDNA copy number and in the reduction of petite formation, strong overexpression has an opposite effect, leading to the loss of mtDNA preceded by the increase in numbers of Holliday junctions (MacAlpine et al., 1998, Zelenaya-Troitskaya et al., 1998, O'Rourke et al., 2002). Therefore Abf2p besides organising mtDNA into nucleoids also mediates inheritance through recombination.

Msh1p

This protein is one of the six *S. cerevisiae* homologues of the *E. coli* mismatch repair enzyme MutS (reviewed in Kolodner and Marsischky, 1999). Unlike other yeast MutS homologues, its localisation into mitochondria has been demonstrated both *in vitro* and *in vivo* through cleavable MTS (Chi and Kolodner, 1994a). Msh1p functions as a mitochondrial repair enzyme, without which yeast is unable to support the maintenance of wt mt genome. The loss of the *MSH1* gene is accompanied with the formation of ρ^- genomes within 20 generations (Reenan and Kolodner, 1992a). Even strains which are hetero-zygotic for *MSH1* deletion display 7-fold increased frequency of point mutation formation, demonstrating the dosage-dependent effect similar to Mip1p (Reenan and Kolodner, 1992b). Like MutS, Msh1p has the ability to bind regions of mismatch and this activity is modulated by ATP-binding (Chi and Kolodner, 1994b).

Cce1p (Mgt1p)

Mgt1p was originally identified as a gene whose deletion leads to loss of suppressivity of hs ρ^- genomes in matings with wt strains (Zweifel & Fangman, 1991). The product of *MGT1* was identified as a cruciform cutting endonuclease (Cce1p) and was shown to localise in mitochondria (Kleff et al., 1992). Purified protein was demonstrated to react specifically with branched DNA structures, four-armed cruciforms being the preferred substrates (Kupfer & Kemper, 1996).

In addition to loss of biased inheritance of hs ρ^- genomes, *CCE1* deletion is accompanied with appearance of fewer and larger nucleoids than wt and decreased mobility of mtDNA (Lockshon et al., 1995). This decrease in mobility was accompanied with increase in Holliday junctions. Therefore the loss of Cce1p leads to aggregation of mtDNA by not resolving recombination junctions. As expected, this effect is stronger in hs strains, where greater numbers of repeat sequences cause more efficient recombination than in wt strain. Recombination-joined mtDNA structures are less efficiently segregated during mitotic division, resulting in loss of hs ρ^- genomes (Lockshon et al., 1995). Deletion of *CCE1* was also found to influence inheritance of respiration-competent mtDNA mutants (Piskur, 1997). Therefore the effect of Cce1p is not hs ρ^- genome specific and is likely to influence inheritance by regulating the available number of segregation-competent mtDNA structures.

Mgm101p

Mgm101p was identified as a factor required for wt mtDNA propagation (Chen et al., 1993). Its homologue is essential for viability in the petite-negative yeast *K. lactis*. Mgm101p possesses the ability to bind DNA and in case of its loss mtDNA becomes hypersensitive for oxidative damage (Meeusen et al., 1999). Although sensitivity of mtDNA to damage can be modulated indirectly by packaging (as it is probably the case with Abf2p), *MGM101* deletion is not accompanied with major changes in nucleoid morphology (Meeusen et al., 1999). This suggests that Mgm101p has probably a more straightforward function in DNA damage repair. A distinct feature of Mgm101p is that while it is required for maintenance of wt and ori-devoid ρ^- mt genomes, this protein is dispensable for hypersuppressive ρ^- genomes (Zuo et al., 2002). Moreover, co-alignment of Mgm101p with a subset of nucleoids which actively incorporate nucleotides further underlines its involvement in mtDNA maintenance (Meeusen and Nunnari, 2003).

Mhr1

By using a novel screen to identify proteins involved in recombination of mtDNA, Ling and Shibata described Mhr1p, whose deletion gives rise to cells being defective in recombination. Furthermore, in mhr1p-deficient cells wt mtDNA is gradually replaced by ρ^- and ρ^0 at higher temperature (Ling and Shibata, 1995). This protein plays a role in shielding of mtDNA from spontaneous oxidative damage, as malonic acid, a compound that decreases oxidative damage, inhibits the formation of petite mutants in *Δmhr1*-strain (Ling et al., 2000). Mhr1p has been shown to stimulate heteroduplex formation by pairing single-strand with duplex DNA (Ling and Shibata 2002). Therefore Mhr1p functions in a recombination-directed repair pathway of mtDNA. However, this protein has also another function in mtDNA transmission as

demonstrated by lack of mtDNA in buds and therefore high fraction of ρ^0 cells in *Δmhr1*-strain (Ling and Shibata, 2002). As described above, mtDNA is organised in *S. cerevisiae* as concatemers. Mhr1p influences this organisation. In case of overexpression the formation of concatemers is enhanced while deletion results in suppression (Ling and Shibata 2004). Mhr1p therefore promotes the mtDNA transmission into buds through formation of concatemeric DNA molecules. Whether Mhr1p participates in formation of concatemers directly by linking mtDNA molecules or the influence is instigated through some other mechanism, remains to be clarified.

Ilv5p

First identified as an enzyme required for branched-chain amino acid synthesis, Ilv5p is an example of a bifunctional protein in mitochondria. Ilv5p involvement in mtDNA maintenance was identified by its ability to suppress *abf2* conditional allele and the instability of wt mtDNA in *Δilv5* background (Zelenaya-Troitskaya et al., 1995). This protein provides a link between the metabolic state of the cell and mitochondrial inheritance, since a general amino acid control pathway (GCN) regulates the number of nucleoids through Ilv5p (MacAlpine et al., 2000). Activated GCN causes the increase in nucleoid numbers. This influence is instigated through intramolecular recombination, as the number of short oligomers in ρ^- strains is increased upon GCN activation while the overall mtDNA amount remains constant (MacAlpine et al., 2000). Furthermore, synergy exists with recombination-involved proteins Abf2p and Cce1p, as both are required for an Ilv5p-dependant effect (MacAlpine et al., 2000). The instability of mtDNA in *Δilv5* strains is not caused by a defect in an amino acid synthesis pathway (Zelenaya-Troitskaya et al., 1995). Moreover, mutations affecting either a metabolic pathway or mtDNA stability cluster to separate regions in Ilv5p (Bateman et al., 2002a). Ilv5p mutants defective in mtDNA maintenance have aberrant intramitochondrial localisation and are targeted to proteolytic degradation (Bateman et al., 2002b). Since an *E. coli* Ilv5p homologue, when transported into mitochondria, displays a similar phenotype as Ilv5p mutants, being defective in mtDNA maintenance, the latter function was probably acquired after the endosymbiosis event (Bateman et al., 2002a).

Aco1p

Another example of a bifunctional protein is Aco1p, a metabolic enzyme involved in the citric acid cycle (Chen et al. 2005). Upon Aco1p loss ρ^0 cells are formed. Its enzymatic activity is dispensable for mtDNA maintenance if mutant proteins are expressed at elevated levels (Chen et al., 2005). As described above, mtDNA from cells lacking Abf2p is especially sensitive to damage (Newman et al., 1996, Chen et al., 2005). Aco1p expression can protect mtDNA

damage in *Δabf2* cells inflicted by the intercalating agent ethidium bromide. Furthermore, Aco1p expression can also rescue the mtDNA loss observed in *Δabf2* cells grown in non-fermentable medium (Chen et al., 2005). This suggests that Aco1p and Abf2p have partially overlapping functions, probably in mtDNA packaging into nucleoids.

Hsp60p

The mitochondrial chaperonine and member of nucleoids Hsp60p is required in addition to folding of transported proteins also for segregation of nucleoids. This is demonstrated by conditional alleles which do not cause defects in chaperonin activity but are unable to support wt mtDNA (Kaufman et al., 2000). Older cells carrying a conditional *hsp60-ts* allele contain more mtDNA than younger cells at non-permissive temperature (Kaufman et al. 2003). This gradual mtDNA loss is accompanied by altered nucleoid morphology that is thought to be caused by a defect in nucleoid transmission. Beside being a nucleoid component, Hsp60p has been reported to bind to active origins in strand-specific manner (Kaufman et al., 2000). ρ^- genomes containing active origins are unstable in the absence of Hsp60p, whereas neutral ρ^- mt genomes remain unaffected. Moreover, this instability is rescued upon deletion of *RPO41*, demonstrating the requirement of transcription for Hsp60p effect (Kaufman et al., 2003). Since mtDNA is present in *hsp60-ts* cells, it is likely that Hsp60p functions in directing the transmission of mtDNA rather than directly influencing synthesis.

2.4. *S. cerevisiae* mitochondrial helicases

Several transactions like recombination, replication and transcription require separation of complementary strands of nucleic acids. This process is catalysed by a diverse group of enzymes called helicases. According to conserved helicase motifs helicases can be classified into five groups; two families and three larger superfamilies (Gorbalenya and Koonin, 1993). Up to date only two DNA helicases have been characterised in yeast mitochondria, Pif1p and Hmi1p, that are both members of the first superfamily (SF1). Studies of the mitochondrial proteome have identified another SF1 helicase, Rrm3p, in mitochondria (Prokisch et al., 2004). This helicase has opposing effects on telomere and rDNA replication to its close homologue Pif1p in the nucleus (Ivessa et al., 2000, Ivessa et al., 2002). Although Rrm3p is able to partially rescue the petite-induction phenotype of *PIF1* deletion (O'Rourke et al., 2005), evidence for direct involvement in mtDNA metabolism is lacking. Therefore Rrm3p will not be included to the list of mitochondrial helicases.

Pif1p

Pif1p is a 5'–3' directional helicase and a member of SF1 (Foury & Lahaye, 1987, Lahaye et al., 1991). Similar to Cdc9p, two different isoforms that have separate nuclear and mitochondrial functions are expressed from the same gene (Schulz & Zakian, 1994). The absence of the nuclear isoform influences the length of telomeres and replication of rDNA loci (Schulz & Zakian, 1994, Zhou et al., 2000). In mitochondria Pif1p was initially found to be nonessential for vegetative growth but required for recombination in matings between ρ^+ and a subset of ρ^- strains (Foury & Kolodynski, 1983, Foury & Van Dyck, 1985). Pif1p is a monomeric distributive helicase that unwinds forked substrates more efficiently than substrates with simple single-stranded overhangs (Lahaye et al. 1993). Preferential usage of forked substrates that resemble recombinational intermediates further supports the role of Pif1p in recombination.

In addition to recombination, Pif1p was found to be involved in mtDNA repair (Foury & Kolodynski, 1983, O'Rourke et al., 2002, Doudican et al., 2005). In *Δpif1* strains mtDNA repair is deficient and petite colony formation is increased at elevated temperatures (Foury & Kolodynski, 1983, Van Dyck et al., 1992). Effects of *PIF1* deletion are augmented in *Δngt1* background (O'Rourke et al., 2002). Ngt1p is a DNA glycosylase involved in base excision repair of oxidatively damaged mtDNA. When a *Δpif1Δngt1* mutant is accompanied with the loss of Sod2p, a major scavenger protein of radical oxygen species, mtDNA is lost altogether (Doudican et al., 2005). This effect in base-excision repair was shown to be independent of Cce1p and Nuc1p, factors involved in mtDNA recombination (O'Rourke et al., 2002). These results underline the role of Pif1p in mtDNA repair, although its exact role remains an open question.

Pif1p was also found to influence mtDNA copy number, since upon its deletion the number of mtDNA molecules decreases (Taylor et al., 2005). This effect in copy number regulation is linked to dNTP levels controlled by Mec1/Rad53 pathway (O'Rourke et al., 2005, Taylor et al., 2005). It has been demonstrated that mtDNA replication is dependent on cellular dNTP pools, and over-expression of ribonucleotide reductase Rnr1p can rescue the petite-induction by mutated *MIP1* allele (Lecrenier & Foury, 1995). Increase in dNTP levels caused either by overexpression of Rnr1p or deletion of the Rnr1p inhibitor protein Sml1p complements the decrease of mtDNA copy number in *Δpif1* strain (Taylor et al., 2005). This effect is independent of Abf2p function, whose expression levels also affect the amount of mtDNA (Zelenaya-Troitskaya et al., 1998, Taylor et al., 2005). Although deletion of *SML1* restores original copy number in a *Δpif1* strain, it only partially rescues the petite-induction phenotype (Taylor et al., 2005). This indicates that copy number regulation and petite-induction are separate processes. Considering the different roles of Pif1 discussed above, this helicase might therefore participate in several processes that influence mtDNA stability.

Hmi1p

The second mitochondrial helicase, the protein Hmi1p is a member of SF1 as well, and a close homologue of the nuclear helicase Srs2p. The latter protein has homologues in several yeast taxonomic groups, but according to sequence comparison Hmi1p is present only in members of the family of *Saccharomycetaceae* (see Results). The *HMI1* gene is probably formed by a duplication event of the original *SRS2* gene that took place before *Saccharomycetaceae* branched to different species (Richard et al., 2005). A unique feature of Hmi1p is the unusual C-terminal location of the otherwise canonical mitochondrial targeting sequence (Lee et al., 1999, Sedman et al., 2000). This C-terminal sequence of Hmi1p has the ability to form an amphipathic α-helix and is able to translocate a passenger protein into mitochondria *in organello* both in C→N and N→C terminal fashion (Lee et al., 1999). Furthermore, its requirement for mitochondrial targeting of Hmi1p has been demonstrated *in vivo*, where a strictly mitochondrial location was observed for over-expressed full-length Hmi1p (Sedman et al. 2000). The reason why Hmi1p has not been observed in the mitochondrial proteome (Prokisch et al. 2004) is probably due to its low endogenous expression level (T. Sedman, unpublished results).

Detailed studies of Hmi1p helicase properties revealed that it is a 3'–5' directional helicase which is monomeric in solution (Kuusk et al., 2005). Similar to Pif1p it is a distributive helicase, unwinding efficiently only relatively short DNA duplexes. It also utilizes most efficiently ATP/dATP as nucleotide cofactor. Hmi1p preferentially utilizes forked and flap structures, as demonstrated by decreased requirement for 3'-overhang in such substrates compared to single-overhang substrates. A specific helicase motif was also shown to be vital for Hmi1p function, as a mutant where a invariant glutamate was substituted against glutamine (E211Q) had lost helicase activity (Ref.II). Studies by another research group have confirmed these results (Monroe et al., 2005).

Hmi1p has been implicated in two processes, pseudohyphal growth and mtDNA maintenance (Lorenz et al., 2000, Sedman et al., 2000). Pseudohyphal (PH) growth of *S. cerevisiae* is similar to hyphal growth in fungal parasites, a phenomenon vital for virulence. As involvement of Hmi1p in pseudohyphal growth is poorly characterized, it is unclear, whether mitochondrial localisation is required for this effect. Hmi1p-s role in maintenance of wt mtDNA is more extensively characterized. Upon its loss a mixture of neutral and hyper-suppressive ρ^- genomes is formed (Sedman et al. 2000). Involvement in mtDNA transcription was ruled out by presence of mitochondrial RNA in *Δhmi1* strains and inability of Hmi1p to utilize RNA as nucleic acid cofactor (Sedman et al., 2000, Monroe et al., 2005). Its further effects on mtDNA are the topic of research in this thesis.

In its promoter Hmi1p has 4–5 binding sites for Gcn4p, a regulator of amino acid biosynthesis (Hinnebusch, 1988, Schuldiner et al., 1998). Gcn4p also

controls the expression of Ilv5p, a bifunctional protein involved in branched-chain amino acid synthesis and regulation of mtDNA inheritance (MacAlpine et al., 2000). Therefore it is tempting to consider the role of Hmi1p in modulating mtDNA metabolism in response to metabolic signalling. However, direct evidence for such a phenomenon is lacking. Gcn4p regulation has also been demonstrated to control hyphal growth in *C. albicans*, a phenomenon similar to PH growth in *S. cerevisiae* (Tripathi et al., 2002). Therefore possible GCN4 regulation of Hmi1p might be linked to a PH growth effect rather than mtDNA maintenance.

3. RESULTS AND DISCUSSION

3.1. Objectives of this study

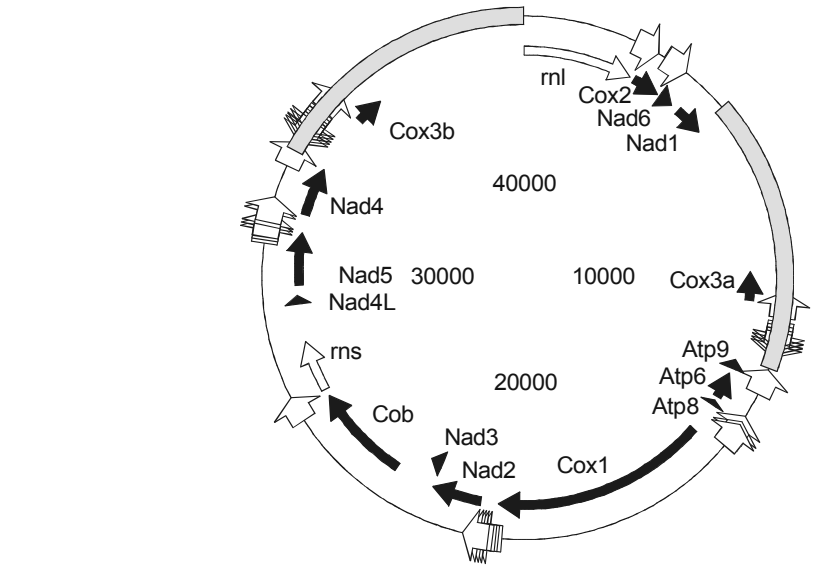
Although ScHmi1p has been described as a mitochondrial DNA helicase in *S. cerevisiae*, its precise biological role has remained obscure. Our goal was to elucidate its role in mtDNA metabolism. For that purporse different genetical experiments were performed in *S. cerevisiae*. We also addressed the issue of the existance of ScHmi1p homologues in a phylogenetically related species. The homologue in the medically important fungal species *C. albicans* was further investigated with the aim to contribute to the understanding of this protein's function and of mtDNA maintenance in general. The organisation of *C. albicans* mtDNA was also characterized.

3.2. Mitochondrial DNA organisation in *C. albicans* is similar to *S. cerevisiae*

C. albicans is like *S. cerevisiae* an unicellular yeast species, but unlike free-living baker's yeast it is a major fungal parasite in humans. Being mainly a mucosal commensal or a mild parasite, it can cause life-threatening infections in immunocompromised patients. *C. albicans* has several genetical and ecological differences from *S. cerevisiae*: this petite-negative yeast is an obligatory diploid without a similar sexual cycle (reviewed in Bennett & Johnson, 2005). To explore whether deviations between these two yeasts also extend to organisation of mtDNA, we conducted experiments to study the topology of *C. albicans* mtDNA.

C. albicans mt genome is half the size of *S. cerevisiae* but with approximately similar gene content (Figure 3). It contains a 6,8 kbp inverted duplication that carries 5 tRNA genes and one structural gene, *cox3* (Figure 3). Our results demonstrated that like in baker's yeast, the RFLM of *C. albicans* mt genome is circular (Figure 7E in I). To address the question of *in vivo* mt genome structure, mtDNA was subjected to separation by PFGE. Hybridisation with a mtDNA-specific probe demonstrated the presence of at least two mtDNA subfractions: linear polydisperse molecules migrating between 30 and 100 kbp and a well-bound immobile molecules (Figure 7D in I). Medium size of the first fraction was around 40 kbp which was the approximate size of the mt genome monomer. The well-bound fraction contained molecules that were unable to move into gel and remained in the well. Under the conditions chosen for this particular PFGE even very large linear molecules could enter the gel, as demonstrated by migration of *C. albicans* chromosomes (1–4 Mbp) in the compression zone (data not shown). Therefore the immobility of the well-bound

A



B

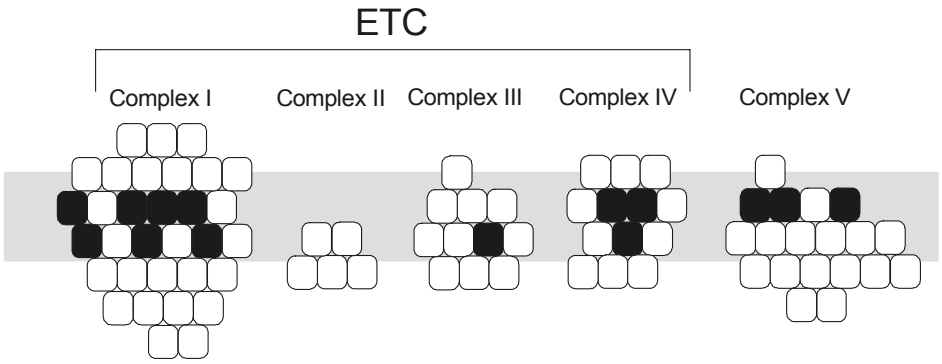


Figure 3. Coding capacity of *C. albicans* mt genome. A. Circular map of *C. albicans* mt genome. Short open arrows – tRNA genes, longer open arrows – rRNA genes, solid arrows – protein genes. Repeat unit is coloured in gray. B. Schematic depiction of ETC complexes and ATP synthetase (complex V). Solid rectangles are polypeptides coded by mt genome, open rectangles – polypeptides coded by nucleus.

fraction is probably caused by the complex structure of these molecules and not of their size. Indeed, according to moving-picture analysis, the well-bound fraction of *S. cerevisiae* mtDNA has been shown to contain complex DNA structures (Bendich, 1996). Taking into account the circularity of RFLM, *C. albicans* mtDNA is probably organised in a similar head-to-tail concatemeric

fashion as reported for *S. cerevisiae*. Even on overexposed blots we did not observe the fraction of circular mtDNA forms that have been reported for *S. cerevisiae* (data not shown). However, provided the scarcity of circular molecules and possible degradation into linear forms, circular mtDNA molecules might have escaped detection.

3.3. Behaviour of ScHmi1p-s conditional mutants

A library of mutated *ScHMI1* clones was generated by error-prone PCR method. Temperature-sensitive mutants were isolated from this library by screening for viability on non-fermentable medium at 23° and 37°. Eight different *ScHMI1* clones were isolated which were unable to support wt mt genome upon shift to non-permissive temperature and therefore had temperature-sensitive function. Mutations causing amino-acid substitutions in these alleles are shown in Table 2.

Table 2. Amino acid substitutions caused by mutations in ScHMI1 conditional alleles. Substitutions within helicase motifs are shown in bold.

Conditional allele	Mutations
hmi1-1	N109D, Q296L, T338P, I438M, **L594V**, K633R
hmi1-2	T33M, M225L, S254P, Q296L, T338P, I438M, R636G
hmi1-5	I280V, Q296R, T507R, K530R, M649R
hmi1-6	**D209V**, L308P, D324E, D325A, K685R
hmi1-7	**E52G**, D209E, F654S
hmi1-8	M629R
hmi1-9	L72P, **I208N**, S451P, N461K, Y658H
hmi1-10	E17G, I84N, L449P, E535G, S590F

Series of strains were generated by exchanging endogenous *ScHMI1* against conditional *Schmi1* alleles using ARS/CEN plasmids and 5'-FOA counter-selection. The disparities in size of colonies formed at 37° reflect the differences in speed of wt mtDNA loss (Figure 5A). However, prolonged exposure (24–48h) to non-permissive temperature resulted in total loss of respiration in all mutant strains (data not shown). The random mutations did not demonstrate clear clustering to specific regions (Figure 5B).

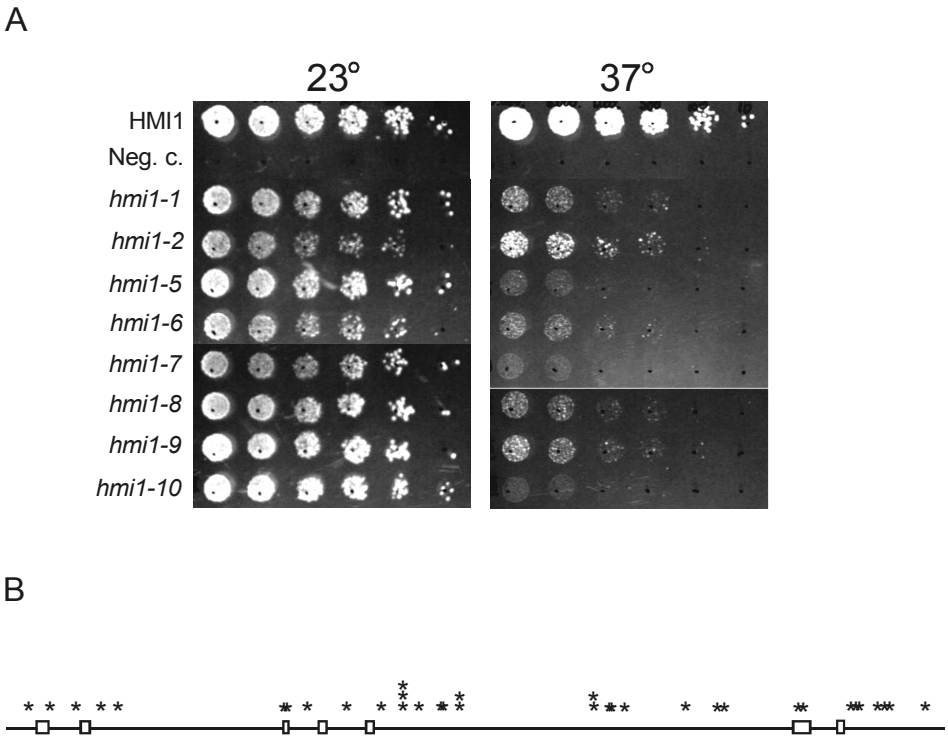


Figure 5. Behaviour of ScHMI1 conditional alleles. A. Growth of the alleles on glycerol-containing medium at permissive (23°) and non-permissive (37°) temperatures. B. Summarisation of mutations of all eight conditional *Schmi1* alleles. Open squares are helicase motifs and asterisks mark the position of amino-acid substitutions. Stacked asterisks denote mutations that are shared by more than one allele.

Changes in mtDNA topology in the conditional mutant *hmi1-5* were addressed by one-dimensional gel analysis. Shift to non-permissive temperature resulted in appearance of substantially shorter DNA fragments compared to mtDNA isolated from wt or *hmi1-5* at permissive temperature as revealed by hybridization with *cox2*-probe (Figure 4B in III). This indicates that the loss of ScHmi1p function was accompanied with the fragmentation of wt mtDNA. A similar but more detailed experiment reveals that this fragmentation is temporary (Figure 6). After prolonged incubation at non-permissive temperature, when ρ^- genomes have replaced ρ^+ genomes (Figure 4A in III), the mobility of mtDNA is similar to that of wt strain. Therefore the observed fragmentation represents the transition state from ρ^+ to ρ^- genomes caused by loss of ScHmi1p function. This effect might either be direct, caused for example by inefficient replication that generates truncated products or indirect like degradation of unsupported ρ^+ mtDNA by nucleases.

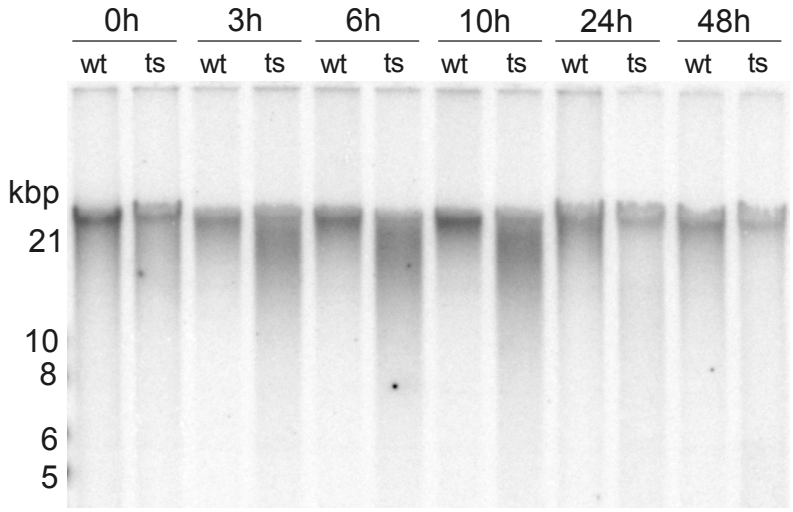


Figure 6. Fragmentation caused by loss of ScHmi1p is non-persistent. Analysis of mtDNA from *S. cerevisiae* species with wt and *hmi1-5* alleles (wt and ts respectively). DNA levels were normalised according to nuclear DNA. Hours denote time from shift to non-permissive temperature.

To find genetic interaction partners of ScHmi1p, a screen for high-copy suppressors was carried out with ts mutants *hmi1-5* and *hmi1-7*. Mutants were chosen according to the stability of wt mtDNA at 23° and the rate of wt mtDNA loss upon shift to 37°. A genomic library of DNA of *S. cerevisiae* (strain w303a) in the 2µ vector pRS425 was generated. Clones that were able to suppress ScHmi1 ts-phenotype were scored based on the ability to grow on non-fermentable medium at 37°. Similar genetic screens have successfully identified novel genes that participate in mtDNA maintenance (Van Dyck et al., 1992, Zelenaya-Troitskaya et al., 1995). No novel genes beside *ScHMI1* itself were identified by this screen. A possibility for insufficient expression difference between ARS/CEN and 2µ vectors exists, although similar approach has been successfully used before, for example to identify yeast frataxin homologue as a high-copy suppressor of mitochondrial intermediate peptidase (Branda et al., 1999).

3.4. ScHmi1 ATPase activity is not essential for wt mtDNA maintenance

Previous studies have established that ScHmi1p is required for wt, but not for hypersuppressive (hs) or neutral ρ^- mt genomes (Sedman et al., 2000). We tested if ability to unwind DNA strands is necessary for this effect by substituting conserved amino acid residues vital for helicase activity. For that purpose mutants were constructed where lysine in position 32 and glutamate in position 211 were substituted against methionine and glutamine respectively (mutations K32M and E211Q). These two residues are located in Walker A and B motifs and are essential for nucleotide cofactor binding and hydrolysis (reviewed in Caruthers & McKay, 2002). Without energy from ATP hydrolysis helicases are unable to undergo conformational changes and processively separate nucleic acid strands. The E211Q variant was analysed for enzymatic activity and was confirmed to be deficient in helicase activity. A complementation assay for analysis of ScHmi1p mutants was developed based on plasmid-shuffling procedure and the colour of yeast colonies. *Ade2* mutation present in the original strain renders respiratory competent cells red while respiratory deficient cells are white. According to the state of mtDNA, three different types of colonies can arise on glucose: fully respiratory-competent red colonies, white respiratory-deficient colonies and sectored colonies with mixed populations of cells. Loss of ScHmi1p produced only white colonies, indicating the failure to support wt mt genome. However, mutated K32M and E211Q proteins did not lead to total loss of respiratory competence (Figure 3D in III). Instead small fraction (2–5%) of red colonies was present along with a substantial amount of sectored colonies. This indicates that although being inefficient, complementation by Hmi1p mutants without helicase activity still occurs. Further proof for function of Hmi1p other than nucleic acid unwinding came from its effect on hs ρ^- mtDNA. Although ρ^- mt genomes can be maintained without ScHmi1p, its presence causes the lengthening of mtDNA and appearance of ssDNA in ρ^- strains. Similar lengthening of ρ^- mtDNA concatemers was also observed with neutral ρ^- strains, (T. Sedman, unpublished results). Synthesis of longer molecules was independent of helicase activity, since mutant proteins K32M and E211Q displayed similar effect. This suggests that the auxiliary action of Hmi1p helicase-deficient mutants observed in 5'-FOA-assays is to facilitate the synthesis of longer mtDNA molecules.

3.5. Hmi1p is conserved throughout the family of *Saccharomycetaceae*

Richard et al., 2005 propose that the ScHmi1p protein is the result of a gene duplication event that produced Hmi1p and Srs2p. Homologues of Hmi1p were reported by sequence comparison in yeast species *K. lactis*, *C. glabrata* and *Debaryomyces hansenii*. However, due to the large number of helicases present in the yeast genome (Shiratori et al., 1999), the mere existence of conserved helicase motifs cannot be treated as an exhaustive proof of the presence of mitochondrial helicases with similar function. To identify possible ScHmi1p homologues, we analysed gene sequences similar to *ScHMI1* for the presence of mitochondrial targeting signals (MTS).

Genomes of closely related yeasts from *Saccharomyces sensu-stricto* and *sensu-lato* groups code for ScHmi1p homologues with only minor differences (data not shown). In addition to proteins from *K. lactis*, *C. glabrata* and *D. hansenii*, *ORF 19.7661* from *Candida albicans* displayed significant homology (44%) to ScHmi1 (Figure 7A). All four proteins were analysed for possible C-terminal mitochondrial targeting signals. Although there is no primary amino acid sequence consensus for MTS, certain general features are characteristic for canonical MTS. These features include abundance of multiple positively charged and hydroxylated amino acids and formation of amphipathic α-helix. The C-terminus of homologous proteins from species mentioned above was poorly conserved; nevertheless characteristic clustering of positively charged and hydrophobic residues to opposite sides of a putative helix was evident in all cases (Figure 7B).

Provided their overall homology and presence of putative MTS these proteins are probable homologues of Hmi1p, representing mitochondrial helicases in yeast species within *Saccharomycetaceae* family. The putative gene duplication event of *SRS2* and *HMI1* happened therefore before separation of *Saccharomyces* and *Kluyveromyces* genera, thus being not a consequence of a proposed genome-wide duplication event of *S. cerevisiae* (Wolfe & Shields, 1997).

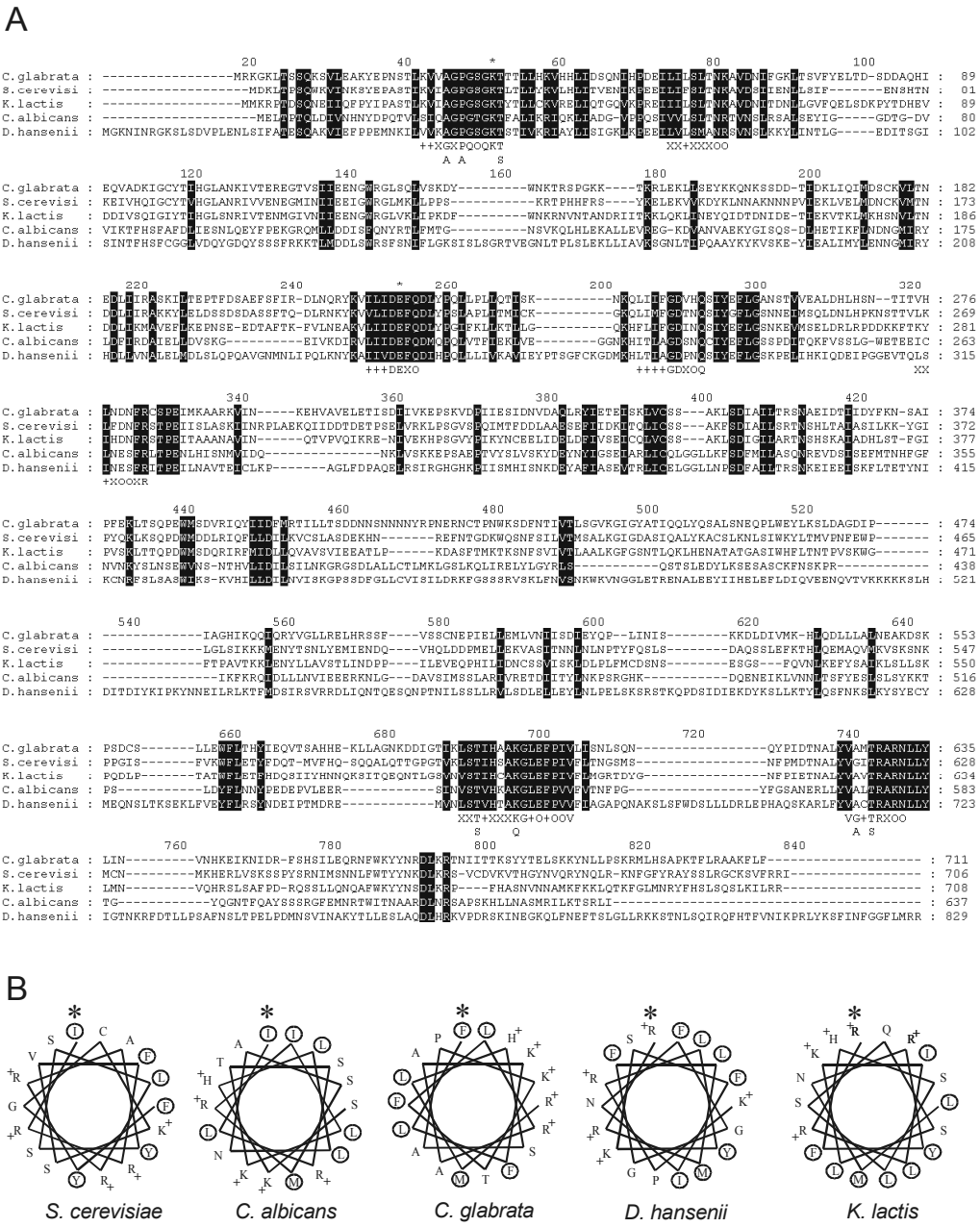


Figure 7. *In silico* analysis of Hmi1p-like proteins. A. Alignment of protein sequences. Identities and strong similarities are coloured in black. Consensus sequences of SF1 helicase motifs are below the alignment (+, hydrophobic residue; O, polar residue; X, any residue). Asterisks mark the conserved residues in Walker A and B motifs. B. Helical wheel diagrams of last C-terminal 18 amino acids. Positive amino acids are marked with the plus sign and hydrophobic ones encircled. Asterisks mark the last amino acids. Alignments were generated with GeneDoc (Nicholas et al., 1997).

3.6. *C. albicans* protein encoded by *ORF 19.7661* is the functional homologue of ScHmi1p

We asked if *C. albicans* protein encoded by *ORF 19.7661* that displays significant (~44%) sequence homology with ScHmi1 and possesses a putative C-terminal MTS is able to rescue the ρ^- formation phenotype associated with ScHmi1p loss. For expression in *S. cerevisiae* four CTG codons were substituted against TCG codons in the *ORF 19.7661* sequence to compensate for the difference in the genetic code between *S. cerevisiae* and *C. albicans*. The modified *ORF 19.7661* was placed under the control of *ScHMI1* promoter and terminator elements and analysed for complementation in a plasmid-shuffling experiment. Indeed the *C. albicans ORF 19.7661* was partially able to rescue *ScHMI1* deletion in a dosage-dependent manner (Figure 1 in I). Complementation was achieved only when the *C. albicans* protein was over-expressed from a 2μ vector. Expression from a ARS/CEN vector was not sufficient for this effect. Since enzymatically inactive ScHmi1p was still able to partially support wt mtDNA, it is possible that a similar effect could cause the observed limited complementation by the *ORF 19.7661*-encoded protein. Although being unable to perform enzymatic transactions, the *ORF 19.7661*-encoded protein might still guarantee the survival of respiratory competent cells with impaired efficiency similar to ScHmi1p mutants K32M and E211Q. To address this problem, conserved amino acids at positions vital for helicase activity were mutated in the *C. albicans* protein and resulting mutants (K31M and E204Q) analysed for complementation in *S. cerevisiae*. For this experiment the reduced complementation by wt *ORF 19.7661*-encoded protein was regarded as maximum. Neither of these mutants was able to support the maintenance of wt mtDNA (data not shown). This indicates that intact ATPase activity is necessary for complementation of *ScHMI1* deletion by the *ORF 19.7661*-encoded protein, thereby demonstrating the deficiency of the *C. albicans* protein in a putative structural role that ScHmi1p has. Another possibility is that the observed complementation effect is caused by non-specific helicase activity. To study if any helicase activity was able to support maintenance of mtDNA in the absence of ScHmi1p, the helicase UvrD, a member of SF1 was fused to ScHmi1p MTS and studied for possible complementation effect. This UvrD construct was unable to rescue loss of ScHmi1p, thereby demonstrating that complementation by the product of the *C. albicans ORF 19.7661* was specific for the given protein. Sequence homology, putative C-terminal MTS and specificity in complementation identify this protein as a functional homologue of ScHmi1p in *C. albicans* is therefore referred to as CaHmi1p.

3.7. Intracellular targeting of CaHmi1p and ScHmi1p is similar in *S. cerevisiae*

A unique feature of ScHmi1p is the unusual localisation of the mitochondrial targeting signal (MTS) (Sedman et al., 2000). Analysis of the C-terminal end sequence of CaHmi1p demonstrated the presence of a possible MTS (Figure 5B). To study the role of the C-terminus as a putative targeting sequence, GFP was fused into the N-terminus of CaHmi1p. In addition to wt, three mutants without 5, 10 or 16 C-terminal amino acid residues were constructed. Resulting ORF-s were placed under the control of a strong, inducible *MET3* promoter and integrated into *RP10* locus of *C. albicans* genome. Expression of the chimeric proteins was induced by methionine and cysteine free medium and their localisation was studied by fluorescence microscopy. In case of full-length and Δ5 CaHmi1p fusions, GFP signal co-localised exactly with the mitochondrial reticulum visualised with MitoTracker Red (MR) (Figure 2 in I). Distribution of GFP-CaHmi1p was uniform throughout the mitochondrial space without concentration into specific foci as it has been observed with some nucleoid-associated proteins like Abf2p (Okamoto et al., 1998). However, this might be due to over-expression driven by *MET3* promoter and might not reflect the pattern of endogenous CaHmi1p localisation. Co-localisation with mitochondria was not detected with Δ10 and Δ16 C-terminal deletions, demonstrating the requirement of C-terminal sequence for mitochondrial targeting. These results were in accordance with localisation studies of ScHmi1p (Sedman et al., 2000). In both cases deletion of 5 C-terminal amino acids from MTS did not have an effect on localisation. The unifying feature of all canonical MTS, as mentioned above, is the ability to form an amphipathic α-helix. Deletion of 5 C-terminal amino acids might not disrupt this structure to such an extent to render the MTS non-functional.

More detailed localisation studies with CaHmi1p were carried out in *S. cerevisiae* that has several markers for mitochondrial subcompartements. Localisation pattern of GFP fusions with full-length and C-terminal CaHmi1p deletions in *S. cerevisiae* were identical to their localisation in *C. albicans* (data not shown). As CaHmi1p protein tagged with a 10 residues long E2 peptide was able to complement *ScHMI1* deletion as efficiently as wt protein, the N-terminal E2 immunotag proved not to interfere with CaHmi1 sorting (Figure 1 in I). Purified mitochondria and mitoplasts (mitochondria without outer membrane) were subjected to proteinase K treatment with or without membrane-disrupting agent (SDS). The integral inner membrane protein D-lactate dehydrogenase and the matrix protein and co-chaperon Mge1p were used as compartment markers. E2-CaHmi1p was digested by proteinase K only if the inner membrane was disrupted by SDS. Extractions of the membrane fraction with TE and Na_2CO_3 demonstrated that like Mge1p, E2-CaHmi1p was found to be loosely associated with the inner membrane (Figure 3 in I). These

results indicate that E2-CaHmi1p is a peripheral inner membrane protein localised in the matrix. Submitochondrial localisation of CaHmi1p is therefore identical to ScHmi1p in *S. cerevisiae* (Sedman et al., 2000). This also eliminates the possibility that the decreased efficiency observed in complementation studies was caused by impaired localisation in *S. cerevisiae*.

3.8. CaHmi1p purification and substrate-specificity

CaHmi1p was overexpressed and purified from *E. coli* as a fusion protein with N-terminal His-tag. Recombinant protein was first enriched from cell lysate by affinity chromatography on Ni-NTA matrix. Crude protein preparation was subjected to subsequent purification by ion-exchange chromatography on S- and Q-Sepharose. Eluted Q-Sepharose fraction containing CaHmi1p was frozen in liquid nitrogen and stored at –70° C, where no apparent decrease in activity was observed for at least 4 months. The purified fraction contained no major proteins other than CaHmi1p (Figure 8).

For *in vitro* studies a series of double-stranded DNA substrates were generated with varying ssDNA overhang length and orientation. As expected, CaHmi1p was unable to resolve DNA substrate with blunt ends (Figure 4 in I). The directionality of helicase movement coupled with strand unwinding determines whether helicase follows 3'–5' or 5'–3' direction.

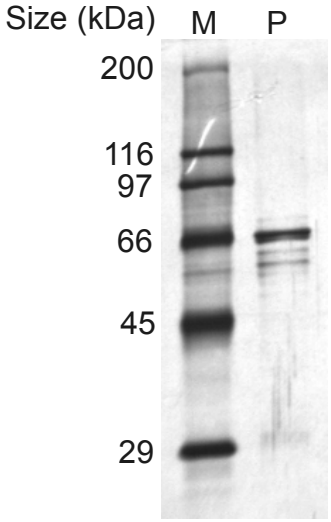


Figure 8. Silver-stained gel of purified CaHmi1p protein. M – molecular weight marker, the size of the bands are shown in left. P – Recombinant CaHmi1p protein after purification through consecutive affinity and ion-exchange columns.

Experiments with 5'-overhang substrate proved to be as unsuccessful as with blunt-ended substrates. On the other hand, DNA strands from substrates with 3'-overhang were efficiently displaced in presence of CaHmi1 and ATP. This indicates that CaHmi1p is a 3'–5' helicase that utilizes nucleotide cofactor hydrolysis to catalyze unwinding of DNA duplex. To determine the specific requirements for 3'-ssDNA overhang, CaHmi1p unwinding ability was analysed by using a series of substrates with different 3'-overhang length. This protein was found to unwind substrates with single-stranded overhangs from 22 to 13 nt with no apparent difference. CaHmi1p was even able, although less efficiently, to unwind substrates with only 9 and 5 nt overhangs. Therefore the requirements of the *C. albicans* helicase for the length of 3'-overhangs was less stringent than for ScHmi1p, where substrates with 16 nt overhang were not unwound (Figure 5 in II). By using a substrate with 22 nt ssDNA overhang the nucleotide cofactor usage was studied. In addition to ATP and dATP, which were the preferential cofactors, CaHmi1p was able to utilize to some extent also CTP and dCTP, the latter more efficiently than the former. In conclusion, the general unwinding characteristics of CaHmi1p and its nucleotide cofactor utilisation are similar to ScHmi1p.

3.9. CaHMI1 deletion strain displays retarded growth and altered nucleoid pattern

To achieve the disruption of both genomic alleles of *CaHMI1*, an integration/excision cassette developed by Morschhauser and colleagues was used (Wirsching et al., 2000). Deletion of one functional allele (strain PJ53) did not cause any observable changes in growth kinetics (data not shown). Surprisingly, disruption of both alleles proved to be not lethal in this petite-negative yeast, the corresponding strain PJ387 was still able to grow on fermentable and on non-fermentable medium (Figure 5B in I). However, loss of both *CaHMI1* alleles was associated with slow growth phenotype (Figure 5C in I). Strain PJ387 exhibited a longer doubling time than the wt CaI4 strain, this effect being more evident when the cells were grown on non-fermentable medium. This suggested defects in mtDNA maintenance process, since the wt mt genome is required for oxidative phosphorylation.

Analysis of DAPI-stained *C. albicans* cells revealed that mtDNA is organised into nucleoids as in other yeasts. No differences were observed in nucleoid morphology between wt and heterozygotic deletion strain. The loss of both *CaHMI1* alleles, however, caused sharp decrease in nucleoid brightness, indicating a possible drop in the amount of mtDNA (Figure 6 in I). To study the reversibility of the altered nucleoid pattern brought about by CaHmi1p deletion, the *CaHMI1* ORF with 400 bp of the downstream region and under control of the *MET3* promoter was inserted into the genome of strain PJ387, resulting in

strain PJ142. Induction of *MET3-CaHMI1* by omitting methionine and cysteine from the medium resulted in a nucleoid pattern that resembled neither wt nor strain PJ387. Although brightness of nucleoids was restored, nucleoids displayed a novel morphology. Instead of concentrating into punctuate foci, the DAPI signal was observable as thread-like structures, mimicking the mitochondrial staining pattern by MitoTracker Red. Therefore it is plausible, that mtDNA was uniformly dispersed throughout mitochondrial space in strain PJ142 under these conditions. This effect was dependent on CaHmi1p expression, as repression of *MET3* promoter resulted in a nucleoid pattern similar to strain PJ387. No alterations in mitochondrial morphology visualised by MitoTracker Red were observed (data not shown). The phenotypic effects of strains with modified *CaHMI1* loci are summarised in Table 3. Above results indicate that although CaHmi1p loss influences mtDNA maintenance, it is not lethal.

Table 3. Genotypes and phenotypes of *C. albicans* strains with modified *CaHMI1* loci, shown here alongside the parent strain CaI4.

Strain	Genotype	Viability	Growth	Nucleoids	mtDNA	Mito-hondria
CaI4	*Δura3::imm434*/ *Δura3::imm434*	Viable	Normal	Normal	Normal	Normal
PJ53	*Δura3::imm434* /*Δura3::imm434* *Δcahmi1*/*CaHMI1*	Viable	Normal	Normal	Normal	Normal
PJ387	*Δura3::imm434* /*Δura3::imm434* *Δcahmi1*/*Δcahmi1*	Viable	Slow	Aberrant	Fragmented	Normal
PJ142	*Δura3::imm434*/ *Δura3::imm434* *Δcahmi1*/*Δcahmi1* *Δrp10*::*CaMET3-CaHMI*/RP10	Viable	Normal	Aberrant	Normal	Normal

3.10. CaHmi1p affects mtDNA levels in reversible manner

The CaHmi1p-dependant decline in DAPI nucleoid signal may be caused by decrease in mtDNA mass. Therefore quantification of mtDNA levels was carried out in the presence and the absence of functional CaHmi1p. Measurements were performed with five probes that mapped to structural genes located on different regions on the *C. albicans* mt genome (Figure 7B in I). The signal of each probe was compared against a signal of the nuclear *MIP1* gene that served as a reference. As was the case with growth rate and nucleoid morphology, no significant changes were observed between wt and heterozygous strain (Figure 8 in I). In strain PJ387, however, most probes displayed a three- to fivefold decrease in mtDNA mass. Thus the decrease in nucleoid signal may be at least partially assigned to overall reduction in mtDNA amount. Surprisingly, this decrease was not uniform throughout the mt genome since a probe specific for the *cox2* gene displayed mtDNA levels similar to wt strain. A reintegrated *CaHMI1* copy restored mtDNA mass to roughly an equal level as wt strain with all probes used, indicating the reversible nature of this phenomenon.

Upon ρ^- genome formation in *S. cerevisiae* no drop in overall mtDNA amount per cell is observed. The subgenomic fragment that makes up the ρ^- genome is amplified to equal levels of wt mtDNA. Therefore the CaHmi1p effect differs from its *S. cerevisiae* homologue, indicating further differences in mtDNA fate after helicase loss between these species. Loss of *C. albicans* mt genome is not uniform but rather region-specific, as demonstrated by elevated levels of *cox2* region. The unexpected amplification of *cox2*-region can be explained by two scenarios. The *C. albicans* wt mt genome was shown to be a continious DNA molecule (Figure 7E in I). If this *in vivo* organisation does not change upon CaHmi1p loss, amplification of certain regions can only be achieved by expansion of repeats containing these regions within the molecule. Alternatively, breakdown of continuous DNA might produce separate molecules comprising different regions of mt genome. Differences in amounts of these physically separated molecules might result in alterations of mtDNA levels. In any case, changes in mtDNA amount accompanying CaHmi1p loss are fully reversible by exogenous CaHmi1p expression.

3.11. MtDNA becomes fragmented upon CaHmi1p loss

Uncut mtDNA from strains CaI4, PJ53, PJ387 and PJ142 were subjected to one-dimensional gel electrophoresis and hybridised with the same probes that were used in mtDNA mass analysis (Figure 7B in I). No dosage-dependant effect on mtDNA was observed between CaI4 and PJ53 strains similar to other phenotypic effects of CaHmi1p loss. Surprisingly, mtDNA in the CaHmi1p-deficient strain was fragmented, displaying numerous smaller fragments than full-length mt genome. Moreover, the pattern of fragmentation was different for every region tested, demonstrating differences in molecules formed after CaHmi1p loss. This variability could only occur if novel molecules containing different regions are physically separated from each other. Therefore the mtDNA sequence determines the specific pattern of a given region after CaHmi1p loss. Another remarkable feature is the simultaneous existence of several DNA molecules. However, it is not clear whether they represent heteroplasmy within one cell or different clonal populations. These molecules with different characteristics are maintained for a prolonged period of time without any tendency toward homogenisation (data not shown). Exogenous expression of CaHmi1p restores the original mtDNA state (Figure 7 in I).

2D gel electrophoresis analysis using a *cox2*-probe supported these results by demonstrating the shortening of overall mtDNA in strain PJ387 compared to wt (Figure 9 in I, A and B). There are also two separate populations of branched molecules in strain PJ387, one originating approximately from the region where shorter fragments were located. More detailed analysis of this region failed to identify any circular DNA forms both in wt and CaHmi1p-deficient strains (Figure 9 in I, C and D).

Prolonged exposure of PJ387 mtDNA signal revealed a minor fraction of molecules migrating in the compression zone in conventional one-dimensional gel (Figure 7C in I). This indicates the presence of substantially longer molecules than subgenomic fragments that make up the majority of PJ387 mtDNA. Similar results were obtained with PFGE analysis where a minor portion of well-bound signal was also observed. It is possible that molecules with complex structures of this fraction become sheared during isolation procedures and migrate in compression zone in one-dimensional gel electrophoresis. Whatever case, in the background of extensive mtDNA breakdown in strain PJ387 a small percentage of molecules with wt-like appearance is still present.

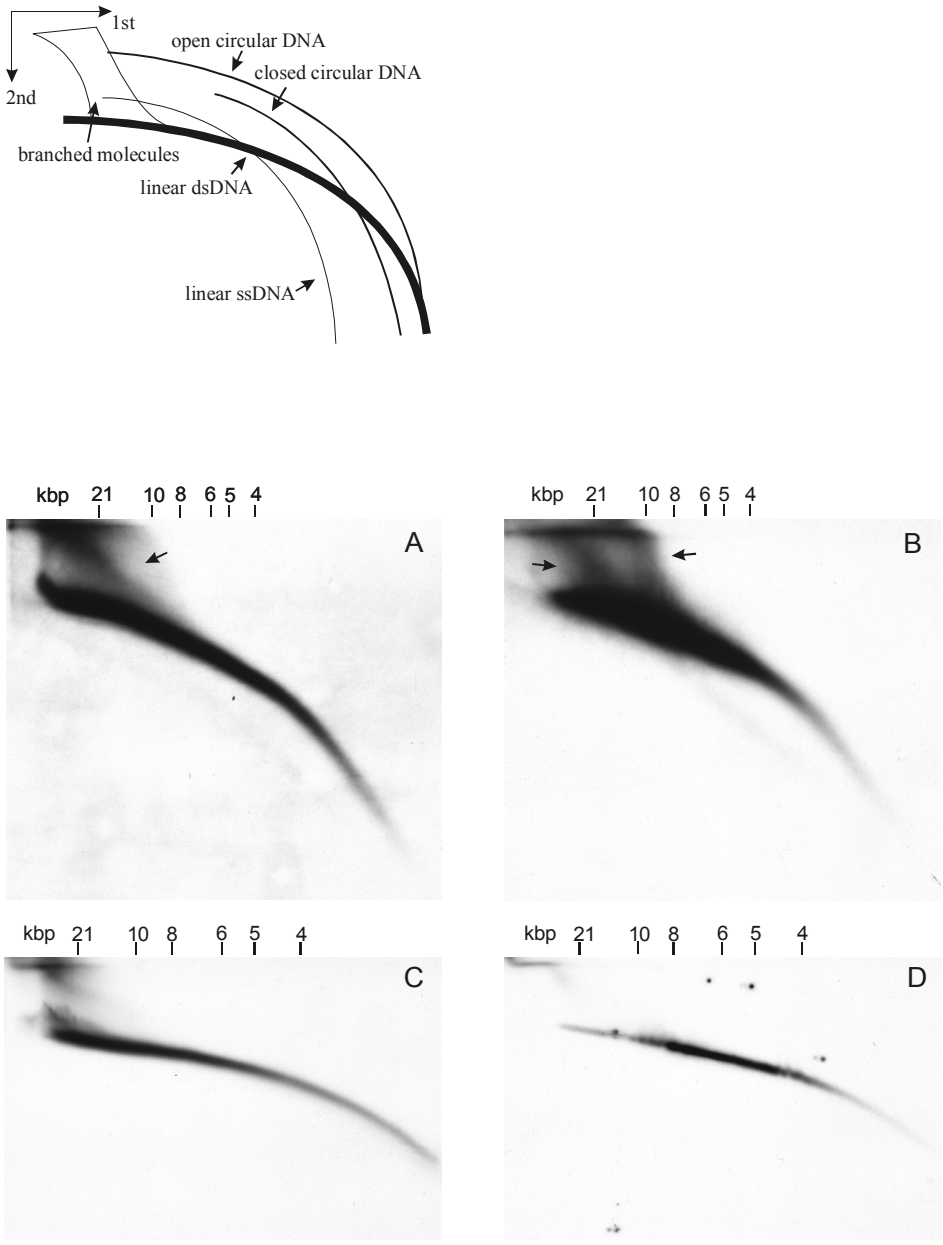


Figure 9. Uncut mtDNA of strains CaI4 (A and C) and PJ387 (B and D) in 2D gel electrophoresis visualised by *cox2*-probe. Electrophoresis parameters for A and B are described in Ref. I. Conditions for C and D are as follows: 1st dimension – 0,58V/cm for 48h at room temperature, 2nd dimension – 3V/cm for 14h at 4°C in presence of ethidium bromide (300 ng/ml). Arrows point to populations of branched DNA molecules. The DNA size marker bands are shown above each individual blot.

3.12. Discussion

Although ScHmi1p has been established as a mitochondrial DNA helicase in *S. cerevisiae*, its precise biological role has remained obscure. We sought to elucidate its function by performing different *in vivo* screens. The conditional alleles are useful tools in observing the time-course effects of loss of protein function and also in search of genetical interaction partners. Unexpectedly, the two mutant alleles analysed yielded no multicopy suppressors other than endogenous ScHmi1p. The strategy of high-copy suppressor screens relies on expression difference between the conditional allele and a possible rescue factor. A plasmid-borne system might therefore not be sensitive enough to reveal all possible genetic interactions. However, a similar approach has been successfully used before, for example to identify a frataxin homologue in yeast as a high-copy suppressor of a mitochondrial intermediate peptidase (Branda et al., 1999). The inability to isolate multicopy suppressors may also be the specific feature of mutants selected for the screen or a general feature of the helicase ScHmi1p.

Another *in vivo* screen described the ability of mutations where helicase activity was disrupted to still support maintenance of the wt mt genome, albeit with decreased efficiency. Thus the separation of DNA strands is not the only task carried out by ScHmi1p. For example, by participating in assembly of a putative macromolecular complex required for mtDNA synthesis, enzymatically inactive Hmi1p may still guarantee the survival of wt mtDNA. In that case the absent helicase activity may be complemented by another helicase. Indirect evidence supporting this hypothesis comes from the fact that reintroduction of wt ScHmi1p into a ρ^- strain causes the increase in length of ρ^- concatemers. Surprisingly, the same effect was observed when helicase-deficient mutants were used. In ρ^- strains this helicase-independent effect is therefore reflected by increased processivity of replication producing longer concatemeres than a system devoid of ScHmi1p. These results support the view of ScHmi1p being the processivity factor of wt mtDNA synthesis. Presence of ssDNA in ρ^- strains, however, required enzymatically active protein. This might point to yet another change in the mode of replication of ρ^- genomes brought about by the helicase activity of ScHmi1p.

The opportunistic pathogen *C. albicans* has several important differences from baker's yeast as for example being obligatory diploid and petite-negative. At the same time, nevertheless, its coding capacity and *in vivo* organisation of mtDNA is remarkably similar to *S. cerevisiae*. It also has several homologues of mtDNA maintenance factors as demonstrated by sequence alignments (Nosek et al., 2006). This suggests that differences in the mtDNA maintenance system cannot be too great. Sequence alignment, complementation studies, submitochondrial localisation and biochemial experiments *in vitro* identified the homologue of ScHmi1p in *C. albicans*, termed CaHmi1p. By demonstrating the viability of *CaHMI1* deletion in *C. albicans*, we established that this protein is not essential for wt mtDNA. However, maintenance of mtDNA is affected in

the absence of CaHmi1p, as indicated by changes in mtDNA appearance and altered nucleoid pattern. In *S. cerevisiae* ScHmi1p expression also influences the nucleoid number in a hs ρ^- strain. As overall mtDNA amounts remained the same in hs *S. cerevisiae* strains, rearrangements in the nucleoid pattern were caused by sorting a larger number of individual mtDNA molecules into separate nucleoids. CaHmi1p loss had an opposite effect with regard to nucleoid brightness and did not cause changes in the approximate nucleoid number per cell. This may indicate that the CaHmi1p effect on nucleoids is related only to changes in mtDNA amount and nucleoid organisation remains unaltered. However, nucleoids do display changed appearance in strain PJ142. Relations between changes of nucleoid morphology and growth environment have been reported in *S. cerevisiae* (MacAlpine et al., 2000). No changes in nucleoid appearance of *C. albicans* wt strain were evident between *MET3* repressed and unrepressed conditions (data not shown). Therefore the inability for normal mtDNA segregation into nucleoids is dependant on Cami1p expression. Aberrant nucleoids may escape detection in absence of CaHmi1p due to decreased DAPI signal in strain PJ387. Thread-like distribution of mtDNA may also be an artefact caused by over-expression of CaHmi1p. The helicase Rep, a member of SF1, is a non-specific DNA helicase with the ability to bind DNA regardless of its sequence (Wong et al., 1992). If the same applies for CaHmi1p, then the excess of this enzyme may disrupt the normal nucleoid structure and cause mtDNA to distribute throughout the mitochondrial space. However, altered nucleoid morphology did not affect notably mtDNA inheritance, as the growth rate of strain PJ142 was not decreased compared to wt (data not shown).

In *S. cerevisiae* loss of ScHmi1p causes shift from wt to a new mtDNA maintenance system, which is unable to uphold wt mtDNA. This system is able to maintain only ρ^- genomes, which regardless of their wt-like physical appearance (Bendich, 1996) contain only one concatemerically arranged fragment of wt mt genome. Since transition from wt to ρ^- mt genome is initiated without any gain-of-function effect in *S. cerevisiae*, the wt genome can sustain both maintenance modes. Analogous capability to support multiple maintenance systems may also be the property of *C. albicans*. Observed fragmentation of mtDNA in this organism after deletion of *CaHMI1* is similar to the mtDNA phenotype of the *S. cerevisiae* strain with the conditional *hmi1-5* allele at non-permissive temperature. But if in *S. cerevisiae* this fragmentation represented the transition state between wt and ρ^- mt genomes, then in *C. albicans* constant heterogeneity of fragment sizes and amount persisted. This means that these fragments are constantly regenerated and therefore are not the products of unsupported mtDNA degradation. From the pool of fragments formed after loss of CaHmi1p, the *cox2*-region was especially conspicuous with wt-like mtDNA levels and characteristic fragmentation pattern. If the probes for *atp6*, *cox3*, *nad2* and *nad5* regions display rather uniform distribution of polydisperse molecules, the pattern of *cox2* fragmentation is distinguished by relatively

strong fragments. This indicates the presence of molecules with discreet lengths. The *cox2*-region is also unique in sense of location since it is situated between inverted repeat sequences (Figure 7A in III). Therefore fragments comprising *cox2* and extending to flanking repeat sequences have complementary regions at both ends. This might lead to circularisation of linear molecules by homologous recombination and formation of a circular molecule pool independent of the rest of the mtDNA. Subsequent amplification, perhaps by rolling-circle mode of replication, may cause the observed increase in mtDNA levels. Simultaneous existence of a linear mt genome and extragenomic circular molecules that are replicated by rolling-circle (t-circles) have been observed in another *Candida* species, *C. parapsilosis* (Nosek et al., 2005). However, 2D analysis did not reveal any DNA forms that could be interpreted as circular DNA molecules neither from wt nor PJ387 strains (Figure 7). Moreover, in strain PJ387 a second population of branched molecules can be seen originating from the linear arc between 8 and 12 kbp. This refers to the presence of free DNA ends, a prerequisite for formation of branched structures through recombination, in a region were the strongest bands in one-dimensional *cox2*-probed blots are concentrated. These results suggest that novel mtDNA molecules formed after loss of CaHmi1p are of linear topology.

Replication of linear DNA units without compensation mechanism causes the gradual reduction of 3'-ends. Since smaller molecules formed upon CaHmi1p deletion are linear, runaway replication in the absence of such a mechanism would lead to shortening and eventually loss of subgenomic fragments. Although linear mt genomes with various end-replication-problem solving mechanisms are widely distributed and present in several taxonomic groups, it is unlikely that such a shift in the replication mode is initiated by the loss of a helicase. A more plausible explanation would be that these linear subgenomic fragments are generated by replication. A candidate for a template is the observed minor fraction of wt-length mtDNA species in strain PJ387. Similarly, PFGE analysis revealed that in addition to shortened polydisperse fragments strain PJ387 also contained a small fraction of molecules with topology similar to wt. This smaller fraction can either represent a mixture of subgenomic fragments organised as concatemers that have acquired wt-like topology (similar to ρ^- genomes) or "true" wt mtDNA. Although based on existing data concatemers cannot be ruled out, the petite-negative nature of *C. albicans* favours the presence of wt mtDNA fraction. Provided the relatively high gene content of the *C. albicans* mt genome, transcription from polydisperse fragments would generate numerous truncated non-functional polypeptides instead of normal proteins. The relatively mild growth defect of strain PJ387 (Figure 5C in I) suggests mostly intact ETC and F_0F_1 ATPase complexes and supports the presence of uninterrupted transcription templates.

The wt-like abundance of *cox2*-marked linear molecules in strain PJ387 raises the question of their difference from other fragments. One possible explanation is the vicinity of an active replication origin (or origins). Frequent

firing of an adjacent origin would explain the amplification of this particular region. The interesting question is why this amplification is limited to sequences in the vicinity of the *cox2* gene in the absence of CaHmi1p. Quantification has shown that most distant regions from *cox2* also display lowest mtDNA levels. If there is an origin close to *cox2*, then this data may point to processivity problems of the DNA synthesis machinery. As demonstrated in *E. coli*, arrested replication forks are vulnerable structures to double-strand breaks (Michel et al., 1997). In yeast stalled nuclear replication forks are stabilised by intra-S phase checkpoint response (Lopes et al., 2004), cells deficient of this Mec1/Rad53 pathway accumulate aberrant DNA structures prone to erosion by ExoI nuclease (Cotta-Ramusino et al., 2005). Replication fork blocks (RFB) can be caused by collision with protein-DNA structures or by complex DNA structures like those formed on centromeres (Greenfeder & Newlon, 1992, Deshpande & Newlon, 1996, Ivessa et al., 2003). Accumulation of paused forks on mtDNA in an environment of high oxidative stress is likely to lead to DNA degradation. Since sequences like for example tRNA genes, where replication might stall, are present throughout the genome (Jones et al., 2004), DNA replication stopping at these structures can generate a series of fragments with characteristic patterns for every given region. The helicase Rrm3p has a function in resolving such structures during genomic DNA replication (Ivessa et al., 2002, Ivessa et al., 2003). Although not being a replicative helicase, Rrm3p facilitates the proceeding of replication forks through certain regions. In the absence of this helicase replication fork movement frequently stalls followed by formation of double-stranded breaks (Ivessa et al., 2003). ScHmi1p has been shown to bind stems of DNA hairpins (Kuusk et al., submitted), which are potential replication fork-stalling structures. We propose a similar auxiliary function for Hmi1p by analogy to Rrm3p in mtDNA replication. According to this model Hmi1p is not the elongative helicase in replication but facilitates the progression of the replication fork by overcoming RFB-s. In the absence of Hmi1p mtDNA replication would frequently stall at these barriers. In *S. cerevisiae* impaired wt-like replication is substituted by a ρ^- maintenance system. However, as discussed above, Hmi1p can still facilitate the synthesis of longer molecules in ρ^- background. In *C. albicans* products of inefficient replication will increase, giving rise to a specific fragmentation pattern. This difference in response to loss of Hmi1p is probably caused by requirement for the full coding potential of the entire mt genome. Therefore the selective pressure applied by ρ^0 lethality distinguishes the fate of *C. albicans* mtDNA from that in *S. cerevisiae*. This difference can be exploited by studies of other mtDNA factors, since it allows observing the changes of wt mtDNA in response to presence of mtDNA maintenance factors. As described above, deletion of several mtDNA maintenance factors will lead to the formation of ρ^- genomes in *S. cerevisiae*. This limits the observations of their effects on non-wt maintenance systems. Therefore *C. albicans* is a useful model system for mtDNA maintenance studies, especially when coupled with genetical data from *S. cerevisiae*.

4. CONCLUSIONS

1. Organisation of mtDNA is similar in *S. cerevisiae* and *C. albicans.*

2. ScHmi1p has an auxiliary function in mtDNA maintenance in addition to helicase activity.

3. CaHmi1p is the functional homologue of ScHmi1p with similar features.

4. CaHmi1p deletion is non-lethal in *C. albicans*.

5. CaHmi1p is required for wt mtDNA organisation and nucleoid pattern.

6. Effects of CaHmi1p deletion on mtDNA organisation are actively reversible.

REFERENCES

Aoki, S., Ito-Kuwa, S. (1987) Induction of petite mutation with acriflavine and elevated temperature in *Candida albicans*. *J Med Vet Mycol*, **25**, 269–277.

Azpiroz, R., Butow, R.A. (1993) Patterns of mitochondrial sorting in yeast zygotes. *Mol Biol Cell*, **4**, 21–36.

Baldacci, G., Bernardi, G. (1982) Replication origins are associated with transcription initiation sequences in the mitochondrial genome of yeast. *EMBO J*, **1**, 987–994.

Baldacci, G., Cherif-Zahar, B., Bernardi, G. (1984) The initiation of DNA replication in the mitochondrial genome of yeast. *EMBO J*, **3**, 2115–2120.

Bateman, J.M., Perlman, P.S., Butow, R.A. (2002)a Mutational bisection of the mitochondrial DNA stability and amino acid biosynthetic functions of Ilv5p of budding yeast. *Genetics*, **161**, 1043–1052.

Bateman, J.M., Iacovino, M., Perlman, P.S., Butow, R.A. (2002)b Mitochondrial DNA instability mutants of the bifunctional protein Ilv5p have altered organization in mitochondria and are targeted for degradation by Hsp78 and the Pim1p protease. *J Biol Chem*, **277**, 47946–47953.

Bendich, A.J. (1996) Structural analysis of mitochondrial DNA molecules from fungi and plants using moving pictures and pulsed-field gel electrophoresis. *J Mol Biol*, **255**, 564–588.

Bennett, R.J., Johnson, A.D. (2005) Mating in *Candida albicans* and the search for a sexual cycle. *Annu Rev Microbiol*, **59**, 233–255.

Blanc, H., Dujon, B. (1980) Replicator regions of the yeast mitochondrial DNA responsible for suppressiveness. *Proc Natl Acad Sci USA*, **77**, 3942–3946.

Blanco, L., Bernard, A., Salas, M. (1991) MIP1 DNA polymerase of *S. cerevisiae*: structural similarity with the E. coli DNA polymerase I-type enzymes. *Nucleic Acids Res*, **19**, 955.

Boldogh, I.R., Nowakowski, D.R., Yang, H.C., Chung, H., Karmon, S., Royes, P., Pon, L.A. (2003) A protein complex containing Mdm10p, Mdm12p, and Mmm1p links mitochondrial membranes and DNA to the cytoskeleton-based segregation machinery. *Mol Biol Cell*, **14**, 4618–4627.

Branda, S.S., Yang, Z.Y., Chew, A., Isaya, G. (1999) Mitochondrial intermediate peptidase and the yeast frataxin homolog together maintain mitochondrial iron homeostasis in *Saccharomyces cerevisiae*. *Hum Mol Genet*, **8**, 1099–1110.

Bui, E.T., Bradley, P.J., Johnson P.J. (1996) A common evolutionary origin for mitochondria and hydrogenosomes. *Proc Natl Acad Sci USA*, **93**, 9651–9656.

Bulder, C.J. (1964) Induction of petite mutation and inhibition of synthesis of respiratory enzymes in various yeasts. *Antonie van Leeuwenhoek*, **30**, 1–9.

Burgess, S.M., Delannoy, M., Jensen, R.E. (1994) MMM1 encodes a mitochondrial outer membrane protein essential for establishing and maintaining the structure of yeast mitochondria. *J Cell Biol*, **126**, 1375–1391.

Caruthers, J.M., McKay, D.B. (2002) Helicase structure and mechanism. *Curr Opin Struct Biol*, **12**, 123–133.

Chen, X.J., Clark-Walker, G.D. (1993) Mutations in MGI genes convert *Kluyveromyces lactis* into a petite-positive yeast. *Genetics*, **133**, 517–525.

Chen, X.J., Clark-Walker, G.D. (1995) Specific mutations in alpha- and gamma-subunits of F1-ATPase affect mitochondrial genome integrity in the petite-negative yeast *Kluyveromyces lactis*. *EMBO J*, **14**, 3277–3286.

Chen, X.J., Clark-Walker, G.D. (1996) The mitochondrial genome integrity gene, MGI1, of *Kluyveromyces lactis* encodes the beta-subunit of F1-ATPase. *Genetics*, **144**, 1445–1454.

Chen, X.J., Clark-Walker, G.D. (1999) Alpha and beta subunits of F1-ATPase are required for survival of petite mutants in *Saccharomyces cerevisiae*. *Mol Gen Genet*, **262**, 898–908.

Chen, X.J., Clark-Walker, G.D. (2000) The petite mutation in yeasts: 50 years on. *Int Rev Cytol*, **194**, 197–238.

Chen, X.J., Guan, M.X., Clark-Walker, G.D. (1993) MGM101, a nuclear gene involved in maintenance of the mitochondrial genome in *Saccharomyces cerevisiae*. *Nucleic Acids Res*, **21**, 3473–3477.

Chen, X.J., Hansbro, P.M., Clark-Walker, G.D. (1998) Suppression of rho0 lethality by mitochondrial ATP synthase F1 mutations in *Kluyveromyces lactis* occurs in the absence of F0. *Mol Gen Genet*, **259**, 457–467.

Chen, X.J., Wang, X., Kaufman, B.A., Butow, R.A. (2005) Aconitase couples metabolic regulation to mitochondrial DNA maintenance. *Science*, **307**, 714–717.

Chi, N.W., Kolodner, R.D. (1994)a Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches. *J Biol Chem*, **269**, 29984–29992.

Chi, N.W., Kolodner, R.D. (1994)b The effect of DNA mismatches on the ATPase activity of MSH1, a protein in yeast mitochondria that recognizes DNA mismatches. *J Biol Chem*, **269**, 29993–29997.

Clark-Walker, G.D., Chen, X.J. (2001) Dual mutations reveal interactions between components of oxidative phosphorylation in *Kluyveromyces lactis*. *Genetics*, **159**, 929–938.

Clark-Walker, G.D., Francois, F., Chen X.J., Vieira Da Silva, M.R., Claisse, M.L. (1997) Mitochondrial ATP synthase subunit 9 is not required for viability of the petite-negative yeast *Kluyveromyces lactis*. *Curr Genet*, **31**, 488–493.

Clark-Walker, G.D., Hansbro, P.M., Gibson, F., Chen, X.J. (2000) Mutant residues suppressing rho(0)-lethality in *Kluyveromyces lactis* occur at contact sites between subunits of F(1)-ATPase. *Biochim Biophys Acta*, **1478**, 125–137.

Cotta-Ramusino, C., Fachinetti, D., Lucca, C., Doksani, Y., Lopes, M., Sogo, J., Foiani, M. (2005) Exo1 processes stalled replication forks and counteracts fork reversal in checkpoint-defective cells. *Mol Cell*, **17**, 153–159.

Cummings, D.J., McNally, K.M., Domenico, J.M., Matsuura, E.T. (1990) The complete DNA sequence of the mitochondrial genome of *Podospora anserina*. *Curr Genet*, **17**, 375–402.

Deshpande, A.M., Newlon, C.S. (1996) DNA replication fork pause sites dependent on transcription. *Science*, **272**, 1030–1033.

Defontaine, A., Bouchara, J.P., Declerk, P., Planchenault, P., Chabasse, D., Hallet, J.N. (1999) In-vitro resistance to azoles associated with mitochondrial DNA deficiency in *Candida glabrata*. *J Med Microbiol*, **48**, 663–670.

Desai, S.D., Pasupathy K., Chetty, K.G., Pradhan, D.S. (1989) Evidence for the presence of DNA primase in mitochondria of *Saccharomyces cerevisiae*. *Biochem Biophys Res Commun*, **160**, 525–534.

Diffley, J.S., Stillman, B. (1991) A close relative of the nuclear, chromosomal high-mobility group protein HMG1 in yeast mitochondria. *Proc Natl Acad Sci USA*, **88**, 7864–7868.

Diffley, J.S., Stillman, B. (1992) DNA binding properties of an HMG1-related protein from yeast mitochondria. *J Biol Chem*, **267**, 3368–3374.

Dimmer, K.S., Jakobs, S., Vogel, F., Altmann, K., Westermann, B. (2005) Mdm31 and Mdm32 are inner membrane proteins required for maintenance of mitochondrial shape and stability of mitochondrial DNA nucleoids in yeast. *J Cell Biol*, **168**, 103–115.

Dinouel, N., Drissi, R., Miyakawa, I., Sor, F., Rousset, S., Fukuhara, H. (1993) Linear mitochondrial DNAs of yeasts: closed-loop structure of the termini and possible linear-circular conversion mechanisms. *Mol Cell Biol*, **13**, 2315–2323.

Donahue, S.L., Corner, B.E., Bordone, L., Campbell, C. (2001) Mitochondrial DNA ligase function in *Saccharomyces cerevisiae*. *Nucleic Acids Res*, **29**, 1582–1589.

Doudican, N.A., Song, B., Shadel, G.S., Doetsch, P.W. (2005) Oxidative DNA damage causes mitochondrial genomic instability in *Saccharomyces cerevisiae*. *Mol Cell Biol*, **25**, 5196–5204.

Dujon, B. (1981) Mitochondrial genetics and functions. In: The molecular biology of the yeast *Saccharomyces*. Strathern, J.N., Jones, E.W., Broachs, J.R., editors. Cold Spring Harbour Laboratory Press, Cold Spring Harbour, New York, 505–635.

Fangman, W.L., Henly, J.W., Brewer, B.J. (1990) RPO41-independent maintenance of [rho-] mitochondrial DNA in *Saccharomyces cerevisiae*. *Mol Cell Biol*, **10**, 10–15.

Fangman, W.L., Henly, J.W., Churchill, G., Brewer, B.J. (1989) Stable maintenance of a 35-base-pair yeast mitochondrial genome. *Mol Cell Biol*, **9**, 1917–1921.

Faugeron-Fonty, G., Le Van Kim, C., de Zamaroczy, M., Goursot, R., Bernardi, G. (1984) A comparative study of the ori sequences from the mitochondrial genomes of twenty wild-type yeast strains. *Gene*, **32**, 459–473.

Fernet, C.S., Clark-Walker, G.D., Claisse, M.L. (2002) The mitochondrial genome can be altered or lost without lethal effect in the petite-negative yeast *Debaryomyces* (*Schwanniomyces*) *occidentalis*. *Curr Genet*, **42**, 94–102.

Foury, F. (1989) Cloning and sequencing of the nuclear gene MIP1 encoding the catalytic subunit of the yeast mitochondrial DNA polymerase. *J Biol Chem*, **264**, 20552–20560.

Foury, F., Dyck E.V. (1985) A PIF-dependent recombinogenic signal in the mitochondrial DNA of yeast. *EMBO J*, **4**, 3525–3530.

Foury, F., Kolodynski, J. (1983) pif mutation blocks recombination between mitochondrial rho+ and rho- genomes having tandemly arrayed repeat units in *Saccharomyces cerevisiae*. *Proc Natl Acad Sci*, **80**, 5345–5349.

Foury, F., Lahaye, A. (1987) Cloning and sequencing of the PIF gene involved in repair and recombination of yeast mitochondrial DNA. *EMBO J*, **6**, 1441–1449.

Foury, F., Roganti, T., Lecrenier, N., Purnelle, B. (1998) The complete sequence of the mitochondrial genome of *Saccharomyces cerevisiae*. *FEBS Lett*, **440**, 325–331.

Foury, F., Vanderstraeten, S. (1992) Yeast mitochondrial DNA mutators with deficient proofreading exonucleolytic activity. *EMBO J*, **11**, 2717–2726.

Friddle, R.W., Klare, J.E., Martin, S.S., Corzett, M., Balhorn, R., Baldwin, E.P., Baskin, R.J., Noy, A. (2004) Mechanism of DNA compaction by yeast mitochondrial protein Abf2p. *Biophys J*, **86**, 1632–1639.

Fukuhara, H., Sor, F., Drissi, R., Dinouel, N., Miyakawa, I., Rousset, S., Viola, AM. (1993) Linear mitochondrial DNAs of yeasts: frequency of occurrence and general features. *Mol Cell Biol*, **13**, 2309–2314.

Genga, A., Bianchi, L., Foury, F. (1986) A nuclear mutant of *Saccharomyces cerevisiae* deficient in mitochondrial DNA replication and polymerase activity. *J Biol Chem*, **261**, 9328–9332.

Giraud, M.F., Velours, J. (1997) The absence of the mitochondrial ATP synthase delta subunit promotes a slow growth phenotype of rho- yeast cells by a lack of assembly of the catalytic sector F1. *Eur J Biochem*, **245**, 813–818.

Gorbalenya, A.E., Koonin, E.V. (1993) Hleicases- amino acid sequence comparisons and structure-function relationships. *Curr Op Struct Biol*, **3**, 419–429.

Graves, T., Dante, M., Eisenhour, L., Christianson, T.W. (1998) Precise mapping and characterization of the RNA primers of DNA replication for a yeast hyper-suppressive petite by in vitro capping with guanylyltransferase. *Nucleic Acids Res*, **26**, 1309–1316.

Gray, M.W., Burger, G., Lang, B.F. (1999) Mitochondrial evolution. *Science*, **283**, 1476–1481.

Greenfeder, S.A., Newlon, C.S. (1992) Replication forks pause at yeast centromeres. *Mol Biol Cell*, **12**, 4056–4066.

Greenleaf, A.L., Kelly, J.L., Lehman, I.R. (1986) Yeast RPO41 gene product is required for transcription and maintenance of the mitochondrial genome. *Proc Natl Acad Sci USA*, **83**, 3391–3394.

Herman, A.I., Griffin, P.S. (1968) Respiratory-deficient mutants in *Saccharomyces lactis*. *J Bacteriol*, **96**, 457–461.

Hinnebusch, A.G., Jackson, B.M., Mueller, P.P. (1988) Evidence for regulation of reinitiation in translational control of GCN4 mRNA. *Proc Natl Acad Sci USA*, **85**, 7279–7283.

Hobbs, A.E., Srinivasan, M., McCaffery, J.M., Jensen, R.E. (2001) Mmm1p, a mitochondrial outer membrane protein, is connected to mitochondrial DNA (mtDNA) nucleoids and required for mtDNA stability. *J Cell Biol*, **152**, 401–410.

Hollenberg, C.P., Borst, P., Thuring, R.W., van Bruggen, E.F. (1969) Size, structure and genetic complexity of yeast mitochondrial DNA. *Biochim Biophys Acta,* **186**, 417–419.

Horner, D.S., Hirt, R.P., Kilvington, S., Lloyd, D. (1996) Molecular data suggest an early acquisition of the mitochondrion endosymbiont. *Proc Biol Sci*, **263**, 1053–1059.

Ito, J., Braithwaite, D.K. (1990) Yeast mitochondrial DNA polymerase is related to the family A DNA polymerases. *Nucleic Acids Res*, **18**, 6716.

Ivessa, A.S., Lenzmeier, B.A., Bessler, J.B., Goudsouzian, L.K., Schnakenberg, S.L., Zakian, V.A. (2003) The *Saccharomyces cerevisiae* helicase Rrm3p facilitates replication past nonhistone protein-DNA complexes. *Mol Cell*, **12**, 1525–1536.

Ivessa, A.S., Zhou, J.Q., Schulz, V.P., Monson, E.K., Zakian, V.A. (2002) *Saccharomyces* Rrm3p, a 5' to 3' DNA helicase that promotes replication fork progression through telomeric and subtelomeric DNA. *Genes Dev*, **16**, 1383–1396.

Ivessa, A.S., Zhou, J.Q., Zakian, V.A. (2000) The Saccharomyces Pif1p DNA helicase and the highly related Rrm3p have opposite effects on replication fork progression in ribosomal DNA. *Cell*, **100**, 479–489.

Jacobs, M.A., Payne, S.R., Bendich, A.J. (1996) Moving pictures and pulsed-field gel electrophoresis show only linear mitochondrial DNA molecules from yeasts with linear-mapping and circular-mapping mitochondrial genomes. *Curr Genet*, **30**, 3–11.

Jones, T., Federspiel, N.A., Chibana, H., Dungan, J., Kalman, S., Magee, B.B., Newport, G., Thorstenson, Y.R., Agabian, N., Magee, P.T., Davis, R.W., Scherer, S. (2004) The diploid genome sequence of Candida albicans. *Proc Natl Acad Sci USA*, **101**, 7329–7334.

Kao, L.R., Megraw, T.L., Chae, C.B. (1993) Essential role of the HMG domain in the function of yeast mitochondrial histone HM: functional complementation of HM by the nuclear nonhistone protein NHP6A. *Proc Natl Acad Sci USA*, **90**, 5598–5602.

Kaufman, B.A., Kolesar, J.E., Perlman, P.S., Butow, R.A. (2003) A function for the mitochondrial chaperonin Hsp60 in the structure and transmission of mitochondrial DNA nucleoids in *Saccharomyces cerevisiae*. *J Cell Biol*, **163**, 457–461.

Kaufman, B.A., Newman, S.M., Hallberg, R.L., Slaughter, C.A., Perlman, P.S., Butow, R.A. (2000) In organello formaldehyde crosslinking of proteins to mtDNA: identification of bifunctional proteins. *Proc Natl Acad Sci USA*, **97**, 7772–7777.

Kerscher, S., Durstewitz, G., Casaregola, S., Gaillardin, G., Brandt, U. (2001) The complete mitochondrial genome of *Yarrowia lipolytica*. *Comp Funct Genom,* **2**, 80–90.

Kispal, G., Sipos, K., Lange, H., Fekete, Z., Bedekovics, T., Janaky, T., Bassler, J., Aquilar Netz, D.J., Balke, J., Rotte, C., Lill, R. (2005) Biogenesis of cytosolic ribosomes requires the essential iron-sulphur protein Rli1p and mitochondria. *EMBO J*, **24**, 589–598.

Kleff, S., Kemper, B., Sternglanz, R. (1992) Identification and characterization of yeast mutants and the gene for a cruciform cutting endonuclease. *EMBO J*, **11**, 699–704.

Kolodner, R.D., Marsischky, G.T. (1999) Eukaryotic DNA mismatch repair. *Curr Opin Genet Dev*, **9**, 89–96.

Koprowski, P., Fikus, M.U., Mieczkowski, P., Ciesla, Z. (2002) A dominant mitochondrial mutator phenotype of *Saccharomyces cerevisiae* conferred by msh1 alleles altered in the sequence encoding the ATP-binding domain. *Mol Genet Genomics*, **266**, 988–994.

Koszul, R., Malpertuy, A., Frangeul, L., Bouchier, C., Wincker, P., Thierry, A., Duthoy, S., Ferris, S., Hennequin, C., Dujon, B. (2003) The complete mitochondrial genome sequence of the pathogenic yeast *Candida* (*Torulopsis*) *glabrata*. *FEBS Lett*, **534**, 39–48.

Kupfer, C., Kemper, P. (1996) Reactions of mitochondrial cruciform cutting endonuclease 1 (CCE1) of yeast *Saccharomyces cerevisiae* with branched DNAs *in vitro*. *Eur J Biochem*, **238**, 77–87.

Kuusk, S., Sedman, T., Aun, A., Sedman, J. (2006) Hmi1p structure- and sequence-specific binding to ori/rep elements. *Submitted*.

Lahaye, A., Leterme, S., Foury, F. (1993) PIF1 DNA helicase from *Saccharomyces cerevisiae*. Biochemical characterization of the enzyme. *J Biol Chem*, **268**, 26155–26166.

Lahaye, A., Stahl, L., Thines-Sempoux, D., Foury, F. (1991) PIF1: a DNA helicase in yeast mitochondria. *EMBO J*, **10**, 997–1007.

Lang, B.F., Ahne, F., Distler, S., Trinkl, H., Kaudewitz, F., Wolf K. (1983) In: Mitochondria 1983,"Nucleo-mitochondrial interactions". Schweyen et al. (eds.), Walter de Gruyter Berlin-New York, pp 313–329.

Lawson, J.E., Gawaz, M., Klingenberg, M., Douglas, M.G. (1990) Structure-function studies of adenine nucleotide transport in mitochondria. I. Construction and genetic

analysis of yeast mutants encoding the ADP/ATP carrier protein of mitochondria. *J Biol Chem*, **265**, 14195–14201.

Lazzarino, D.A., Boldogh, I., Smith, M.G., Rosand, J., Pon L.A. (1994) Yeast mitochondria contain ATP-sensitive, reversible actin-binding activity. *Mol Biol Cell*, **5**, 807–818.

Lee, C.M., Sedman, J., Neupert, W., Stuart, R.A. (1999) The DNA helicase, Hmi1p, is transported into mitochondria by a C-terminal cleavable targeting signal. *J Biol Chem*, **274**, 20937–20942.

Lecrenier, N., Foury, F. (1995) Overexpression of the RNR1 gene rescues *Saccharomyces cerevisiae* mutants in the mitochondrial DNA polymerase-encoding MIP1 gene. *Mol Gen Genet*, **249**, 1–7.

Ling, F., Makishima, F., Morishima, N., Shibata, T. (1995) A nuclear mutation defective in mitochondrial recombination in yeast. *EMBO J*, **14**, 4090–4101.

Ling, F., Morioka, H., Ohtsuka, E., Shibata, T. (2000) A role for MHR1, a gene required for mitochondrial genetic recombination, in the repair of damage spontaneously introduced in yeast mtDNA. *Nucleic Acids Res*, **28**, 4956–4963.

Ling, F., Shibata, T. (2002) Recombination-dependent mtDNA partitioning: in vivo role of Mhr1p to promote pairing of homologous DNA. *EMBO J*, **21**, 4730–4740.

Ling, F., Shibata, T. (2004) Mhr1p-dependent concatemeric mitochondrial DNA formation for generating yeast mitochondrial homoplasmic cells. *Mol Biol Cell*, **15**, 310–322.

Lisowsky, T, Michaelis, G. (1988) A nuclear gene essential for mitochondrial replication suppresses a defect of mitochondrial transcription in *Saccharomyces cerevisiae*. *Mol Gen Genet*, **214**, 218–223.

Lorenz, M.C., Cutler, N.S., Heitman, J. (2000) Characterization of alcohol-induced filamentous growth in *Saccharomyces cerevisiae*. *Mol Biol Cell*, **11**, 183–199.

Lockshon, D., Zweifel, S.G., Freeman-Cook, L.L., Lorimer, H.E., Brewer, B.J., Fangman, W.L. (1995) A role for recombination junctions in the segregation of mitochondrial DNA in yeast. *Cell*, **81**, 947–955.

Lorimer, H.D., Brewer, B.J., Fangman, W.L. (1995) A test of the transcription model for biased inheritance of yeast mitochondrial DNA. *Mol Cell Biol*, **15**, 4803–4809.

MacAlpine, D.M., Kolesar, J., Okamoto, K., Butow, R.A., Perlman, P.S. (2001) Replication and preferential inheritance of hypersuppressive petite mitochondrial DNA. *EMBO J*, **20**, 1807–1817.

MacAlpine, D.M., Perlman, P.S., Butow, R.A. (1998) The high mobility group protein Abf2p influences the level of yeast mitochondrial DNA recombination intermediates *in vivo*. *Proc Natl Acad Sci USA*, **95**, 6739–6743.

MacAlpine, D.M., Perlman, P.S., Butow, R.A. (2000) The numbers of individual mitochondrial DNA molecules and mitochondrial DNA nucleoids in yeast are co-regulated by the general amino acid control pathway. *EMBO J*, **19**, 767–775.

Maleszka, R., Skelly, P.J., Clark-Walker, G.D. (1991) Rolling circle replication of DNA in yeast mitochondria. *EMBO J*, **10**, 3923–3929.

Mangus, D.A., Jahn, S.H., Jaehning, J.A. (1994) Release of the yeast mitochondrial RNA polymerase specificity factor from transcription complexes. *J Biol Chem*, **269**, 26568–26574.

Masters, B.S., Stohl, L.L., Clayton, D.A. (1987) Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell*, **51**, 89–99.

Matsunaga, M., Jaehning, J.A. (2004) Intrinsic promoter recognition by a "core" RNA polymerase. *J Biol Chem*, **279**, 44239–44242.

Meeusen, S., Nunnari, J. (2003) Evidence for a two membrane-spanning autonomous mitochondrial DNA replisome. *J Cell Biol*, **163**, 503–510.

Meeusen, S., Tieu, Q., Wong, E., Weiss, E., Schieltz, D., Yates, J.R., Nunnari, J. (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**, 291–304.

Megraw, T.L., Chae, C.B. (1993) Functional complementarity between the HMG1-like yeast mitochondrial histone HM and the bacterial histone-like protein HU. *J Biol Chem*, **268**, 12758–12763.

Michel, B, Ehrlich, S.D., Uzest, M. (1997) DNA double-strand breaks caused by replication arrest. *EMBO J*, **16**, 430–438.

Miyakawa, I., Sando, N., Kawano, S., Nakamura, S., Kuroiwa, T. (1987) Isolation of morphologically intact mitochondrial nucleoids from the yeast, *Saccharomyces cerevisiae*. *J Cell Sci*, **88**, 431–439.

Moller, K., Olsson, L., Piskur, J. (2001) Ability for anaerobic growth is not sufficient for development of the petite phenotype in *Saccharomyces kluyveri*. *J Bacteriol*, **183**, 2485–2489.

Monroe, D.S. Jr., Leitzel, A.K., Klein, H.L., Matson, S.W., Biochemical and genetic characterization of Hmi1p, a yeast DNA helicase involved in the maintenance of mitochondrial DNA. *Yeast*, **22**, 1269–1286.

Newman, S.M., Zelenaya-Troitskaya, O., Perlman, P.S., Butow, R.A. (1996) Analysis of mitochondrial DNA nucleoids in wild-type and a mutant strain of *Saccharomyces cerevisiae* that lacks the mitochondrial HMG box protein Abf2p. *Nucleic Acids Res*, **24**, 386–393.

Nicholas, K.B., Nicholas H.B. Jr., and Deerfield, D.W. II. (1997) GeneDoc: analysis and visualization of genetic variation. *EMBNEW NEWS*, **4**, 14.

Nosek J., Dinouel, N., Kovac, L., Fukuhara, H. (1995) Linear mitochondrial DNAs from yeasts: telomeres with large tandem repetitions. *Mol Gen Genet*, **247**, 61–72.

Nosek, J., Novotna, M., Hlavatovicova, Z., Ussery, D.W., Fajkus, J., Tomaska, L. (2004) Complete DNA sequence of the linear mitochondrial genome of the pathogenic yeast *Candida parapsilosis*. *Mol Genet Genomics*, **272**, 173–180.

Nosek J., Rycovska, A., Makhov, A.M., Griffith, JD., Tomaska, L. (2005) Amplification of telomeric arrays via rolling-circle mechanism. *J Biol Chem*, **280**, 10840–10845.

Nosek, J., Tomaska, L. (2003) Mitochondrial genome diversity: evolution of the molecular architecture and replication strategy. *Curr Genet*, **44**, 73–84.

Nosek, J., Tomaska, L., Bolotin-Fukuhara, M., Miyakawa, I. (2006) Mitochondrial chromosome structure: an insight from analysis of complete yeast genomes. *FEMS Yeast Res*, **6**, 356–370.

Nosek, J., Tomaska, L., Fukuhara, H., Suyama, Y., Kovac, L. (1998) Linear mitochondrial genomes: 30 years down the line. *Trends Genet*, **14**, 184–188.

Nosek, J., Tomaska, L., Pagacova, B., Fukuhara, H. (1999) Mitochondrial telomere-binding protein from Candida parapsilosis suggests an evolutionary adaptation of a nonspecific single-stranded DNA-binding protein. *J Biol Chem*, **274**, 8850–8857.

Nunnari, J., Marshall, W.F., Straight, A., Murray, A., Sedat, J.W., Walter, P. (1997) Mitochondrial transmission during mating in Saccharomyces cerevisiae is

determined by mitochondrial fusion and fission and the intramitochondrial segregation of mitochondrial DNA. *Mol Biol Cell*, **8**, 1233–1242.

Okamoto, K., Perlman, P.S., Butow, R.A. (1998) The sorting of mitochondrial DNA and mitochondrial proteins in zygotes: preferential transmission of mitochondrial DNA to the medial bud. *J Cell Biol*, **142**, 613–623.

O'Rourke, T.W., Doudican, N.A., Mackereth, M.D., Doetsch, P.W., Shadel, G.S. (2002) Mitochondrial dysfunction due to oxidative mitochondrial DNA damage is reduced through cooperative actions of diverse proteins. *Mol Cell Biol*, **22**, 4086–4093.

O'Rourke, T.W., Doudican, N.A., Zhang, H., Eaton, J.S., Doetsch, P.W., Shadel, G.S. (2005) Differential involvement of the related DNA helicases Pif1p and Rrm3p in mtDNA point mutagenesis and stability. *Gene*, **354**, 86–92.

Osinga, K.A., de Vries, E., Van der Horst, G., Tabak, H.F. (1984) Processing of yeast mitochondrial messenger RNAs at a conserved dodecamer sequence. *EMBO J*, **3**, 829–834.

Parisi, M.A., Xu, B., Clayton, D.A. (1993) A human mitochondrial transcriptional activator can functionally replace a yeast mitochondrial HMG-box protein both in vivo and *in vitro*. *Mol Cell Biol*, **13**, 1951–1961.

Pel, H.J., Rosenfeld, S., Bolotin-Fukuhara, M. (1996) The nuclear *Kluyveromyces lactis* MRF1 gene encodes a mitochondrial class I peptide chain release factor that is important for cell viability. *Curr Genet*, **30**, 19–28.

Peteresen, R.F., Langkjaer, R.B., Hvidtfeldt, J., Gartner, J., Palmen, W., Ussery, D.W., Piskur, J. (2002) Inheritance and organisation of the mitochondrial genome differ between two *Saccharomyces* yeasts. *J Mol Biol*, **318**, 627–636.

Piskur, J. (1997) The transmission disadvantage of yeast mitochondrial intergenic mutants is eliminated in the mgt1 (cce1) background. *J Bacteriol*, **179**, 5614–5617.

Prokisch, H., Scharfe, C., Camp, D.G. 2nd, Xiao, W., David, L., Andreoli, C., Monroe, M.E., Moore, R.J., Gritsenko, M.A., Kozany, C., Hixson, K.K., Mottaz, H.M., Zischka, H., Ueffing, M., Herman, Z.S., Davis, R.W., Meitinger, T., Oefner, P.J., Smith, R.D., Steinmetz, L.M. (2004) Integrative analysis of the mitochondrial proteome in yeast. *PLoS Biol*, **2**, e160.

Reenan, R.A., Kolodner, R.D. (1992)a Isolation and characterization of two *Saccharomyces cerevisiae* genes encoding homologs of the bacterial HexA and MutS mismatch repair proteins. *Genetics*, **132**, 963–973.

Reenan, R.A., Kolodner, R.D. (1992)b Characterization of insertion mutations in the *Saccharomyces cerevisiae* MSH1 and MSH2 genes: evidence for separate mitochondrial and nuclear functions. *Genetics*, **132**, 975–985.

Richard, G.F., Kerrest, A., Lafontaine, I., Dujon, B. (2005) Comparative genomics of hemiascomycete yeasts: genes involved in DNA replication, repair, and recombination. *Mol Biol Evol*, **22**, 1011–1023.

Rickwood, D., Chambers, J.A., Barat, M. (1981) Isolation and preliminary characterisation of DNA-protein complexes from the mitochondria of *Saccharomyces cerevisiae*. *Exp Cell Res*, **133**, 1–13.

Ropp, P.A., Copeland, W.C. (1995) Characterization of a new DNA polymerase from *Schizosaccharomyces pombe*: a probable homologue of the *Saccharomyces cerevisiae* DNA polymerase gamma. *Gene*, **165**, 103–107.

Rycovska, A., Valach, M., Tomaska, L., Bolotin-Fukuhara, M., Nosek, J. (2004) Linear versus circular mitochondrial genomes: intraspecies variability of

mitochondrial genome architecture in Candida parapsilosis. *Microbiology*, **150**, 1571–1580.

Schapira, A.H. (2006) Mitochondrial disease. *Lancet*, **368**, 70–82.

Schinkel, A.H., Koerkamp, M.J., Touw, E.P., Tabak, H.F. (1987) Specificity factor of yeast mitochondrial RNA polymerase. Purification and interaction with core RNA polymerase. *J Biol Chem*, **262**, 12785–12791.

Schubot, F.D., Chen, C.J., Rose, J.P., Dailey, T.A., Dailey, H.A., Wang, B.C. (2001) Crystal structure of the transcription factor sc-mtTFB offers insights into mitochondrial transcription. *Protein Sci*, **10**, 1980–1988.

Schuldiner, O., Yanover, C., Benvenisty, N. (1998) Computer analysis of the entire budding yeast genome for putative targets of the GCN4 transcription factor. *Curr Genet*, **33**, 16–20.

Schulz, V.P., Zakian, V.A. (1994) The saccharomyces PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. *Cell*, **76**, 145–155.

Shiratori, A., Shibata, T., Arisawa, M., Hanaoka, F., Murakami, Y., Eki, T. (1999) Systematic identification, classification, and characterization of the open reading frames which encode novel helicase-related proteins in Saccharomyces cerevisiae by gene disruption and Northern analysis. *Yeast*, **15**, 219-253.

Sedman, T., Kuusk, S., Kivi, S., Sedman, J. (2000) A DNA helicase required for maintenance of the functional mitochondrial genome in *Saccharomyces cerevisiae*. *Mol Cell Biol*, **20**, 1816–1824.

Sekito, T., Okamoto, K., Kitano, H., Yoshida, K. (1995) The complete mitochondrial DNA sequence of *Hansenula wingei* reveals new characteristics of yeast mitochondria. *Curr Genet*, **28**, 39–53.

Sickmann, A., Reinders, J., Wagner, Y., Joppich, C., Zahedi, R., Meyer, H.E., Schonfisch, B., Perschil, I., Chacinska, A., Guiard, B., Rehling, P., Pfanner, N., Meisinger, C. (2003) The proteome of *Saccharomyces cerevisiae* mitochondria. *Proc Natl Acad Sci USA*, **100**, 13207–13212.

Simon, V.R., Karmon, S.L., Pon, L.A. (1997) Mitochondrial inheritance: cell cycle and actin cable dependence of polarized mitochondrial movements in *Saccharomyces cerevisiae*. *Cell Motil Cytoskeleton*, **37**, 199–210.

Simon, V.R., Swayne, T.C., Pon, L.A. (1995) Actin-dependent mitochondrial motility in mitotic yeast and cell-free systems: identification of a motor activity on the mitochondrial surface. *J Cell Biol*, **130**, 345–354.

Skelly, P.J and Maleszka, R. (1989) Isolation of mitochondrial DNA using pulsed field gel electrophoresis. *Nucleic Acids Res*, **17**, 7537.

Sogo, J.M., Lopes, M., Foiani, M. (2002) Fork reversal and ssDNA accumulation at stalled replication forks owing to checkpoint defects. *Science*, **297**, 599–602.

Stohl, L.L., Clayton, D.A. (1992) *Saccharomyces cerevisiae* contains an RNase MRP that cleaves at a conserved mitochondrial RNA sequence implicated in replication priming. *Mol Cell Biol*, **12**, 2561–2569.

Taylor, S.D., Zhang, H., Eaton, J.S., Rodeheffer, M.S., Lebedeva, M.A., O'Rourke, T.W., Siede, W., Shadel, G.S. (2005) The conserved Mec1/Rad53 nuclear checkpoint pathway regulates mitochondrial DNA copy number in *Saccharomyces cerevisiae*. *Mol Biol Cell*, **16**, 3010–3018.

Thomas, J.O., Travers, A.A. (2001) HMG1 and 2, and related 'architectural' DNA-binding proteins. *Trends Biochem Sci*, **26**, 167–174.

Tomaska, L., Nosek, J., Fukuhara, H. (1997) Identification of a putative mitochondrial telomere-binding protein of the yeast *Candida parapsilosis*. *J Biol Chem*, **272**, 3049–3056.

Tomaska, L., Nosek, J., Makhov, A.M., Pastorakova, A., Griffith, J.D. (2000) Extragenomic double-stranded DNA circles in yeast with linear mitochondrial genomes: potential involvement in telomere maintenance. *Nucleic Acids Res*, **28**, 4479–4487.

Tomaska, L., Makhov, A.M., Nosek, J., Kucejova, B., Griffith, J.D. (2001) Electron microscopic analysis supports a dual role for the mitochondrial telomere-binding protein of *Candida parapsilosis*. *J Mol Biol*, **305**, 61–69.

Tripathi, G., Wiltshire, C., Macaskill, S., Tournu, H., Budge, S., Brown, A.J. (2002) Gcn4 co-ordinates morphogenetic and metabolic responses to amino acid starvation in *Candida albicans*. *EMBO J*, **21**, 5448–5456.

Van Dyck, E., Clayton, D.A. (1998) Transcription-dependent DNA transactions in the mitochondrial genome of a yeast hypersuppressive petite mutant. *Mol Cell Biol*, **18**, 2976–2985.

Van Dyck, E., Foury, F., Stillman, B., Brill, S.J. (1992) A single-stranded DNA binding protein required for mitochondrial DNA replication in *S. cerevisiae* is homologous to *E. coli* SSB. *EMBO J*, **11**, 3421–3430.

Willer, M., Rainey, M., Pullen, T., Stirling, C.J. (1999) The yeast CDC9 gene encodes both a nuclear and a mitochondrial form of DNA ligase I. *Curr Biol*, **9**, 1085–1094.

Williamson, D., (2002) The curious history of yeast mitochondrial DNA. *Nat Rev Genet*, **3**, 475–481.

Williamson, D.H. (1976) Packaging and recombination of mitochondrial DNA in vegetatively growing yeast cells. In: Bandlow, W., Schweyen, R.J., Thomas, D.Y., Wolf, K. Genetics, biogenesis and bioenergetics of mitochondria. Walter de Gruyter, Berlin, pp. 99–115.

Williamson, D.H., Fennell, D.J. (1979) Visualization of yeast mitochondrial DNA with the fluorescent stain "DAPI". *Methods Enzymol*, **56**, 728–733.

Wirsching, S., Michael, S., Morschhauser, J.(2000) Targeted gene disruption in *Candida albicans* wild-type strains: the role of the MDR1 gene in fluconazole resistance of clinical *Candida albicans* isolates. *Mol Microbiol*, **36**, 856–865.

Wolf, K., Sebald-Althaus, M., Schweyen, R.J., Kaudewitz, F. (1971) Respiration deficient mutants of *Schizosaccharomyces Pombe I*. *Mol Gen Genet*, **110**, 101–109.

Wolfe, K.H., Shields, D.C. (1997) Molecular evidence for an ancient duplication of the entire yeast genome. *Nature*, **387**, 708–713.

Xu, B, Clayton, D.A. (1995) A persistent RNA-DNA hybrid is formed during transcription at a phylogenetically conserved mitochondrial DNA sequence. *Mol Cell Biol*, **15**, 580–589.

Yarunin, A., Panse, V.G., Petfalski, E., Dez, C., Tollervey, D., Hurt, E.C. (2005) Functional link between ribosome formation and biogenesis of iron-sulfur proteins. *EMBO J*, **24**, 580–588.

Youngman, M.J., Hobbs, A.E., Burgess, S.M., Srinivasan, M., Jensen, R.E. (2004) Mmm2p, a mitochondrial outer membrane protein required for yeast mitochondrial shape and maintenance of mtDNA nucleoids. *J Cell Biol*, **164**, 677–688.

de Zamaroczy, M., Faugeron-Fonty, G., Baldacci, G., Goursot, R., Bernardi, G. (1984) The ori sequences of the mitochondrial genome of a wild-type yeast strain: number, location, orientation and structure. *Gene*, **32**, 439–457.

de Zamaroczy, M., Marotta, R., Faugeron-Fonty, G., Goursot, R., Mangin, M., Baldacci, G., Bernardi, G. (1981) The origins of replication of the yeast mitochondrial genome and the phenomenon of suppressivity. *Nature*, **292**, 75–78.

Zelenaya-Troitskaya, O., Newman, S.M., Okamoto, K., Perlman, P.S., Butow, R.A. (1998) Functions of the high mobility group protein, Abf2p, in mitochondrial DNA segregation, recombination and copy number in *Saccharomyces cerevisiae*. *Genetics*, **148**, 1763–1776.

Zelenaya-Troitskaya, O., Perlman, P.S., Butow, R.A. (1995) An enzyme in yeast mitochondria that catalyzes a step in branched-chain amino acid biosynthesis also functions in mitochondrial DNA stability. *EMBO J*, **14**, 3268–3276.

Zhou, J., Monson, E.K., Teng, S.C., Schulz, V.P., Zakian, V.A. (2000) Pif1p helicase, a catalytic inhibitor of telomerase in yeast. *Science*, **289**, 771–774.

Zinn, A.R., Pohlman, J.K., Perlman, P.S., Butow, R.A. (1987) Kinetic and segregational analysis of mitochondrial DNA recombination in yeast. *Plasmid*, **17**, 248–256.

Zivanovic, Y., Wincker, P., Vacherie, B., Bolotin-Fukuhara, M., Fukuhara, H. (2005) Complete nucleotide sequence of the mitochondrial DNA from *Kluyveromyces lactis*. *FEMS Yeast Res*, **5**, 315–322.

Zuo, X.M., Clark-Walker, G.D., Chen, X.J. (2002) The mitochondrial nucleoid protein, Mgm101p, of *Saccharomyces cerevisiae* is involved in the maintenance of rho(+) and ori/rep-devoid petite genomes but is not required for hypersuppressive rho(–) mtDNA. *Genetics*, **160**, 1389–1400.

Zweifel, S.G., Fangman, W.L. (1991) A nuclear mutation reversing a biased transmission of yeast mitochondrial DNA. *Genetics*, **128**, 241–249.

SUMMARY IN ESTONIAN

Mitokondriaalse helikaasi Hmi1p uuringud pärmides *Candida albicans* ja *Saccharomyces cerevisiae*.

Mitokonder on ainus organell mittefotosünteesivas eukarüootses rakus mis omab omaenda DNA-d. See mitokondriaalne DNA (mtDNA) on jäänuk mitokondri eellase genoomist. Sümbioosse seose tekkimise ja süvenemisega eukarüoodi ja mitokondri eellaste vahel kaasnes mitokondriaalse genoomi järkjärguline vähenemine, geenid kas inkorporeeriti tuuma genoomi või kaotati kui ebavajalikud. Sellest hoolimata sisaldab erinevate organismide mt genoom teatud arvu geene, mis kodeerivad enamasti piiratud arvu elektronide transpordiahela komplekside subühikuid ja translatsiooniks vajalikke RNA-sid. Seega on mtDNA säilitus- ja taastootmismehhanismide veatu kulgemine vajalik membraanpotensiaali tagamiseks mitokondris ja seeläbi oluline eukarüootsete organismide ellujäämiseks.

Ainuraksed organismid hõimkonnast *Ascomycota* ehk pärmid ühendavad eukarüootseid mehhanisme võimsate geneetiliste meetoditega, mistõttu neid kasutatakse laialdaselt mudelorganismidena. Kõige paremini on mitokondriaalne süsteem iseloomustatud hariliku pagaripärmi (*Saccharomyces cerevisiae*) puhul, seda suuresti tänu hästi väljatöötatud geneetiliste katsesüsteemide olemasolule ja oma võimele jääda ellu ilma funktsionaalse mtDNA-ta. Sellisel juhul ei sisalda *S. cerevisiae* kas üldse mtDNA-d või omab mutantset mt genoomi, mis koosneb tandeemselt korratud lühikesest metsik-tüüpi (wt) mt genoomi fragmendist. See omadus ehk petite-positiivsus eristab teda enamikust teistest pärmidest, rääkimata hulkraksetest organismidest. Seetõttu ei pruugi *S. cerevisiae* mtDNA säilitamise eripärad olla kuigi lihtsalt ülekantavad teistele eukarüootidele. Pärm *Candida albicans* on seevastu petite-negatiivne organism, vajades elutegevuseks täielikku wt mtDNA kodeerimismahtu. Samuti on ta peamine seenparasiit inimestel, põhjustades nõrgestatud immuunsüsteemiga isikutel eluohtlikke infektsioone.

Geneetilise info replikatsiooniks ja transkriptsiooniks on vajalik DNA ahelate eraldamine teineteisest. Seda protsessi katalüüsivaid valke nimetatakse helikaasideks. Praeguseks on teada kahe DNA helikaasi olemasolu pärmi mitokondris – Pif1p ja Hmi1p. Käesoleva töö eesmärgiks oli heita valgust Hmi1p rollile mtDNA säilitamisel, uurides selleks mudelorganismide *S. cerevisiae* ja *C. albicansi* valke (ScHmi1p ja CaHmi1p) ja nendega seotud muutusi mtDNA-s. Käesoleva töö tulemused on kokkuvõtvalt järgnevad:

1. Hoolimata järjestuse erinevustest on *C. albicansi* mtDNA organiseeritud samasuguse topoloogiaga molekulide rühmadesse nagu *S. cerevisiae* mtDNA. See lubab eeldada, et antud organismide mtDNA säilitussüsteemide erinevused ei ole arvatavasti väga suured.

2. Mutatsioonid, mis põhjustavad ScHmi1p temperatuuritundlikust, ei klasterdu ühtegi kindlasse regiooni. ScHmi1p väljalülitamine konditsionaalsete mutantide abil põhjustab transientset mtDNA fragmenteerumist. Mutantse mt genoomi osakaalu suurenemisel rakkude populatsioonis fragmenteerunud fraktsioon kaob.
3. Kuigi teatud konserveerunud aminohapete muteerumisel kaob ScHmi1p helikaasne aktiivsus, on antud mutatsioone (K32M ja E211Q) sisaldavad valgud võimelised madala efektiivsusega tagama wt mtDNA säilumise. Samuti põhjustavad nad sarnaselt wt ScHmi1p-ga mutantsete ρ^- mt genoomide konkatemeeride pikenemist. Samas ei ole täheldatav K32M ja E211Q mutantide puhul üksikahelalise DNA (ssDNA) olemasolu ρ^- süsteemis.
4. Hmi1p homoloog on esindatud *Saccharomycetaceae* sugukonda kuuluvates pärmides. Antud homolooge ühendab peale aminohappelise järjestuse sarnasuse veel potensiaalse C-terminaalse mitokondriaalse lokalisatsiooni signaali olemasolu.
5. Valk, mida kodeeritakse *C. albicansi* ORF 19.7661-e poolt on võimeline komplementeerima ScHmi1 deletsiooni ning on seega antud valgu funktsionaalne ortoloog *C. albicansis* ehk CaHmi1p.
6. CaHmi1p transporditakse mitokondrisse C-terminaalse lokalisatsiooni signaali abil nii *C. albicansis* kui ka *S. cerevisiaes*. Samuti on CaHmi1p submitokondriaalne lokalisatsioon pagaripärmi mitokondris on identne ScHmi1-ga.
7. Puhastatud CaHmi1p valk omab helikaasset aktiivsust. Sarnaselt ScHmi1-le vajab antud valk seostumiseks ssDNA regiooni ja omab 3'–5' orientatsiooni.
8. *CaHMI1* deletsioon ei ole letaalne, kuid põhjustab aeglasemat kasvu võrreldes wt tüvega. Sellega kaasnevad muutused mitokondri nukleoidide DAPI-signaalis. Eksogeenne CaHmi1p üleekspressioon ei taasta algupärast nukleoidide mustrit.
9. *CaHMI1* deletsioon põhjustab järjestus-spetsiifilist mtDNA koguse vähenemist. *Cox2* geeni regiooni DNA tase ei muutu *CaHMI1* deletsioonist sõltuvalt. Algne mtDNA tase on taastatav eksogeense CaHmi1p ekspressiooniga.
10. Vastusena CaHmi1p kadumisele toimub enamiku mtDNA lagunemine lineaarseteks fragmentideks. See efekt on sarnaselt mtDNA koguse muutustele järjestuspetsiifiline ja püsiv *Δcahmi1* tüves. Kõrvuti subgenoomsete fragmentidega esineb ka väike kogus wt-sarnase topoloogiaga mtDNA-d. *CaHMI1* reintroduktsioon taastab wt topoloogia.

Varasemast oli teada, et ScHmi1 on vajalik ρ^+ kuid mitte ρ^- mtDNA säilimiseks. Ensümaatiliselt inaktiivsed mutandid on võimelised teatud määral tagama wt mt genoomi säilimise. Seega ei ole helikaasne aktiivsus antud valgu ainuke ülesanne. ScHmi1p olemasolu sõltumata ensümaatilisest aktiivsusest

avaldab mõju ka ρ⁻ mtDNA topoloogiale, põhjustades konkatemeersete molekulide pikenemist. Antud andmed viitavad helikaassest aktiivsusest sõltumatule ScHmi1p rollile pikkade mtDNA molekulide sünteesil.

MtDNA fragmenteerumine *C. albicansis* vastusena *CaHMI1* deletsioonile on analoogne ScHmi1 konditsionaalsete mutantide fenotüübiga. Kuid erinevalt pagaripärmist on see fragmenteerumine ajas püsiv. See viitab subgenoomsete fragmentide pidevale taastootmisele. Sellisel viisil tekkivate fragmentide pikkused kui ka kogused on järjestusspetsiifilised. Kuna antud fragmendid on lineaarse topoloogiaga, ei esinda nad tõenäoliselt ainukest replikatsiooni matriitsi. Vastasel korral põhjustaks replikatsioon lineaarsetelt fragmentidelt kompensatsioonimehhanismi puudumise tõttu 3' otste järk-järgulist vähenemist. Selle matriitsi rolli võib täita wt mtDNA fraktsioon, mis on väheses koguses täheldatav ka *CaHMI1* deletsiooni korral. Antud hüpoteesi toetab ka fenotüübi pööratavus, sest CaHmi1 eksogeense ekspressiooni korral deletsiooni tüves taastub algne mtDNA kogus ja organisatsioon. Samuti on tähelepanuväärne tekkivate fragmentide tasemete järjestusspetsiifilised erinevused. Kuna teistest kõrgema tasemega *cox2*-fraktsiooni molekulid on samuti lineaarsed, tulenevad erinevused mtDNA kogustes pigem järjestusest kui muutustest subgenoomsete fragmentide topoloogias. See fenomen võib viidata replikatsiooni alguspunktide asetsemisele *C. albicansi* genoomis. Juhul kui *cox2*-fraktsioon amplifitseerub tänu lähedalasetsevale replikatsiooni alguspunktile, võib pidada eemalasetsevate regioonide taseme languse põhjuseks replikatsiooni protsessiivsuse kadu. Seega võib CaHmi1p roll mtDNA sünteesil olla seotud replikatsioonikahvli liikumisega. Antud valgu puudumisel oleks selle hüpoteesi järgi replikatsioon tugevalt häiritud, põhjustades ebatäielike sünteesiproduktide kuhjumist. Selline hüpotees on kooskõlas ScHmi1 andmetega, mis samuti viitavad protsessiivsuse muutustele ρ⁻ mtDNA säilitamisel.

ACKNOWLEDGEMENTS

First I would like to thank my supervisor Juhan Sedman for guidance since the beginning of my scientifical career in his lab. My gratitude also belongs to every other person in our group.

I wish to thank Aare Abroi and Arnold Kristjuhan for useful comments on this thesis. I am deep in debt to Joachim M Gerhold for critical reading of the manuscript and english language corrections. I am grateful to Ülo Maiväli and Jaanus Remme for useful suggestions on the thesis and conversiations on interesting scientifical topics over a cup of tea.

My brother Arvi Jõers deserves my gratitude for suggestions on the thesis and for being a big brother which sometimes can be a demanding task. Special thanks go to Aare Verliin for those several fishing trips and to Andres Hiiemäe for holding on to rope.

Last but not least I would like to thank my parents for their support over the years.

PUBLICATIONS

Joers, P., Gerhold, J.M., Sedman, T., Kuusk, S., Sedman J. (2006)
The helicase CaHmi1p is required for wild-type mitochondrial
DNA organization in *Candida albicans*, *FEMS Yeast Res*, accepted.

Kuusk, S., Sedman, T., **Joers, P**., Sedman, J. (2005)
Hmi1p from *Saccharomyces cerevisiae* mitochondria
is a structure-specific DNA helicase. *J Biol Chem*, **280**, 24322–24329.

Sedman, T., **Joers, P**., Kuusk, S., Sedman, J. (2005)
Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial
DNA molecules in yeast *Saccharomyces cerevisiae*. *Curr Genet*, **47**, 213–222.

Curriculum vitae

Priit Jõers

Date of birth: 1976.05.05. Tallinn
Citizenship: Estonian
Family status: Single
Work address: Institute of Molecular and Cell Biology, Riia 23 Tartu 51010, Estonia
e-mail: pjoers@ebc.ee

Education and professional employment

1994	Õismäe Humanitarian Gymnasium
1994–1998	B.Sc. student in Tartu University, Department of Virology. B.Sc. degree in 1998
1998–2000	M.Sc. student in Tartu University, Department of Molecular Biology, M.Sc degree in 2000.
2000–2005	Ph.D. student in Tartu University, Department of Biochemistry.
2002–2003	extraordinary research scientist in Institute of Molecular and Cell Biology
Since 2003	research scientist in Institute of Molecular and Cell Biology
2005–2006	extraordinary research scientist in Institute of Technology, Tartu University

Scientific work

Since 1998 my main research interests have been maintenance and synthesis of mtDNA in various microorganisms.

Curriculum vitae

Priit Jõers

Sünniaeg ja -koht: 1976.05.05. Tallinn
Kodakondsus: Eesti
Perekonnaseis: Vallaline
Aadress tööl: TÜMRI, Riia 23 Tartu 51010, Eesti
e-post: pjoers@ebc.ee

Haridus ja erialane teenistuskäik

1994	Õismäe Humanitaargümnaasium
1994–1998	TÜ bakalaureuseõpe, bakalaureusekraad 1998 kevadel
1998–2000	TÜ bioloogia-geograafia teaduskonna molekulaarbioloogia magistriõpe, magistrikraad kaitstud 2000 kevadel
2000–2005	TÜ bioloogia-geograafia teaduskonna biokeemia doktoriõpe
2002–2003	erakorraline teadur TÜ MRI-s
Alates 2003	korraline teadur TÜ MRI-s
2005–2006	erakorraline teadur TÜ Tehnoloogia Instituudis

Teaduslik ja arendustegevus

Alates 1998 aastast on uurimistöö põhisuunaks olnud mitokondriaalse DNA replikatsiooni ja säilitusmehhanismide uurimine.