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

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Supervisors: Professor Juhan Sedman, PhD
Chair of General and Microbial Biochemistry,
Institute of Molecular and Cell Biology,
University of Tartu, Tartu, Estonia

Associate Professor Priit Jõers, PhD
Chair of General and Microbial Biochemistry,
Institute of Molecular and Cell Biology,
University of Tartu, Tartu, Estonia

Reviewer: Professor Arnold Kristjuhan, PhD
Chair of Cell Biology,
Institute of Molecular and Cell Biology,
University of Tartu, Tartu, Estonia

Opponent: Professor Pawel Golik, PhD
Institute of Genetics and Biotechnology, Faculty of Biology,
University of Warsaw, Warsaw, Poland

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LIST OF ORIGINAL PUBLICATIONS

The following original publications will be referred to in the text by their Roman numerals:

I Gaidutšik, I., Sedman, T., Sillamaa, S., & Sedman, J. (2016). Irc3 is a mitochondrial DNA branch migration enzyme. *Scientific Reports*, *6*, 26414. https://doi.org/10.1038/srep26414

II Piljukov, V.-J., Sillamaa, S., Sedman, T., Garber, N., Rätsep, M., Freiberg, A., & Sedman, J. (2023). Mitochondrial Irc3 helicase of the thermotolerant yeast *Ogataea polymorpha* displays dual DNA- and RNA-stimulated ATPase activity. *Mitochondrion*, *69*, 130–139. https:// doi.org/10.1016/j.mito.2023.02.004

III Sillamaa, S., Piljukov, V.-J., Vaask, I., Sedman, T., Jõers, P., & Sedman, J. (2023). UvrD-like helicase Hmi1 Has an ATP independent role in yeast mitochondrial DNA maintenance. *DNA Repair*, *132*, 103582. https://doi.org/10.1016/j.dnarep.2023.103582

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My contribution to the articles is as follows:

Ref I Performed *in vivo* experiments and *in silico* analysis, participated in the data analysis and manuscript editing.

Ref II Participated *in silico* analysis, experimental data analysis and was involved in manuscript writing.

Ref III Designed and performed the experiments, analyzed data, and wrote the first version of the manuscript.

ABBREVIATIONS

2D-NAGE	neutral two-dimensional agarose gel
BER	base excision repair
D-loop	displacement loop
DSB	double-stranded break
ETC	electron transport chain
FOA	5-fluoroorotic acid
G4	G-quadruplex
HR	homologous recombination
HS	hypersuppressive
MMR	mismatch repair
MMS	methyl methanesulfonate
mtDNA	mitochondrial DNA
NHEJ	non-homologous end joining
ori	origin of replication
op	*Ogataea polymorpha*
OXPHOS	oxidative phosphorylation
PFGE	pulsed-field gel electrophoresis
RT-PCR	reverse transcription PCR
RCR	rolling circle replication
R-loop	RNA:DNA hybrid loop
ROS	reactive oxygen species
sc	*Saccharomyces cerevisiae*
SF	superfamily (of helicases)

1 INTRODUCTION

Mitochondria have a wide range of complex roles in cellular signaling and metabolic processes, making them an integral part of the metabolic network (Picard & Shirihai, 2022). Historically, mitochondria are best known for their role in oxidative phosphorylation (OXPHOS) but have also important roles in the induction of apoptosis, innate immunity signaling and in a wide spectrum of oxidation and reduction of different metabolites. Since electron transport chain complexes (ETC) consist of subunits encoded by nuclear and mitochondrial genomes, a regulated integration of both expression systems is required for the assembly (Vercellino & Sazanov, 2022). Although most of the mitochondrial proteins (roughly 1000) are encoded by the nuclear genome and transported to the mitochondria, mitochondria are semiautonomous organelles with their own mitochondrial DNA (mtDNA) that encodes 35 genes in *Saccharomyces cerevisiae* (37 in humans) including a handful of ETC subunits and additional rRNAs and tRNAs needed for the maintenance of its own genome and protein synthesis (Foury *et al.,* 1998; Morgenstern *et al.,* 2017). The inability to effectively maintain and repair the mtDNA can lead to the mtDNA related deficiencies that in humans often manifest through different diseases, including late-onset diseases, like chronic progressive external ophthalmoplegia (Nissanka & Moraes, 2018; Suomalainen & Battersby, 2018). The reason some diseases manifest later in life is believed to be connected to the fact that mtDNA is present in multiple copies in the cell. A single mutation only leads to the rise of heteroplasmy which must be fixed over time in the majority of mtDNA copies, to have a significant impact on mtDNA maintenance. The age-driven accumulation of mutations has been associated with the higher concentration of reactive oxygen species (ROS) in the mitochondria, but the mutation load characteristics of aging indicate, that the accumulation of the replication and repair errors also have an important role in this process (Kennedy *et al.,* 2013; Nissanka & Moraes, 2018).

One such group of proteins related to the replication errors and shown to be crucial for the mtDNA maintenance are DNA helicases. Helicases are motor enzymes that are found from viruses to the humans and are involved in a variety of different DNA- and RNA-associated functions, from the replication to the splicing of introns and ribosome assembly (Brosh & Matson, 2020; Bohnsack *et al.,* 2023). Interestingly, eminent differences among mtDNA helicases in humans and yeast are observed. Remarkably, *S. cerevisiae* lacks the homolog of the mitochondrial replicative helicase Twinkle but has two yeast-specific DNA helicases, Hmi1 and Irc3, both of which are shown to be either essential or crucial for mtDNA maintenance (Sedman *et al.,* 2000, 2014). Further characterization of the function of these two helicases in the yeast mitochondria would contribute to a more extensive understanding of the mechanisms of how yeast mtDNA is maintained and how exactly the mitochondrial helicases are involved in mitochondrial metabolism.

The review of the literature of this thesis has been divided into two parts. The first part provides an overview of *S. cerevisiae* mtDNA and its mechanism of maintenance. The second part offers a general overview of helicases, focusing on *S. cerevisiae* mtDNA helicases. The experimental part of this thesis focuses on the function of two helicases, Irc3 and Hmi1, in mtDNA maintenance.

2 REVIEW OF LITERATURE

2.1 Mitochondrial DNA maintenance

2.1.1 Yeast mitochondrial genome and DNA topology

The mtDNA size varies greatly in eukaryotes from the compact human mitochondrial genome of 16.6 kb to 11.3 Mb in plants (Anderson *et al.,* 1981; Sloan *et al.,* 2012). Different species also exhibit a range of different topological conformations of mtDNA, ranging from simple circular and linear molecules to heterogeneous complex systems such as the Trypanosoma genus, where large mtDNA circles (maxicircles) are conjoined with thousands of smaller circular molecules (minicircles) into a complex network (Maleszka *et al.,* 1991; Bendich, 1996; Oldenburg & Bendich, 1996; Jensen & Englund, 2012). The baker's yeast (*S. cerevisiae*) belongs to the phylum *Ascomycota* under the class of *Saccharomycotina*. Even within this class, the mtDNA topology varies a great deal, ranging from circular and simple linear molecules to polydisperse linear networks of branched molecules (Valach *et al.,* 2011). The variability is not restricted to disparate clades but can exist between closely related species such as the *Candida* family. This family has species with circular mtDNA, like *C. orthopsilosis*, *C. jiufengensis, C. alai*, but also has species with different linear topologies, such as the *C. albicans* linear branched mtDNA or *C. parapsilosis, C. viswanathii, C. frijolesensis* that have adapted different mechanisms for the linear molecule termination (Kosa *et al.,* 2006; Gerhold *et al.,* 2010; Valach *et al.,* 2011).

For some time, *S. cerevisiae* mtDNA was considered to have a circular topology until the PFGE (pulsed-field gel electrophoresis) enabled to determine that most of the molecules in *S. cerevisiae* mitochondria are actually linear (Skelly & Maleszka, 1989; Maleszka *et al.,* 1991; Bendich, 1996; Williamson, 2002). It has been estimated that in the haploid *S. cerevisiae* cell, there are approximately 20–50 copies of the mtDNA, and its genome was also the first one fully sequenced among yeasts (FY1679 mitochondrial genome of 85 779 base pair) (Foury *et al.,* 1998; Williamson, 2002; Göke *et al.,* 2020). *S. cerevisiae* mtDNA has a high A+T composition (approximately 83%), with characteristic G+C rich clusters including gene coding regions that encode 8 OXPHOS proteins: Cox1, Cox2, Cox3 (cytochrome c oxidase subunits), Atp6, Atp8, Atp9 (ATP synthase subunits), Cob (ubiquinol-cytochrome c oxidoreductase subunit) plus a ribosomal protein Var1, two rRNAs (21S and 15S), 24 tRNAs and the RNA subunit of the RNase P (Foury *et al.,* 1998). Although the number of *S. cerevisiae* 35 mitochondrial genes is comparable to human 37 genes, its mitochondrial genome is significantly larger 85.8 kb, and there are considerable differences in the *S. cerevisiae* mitochondrial genome architecture, such as the presence of introns (in *COX1*, *COB*, and *21SRRNA* genes) and eight origins of replications (ori) (two in humans) (Anderson *et al.,* 1981; Foury *et al.,* 1998). Four origins (Ori1, 2, 3, 5) are considered active due to their intact RNA polymerase promoter capable of initiating

transcription (Baldacci & Bernardi, 1982; de Zamaroczy *et al.,* 1984). In addition, *S. cerevisiae* lacks the ETC complex I (NADH dehydrogenase complex) and its genes, which are encoded by the human mitochondrial genome and also by many other yeast families, such as *Candida* and *Kluyveromyces* (Anderson *et al.,* 1981; Nosek & Fukuhara, 1994; Foury *et al.,* 1998; Zivanovic *et al.,* 2005).

Instead of "classic" histones, the mitochondrial protein Abf2 binds the mtDNA in every ~10–20 base pair and bends it 90° to compact the mtDNA (Diffley & Stillman, 1991; Chakraborty *et al.,* 2017). This structural complex with additional proteins forms a structure called a nucleoid (Miyakawa *et al.,* 1987). The number, size and protein content of the nucleoids can vary depending on the growth conditions, but under the aerobic conditions the haploid *S. cerevisiae* has shown to contain an average of 42 nucleoids (Chen & Butow, 2005; Meeusen & Nunnari, 2003). The nucleoid structures associate with the mtDNA replisome for the replication of the mtDNA and ensure the proper mtDNA segregation during the cell division (Okamoto *et al.,* 1998; Meeusen & Nunnari, 2003). In addition, the nucleoid structure seems to have an important role in regulating the mtDNA gene expression in response to metabolic signaling. The binding of Abf2 with mtDNA has been shown to depend on the growth conditions, as under the fermentative conditions, the mtDNA is tightly packed and harder to access (Kucej *et al.,* 2008). Under the non-fermentative conditions, Abf2 binds more sparsely with the mtDNA, presumed to allow active gene expression (Kucej *et al.,* 2008). Similar compaction of mtDNA has been observed in the studies with mammalian cells, as the higher concentration of the mammalian mitochondrial HMG-like protein TFAM leads to the formation tighter nucleoids (Kukat *et al.,* 2015). *In vitro* studies have confirmed that the more tightly packed nucleoid does diminish the level of replication and transcription, indicating that the changes in nucleoid structure can regulate these processes (Farge *et al.,* 2014). In addition, the 38 potential nucleoid-associated proteins include chaperones and bifunctional metabolic enzymes, like Hsp60, Ilv5, and Aco1, that link the mtDNA stability and nucleoid composition to the metabolic state of the cell (Kaufman *et al.,* 2000; MacAlpine *et al.,* 2000; Chen *et al.,* 2005; Kucej & Butow, 2007; Miyakawa, 2017). The increased recruitment of Hsp60 and Ilv5 to the nucleoid have shown to occur under the repressed respiration and amino acid starvation conditions, respectively, as the Aco1 has been shown to partially complement the loss of the Abf2 protein and proposed to protect the mtDNA from the ssDNA breaks (Chen *et al.,* 2005, 2007; Kucej *et al.,* 2008). Altogether, this indicates a regulatory mechanism in yeast cells that allows the protection and propagation of the mtDNA to be regulated according to the metabolic fitness of the cell.

2.1.2 Mitochondrial DNA maintenance in yeast

The mitochondrial function and the maintenance of mtDNA are primarily carried out by the proteins encoded by the nuclear genome. Although in *S. cerevisiae*, the proteins associated with mitochondrial functioning are determined to be in the thousands, the high-confidence proteome is estimated to consist of at least

900 proteins (Morgenstern *et al.,* 2017; Vögtle *et al.,* 2017; Schulte *et al.,* 2023). Some of these proteins have a dual localization to the nucleus and mitochondria, where they can have different functions, as shown for the mitochondrial helicase Pif1 protein (Zhou *et al.,* 2000). The proteins located in the mitochondria can often be identified by the presence of an N-terminal mitochondrial signaling peptide, which has a characteristic positive pre-sequence, forming amphipathic α-helices (von Heijne, 1986). However, it has been determined that the mitochondrial proteins may also have a C-terminal targeting signal, such as in the case of the mitochondrial helicase Hmi1 or internal mitochondrial signaling sequences as in the case of ribosomal proteins (Lee *et al.,* 1999; Bykov *et al.,* 2022).

Approximately 25% of the yeast mitochondrial proteome has been shown to be necessary for mtDNA maintenance and gene expression (Sickmann *et al.,* 2003). *S. cerevisiae* has a great advantage in studying the proteins necessary for mtDNA maintenance, as it is a facultative anaerobe and *petite*-positive yeast that can survive on the fermentable carbon source without the mtDNA. This is due to its capacity to uphold membrane gradients even without functional ETC complexes, achieved through efficient ATP hydrolysis by the "reverse" reaction of the ATP synthase complex (Giraud & Velours, 1997; Stenger *et al.,* 2020). Albeit being energetically highly inefficient, it ensures the import and export of components from and to the mitochondria, some of which are essential for cells. The loss of mtDNA can be caused by the variety of mutations in different nuclear genes, termed PET genes, and lead to the rise of smaller and slower-growing molecules called "*petite*" mutants (Tzagoloff & Dieckmann, 1990). Cytoplasmic *petite* mutants are a group of mutants that can be caused either by the complete loss of mtDNA (rho^0) or most of its sequence, in which case only a small fragment of the original mtDNA is retained in tandem repeats (rho^-) (Goldring *et al.,* 1970; Dujon, 2020). In contrast to human mtDNA, the yeast mtDNA is inherited biparentally during non-vegetative reproduction but tends to homogenize rather quickly (Ling & Shibata, 2004). This homoplasmy is especially quick to arise for the rho^- mutants, which exhibit a phenomenon called "suppressiveness", based on its genetic inheritance pattern when crossed with the wild-type (wt) cells (Ephrussi *et al.,* 1955). Rho^- strains are divided into neutral and hypersuppressive (HS) rho^- mutants, based on the observation that when crossed with wt cells, the progeny predominantly results with either a wt mitochondrial genome or a rho^- genome, respectively (Ephrussi *et al.,* 1955; Blanc & Dujon, 1980). The exact mechanism of "suppressiveness" is still not fully understood, but it has been proposed to be linked to the mtDNA segregation and replication processes. One of the proteins shown to be important for maintaining hypersuppressiveness is the mitochondrial resolvase Cce1 (Zweifel & Fangman, 1991). The absence of Cce1 in the HS rho^- strains leads to the impaired segregation of the mtDNA, which is thought to be linked to the decreased hypersupressivness of these HS rho^- strains (Zweifel & Fangman, 1991; Lockshon *et al.,* 1995; Piskur, 1997). One of the genetic characteristics of the HS strains is that they always contain an active *ori* sequence, which has led to the proposal that the mtDNA with short tandemly organized repeats could have the replication advantage (Blanc & Dujon, 1980;

MacAlpine *et al.,* 2001). Later studies have shown that the hypersuppressivness depends on the activity of RNA polymerase, which in turn can be suppressed by the mitochondrial Pet127 protein (Corbi & Amon, 2021). Pet127 is a yeast 5'-3' exoribonuclease that has been shown to participate in the post-transcriptional processing of RNA, and influence transcript levels in the cells (Wiesenberger & Fox, 1997; Fekete *et al.,* 2008; Łabędzka-Dmoch *et al.,* 2022).

It is important to emphasize that mtDNA maintenance is a complex system that goes beyond the proteins directly involved in DNA replication and repair (see chapter 2.1.3 and 2.1.4). It was shown quite early that the inhibition of protein synthesis by cold or antibiotics, leads to the mtDNA loss in *S. cerevisiae* (Weislogel & Butow, 1970). Deletions of different genes of proteins involved in protein synthesis, as well as components of the mitochondrial ribosome small and large subunits, also lead to the loss of mtDNA (Myers *et al.,* 1985; Merz & Westermann, 2009; Stenger *et al.,* 2020). In *S. cerevisiae*, translation seems to be linked with post-transcriptional modifications through the complex named MIOREX (mitochondrial organization of gene expression complexes), which combines the mitochondrial ribosomes but also different proteins needed for RNA metabolism (Kehrein *et al.,* 2015). Some MIOREX complexes also interact with the mtDNA nucleoids in the cell, indicating the broader connection and regulation of the mtDNA maintenance, replication and gene expression (Kehrein *et al.,* 2015). The direct coupling of the transcription and replication, like observed in mammalian cells through the transcription-dependent priming of the replication, is still being investigated in *S. cerevisiae* (see chapter 2.1.3). However, the importance of the transcription, the maturation of the transcripts, and their balanced level in the cell have been shown to have a critical role in mtDNA maintenance (reviewed in Golik, 2024). The role of mitochondrial RNA helicases in gene expression is further elaborated in chapter 2.2.4.4.

2.1.3 Mitochondrial DNA replication in *S. cerevisiae*

In the cell, mtDNA is packed into the nucleoid structure, which in turn associates with the two-membrane spanning structure thought to be a mitochondrial replisome complex, that contains the mtDNA polymerase Mip1 and the mtDNA binding protein Mgm101 (Meeusen & Nunnari, 2003). However, so far, the only two proteins essential for the mtDNA replication and maintenance both in *rho*$^{+}$ and *rho*$^{-}$ strains are shown to be Mip1 and the mitochondrial single-stranded binding protein Rim1 (Foury, 1989; Van Dyck *et al.,* 1992). Over the years, the studies of the replication in *S. cerevisiae* have strongly focused on the issue of how this replication is initiated and which proteins are involved in this process. Several pieces of evidence have been presented in favor of the transcription-dependent and the recombination-dependent replication initiation mechanisms (Fig. 1).

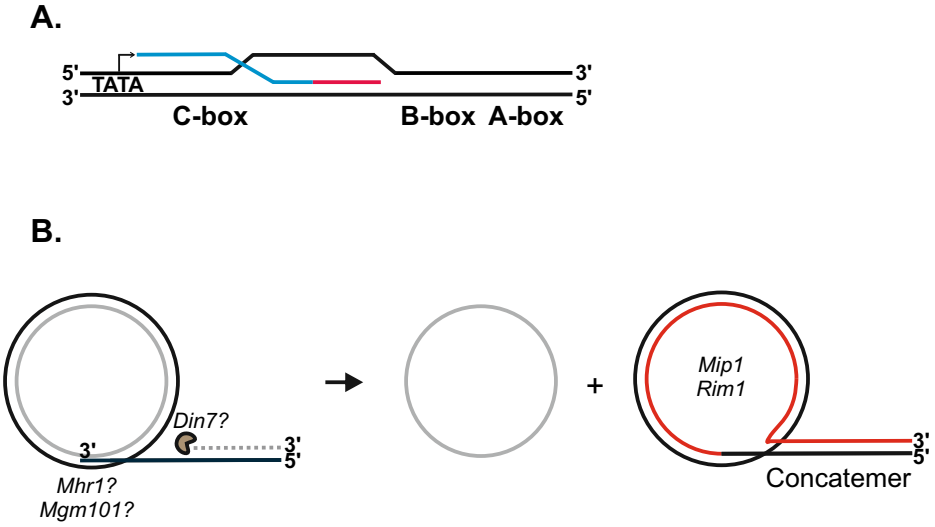

Figure 1. Replication initiation models proposed for *S. cerevisiae* mtDNA. A) The schematic representation of the origin of replication (*ori*) and the possible replication initiation site (based on data from Baldacci *et al.,* 1984; de Zamaroczy *et al.,* 1984). The A-, B-, and C-box locations are indicated, and TATA represents the promoter region. RNA primer is blue, and newly synthesized DNA is red **B)** The rolling circle replication (RCR) model where the recombination-dependent mechanism initiates the replication (based on data from Ling *et al.,* 2013; Chen & Clark-Walker, 2018). The replication could be initiated by the Mhr1 or Mgm101, using the 3' overhang of a double-stranded DNA or a 3' end of the single-stranded DNA, respectively. In the case of a double-stranded break (DSB), this overhang is proposed to be produced by the nuclease Din7. The DNA is replicated by the DNA polymerase Mip1, in the presence of mitochondrial single-stranded protein Rim1. The lagging strand is proposed to be synthesized through the strand switch and the formation of the concatemer. Newly synthesized DNA is displayed in red.

Transcription-dependent replication initiation

The transcription-dependent initiation model couples the mtDNA replication with the mitochondrial transcription and the mitochondrial RNA polymerase Rpo41. Rpo41 is a single-subunit RNA polymerase that shares homology with the bacteriophage T3 and T7 RNA polymerases, and is essential for mitochondrial transcription and maintenance of wt mitochondrial genome (Greenleaf *et al.,* 1986; Kelly & Lehman, 1986; Masters *et al.,* 1987). The binding of Rpo41 with the autoinhibited transcription factor Mtf1 (homolog of human TFB2M) leads to the initiation of the promoter-specific transcription from double-stranded DNA (Jang & Jaehning, 1991; Yang *et al.,* 2015; Basu *et al.,* 2020). The initiation complex goes through conformational changes and DNA scrunching, leading to the elongation complex formation (Sohn *et al.,* 2020). In human mitochondria, RNA synthesis by the mitochondrial RNA polymerase POLRMT for replication

initiation and transcription both start from the LSP (light strand promoter), which is located downstream of the Ori_H (Chang *et al.,* 1985; Chang & Clayton, 1985; Wanrooij *et al.,* 2008). For mtDNA replication, RNA transcription is prematurely terminated by the RNA:DNA hybrid loop (R-loop) formed of the transcribed RNA and proposedly by the G-quadruplex (G4) structure formed by the mtDNA (Xu & Clayton, 1996; Wanrooij *et al.,* 2012; Posse *et al.,* 2019). These structures must be processed by RNase H1 to create a 3'-end needed for a human DNA polymerase γ (Polγ) to prime mtDNA replication (Posse *et al.,* 2019). In *S. cerevisiae*, the *ori* sequences have a similar structure containing the RNA polymerase promoter sequence, followed by three GC-rich conserved sequence blocks (CSB) named C, B, and A-box and the R-loop is shown to form by the C-box (similar to human CSB2) (Baldacci *et al.,* 1984; de Zamaroczy *et al.,* 1984; Xu & Clayton, 1995). Rpo41 and its accessory factor Mtf1 have been shown to have primase activity and are able to generate the RNA primers on ssDNA and dsDNA, which can further be used for DNA synthesis by the Mip1 (Sanchez-Sandoval *et al.,* 2015; Ramachandran *et al.,* 2016). In addition, the *in vitro* studies showed that RNA primers needed for replication could be synthesized both from the ori promoters and from the gene promoters (Sanchez-Sandoval *et al.,* 2015).

In human cells, the heavy strand synthesis leaves Ori_L single-stranded, forming a stem-loop structure that is concurrently bound by the replicative mitochondrial polymerase Polγ and POLRMT, triggering the transcript slippage from the poly(T) tract, leading to the initiation of DNA replication (Wanrooij *et al.,* 2008; Fusté *et al.,* 2010, 2014; Sarfallah *et al.,* 2021). In *S. cerevisiae*, the Rpo41-Mtf1 complex has shown to be an effective primase on the ssDNA in the presence of the Rim1 *in vitro* and able to initiate replication from the 10–12nt RNA transcript (Ramachandran *et al.,* 2016). This transcript contains a polypyrimidine tract and has led to the proposal that transcription-dependent initiation might play a role in the lagging strand synthesis and replication reinitiation in yeast (Ramachandran *et al.,* 2016).

Recombination-dependent replication initiation

First hints that the transcription-dependent initiation mechanism could not be the only replication initiation mechanism possible in *S. cerevisiae* emerged quite early because of the studies conducted on *rho*$^-$ mutants. Neutral *rho*$^-$ strains that do not contain the *ori* sequences or gene promoter region are still able to maintain the mtDNA (Fangman *et al.,* 1989). In addition, the presence of Rpo41 is not essential for the mtDNA maintenance in *rho*$^-$ mutants (Fangman *et al.,* 1990). The fact that transcription is dispensable in the *rho*$^-$ strains and that *S. cerevisiae* mitochondria are prone to a high level of recombination, led to the proposal of an alternative recombination-dependent initiation mechanism (Dujon *et al.,* 1974; Fangman *et al.,* 1990; Fritsch *et al.,* 2014).

The studies with the HS *rho*$^-$ *petite* mutant, containing the ori5 (1.1 kb head-to-tail tandem repeat) sequence, showed that the DSB could be introduced inside the ori5 by the mtDNA glycosylase Ntg1 (Ling *et al.,* 2007; Hori *et al.,*

2009). In this DSB-dependent replication initiation model the Din7 nuclease is proposed to processes the DSB and creates 3'-single stranded DNA tail, which can then be used by the Mhr1 for homologous pairing to initiate the replication (Fig. 1B) (Ling & Shibata, 2002; Ling *et al.,* 2013).The DSB-dependent replication model has also been investigated in the *S. cerevisiae rho*$^{+}$ cells, where it was shown that the bacterial Ku protein imported to the mitochondria can bind and block the Ntg1-dependent ori5 DSB, which leads to the loss of mtDNA, possibly by hindering the mtDNA replication (Prasai *et al.,* 2017). Under normal cellular conditions, Mhr1 could bind these DSB in *S. cerevisiae* mitochondria (Ling *et al.,* 2007; Prasai *et al.,* 2018).

An alternative model has been proposed involving the mitochondrial Mgm101 protein, which is essential for maintaining the wt and neutral *rho*$^{-}$ mtDNA, but is dispensable for the HS *rho*$^{-}$ strain mtDNA maintenance (Chen *et al.,* 1993; Zuo *et al.,* 2002). Mgm101 is shown to be a part of the nucleoid and replisome complex and directly binds with mtDNA (Meeusen *et al.,* 1999; Kaufman *et al.,* 2000; Meeusen & Nunnari, 2003). In *S. cerevisiae* and *C. parapsilosis*, Mgm101 has been shown to be important for recombination, as it possesses strand annealing and strand invasion activities (Mbantenkhu *et al.,* 2011; Pevala *et al.,* 2016). These results have led to the proposal that Mgm101 (Fig. 1B) might initiate the rolling circle replication (RCR) with an invading single-stranded DNA molecule, which has a 3' overhang usable by Mip1 for DNA replication (Chen & Clark-Walker, 2018).

In conclusion, the protein set of *S. cerevisiae* could enable the mtDNA replication to be initiated by several mechanisms. Different studies have speculated that in the wt yeast, both mechanisms might exist simultaneously. Recombination-dependent replication initiation would be used for the leading-strand replication, while Rpo41 would prime the synthesis of the lagging strand or be needed for the replication reinitiation (Gerhold *et al.,* 2010; Sanchez-Sandoval *et al.,* 2015; Ramachandran *et al.,* 2016).

Elongation

In *S. cerevisiae*, the mtDNA replication elongation depends on the mtDNA polymerase Mip1 (Genga *et al.,* 1986; Foury, 1989). Unlike its mammalian homologs, Mip1 is processive as a single-subunit enzyme and has a C-terminal extension characteristic to the fungal mtDNA polymerases, which in *S. cerevisiae* is shown to be essential for polymerase and strand-displacement activity (balances the synthesis and degradation) (Lucas *et al.,* 2004; Young *et al.,* 2006; Viikov *et al.,* 2011, 2012; Trasviña-Arenas *et al.,* 2019). In addition to the central polymerization domain, Mip1 also has the 3'-5' exonuclease domain responsible for the exonucleolytic proofreading activity and the N-terminal extension essential for nucleotide incorporation (Foury & Vanderstraeten, 1992; Trasviña-Arenas *et al.,* 2019). As the replication progresses, the single-stranded binding protein Rim1 dimerizes, binds the ssDNA, leading to the formation of homo-tetramers (Van Dyck *et al.,* 1992; Ramanagoudr-Bhojappa *et al.,* 2013; Singh *et al.,* 2018).

The exact replication mode in *S. cerevisiae* is still a matter of debate. Although most of the mtDNA molecules in *S. cerevisiae* are branched and linear, the observation of a small number of circular molecules and the topology studies with the wt *Torulopsis glabrata* and *S. cerevisiae rho*$^{-}$ mutant strains have led to the proposal that RCR could be the replication mechanism of mtDNA in yeasts (Maleszka *et al.,* 1991; Bendich, 1996; Ling & Shibata, 2004). Indeed, there are examples of *Schizosaccharomyces pombe* and *Caenorhabditis elegans* that use the RCR replication for their mtDNA replication (Han & Stachow, 1994; Lewis *et al.,* 2015). The neutral two-dimensional agarose gel (2D-NAGE) topology studies with the *S. cerevisiae rho*$^{-}$ strains further confirmed the presence of circular ssDNA monomers that could represent the RCR or asymmetric strand displacement replication intermediates (MacAlpine *et al.,* 2001). It has been proposed that the quick rise of the homoplasmy in yeast could be explained by a model, where the concatemers are synthesized during RCR and selectively transmitted to the daughter cells where they are to be processed to circular monomers (Ling & Shibata, 2002, 2004). The RCR has also gained support from the matter that, so far, RNase H and topoisomerase have not been described in the yeast mitochondria, which would be required for processing the RNA primer and for dealing with the topological constraints during the replication, respectively (Chen & Clark-Walker, 2018).

However, the mode of elongation of wt mtDNA has remained elusive, mostly due to the difficulties of observing full-length replication intermediates of molecules as large as *S. cerevisiae* mtDNA. Furthermore, studies with *petite*-negative yeast have implicated that coupled or uncoupled replication of the branched and linear mtDNA of *C. albicans* and *C. parapsilosis* occurs (Gerhold *et al.,* 2010, 2014). In these yeasts, replication is thought to be initiated through a recombination-dependent mechanism via strand invasion events all over the mtDNA, although initiation hotspots are near the inverted repeats in *C. albicans* and near the telomeres in *C. parapsilosis*.

How mtDNA replication is precisely terminated in the *S. cerevisiae* mitochondria relies strongly on the replication mode and must be determined by future studies. However, this process likely involves the mtDNA ligase Cdc9 and the strand-displacement activity of Mip1 (Donahue *et al.,* 2001; Viikov *et al.,* 2011). In addition, it is also plausible that some nucleases would be needed, dependent on the replication mechanism. For example, a recombination junction resolvase Cce1 or a potential flap-structures modifying nuclease, Exo5, which is essential in *rho*$^{+}$ cells for *S. cerevisiae* mtDNA maintenance (Kleff *et al.,* 1992; Burgers *et al.,* 2010).

2.1.4 Mitochondrial DNA repair in *S. cerevisiae*

Compared to nuclear DNA, mtDNA has a higher mutation rate and is thought to be more prone to DNA damage. In *S. cerevisiae*, the average of base substitutions per site per cell division is estimated to be approximately 12.2×10^{-9}, which is 37 times higher than the estimated average for the nuclear genome (Lynch *et al.,*

2008). mtDNA is more prone to oxidative damage; for example, the accumulation of the oxidized DNA product 8-hydroxydeoxyguanosine has shown to be 16 times higher in the rat liver cells' mtDNA, compared to the nuclear DNA (Richter *et al.,* 1988). It is thought to be related to the matter that mitochondria themselves are a major producer of the cellular ROS, which arise from reactions with free electrons from the ETC complexes (Nissanka & Moraes, 2018). Damage can also occur as a result of DNA damaging agents, as they have been shown to increase the levels of different ROS species or directly damage the DNA, such as the accumulation of pyrimidine-dimers by the UV damage (Prakash, 1975; Rowe *et al.,* 2008). In addition, mutations can also be introduced by replication machinery, as mtDNA polymerase Mip1 integrates ribonucleotides into the mtDNA, which are not effectively removed and the mutations in Mip1, lead to error-prone replication (Foury & Vanderstraeten, 1992; Wanrooij *et al.,* 2017) (Fig. 2).

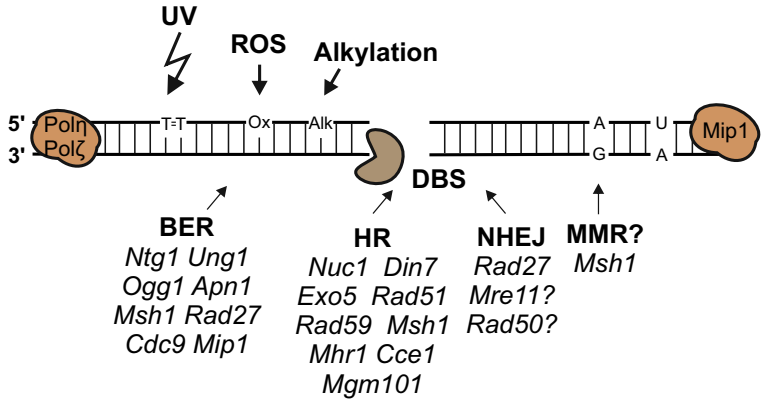

Figure 2. The schematic summary of the DNA damage and repair mechanism discussed in this chapter. UV radiation, reactive oxygen species (ROS), alkylating agents, and replication errors lead to mtDNA damage, including double-stranded breaks (DSB). Error-prone polymerases Polη and Polζ can progress the synthesis through damaged positions. Different DNA damage repair pathways are indicated: base excision repair (BER), homologous recombination (HR), non-homologous end joining (NHEJ), and mismatch repair (MMR). Proteins potentially involved in different damage repair pathways, which are discussed in this chapter, are listed. Proteins or repair pathways for which the presence or function in mitochondria is still disputed are indicated with a question mark (?).

The multi-copy nature of the mitochondrial genome has an advantage; a single mutational event does not have the same potential impact on gene expression as it does in the nuclear genome. In mammalian cells, it has been shown that the multi-copy nature of the mtDNA allows to deal with the deleterious effects of DSBs through excessive degradation of damaged mtDNA (Shokolenko *et al.,* 2009, 2013; Moretton *et al.,* 2017). This degradation has shown to be dependent on proteins involved in replication, such as the mammalian mtDNA polymerase POLG, the replicative helicase TWINKLE, and the exonuclease MGME1 (Peeva *et al.,* 2018).

In *S. cerevisiae* mitochondria DSB repair has shown to involve non-homologous end-joining (NHEJ) or homologous recombination (HR) (Kalifa *et al*., 2012). In *S. cerevisiae*, the endonuclease, Rad27 locates into the mitochondria and is proposed among other repair pathways to have a role in mitochondrial DSB repair, possibly in NHEJ (Kalifa *et al*., 2009; Nagarajan *et al*., 2017). In the nucleus, Rad27 has been shown to participate in NHEJ by processing the 5' DNA flaps created during end-joining (Wu *et al*., 1999; Tseng & Tomkinson, 2004). The involvement of other proteins in mitochondrial NHEJ has remained controversial. While nuclear NHEJ Ku70/Ku80 and MRX (Mre11, Rad50, Xrs2) complex proteins have been implicated in mtDNA repair, their localization to the mitochondria has remained questionable (Sickmann *et al*., 2003; Kalifa *et al*., 2012; Morgenstern *et al*., 2017; Vögtle *et al*., 2017; Schulte *et al*., 2023). It has been shown that Ku80 does not localize to the mitochondria; instead, mitochondrial recombination protein Mhr1 binds close to the DSB, indicating its role in DSB repair (Prasai *et al*., 2018).

The role of error-free HR-dependent repair is more complicated in the mitochondria, as it is thought to be related to the HR-driven replication of *S. cerevisiae* mtDNA (see chapter 2.1.3). To process the DSB, either for recombination-dependent replication or repair, the free 3'-single-stranded tail must be created. Nuc1 (homolog to human EndoG and ExoG) is a major nuclease in the mitochondria, which exhibits endonuclease and exonuclease activity of 5'-3' on dsDNA (Dake *et al*., 1988; S. Büttner *et al*., 2007). In addition, mitochondrial 5'-3' exonuclease Din7 can directly process double-stranded DNA and create a suitable 3'-single-stranded DNA tail for a HR or serve as a primer for the replication (Ling *et al.,* 2013). It has been proposed, that the presence of elevated levels of Din7 preferably leads to replication instead of recombinational repair (Ling *et al*., 2013). The homologous pairing could be mediated by the mitochondrial recombinases Mhr1 and Mgm101 (as discussed in chapter 2.1.3). During HR, Holliday junctions form, which must be resolved by a resolvase, such as the cruciform cutting enzyme Cce1 (Kleff *et al*., 1992; Kupfer & Kemper, 1996). In addition, it has been shown that nuclear HR proteins Rad51 and Rad59 are located in the mitochondria, and the loss of these proteins, together with Rad52, affects mitochondrial DSB repair (Stein *et al*., 2015). Other mitochondrial repair proteins have been shown to affect recombination, such as Ntg1 and Msh1, which both elevate recombination events (Phadnis *et al.,* 2006; Kaniak *et al.,* 2009).

S. cerevisiae mitochondria share base-excision repair (BER) pathway proteins Ogg1, Ung1, Ntg1, and Apn1 with the nucleus, which can recognize and repair bases modified by oxidative damage (Alseth *et al.,* 1999; Chatterjee & Singh, 2001; Singh *et al.,* 2001; Vongsamphanh *et al.,* 2001). The bifunctional Ogg1 and Ntg1 have both glycosylase and apurinic/apyrimidinic lyase activity and can recognize and process the oxidized DNA bases. Ogg1 mainly deals with the frequently occurring 8-oxoguanine, while Ntg1 recognizes a wide variety of oxidized pyrimidines and purines and can process abasic sites (van der Kemp *et al.,* 1996; Sandigursky *et al.,* 1997; Sentürker *et al.,* 1998; Alseth *et al.,* 1999; Meadows *et al.,* 2003). The glycosylase Ung1 is needed to remove deaminated

cytosine and misincorporated uracil bases (Impellizzeri *et al.,* 1991; Chatterjee & Singh, 2001). After the excision of the damaged nucleotide, the Apn1 apurinic/ apyrimidinic endonuclease/ 3'-diesterase creates the 3'-OH, which can then be utilized for gap filling by the mtDNA polymerase Mip1 and mitochondrial ligase Cdc9 (Johnson & Demple, 1988; Ramotar *et al.,* 1991; Donahue *et al.,* 2001; Viikov *et al.,* 2011). In human mitochondria, both the short- and long-patch BER are shown to be present. In short-patch BER, human mitochondrial Polγ replaces one nucleotide and posesses a 5' deoxyribose phosphate lyase activity needed to generate the product, which can then be ligated (Longley *et al.,* 1998). In long-patch BER several nucleotides are replaced, creating a 5' flap, which must be removed by 5' exo/ endonuclease, proposed to be hFEN1, hDNA2, hEXOG, hMGME1 or a combination of them in humans (Akbari *et al.,* 2008; Liu *et al.,* 2008; Szczesny *et al.,* 2008; Zheng *et al.,* 2008; Uhler *et al.,* 2016; Urrutia *et al.,* 2022; Szymanski *et al.,* 2022). Rad27, a homologue of human FEN1, has been proposed to have a similar role in the mitochondrial long-patch BER in yeast (Kalifa *et al.,* 2009).

The homolog of bacterial mismatch repair (MMR) protein MutS, Msh1, localizes to the mitochondria and can bind the mismatches (Chi & Kolodner, 1994). However, the role of MMR in *S. cerevisiae* mitochondria has remained unclear, since Msh1 is the only MMR protein detected in mitochondria so far and has a genetical interaction with Ogg1, Ntg1, and Apn1 proteins, indicating a possible function in the oxidative damage repair and BER pathway (Dzierzbicki *et al.,* 2004; Pogorzala *et al.,* 2009). Msh1 has also been shown to affect recombination and be important for mtDNA transmission (Mookerjee *et al.,* 2005; Mookerjee & Sia, 2006; Kaniak *et al.,* 2009).

Mitochondria are thought to lack the classical nucleotide-excision repair pathway, as the mtDNA accumulates UV damage-induced pyrimidine-dimers in mammalian and yeast cells (Clayton *et al.,* 1974; Prakash, 1975). mtDNA lesions, which cannot be repaired by the mitochondrial repair system, such as UV-damage induced pyrimidine-dimers, can be bypassed by the error-prone polymerases like polymerase ζ and η, described in yeast mitochondria (H. Zhang *et al.,* 2006; Kalifa & Sia, 2007; Chatterjee *et al.,* 2013).

2.2 Mitochondrial helicases

2.2.1 Introduction to helicases

Helicases were first described in 1976 as nucleoside triphosphate-dependent motor enzymes that can unwind different nucleic acid substrates (Abdel-Monem *et al.,* 1976; Abdel-Monem & Hoffmann-Berling, 1976). Their biochemical functions are multifarious, including unwinding double-stranded nucleic acids, resolving branched or higher structures, resolving Holliday junctions, unwinding R-loops and displacement loops (D-loop), and displacing proteins from the nucleic acids via translocation (Brosh & Matson, 2020; Bohnsack *et al.,* 2023). These activities make helicases essential for replication, transcription, translation, repair, and are

also required for remodeling chromatin, regulating the length of telomeres, RNA processing, splicing, and ribosome synthesis (Brosh & Matson, 2020; Bohnsack *et al.,* 2023).

Helicases and translocases are divided into six superfamilies (SF) based on their conserved amino acid motifs, the largest being SF1 and SF2, which contain mono- and oligomeric helicases and SF3-6 hexameric helicases (Gorbalenya & Koonin, 1993; Singleton *et al.,* 2007). For SF1 and SF2, seven conserved helicase motifs (I, Ia, II–VI) were described first (Gorbalenya *et al.,* 1989; Gorbalenya & Koonin, 1993). Later, the additional motifs were determined, which help to divide the helicases into families within the superfamilies (Fairman-Williams *et al.,* 2010). Comparative sequence analysis of helicases has determined that SF1 contains three different helicase families (UvrD/Rep, Pif1-, and Upf1-like), while SF2 contains nine families (RecG-, RecQ-, Ski2-, RIG-I-like, Rad3/XPD, T1R, Swi/Snf, DEAD-box and DEAH/RHA) (Fairman-Williams *et al.,* 2010). Helicases and translocases are also classified based on their choice of substrate as type α (ssDNA) and type β (dsDNA), and based on the polarity of translocation as type A (3'-5') and type B (5'-3') (Singleton *et al.,* 2007). Helicases can also be active or passive, based on the unwinding mechanism of the nucleic-acid substrates. Respectively, protein binding with the nucleic acid leads to the thermal fraying of the dsDNA by a passive mechanism or the breaking of the hydrogen bonds at the expense of ATP hydrolysis during an active mechanism (Lohman, 1992; Manosas *et al.,* 2010).

In addition, helicases also differ in their catalytic rate, step size, and processivity which defines the speed and the mode of movement, or whether one catalytic cycle (distributive enzyme) or many cycles (processive enzyme) is catalyzed before the enzyme disassociates from the nucleic acid (Singleton *et al.,* 2007). However, these properties are not always static and can depend on different factors, like the oligomerization state and the accessory proteins. For example, SF1 helicase *Escherichia coli* Rep has different unwinding and translocation activity whether as a monomer or dimer, and is also dependent of the processivity factor PriC (Cheng *et al.,* 2001; Heller & Marians, 2005; Nguyen *et al.,* 2021). In addition, Rep activity is also controlled by its tertiary structure, as the 2B domain of Rep helicase regulates the translocation, DNA unwinding rate and processivity (Brendza *et al.,* 2005; Makurath *et al.,* 2019).

2.2.2 Tertiary structure of helicases and helicase motifs

The first helicase protein crystal structure was published for SF1 member *Geobacillus stearothermophilus* (in earlier studies referred as *Bacillus stearothermophilus*) PcrA (Subramanya *et al.,* 1996; Velankar *et al.,* 1999), followed by other crystal structures of the SF1 members, such as bacterial Rep and UvrD and SF2 members Hepatitis C virus helicase NS3, *S. cerevisiae* eIF4A and bacterial RecG (Korolev *et al.,* 1997; Kim *et al.,* 1998; Benz *et al.,* 1999; Caruthers *et al.,* 2000; Singleton *et al.,* 2001; Lee & Yang, 2006). Based on these crystal structures, it was determined that the "helicase core" consists of two RecA-like

domains, which in SF1 are both divided into two subdomains (1A, 1B, 2A, 2B), while SF2 helicases have two RecA-like domains and at least one extra domain (Fig. 3A). The RecA-like domains contain the conserved helicase motifs, which form the NTP binding pocket and nucleic acid binding channel. The NTP is located between the RecA domains and the γ-phosphate is directed towards the nucleic acid, which is located in the central channel of the protein, surrounded and bound by the amino acid residues of the nucleic acid binding motifs (Korolev *et al.,* 1997; Kim *et al.,* 1998; Velankar *et al.,* 1999; Singleton *et al.,* 2001; Lee & Yang, 2006).

The NTP binding pocket is formed of amino acid residues belonging to the motifs Q, I, II, III, VI (Soultanas *et al.,* 1999). Motifs I and II, which are also referred to as Walker A and B motifs, have been shown to be conserved in different enzymes, using energy derived from ATP hydrolysis (Walker *et al.,* 1982). The highly conserved G-K-T/S (glycine-lysine-threonine/serine) sequence in motif I participates in ATP hydrolysis; more specifically, lysine has been shown to bind the β-phosphate, while threonine coordinates the Mg^{2+} ion, and their mutations lead to the strongly reduced ATPase activity and inactivation of the protein function (Fig. 3B) (Rozen *et al.,* 1989; George *et al.,* 1994; Soultanas *et al.,* 1999; Velankar *et al.,* 1999).

Motif II contains highly conserved neighboring aspartic acid and glutamic acid, which bind to a Mg^{2+} ion, and the glutamic acid possibly participates in the deprotonation of the water molecule (Soultanas *et al.,* 1999; Velankar *et al.,* 1999; Lee & Yang, 2006). These amino acid residues are crucial for ATP hydrolysis and helicase activity, but do not seem to interfere with the ability to bind ATP and ssDNA (Pause & Sonenberg, 1992; Brosh & Matson, 1996). Motif VI has a structural role, as it contains a conserved arginine, which contacts the γ-phosphate and histidine/glutamine, that interacts with motif II to form a salt bridge (Korolev *et al.,* 1997; Kim *et al.,* 1998; Caruthers *et al.,* 2000; Lee & Yang, 2006). Mutations in motif VI lead to decreased ATP hydrolysis and helicase activity (Pause & Sonenberg, 1992; Gross & Shuman, 1996; Kim *et al.,* 1997; Hall *et al.,* 1998). The later-identified Q-motif is conserved in helicases that use ATP as a cofactor, as the highly conserved glutamine is shown to interact with adenine base, which is important for the catalytic activity of the helicases (Tanner *et al.,* 2003; Cordin *et al.,* 2004; Lee & Yang, 2006; Fairman-Williams *et al.,* 2010; Wu *et al.,* 2012). Motif III has been thought to have a central role in coordinating the concurrent binding of the nucleotide triphosphate and the nucleic acid substrate, to enable the helicase activity of the protein (Brosh & Matson, 1996; Banroques *et al.,* 2010). Different point mutations of motif III abolish or severely reduce the helicase activity (Pause & Sonenberg, 1992; Sharples *et al.,* 1994; Dillingham *et al.,* 1999; Banroques *et al.,* 2010). It is important to note that this motif has the most distinctive difference in their conserved amino acid residues sequence and, therefore, is used for distinguishing between the SF1 and SF2 helicases (Fig. 3B) (Fairman-Williams *et al.,* 2010). Motif III of SF2 helicases is short and has, in most cases, conserved a T/SAT (threonine/serine-alanine-threonine) amino acid residues sequence, in comparison to SF1 family helicases which have a more extended motif

containing different aromatic and positively charged amino acid residues (Fairman-Williams *et al.,* 2010). In addition, unlike SF2, the SF1 helicases have an extra motif IIIa (in some articles referred to as motif IV) that participates in ATP binding (Hall & Matson, 1997; Fairman-Williams *et al.,* 2010). The most comprehensive studies with motif III were conducted with the SF1 UvrD-like family helicases. Crystal structures have determined that motif III structurally forms a loop where the conserved threonine, tryptophan (or phenylalanine), and arginine have shown to be important for DNA substrate binding, as the glutamine interacts with γ-phosphate of ATP (Korolev *et al.,* 1997; Velankar *et al.,* 1999; Lee & Yang, 2006). The biochemical characterizations have shown that glutamine is important for ATP hydrolysis and helicase activity but does not interfere with DNA binding (Brosh & Matson, 1997; Dillingham *et al.,* 1999). The tryptophan and arginine of motif III do not interfere with the ATPase activity, but are important for substrate binding and helicase activity, similar to threonine, which in addition affects the ATP hydrolysis (Dillingham *et al.,* 1999, 2001). One of the aspartic acids in motif III has been shown to be an important factor in the formation of the active substrate complex and the interaction of ATP and DNA binding (Brosh & Matson, 1996).

Motifs Ia, IV, and V are considered nucleic acid binding motifs, as in SF1 and SF2, amino acid residues from these motifs localize in the nucleic acid binding channel and bind directly with the nucleic acids, however, in the SF1 family, motif V also binds ATP (Kim *et al.,* 1998; Velankar *et al.,* 1999; Lee & Yang, 2006). Different point mutations in these motifs lead to decreased nucleic acid binding and helicase activity (Graves-Woodward & Weller, 1996; Dillingham *et al.,* 2001; Marintcheva & Weller, 2003; Banroques *et al.,* 2008; Guo *et al.,* 2014). Motifs Ia, IV, and V are less conserved among the helicase families (Fig. 3B), indicating the specialization on different substrates and binding modes (Fairman-Williams *et al.,* 2010).

While the core helicase domain containing helicase motifs is relatively conserved in different helicases, specific functions are often related to additional C- and N-terminal domains characteristic to that specific family (Fairman-Williams *et al.,* 2010). The most prominent examples are RecQ family helicases, which have two C-terminal domains: the RQC (RecQ-C-terminal) and the HRDC (Helicase-and-RNaseD-like-C-terminal) domain. These domains ensure binding with the DNA structures, such as, ssDNA, dsDNA, G4 structures, and Holliday junctions, depending on the specificity of the RecQ family member helicase (Liu *et al.,* 1999, 2004; Bernstein & Keck, 2005; Guo *et al.,* 2005; Wu *et al.,* 2005). In addition, the RQC domain consists of a Zn-binding domain and a WH (winged-helix) domain, the last of which also physically participates in the DNA unwinding processes forming a “pin” structure (see chapter 2.2.3), and is essential for oligomerization (Kitano *et al.,* 2010; Lucic *et al.,* 2011). The N-terminal domains of Rad5 and SMARCAL1, the HIRAN and two HARP domains, respectively, are shown to be critical for binding specific DNA substrates and essential for branch migration and fork regression activity (Bétous *et al.,* 2012; Shin *et al.,* 2018).

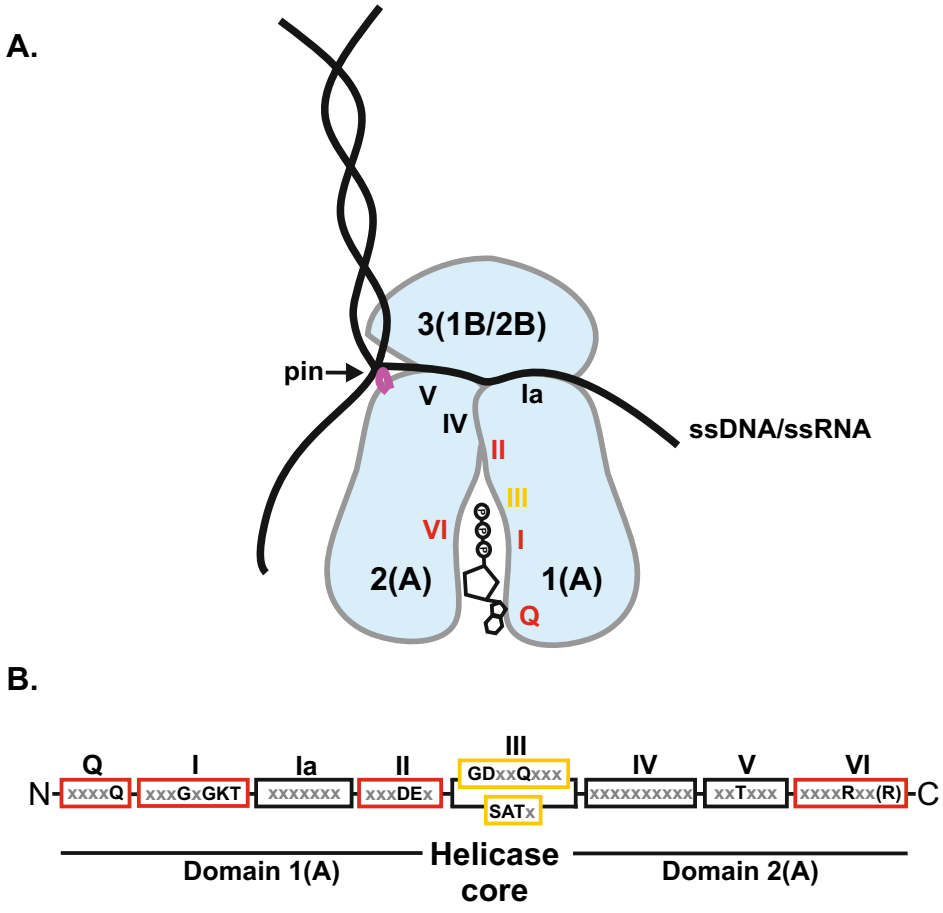

Figure 3. A simplified schematic representation of the helicase structure and the helicase motifs. A) The generalized helicase structure (not to scale) with the nucleic acid and ATP binding sites indicated. The RecA domains 1 (1A for SF1) and 2 (2A for SF1) and domain 3 (1B/2B for SF1) are shown. The main helicase motifs are indicated by their approximate locations: red- motifs involved in the ATP binding, black- nucleic acid binding, and yellow- both. The helicases can use a "pin" structure to separate the two strands of dsDNA/RNA physically. The example of a location of the "pin" structure for the *E. coli* UvrD helicase is indicated (pink). **B)** The conserved helicase core and its motifs, the color code is the same as in panel A. Selection of the most conserved amino acid residues in both SF1 and SF2 are indicated. For motif III, the representative set of amino acid residues conserved in this motif for both families are indicated as SF1 (upper box) and SF2 (lower box). The gray x indicates the positions in the motif where the amino acid residues are less conserved. Note that the sequence and conserved amino acid residues can vary greatly from one family to another. In addition, most helicase families contain an additional N- or C-terminal domains that are conserved within the specific helicase family. Figure is drawn, based on the data from Lee & Yang, 2006; Fairman-Williams *et al.,* 2010.

2.2.3 Nucleic acid unwinding and translocation

The aforementioned "pin" structure is proposed to function in nucleic acid unwinding, as the structure separates the duplex DNA using the translocating energy (Byrd & Raney, 2012). The "pin" structure is a β-hairpin that positions inside the helicase core or as an additional N- or C-terminal domain, present in different helicase family members, like Hel308, UvrD, RecQ (Lee & Yang, 2006; K. Büttner *et al.,* 2007; A. C. W. Pike *et al.,* 2009; Fairman-Williams *et al.,* 2010; Lucic *et al.,* 2011; Byrd & Raney, 2012). In some cases, a whole domain is characterized, such as the RecG helicase family "wedge domain," that contains amino acid residues needed for binding and positioning of the branched DNA molecules to ensure the highly processive unwinding of the DNA (Singleton *et al.,* 2001; Briggs *et al.,* 2005). Based on the single-molecule studies with UvrD, a general model for the non-hexameric helicase, is proposed that helicases unwind 1 base pair of DNA per ATP hydrolysis, but the separated DNA strands on each side of the protein bind with structural amino acid residues, leading to the DNA sequestering and looping until the unwound strand is released, on average, every 3 base pair (Carney *et al.,* 2021).

To unwind the nucleic acid duplexes, the helicase usually must translocate on the nucleic acid until it reaches the DNA junction. Different mechanistic models have been proposed for translocation, including the powerstroke mechanism "Inchworm" and the "Brownian motor" mechanism (Fig. 4).

The powerstroke mechanism assumes that the monomeric protein has two nucleic acid binding regions or that the protein is at least dimeric, and each monomer contains one nucleic acid binding region (Yarranton & Gefter, 1979; Wong & Lohman, 1992). The studies on SF1A helicases UvrD, PcrA, and Rep, led to the proposal of the "inchworm" mechanism, which is considered to be applicable for most of the monomeric SF1 helicases (Yarranton & Gefter, 1979; Bird *et al.,* 1998; Velankar *et al.,* 1999; Lee & Yang, 2006). In this model, the binding of ATP with the protein leads to the strong binding of domain two with the nucleic acid and to the sliding of domain one closer to domain two (Fig. 4A). As the ATP hydrolyzes, domain one binds strongly to the nucleic acid, and the connection of domain two weakens and leads to the sliding of domain two further towards the 5' end of the nucleic acid. For SF1B helicases, the overall translocation occurs similarly with some underlying differences. To move towards the 3' end of nucleic acid, domain one moves ahead, and the binding of ATP strengthens the grip of domain one with the nucleic acid instead of domain two (Saikrishnan *et al.,* 2009). In addition, there are differences in the structural mode of the translocation for both enzyme groups, as the amino acid residues required for binding the nucleic acid are located differently. The SF1B enzymes mostly bind the nucleic acid phosphate backbone instead of the bases, as described for SF1A enzymes (Saikrishnan *et al.,* 2009).

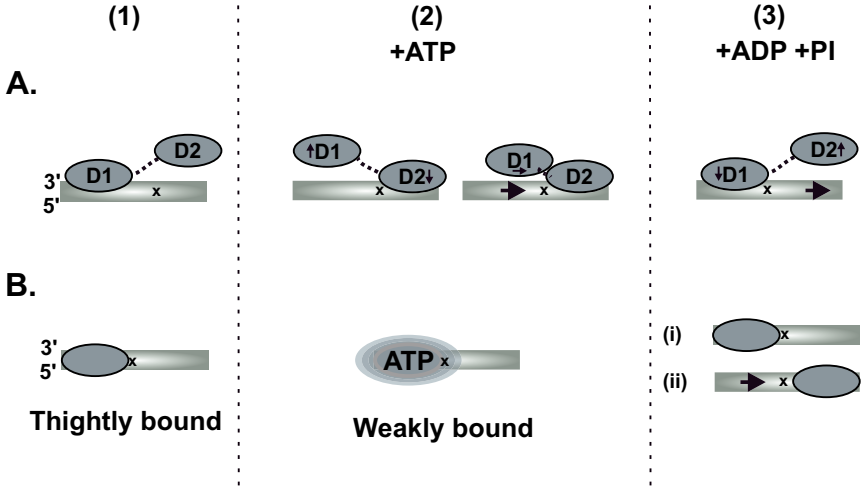

Figure 4. The "Inchworm" and "Brownian motor" models of translocation. For both models, three states are indicated: (1) the original position of the protein, (2) the binding of ATP, and (3) the ATP hydrolysis. **A)** "Inchworm" mechanism (Velankar *et al.,* 1999), where the protein has two nucleic acid binding sites, one in each domain. Helicase domain two is moving ahead for the SF1A helicases (3'-5' translocases). ATP binding makes domain two bind to the nucleic acid; simultaneously domain one is released and brought closer to domain two. Once ATP is hydrolyzed, domain one binds to the nucleic acid, and domain two is released and able to slide forward. **B)** In the "Brownian motor" model (Levin *et al.,* 2005), the protein has one nucleic acid binding site. The protein strongly binds nucleic acid in the energy minimum state. ATP binding results in the weakly bound state, enabling the movement of the protein. ATP hydrolysis restores the low energy state, making the protein bind tightly either (i) at the same position or (ii) one step further. Note that this figure describes the 3'-5' translocation, and the DNA is placed opposite of the typically occurring 5'-3' direction.

The studies on SF2 helicase NS3 have led to the proposal of a "Brownian motor" mechanism, according to which the protein only has one binding site with the nucleic acid, and the translocation movement is a stochastic process based on the energetic switch between two different conformations of the enzyme (distinguished by weakly and tightly bound conformations) (Levin *et al.,* 2003, 2005). In this model, the protein binds tightly with the nucleic acid in the absence of ATP, being trapped in the energy well. The binding of ATP leads to the weakly bound conformation of the protein, allowing the movement on the nucleic acid, followed shortly by ATP hydrolysis restoring the tightly bound conformation (Fig. 4B). This action results in the enzyme translocating one step forward or remaining in the same position.

However, for some SF2 helicases, the translocation does not seem vital for their helicase activity (Yang *et al.,* 2007). For DEAD-box proteins, the translocation-independent local strand separation mechanism of RNA unwinding has

been described (Yang & Jankowsky, 2006; Yang *et al.,* 2007). DEAD-box proteins use the ATP binding energy of one ATP to directly bind and unwind the DNA-RNA duplexes that are many nucleotides long (Chen *et al.,* 2008).

2.2.4 *S. cerevisiae* mitochondrial helicases

In *S. cerevisiae*, at least six mitochondrial helicases have been experimentally described, three of them are Pif1, Irc3, Hmi1 that have DNA helicase activities and three of them are RNA helicases Suv3, Mrh4 and Mss116 (Séraphin *et al.,* 1989; Lahaye, 1991; Stepien *et al.,* 1992; Sedman *et al.,* 2000; Schmidt *et al.,* 2002; Sedman *et al.,* 2014). It must be noted, however, that these might not represent the full helicase complement functioning in the mitochondria. Lately, it has been shown that a minor fraction of nuclear DNA helicase Mph1 of the RIG-I-like (sometimes also referred to as FANCM-like) family is present in the mitochondria and is important for mtDNA maintenance under stress conditions (Bernal *et al.,* 2019). This raises the possibility that some proteins, including helicases, currently identified as "nuclear," can have additional functions in mitochondria and underline the complexity of mtDNA homeostasis dynamics. The *S. cerevisiae* mtDNA helicases differ quite significantly from the set of DNA helicases described to function in the mammalian mitochondria (reviewed in Peter & Falkenberg, 2020). In mammalian cells, the replicative DNA helicase TWINKLE is present and has been shown to be essential for DNA replication (Spelbrink *et al.,* 2001; Korhonen *et al.,* 2003). However, *S. cerevisiae* mitochondria does not seem to contain the orthologs of TWINKLE or any hexameric helicases (Morgenstern *et al.,* 2017; Vögtle *et al.,* 2017; Schulte *et al.,* 2023). In addition, two other mammalian helicases, RECQL4 and DNA2, are shown to have a dual localization to the nucleus and mitochondria, which have homologs in yeasts but are so far not described in the yeast mitochondria (Duxin *et al.,* 2009; Croteau *et al.,* 2012). In mammalian cells both are shown to be important for mtDNA stability, as the RECQL4 has been shown to reduce mtDNA damage, and DNA2 has been shown to interact with the mitochondrial replication machinery and is proposed to have a role in mtDNA replication and repair, likely through the processing of nucleic acid flap structures (Zheng *et al.,* 2008; Duxin *et al.,* 2009; Croteau *et al.,* 2012).

2.2.4.1 Pif1

Pif1 (*petite* integrated frequency) was first described in a study screening for proteins important for recombination between the *S. cerevisiae rho*$^{+}$ and *rho*$^{-}$ strains' mtDNA and was the first identified mtDNA helicase (Foury & Kolodynski, 1983; Foury & Lahaye, 1987; Lahaye, 1991). Pif1 is a SF1 DNA helicase that is widely conserved across organisms (Lahaye, 1991; D.-H. Zhang *et al.,* 2006; Bochman *et al.,* 2011). In yeast and humans, it has two isoforms, one of which is localized to the nucleus and the other to the mitochondria (Lahaye, 1991; Zhou *et al.,* 2000; D.-H. Zhang *et al.,* 2006; Futami *et al.,* 2007).

Pif1 is a monomeric ATP- and ssDNA-dependent 5'-3' direction helicase able to unwind fork structures, R-loops, and is especially known for its activity to translocate on DNA and temporarily unwind G4 structures (Lahaye, 1991; Lahaye *et al.,* 1993; Boulé & Zakian, 2007; Zhou *et al.,* 2014). The DNA G4s are structures where, through the Hoogsteen pairing, four guanines bind each other as a planar, and these planes, in return, can stack onto each other. The formed secondary structures can block the replication fork progression or affect the gene expression through modeling the chromatin structure, R-loop stability, or transcription activation, as both the unbalanced formation and resolution can lead to genome instability (Dahan *et al.,* 2018; Robinson *et al.,* 2021). It has been shown that in the nucleus Pif1 binds G4s *in vivo,* and this interaction is important for the replication progression and maintaining genome stability (Paeschke *et al.,* 2011, 2013). In addition, Pif1 interacts with PCNA to promote the Polδ-mediated break-induced replication and facilitate the replication fork progression of lagging strand through G4s (Buzovetsky *et al.,* 2017; Dahan *et al.,* 2018). The G4 structures are also shown to be present in the mammalian mitochondria, where they affect the mtDNA replication, and in *S. cerevisiae*, the G4 motifs are predicted to occur 10-fold more often in mtDNA than in nuclear DNA, leading to the proposal that Pif1 could also unwind G4 structures of mtDNA (Capra *et al.,* 2010; Doimo *et al.,* 2023). This idea is further supported by the *in vitro* experiments, which showed that Pif1 stimulates the mtDNA polymerase Mip1's ability to resolve G4 structures (Sparks *et al.,* 2019).

In mitochondria, the absence of *PIF1* leads to a lowered level of recombination, UV sensitivity, and mtDNA loss during the growth in glucose-containing media (Foury & Kolodynski, 1983; O'Rourke *et al.,* 2005). Genetic studies have implied that Pif1 also has a recombination-independent role in oxidative damage induced mtDNA repair (O'Rourke *et al.,* 2002; Doudican *et al.,* 2005). The *PIF1* deletion strain can grow on the non-fermentable glycerol-containing media but loses its mtDNA at higher temperatures (like 36 °C) (Van Dyck *et al.,* 1992). This loss of mtDNA can be partially rescued by the overexpression of the mitochondrial single-strand binding protein Rim1, which interacts with Pif1 and increases its otherwise low helicase processivity up to 5-fold, indicating that Rim1 might be its processivity factor (Van Dyck *et al.,* 1992; Ramanagoudr-Bhojappa *et al.,* 2013; Zybailov *et al.,* 2015). This has led to the proposal, that Pif1 might be involved in the replication processes, which has been further supported by the observations that Pif1 directly interacts with mtDNA and has shown to have an important role in mtDNA DSB processing, the dNTP incorporation and fork progression (Cheng *et al.,* 2007, 2009). The effect of the loss of Pif1 mitochondrial function can be suppressed by the additional loss of the Pif1 nuclear paralog Rrm3 helicase, a phenomenon proposed to be associated with the dNTP pool regulation (O'Rourke *et al.,* 2005; Cheng *et al.,* 2009). This result, together with the observations that mtDNA can be replicated in the absence of Pif1, indicates that the role of Pif1 in mtDNA replication must be dispensable.

In the nucleus, many different functions of Pif1 have been described, including the regulation of telomeres, lagging strand synthesis, and promoting

replication through different DNA regions (reviewed in Malone *et al.,* 2022). Pif1 mediates the balanced length of telomeres and prevents the addition of telomeres at the end of DSB by inhibiting telomerase activity (Boulé *et al.,* 2005; Zhou *et al.,* 2000; Makovets & Blackburn, 2009). Pif1 also participates in lagging strand Okazaki fragment synthesis with Polδ, where it stimulates the strand displacement and the formation of longer flaps which can further be processed by DNA2 (Budd *et al.,* 2006; Rossi *et al.,* 2008; J. E. Pike *et al.,* 2009).

2.2.4.2 Mitochondrial DNA helicase Irc3 and its potential functional analogues

Early large-scale studies identified Irc3 (Increased Recombination Centers) as a protein that localizes to the mitochondria and was also identified in large-scale screen of genes affecting the expression of the recombination protein Rad52 (Sickmann *et al.,* 2003; Alvaro *et al.,* 2007). *S. cerevisiae* Irc3 ($Irc3_{sc}$) is a mitochondrial ATP-dependent helicase stimulated by dsDNA and shown to be important for mtDNA maintenance (Sedman *et al.,* 2014). In addition, our recent studies show that the *Ogataea polymorpha* Irc3 ($Irc3_{op}$) ATPase activity is also stimulated by RNA (see chapter 5.3), pointing towards the possibility that this helicase might also have a role in RNA metabolism, which is further underlined by the fact, that recent studies with $Irc3_{sc}$ suggested that $Irc3_{sc}$ might have a role in translation elongation (Kaur & Datta, 2021).

However, the result part of this thesis will mainly concentrate on the Irc3 function in mtDNA maintenance. The loss of $Irc3_{sc}$ protein leads to drastic loss of mtDNA and accumulation of dsDNA breaks and branched molecules, indicating the role of $Irc3_{sc}$ in mtDNA metabolism (Sedman *et al.,* 2014). Our group has further characterized the DNA substrate usage of $Irc3_{sc}$, which showed a preference towards the three-way branched, fork-like, and four-way branched, Holliday-junction-like substrates, which is comprehensively discussed in the results part of this thesis (see chapter 5.3). Further studies have determined that the $Irc3_{sc}$ C-terminal part of the protein ensures a specificity for binding and unwinding of branched DNA molecules (Sedman *et al.,* 2017). $Irc3_{sc}$ is active as a monomeric protein that binds with the branch points of mtDNA molecules and can translocate along the double-stranded DNA using ATP energy for unwinding the DNA substrates (Sedman *et al.,* 2017; Piljukov *et al.,* 2020). The results of this thesis indicate that RecG could be the functional analog of $Irc3_{sc}$ (see chapter 5.1). Other functional analogs have been proposed for RecG, like the human helicases SMARCAL1 (Swi2/Snf2 helicase family) and bacteriophage T4 helicase UvsW which also has moderate homology to $Irc3_{sc}$ (see chapter 5.1) (Carles-Kinch *et al.,* 1997; Bétous *et al.,* 2013).

RecG, UvsW, and SMARCAL1 share many similar biochemical properties, such as the preferential binding of structures resembling replication forks and Holliday junctions (Lloyd & Sharples, 1993; Whitby *et al.,* 1994; Whitby & Lloyd, 1998; McGlynn & Lloyd, 2000; Singleton *et al.,* 2001; McGlynn & Lloyd, 2001; Nelson & Benkovic, 2007; Webb *et al.,* 2007; Bétous *et al.,* 2012). They are able

to initiate the fork regression and branch migration to avoid harmful effects caused by the accumulation of these structures due to aberrant DNA transactions (Whitby *et al.,* 1993, 1994; McGlynn & Lloyd, 2001; Singleton *et al.,* 2001; Webb *et al.,* 2007; Long & Kreuzer, 2009; Bétous *et al.,* 2012). These helicases unwind D-loop structures, which indicates their role in recombination-dependent processes (McGlynn *et al.,* 1997; Nelson & Benkovic, 2007; Ciccia *et al.,* 2012). For example, the recombinational role of UvsW as a part of UvsXYW (analog of eukaryotic Rad51/Rad52/Rad54 complex), has been shown (Gajewski *et al.,* 2011, 2016). In addition, these helicases unwind R-loops and suppress their accumulation in the cells to avoid the instability of DNA maintenance (Vincent *et al.,* 1996; Fukuoh *et al.,* 1997; Nelson & Benkovic, 2007; Hodson *et al.,* 2022). Based on human SMARCAL, FANCM, and ZRANB3 translocases, it has been proposed, that independent from the helicase activity, the translocases migrate to the branch point of the R-loop and displace the RNA from the R-loop, rather than unwinding it (Hodson *et al.,* 2022). In bacteriophages, the R-loop unwinding by the UvsW helicase mediates the progression of late-phase recombination-dependent replication (Dudas & Kreuzer, 2001).

Based on RecG and UvsW, a general fork regression model for the SF2 family helicases has been proposed, where the helicase core is translocating on the parental duplex DNA, while the structural part of the protein binds with the branch point and fork arms to rewind the DNA, even in the presence of a denaturing force via active helicase mechanism (Manosas *et al.,* 2013). It has been shown that RecG physically interacts with the SSB C-terminal tip, which helps RecG to load to the stalled replication fork and undergo conformational changes needed for translocation on the dsDNA (Sun *et al.,* 2015; Yu *et al.,* 2016; Sun *et al.,* 2018; Bonde *et al.,* 2023). The binding of RecG prevents the accidental unwinding of the stalled fork by helicase PriA (Tanaka & Masai, 2006). RecG proceeds with the simultaneous unwinding and rewinding to form a "chicken foot"/ Holliday junction-like structure, which migrates away from the stalling point and allows the repair process to occur (Manosas *et al.,* 2013) (Fig. 5). The accumulation of replication fork-like structures *in vivo*, in the absence of RecG, has led to the conclusion that RecG is able to turn these structures into Holliday junction-like structures, and these are further processed and resolved by the RuvC or RuvAB complex (McGlynn & Lloyd, 2001; Gupta *et al.,* 2014; Mawer & Leach, 2014).

In addition, the *in vivo* function of RecG is important for the DSB processing and for preventing the over-replication of DNA, especially at the DSB and terminus region of the chromosome (Meddows *et al.,* 2004; Rudolph *et al.,* 2009, 2013; Azeroglu *et al.,* 2016). As these effects are PriA dependent, RecG is proposed to mediate the correct function of PriA through its ability to process 3'-flaps and D-loops, which mediate the correct binding of PriA and limits the re-replication events (Rudolph *et al.,* 2010, 2013; Azeroglu *et al.,* 2016).

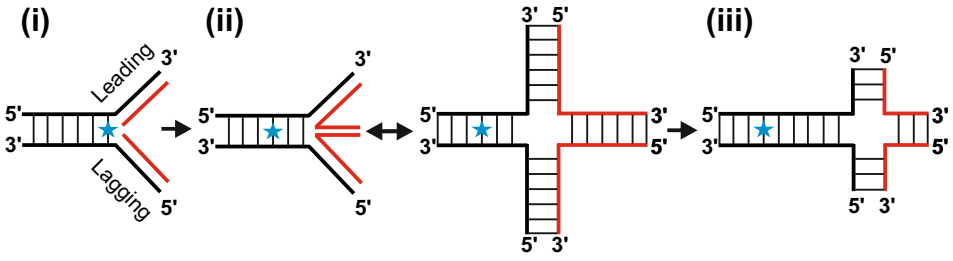

Figure 5. Fork regression and branch migration. A simplified schematic representation of how (I) the stalled replication fork substrate, (II) can be regressed and turned into the four-way (Holliday junction) structure, and then (III) further branch migrated. A star indicates the DNA damage location.

It is important to underline that branch migration ability is not limited to these helicases and is also widely present among other SF2 helicase families. For example, the RecQ family helicases, *E.coli* RecQ, *S. cerevisiae* Sgs1 and human RECQL1, BLM and WRN, among other functions, have been shown to bind with Holliday junctions and branch migrate (Harmon & Kowalczykowski, 1998; Constantinou *et al.,* 2000; Karow *et al.,* 2000; LeRoy *et al.,* 2005; Cejka & Kowalczykowski, 2010). In addition, the branch migration activity is present for the RIG-I-like family members; *S. cerevisiae* Mph1 and its human ortholog FANCM and Swi2/Snf2 helicase family members; yeast Rad5 and Rad54, human HLTF, ZRANB3 and RAD54 (Bugreev *et al.,* 2006; Blastyák *et al.,* 2007; Gari *et al.,* 2008; Zheng *et al.,* 2011; Halder *et al.,* 2022).

2.2.4.3 Mitochondrial DNA helicase Hmi1 and other UvrD-like family helicases

UvrD-like family helicases are SF1A helicases, and the family consists of helicases like UvrD, PcrA, Rep, Srs2 and RecB, which are involved with a wide range of different functions in replication, recombination, repair, and transcription (Fairman-Williams *et al.,* 2010). PcrA and Rep were one of the first helicases that had their crystal structures solved, contributing to the understanding of the substrate binding and the conformational changes that follow the ATP hydrolysis, leading to the proposal of the "inchworm" translocation mechanism (see chapter 2.2.3). The family is named after its most prototypic member, the *E. coli* UvrD helicase, which was first identified and named after the UV sensitivity of its mutant (Ogawa et al., 1968). Helicases in this family have quite multifarious functions, illustrated by the fact that the UvrD itself has shown to have a role in DNA replication, recombination and repair, participating in the NER and MMR (Kuemmerle & Masker, 1980; Lahue *et al.,* 1989; Washburn & Kushner, 1991; Brosh & Matson, 1995; Veaute *et al.,* 2005).

The mtDNA helicase Hmi1 also belongs to the UvrD-like family helicases, shares moderate homology with the other family members, and is the only family member shown to localize to the mitochondria (Sedman *et al.,* 2000; Fairman-

Williams *et al.,* 2010). Hmi1 protein is mostly the helicase core, with a short C-terminal part of the protein that also contains a unique and rare approximately 36 amino acid residues long C-terminal mitochondrial localization signal (Lee *et al.,* 1999; Sedman *et al.,* 2000). Hmi1 is a ssDNA-dependent helicase that uses primarily ATP energy and Mg^{2+} for its unwinding activity, and is thought to be a monomeric distributive DNA helicase, which can translocate through a duplex DNA for about 30 nucleotides (Sedman *et al.,* 2000; Kuusk *et al.,* 2005; Monroe *et al.,* 2005). Hmi1 efficiently unwinds duplex DNA with at least 19nt 3'-ssDNA overhang, different branched structures, such as the fork-like DNA substrates and flap structures with shorter 3' overhangs and shows little activity on the bubble and D-loop substrates with and without the ssDNA overhang (Kuusk *et al.,* 2005).

The *in vivo* studies have shown that the Hmi1 function is important for maintaining mtDNA in the *petite*-positive *S. cerevisiae*, where it is essential for maintaining the complete mitochondrial genome (Sedman *et al.,* 2000). However, *rho*$^{-}$ genome, coding a small tandemly repeated fragment of the original mtDNA that maintains transcription, can be maintained in the *hmi1Δ* background (Sedman *et al.,* 2000). In *petite*-negative *C. albicans*, the deletion of the *HMI1* gene leads to instability and lowered levels of mtDNA but is not essential for survival of the mitochondrial genome (Jõers *et al.,* 2007). Further studies with the *S. cerevisiae* *rho*$^{-}$ strains showed that the loss of Hmi1 leads to the accumulation of shorter concatemers, but this effect is suppressed in the strains lacking the transcription through the deletion of the mitochondrial RNA polymerase Rpo41 (Sedman *et al.,* 2005). Interestingly, abolishing the Hmi1 helicase and ATPase activity with a point mutation E211Q does not lead to the complete loss of Hmi1 function (Sedman *et al.,* 2005).

Based on the early studies, possible functions in replication and recombination were proposed for the Hmi1. The fact that Hmi1 is essential for wt mtDNA maintenance indicated that Hmi1 might participate in the replication (Sedman *et al.,* 2000). Indeed, other UvrD-like helicase family members, UvrD, PcrA, and Rep, are shown to be involved in replication processes (Lane & Denhardt, 1974; Scott *et al.,* 1977; Petit *et al.,* 1998; Bruand & Ehrlich, 2000). Both UvrD and Rep promote the replication in bacteria, but Rep directly interacts with the replisome, loading the replicative DNA helicase DnaB to the lagging strand in cooperation with PriC (Heller & Marians, 2005; Guy *et al.,* 2009). UvrD is likely to be more involved with promoting the replication through protein displacement, which is in accordance with the fact that UvrD, PcrA, and another family member *B. subtilis* HelD, have been shown to directly interact with RNA polymerase (Guy *et al.,* 2009; Gwynn *et al.,* 2013; Wiedermannová *et al.,* 2014). For UvrD and PcrA, roles in RNA polymerase backtracking and R-loop suppression have been proposed, as HelD is shown to remove the stalled RNA polymerase from the DNA (Epshtein *et al.,* 2014; Newing *et al.,* 2020; Urrutia-Irazabal *et al.,* 2021).

The branched substrate specificity and the slight accumulation of the branched molecules in *rho*$^{-}$ mtDNA, caused by the loss of Hmi1, have also indicated a possible role in recombination (Kuusk *et al.,* 2005; Sedman *et al.,* 2005). This

could indicate the functional similarities with the *S. cerevisiae* nuclear prototypic recombination protein Srs2. Srs2 localizes to the recombination centers, physically interacts with Rad51, and has the ability to remove the Rad51 nucleofilament from DNA in the helicase activity-dependent way (Krejci *et al.,* 2003; Veaute *et al.,* 2003; Krejci *et al.,* 2004; Burgess *et al.,* 2009). Nevertheless, Srs2 is also recruited to the replication fork by the sumoylated PCNA and this interaction regulates the recruitment of other replication factors in an ATP-independent manner (Papouli *et al.,* 2005; Pfander *et al.,* 2005; León Ortiz *et al.,* 2011). Functional homologs of Srs2 and UvrD-like family members, human HFBH1 and *S. pombe* Fbh1 have a similar function in the recombination, and they are shown to interact with PCNA (Fugger *et al.,* 2009; Bacquin *et al.,* 2013; Tsutsui *et al.,* 2014).

As mentioned above, one of the closest homologs of Hmi1, UvrD functions in different repair pathways, and Srs2 cooperates with ExoI nuclease to remove accidentally integrated ribonucleotides from the DNA (Potenski *et al.,* 2014). In addition, another UvrD-like helicase family member RecB is a part of the well-known RecBCD complex, which participates in DSB-induced HR-dependent repair (reviewed in Amundsen & Smith, 2023).

2.2.4.4 *S. cerevisiae* mitochondrial RNA helicases

In *S. cerevisiae*, three RNA helicases, Suv3, Mss116, and Mrh4 have been described that belong to the SF2 and are important for maintaining mtDNA (Séraphin *et al.,* 1989; Stepien *et al.,* 1992; Schmidt *et al.,* 2002; Fairman-Williams *et al.,* 2010). In yeast, Suv3 forms a mitochondrial RNA degradation complex with Dss1 (3'-5' ribonuclease), which controls the RNA turnover and is important for mRNA maturation surveillance (Margossian *et al.,* 1996; Dziembowski *et al.,* 2003; Łabędzka-Dmoch *et al.,* 2021). The complex activity depends on its structure and cooperation of the Suv3 and Dss1, which is especially important for the degradation of the secondary structured RNAs (Razew *et al.,* 2018). Deletion of the mitochondrial RNA degradation complex components in *S. cerevisiae* leads to altered transcript levels in the cells, affects translation, and results in respiration deficiency (Stepien *et al.,* 1992; Dmochowska *et al.,* 1995; Dziembowski *et al.,* 1998). However, this respiration deficiency can be rescued with a lower transcription level, indicating that the balanced transcript level is important for mtDNA maintenance (Rogowska *et al.,* 2006). In yeast, Suv3 is also proposed to directly influence mtDNA replication through interaction with the active *ori* (Guo *et al.,* 2011). Similar observations have been made for the human mitochondria, where SUV3 has been determined as a mitochondrial nucleoid protein and is also part of a human mitochondrial RNA degradation complex with human PNPase (polynucleotide phosphorylase) (Bogenhagen *et al.,* 2008; Szczesny *et al.,* 2010; Borowski *et al.,* 2013). In addition to its role in the surveillance of human mitochondrial RNA metabolism, the inactivation of mitochondrial RNA degradation complex also leads to the accumulation of R-loops, mainly at the replication

initiation, noncoding region of the mtDNA, causing mtDNA replication and maintenance defects (Silva *et al.,* 2018).

Mitochondrial RNA helicase Mss116, is shown to act as a transcription elongation factor that physically interacts with Rpo41 (Markov *et al.,* 2009, 2014). Its activity seems especially important under stress conditions for stabilizing the transcription elongation complex to maintain efficient transcription (Markov *et al.,* 2014). In addition, Mss116 also has a vital role in the post-transcriptional splicing of all the *S. cerevisiae* mtDNA introns as an ATP-dependent RNA chaperone (Séraphin *et al.,* 1989; Huang *et al.,* 2005). In *S. cerevisiae* mitochondria, mRNA translation is initiated through the interaction of specific translation activators with the 5' UTR of the mRNA (absent in mammalian mitochondrial transcripts) (reviewed in Kummer & Ban, 2021). Mss116 is shown to interact with the translation activator protein Pet309 and is important for ensuring the translation of the Cox1 protein (De Silva *et al.,* 2017). Altogether underlining the idea that one helicase can have a diverse role in maintaining the function of mitochondria and can affect different key processes in gene expression.

Furthermore, both DEAD-box family mitochondrial RNA helicases Mss116 and Mrh4 have shown to be involved in the mitochondrial ribosome large subunit assembly (De Silva *et al.,* 2013, 2017). Compared to bacterial ribosomes, mitochondrial ribosomes have a higher number of ribosomal proteins, and 5S rRNA is entirely substituted by them (Amunts *et al.,* 2014; Desai *et al.,* 2017). In *S. cerevisiae*, the mitochondrial ribosomes are assembled of 54S large subunits (21S rRNA and 39 proteins) and 37S small subunits (15S rRNA and 34 proteins) (Amunts *et al.,* 2014; Desai *et al.,* 2017). The RNA helicase Mrh4 participates in the mitoribosome biogenesis, specifically interacting with the 21S rRNA and participating in large subunit assembly (De Silva *et al.,* 2013). Helicase DDX28 has been proposed in human mitochondria as a possible ortholog of Mrh4. Although DDX28 does not complement Mrh4 *in vivo*, it has a similar function in human mitochondrial ribosome large subunit assembly (De Silva *et al.,* 2013; Tu & Barrientos, 2015).

3 AIMS OF THE STUDY

The mitochondrial proteins ensuring mtDNA maintenance have been studied in different model organisms to establish their specific functions. Not all these proteins are conserved throughout the eukaryotes, like the yeast mitochondrial helicases Irc3 and Hmi1. Determining the function of these yeast-specific proteins would help to understand the mechanism of mtDNA maintenance in yeast and establish the similarities and differences with other organisms. To determine the function of Irc3 and Hmi1, their substrate usage *in vitro* and their role in the mtDNA maintenance *in vivo* were assessed.

The experimental aims of this study were:

1. To study the function of Irc3 *in vivo* and its role in a branched DNA molecule metabolism.
2. *In silico* analysis of the *S. cerevisiae* Irc3 homologs.
3. To analyze the ATP hydrolysis dependent and independent role of Hmi1 in mtDNA maintenance, using the Hmi1 mutant strains with impaired ATP hydrolysis and ssDNA binding activity.

4 METHODS

Helicases are a large group of motor enzymes participating in a wide variety of cellular activities. Establishing their biochemical properties and studying the phenotypic effects of their loss or mutations *in vivo* helps specify their role in the cell. This thesis aimed to further study the function of mtDNA helicases Irc3 and Hmi1 and to describe the protein characteristics with different approaches (Ref I, II, III).

4.1 *In silico* methods used in this thesis

Discovering the putative helicases coded by the genome is simplified by their highly conserved helicase core containing the helicase motifs I–VI (see chapter 2.2.1 and 2.2.2). In addition, these conserved motifs are used to designate the helicases to different families, which often help to predict the possible substrate usage and function, based on the studies of the representative members of the family (Fairman-Williams *et al.,* 2010). As a part of this thesis, the evolutionary relationships and possible orthologous of Irc3 in a broader range of organisms were identified based on their conservation of the helicase core region (Ref I). In addition, the classical alignment studies of *S. cerevisiae* $Irc3_{sc}$ with Irc3-like proteins from other yeasts and with the orthologous proteins from different organisms were used (Ref I, II). The sequence alignment of the homologous proteins of Hmi1 from the UvrD-like helicase family was used to specify putative functional amino acid residues of Hmi1 to be tested (Ref III).

4.2 The construction of Hmi1 point mutants and their biochemical characterization

The helicase motifs contain conserved amino acid residues, which are important for binding the nucleic acid substrate or hydrolyzing the nucleotide triphosphate (see chapter 2.2.2). Although several motifs are important for one of the functions, the studies with UvrD-like family helicases have shown that motif III links the ssDNA and ATP hydrolysis/binding (Brosh & Matson, 1995, 1996; Korolev *et al.,* 1997; Dillingham *et al.,* 1999; Velankar *et al.,* 1999; Dillingham *et al.,* 2001; Lee & Yang, 2006). The role of several individual amino acid residues of motif III has been biochemically characterized for *E. coli* UvrD and *G. stearothermophilus* PcrA, some of these are also conserved in Hmi1 (Ref III, Fig. 1A). Since the motif III of Hmi1 seemed a good candidate region for mutating the conserved amino acid residues, which would lead to either lowering the ATPase or ssDNA binding activity, we created a set of single amino acid residue mutations, either predicted to be involved in the ATP binding/hydrolysis (D237N, Q240E, Q240N) or ssDNA binding (Y243A and F245A) (Ref III, Fig. 1A). In addition we mutated the serine in position 241, which is conserved, but to our knowledge it has not

been studied before in UvrD or PcrA, and included mutants K32M and E211Q of the motif I and II, previously constructed in our laboratory (Kuusk *et al.,* 2005; Sedman *et al.,* 2005). To assess if the mutants retain the ATPase and ssDNA binding activity *in vitro*, Hmi1 and its mutants were expressed and purified as a GST-Hmi1 fusion proteins using the Glutathione Sepharose matrix (Ref III; Kuusk *et al.,* 2005). The protein binding with ssDNA was determined by measuring the complex formation of Hmi1 and ssDNA by fluorescence anisotropy. The ATPase activity of helicases was analyzed using NADH oxidation coupled spectrophotometric assay.

4.3 Methods used for determining the *in vivo* function of Irc3 and Hmi1

The unique ability of *S. cerevisiae* to survive without mtDNA using fermentation, enables the study of the proteins, which in other model systems would lead to the loss of mtDNA and cell death. To clarify the function of Irc3 and Hmi1 in the cell, we evaluated the effects of their deletion or the mutations of the protein on yeast growth on a fermentable (glucose) and non-fermentable (glycerol) carbon source (Ref I, II, III).

Methods used for the assessment of the respiratory active cells

We assessed the mtDNA stability of mutant strains gained using different methods. For the complementation of the $Irc3_{sc}$ function by the bacterial RecG and $Irc3_{op}$, mitochondrially targeted RecG and $Irc3_{op}$ encoded by plasmid were introduced into *S. cerevisiae* W303 strain deleted for *irc3* (Ref I, II). The *recG* gene was cloned into the yeast centromeric plasmid pRS315, and the yeast CYC1 promoter and the mitochondrial signal of *S. cerevisiae CIT1* gene were added to ensure the expression in yeast and transport of the protein to the mitochondria, respectively.

The strains overexpressing Hmi1 in the wt background were obtained through the transformation of the wt W303α with either the plasmid encoding the wt or mutant Hmi1. The haploid Hmi1 strains with the mutation introduced into the chromosomal context were constructed using a diploid strain, where the mutations were introduced into one allele of the *HMI1* gene. In diploid strains, one wt *HMI1* gene allele copy is sufficient to maintain the mitochondrial genome; the mutant haploid strains were gained and assessed with the tetrad dissection.

The strains described above were assessed for mtDNA stability. The respiratory active cells were first selected by growing the strains in the non-fermentable medium containing glycerol and then released to the fermentable medium. The stability was either assessed over a 20h growth period in an exponential growth phase or in a stationary phase for a longer period. The proportion of respiratory active colonies was calculated either using the relative survival of the colonies on glycerol and glucose-containing medium (Ref I) or on the glycerol-containing medium, which contained low levels (0.1%) of glucose (Ref II, III). This low

glucose medium allows all the cells to grow into a colony, but only the cells able to use glycerol and, therefore are respiratory active, can form normal-sized colonies.

mtDNA maintenance proficiency was also tested with the 5-fluoroorotic acid (FOA) assay, with strains where the *HMI1* gene was deleted from the chromosome and only expressed from a plasmid carrying the wt *HMI1* gene. The mutant strains obtained by shuffling the wt *HMI1* gene-carrying plasmid with the mutant *hmi1* gene-carrying plasmid. Following 3–4 days of growth on medium containing glucose, the rise of respiratory defective cells according to the color of yeast colonies was assessed. The *S. cerevisiae* laboratory strain W303 has the advantage of forming red colonies in the presence of respiration caused by the *ade2* mutation. This allows for differentiation between the red colonies (respiratory active cells), white colonies (respiration incompetent mutant cells), or mixed colonies (containing a mixture of these cells) on the fermentable glucose-containing medium.

Treatment of mutant strains, with different DNA damaging agents can indicate the involvement of mutated gene products in different repair or recombination pathways. We tested the effect of methyl methanesulfonate (MMS), hydrogen peroxide (H_2O_2), and UV-C on the Hmi1 mutant strains. As a control, we included the w303α *sod2Δ* pRS315 strain, which in our assay and earlier studies was shown to be sensitive to the DNA damaging agents (Lee *et al.,* 2001; Kim *et al.,* 2005).

Methods used for the assessment of the mtDNA stability and gene expression

The impact of different deletions and mutations on the mtDNA was further assessed using different methods. The *rho*$^{-}$ mutant strain, a11, which contains approximately a 1.8 kb tandem-repeated mtDNA fragment containing the active *ori3* sequence, enabled us to study the mtDNA topology changes of the strain lacking $Irc3_{sc}$ and the genetic interaction with mitochondrial resolvase Cce1 using the 2D-NAGE (Ref I). The mtDNA copy number was determined using quantitative PCR and Southern blot methods, and the morphology and topology changes in mtDNA were visualized using a fluorescence microscope and different agarose gel-based methods (Ref I, III). Hmi1 and its mutants' effect on transcription was studied using reverse transcription PCR (RT-PCR), and gene expression was analyzed with Western Blot for the strains grown under the non-fermentative conditions where protein synthesis is present and the rise of *rho*$^{-}$ cells is limited (Ref III).

Specific materials used and the exact experimental conditions are more thoroughly elaborated in the Material and Methods section of the particular articles (Ref I, II, and III). Experimental data acquired using these methods and their meaning in the broader cellular context are further discussed in the following chapters.

5 RESULTS AND DISCUSSION

5.1 *S. cerevisiae* Irc3 participates in the metabolism of branched mtDNA molecules (Ref I)

Helicases are divided into families based on their conservation in amino acid sequences. Irc3 has a distinctive SAT sequence in motif III characteristic of SF2 helicases, but its classification into the exact helicase family within SF2 has remained elusive (Sedman *et al.,* 2014, Ref I). Earlier homology analysis has assigned *S. cerevisiae* Irc3 ($Irc3_{sc}$) as a putative RNA helicase to the DEAD-box helicase family based on the conservation of the helicase core (de la Cruz *et al.,* 1999). However, the biochemical studies revealed that the ATPase activity of $Irc3_{sc}$ is stimulated by DNA (Sedman *et al.,* 2014). The experimental data indicated that $Irc3_{sc}$ has an important role in mtDNA maintenance, and the absence of $Irc3_{sc}$ leads to the accumulation of dsDNA breaks and the formation of the *rho*$^-$ yeast mutants (Sedman *et al.,* 2014).

To further clarify the *in vivo* role of $Irc3_{sc}$ in mtDNA maintenance, we studied the effects of $Irc3_{sc}$ loss on mtDNA replication and repair intermediates using 2D-NAGE (Ref I). Direct studies of the *S. cerevisiae* mtDNA with 2D-NAGE have so far been mostly conducted with the *rho*$^-$ strains due to the compact mtDNA size and high detection specificity. The analysis of the restriction enzyme digested, a11 1.8 kb, mtDNA detected different replication and topology intermediates, such as regular branched molecules like the Y-shaped replication fork and the X-shaped Holliday junction, as well as larger irregular branched structures (partially single-stranded), which move as a cloud on the upper part of the gel (Ref I, Fig. 1A, B). The analysis of mtDNA showed that compared to the strain expressing *IRC3* gene, the *irc3Δ* strain has a substantially lower amount of the regular Y- and X-shaped molecules and the amount of irregular branched molecules is increased. In *S. cerevisiae*, these regular branched molecules can be resolved by the mitochondrial resolvase Cce1, and their accumulation in the absence of Cce1 is clearly visible on the 2D-NAGE (Kupfer & Kemper, 1996; Ref I, Fig. 1C, D). The *Δirc3*/*Δcce1* double mutant strain also accumulates these molecules, but to a lesser extent, which indicates that $Irc3_{sc}$ is most likely involved in the immediate processing of these molecules (e.g., fork reversal and branch migration) rather than their resolution (Ref I, Fig. 1C, D). In addition, the mtDNA copy-number analysis for the same strains indicated that the deletion of $IRC3_{sc}$ leads to a noticeable loss of mtDNA, which further indicates direct participation of $Irc3_{sc}$ in the mtDNA maintenance (Ref I, Sup. Fig. 4). As mentioned above, previous *in vivo* studies have shown that the loss of $Irc3_{sc}$ leads to the accumulation of the DSBs (Sedman *et al.,* 2014). It is plausible that the processing of the branched molecules by $Irc3_{sc}$ is important to avoid the accumulation of DSBs and could indicate the role of $Irc3_{sc}$ in HR. In the strains lacking Irc3, the amount of regular replication intermediates seems to decrease instead of accumulating, which could indicate that $Irc3_{sc}$ functions upstream of Cce1, creating the molecules resolved by Cce1.

The observation that $Irc3_{sc}$ affects the formation of branched mtDNA molecules was further confirmed by *in vitro* studies, which indicated that $Irc3_{sc}$ has an affinity for and can unwind the branched Y- and X-shaped molecules (see chapter 5.3). Similar substrate specificity has been described for the bacterial RecG and its functional analogs, the T4 bacteriophage UvsW, and mammalian SMARCAL1 (see chapter 2.2.4.2). In addition, the RecG homolog in plants was described to function in the mitochondria and chloroplast, being important for recombination-dependent repair and suppressing unwanted recombination events (Odahara *et al.,* 2015; Wallet *et al.,* 2015). The plant RECG was shown to complement the bacterial RecG, indicating the functional similarities across the species (Odahara *et al.,* 2015; Wallet *et al.,* 2015).

For this reason, we decided to test if the *E. coli* RecG can complement $Irc3_{sc}$ *in vivo*. The deletion of the *IRC3* gene leads to the rapid loss of mtDNA and respiration, which can be detected through the inability to grow on non-fermentable media. We decided to test if the bacterial *recG* gene can improve the respiration activity of the *irc3Δ* strain cells. The strain w303a *irc3Δ* was transformed with either empty pRS315 (negative control) or pRS315 expressing *IRC3* or *E. coli* r*ecG*. First, the ability of RecG to improve the growth rate of the *irc3Δ* strain in glycerol was tested. We indeed observed a significant increase in the growth rate of the strain containing RecG compared to the strain lacking both $Irc3_{sc}$ and RecG (Ref I, Fig. 5B). Next, the ability of the same strains to maintain its mtDNA in fermentative conditions was tested. RecG was partially able to recover the fraction of the respiratory competent cells compared to the *irc3Δ* strain, as during 20 hours of exponential growth in the fermentable medium, the proportion of the respiratory active cells of the strain lacking the $Irc3_{sc}$ and RecG dropped from 2.7% to 0.1% and for the strain expressing RecG from 23.6% to 11% (Ref I, Fig. 5C–E). Similar results were also confirmed with the stationary growth over the course of 168 hours in glucose-containing medium, as RecG was able to complement the $Irc3_{sc}$ function compared to the strain lacking $Irc3_{sc}$ (Ref I, Fig. 5C–E; Ref II, Fig. 1C). The w303 *irc3Δ* strain mtDNA exhibits fragmentation and accumulation of smaller mtDNA fragments, but the expression of a plasmid carrying the wt $Irc3_{sc}$ or RecG leads to the 5-fold decrease in the amount of small mtDNA fragments (0.2–0.5 kb) (Ref I, Fig. 5F, G). RecG seems to be able to retain mtDNA integrity similarly to the $Irc3_{sc}$, and it is feasible that their complementation results from the Irc3 function in mtDNA metabolism.

Since $Irc3_{sc}$ function was partially complemented by the RecG protein *in vivo*, we decided to conduct the phylogenesis study with a representative set of proteins with similar activities in *S. cerevisiae*, *E. coli*, *H. sapiens*, the RecG functional analog UvsW from the bacteriophage T4, and the class I restriction enzymes as defined by the preliminary studies, to have a similarity with the $Irc3_{sc}$ (Ref I). Based on the alignment of the helicase core sequences, $Irc3_{sc}$ seems to be evolutionarily closest with the bacteriophage T4 branch migration enzyme UvsW (Ref I, Sup. Fig. 1). A direct comparison of the sequences showed that $Irc3_{sc}$ is 22,92% identical to UvsW and 20,16% to RecG (UvsW and RecG have an identity % 18.67) (The UniProt Consortium, 2023). Similarity with the

bacteriophage T4 helicase UvsW is not surprising, as different mitochondrial proteins have been proposed to have an evolutionary bacteriophage origin, indicating possible similarities in their DNA replication and transcription systems (Shutt & Gray, 2006). Recently, a putative human RNA helicase was proposed as a possible $Irc3_{sc}$ ortholog in mammals (GenBank accession no. BAG50877) (Kaur & Datta, 2021). This putative RNA helicase sequence is identical to the C-terminal part of the human DDX52 helicase (413 aa out of 599 aa of human DDX52), indicating it probably represents a DDX52 partial fragment (or alternative transcript of DDX52). Human DDX52, however, has a proposed ortholog in *S. cerevisiae*, the nuclear RNA helicase Rox1, which has a 42.02% identity with human DDX52, as the sequence identity of $Irc3_{sc}$ is only 26.43% (The UniProt Consortium, 2023).

5.2 Irc3-like helicases are conserved in different yeast species (Ref II)

Although Irc3-like helicases are not found in other eukaryotes, they are conserved in the fungi kingdom (Ref II). Several Irc3-like protein sequences were identified among ascomycete and basidiomycete fungi using the BLAST analysis. The representative alignment (Ref II, Fig. 1A) showed that for the $Irc3_{sc}$ and other putative Irc3-like helicases, the helicase core is located in the N-terminal part of the protein, and the putative mitochondrial signal is present in all these proteins, indicating a possible conserved mitochondrial function of this protein. In addition, the Irc3-like helicases have an extra C-terminal region, which is variable in length and less conserved than the N-terminal helicase core (Ref II, Fig. 1A). The structure predictions using AlphaFold have led to the proposal that the C-terminal region of the Irc3-like helicases could structurally resemble the C-terminal domain of the Ski2-like subfamily helicases, called the first domain (Piljukov, 2023). Functional studies with the $Irc3_{sc}$ mutants have shown that the C-terminal part of the protein is most likely involved in DNA substrate binding (Sedman *et al.,* 2017).

Our *in vitro* studies with *S. cerevisiae* Irc3 had technical limitations caused by the thermal instability of the protein. To overcome these limitations and to study the functional conservation of Irc3 in different yeast species, the Irc3 protein was purified from the thermostable yeast *O. polymorpha* ($Irc3_{op}$). Protein thermostability has been associated with shorter sequence lengths and smaller amounts of aspartic acid residues (Leuenberger *et al.,* 2017). Indeed, $Irc3_{op}$ protein is smaller compared to the $Irc3_{sc}$ ($Irc3_{sc}$ 689 and $Irc3_{op}$ 622 amino acid residues), contains a smaller number of aspartic acid residues ($Irc3_{sc}$ 48 and $Irc3_{op}$ 34 amino acid residues) and is enriched in prolines ($Irc3_{sc}$ 15 and $Irc3_{op}$ 21 amino acid residues) (Ref II, Fig. 1A, Sup. Fig. 1). As expected, the ATPase activity profile on different temperatures and the circular dichroism analysis of the purified $Irc3_{op}$ confirmed that the protein is stable at elevated temperatures and has an optimal ATP hydrolysis activity at 41 °C (Ref II, Fig. 2). We also confirmed that $Irc3_{op}$ can complement the mitochondrial function of $Irc3_{sc}$. Following the 168h of

growth in a glucose-containing medium, an *S. cerevisiae* strain expressing $Irc3_{op}$ encoded by a plasmid in the *irc3Δ* background retained approximately 60% of the respiratory-active cells compared to the strain expressing $Irc3_{sc}$ encoded by plasmid (Ref II, Fig. 1C). In comparison, only 5% of the respiratory active cells were retained for the strain completely lacking Irc3. These results indicate that $Irc3_{op}$ and $Irc3_{sc}$ are functional homologs, but there might also be some functional diversity between them.

5.3 The possible role of Irc3 in yeast mitochondria

For both $Irc3_{sc}$ and $Irc3_{op}$, it has been shown that they need 13–21 base pair to efficiently bind with DNA (Sedman *et al.,* 2017; Ref II, Fig. 4A). The k_{cat} of ATP hydrolysis (ATPase activity) positively correlates with the dsDNA cofactor length (21–75 base pairs) and the K_{DNA} values only change 2–3 fold (Sedman *et al.,* 2017; Ref II, Fig. 4). This dependence is thought to be characteristic of processive translocase enzymes, indicating that both Irc3 proteins could translocate along the DNA lattice (Young *et al.,* 1994; Fischer *et al.,* 2007; Fishburn *et al.,* 2015). Further studies with the $Irc3_{sc}$ have confirmed that the protein is a monomer, able to translocate along the DNA in an ATP-dependent manner and displace an oligonucleotide from the triple-stranded DNA structures (Sedman *et al.,* 2017; Piljukov *et al.,* 2020). For this activity, the N-terminal helicase core and the C-terminal part of the protein are both necessary (Sedman *et al.,* 2017).

Biochemical characterization of the $Irc3_{sc}$ and $Irc3_{op}$ has shown that both proteins hydrolyze ATP in the presence of dsDNA (Sedman *et al.,* 2014; Ref II, Fig. 3). However, both Irc3 proteins bind with higher affinity to the branched DNA substrates that mimic the fork-like structures (Y-shaped) (Fig. 6B). The ATPase activity of $Irc3_{op}$ is efficiently stimulated by the fork-like DNA substrate FK50DNA ($k_{cat} = 43 \pm 1.6\ s^{-1}$) which is 3-times higher compared to the regular 50 nucleotide long double-stranded substrate DS50DNA ($k_{cat} = 16 \pm 0.3\ s^{-1}$) (Ref II, Fig. 5A). Moreover, while the fork-like substrate increases the ATPase activity of the $Irc3_{sc}$, it is further increased in the presence of the four-way junctions (X-shaped) that mimic the Holliday junction structures (Fig. 6A), especially with a cofactor, which has homologous branches and permits the branch migration (Ref I, Fig. 2C).

Furthermore, it was shown that $Irc3_{sc}$ has a helicase activity, which can unwind the replication fork-like structures (Ref I, Fig. 3C–F). Additional studies with the $Irc3_{sc}$ have revealed that the preferred fork-like substrate is the fork with the nascent lagging strand, lacking the nascent leading strand (Fig. 6B), which is unwound approximately 10-times more efficiently than the fork with the nascent leading strand (Piljukov *et al.,* 2020). In addition, the fork-like substrates with double-stranded arms were tested. $Irc3_{sc}$ unwounded more efficiently and faster the substrate that has homologous arms and can undergo fork regression, than the substrate with non-homologous arms, unable to do so (Ref I, Fig. 4 A, B, E). Similar preference was observed when comparing the unwinding of the Holliday

junction-like substrates, as $Irc3_{sc}$ unwinds more efficiently and faster the substrate with homologous arms and is therefore able to branch migrate compared to the immobile structure (Ref I, Fig. 4C, D, F). This indicates that $Irc3_{sc}$ might also be able to promote fork regression and branch migration *in vivo*.

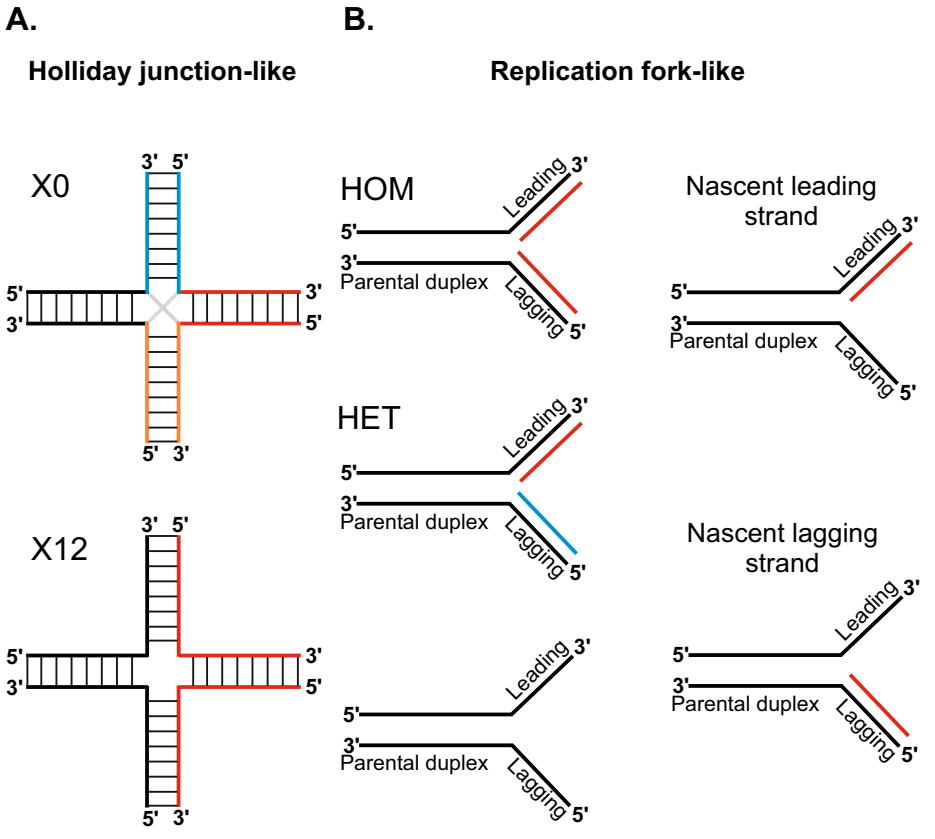

Figure 6. Different branched substrates used for the study of $Irc3_{sc}$. For the branched substrates (Ref I), the colors of the strands indicate the strands that are homologous and able to anneal. **A)** Holliday junction-like structures with a non-movable core (X0) and a movable core (X12). For the X0 substrate, the branch migration cannot occur because all four strands are non-homologous. **B)** Different replication fork-like substrates. HOM-fork with newly synthesized homologous strands that can be used for fork regression. HET-fork with newly synthesized non-homologous strands that cannot be subjected to fork regression. Replication fork-like substrates with only one newly synthesized (nascent) leading or lagging strand are shown.

Many prominent helicase families, which include members with branch migration and fork regression activity, like the RecQ, RecG, and SWI/SNF family, have extra N- or C-terminal domains conserved within the family (see chapter 2.2.2). These additional domains have been shown to be important for the binding

of different branched structures to provide their recognition and unwinding. The Irc3-like helicases also have the conserved C-terminal extra region, which could possibly have a similar role (Ref II, Fig. 1A). For the $Irc3_{sc}$, the deletion of 11 amino acid residues from the C-terminus led to the rapid loss of the respiration competent colonies (Sedman *et al.,* 2017). In addition, the Irc3 mutant Irc3-ΔC (aa 29-482), which only has the N-terminal helicase core, is unable to form a complex with the Holliday junction-like substrate, and its ATPase activity is stimulated to similar extent both by regular dsDNA and branched DNA, in contrast to the wt $Irc3_{sc}$, which is more efficiently stimulated by the branched substrate (Sedman *et al.,* 2017). These results indicate that the C-terminal domain of $Irc3_{sc}$ and its ability to bind specific branched mtDNA structures is crucial for $Irc3_{sc}$ function in mtDNA maintenance. It is conceivable that the C-terminal parts of the $Irc3_{sc}$ orthologs in other yeast species have a similar function.

The DNase I protection studies determined that $Irc3_{sc}$ prefers to unwind fork-like substrates that lack the nascent leading strand, and the protein preferably binds with either the lagging strand or parental duplex in close vicinity to the branch point (Piljukov *et al.,* 2020). These results underline the similarities to RecG, which also binds to the branch point and has been shown to be active on the fork-like substrates, with the highest affinity towards the fork with the nascent lagging strand (McGlynn & Lloyd, 2001; Singleton *et al.,* 2001; Wahab *et al.,* 2013). It is important to mention that for the RecG, the activity on these substrates is enhanced by the presence of a single-stranded binding protein (Wahab *et al.,* 2013), an effect that should also be tested for the Irc3-like proteins in the future. RecG and Irc3 both also seem to have at least some overlap in their *in vivo* functions, as RecG can partially complement $Irc3_{sc,}$ and they both are indicated to be involved in the DSB metabolism (see chapters 2.2.4.2 and 5.1). In bacterial systems, RecG is thought to function in the initial response to stalled replication forks and to mainly modify the fork-like structures by creating the Holliday junction-like structures, which are cleaved by RuvC or further processed by the RuvAB complex (Wahab *et al.,* 2013; Gupta *et al.,* 2014; Mawer & Leach, 2014). Our studies indicate that $Irc3_{sc}$ might have a similar role in remodeling these replication fork-like and Holliday junction-like structures in the cell, enabling them to be resolved by Cce1 and, thereby reducing the risk of accumulating DSB, which could lead to the instability and loss of mtDNA in the cell. The branch migration activity of Irc3 might be necessary for migrating the branch to the specific sequence suitable for the Cce1 cleaving (Schofield *et al.,* 1998). However, the studies with the double mutant strain lacking both Irc3 and Cce1 showed the presence of branched mtDNA molecules, indicating that $Irc3_{sc}$ might not be the only protein capable of processing branched mtDNA molecules in mitochondria (see chapter 5.1). Lately, another branch migration helicase in *S. cerevisiae* Mph1 has been shown to locate to the mitochondria, which indicates that other proteins might help to compensate for this function in the absence of $Irc3_{sc}$ (Zheng *et al.,* 2011; Bernal *et al.,* 2019).

It is conceivable to speculate that in addition to the possible role in replication and recombination through branch migration and unwinding replication fork-like

structures, Irc3 might have an additional function in the cell. The branch migration enzyme RecG and its functional analogs, UvsW and SMARCAL are also shown to resolve R-loops and to prevent their accumulation to maintain DNA stability (see chapter 2.2.4.2). Our study with the $Irc3_{op}$ raises new possibilities about the activity of Irc3-like helicases on RNA substrates. The early studies with the $Irc3_{sc}$ showed no ATPase activity with the whole mitochondrial RNA extract purified from the *S. cerevisiae* mitochondria or with the exogenous RNA substrates (Sedman, *et al.,* 2017). In contrast, the ATPase assays of $Irc3_{op}$ in the presence of synthetic RNA cofactors showed that RNA can stimulate the ATPase activity of $Irc3_{op}$ (Ref II, Fig. 5B). However, the ATPase activity induced by the 50 nucleotide dsRNA substrate DS50RNA, which had the highest stimulatory effect (k_{cat} = 3.9 ± 0.3 s^{-1}), was still more than 4-times lower compared to the identical dsDNA substrate DS50DNA (k_{cat} = 16 ± 0.3 s^{-1}) and approximately 11-times lower compared to the most active fork-like DNA substrate FK50DNA (k_{cat} = 43 ± 1.6 s^{-1}) (Ref II, Fig. 5B). In addition, the endogenous rRNA and tRNA were purified from the *O. polymorpha* to determine if Irc3 might need the specific structured RNA for an optimal ATP hydrolysis stimulation. Indeed, the native RNAs, such as rRNA (k_{cat} = 12.3 ± 0.4 s^{-1}) and tRNA (k_{cat} = 12.3 ± 0.6 s^{-1}), stimulated $Irc3_{op}$ ATPase activity approximately 3-times better than the dsRNA substrate but are still lower than the activities observed for dsDNA and fork-like DNA substrates (Ref II, Fig. 5C and Table 1). Another difference between the $Irc3_{sc}$ and $Irc3_{op}$ seems to be the weak stimulation of ATPase activity by the ssDNA (Ref II, Fig. 5A and Table 1). Fluorescence anisotropy analysis of the DNA-$Irc3_{op}$ complex formation revealed that although ssDNA stimulates the ATP hydrolysis poorly, the protein binds ssDNA more tightly (K_d approximately 20nM) than dsDNA (K_d approximately 40nM) (Ref II, Fig. 6A). In addition, the competition study with ssDNA and forked DNA revealed that ssDNA efficiently competes with fork-like DNA for binding with $Irc3_{op}$ and acts as an inhibitor for ATP hydrolysis activity (Ref II, Fig. 6D). This might indicate the role of ssDNA in regulating the Irc3-like helicases' cellular activity. One possible option is that the differences in the stimulation of ATPase activity between $Irc3_{sc}$ and $Irc3_{op}$ and the fact that $Irc3_{sc}$ showed no stimulation by ssDNA and RNA may have resulted from the use of different methods for detecting the stimulation of ATPase activity of these proteins. Since the ATPase activity measured for $Irc3_{op}$ with NADH oxidation coupled spectrophotometric assay enabled us to detect lower ATP hydrolysis activities than the assay using charcoal conducted with $Irc3_{sc}$, it remains to be clarified in the future whether, $Irc3_{sc}$ also exhibits low-level activities with ssDNA or RNA substrates. Further studies could determine if Irc3-like helicases have a similar role with RecG and its functional analogs in the R-loop metabolism, or if the ATPase activation by structured RNA is conserved and indicates a role in gene expression. Indeed, $Irc3_{sc}$ has shown to have a genetic interaction with Rpo41, as the mutant strain lacking both proteins preserves the replication intermediates, that are lost in the strain lacking only $Irc3_{sc}$, however the overall loss of mtDNA is higher (Sedman *et al.,* 2014). In addition, a study by Kaur and Datta, 2021 has suggested that $Irc3_{sc}$ might also have an impact on tran-

scription and translation processes. This study indicated that the deletion of *IRC3*$_{sc}$ leads to reduced transcription of the *COX1*, *ATP8*, and *ATP6* polycistronic transcript and to an overall reduction of translation under the fermentative conditions. Irc3$_{sc}$ cofractionated with the mitochondrial small ribosomal subunits, and gene expression studies indicated that Irc3$_{sc}$ could be involved in the translation elongation process (Kaur & Datta, 2021). These results indicate that Irc3-like helicases may have a broader role in overall mitochondrial metabolism, and further studies could help us understand how these different functions are linked in the mitochondria.

5.4 The ATP-independent function of Hmi1 (Ref III)

Based on the amino acid residues sequence alignment studies, Hmi1 is an SF1 helicase and belongs to the UvrD-like helicase family and it is most homologous to *G. stearothermophilus* PcrA, *S. pombe* Fbh1, *E. coli* UvrD and *S. cerevisiae* Srs2 (Fairman-Williams *et al.,* 2010; The UniProt Consortium, 2023). Similarly to the other UvrD-like family members, Hmi1 is a ssDNA-dependent, 3'-5' directional helicase (Kuusk *et al.,* 2005; Singleton *et al.,* 2007). There are two *S. cerevisiae* helicases appointed to this family- Srs2, which has a multifarious role in the maintenance of nuclear DNA (see chapter 2.2.4.3), and Hmi1, which functions in the mitochondria (Sedman *et al.,* 2000). Hmi1 orthologs have so far not been described in the higher eukaryotes but are annotated in other yeast species, and to some extent studied in *C. albicans* (Richard *et al.,* 2005; Jõers *et al.,* 2007).

Hmi1 is a 3'-ssDNA-dependent helicase, which means that, similarly to the other DNA helicases, Hmi1 binds to the substrate ssDNA and ATP simultaneously (Kuusk *et al.,* 2005). The ability to bind substrates like ATP and DNA is mediated through specific structural elements and is necessary for the proper functioning of the helicase. Disruption of the *HMI1* gene leads to the loss of respiratory active cells (Sedman *et al.,* 2000). However, Hmi1, does not completely lose its mitochondrial function when the ATP hydrolysis activity needed to unwind the double-stranded DNA is severely reduced by the mutation E211Q (Sedman *et al.,* 2005). This indicates that Hmi1 might also have ATP-independent functions, which could be related to substrate DNA (ssDNA) binding.

5.4.1 Hmi1 motif III links the ATP hydrolysis and ssDNA binding ability (Ref III)

As described above (chapter 2.2.2 and 4.2) helicase motif III has shown to have amino acid residues important for both ATP hydrolysis and ssDNA binding activity and the amino acid residues in motif III, are quite conserved for UvrD-like family helicases (Ref III, Fig. 1A). To study the ATP- and ssDNA-dependent function individually, we constructed, in addition to the previously characterized motif I and II mutants K32M and E211Q, the possible separation-of-function

mutants of motif III. The mutant strains D237N, Q240E, Q240N are potentially involved in the ATP binding/hydrolysis, Y243A and F245A potentially involved in ssDNA binding, and in addition S241A (see chapter 4.2 and Ref III, Fig. 1A). The wt and mutant proteins constructed were expressed in the bacterial system and purified as GST-Hmi1 fusion proteins (see chapter 4.2) and their ATPase and ssDNA activities were determined (Ref III, Table 1 and Sup. Fig. 4).

The recombinant GST-Hmi1 mutants Y243A and F245A, predicted to affect ssDNA binding, were stable and purified similarly to the protein with the single amino acid residue substitution S241A and the wt protein (Ref III, Sup. Fig. 3). We characterized their ATPase and ssDNA binding activities and determined that F245A indeed displays the ATPase activity comparable to the wild type but has approximately 5-fold decreased ssDNA binding ability (Ref III, Table 1 and Sup. Fig. 4). A similar effect is observed with PcrA, where the aromatic tryptophan substitution W259A in the equivalent position also shows severe defect of ssDNA binding, but retains the ATP hydrolysis activity (Dillingham *et al.,* 1999). Interestingly, for Srs2, the equivalent mutation, F285A, has different biochemical characteristics, as the mutation leads to decreased ATP hydrolysis activity (51% of the wt levels) and only a 1.4-fold reduction of ssDNA binding (Meir *et al.,* 2023). The ATPase activity of Y243A of Hmi1 is less than 5% compared to the wild type and the affinity for ssDNA is decreased approximately 3-fold (Ref III, Table 1 and Sup. Fig. 4). The equivalent of the Y243A mutant for the PcrA protein, Y257A, shows similar biochemical properties, a slight decrease of ssDNA binding and noticeably (7-fold) lower ATPase activity compared to the wt protein (Dillingham *et al.,* 2001). However, the equivalent mutation in Srs2 Y283A has only a slight effect on ssDNA binding and ATPase activity (Meir *et al.,* 2023). These results indicate that the biochemical characteristics of these ssDNA binding amino acid residues of Hmi1 are more like the PcrA than the Srs2 protein, possibly indicating a more similar functional role in the cell. In the crystal structures of PcrA, UvrD, and Rep, both of these aromatic amino acid residues (tyrosine and tryptophan) are located in the ssDNA binding channel (in positions 257 and 259 for PcrA, 254 and 256 for UvrD and 248 and 250 for Rep protein) (Korolev *et al.,* 1997; Velankar *et al.,* 1999; Lee & Yang, 2006). Structural studies further indicate that in this channel, these amino acid residues stack with the ssDNA nucleobases to stabilize and allow directional movement on the substrate DNA.

The recombinant protein of GST-Hmi1-S241A mutant retained the wt ATP hydrolysis/binding and ssDNA binding activity (Ref III, Table 1, and Sup. Fig. 4). This underlines the fact that not the whole motif III, but specific conserved amino acid residues in this motif, bind directly with the substrate and are essential for the function of Hmi1. Unfortunately, the mutants of motif III amino acid residues Q240 and D237 and motif I K32M, which are predicted to be involved in the ATP binding, demonstrated noticeable fragmentation in the recombinant protein expression system that hampered their biochemical characterization. Based on the crystal structures of UvrD, PcrA, and Rep, the lysine (K) conserved in motif I is directly binding the β-phosphate and glutamine (Q) in

motif III positions near the γ-phosphate (Korolev *et al.,* 1997; Velankar *et al.,* 1999; Lee & Yang, 2006). The glutamine (Q) directly contacts the γ-phosphate in the PcrA crystal structure, but in the UvrD crystal structure, it is proposed to position the water molecule for the nucleophilic attack (Velankar *et al.,* 1999; Lee & Yang, 2006). Although we were not able to determine the *in vitro* effect of the mutations of Q240E and Q240N on the Hmi1 protein, the equivalent mutations in the homologous *G. stearothermophilus* PcrA are shown to lead to the decreased ATPase activity, but can bind ssDNA comparable to the wt protein (Dillingham *et al.,* 1999). The D237N equivalent mutant in the UvrD protein, D248N, leads to reduced binding of ssDNA in the absence of ATP and vice versa but it can efficiently form a ternary complex (Brosh & Matson, 1996). Based on this result, it has been proposed, that aspartic acid is important for stabilizing the substrate complexes and is needed for efficient helicase activity on long duplexes (Brosh & Matson, 1996). For the UvrD protein, the equivalent mutant of K32M, K35M, has been shown to lack helicase activity and to have a severely decreased ATPase activity (George *et al.,* 1994).

In conclusion, we were not able to purify and test any of the mutants of motif III, which would only affect ATP hydrolysis and not ssDNA binding, and for this reason, the E211Q mutant of motif II was chosen as a representative impaired ATPase mutant (Ref III). Based on the UvrD crystal structure, glutamic acid binds a Mg^{2+} ion and could participate in a nucleophilic attack of the water molecule during ATP hydrolysis (Lee & Yang, 2006). In the previous and current study, the mutant E211Q led to the disruption of ATP hydrolysis but preserved the DNA binding ability (Ref III, Table 1, and Sup. Fig. 4; Kuusk *et al.,* 2005). This is consistent with the biochemical characterization of the equivalent mutation in UvrD (E221Q) and PcrA (E224A), both of which have severely reduced ATPase activity (0.2% and 1.4% compared to the wild type, respectively) (Brosh & Matson, 1995; Soultanas *et al.,* 1999). To check the co-dependency of the ATPase and ssDNA binding activity, we also constructed a double mutant E211Q/F245A, which, as expected, resulted in severely decreased ATPase activity and ssDNA binding (Ref III, Table 1, and Sup. Fig. 4).

5.4.2 The effect of Hmi1 mutations on the maintenance of respiration (Ref III)

Previous studies by our group showed that the deletion of the Hmi1 protein leads to the rapid loss of functional mitochondrial genome and the rise of *rho*$^{-}$ and *rho*^{0} mutant cells (Sedman *et al.,* 2000). However, the inactivation of the ATPase activity through the E211Q or K32M mutations did not lead to a complete loss-of-function phenotype (Sedman *et al.,* 2005). Therefore, we determined whether the mutants, with abolished ATPase activity and ssDNA binding affinity, have similar effects on respiration. We assessed mtDNA stability through the fraction of the respiratory active colonies and yeast growth on non-fermentable media in three different ways: in FOA assay (1), where the wt *HMI1* or a gene carrying a

point-mutation is expressed only from the plasmid (*hmi1Δ* background); tetrad dissection (2) and mtDNA stability assay (3), where the mutation is inserted into the native chromosomal context (Ref III and chapter 4.3).

In all three assays (see table 1), the mutants S241A and Q240N are quite similar to the wt, indicating that these mutants have the least effect on the functioning of the Hmi1 *in vivo*. The mutants D237N and Q240E exhibited a slight loss of respiration activity compared to the wt strain in FOA assay, but the colonies grown from the spores with mutant *hmi1* alleles displayed a mixture of respiring and non-respiring cells, indicating a gradual loss of functional mtDNA (Ref III, Sup. Fig. 1). Although they maintained respiratory active cells comparable to the wt strain during growth in the glycerol-containing medium, they progressively lost their respiration activity in the glucose-containing medium after 20h exponential growth (Ref III, Fig. 1C). This indicates that over time, the ability to support respiration diminishes for these mutants in the fermentable medium. The fact that Q240E has a more substantial impact on the respiration activity is not surprising, as for the equivalent mutation in the PcrA helicase, the substitution of glutamine with the negatively charged glutamic acids, has a more severe effect on the helicase activity (Dillingham *et al.,* 1999).

Table 1. The summary of the average results obtained from different *in vivo* experiments*

Strain	(1) FOA assay (% of red colonies)	(2) Tetrad dissection	(3) Glycerol 0h (%)	(3) Glucose after 20h (%)	Protein expression *in vivo* (%)
wt	37.5 (±5.1)	+	90 (±6.4)	94.7 (±7.3)	100%
K32M	31.8 (±4.4)	+/–	73.4 (±3.9)	13.7 (±6.9)	45.9 (±7.7)
E211Q	23.4 (±3.4)	+/–	69.2 (±7.7)	34.1 (±7.6)	79.9 (±18.9)
D237N	30.7 (±7.9)	+/–	76.1 (±20.5)	48.3 (±10.3)	67.8 (±10.2)
Q240E	27.4 (±3.8)	+/–	73.0 (±11.0)	55.3 (±5.4)	66 (±21.3)
Q240N	35.1 (±9.0)	+	88.4 (±6.7)	73.5 (±16.8)	51.7 (±5.5)
S241A	35.5 (±4.7)	+	95.3 (±2.3)	95.3 (±2.7)	61.5 (±6.5)
Y243A	4.6 (±0.2)	+/–	38.6 (±2.4)	13.5 (±3.4)	71.6 (±34.8)
F245A	11 (±2.1)	+/–	72.1 (±4.4)	20.8 (±5.5)	86.3 (±24.7)
E211Q/F245A	0.3 (±0.4)	–	11.7 (±6.4)	0	90.7 (±8.8)

*The graphs, figures, and tables containing the raw data, error rates, and the p-values can be found in Ref III. The pink color indicates a statistically significant change in the result compared to the wt strain. + (Red mutant tetrad), +/– (Sectored mutant tetrad), – (white mutant tetrad) on the glucose containing plate.

In the same assays, the mutants K32M, E211Q, Y243A, F245A, and the double mutant E211Q/F245A showed a significant loss of respiration compared to the wt strain (except the K32M in FOA assay). However, this was especially noticeable for the Y243A, F245A, and the double mutant E211Q/F245A. In the FOA assay, the strains Y243A, F245A E211Q/F245A retained only 4.6%, 11%, and 0.3% of respiratory active (red) colonies respectively, (Ref III, Fig. 1B and Sup. Table 4). This result correlates with the result that these mutants also had the most decreased ssDNA binding activity. Already at the 0h time point, the mutant strains K32M, E211Q, Y243A, F245A, and E211Q/F245A lost a significant fraction of the respiratory active cells (see Table 1), indicating a rapid loss of respiration on the low glucose containing plates. The fraction of the respiratory active cells decreased even more during the growth in glucose. The respiration activity loss was especially prominent for the mutant E211Q/F245A, which has both ATPase and ssDNA binding severely decreased. This strain had severe difficulties with growing on the non-fermentable medium (11.7% respiratory active colonies), and no respiratory active cells were present after 10 hours of growth in the fermentable medium (Ref III, Fig. 1C). These results indicate that both the ATPase activity and ssDNA binding are important and seem to have an additive effect on maintaining respiration. Note that we could isolate four viable spores of all the strains with point mutations on the glycerol-containing medium. Thus, under the non-fermentative conditions, where the cells must maintain mtDNA to survive, all the Hmi1 point mutants can support Hmi1's essential function in mtDNA maintenance (Ref III, Sup. Fig. 1).

We also measured the expression level of Hmi1 mutant proteins in the cell to ensure that these mutant proteins were expressed properly *in vivo* and to support the idea that the phenotypic effects were really caused by the loss of function. We observed that the mutants that we were not able to purify as a recombinant protein in the bacterial system -K32M, D237N, Q240E, Q240N- also had a lower expression *in vivo* (Ref III, Table 1, and Sup. Fig. 2A). However, the expression of Hmi1 mutant S241A was also lower *in vivo* compared to the wt, but this mutant acts in all our assays similarly to the wt. The mutant proteins with the most severe phenotype defect, like E211Q, Y243A, F245A, and E211Q/F245A, are expressed in yeast similarly to the wt Hmi1 (Ref III, Table 1, and Sup. Fig. 2A), indicating that the phenotypic effect is caused by the loss of function rather than by the loss-of-expression.

We also evaluated the dominant-negative interactions of Hmi1 mutants by overexpressing the plasmid with wt *HMI1* or its mutant gene in the w303α background but did not observe any significant loss of respiration over the 140 hours of continuous growth in the glucose-containing medium (Ref III, Fig. 2). This experiment further validates that the loss of respiratory activity in different *in vivo* assays results from direct effect of mutations on the biochemical function of the helicase protein, caused by the mutation rather than the toxic accumulation of the mutant protein. These results also indicate that there is no competitional binding or interaction of the wt and mutant proteins, which could affect the Hmi1 function considerably. In addition, these results imply that Hmi1 most likely functions as

a monomer in the cell, as previously proposed by the *in vitro* studies (Monroe *et al.,* 2005; Sedman *et al.,* 2005). However, this result underlines the functional difference of Hmi1, PcrA, and UvrD, as for the equivalent ATP hydrolysis mutants of PcrA (E224Q) and UvrD (K35M and E221Q), overexpression has been shown to lead to the dominant-negative effect (George *et al.,* 1994; Brosh & Matson, 1995; Urrutia-Irazabal *et al.,* 2021).

5.4.3 The effect of defective Hmi1 ATPase activity and ssDNA binding on mtDNA (Ref III)

To further discern the possible roles of the Hmi1 ATPase activity and ssDNA binding affinity in the mitochondria, we chose two different separation-of-function mutants for further study. In the strain w303α Hmi1 E211Q, Hmi1 is ATPase deficient but retains the ssDNA binding activity comparable to the wt strain. In contrast, the mutant strain w303α Hmi1 F245A, Hmi1 has the wt ATPase activity but has severely decreased ssDNA binding activity. Compared to the *hmi1* deletion strain, these mutants retain their mtDNA and can grow in non-fermentable media, which enabled us to study the effect of these mutants on the full genome of mtDNA, under conditions where mitochondrial transcription and translation are essential for the cell. Previous studies of our lab with *petite*-negative *C. albicans* strain demonstrated that in this organism, Hmi1 function is not essential for cell survival and for mtDNA maintenance, but the deletion of *HMI1* leads to remarkable fragmentation of mtDNA (Jõers *et al.,* 2007). However, in *S. cerevisiae*, we did not observe any changes in the overall morphology or fragmentation of mtDNA in the four regions tested (*COX1*, *COB*, *21S RRNA*, and *COX2*) (Ref III, Fig. 5A, C). This indicates that in the w303α Hmi1 E211Q and F245A mutants, no detectable large mtDNA rearrangements occurred, which could explain the loss of respiratory activity for these strains.

However, we did observe a change in the overall topology of mtDNA with the PFGE, as the mutant strains showed a change in the size distribution of mtDNA. In the mutant strains w303α Hmi1 E211Q and especially in the w303α Hmi1 F245A strain, an accumulation of mtDNA fragments, which were shorter than in the wt strain, was observed (Ref III, Fig. 5D, E). In addition, although we expected to see a mtDNA copy-number decrease in these mutants, we observed a copy-number increase up to 1.39-fold, and the rise was significant for the *21S RRNA* genes in both mutant strains E211Q and F245A and for the *COX2* gene in the F245A strain (Ref III, Fig. 5B). However, the slight increase of mtDNA copy-number and accumulation of smaller mtDNA molecules possibly does not explain the strong growth defective phenotype of these loss-of-function mutants. For this reason, we also tested the effect of the decreased ATPase and ssDNA activity on the mitochondrial gene expression and discovered that in both strains, the synthesis of the Cox2 protein, encoded by the mtDNA, is decreased, especially in the w303α Hmi1 F245A strain (Ref III, Fig. 6A).

Previous studies with the *rho*$^{-}$ strains HS61 by our laboratory have shown that the loss of Hmi1 does not abolish transcription and, therefore, is not essential for

transcription (Sedman *et al.,* 2000). However, the loss of Hmi1 in this strain leads to the shortening of double-stranded mtDNA concatemers. The length of linear concatemeric mtDNA molecules is partially restored in the mitochondrial RNA polymerase (Rpo41) deletion background (Sedman *et al.,* 2005). These results indicated that the loss of Hmi1 functions has a more severe effect on mtDNA in the presence of transcription, suggesting a direct role of Hmi1 in transcription or an indirect role in processing mtDNA in the transcription-dependent processes. This led us to question if the changes in transcription activity of mtDNA might cause the change in the gene expression. Using RT-PCR, we analyzed the steady-state transcription of the *COX2* gene in the Hmi1 mutants with decreased ATPase and ssDNA binding activity under the non-fermentative conditions. We observed no significant loss of mRNA transcript levels (Ref III, Fig. 6B). On the contrary, the steady-state mRNA levels of the mitochondrial *COX2* gene were slightly elevated for w303α Hmi1 E211Q and F245A strains, which might result from the fact that in the mutant strains the mtDNA copy-number of this specific gene is also higher compared to the wt strain (Ref III, Fig. 5B, 6B).

5.4.4 The role of Hmi1 in the damage induced mtDNA repair (Ref III)

In contrast to the Hmi1 deletion strain, the separation-of-function strains can maintain the complete mitochondrial genome. For this reason, we next tested the effect of DNA damage on the maintenance of respiration in these mutants. We tested the response of different repair pathways by incubating yeasts on plates containing MMS, H_2O_2, or plates radiated with UV-C right after plating.

MMS can alkylate guanine, adenine, and cytosine bases, which can lead to replication fork stalling and to the formation of DSB that could be repaired through the HR-dependent mechanism (Lundin *et al.,* 2005; Groth *et al.,* 2010; Yang *et al.,* 2010; Ma *et al.,* 2011). Several recombination proteins, like the RAD-epistasis group proteins and UvrD-like family helicases Srs2, UvrD, PcrA, and HelD, are shown to be necessary for repairing MMS-induced damage in the cell nucleus (Mendonca *et al.,* 1993; Dong & Fasullo, 2003; Bronstein *et al.,* 2018; Moreno-del Álamo *et al.,* 2021). In mitochondria, the nuclease Din7 expression is upregulated in the presence of MMS, and the endonuclease Apn1 is shown to be important for mtDNA repair after the MMS damage (Mieczkowski *et al.,* 1997; Acevedo-Torres *et al.,* 2009). We tested the effect of MMS on Hmi1 mutant strains but detected no visible growth defect (Ref III, Fig. 3, Sup. Fig. 5, 6). For Srs2 helicase, MMS-induced damage defect is observed under the conditions where Srs2 is overexpressed (Bronstein *et al.,* 2018). For this reason, we also tested the effect of Hmi1 overexpression but observed no difference between the strains overexpressing the wt *HMI1* or mutant *hmi1* in a strain where the wt copy of the gene was also expressed from the endogenous background (Ref III, Fig. 3A, Sup. Fig. 5A). Furthermore, the Hmi1 overexpressed from the high copy number 2μm plasmid (pRS425) did not either elevate the frequency of *petite* cell formation (Fig. 7).

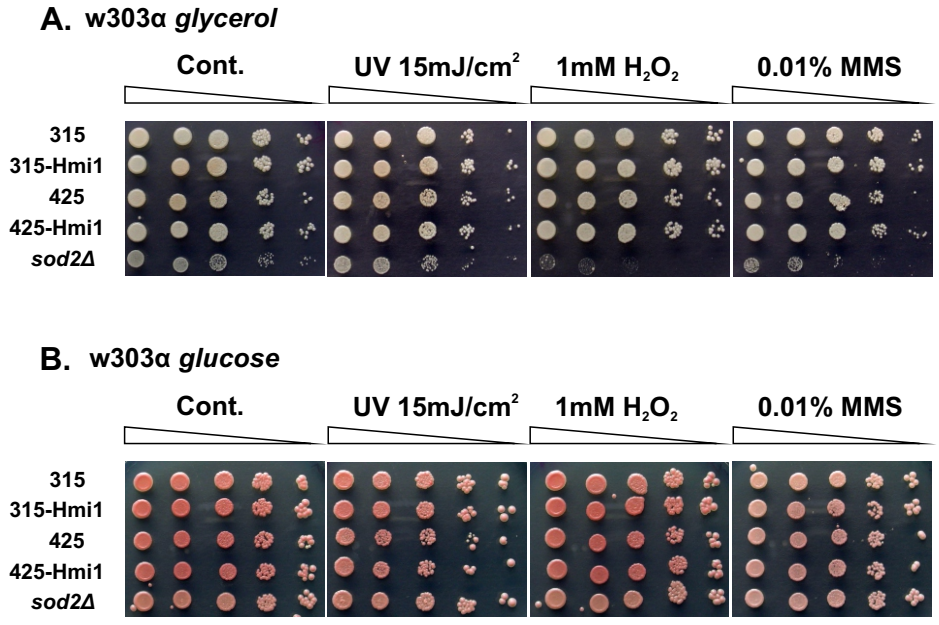

Figure 7. The overexpression of Hmi1 does not lead to the loss of respiration under DNA damaging conditions. The W303α strain expressing either the empty or Hmi1 expressing pRS315 and pRS425 plasmid and the control strain with *sod2Δ*. The serial dilutions of the cells 5000, 2500, 500, 50, 10 were spotted on the SC-Leu glycerol (A) or glucose (B) containing plates, which contained 1mM H_2O_2, 0.01% MMS or were treated with 15mJ/cm^2 UV-C, as indicated.

The oxidative damage by H_2O_2 leads to the rise of oxidatively modified DNA bases and ssDNA/dsDNA breaks, which result in a wide range of different DNA alterations, from single base mutation to recombinational crossovers (Aruoma *et al.,* 1989; Zhang *et al.,* 2019). In *S. cerevisiae* wt and *rho*$^-$ strains, the mtDNA copy-number has been shown to increase as a result of H_2O_2 treatment, and in *rho*$^-$ strains, this leads to elevated DSB levels and induces the complete loss of mtDNA (Doudican *et al.,* 2005; Hori *et al.,* 2009). The H_2O_2-induced damage to mtDNA has been shown to be elevated in the strains lacking proteins, such as Ntg1, Mhr1, and Din7, that are proposed to be involved in recombination-dependent repair and replication pathway (Hori *et al.,* 2009; Ling *et al.,* 2013). In addition, the loss of respiration-competent cells of the yeast strain, lacking both Pif1 and Ntg1, is elevated under the oxidative stress caused by the combination of H_2O_2 and antimycin (Doudican *et al.,* 2005). To test if Hmi1 ATP or ssDNA binding activity might affect the oxidative damage-induced repair, we tested the survival of the mutant strains and their maintenance of respiration on the plates containing H_2O_2. However, we did not observe any effect of H_2O_2 on the Hmi1 mutant strains compared to the strains growing on the plates with no H_2O_2, indicating that Hmi1 is not essential for oxidative damage repair.

Finally, we tested the effect of UV-induced damage since the UvrD-like helicase family members UvrD and PcrA are shown to have a role in nucleotide excision repair (Kuemmerle & Masker, 1980; Washburn & Kushner, 1991; Petit *et al.,* 1998). The most common consequence of the UV induced damage is the induction of cyclobutane-pyrimidine dimers, and to a lesser extent, 6–4 photo-products formation in DNA that are not directly repaired in yeast mitochondria and could cause replication stalling (Prakash, 1975; Douki *et al.,* 2000; Torregrosa-Muñumer *et al.,* 2015). Surprisingly, we observed the sensitivity to the UV-C, but only for the strains w303α Hmi1 F245A, w303α Hmi1 Y243A, and the double mutant w303α Hmi1 E211Q/F245A, which have impaired ssDNA substrate binding ability (Ref III, Fig. 3 and 4, Sup. Fig. 5, 6). To further confirm this result, we conducted a quantitative assay where the survival of the cells was counted on the glycerol-containing medium, comparing the number of colonies formed on the untreated plate and the plates irradiated with different UV-C dosages. These results further confirmed that the mutant E211Q has a similar survival to the wt strain, but at the higher UV-C dosage, the mutant F245A has a noticeable effect on cell survival, and the double mutant E211Q/F245A UV sensitivity is even higher compared to the single mutant (Ref III, Fig. 4C). This phenotype differs from the observation made with bacterial UvrD, where UvrD E221Q, the equivalent mutation of Hmi1 E211Q, has been shown to be sensitive to UV-damage, indicating that ATPase and helicase activity are important for UvrD excision repair functions (Brosh & Matson, 1995).

Studies on *S. cerevisiae* mtDNA have indicated that NER is absent in the mitochondria, since the pyrimidine-dimers are not repaired (Prakash, 1975). Therefore, the role of Hmi1, if participating in UV damage repair, would use a different mechanism to repair or overcome UV-induced damage. The impact of UV on mtDNA has been more thoroughly studied in the mammalian cells. Low doses of UV lead to the accumulation of branched molecules in the mammalian cells, including Holliday junction-like structures and fork-like structures, which has led to a proposal that UV damage possibly amplifies replication stalling in the mitochondria (Torregrosa-Muñumer *et al.,* 2015). UV-damage might have a similar effect on *S. cerevisiae* mtDNA, and this could indicate that the Hmi1 ssDNA binding ability might be necessary for dealing with stalled replication forks.

5.5 The possible role of Hmi1 in yeast mitochondria

The results of this thesis show that the function of Hmi1 does not rely solely on its ATP hydrolysis and helicase activity in the cell, as the mutant protein, unable to hydrolyze ATP, is still able to partially contribute to the maintenance of the fully functional mtDNA (Kuusk *et al.,* 2005; Sedman *et al.,* 2005; Ref III). Previous studies have proposed that the Hmi1 function requiring ATP hydrolysis might be involved in recombinational processes, as Hmi1 prefers to unwind dsDNA with 3'-ssDNA overhang or 3'-flap structure *in vitro* (Kuusk *et al.,* 2005). The ATP hydrolysis-dependent function of Hmi1 might be only needed during

specific repair or recombination events, which do not occur with every single molecule of the mtDNA and are not essential for mtDNA replication. This would be in accordance with the observation that the ATPase-deficient mutants have an impact on maintaining mtDNA over a longer period of time (Ref III, Fig. 1C). It can also not be ruled out that the ATP hydrolysis-dependent function can be complemented by other helicases, or that Hmi1 forms a complex with other proteins that are at least partially able to provide the necessary ATP hydrolysis energy. For example, studies on PcrA have suggested that for the replacement of RecA from the DNA, the ATPase activity of RecA is essential, but the ATPase activity of PcrA could be dispensable (Anand *et al.,* 2007; Fagerburg *et al.,* 2012).

As the rise of *rho*$^{-}$ strains is proposed to be associated with the inaccurate HR events (Gaillard *et al.,* 1980), it could be possible that Hmi1 might participate in the suppression of recombination events, such as the nuclear Srs2 (see chapter 2.2.4.3). It currently seems to be rather unlikely as, so far, no significant effect on the respiratory activity in the cells overexpressing Hmi1 under normal or DNA-damaging conditions has been observed (see chapter 5.4.4). The yeast nuclear Srs2 has also been proposed to regulate the Rad51-dependent repair synthesis at the replication forks (Burkovics *et al.,* 2013). In this model, the SUMOylated PCNA is shown to recruit the Srs2, that is able to inhibit the D-loop extension in ATP hydrolysis independent manner (Burkovics *et al.,* 2013). It is possible that Hmi1 also has a regulatory role. This would explain why the double mutant E211Q/F245A, which seems to be expressed comparable to the wt protein in the cell, while being ATPase inactive with severely diminished ssDNA binding, can survive on the non-fermentable media, unlike the *hmi1Δ* strain (see chapters 5.4.1, 5.4.2). We can also not rule out the possibility, that this is a ssDNA binding dependent function, as under cellular conditions the ssDNA binding activity of E211Q/F245A might be sufficient for the essential Hmi1 functioning.

The studies based on the mtDNA topology have revealed a role of Hmi1 in the maintenance of longer concatemers in *S. cerevisiae rho*$^{-}$ strains and of the full-length mtDNA in *C. albicans,* which has led to the proposal that Hmi1 might be necessary for replication fork progression or replication reinitiation (Sedman *et al.,* 2005; Jõers *et al.,* 2007). Studies with mammalian mtDNA have demonstrated that replication stalling caused by induced damage leads to replication reinitiation by the primase-polymerase Primpol, or stalled forks can be processed through degradation by MGME1 or a recombination-dependent mechanism (Torregrosa-Muñumer *et al.,* 2017, 2019). As discussed above (see chapter 5.4.4) in mammalian cells this replication fork stalling can be caused by the UV-damage (Torregrosa-Muñumer *et al.,* 2015). If UV-damage has a similar effect in the yeast mitochondria, our results, showing that Hmi1 ssDNA binding is important for dealing with the UV-damage, would further argue for the role of Hmi1 in replication reinitiation. However, the replication fork stalling also occurs under the normal growth condition, because of the collision of the replisome and transcription machinery. UvrD-like family helicases *E. coli* UvrD, and its ortholog in *B. subtilis*, PcrA, have been shown to have a role in resolving stalled replication-transcription conflicts, and different models have been proposed to explain how

the enzymes might interact with RNA polymerase to resolve these conflicts and suppress R-loops during transcription (Epshtein *et al.,* 2014; Merrikh *et al.,* 2015; Hawkins *et al.,* 2019; Urrutia-Irazabal *et al.,* 2021). For *B. subtilis* PcrA it has been shown, that the suppression of R-loops is dependent on the ATP hydrolysis (Urrutia-Irazabal *et al.,* 2021). If Hmi1 participates in the processing of the stalled replication forks, including polymerase collisions, it would explain why the loss of Hmi1 function is less pronounced in the *rho*$^{-}$ strains lacking transcription, as we observed no direct effect of the ATP-dependent and independent function on steady-state mRNA levels (Sedman *et al.,* 2005; Ref III, Fig. 6B). It is important to note that the steady-state mRNA levels only provide us with confirmation that transcription is active in these strains and that the lowered Cox2 protein level cannot be directly linked to the diminished mRNA expression. Future studies will have to determine if mRNAs are correctly transcribed in the Hmi1 mutant strain or if Hmi1 has a role in downstream activities in the cell, which could explain the observed decrease in mitochondrial Cox2 protein level.

6 CONCLUSIONS

It has been known from early studies that mitochondrial protein synthesis can also affect mtDNA maintenance, complicating the verification of whether proteins have a direct function on mtDNA or an indirect role in mtDNA maintenance (see chapter 2.1.2). Mitochondrial helicases are actively involved and affect mtDNA maintenance through various cellular processes. The deletion of mitochondrial RNA helicases Suv3, Mrh4, and Mss116 leads to the loss of respiration, and Suv3 has been proposed to have a role in mtDNA replication (Guo *et al.,* 2011; Sedman *et al.,* 2014). The DNA helicase Pif1 has been shown to have a role in mtDNA maintenance, possibly in replication or in recombination-dependent processes (see chapter 2.2.4.1). This thesis further expands the knowledge about yeast-specific mtDNA helicases Irc3 and Hmi1 and their possible multifarious roles in yeast mitochondria.

The main results of this work can be summarized as follows:

1. *S. cerevisiae* ($Irc3_{sc}$) is involved in the creation/processing of replication fork and Holliday junction-like DNA structures *in vivo*. These results are in accordance with the fact that *in vitro* $Irc3_{sc}$ is a DNA helicase that preferably binds branched DNA molecules and exhibits fork reversal and branch migration activities. These activities indicate its similar function to bacterial RecG, which is also able to partially complement $Irc3_{sc}$ *in vivo*.
2. Irc3-like helicases appear to be fungi-specific helicases that potentially function in mitochondria and have specific conserved C-terminal domains.
3. *O. polymorpha* Irc3 ($Irc3_{op}$) is a homolog of $Irc3_{sc}$ that can partially complement the lack of $Irc3_{sc}$ and shares similar DNA-stimulated ATPase activity, indicating their functional similarities. However, the ATPase activity of $Irc3_{op}$ is also stimulated by RNA, which raises a new possible role in RNA metabolism for Irc3-like helicases.
4. The ATP hydrolysis and ssDNA binding activity are important for the functioning of the Hmi1 protein. However, the ATP hydrolysis function is not essential for Hmi1 *in vivo*, indicating an ATP hydrolysis-independent role in mtDNA maintenance.
5. Experiments with mutant strains that have significantly decreased ssDNA binding indicate that Hmi1 might have a role in damage-induced repair or replication reinitiation, and the decreased functionality influences mitochondrial gene expression.

In conclusion, this thesis further underlines the idea that mitochondrial helicases have multifarious roles and possibly function in different steps of mitochondrial metabolism. In addition to the ATPase-dependent and independent role of Hmi1 and their effects on gene expression, this thesis also characterized the direct role

of Irc3 in maintaining mtDNA and discusses its potential role as an RNA helicase (see chapter 5.3). Studies with yeast-specific helicases indicate that although these proteins do not have very close homologs in higher eukaryotes, these functions seem to be fulfilled by other helicases and possible functional analogs. However, it is likely that differences in the set of DNA helicases present in yeast mitochondria compared to higher eukaryotes reflect potential differences in mtDNA replication mechanisms and the usage of recombinational events on mtDNA maintenance, which needs to be clarified through future studies.

SUMMARY IN ESTONIAN

Helikaaside Hmi1 ja Irc3 roll pärmi mitokondriaalse DNA säilitamisel

Mitokonder osaleb mitmetes olulistes raku funktsioonides, millest kõige tuntum on oksüdatiivne fosforüleerimine, mis tagab eukarüootse raku hingamise. Enamus mitokondri valke sünteesitakse raku tuumas, aga hingamisahelas osalevad ka mitokondri genoomi alusel sünteesitud komponendid. Sõltuvalt tüvest on *S. cerevisiae* mitokondriaalne DNA umbes 85 tuhat aluspaari pikk ja kodeerib 8-t hingamisahelas vajalikku valku ning lisaks nende valkude sünteesimiseks vajalikke rRNA ja tRNA-sid (Foury *et al.,* 1998). Mitokondriaalse DNA säilimine on hädavajalik hingamisahela toimimiseks ja mitokondriaalses DNA-s tekkivad vead häirivad mitokondri funktsiooni, mis inimestel avalduvad tihti haigustena (Suomalainen & Battersby, 2018). *S. cerevisiae* on fakultatiivne anaeroob ja suudab fermentatiivsel söötmel ellu jääda ka ilma mitokondriaalse DNA-ta ning on seetõttu osutunud oluliseks mudelorganismiks mitokondriaalse DNA säilitamisega seotud valkude uurimises. Mitokondriaalse DNA säilitamine on kompleksne protsess, milles lisaks DNA replikatsioonile ja parandamisele on väga oluline roll ka geenieekspressioonil. Üks grupp olulisi valke, mis tagavad mitokndriaalse DNA säilimise, on mitokondriaalsed helikaasid. Helikaasid on mootorvalgud, mis kasutades ATP hüdrolüüsi energiat suudavad lahti harutada erinevaid DNA ja RNA molekule või modifitseerida neid tagamaks nende korrektse säilimise ja edasipärandumise rakus (Brosh & Matson, 2020; Bohnsack *et al.,* 2023). *S. cerevisiae* mitokondris on kirjeldatud DNA helikaaside Pif1, Hmi1, Irc3 ja RNA helikaaside Suv3, Mss116, Mrh4 funktsioone.

Doktoritöö põhiliseks eesmärgiks on selgitada välja Hmi1 ja Irc3 roll pärmi mitokondriaalse DNA säilimises ja nende helikaaside üldisem funktsioon mitokondris. Varasemalt on näidatud, et *S. cerevisiae* Irc3 ($Irc3_{sc}$) on helikaas, mis on võimeline lahti harutama kaheahelalist DNA-d ja mille puudumisel hakkavad rakus kuhjuma kaheahelalised katked (Sedman *et al.,* 2014). $Irc3_{sc}$ omab lisaks konserveerunud helikaasi domeenile ka eraldi C-terminaalset domeeni, mis on oluline hargnenud molekulide sidumiseks (Sedman *et al.,* 2017). Lisaks sellele on näidatud, et $Irc3_{sc}$ funktsioon mõjutab ka mitokondriaalse DNA transkriptsiooni ja translatsiooni elongatsiooni (Kaur & Datta, 2021). Hmi1 on helikaas, mis on essentsiaalne funktsionaalse mitokondriaalse DNA säilimiseks ja suudab lahti harutada molekule, mis omavad 3'-üheahelalist DNA otsa (Sedman *et al.,* 2000; Kuusk *et al.,* 2005).

Rakusisese funktsiooni täpsemaks uurimiseks tehti kindlaks, kuidas mõjutab Irc3 ja Hmi1 valkude puudumine või Hmi1 mutantse valgu ekspresioon raku hingamisvõimet ja elumust tavalistes kasvutingimustes ning uuriti Hmi1 elumust erinevate DNA-d kahjustavate ühendite olemasolul. Lisaks sellele uuriti mõju mtDNA topoloogiale, koopiaarvule ja geenieskpresioonile.

Doktoritöö kõige olulisemad tulemused ja järeldused on järgmised:

1. *S. cerevisiae* Irc3 on DNA helikaas, mille puudumisel hakkavad pärmirakus kuhjuma hargnenud DNA molekulid ja replikatsiooni kahvlid. See on kooskõlas *in vitro* katsetega, mis näitasid, et $Irc3_{sc}$ eelistab siduda ja lahti harutada hargnenud DNA molekule ja omab *in vitro* replikatsioonikahvli ümberpööramise ja hargenud ahela mööda DNA-d liigutamise aktiivsust. Need aktiivsused on sarnased *E. coli* RecG valguga ja katsetest selgus, et RecG on võimeline osaliselt komplementeerima $Irc3_{sc}$ puudumist.
2. Irc3 sarnased helikaase leidub erinevates pärmides ja nende konserveerunud järjestuse põhjal on alust arvata, et need võiksid lokaliseeruda mitokondrisse ka teistes pärmides nagu on varasemalt näidatud *S. cerevisiae* Irc3 puhul. Nendes valkudes on konserveerunud C-terminaalne domeen, mida seostatakse hargnenud ahelaga molekulide sidumise ja lahtiharutamisega.
3. *O. polymorpha* Irc3 ($Irc3_{op}$) on võimeline osaliselt komplementeerima $Irc3_{sc}$ puudumist ja neil on sarnane DNA poolt stimuleeritud aktiivsus, mis viitab nende sarnasele funktsioonile. Lisaks sellele on $Irc3_{op}$ ATPaasne aktiivsust stimuleeritud ka RNA poolt. Selle uue tulemuse valguses on põhjust arvata, et Irc3-ga sarnastel helikaasidel võib olla roll ka RNA molekulide metabolismis, mis oleks kooskõlas Kaur ja Data, 2021 tulemustega.
4. Katsed *S. cerevisiae* Hmi1-ga kinnitasid, et ATP hüdrolüüs ja üheahelalise DNA sidumine on mõlemad olulised hingamisvõimeliste rakkude säilimiseks ja Hmi1 funktsiooni tagamiseks. Erinevalt mitmetest teistest helikaasidest, ei vii Hmi1 ATPasse aktiivuse drastiline vähenemine valgu täieliku funktsiooni kadumiseni. See viitab sellele, et Hmi1 omab ka ATP hüdrolüüsist sõltumatut funktsiooni rakus.
5. Katsed mutantidega, millel on oluliselt vähenenud üheahelalise DNA substraadi sidumise võime, näitasid, et Hmi1 võib olla roll UV-kiirgusest tulenevate DNA kahjustuste parandamises või replikatsiooni reinitsieerimises. Lisaks mõjutab üheahelalise DNA sidumisega seotud aktiivsuse langemine ka mitokondriaalset geenieksprессiooni.

Käesoleva doktoritöö tulemused, koos varem publitseeritud tulemustega näitavad, et mitokondriaalsed helikaasid Hmi1 ja Irc3 on multifunktsionaalsed valgud, mis mõjutavad mitokondri metabolismi mitmel eri moel ja mis ilmestab mitokondriaalse DNA säilimise keerukust.

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PUBLICATIONS

CURRICULUM VITAE

Name:	Sirelin Sillamaa
Date of birth:	05.12.1993
E-mail:	sirelin.sillamaa@gmail.com

Education

Since 2018	University of Tartu, PhD studies (Molecular- and Cell biology)
2018	University of Tartu, MSc (Gene Technology)
2016	University of Tartu, BSc (Gene Technology)
2013	Tallinn German Gymnasium

Professional career

01.10.2022–29.02.2023	Junior Research Fellow (Biochemistry), Institute of Molecular and Cell Biology, University of Tartu
01.09.2020–31.08.2022	Junior Research Fellow (Biomedicine and Biotechnology), Institute of Molecular and Cell Biology, University of Tartu
14.03.2022–15.07.2022	Project Manager, Centre for Entrepreneurship and Innovation, University of Tartu
01.07.2016–31.08.2016	Bioanalyst, Institute of Molecular and Cell Biology, University of Tartu

Research and development work

Main fields of research

CERCS RESEARCH FIELD: P320 Nucleic acids, protein synthesis

S. cerevisiae mitochondrial helicases, mitochondrial DNA maintenance and topology

List of publications

Sillamaa, S., Piljukov, V.-J., Vaask, I., Sedman, T., Jõers, P., & Sedman, J. (2023). UvrD-like helicase Hmi1 Has an ATP independent role in yeast mitochondrial DNA maintenance. *DNA Repair*, *132*, 103582. https://doi.org/10.1016/j.dnarep.2023.103582

Piljukov, V.-J., **Sillamaa, S.**, Sedman, T., Garber, N., Rätsep, M., Freiberg, A., & Sedman, J. (2023). Mitochondrial Irc3 helicase of the thermotolerant yeast *Ogataea polymorpha* displays dual DNA- and RNA-stimulated ATPase activity. *Mitochondrion*, *69*, 130–139. https://doi.org/10.1016/j.mito.2023.02.004

Asanović, I., Strandback, E., Kroupova, A., Pasajlic, D., Meinhart, A., Tsung-Pin, P., Djokovic, N., Anrather, D., Schuetz, T., Suskiewicz, M.J., **Sillamaa, S.**, Köcher, T., Beveridge, R., Nikolic, K., Schleiffer, A., Jinek, M., Hartl, M., Clausen, T., Penninger, J., Macheroux, P., Weitzer, S., Martinez, J. (2021). The oxidoreductase PYROXD1 uses NAD(P)+ as an antioxidant to sustain tRNA ligase activity in pre-tRNA splicing and unfolded protein response. *Molecular Cell*, *81*(12), 2520–2532.e16. https://doi.org/10.1016/j.molcel.2021.04.007

Sedman, T., Garber, N., Gaidutšik, I., **Sillamaa, S.**, Paats, J., Piljukov, V. J., & Sedman, J. (2017). Mitochondrial helicase Irc3 translocates along double-stranded DNA. *FEBS Letters*, *591*(23), 3831–3841. https://doi.org/10.1002/1873-3468.12903

Gaidutšik, I., Sedman, T., **Sillamaa, S.**, & Sedman, J. (2016). Irc3 is a mitochondrial DNA branch migration enzyme. *Scientific Reports*, *6*, 26414. https:// doi.org/10.1038/srep26414

Scholarships

2017 Rotalia Foundation scholarship

2016 Estonian National Contest for University Students; first place in the bio- and environment science category for Bachelor's thesis

Other administrative and professional activities

Member of the Estonian Bioscience Students' Association (2013–2020)
Member of the University of Tartu student union (2015–2021)
Member of the Estonian Biochemical Society (since 2019)
Science popularization with Mobile Bioclass and Biology workshop (University of Tartu Youth Academy)

Teaching and supervision at the University of Tartu

Practical Course in Biochemistry
Supervisor of two BSc theses and one secondary school student project

ELULOOKIRJELDUS

Nimi:	Sirelin Sillamaa
Sünniaeg:	05.12.1993
E-post:	sirelin.sillamaa@gmail.com

Haridus

Alates 2018	Tartu Ülikool, doktoriõpe (molekulaar- ja rakubioloogia)
2018	Tartu Ülikool, MSc (geenitehnoloogia)
2016	Tartu Ülikool, BSc (geenitehnoloogia)
2013	Tallinna Saksa Gümnaasium, keskharidus

Teenistuskäik

01.10.2022–29.02.2023	biokeemia nooremteadur, molekulaar- ja rakubioloogia instituut, Tartu Ülikool
01.09.2020–31.08.2022	biomeditsiini ja biotehnoloogia nooremteadur, molekulaar- ja rakubioloogia instituut, Tartu Ülikool
14.03.2022–15.07.2022	teaduskiirendi projektijuht, ettevõtlus- ja innovatsioonikeskus, Tartu Ülikool
01.07.2016–31.08.2016	bioanalüütik, molekulaar- ja rakubioloogia instituut, Tartu Ülikool

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S. cerevisiae mitokondriaalsed helikaasid, mitokondriaalse DNA säilitamine ja topoloogia

Publikatsioonide loetelu

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