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Replication and Recombination of mitochondrial DNA in Yeast

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This thesis is based on the following original publications which will be referred to in the text by their Roman numerals:

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My contributions to the articles were as follows:

I. Designed and performed experiments, analysed experimental data and participated in the writing.

II. Participated in data analyses and writing.

III. Designed and performed experiments, analysed experimental data and participated in the writing.

ABBREVIATIONS

2D-AGE	two dimensional agarose gel electrophoresis
ATP	adenosine triphosphate
BND-cellulose	benzoylated naphthoylated diethylaminoethyl cellulose
bp	base pair
CTP	cytidine triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
DAPI	4´,6-diamidino-2-phenylindole
D-loop	displacement loop
DNA	deoxyribonucleic acid
ds	double stranded
ETC	electron transfer chain
GFP	green-fluorescent-protein
GST	glutathione S-transferase
HMG-box	High Mobility Group-box
HR	homologous recombination
IR	inverted repeat
kb	kilo bases
LCR	large coding region
mt	mitochondrial
nt	nucleotide
O_H	heavy-strand replication origin
O_L	light-strand replication origin
ORF	open reading frame
ori/rep	origin of replication
OXPHOS	oxidative phosphorylation
PFGE	pulsed field gel electrophoresis
POLRMT	mitochondrial RNA polymerase
qRT-PCR	quantitative real time polymerase chain reaction
RC	rolling circle
RDR	recombination driven replication
RITOLS	RNA incorporation throughout the lagging-strand
R-loop	RNA-loop
RNA	ribonucleic acid
SCR	small coding region
SEI	single end invasion
ss	single stranded
TFAM	mitochondrial transcription factor A
wt	wild type

1. INTRODUCTION

In eukaryotes, a main function of mitochondria is the production of ATP as major source of energy. ATP is generated by the means of oxidative phosphorylation (OXPHOS). This requires flawless function of the electron transfer chain (ETC) and key components of the ETC´s protein complexes are encoded in the mitochondrial DNA (mtDNA). Therefore, accurate mtDNA maintenance is essential, because defects or alterations may cause severe diseases in humans or mammals (Holt et al., 1988; Spinazzola et al., 2006), and lead to partial or complete loss of mtDNA in yeast.

mtDNA appears in form of protein-DNA complexes, by analogy to prokaryotic DNA-organising structures referred to as nucleoids (reviewed in Chen and Butow, 2005). But since the first visualizations of mtDNA in the 1960ies (Sinclair and Stevens, 1966; van Bruggen et al., 1966), it has become increasingly clear that mt genomes between plants, animals/humans and fungi differ not only in size but also in organisation and maintenance (reviewed in Williamson, 2002). While mtDNA in mammals exists as ~16.5 kb covalently closed circles, in plants and yeasts large complex networks prevail. The unit-sizes of the latter range from ~20 kb in *Schizosaccharomyces pombe* up to ~80 kb in *Saccharomyces cerevisiae* and 200–2400 kb in different angiosperms (Ward et al., 1981; Lang et al., 1983; Foury et al., 1998; MacKenzie and McIntosh, 1999).

Numerous factors involved in mtDNA maintenance were identified in yeast and homologs of these were found in other eukaryotes (reviewed in Chen and Butow, 2005). However, models to describe mtDNA replication were best developed and studied in vertebrate mitochondria (Kirschner et al., 1968; Clayton, 1982; Holt et al., 2000; Yang et al., 2002; Bowmaker et al., 2003; Reyes et al., 2005; Yasukawa et al., 2005; Yasukawa et al., 2006).

Analyses of mtDNA in the best-studied yeast model organism *S. cerevisiae* are limited by the fact that disruption of factors influencing mtDNA maintenance leads to loss of or heavy alteration of the genome. Such altered genomes are yet maintained in the absence of certain enzymes needed for wild type mtDNA metabolism. The majority of studies in *S. cerevisiae* were conducted in so-called *rho* mutants, which harbour heavily altered (non-wild type) mtDNA. Based on the obtained data, rolling circle (RC) was proposed as mode of replication. However, due to the above mentioned alterations to the mtDNA it is not clear to what extent these findings are applicable to wild type mtDNA replication.

Different from *S. cerevisiae*, *Candida albicans* belongs to the group of yeast that are stringently dependent on functional mtDNA. Although far less well studied than *S. cerevisiae*, *C. albicans* therefore potentially represents a valuable tool to study wild type mtDNA metabolism.

The literature overview in this thesis describes organisation, topology and metabolism of yeast mtDNA emphasising common features and differences between *C. albicans* and *S. cerevisiae*. It further gives an overview of existing

models of mtDNA replication including a brief description of known factors involved in DNA maintenance in mitochondria. The major focus of my research was on topological analyses of metabolic intermediates of *C. albicans* mtDNA in an effort to define its mode (or the modes) of replication. Topological analyses are supported by data on two proteins, the helicase Hmi1p and the DNA-binding HMG-box protein Gcf1, both involved in the mtDNA metabolism in *C. albicans*. The role of DNA recombination in yeast mitochondria will be discussed in the context of mtDNA replication and the overall topology of yeast mtDNA.

2. REVIEW OF LITERATURE

2.1. Mitochondrial DNA in Yeast

2.1.1. mtDNA Organization and Topology

Mitochondria are compartments in most eukaryotic cells and their major function is the production of energy in form of ATP. According to the endosymbiotic theory of mitochondrial origin, the ancestral free-living form of mitochondria was a α-proteobacterium. This theory got major support by the discovery of mtDNA, which is today regarded as a remnant of the former symbiont´s genome (for review see Gray et al., 1999; Lang et al., 1999). In all mitochondria containing eukaryotes, mtDNA has the essential role to encode key proteins crucial for energy production. The coding capacity of most known mt genomes generally comprises 2 ribosomal RNAs, several tRNAs and 13 to 14 proteins of different complexes essential for OXPHOS. The gene content may vary however, since one of the largest coding capacities of mt genomes is observed in the protozoon *Reclinomonas americana* which harbours 97 genes (Lang et al., 1997). The best studied yeast model organism *Saccharomyces cerevisiae* and the model organism in this present thesis *Candida albicans* are both fungi that belong to the division *Ascomycota* and the subphylum *Saccharomycotina*. Although the coding capacities of the mt genome in both yeasts are similar, their sizes differ from each other. Depending on the strain analysed, mtDNA in *S. cerevisiae* has a unit size of ~75–85 kb (Foury et al., 1998) while in *C. albicans* it is ~40 kb (Anderson et al., 2001). This high variability in genome unit sizes in different yeasts can range from as little as ~ 20 kb (e.g. *Schizosaccharomyces pombe*) to as much as ~100 kb (e.g. *Podospora anserina*).

As much as the coding capacity, but especially the unit sizes of different mt genomes can vary, their overall topology as well shows a high degree of variability. The discovery of covalently closed circular molecules of the ~ 16 kb mtDNA in chicken, mice and cows nearly 5 decades ago lead to the general belief that mtDNA in all eukaryotes has to be circular, thus of course in yeast as well (Williamson, 2002). This dogmatic view was challenged by topological analyses in different yeasts (Bendich, 1996; Jacobs et al., 1996; Maleszka, 1993; Nosek et al., 1998; Nosek and Tomaska, 2003). These analyses revealed that in *S. cerevisiae* mtDNA forms a network of complex branched molecules and only a minor fraction of circular molecules are detected. *Candida glabrata* mtDNA has a unit size of ~19 kb but mainly linear molecules of sizes between 50–100 kb (2–7 genome units) and, similarly to *S. cerevisiae*, only a small fraction of molecules is present in circular form. The mtDNA in another *Candida* species, *Candida parapsilosis*, is predominantly present as one unit (~35kb) linear molecules. Interestingly, both ends of this linear genome carry telomere-like sequences (Nosek et al., 1995; Nosek et al., 1998) whose precise role in the maintenance of *C. parapsilosis* mtDNA is still being investigated.

2.1.2. mt Nucleoids and Proteins Involved in mtDNA Metabolism

MtDNA is polyploid with several 10 to 1000 copies per cell, depending on the organism, tissue and/or metabolic state of the cell. These multiple copies are organized into DNA-protein complexes which are termed nucleoids in analogy to the bacterial chromosome. The mt nucleoids are suggested represent the segregational units of mtDNA (Lockshon et al., 1995; Nunnari et al., 1997; MacAlpine et al., 2000).

Since the number of estimated mtDNA copies is generally larger than the numbers of nucleoids per cell, individual nucleoids are believed to harbour several copies of the genome. As discussed based on findings from plant organelle DNA (both, mitochondrial and chloroplastidal), in many organisms it does not seem to be fully understood whether the organelle DNA is present as finished chromosomes, as they are known in nuclei, or as unfinished DNA molecules, which in their sum have to ensure entire and unharmed presence of the genome (Bendich, 2007). It therefore remains to be elucidated whether nucleoids (at least in plants and fungi) contain finished, unfinished or both types of mtDNA copies.

In yeast, the composition of mt nucleoids includes various proteins that are involved in DNA replication, transcription, repair, but also DNA maintenance in terms of organisation (e.g. bending, packaging) and segregation. An overview of well characterized proteins of mtDNA maintenance in yeast and their assigned functions is given in Table 1. By formaldehyde cross-linking, some proteins, which are listed in Table 1, have been identified as being components of the mt nucleoid in *S. cerevisiae* (Kaufman et al., 2000), one of the most prominent being the HMG-box protein Abf2. Abf2 is discussed as one major DNA packaging protein of mtDNA having the ability to bind, bend and wrap DNA (Diffley and Stillman, 1991; Diffley and Stillman, 1992; Zelenaya-Troitskaya et al., 1998; Friddle et al., 2004; Stigter, 2004). Under changing metabolic conditions, Abf2 is accompanied by Ilv5 and/or Aco1 in nucleoid formation (Chen et al., 2005). The latter are of special interest since these two proteins are bifunctional. Ilv5 was initially identified as an enzyme of branched-chain amino acid synthesis. It was later found to influence mtDNA stability (Zelenaya-Troitskaya et al., 1995) having a synergistic effect with Abf2 and the resolvase Cce1 (MacAlpine et al., 1998). Aco1 is an aconitase participating in the Krebs-cycle, and it has later been described to protect mtDNA damage in Abf2 deficient cells suggesting similar functions of both proteins (Chen et al., 2005).

Table 1. Proteins associated with mtDNA maintenance in yeast and their functions.

yeast mtDNA maintenance protein	function	Source(es)
Abf2[1]	DNA binding, bending, wrapping, packaging	Diffley and Stillman, 1991; Diffley and Stillman, 1992; Kaufman et al., 2000; Friddle et al., 2004
Mip1	DNA polymerase	Ropp and Copeland, 1995; Foury, 1989
Rim1[1]	ssDNA binding protein	Van et al., 1992
Mhr1	recombinase	Ling et al., 1995; Ling and Shibata, 2002
Cce1	resolvase	Zweifel and Fangman, 1991; Kleff et al., 1992; Lockshon et al., 1995
Exo5 (DEM1)	ssDNA 5′-exonuclease	Burgers et al., 1988; Burgers et al., 2010
Hmi1	3′-5′-DNA helicase	Lee et al., 1999; Sedman et al., 2000; Kuusk et al., 2005; Sedman et al., 2005
Pif1	5′-3′- DNA helicase	Foury and Lahaye, 1987; Lahaye et al., 1991
Rpo41	RNA polymerase	Greenleaf et al., 1986; Kelly and Lehman, 1986
Mtf1	Rpo41 specificity factor/sigma factor	Schinkel et al., 1987; Mangus et al., 1994
Cdc9	ligase	Willer et al., 1999; Donahue et al., 2001
Mgm101[1]	DNA binding, propagation, repair	Chen et al., 1993; Meeusen et al., 1999
Msh1	mismatch repair	Reenan and Kolodner, 1992a; Reenan and Kolodner, 1992b
Hsp60[1]	mt nucleoid segregation	Kaufman et al., 2000
Ilv5[1]	amino acid biosynthesis/ mtDNA stability	Zelenaya-Troitskaya et al., 1995
Aco1[1]	aconitase/mtDNA maintenance	Chen et al., 2005

[1] identified as nucleoid component in formaldehyde cross-linking (Kaufman et al., 2000)

Based on findings in *Saccharomyces cerevisiae*, more than 30 proteins in different organisms have been assigned to mtDNA maintenance, prominent examples being the DNA polymerases Mip1 (Genga et al., 1986) in *S. cerevisiae* and Polγ in mammals (Tibbetts and Vinograd, 1973), the packaging factors Abf2 from yeast (Diffley and Stillman, 1991) and its counterpart in humans TFAM

(Fisher and Clayton, 1988) which is furthermore involved in transcription, and helicases like Hmi1 (Kuusk et al., 2005; Sedman et al., 2005) in yeast, Pif1 which is conserved from yeast to man (Lahaye et al., 1991; Bessler et al., 2001) or Twinkle in humans (Spelbrink et al., 2001). Identification of these proteins´ functions in *S. cerevisiae* was widely based on changes to the mtDNA or on genetic screens.

2.1.3. *Rho* mutants

A rare feature among mitochondria containing eukaryotes is the ability to survive heavy alteration or total loss of mtDNA as e.g. exhibited by a relatively small group of yeasts that is referred to as "petite-positive". Under laboratory conditions, animal cells lacking mtDNA and, thus, respiratory activity can be generated (Desjardins et al., 1985; King and Attardi, 1989). In the presence of fermentable carbon sources, "petite-positive" yeasts are not dependent on mitochondrially derived ATP, but are nonetheless reduced in growth speed and colony size, therefore forming small (French "petite") colonies (Ephrussi et al., 1949). Historically, the unidentified cytoplasmic factor that was believed to cause this phenotype was termed "rho" (ρ). Later the formation of petite colonies was linked to the state of the mtDNA while the nomenclature was kept. Therefore, wild type mtDNA in petite positive yeasts is termed rho^+, complete loss of mtDNA is referred to as rho^0 and heavy alterations as rho^-. The alteration of rho^+ to rho^-/rho^0 mtDNA is a naturally occurring phenomenon with rho mutants forming at a frequency of 1–2 % cells per generation in laboratory strains of *S. cerevisiae* (e.g. Baruffini et al., 2007). Their formation may be increased by chemical treatment as e.g. ethidium bromide (Slonimski et al., 1968), by irradiation with UV-light (Deutsch et al., 1974) or biotic factors like e.g. starvation and suboptimal growth temperatures (Butow et al., 1973; Barclay and Little, 1978). Rho-mutants, however, may also form due to disruption of proteins (Table 1) which are directly or indirectly involved in mitochondrial metabolism (e.g. Lawson et al., 1990; Chen and Clark-Walker, 1999), mtDNA maintenance, mt protein synthesis or mt transcription (for review see e.g. Contamine and Picard, 2000).

Rho- mtDNA is mainly maintained as concatemeric direct or inverted repeats of relatively short (a few hundred to a few thousand bp) remnant sequences, which are amplified to reach approximately the mass of wild type rho^+ mtDNA. Rho^- mt genomes can be divided into two subclasses according to their ability to outcompete rho^+ mtDNA in genetic crosses. If the percentage of rho^- mtDNA containing progeny is lower or equals to rho^+, they are referred to as neutral rho^-, if the number of rho^- progeny exceeds it, they are termed suppressive. Herein, a difference is made between suppressive rho^- mutants, outnumbering rho^+ by up to 95 %, and hypersuppressive one which occur at frequencies higher than 95 %. However, supressivity is not determined by transmission of mtDNA (MacAlpine et al., 2001) but is rather caused by

replicational or segregational advantage of hypersuppressive genomes over wild type.

Hypersupressivity requires the presence of a *ori/rep* sequence which contains an active promoter (Blanc and Dujon, 1980). These sequences have been proposed to be cis-acting elements in the mtDNA of yeast with a role in replication initiation as will be described in more detail further down in this section.

2.2. Replication Models of mtDNA

2.2.1. mtDNA replication in vertebrates

Early works on rat liver mitochondria identified Cairns-like DNA intermediates (Cairns, 1963) and therefore conventional theta replication appeared to be the mode of mtDNA replication at least in animals (Kirschner et al., 1968; Wolstenholme et al., 1973a; Wolstenholme et al., 1973b). Following experimental data established a seemingly irrevocable paradigm suggesting a strand-displacement model with distinctive separate leading and lagging strand replication origins, O_H and O_L (Fig. 1; e.g. Robberson et al., 1972; Clayton, 1982). According to this model, unidirectional replication of leading strand DNA would proceed up to the lagging strand origin O_L at approximately $2/3^{rd}$ of the mtDNA molecule, displacing the lagging strand as ssDNA. This asynchronous leading- and lagging-strand-displacement model was challenged by more recent findings which produced a unidirectional and strand-coupled replication model from a defined replication origin (Fig. 1; Holt et al., 2000), extensive incorporation of RNA throughout the lagging strand (RITOLS, Yang et al., 2002; Yasukawa et al., 2006) such as bidirectional, strand-coupled replication from a defined origin of replication but also from various sites along mtDNA (Reyes et al., 2005; Yasukawa et al., 2005). This newer strand-synchronous model was introduced and is being discussed as an alternative to the established strand-displacement model. Comparison of mtDNA prepared from tissue and from cultured cells revealed that coupled DNA synthesis of leading and lagging strand preferably occurs in cultured cells (Yasukawa et al., 2005). Identification and mapping of RITOLS (Fig. 1; Yang et al., 2002; Yasukawa et al., 2006) suggested that the lagging strand, initially believed to be single stranded and at most protected by single strand binding protein, was covered by RNA prior to being replaced by DNA. A detailed description of DNA preparation methods (Pohjoismaki et al., 2010) shows that the interpretation of topological analyses is to a large extent dependent on extraction methods of nucleic acids, thus suggesting that earlier findings may have been distorted due to preparation artefacts. A yet remaining but important detail of priming lagging strand replication was recently solved, because the mtRNA polymerase POLRMT was described to exhibit origin-specific primase function in mammalian mitochondria (Wanrooij et al., 2008; Fuste et al., 2010). Most remarkably, the lately described topology and replication intermediates of human heart mtDNA in comparison to

cultured cells and mtDNA from other human tissues indicate parallel occurrence of different tissue depended replication mechanisms (Pohjoismaki et al., 2009). In human heart, replication intermediates indicative of theta mode coupled DNA synthesis or RITOLS were absent. The complex tangled topology in this tissue and its DNA intermediates were discussed to possibly arise from molecular recombination, thus replication driven replication (RDR).

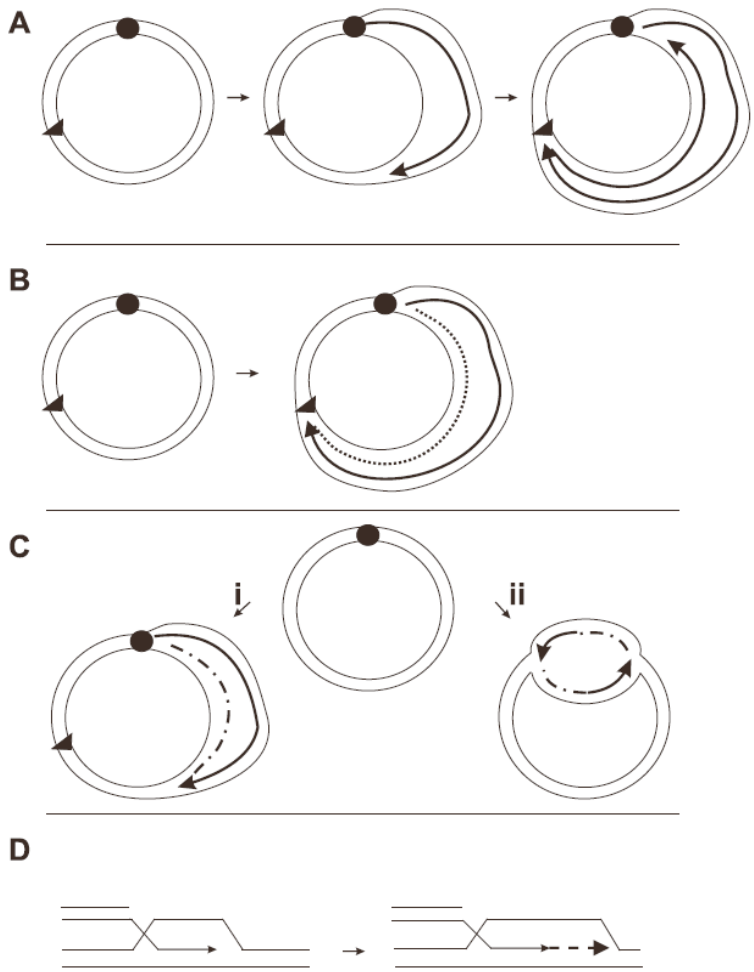

Figure 1: (A) The "orthodox" model of leading and lagging strand synthesis. Leading strand synthesis initiates at O_H (filled circle) and only when it reaches O_L (filled rectangle) lagging strand synthesis is initiated. (B) The unreplicated lagging strand was found to be protected by incorporation of RNA (dotted line) up to O_L and occasionally also full length [RITOLS]. (C) Coupled leading- and lagging strand synthesis are initiated at a specific origin (filled circle) and can proceed either uni-directionally (i) or bi-directionally (ii). (D) Strand-invasion during homologous recombination can provide a primer for DNA synthesis, similarly to what has been demonstrated for phage T4 replication.

2.2.2. mtDNA replication in yeast

In yeast, most studies on mtDNA replication have been focused on *S. cerevisiae*, where many factors involved in DNA maintenance were identified as outlined above (Table 1). In dependence on the observed yeast strain, 7–8 sequences, interpreted as replication origins ("*ori/rep*"), have been assigned to the mtDNA of *S. cerevisiae* (deZamaroczy M. et al., 1981; Schmitt and Clayton, 1993; Foury et al., 1998). These sequences have an average length of 300 bp. They harbour three GC-rich islands, termed A-, B- and C-box, which are fully conserved. Boxes A and B are believed to form a hairpin structure which is separated from the C-box by an AT-rich sequence stretch (Fig 2). Only 3–4 of these *ori/rep* sequences are believed to be active (reviewed in Lecrenier and Foury, 2000) depending on the presence of a mitochondrial RNA polymerase promoter consensus sequence, ATATAAGTA, at nt position -1 of the C-box. (Baldacci and Bernardi, 1982; Osinga et al., 1982).

However, topological evidence for involvement of these *ori/rep* sequences in replication initiation of yeast mtDNA similarly to what has been demonstrated the vertebrate system is missing. Furthermore, the majority of mtDNA maintenance studies in *S. cerevisiae* were performed in *rho*$^-$ cells (see above) where only a small fragment of the 80 kb wt genome is maintained (for review see e.g. Bernardi, 2005; Chen and Butow, 2005). As previously explained, in *S. cerevisiae* mtDNA sequence losses are non-lethal. Although this provides a valuable tool in identification of e.g. maintenance factors, formation of *rho*$^-$ cells imposes limitations to studies of wild-type mtDNA topology in *S. cerevisiae*. A distinctive class of *rho*$^-$ mutants, the hypersuppressive *rho*$^-$ strains, retain an active *ori/rep* element on their remnant mtDNA and transcription from the associated promoter is required for preferential inheritance of these hypersuppressive *rho*$^-$ genomes in mating assays. This lead to the proposal that transcription is used to prime bidirectional replication from *ori/rep* elements in yeast mtDNA (Baldacci and Bernardi, 1982; Baldacci et al., 1984). Although RNA primed DNA strands have been detected in *rho*$^-$ yeast mtDNA preparations (Baldacci et al., 1984; Xu and Clayton, 1995; Graves et al., 1998), the role of transcription is questionable, because *rho*$^-$ genomes can be maintained in strains where the mitochondrial RNA polymerase Rpo41 has been deleted (Fangman et al., 1990).

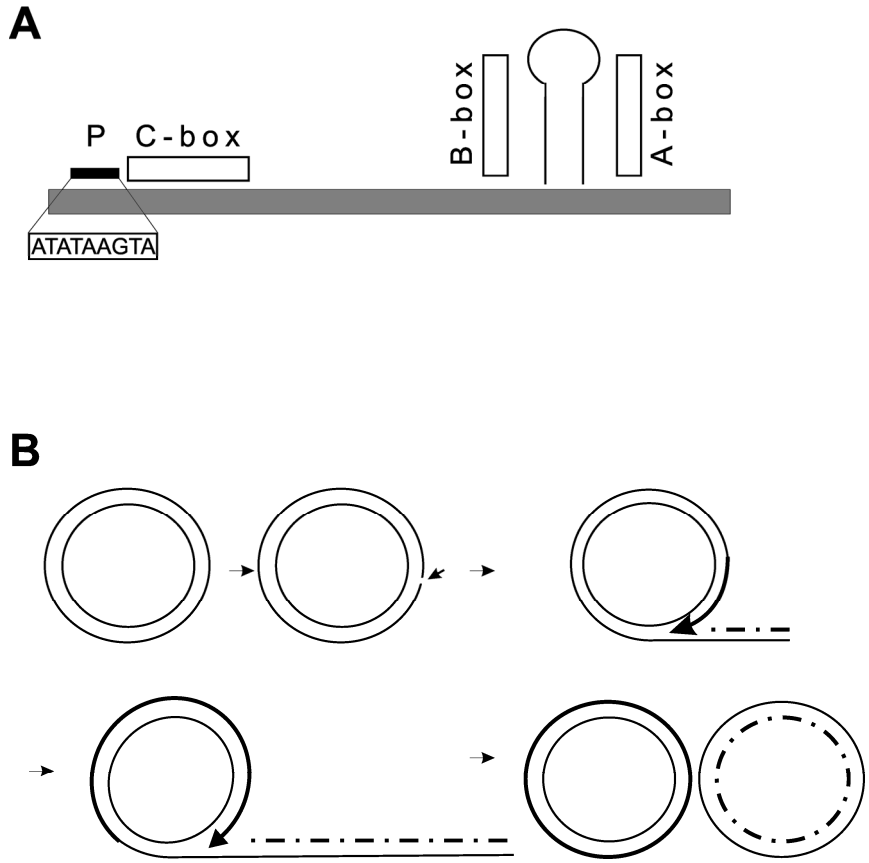

Figure 2: (A) Scheme of a *S. cerevisiae*-like *ori/rep* element. The GC-rich boxes A and B potentially form a hairpin structure. An AT-rich sequence stretch separates the A- and B-boxes from the GC-rich C box which is accompanied by a promoter (P). (B) Rolling circle replication may be initiated by introducing a nick into the circular template providing a 3´-end to prime synthesis. The displaced lagging strand can be replicated via Okazaki-fragment formation.

In mtDNA preparations of yeast, molecules of sigma-like rolling circle replication have been detected (Fig. 2; Maleszka et al., 1991). However, the mechanism (or mechanisms) explaining the origin of RC replication structures are still under discussion and recent research in the field has introduced further evidence for (homologous) recombination (Ling et al., 2000; Ling and Shibata, 2002) that could readily be linked to a replication model resembling that of bacteriophage T4 (Mosig, 1998; Kreuzer, 2000). The linear genome of T4 is initially replicated from an origin where an R-loop or RNA-DNA hybrid provides the first primer for leading strand synthesis and lagging strand synthesis is initiated shortly after this via Okazaki-fragment formation. Progression of these first

strand-coupled replication forks to the ends of the T4 chromosome will lead to completion of leading strand synthesis, leaving the lagging strands with 3´-ssDNA overhangs. These 3´-ssDNA ends can invade homologous duplex DNA molecules, thus generating D-loop-like structures providing primers for following rounds of replication (Mosig, 1998; Kreuzer, 2000). Generally, molecular recombination has an important role in DNA maintenance by DNA repair mechanisms e.g. with homologous exchange of damaged by undamaged DNA (Paques and Haber, 1999), but also in DNA replication in the recovery of replication fork collapses followed by replication restart (e.g. Rothstein et al., 2000).

Although such a recombination driven replication (RDR) mechanism is plausible for mtDNA replication in yeast, the low abundance or complete absence of circular molecules from many different mtDNA preparations imposes questions on the validity of the RDR initiated RC-replication model for all yeast species.

The yeast *Candida albicans* is part of the human gastrointestinal flora, but is at the same time a human pathogen that can proliferate to cause skin and mucosal infections with sever implications for immunocompromised individuals (for review see Noble and Johnson, 2007). Unlike *S. cerevisiae*, *C. albicans* belongs to the group of petite negative yeasts which cannot afford irreversible damage or loss of its mtDNA and therefore is a valuable tool to study the topology of replication intermediates in wt mtDNA. Close homologs to *S. cerevisiae* mtDNA maintenance and replication proteins can be found in *C. albicans* suggesting close mechanistic similarities in both yeasts (Wardleworth et al., 2000; Nosek et al., 2006; Ref. II and Ref III in this thesis).

3. RESULTS

3.1. Objectives of this study

Data on the topology of mtDNA in yeast of the recent 15 years have contributed to soften the view that all mtDNA throughout eukaryotes have the same shape and are maintained by identical mechanisms. However, within fungi, *S. cerevisiae* has been established as the major model organism and replication models were established, being treated almost dogmatically. The present study was aimed at analysing the topology of DNA intermediates in yeast, using *C. albicans* as model organism. Characterisations of two mitochondrial proteins from this yeast were employed to make a first step towards establishing an additional and new model of mtDNA replication for yeast mitochondria.

3.2. Organisation and topology of mtDNA in *C. albicans*

The mtDNA *C. albicans* has a one-unit size of 40.4 kb (Anderson et al., 2001). The mt genome consists of 13 OXPHOS components, 2 rRNA subunits and several tRNAs genes, which are clustered into two major coding regions, LCR (Large Coding Region) and SCR (Small Coding Region) (Ref. I, Fig. 1A). SCR and LCR are separated by a 2 x 7 kb inverted repeat (Wills et al., 1985; Shaw et al., 1989), here termed IRa and IRb (Inverted Repeat a; Inverted Repeat b). *Cox3* and a cluster of 5 tRNA genes are the only genes found within the inverted repeat leaving a major part (~5,6 kb) of IRa and IRb non-coding. Restriction-fragment analyses with enzymes that cut once or twice per one unit showed that SCR and LCR occur in switched orientations relatively to each other (Ref. I, Fig. 1B and 1D). Cleavage with NcoI-HindIII, NcoI-LguI or NcoI-Esp3 (Ref. I, Fig. 1B and D) were expected to produce two bands (stars in Fig. 1B) by hybridisation to probe *cox3*. Each digest gave four prominent bands (Fig. 1B). Size-alignment of the two additional bands revealed the switched relative orientations of SCR and LCR (Ref. I, black arrows in Fig. 1D). Since the relative intensities of the four major bands in each lane were equal, both orientations obviously appear at equal rates within DNA preparations. Whether these different orientations occur within one mitochondrial network or whether they are cell specific and are simultaneously detected within DNA preparations from a heterogeneous culture stays obscure. However, since homoplasmy is normally established rather quickly (Dujon, 1981), it seems logical to assume that relative orientation switches between LCR and SCR are found within one mt network.

Although the mtDNA of *C. albicans* produces a circular map in restriction-fragment analysis (Wills et al., 1985; Jones et al., 2004), circular molecules were not detected in PFGE analyses (Ref. I, Fig. 1C and Ref. II, Fig. 7D). Most

of the mtDNA signal was detected in the well and as a smear of molecules that align with linear DNA marker fragments of 12.2 to 48.5 kb (Ref. I, Fig. 1C, lane 1–7, Ref. I, Fig. S1C, Ref. II, Fig. 7D). The smear of molecules was partially resistant to restriction enzyme cleavage and was also observed in one-dimensional gel electrophoresis (e.g. Fig. 1B), but also in 2D-AGE ("cloud", Ref. I, Fig. 2,3,4, Ref.III, Fig. 5).

In order to exclude preparation artefacts, agarose embedded cells, embedded mitochondria and purified mtDNA were compared in the analysis. Purified mtDNA gave significantly lower signals of the well-bound fraction (Ref. I, Fig. 1C lanes 5, 6, 7) than embedded whole cells (lanes 1, 2) or mitochondria (lanes 3, 4). This is evidently due to breakage of large complex DNA molecules in the course of preparation. As a result, more mtDNA that migrates as a smear of molecules, is observed with all purified mtDNA (Ref. I, Fig. 1C and Ref. II, Fig. 7D). This effect has been observed in earlier PFGE analyses of *Neurospora crassa* mtDNA where *in gelo* and in-liquid preparations were analysed simultaneously (Bendich, 1996).

The mtDNA of *N. crassa* has a unit-size of 67 kb and is thus comparable to *C. albicans* mtDNA. As demonstrated by Bendich, 1996, *N. crassa* mtDNA contains 1% or less supercoiled circular molecules, which are detected as a faint signal between the well and the compression zone (cz). Relaxed circular molecules are immobile in PFGE and would therefore stay well-bound. *C. albicans* mtDNA did not produce signals comparable to those of *N. crassa* even after 3 day exposure to phosphorimager screens. Such an extended exposure to phosphorimager screens imposes the physical limit of detection, because the storage phosphor cannot be exited further. At the same time, due to the sensitivity of the screens, 3 day exposures equal 12–15 day exposure to regular X-ray films. Since these measures did not deliver detectable signals between the well and the cz, it is reasonable to conclude that if a fraction of this mtDNA is present in supercoiled circular forms, they make only a very minor fraction.

Different enzyme treatments were employed to check whether *C. albicans* mtDNA contains circular intermediates. Cleavage with the single cutting restriction enzyme NcoI was performed, assuming that this would linearize unit-sized supercoiled or relaxed circles. NcoI cleavage resulted in a down-shift of the hybridisation signal of mobile DNA molecules (Ref. I, Fig. 1C, lanes 2, 4, 6). Instead of ~12 to 48 kb, as observed with untreated mtDNA (lanes 1, 3, 5), signal was now detected between 8 kb and ~40 kb which equals the unit size (40.42 kb). An enhanced signal at ~40 kb could indicate linearization of relaxed circular DNA or cleavage of head-to-tail concatemers from the complex well bound fraction. Head-to-head or tail-to-tail concatemers cleaved with NcoI would have produced linear fragments larger than unit size (~75 kb). However, as unit-sized supercoiled circular molecules were not detected, the 40 kb band could therefore hardly be the result of linearized unit-sized supercoiled circular molecules.

Topoisomerase I (TopoI) catalyzes the relaxation of negatively supercoiled DNA (Kirkegaard and Wang, 1978) and is therefore diagnostic of circular

molecules. TopoI treatment did not lead to major changes in the running patterns (Ref. I, Fig. 1C, lane 7). If yet undetected, and thus scarcely occurring, supercoiled circular molecules were relaxed by TopoI, they would be immobilized and increase the signal of the well-bound fraction. In contrast, TopoI treatment rather resulted in a slight reduction of the signal in the well, but certainly not in an increase (Ref. I, Fig. 1C, lane 7).

Phage T7 endonuclease I (T7EndoI) is reported to recognize and cleave cruciform, branched, nicked and non-perfectly matched DNA structures (Guan et al., 2004). T7EndoI treatment degraded molecules of the well-bound fraction and of sizes larger than 20 kb. Remaining molecules of sizes between ~5 to 20 kb are assumed to be linear dsDNA (Ref. I, Fig. 1C, lane 8). Circular dsDNA can be a substrate for T7EndoI if it contains cruciform structures. However, such cruciforms are discussed to be stably maintained only on supercoiled circular DNA. This was obviously not detected in analyses of *C. albicans* mtDNA. Relaxed circular DNA can be cleaved by T7EndoI at a nicked position. Similarly to NcoI treatment, cleavage of single-nicked monomeric circular molecules would result in a 40 kb band of linear DNA, which did not form upon T7EndoI treatment. Circular molecules containing multiple irregular nicks could be cleaved resulting in fragments of 20 kb and smaller. However, no hybridisation signal could be attributed to finished relaxed or supercoiled circles. Therefore targets into which multiple nicks could be introduced are missing.

T7EndoI treatment removed all signal from the well. Lacking detectable amounts of circular molecules, this significant effect is best explained by molecules of 20 kb and smaller being generated by cleavage of frequently branched molecules. The majority of *C. albicans* mtDNA thus forms a complex and branched network of linear subsets also containing head-to-tail concatemers.

3.3. Replication origin search and analyses of mtDNA replication intermediates by 2D-AGE

In order to determine replication initiation sites of *C. albicans* mtDNA, *in silico* sequence analyses were initially performed. These analyses used either the entire conserved *S. cerevisiae-ori/rep* sequences (Fig. 2) or the separate A-, B- and C-boxes as query. Sequence comparison of the complete mtDNA of *C. albicans* to these queries using BLAST (Altschul et al., 1990) or CLUSTAL X (Thompson et al., 1997) did not return homologous or similar sequences.

In addition, searches for promoter sequences according to a generalized *S. cerevisiae* consensus promoter ATATAAGTA (Osinga et al., 1982) were performed. These returned a *S. cerevisiae*-like promoter -1bp upstream of *rRnl* (Fig. 3 A), the gene encoding the large ribosomal subunit which is located in SCR. Searches for exact matches to the found *C. albicans* sequence AAATAAGTG returned only one more exact match at nt 16671–16663 which is located within the *cox1* sequence. Therefore, further searches were conducted

employing the *C. albicans – S. cerevisiae* consensus ANATAAGTN (Fig. 3 A), where "n" allows for presence of any base. These revealed a total of 41 matches, eight of which are situated in IRa/IRb.

A- and B-boxes of *S. cerevisiae ori/rep*-sequences are predicted to form hairpins (Fig. 2). In addition, the site of initiation of second-strand synthesis in mammalian mtDNA, O_L can potentially form a hairpin stem-loop structure. Although its sequence is poorly conserved throughout mammalian mtDNA, the ability of O_L to form a stem-loop is conserved (Hixson et al., 1986). *In silico* folding using *Vienna M-fold* (Zuker, 2003) was employed to identify putative hairpin structures in *C. albicans* (Fig. 3 B). Sequences at nt 7330–7386 and 38574–38630 respectively, show potential to form a stem-loop structure comparable to previously proposed hairpin formations (deZamaroczy M. et al., 1984).

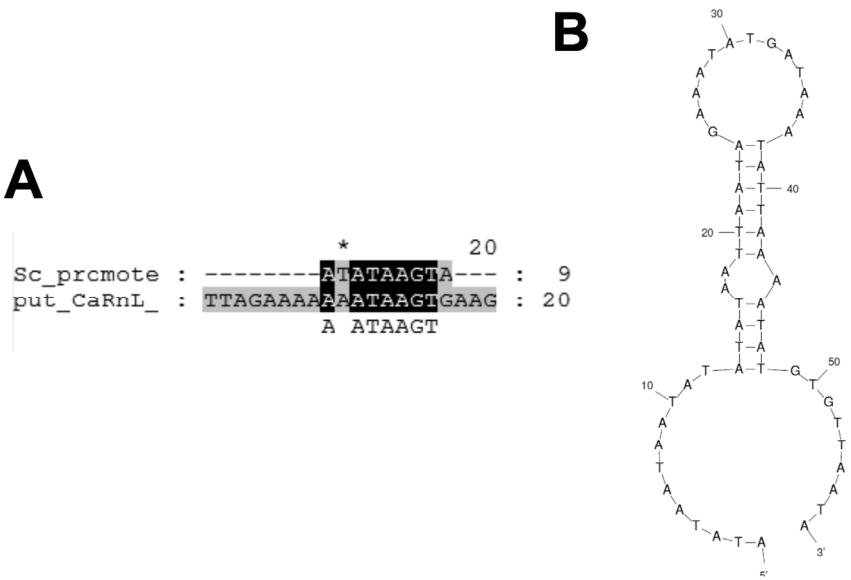

Figure 3: (A) Alignment of the *S. cerevisiae* consensus promoter sequence and the upstream region of *rRnL* returned a *C. albicans-S. cerevisiae* consensus promoter sequence AnATAAGTn. (B) A hairpin structure potentially forming at nt 7330–7386 and 38574–38630 respectively as predicted *in silico* by *Vienna M-fold*.

G and C rich sequence islands, which might qualify as *S. cerevisiae*-like C-boxes, are spread across the mtDNA in *C. albicans*, but none of these structural homologs could convincingly be combined to form a complete *S. cerevisiae*-like *ori/rep*. Therefore, a comprehensive topological analysis was conducted.

Different restriction fragments of the entire mtDNA were analysed by neutral 2D-AGE. Fragments of sizes between 2.5 to 8.3 kb were chosen to overlap by several kb (Ref. I, Fig. S1A, Fig. 2). By 2D-AGE, restriction fragments that contain a replication origin of strand-coupled synthesis (see 2. Overview of

literature) can be detected as “bubble arc” (Ref. I, Fig. 2F, grey dotted line “B”). Replication forks passing any fragment give Y-arc patterns (Ref. I, Fig. 2F “Y”), which extend from the 1N spot of non-replicating molecules to the 2N spot of almost fully replicated molecules. Vertical arcs that emerge from the 2N (Ref. I, Fig. 2F “X”) spot have been shown to consist of four-stranded DNA structures (Holliday-junctions, fork regression structures) and are termed X-arcs (e.g. Lockshon et al., 1995; MacAlpine et al., 1998; Schvartzman and Stasiak, 2004; Pohjoismaki et al., 2009).

None of the restriction fragments of the *C. albicans* mtDNA delivered a bubble arc, but Y-arcs were detected on all fragments. Examples presented in Ref. I are fragments of BamHI-EcoRI probed for *cox3* (nt 8277–11724), EcoRV – *atp6* (nt 12220–15748), EcoRV – *nad2* (nt 19173–23049), SnabI – *cob* (nt 21827–25069) and SnaBI – *nad5* (nt 27735–31334) (Ref. I, Fig. 2A-E). Examples presented in Ref. III are BglII-*cox2* (nt 40026–5934) and EcoRI-*nad2* (nt 16828–22554).

Below the dsDNA, an arc that was resistant to RNAseA and RNAse1 treatments (data not shown) was detected with all probes. As demonstrated further down (3.5) by S1 nuclease treatment this arc consists of ssDNA (Ref. I, Fig. 4).

Prominent X-arcs were detected with all restriction fragments of the mtDNA, and also a blurred, wide-spread arc that extends from Y- and X-structures reaching to the gel-well was detected in all regions (Ref. I, Fig. 2F, Ref. III, Fig. 5G; “C”). Similar cloud-arcs (C-arcs) were detected in studies on mtDNA in yeast and plants (Han and Stachow, 1994; Manchekar et al., 2006). For plant mtDNA, the molecules that migrate along these C-arcs are described to be of complex and branched structure (Manchekar et al., 2006). The preparation of *Schizosaccharomyces pombe* mtDNA employed column enrichment on BND-cellulose, thus indicating that these molecules are rich in ssDNA stretches and/or contain exposed single nucleotides (Han and Stachow, 1994). Pre-studies in the course of this thesis were similarly conducted using BND-cellulose purification of *C. albicans* mtDNA replication intermediates. These similarly showed that molecules clustering in the cloud arc are ssDNA-rich, because they elute only under conditions where strongly bound ssDNA-containing molecules are eluted from the column.

3.4. ssDNA and RNA:DNA duplexes in the mtDNA *C. albicans*

To assess the nature of certain structures which were repeatedly detected on different 2D-AGE radiographs, treatments with DNA and RNA modifying enzymes were employed. Treatments included S1 nuclease to degrade free ssDNA or exposed ssDNA-stretches, RNAseH to remove RNA from RNA: DNA duplexes and RNAseA or RNAse1 respectively, to degrade free RNA.

A 2.3 kb EcoRV fragment (nt 2444 to 4807) probed for *cox2* that is located in SCR and covers a large part of the genes *rRNL* and *cox2* was chosen for this detail study, because it yielded the largest number of interesting additional structures (Ref.I, Fig. 4A-D, interpreted in 4E and Fig. S2).

S1 treatment removed the arc of higher mobility than ds linear DNA, thus showing that it consists of ssDNA (Fig. 4B). RNAseA or RNAse1 treatments did not diminish these molecules, confirming that they are DNA species (data not shown). S1 treatment also removed large parts of the C-arc indicating substantial amounts of exposed ssDNA stretches, overhangs or nicks (Ref. I, Fig. 4A and B). This was in accordance with proposals made on the basis of plant mtDNA analyses (Manchekar et al., 2006) and conclusions drawn from mtDNA preparations of *S. pombe* (Han and Stachow, 1994).

Removal of complex and branched molecules by S1 nuclease lead to formation of X-like vertical arcs extending form two discrete spots on the dsDNA-arc at ~9 and ~11 kb (Ref. I, Fig. 4B and E, Fig. S2). These large X-like arcs appeared only on this particular EcoRV-*cox2* fragment while S1 treatments of different restriction fragments removed C-arcs without generating such features. Spots at 8–9 and 10–11 kb and extending vertical arcs might be the result of dsDNA molecules that are joined at their tips. Such molecules would have enough mobility to run on the linear dsDNA arc similarly to 2N spots. Two homologous dsDNA molecules with sizes of 4.5 or 5.5 kb could form X-shaped Holliday structures and thus explain formation of the observed large X-arcs. Since these molecules are probed for *cox2*, either one of the EcoRV sites (nt 2444 or 4807) could be blocked for cleavage. The other end of the monomeric fragment might result from double strand breaks in IRa and IRb adjacent to SCR (Ref. I, Fig S2). In this case, the extra large X-arcs would provide further evidence for recombination between the inverted repeat sequences.

Different restriction fragments showed a quick moving arc (Q). This structure is of higher mobility than dsDNA and extends as a horizontal bent arc from the 1N spot (Ref. I, Fig. 4A and E, but also Ref. I, Fig. 2B and D). The Q-arc was fully removed by S1 (Ref. I, Fig. 4B and E). Since the running pattern of this arc does not differ very much from the one of ds linear DNA, it is feasible to assume that it consists of molecules with long ssDNA stretches and varying sized portions of dsDNA (Fig. 4).

Another structure was specific to the *cox2*-probed EcoRV fragment. A blurred triangular arc with the shape a flag or pennon of sizes between 2.3–3.5 kb (Ref. I, Fig. 4) was found to be RNAseH sensitive. In adaptation of

findings by (Pohjoismaki et al., 2010), the "flag" might represent R-loop-like structures formed by transcription.

Detection of free ssDNA and of ssDNA-stretches in complex molecules might result from DNA intermediates of uncoupled strand-displacement (SD) synthesis. On the other hand, all analysed fragments contained Y-arcs of passing replication forks, thus pointing to different parallel replication mechanisms in *C. albicans* mitochondria.

3.5. Gcf1p from *C. albicans* influences recombination intermediates and DNA copy-numbers

In Ref. III, Gcf1p from *C. albicans* is described. This protein was identified by *in silico* analyses (Nosek et al., 2006) as a homolog of *S. cerevisiae* Abf2p. However, differing from Abf2p, Gcf1p showed only one region which was clearly identified as HMG sequence motives (Ref. III, Fig. 1). Since *in silico* sequence analyses returned high probabilities for mitochondrial targeting of Gcf1p (Ref. III, ~70 to ~99 %, respectively), GFP-Gcf1 fusion proteins were expressed both, heterologously in *S. cerevisiae* and natively in *C. albicans*. Both constructs in both organisms were clearly targeted to mitochondria, as demonstrated by co-staining with DAPI (Ref. III, Fig. 2).

In order to assess a possible role of Gcf1p in mtDNA maintenance, *C. albicans* mutant strains were generated. Interestingly, attempts to fully knock-out both alleles did not result in a viable strain indicating a vital function of Gcf1p in mtDNA metabolism as *C. albicans* does not tolerate loss of its mt genome. However, while *S. cerevisiae abf2*-knock-out strains rapidly lose their mtDNA when grown on fermentable carbon sources, they retain it when grown on non-fermentable carbon sources. Heterologous expression of Gcf1p in *S. cerevisiae abf2*-deletion strains showed almost complete complementation of loss of Abf2p by Gcf1p (Ref. III, Table 2), therefore further underlining a possible function for Gcf1p in mtDNA maintenance.

Since in *C. albicans* deletion of both GCF1 alleles was unsuccessful, a strain was generated that entirely lacks one functional allele of *GCF1* and carries the other under the control of the *MET3* promoter ($\Delta gcf1/P_{MET3}$-*GCF1*). Quantitative real-time PCR (qRT-PCR) showed that 12 h growth of this strain under expression repressing growth conditions exhibited more than 3,200-fold decrease in levels of *GCF1*-mRNA (Ref. III, Fig. 4B). Further qRT-PCR analyses revealed a correlating decrease in relative mtDNA copy numbers (Ref. III, Fig 4C) after 48 h propagation of the repressed strain (Ref. III, Fig. 4D). Under de-repressed conditions, $\Delta gcf1/P_{MET3}$-*GCF1* contained up to 50% more mtDNA. The elevated relative mtDNA copy numbers clearly correlated with the observed higher *GCF1*-mRNA levels. A similar effect has been observed with moderate over-expression of *S. cerevisiae* Abf2p (Zelenaya-Troitskaya et al., 1998).

2D-AGE was employed to study (a) possible direct influence(s) of Gcf1p on the mtDNA in *C. albicans* (Ref. III, Fig. 5). Both examples presented, BglII-*cox2* (nt 40026–5934) and EcoRI-*nad2* (nt 16828–22554), showed significantly reduced C-arcs and absence of X-arcs in strain Δ*gcf1*/P_{MET3}-*GCF1* that was grown under repressing conditions. Preliminary results of a detailed biochemical characterization of recombinantly expressed Gcf1p confirm the predicted mitochondrial targeting sequence, but differently from initial experiments with a crude protein preparation of GST-tagged Gcf1p, the protein apparently exhibits stronger substrate specificity to cruciform DNA (manuscript in preparation). The topological data and the preliminary protein characterisation together indicate that Gcf1p could stabilize recombination intermediates *in vivo*. The reduction of recombination intermediates in correlation with reduced relative DNA copy numbers of the repressed strain Δ*gcf1*/P_{MET3}-*GCF1* suggests a link between recombination and replication in *C. albicans* mtDNA maintenance. Recombination-driven-replication (RDR) has been proposed for mtDNA *S. cerevisiae* (e.g. Ling et al., 2007) and is a well-studied replication mechanism in bacteriophage T4 (Mosig, 1998; Kreuzer, 2000). It is generally accepted that strand invasion during HR widely employs single-stranded 3´-DNA termini as precursors (Mosig, 1998; Kowalczykowski, 2000; George et al., 2001) although 5´-overhangs are also shown to be invasive (Mazloum and Holloman, 2009). Studies of the bacterial RecBCD complex have shown that such termini can be products of targeted processes. They may also be generated from dsDNA breaks or ends of linear molecules. If RDR is an important mechanism in *C. albicans* mtDNA maintenance, and if it was dependent on dsDNA breaks or ends acting as its precursors, large amounts of shorter than 1N dsDNA molecules should be detected upon digestion. All 2D-AGE radiographs (Ref. I, Fig 2, 3, 4; Ref. III, Fig. 5), show that such shorter linear dsDNA molecules constitute a significant portion of the hybridisation signal per radiograph. Their almost even distribution along the arc of ds linear DNA and their presence in all analyzed regions could indicate that the suggested breaks or ends occur rather randomly. This seems to be supported by PFGE data as presented in this thesis (Ref. I, Fig. 1, Ref. II Fig. 7), and comparable gel-run patterns have been theoretically discussed in a study on plant mtDNA (Oldenburg and Bendich, 2001). However, detection of specific bands in different 1D-AGE (demonstrated further down) indicates frequent but not random formation of ends or breaks.

In order to get a better estimate of the presence of shorter dsDNA molecules, quantifications were made and the results were expressed as percentages of total detected hybridisation signal per each radiograph (Ref. I, Fig. 2D, H).

Out of the total signal, 13 % were detected in the area between 1 and 2N spots including Y- and X-arcs, and 21 % as C-arc signal of molecules larger than 2N and X-arcs. This relative signal distribution reflects the fact that mitochondria from exponentially growing cells show a high rate of DNA synthesis and should therefore contain significant amounts of the detectable replication intermediates. 1N spots of non-replicated dsDNA made 32% of the total signal. Molecules that migrate on the dsDNA-arc lower than 1N gave an average

relative signal of 34 %. This is in accordance with the initial hypothesis of highly abundant potential precursor molecules. If frequently detected shorter dsDNA indeed provide precursor molecules for RDR of mtDNA *in vivo*, their presence on all restriction fragments would point to numerous replication initiations throughout the mtDNA of *C. albicans*.

3.6. The mtDNA of *C. albicans* reveals opposing replication forks

Depending on the invading end, RDR at random positions would most likely produce unidirectional replication forks. In case of simultaneous initiations at different sites, these could eventually oppose each other (Ref. I, Fig. 5A). A modified 2D-AGE approach was used to determine the polarity of replication forks (Friedman and Brewer, 1995; Reyes et al., 2005). 1st dimension gels were essentially run as in regular 2D-AGE. mtDNA separated in 1st-D gel lanes was *in gelo* digested with suitable restriction enzymes followed by separation in 2nd-D gels. As depicted in Ref.I, Fig. 5D, unidirectional forks entering the analyzed restriction fragment will form either Y_a or Y_b arcs.

Examples of fork-direction analyses of fragments from LCR and SCR are shown in Ref. I, Fig. 5B and C. BglII-EcoRI-digested DNA (nt 40023–3665) was treated *in gelo* with EcoRV (nt 2444) and probed for *rRnL* and a DraI fragment (nt 26323–30691), *in gelo* treated with SpeI (nt 27816) was probed for *nad5* (Ref. I, Fig. 5B and C). Both fragments revealed Y-arc patterns of forks passing them in opposing directions. DraI (nt 38293–2391, *in gelo* NcoI, nt 929) and BglII (nt 14400–18855, *in gelo* BshNI, nt 13647) fragments in SCR and LCR, respectively gave the same result. It may therefore be assumed that opposing replication forks are passing the entire mtDNA of *C. albicans*. There are, however, two possible explanations for forks of opposing polarity. Forks could either originate from bidirectional initiations or from opposing unidirectional initiations.

3.7. A novel type of Y-arc like DNA intermediates suggests a direct link between replication and recombination

One part of the previously mentioned *in silico* analyses was covered by a cumulative GC skew analysis (Grigoriev, 1998). This approach for prediction of replication origins and termini is based on analyses of asymmetrical distribution of G and C bases in DNA strands. The skewed GC distribution along DNA is not only discussed to be diagnostic of replication initiation/termination, but can also indicate transcription initiation or recombination hot-spots. This analysis showed local minima in IRa (nt 7310) and IRb (nt 38580) (Ref. I, Fig. S1B).

As to be seen in Fig. 1B in Ref. I, cleavage of mtDNA with single or rare cutting enzymes delivered a ladder of faint bands additionally to major products. Specific ends (or breaks) (Ref. I, Fig. 1B, arrows) could be mapped to the positions of the GC skew minima (Ref. I, Fig. S1B). If these bands represent prominent breaks or ends of linear DNA molecules at position 7310, fragments with sizes of 5.6 kb (nt 7310-HindIII), 22.5 kb (nt 7310-LguI) and 24.7 kb (nt 7310-Esp3) are predicted to form and, indeed were detected by probing for *cox3*. Similarly, a break or end at position 38580 would produce fragments of 14.3 kb (nt 38580-HindIII), 8.7 kb (nt 38580-LguI) and 6.6 kb (nt 38580-Esp3), which were as well found. A band of 6.4 kb in NcoI digested mtDNA separated by PFGE (Ref. I, Fig. S1C) and probed for *cox2*, corresponds to a fragment spanning from NcoI (nt 929) to nt 7310. For plant chloroplast (cp) DNA a mechanism has been proposed that uses ends forming at putative replication origins for RDR. Therefore, and due to the correlation of the GC skew analysis with potentially forming ends or breaks within IRa and IRb, the inverted repeat sequence edged into the focus of a closer analysis.

2D-AGE mapping of IRa and IRb produced a novel type of Y-arcs when restriction fragments covered parts of IRa or IRb and SCR (Ref. I, Fig. 3A-D and F). The formation of these extra small or extra large Y-arcs (Y_{ES} or Y_{EL}) was dependent on the choice of restriction enzymes. In Ref. I, Fig. 3A and B show mtDNA cleaved with DraI (nt 38293–2391) or DraI-NcoI (nt 38293–929) probed for *rRnL*. In addition to regular Y-arcs, the novel Y-structures constituting Y_{ES}-arcs were observed. BamHI-NcoI (nt 38238–929) probed for *rRnL*, produced regular Y- and Y_{EL}-arcs (Ref. I, Fig. 3C).

As depicted in Ref. I, Fig. 3G and H, Y-shaped molecules giving rise to Y_{ES}- and Y_{EL}-arcs can be explained by strand invasion during homologous recombination between IRa and IRb. Invasion of an ssDNA-overhang can form a Y-shaped recombination intermediate. If the invading 3′-end is used as a primer for replication, this invasion intermediate can be turned into a regular Y-shaped fork structure. It has been shown elsewhere that “single end invasion” (SEI) intermediates of nuclear DNA in *S. cerevisiae* co-migrate with Y-arcs (Hunter and Kleckner, 2001).

Restriction fragments of EcoRI-BamHI (nt 3665–7717) that were probed for *nad1* produced an Y_{ES}-pattern as expected and therefore further strengthened the model of homologous recombination between IRa and IRb (Ref. I, Fig. 3D).

A DraI fragment (nt 7662–10538 and nt 35417–38293; probe *rep* [repeat]) that did not yield parts of SCR-sequence but contained IRa/IRb-sequence exclusively, showed regular Y-, X- and C-arcs (Ref. I, Fig. 3E). The analysed region did not reveal Y_{ES}- or Y_{EL}-arcs further stressing that these are only seen if the analysed fragment contains portions of sequence outside of IRa/IRb.

The correlation of GC skew minima, suitable ends or breaks in their vicinity and inverted-repeat-sequence-specific 2D-AGE structures suggested special involvement of IRa/IRb in replication and/or recombination.

3.8. The DNA helicase CaHmi1p influences growth fitness, nucleoid structure and mtDNA stability

Hmi1p is a DNA helicase in mitochondria of the yeast family of Saccharomycetaceae (Joers, 2006). Hmi1p has previously been studied in *S. cerevisiae* (Lee et al., 1999; Sedman et al., 2000; Kuusk et al., 2005; Monroe et al., 2005; Sedman et al., 2005). The protein was shown to bear a C-terminal mitochondrial targeting signal and to be strictly targeted to mitochondria *in vivo*. ScHmi1p is essential for mtDNA maintenance in rho^+ *S. cerevisiae* cells, but is dispensable in rho^- strains. ScHmi1p has 3´-5´ helicase activity, is preferentially active on forked and flap structures and is not required for transcription in mitochondria.

ORF 19.7661 from *C. albicans* was shown to be the functional homolog of ScHmi1p (Ref. II). As ScHmi1p, CaHmi1p was likewise shown to be targeted into mitochondria via a C-terminal targeting signal (Ref. II, Fig 2) and could complement loss of ScHmi1p in *S. cerevisiae* cells (Ref. II, Fig. 1). Furthermore, in submitochondrial localisation studies, CaHmi1p was found to be loosely associated with the inner membrane (Ref. II, Fig. 3) and is thus identical to ScHmi1p (Sedman et al., 2000). Biochemical characterisation of recombinant His-tagged CaHmi1p revealed that, like its counterpart in *S. cerevisiae*, CaHmi1p is a 3´-5´ DNA helicase that uses ATP, dATP and to a smaller extend CTP and dCTP for hydrolysis to catalyze the unwinding reaction (Ref. II, Fig. 4).

To study *in vivo* effects of Hmi1p *C. albicans* mutant strains were generated. Since ScHmi1p is essential for wild type mtDNA maintenance, knock-out of both alleles of *CaHMI1* was expected to be lethal in *C. albicans* (see Introduction and Overview of Literature). Very surprisingly, the strain *Δcahmi1/Δcahmi1* (PJ387) was viable but revealed reduce growth fitness since its doubling time was longer than for wild type cells (Ref. II, Fig. 5). DAPI staining showed altered nucleoid morphology in strain PJ387 since the brightness of nucleoid staining was significantly reduced indicating lowered amounts of mtDNA (Ref. II, Fig.6). This effect was reversible, because re-introduction of *CaHMI1* (strain PJ142) showed restored brightness of its stained nucleoids. The nucleoids, however, did not display their typical wild type-like punctuate appearance but rather formed threaded structures. As staining of mitochondrial membranes with Mito Tracker Red results in a similar pattern, it was assumed that mtDNA now is dispersed throughout the mitochondrial space. Expression of the reintroduced gene was controlled by the *MET3*. Under repressing conditions, simulating the double knock-out of *HMI1*, DAPI staining was observed as in strain PJ387, while under de-repressed growth conditions the DAPI signal was restored. This clearly showed that the observed effects were dependent on presence or absence of CaHmi1p and that loss of the protein directly influences mtDNA maintenance without being lethal to the cell.

Undigested mtDNA from wild type CAI4, PJ387 and the two control strains PJ53 (*Δhmi1/CaHMI1*) and PJ142 (*Δcahmi1/Δcahmi1 Δrp10::CaMET3-*

CaHMI1/RP10) were analysed on 1-dimensiolnal agarose gels and hybridised to probes *cox2*, *cox3*, *atp6*, *nad5* and *nad2* (Ref. II, Fig. 7; see also Ref. I, Fig. 1A). Strain PJ387 revealed fragmentation of its mtDNA into pieces smaller than unit size (40.4 kb) which, however, were stably maintained. PFGE showed that a minor fraction of complex well-bound, but also a weaker smear of large mobile molecules were still present in the *HMI1*-double-knock-out strain (Ref. II, Fig. 7D).

Quantifications of relative mtDNA levels in PJ387 revealed a skewed representation of *cox2*, *cox3*, *atp6*, *nad5* and *nad2* with higher levels of *cox2* than for the other analysed regions and significantly reduced levels for *cox3*, *atp6*, *nad5* and *nad2* (Ref. II, Fig. 8). This intriguing finding prompted for a more detailed analysis of DNA copy number distribution in strain PJ387.

3.9. Relative DNA copy numbers in the *HMI1* mutant PJ387 indicate involvement of IRa/IRb and possibly SCR in replication initiation

In order to get a more detailed overview of mtDNA levels in strain PJ387, relative DNA copy numbers of a total of 19 loci analyzed and compared to wild type strain levels (Ref. I, Fig. 6). As previously (Ref. II, Fig. 8), *cox2* but also *rRnL* and *nad1* in SCR showed higher DNA copy numbers than genes from LCR. A significant peak at *nad1* was observed showing almost twice higher DNA levels than wild type (Ref. I, Fig. 6A). DNA of a defined region of IRa/IRb at positions nt 5540 to nt 6776 and 39210 to 40420 was represented equally to *cox2* and *rRnL*. A drop in DNA copy numbers to wt level was observed between nt positions 6776 to 7565 and 38395 to 39210 of the repeat sequence (Ref. I, Fig. 6B). Analysis of the adjacent IRa/IRb sequence and of LCR demonstrated that copy numbers progressively decreased to ~25 % of wild type.

The significantly altered DNA-accumulation in strain PJ387 that suffers from defective mtDNA maintenance indicates that a defined region in IRa/IRb may serve as zone of elevated replication initiation leading to efficient replication of the nearby SCR even in the absence of Hmi1p. Strand invasion that could provide primers for replication was detected at this zone in form of the novel Y_{ES}- and Y_{EL}-arcs (Ref. I, Fig. 3). GC skew data supports this model (Ref. I, Fig. S1).

4. DISCUSSION

A major part of studies on yeast mtDNA replication were focused on *S. cerevisiae*. Although many DNA maintenance factors were identified, analyses were mostly performed in *rho*$^-$ cells harbouring only a small fragment of the 80 kb wild-type mtDNA (for review see e.g. Contamine and Picard, 2000; Chen and Butow, 2005). This, however, means that studies of wild-type mtDNA topology in *S. cerevisiae* are somewhat limited, and it cannot be taken for granted that generally valid yeast-models can be deducted from analyses in *rho*$^-$ strains. Since close homologs to *S. cerevisiae* mtDNA maintenance proteins are found in *C. albicans* (Wardleworth et al., 2000; Ref. II; Ref. III) it is possible that both yeasts share close mechanistic similarities, making *C. albicans* a potentially useful wild-type model organism.

The works conducted in the course of this dissertation provide evidence that recombination and replication are closely linked processes in the maintenance of mtDNA in *C. albicans*. It is further shown that the topology of this mtDNA is similar to other yeasts, but stands in sharp contrast to vertebrates. This thesis therefore further contributes to the dispersal of the long standing persuasion, which arose with the discovery of circular mtDNA in vertebrate mitochondria and the endosymbiotic theory, that mtDNA in all eukaryotes have similar topologies and must follow equal replication mechanisms.

The best studied model of recombination-driven DNA replication (RDR) is replication in phage T4 (Mosig, 1998; Kreuzer, 2000). Following this model, homologous recombination (HR) has been discussed to initiate mtDNA replication in yeast and plant similarly to T4 (Ling et al., 2000; Ling and Shibata, 2002; Ling et al., 2007; Manchekar et al., 2006), and it has been shown for protist mtDNA that replication coincides with recombination (Preiser et al., 1996). Recent data on human heart mtDNA proposed a HR-like mechanism for non-theta replication (Pohjoismaki et al., 2009).

The formation of Y_{ES}- and Y_{EL} – arcs (Ref. I, Fig. 3) and their changing behaviour in different restriction digests clearly demonstrated homologous recombination between the inverted repeat sequences of the mtDNA in *C. albicans*. The mere detection of homologous DNA strand exchange would not have yet been a proof of RDR, but strand invasion intermediates (as HR intermediates) may provide primers for DNA synthesis, which in turn may be detected as Y-structures (Ref. I, Fig. 3H). Importantly, if invasions during recombination lead to formation of Y-arcs (Y_{ES}, Y_{EL}) which are detectable as special distinctive structures because of the distribution of restriction sites in homologous and non-homologous regions as is the case for IRa/IRb and SCR (Ref. I, Fig. 3I), it is reasonable to suggest that also Y-arcs spanning from 1N to 2N could be initiated by homologous recombination. Holliday-like structures in all regions of the *C. albicans* mtDNA might also be indicative of this, and the data together would point to randomly initiated RDR throughout the entire DNA.

Abf2p from *S. cerevisiae* was shown to bind, bend and compact mtDNA and it has been implemented in mtDNA recombination (Zelenaya-Troitskaya et al.,

1998; Friddle et al., 2004; Stigter, 2004). The Abf2p homolog in *C. albicans* mitochondria is Gcf1p (Ref. III). It has an impact on mtDNA copy numbers and on the formation of recombination intermediates (Ref. III, Fig. 4 and 5). This further supports the hypothesis of tight links between replication and recombination in the maintenance of mtDNA in yeast.

GC skew analysis (Ref. I, Fig. S1), returned local minima diagnostic of replication initiation, transcription starts or recombination hot-spots within IRa/IRb. The genome organization of *C. albicans* suggests that there could be at least two major polycistronic transcripts of SCR and LCR, in accordance with what has been shown or discussed for other yeast mitochondria (Schafer et al., 2005). Since the detected GC skew minima do not form at the hypothetical transcription initiation sites, it is more feasible to focus on either replication initiation or recombination. As Y-structures indicative of HR match with these positions, but no bubble arcs diagnostic of transcription based strand-coupled replication were detected (Ref. I), the conclusion is that the zones of IRa/IRb flanking SCR may represent recombination hot-spots that serve in replication initiation as well. Possibly forming ends or breaks that could be used as precursors for strand invasions within these zones further support this model (Ref. I, Fig. 1B).

Based on the biochemical characterization of Hmi1p from *S. cerevisiae*, it has been suggested that it might have a role in RDR, because it displayed high specificity to flap-structures which may be regarded as recombination intermediates (Kuusk et al., 2005). In a *S. cerevisiae rho*$^-$ strain, Hmi1p was shown to be required for formation of long concatemers (Sedman et al., 2005), and this as well might rely on molecular recombination. The clear over-representation of *cox2* over *cox3*, *atp6*, *nad5* and *nad2* in the *C. albicans hmi1*-mutant strain PJ387 (Ref. II, Fig 8) raised speculation on a putative nearby replication origin. The more detailed mtDNA copy number analyses revealed accumulation of SCR and a defined region in IRa/IRb in comparison to wild type (Ref. I, Fig. 6, Ref. II, Fig. 8). It is possible that frequent replication initiation leads to elevated DNA accumulation at or close to initiation sites under circumstances where DNA maintenance is obviously disturbed. Although the precise function of Hmi1p *in vivo* is yet to be determined, data from strain PJ387 would then suggest that replication initiation takes place but flawless progression is hampered. In any case, the analyses from the *hmi1*-knock-out strain provide another piece of evidence for a special role of IRa/IRb, supposedly in RDR. It is yet unclear if there is a certain sequence motive or perhaps a structural feature (e.g. hairpin) that renders the specific region within the non-coding IRa/IRb especially elevated in PJ387. The conjunction of Y_{ES} and Y_{EL} structures at this region and the elevated copy numbers, however, suggest that it represents a zone of preferred or enhanced recombination that may serve in RDR initiation.

The thorough topological screen of the *C. albicans* mtDNA did not reveal bubble-arcs diagnostic of transcription based, strand-coupled replication initiation. On the other hand, Y-arcs that are generally recognized as replication forks, and that pass the respective analyzed fragment were found in all regions

(Ref. I, Ref. III). As discussed above, these forks may be generated by strand invasion, but clearly, the result is a passing replication fork of strand-coupled synthesis. In addition, analyses of their polarity have shown that forks are passing the fragments in opposing directions (Ref. I, Fig. 5). Taken together the data suggest that mtDNA of *C. albicans* could be replicated strand-coupled, with forks of opposing directions.

Complex and branched molecules that contain exposed ssDNA stretches, but also free ssDNA were detected throughout the mtDNA (Ref. I, Fig. 2 and 4). These suggest that a parallel mechanism exists which either replicates the entire genome asynchronously, or strand-invasion initially produces a limited extend of uncoupled DNA synthesis that is thereafter turned into strand-coupled progression. The latter would appear to be SD synthesis in the beginning, leaving the displaced strand unreplicated, thus forming free ssDNA.

The parallel occurrence of Y-arcs and C-arcs in combination with free ssDNA points to different replication mechanisms active in *C. albicans* mitochondria. But since no replication intermediates could so far clearly be attributed to a mechanism that requires an RNA primer, it is proposed here that the different mechanisms are all based on HR for initiation. Whether the strand-coupled and -uncoupled modes of replication occur simultaneously or in dependence of the cell cycle phases of individual cells remains to be resolved.

Transcription had been discussed to initiate yeast mtDNA replication from promoter sequences associated with the previously described *ori/rep* elements in *S. cerevisiae*. Support for this was gained mostly by experiments that were performed with *rho*$^-$ strains (Baldacci et al., 1984; Xu and Clayton, 1995; Graves et al., 1998). Paradoxically, *rho*$^-$ genomes are stably maintained and replicated in Rpo41-knock-out-strains (Fangman et al., 1990), and also topological evidence, in form of e.g. bubble-arc formation in 2D-AGE, that would demonstrate involvement of the *ori/rep* sequences in replication initiation is lacking.

RDR was alternatively proposed for initiation of *S. cerevisiae* mtDNA (Ling et al., 2007). These authors related RDR to initiation of rolling-circle (RC) replication. RC replication has also been proposed for mtDNA of the yeast *S. pombe* and for certain mitochondrial plasmids from plant (Han and Stachow, 1994; Backert et al., 1997; Backert et al., 1998). In both cases, Y-arcs patterns but neither bubble- nor double Y-arcs were detected.

RC replication is described to initiate from a nick providing a 3´-end as primer for leading strand synthesis, which displaces the lagging strand (Khan, 1997). RC is thus SD synthesis using a circular template DNA. Lacking detectable amounts of circular template (Ref. I, Fig. 1C; Ref. II Fig. 7D), the mtDNA in *C. albicans* is likely to use linear and branched molecules as substrate for SD synthesis, if it occurs. PFGE of T7EndoI-treated DNA samples indicated that *C. albicans* mtDNA might contain nicked DNA stretches (Ref. I, Fig. 1C, refer to description in 3.2 in this thesis). Such nicks could provide initiation points for SD replication similarly to RC replication (Khan, 1997). On

the other hand, SD replication may be initiated by strand invasions or even by annealing of free ssDNA to exposed ssDNA stretches as well.

PFGE (Ref. I, Fig. 1C, Ref. II, Fig 7D) further showed that *C. albicans* mtDNA is mainly present as complex branched molecules. Similar DNA-networks have been detected in mitochondria of different yeasts and plants, but in chloroplasts as well (Maleszka, 1993; Jacobs et al., 1996; Oldenburg and Bendich, 1996; Oldenburg and Bendich, 2001; Oldenburg and Bendich, 2004a; Oldenburg and Bendich, 2004b; Manchekar et al., 2006). The structure of maize chloroplast (cp)DNA has been proposed to result from RDR leading to head-to-tail concatemerization and branching (Bendich, 2007).

C. albicans mtDNA and plant cpDNA appear to share a number of common features (Oldenburg and Bendich, 2004a; Oldenburg and Bendich, 2004b). Besides similar topologies, both organellar DNA carry inverted repeats that are separated by unique coding regions. Although IRa/IRb is mainly non-coding, while the inverted repeat sequence in cpDNA is comprised of coding regions, they both seemingly function in DNA replication. An earlier study mapped replication origins to the ends of the inverted repeat sequences in cpDNA (Kunnimalaiyaan and Nielsen, 1997; Kunnimalaiyaan et al., 1997). Identifying molecule ends forming at the position of these origins, it was later proposed that RDR is initiated using these free ends and the homology of the cpDNA repeats for replication in a transcription independent manner (Oldenburg and Bendich, 2004a; Oldenburg and Bendich, 2004b). It was further discussed that the complex branched topology of cpDNA is the result of RDR. Accordingly, the topology of *C. albicans* mtDNA implicates that RDR is the major replication mechanism. Ends or breaks at positions in IRa/IRb of *C. albicans* mtDNA (Ref. I, Fig. 1B) mapping to GC skew minima (Ref. I, Fig. S1B) and HR initiated Y_{ES} and Y_{EL} structures (Ref. I, Fig. 3) are in line with the cpDNA replication model, thus stressing the similarities between these plastids. The example of cpDNA in combination with data presented in this thesis and findings in mitochondria of plants (Oldenburg and Bendich, 2001; Manchekar et al., 2006) or human heart (Pohjoismaki et al., 2009) together suggest that HR is a universal mechanism in mtDNA replication.

5. CONCLUSIONS

1. The mtDNA in *C. albicans* is mainly present as a complex branched network and circularly permuted linear molecules.
2. Neither finished circular molecules nor circular intermediates were detected in mtDNA preparations of *C. albicans*, thus RC replication is unlikely in this yeast.
3. The formation of Y-structures can be initiated via strand invasions as demonstrated through detection of special extra large or extra small Y-structures, thus providing a direct link between replication and recombination.
4. Abundant recombination structures in form of X-arcs, and complex cloud structures stress the role of recombination for replication in yeast mitochondria.
5. Specific origins of replication that employ a RNA primed strand-coupled initiation are absent from mtDNA in *C. albicans*.
6. The influence of the HMG-box protein Gcf1p from *C. albicans* on mtDNA copy numbers and recombination intermediates further supports the close linkage between replication and recombination.

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SUMMARY IN ESTONIAN

Mitokondriaalse DNA replikatsioon ja rekombinatsioon pärmis

Mitokondrite peamine funktsioon eukarüootsetes organismides on ATP tootmine. Põhiliselt sünteesitakse ATP oksüdatiivsel fosforüleerimisel (OXPHOS), milleks on vajalik funktsionaalne elektronide transportahel (ETC). Osaliselt on ETC valkkompleksid kodeeritud mitokondriaalse genoomi poolt. Seetõttu on mitokondriaalse DNA (mtDNA) täpne säilitamine ülioluline. Defektid või muutused selles võivad põhjustada inimestel või imetajatel tõsiseid haigusi (Holt jt., 1988; Spinazzola jt., 2006), pärmidel aga lõppeda osalise või täieliku mtDNA hävimisega.

mtDNA eksisteerib rakus valk-DNA kompleksidena. Sarnaselt prokarüootsete organismide DNA-d organiseerivatele struktuuridele nimetatakse neid nukleoidideks (nt. Chen ja Butow, 2005). Alates mtDNA esimesest visualiseerimisest 1960-ndatel aastatel (Sinclair ja Stevens, 1966; van Bruggen jt., 1966) on järjest enam saanud selgemaks, et taimede, loomade/inimese ja seente mt genoomid ei erine üksteisest mitte ainult suuruse vaid ka organisatsiooni ja säilitamise osas (nt. Williamson, 2002). Imetajate mtDNA moodustab ~16.5 kb suuruseid rõngasmolekule. Taimedes ja pärmides on valdavad suured kompleksed molekulid, mille üksikute osade suurus varieerub ~20 kb pärmis *Schizosaccharomyces pombe* kuni ~80 kb pärmis *Saccharomyces cerevisiae* ning 200-2400 kb erinevates katteseemnetaimedes (Ward jt., 1981; Lang jt., 1983; Foury jt., 1998; MacKenzie ja McIntosh, 1999).

Mitmed mtDNA säilitamisega seotud faktorid on identifitseeritud esmalt pärmides ja nende homoloogid leitud siis ka teistes eukarüootides (nt. Chen ja Butow, 2005). mtDNA replikatsiooni kirjeldavad mudelid on aga peamiselt loodud ja uuritud imetajatel (Kirschner jt., 1968; Clayton, 1982; Holt jt., 2000; Yang jt., 2002; Bowmaker jt., 2003; Reyes jt., 2005; Yasukawa jt., 2005; Yasukawa jt., 2006).

Pärmidest kõige rohkem uuritud mudelorganismi *S. cerevisiae* mtDNA analüüsimist takistab fakt, et mtDNA säilitamist mõjutavate faktorite segilöömine põhjustab genoomi kadumist või suuri muutusi selles. Selliselt muudetud genoome säilitatakse mõnede metsiktüüpi mtDNA metabolismiks vajalike ensüümide puudumisel. Enamus *S. cerevisiae* uurimusi on viidud läbi nn. *rho* mutantides, mille mtDNA on tugevasti muudetud (mitte-metsiktüüpi). Vastavalt selliselt saadud andmetele pakuti mtDNA replikatsioonimehhanismiks 'veereva ratta mudelit' (RC). Ülalnimetatud muutuste tõttu mtDNA-s ei ole aga selge, mil määral see tulemus kehtib metsiktüüpi mtDNA replikatsiooni puhul.

Erinevalt pärmist *S. cerevisiae* kuulub *Candida albicans* pärmide gruppi, mis sõltuvad rangelt funktsionaalsest mtDNA-st. Seega, kuigi *C. albicans* on kaugelt vähem uuritud kui *S. cerevisiae*, on *C. albicans* potentsiaalselt väga hea mudelorganism metsiktüüpi mtDNA metabolismi uurimiseks.

Minu töö peamine rõhuasetus oli mtDNA metaboolsete vaheproduktide topoloogia uurimine eesmärgiga mõista mtDNA replikatsioonimehhanisme. To-

poloogiliste analüüside tulemusi toetavad ka kahe *C. albicans* mtDNA metabolismis osaleva valgu, helikaasi Hmi1p ja DNA-seoselise valgu Gcf1p funktsionaalse analüüsi andmed. Käesoleva töö tulemused on kokkuvõtvalt järgnevad:

1. *C. albicans* mtDNA eksisteerib peamiselt komplekse hargnenud molekulide võrgustikuna. *C. albicans* mtDNA proovides leidub lineaarseid, kuid mitte ringikujulisi DNA molekule.
2. Kuigi *in silico* analüüsid näitasid üksikute elementide olemasolu, mida peetakse *Saccharomyces cerevisiae* oletatavate replikatsiooni alguspunktide osadeks, ning GC ebaühtlase jaotuse („GC-skew“) analüüsid näitasid *C. albicans* mtDNA mittekodeerivas pööratud korduse teatud piirkondades bakteritele sarnaseid replikatsiooni alguspunkte, ei leitud *C. albicans* mtDNA-s RNA praimeriga alustatavaid sünkroonsele replikatsioonile iseloomulikke replikatsiooni vaheprodukte.
3. Üheahelalise DNA (ssDNA) ja ssDNA lõike sisaldavate keerukate molekulide leidumine proovides viitab asünkroonsele replikatsioonile, kus sünteesitav ahel moodustab kaksikheeliksi matriitsahelaga ning vana komplementaarne ahel jääb ajutiselt üksikahela kujule. Samaaegselt leidus proovides aga ka Y-kujulisi, sünkroonse replikatsiooni kahvlitele iseloomulikke molekule. See näitab, et *C. albicans* mitokondrites eksisteerivad paralleelselt erinevad replikatsiooni mehhanismid.
4. *C. albicans* mtDNA seonduv Gcf1p mõjutab mtDNA koopia-arvu ja rekombinatsiooni vaheprodukte. See viitab rekombinatsiooni seotusele replikatsiooniga. Rohked X-kujulised rekombinatsiooni vaheproduktid ja kaheahelalise DNA otste ja katkemiste kujul eksisteerivad potentsiaalsed homoloogse rekombinatsiooni eellasmolekulid toetavad veelgi seda järeldust.
5. *C. albicans* mtDNA pööratud korduse piirkonna otstes leiti uut tüüpi Y-kujulisi mtDNA vaheprodukte, mis on kas suuremad või väiksemad standartsest Y-kujulisest struktuurist. Täpne kaardistamine näitas, et need struktuurid tekivad homoloogse rekombinatsiooni käigus korduvjärjestuste vahel. Selle rekombinatsiooni jooksul toimuv ahela invasioon võib tekitada DNA sünteesiks vajalikke praimereid ning kahvlikujuliste DNA vaheproduktide moodustumine nende invasioonide tagajärjel näitab otsest seost pärmi mitokondrite replikatsiooni ja rekombinatsiooni vahel.
6. *C. albicans* mutantse tüvega, milles puudus mitokondriaalne helikaas Hmi1p ja milles mtDNA säilitamine oli seetõttu takistatud, läbi viidud mtDNA koopiate arvu analüüs näitas nendes tüvedes metsiktüüpi mtDNA-ga võrreldes suuri muutusi. Teatud mtDNA piirkondades koopiate arv suurenes, teistes aga vähenes oluliselt, millest võib järeldada, et puuduliku replikatsiooni tõttu on koopiate arv olulisemate replikatsiooni alguspiirkondade lähedal. Koopiate arv suurenes ka leitud uut tüüpi Y-kujuliste molekulide läheduses ja *in silico* analüüsi abil ennustatud replikatsiooni alguspiirkondades.
7. *C. albicans* mtDNA replikatsioonikahvlid on erineva polaarsusega, mis on heas kooskõlas homoloogilise rekombinatsiooni poolt initseeritud replikatsiooni mudeliga. Selle kohaselt võib konkreetse replikatsioonikahvli liikumise suund olla ära määratud invaseeruva DNA otsa polaarsusega.

Käesoleva töö käigus kogutud andmed näitavad, et *C. albicans* mtDNA peamine replikatsioonimehhanism on rekombinatsiooni poolt põhjustatud replikatsioon. Ringikujuliste matriitsmolekulide puudumise tõttu on RC selles pärmis replikatsioonimehhanismina tõenäoliselt välistatud. Arvatavasti on rekombinatsiooni poolt põhjustatud replikatsioon peamiseks replikatsioonimehhanismiks mitte ainult *C. albicans* mtDNA puhul, vaid paljude, kui mitte kõigi pärmide mtDNA ning arvatavasti sarnase topoloogiaga kloroplastide DNA jaoks.

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I have gained support of many kinds from friends during decades already, and what wants to be said will be said – in privacy.

There is however a group of people that must be mentioned at this very point: my family. I owe my greatest gratitude to my wife Pille, and recently we got additional support from our son Joosep, to my parents Jutta and Klaus, my brother Markus, to Anna and Clara, my grandparents Erika and Heinrich Martin and of course all my relatives. I am certainly very grateful to my parents-in-law, Elve and Maido, and all relatives here in Estonia for making a second home for me here.

I dedicate this thesis to my family and to the memory of my grand-parents Heinrich Josef and Lucie Schermaul.

PUBLICATIONS

CURRICULUM VITAE

I. General Data

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Language skills:	German, English, French, Greek (basic level), Estonian	
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	2008–...	University of Tartu, Faculty of Natural Sciences, Insititute of Molecular and cell Biology, specialist

II. Scientific activities

Main research interests:

1) Physiological and molecular studies of ADT/ATP transport proteins of plants.
2) Studies of mitochndrial DNA metabolism in yeast.

List of Publications:

- Gerhold, J.M., Aun, A., Sedman, T., Jõers, P., Sedman, J. (2010) Strand Invasion Structures in the Inverted Repeat of *Candida albicans* Mitochondrial DNA Reveal a Role for Homologous Recombination in Replication. Mol. Cell, accepted.
- Visacka, K.; Gerhold, JM.; Petrovicova, J.; Kinsky, S.; Jõers, P.; Nosek, J.; Sedman, J.; Tomaska, L. (2009). Novel subfamily of mitochondrial HMG-box containing proteins: Functional analysis of Gcf1p from Candida albicans. Microbiology, 155(4), 1226–1240.

- Leroch, M.; Neuhaus, H. E.; Kirchberger, S.; Zimmermann, S.; Melzer, M.; Gerhold, J.; Tjaden, J. (2008). Identification of a novel adenine nucleotide transporter in the endoplasmic reticulum of Arabidopsis. Plant Cell, 20, 438–451.
- Jõers, P.; Gerhold, J. M.; Sedman, T.; Kuusk, S.; Sedman, J. (2007). The helicase CaHmi1p is required for wild-type mitochondrial DNA organization in Candida albicans. FEMS Yeast Research, 7(1), 118–130.
- Joachim M. Gerhold, (2004), Diplomarbeit: Physiologische und molekulare Analyse von ADP/ATP-Carriern aus Pflanzen, Technische Universität of Kaiserslautern, Fachbereich Biologie, (supervisors) H. Ekkehard Neuhaus and Joachim Tjaden.

Fellowships/scholarships
SA Archimedes scholarships to attend different conferences 2007–2010

Other professional activities:
- Gordon Research Conference Mitochondria & Chloroplasts, July 11–16, 2010, Il Ciocco Hotel and Resort, Lucca (Barga), Italy, poster-presentation: „Recombination dependent DNA replication in yeast mitochondria"
- IMCB and EBC Annual Conference 2009, December 2009, poster-presentation: „Recombination dependent DNA replication in yeast mitochondria"
- IMCB and EBC Annual Conference 2008, December 2008, presentation: „Mhb1p from Candida albicans – another piece in the puzzle of mtDNA replication"
- Replication Retreat, MRC DUNN, Cambridge April 10–12th, 2008, presentation: "Recombination-dependent replication of mtDNA in *Candida albicans*"
- IMCB and EBC Annual Conference 2007, December 2007, presentation: "Mitochondrial DNA replication in *Candida albicans*"
- Scandomit 2007: Nordic Workshop on Mitochondria, Disease and Ageing, Finnish Metalworkers' Union Institute 'Murikka', Teisko, Finland, 23–25 October 2007, presentation: "Replication of mtDNA via recombination in *Candida albicans*"
- Baltic Sea Area Network on Molecular Cell Biology, June 1–2nd 2006, presentation: "Searching replication origins in mtDNA of *Candida albicans*"
- IMCB and EBC Annual Conference 2005, December 14–15th 2005, presentation: "MtDNA helicases in *Saccharomyces cerevisiae*"

ELULOOKIRJELDUS

I. Üldandmed

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II. Teaduslik ja arendustegevus

Peamised uurimisvaldkonnad:

1) ADT/ATP-trasnpordi valukde füsioloogiline ja molekulaarne analüüs taimedes
2) Mitokondriaalse DNA metabolismi uuringud pärmides

Publikatsioonide loetelu

- Gerhold, J.M., Aun, A., Sedman, T., Jõers, P., Sedman, J. (2010) Strand Invasion Structures in the Inverted Repeat of *Candida albicans* Mitochondrial DNA Reveal a Role for Homologous Recombination in Replication. Mol. Cell, accepted.
- Visacka, K.; Gerhold, JM.; Petrovicova, J.; Kinsky, S.; Jõers, P.; Nosek, J.; Sedman, J.; Tomaska, L. (2009). Novel subfamily of mitochondrial HMG-box containing proteins: Functional analysis of Gcf1p from Candida albicans. Microbiology, 155(4), 1226–1240.

- Leroch, M.; Neuhaus, H. E.; Kirchberger, S.; Zimmermann, S.; Melzer, M.; Gerhold, J.; Tjaden, J. (2008). Identification of a novel adenine nucleotide transporter in the endoplasmic reticulum of Arabidopsis. Plant Cell, 20, 438–451.
- Jõers, P.; Gerhold, J. M.; Sedman, T.; Kuusk, S.; Sedman, J. (2007). The helicase CaHmi1p is required for wild-type mitochondrial DNA organization in Candida albicans. FEMS Yeast Research, 7(1), 118–130.
- Joachim M. Gerhold, (2004), Diplomarbeit: Physiologische und molekulare Analyse von ADP/ATP-Carriern aus Pflanzen, Technische Universität of Kaiserslautern, Fachbereich Biologie, (juh.) H. Ekkehard Neuhaus ja Joachim Tjaden

Saadud uurimistoetused ja stipendiumid
SA Archimedes stipendiumid osalemiseks erinevatel konverentsidel 2007–2010

Muu teaduslik organisatsiooniline ja erialane tegevus
- Gordon Research Conference Mitochondria & Chloroplasts, July 11–16, 2010, Il Ciocco Hotel and Resort, Lucca (Barga), Italy, poster-ettekanne: „Recombination dependent DNA replication in yeast mitochondria“
- IMCB and EBC Annual Conference 2009, December 2009, poster-ettekanne: „Recombination dependent DNA replication in yeast mitochondria“
- IMCB and EBC Annual Conference 2008, December 2008, ettekanne: „Mhb1p from Candida albicans – another piece in the puzzle of mtDNA replication“
- Replication Retreat, MRC DUNN, Cambridge April 10–12th, 2008, ettekanne: “Recombination-dependent replication of mtDNA in *Candida albicans*”
- IMCB and EBC Annual Conference 2007, December 2007, ettekanne: “Mitochondrial DNA replication in *Candida albicans*”
- Scandomit 2007: Nordic Workshop on Mitochondria, Disease and Ageing, Finnish Metalworkers’ Union Institute ‘Murikka’, Teisko, Finland, 23–25 October 2007, ettekanne: “Replication of mtDNA via recombination in *Candida albicans*”
- Baltic Sea Area Network on Molecular Cell Biology, June 1–2nd 2006, ettekanne: “Searching replication origins in mtDNA of *Candida albicans*”
- IMCB and EBC Annual Conference 2005, December 14–15th 2005, ettekanne: “MtDNA helicases in *Saccharomyces cerevisiae*”

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