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# Irc3 is a mitochondrial branch migration enzyme in *Saccharomyces cerevisiae*

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# LIST OF ORIGINAL PUBLICATIONS

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

I Sedman T, Gaidutšik I, Villemson K, Hou Y, Sedman J. (2014). Double-stranded DNA-dependent ATPase Irc3p is directly involved in mitochondrial genome maintenance. Nucleic Acid Research. 42(21):13214–13227.

II Gaidutšik I[1], Sedman T[1], Sillamaa S, Sedman J. (2016). Irc3 is a mitochondrial DNA branch migration enzyme. Scientific Reports. 6: 26414.
[1] Authors contributed equally to this work

III Sedman T[1], Garber N[1], Gaidutšik I, Paats J, Sillamaa S, Piljukov V, Sedman J. (2017). Mitochondrial helicase Irc3 translocates along double-stranded DNA. FEBS Letters. 591:3831–3841
[1] Authors contributed equally to this work

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Ref I Oxford University Press

Ref II Springer Nature

Ref III John Wiley & Sons

My contribution to the articles is as follows:

Ref I Designed and performed the experiments together with Sedman T., participated in the data analysis and writing of the manuscript.

Ref II Designed and performed *in vitro* experiments, analyzed data and was involved in writing of the manuscript.

Ref III Performed *in vitro* experiments and edited the manuscript.

# ABBREVIATIONS

| | | |
|---|---|---|
| aa | – | amino acids |
| ATR | – | ataxia telangiectasia and Rad3-related protein |
| BER | – | base excision repair |
| BS | – | Bloom´s syndrome |
| CTD | – | C-terminal domain |
| D-loop | – | displacement loop |
| DSB | – | double-stranded break |
| DSBR | – | double-strand DNA break repair pathway |
| ICL | – | interstrand crosslinks |
| FA | – | Fanconi anemia |
| GST | – | gluthatione-S-transferase |
| HJ | – | Holliday junction |
| HR | – | Homologous recombination |
| mt | – | mitochondrial |
| MTS | – | mitochondrial targeting sequence |
| NA | – | nucleic acid |
| NHEJ | – | non-homologous end joining |
| NTP | – | nucleoside triphosphate |
| OB-fold | – | oligonucleotide/oligosaccharide binding-fold |
| OXPHOS | – | oxidative phosphorylation |
| R-loop | – | RNA oligonucleotide containing structure analogous to D-loop |
| RDR | – | recombination-dependent replication |
| RBM | – | RPA-binding motif |
| ROS | – | reactive oxygen species |
| RPA | – | Replication protein A |
| SDSA | – | synthesis-dependent strand annealing |
| SCE | – | sister chromatid exchange |
| SF | – | superfamily |

# 1. INTRODUCTION

Mitochondrion is a multifunctional eukaryotic organelle best known for its involvement in energy production. Another characteristic trait of mitochondrion is the presence of its own minuscule genome which encodes several indispensable subunits of the respiratory chain complexes essential for the generation of energy via oxidative phosphorylation (OXPHOS) pathway. For this reason, the loss or damage of the mitochondrial genome has a dramatic impact on the survival of higher eukaryotes (Suomalainen & Battersby, 2017). In contrast, an important single cell model eukaryote, the baker's yeast *Saccharomyces cerevisiae*, can tolerate the absence of mitochondrial (mt) genome and, thus, is a perfect candidate for studying mechanistic aspects of mtDNA maintenance. The mitochondrial genome has limited coding capacity and to build up the functional organelle, more than 900 nuclear proteins are transported into mitochondria in *S. cerevisiae* (Morgenstern *et al.*, 2017). A surprisingly large number of those proteins do not have clearly defined roles yet. In the intricate field of mtDNA research, the description of novel factors involved in various underlying processes such as replication, recombination, repair and segregation will improve a global understanding of genome maintenance mechanism.

Helicases are protein motors driven by the hydrolysis of nucleotide cofactors that are interacting with nucleic acids or nucleic acid-protein complexes and, thus, catalyze various rearrangements in the structure of DNA or RNA. Helicases are ubiquitous enzymes found throughout the tree of life and are involved in all aspects of nucleic acid metabolism. Therefore, it is obvious that helicases are required for the stability and proper expression of the mitochondrial genome. Previously, three mitochondrial helicases Mhr4, Mss116 and Suv3 were found to be involved in mitochondrial gene expression (Bifano *et al.*, 2010; De Silva *et al.*, 2013; Turk & Caprara, 2010) Two helicases were implicated in mtDNA maintenance: Pif1 (Lahaye *et al.*, 1991) and Hmi1 (Sedman *et al.*, 2000). Several years ago a novel, previously not analyzed helicase Irc3 was found to be targeted to mitochondria in a genome-wide effort (Alvaro *et al.*, 2007; Huh *et al.*, 2003) and our group started to scrutinize its role in mitochondria.

It is important to characterize helicase motifs containing proteins because they are able to act on various nucleic acid substrates or utilize specific structures and may not even be *bona fide* helicases. Definitely, all aforementioned properties reflect the biological role of a specific protein. For these reasons, to better define the role of Irc3, together with *in vivo* experiments, the biochemical characterization of purified Irc3 was conducted. In this thesis I am presenting our current understanding of the Irc3 function. The theoretical part of the thesis is focused on helicases, their structure and mechanism. In addition, the biological roles of proteins exhibiting activities similar to Irc3 are described. In the second part of my literature overview I concentrate on mitochondrial genome structure and processes involved in its maintenance. In the experimental part I focus on the results of my efforts on Irc3 protein purification together with its biochemical

and *in vivo* characterization. In the final part, findings presented here will be combined with other our results and the potential role of Irc3 in the mitochondria of baker's yeast will be suggested. Even though the final decision concerning the function of Irc3 helicase is not done, the body of evidences places the protein in the broad field of homologous recombination that is widely used by yeast mitochondria.

# 2. REVIEW OF LITERATURE

## 2.1. SF1 and SF2 helicases

### 2.1.1. General introduction

Helicases are protein motors driven by the hydrolysis of nucleotide cofactor, which are able to interact with nucleic acids (NA) or NA-protein complexes and catalyze various rearrangements in their structures. Helicases are very ubiquitous enzymes found in viruses, archaea, prokaryotes and eukaryotes. This reflects the great importance of these proteins implicated in fundamental processes of DNA and RNA metabolism.

The first DNA helicase to be purified and described was DNA helicase I (Abdel-Monem *et al.*, 1976; Abdel-Monem & Hoffmann-Berling, 1976). Since then a large number of helicases have been characterized in different organisms. Accumulated sequence data and development of bioinformatics tools led to identification of conserved helicase motifs. According to sequence differences between the conserved motifs, helicases were divided into 3 superfamilies (SF) and 2 families (Gorbalenya & Koonin, 1993). This initial study has largely stood the test of time even though, in the following years, due to progress in structural and functional studies, the number of conserved motifs increased to at least 11 and the number of superfamilies to 6, with SF1 and SF2 being the largest (Beyer *et al.*, 2013; Fairman-Williams *et al.*, 2010; Singleton *et al.*, 2007). In my following review I will mainly concentrate on SF1 and especially SF2 proteins as they are closest to the object of my study.

Conserved helicase motifs form two core domains (Figure 1B) involved in the binding and hydrolysis of nucleoside triphosphate (NTP), contacting NA and coupling the produced chemical energy with unwinding or translocation (Hall & Matson, 1999). The central part of SF1 and SF2 helicase is formed by two RecA-like neighboring domains termed in the case of SF2 domain 1 (D1) and domain 2 (D2). The characteristic motifs localize within the domains and are involved in three main actions: 1) hydrolysis of triphosphate (Q, I, II, VI), 2) contacting with NA (Ia, Ib, IV, IVa, V,) and 3) transmitting the energy of hydrolysis to motor function (III, Va) (Figure 1A). The group 1 and 3 motifs are located in the cleft on the surfaces of D1 and D2 domains and the group 2 on outer face of the motor core (Beyer *et al.*, 2013; Fairman-Williams *et al.*, 2010; Singleton *et al.*, 2007).

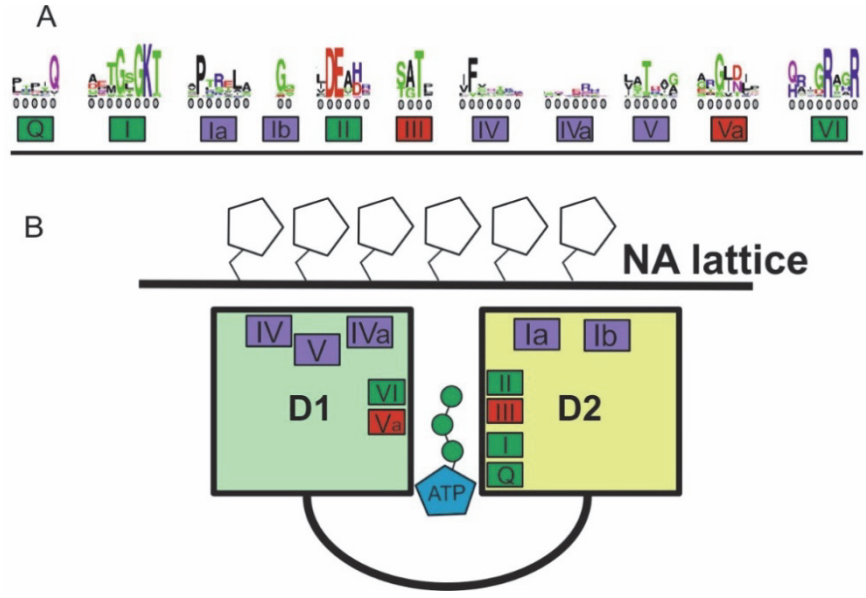


**Figure 1.** The schematic diagram of SF2 motor core and conserved helicase motifs in SF2. (A.) The sequence conservation of helicase motifs in SF2. The size of the single letter acronym reflects a relative level of amino acid conservation. Motifs (Q, I, Ia, Ib, II, III, IV, IVa, V, Va, VI) in green rectangles are involved in NTP binding and hydrolysis, in purple – binding and interacting with NA and in red – connecting these activities. (B.) D1/2 are RecA-like domains with ATP bound in a cleft between them. NA sugar-phosphate backbone is black. The relative positions of motifs are depicted on helicase domains. Adapted from (Beyer *et al.*, 2013; Fairman-Williams *et al.*, 2010)

It is worth mentioning that SF1 and SF2 helicases are very similar and conserved motifs of both superfamilies are folded into analogous core domains. The sequence motifs I, II, VI which are involved in NTP hydrolysis are conserved across both SFs, thus revealing the evolutionary conservation of this process. On the contrary, the motifs involved in the coordination between NTP hydrolysis and NA binding (III, Va) are highly conserved inside each SF, but not across both. In addition, spatial arrangement of these motifs between neighboring motifs also varies. The described differences suggest that the communication between NTP and NA binding is differently organized in SF1 and SF2. The low conservation of NA binding motifs between both SFs also points on diverse mode of interaction with NA inside both superfamilies. Finally, it should be noted that some conserved motifs appear in SF1, but are absent in SF2 and *vice versa* (Fairman-Williams et al., 2010).

Usually, the helicase motor in SF1 and SF2 helicases is surrounded by N- or C-terminal flanking regions. These sequences are typically folded into accessory domain or domains (Fairman-Williams *et al.*, 2010). Accessory domains usually have specific roles such as RNA and DNA binding, protein-protein

interaction, nuclease activity or oligomerization (Bae *et al.*, 1998; Bernstein & Keck, 2003; Klostermeier & Rudolph, 2009; Yoneyama & Fujita, 2008). These additional activities influence or even define the function of helicase (Fairman-Williams *et al.*, 2010). In many cases, accessory domains dictate also the physiological role of helicases by giving them an ability to act on specific NA structures, regulating the interaction with other proteins or recruitment into protein complexes (Shereda *et al.*, 2009; Singleton *et al.*, 2001; Yoneyama & Fujita, 2008).

The helicase core is a motor providing the functional basis for many types of actions. Its presence may not tell anything about the specific mode of action of a protein. It is now obvious, that not all proteins which contain helicase motifs exhibit helicase activity. The *bona fine* helicases are only a subset of the large group of enzymes that are capable to translocate on NA without unwinding or act as energy-dependent switches (Beyer *et al.*, 2013; Fairman-Williams *et al.*, 2010).

## 2.1.2. Mechanistic view on helicase function

### *2.1.2.1. The classification of helicases*

The classification of helicases into (super)families based on their motifs was done in 1993 (Gorbalenya & Koonin, 1993) and it is still in use. However, gathered data in helicase research field showed that sequence based classification does not give any insight into mechanistic behavior and function of protein. As most helicases are able to conduct unidirectional translocation on NA lattice, a new approach to classification based on this mechanistic behavior has been proposed (Singleton *et al.*, 2007). The translocation polarity on NA lattice divides proteins into subgroups A (3´– 5´) and B (5´– 3´). Both, SF1 and SF2 contain representatives of A and B type enzymes. It should be stressed that for most helicases the translocation activity is indispensable property for base separation. However, as previously noted, not all translocases act as helicases.

In addition helicases can be distinguished based on the type of DNA they are able to move on (ssDNA or dsDNA) and are designated as α and β type respectively (Singleton *et al.*, 2007). Interestingly, SF1 appears to have exclusively α-type proteins while SF2 contained both types (α and β) of enzymes (Singleton *et al.*, 2007).

Finally, helicases and translocases can be classified based on the type of nucleic acid substrate. The proteins of the SF1 and SF2 can have specificity to DNA or RNA substrates and in some cases to RNA-DNA hybrids. Several helicases from both superfamilies have been shown to act on both types of NA (Guenther *et al.*, 2009; Tackett *et al.*, 2001). Interestingly, the specialization to DNA or RNA is not conserved inside helicase family. Which structural features or sequence motifs dictate the specificity to distinct type of NA remain to be elucidated (Fairman-Williams *et al.*, 2010).

### 2.1.2.2. Mechanistic characterization of helicases

The helicase translocation or strand separation can be characterized by three main parameters: rate, processivity and step size.

The rate of a helicase is the number of bases translocated or base pairs separated per second. The unwinding and translocation rates of helicases vary from several tens to several hundred per second with some examples of unwinding rates exceeding several thousands of base pairs per second. Helicase activities could be regulated by interaction with different accessory proteins or complexes (Singleton *et al.*, 2007).

Based on processivity, helicases can be characterized as distributive or processive. A distributive enzyme conducts only one catalytic act before dissociating from substrate. A processive enzyme, in contrary, catalyzes multiple cycles before dissociation. Nearly all helicases are to some extent processive enzymes (Singleton *et al.*, 2007). Processivity is a number of bases translocated or unwound per single binding event of a protein. In other words, processivity is the possibility of an enzyme to make a step forward instead of dissociating from NA and is equal to the rate constant of moving forward divided by the sum of the forward moving and dissociation constants. Usually replicative helicases are expected to be highly processive while proteins involved in repair may not exhibit high processivity (Lohman & Bjornson, 1996).

The action of motor proteins, such as helicases could be viewed as a number of repetitive actions referred to as steps. Each step consists of processes such as NTP binding, hydrolysis, conformational changes and etc. The step size could be defined as distance change between the mass center of a protein per ATP molecule hydrolyzed or as a number of base pairs separated (translocated) between two successive rate limiting steps in the unwinding or translocation cycle (Lohman *et al.*, 2008).

### 2.1.2.3. Oligomerization state of SF1 and SF2 helicases

Probably, there is no straight answer concerning the oligomeric state of SF1 and SF2 enzymes. Generally, it is assumed that SF1 and SF2 proteins are monomeric or dimeric. In case of dimeric enzymes, many or even all functions can be performed by monomers (Lohman *et al.*, 2008). The unwinding activities of some helicase monomers are shown to have low processivity (Nanduri *et al.*, 2002). Usually oligomerization of such monomers enhances helicase activity (Byrd & Raney, 2005; Levin *et al.*, 2004). For some cases it is important to differentiate between the helicase and translocase activities, as some SF1 helicases can utilize regulation mechanism by which monomeric forms act only as translocases and dimerization or higher order oligomerization is needed for effective unwinding of NA (Cheng *et al.*, 2001; Maluf *et al.*, 2003). For example, translocation activity could be required for protein displacement and helicase activity might be harmful or useless in this case (Veaute *et al.*, 2005; Veaute *et al.*, 2003).

### *2.1.2.4. Models for helicase translocation*

Based on experimental data two models of helicase translocation on NA have been proposed: stepping motor and Brownian motor.

The stepping model (Figure 2A) implies two nucleic acid binding sites that can bind and release NA lattice and undergo conformational changes under the control of NTP hydrolysis (Velankar *et al.*, 1999; Yarranton & Gefter, 1979). In the stepping model version for monomeric helicases called "inch worm", the translocation event starts when the first tightly bound helicase domain anchors the protein to NA and the second weakly bound domain dissociates from NA to move further. When the second helicase domain tightly binds to NA, the first domain releases NA and moves closer to second domain.

This model could also be applied to dimeric helicases and is referred as rolling model (Wong & Lohman, 1992). In case of rolling model each molecule of dimeric helicase acts as a NA binding unit under the control of a separate NTP hydrolyzing center. The coordinated binding and release of NA by each subunit leads to unidirectional movement on NA. In contrast to the inchworm model, where the relative position of subunits to each other remains unchanged, the rolling model reminds walking with the leading molecule becoming trailing and *vice versa*.

An alternative mechanism for helicase translocation on NA is proposed by Brownian motor model (Figure 2B) (Levin *et al.*, 2005). In comparison to previous model, this mechanism includes two conformations of an enzyme (weakly and tightly bound) and only one NA binding site. When the helicase is tightly bound to NA, the helicase-nucleic acid energy profile becomes sawtooth and the possibility of the helicase to move is low, NTP binding makes conformational changes in protein and loosens the protein-NA contact. This changes the helicase-NA energy profile to shallow. Transient state allows helicase to randomly migrate in both directions. When ATP is hydrolyzed the sawtooth profile restores and this causes the helicase to conduct a step forward (power stroke) and slide down the energy slope. Depending on the location of helicase after transition state, the enzyme will remain in almost the same position or move forward.

It is worth to notice that both translocation models could be applied for single stranded and also for double-stranded NA translocating proteins (β-type), while their motor core interacts with a single strand of NA during their movement (Singleton *et al.*, 2007). The difference between SF1 and SF2 arises from the way how exactly the enzyme contacts the nucleic acid. Biochemical studies and crystal structures of SF2 enzymes support the model in which proteins make contacts with phosphodiester backbone of NA (Beyer *et al.*, 2013; Singleton & Wigley, 2002). In contrast, SF1 enzymes could be divided into SF1A enzymes, which interact with NA via nucleobases, and SF1B – their interaction resembles the mode of SF2 proteins (Raney *et al.*, 2013).

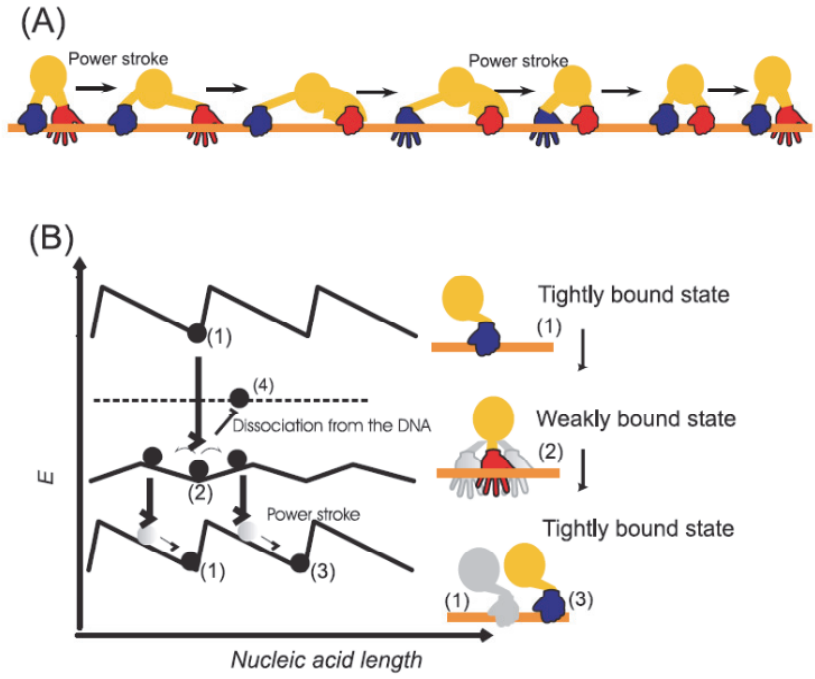


**Figure 2.** Two models of helicase translocation. In the stepping model (A) the first domain of helicase is tightly bound to NA, the second, weakly bound domain dissociates and moves forward. When it restores high affinity to NA, the second domain lowers the affinity to NA and moves ahead. Opened hand resembles weak binding of a domain and closed hand – tight binding. (B) The Brownian model of helicase translocation. (1) In tightly bound state (closed hand) helicase-NA energy profile is sawtooth and helicase is trapped in deep energy well. Weakly bound (opened hand), transient state allows helicase to move forward, backward (2) or even dissociate (4) from NA. When the tight biding of helicase is restored (closed hand), the deep energy profile turns back and helicase moves forward (3) or stays at the same position (1) depending on where it migrated during transition state. Yellow circles depict protein and orange line NA. Adapted from (Patel & Donmez, 2006).

### *2.1.2.5. Nucleic acid unwinding*

Two major mechanisms of NA unwinding by helicase proteins have been proposed. Both mechanisms assume that a helicase translocates on ss nucleic acid by any of the previously described mechanisms. When the helicase encounters a region of ds NA, the duplex separation occurs. Depending on the way the duplex is separated, the unwinding mechanism is defined as passive or active. By using the passive mechanism, a helicase does not interact with ds nucleic acid region and simply waits until the edge of the duplex opens due to thermal fluctuations. Helicase advances to this opening and physically blocks the base pair from reformation. In active unwinding mechanism helicase

interacts with duplex NA and facilitates its unwinding by the destabilization of base pairs (Lohman, 1992). The active model predicts that the unwinding rate of the enzyme can reach the rate of enzyme translocation, while the unwinding rate of passive enzyme is lower compared to translocation due to waiting time on a strand. The described models are not distinct categories, but resemble two extremes with many possibilities in between (Betterton & Julicher, 2005).

The physical mechanism of how destabilization occurs is not largely understood. The active unwinding could be a result of a protein-DNA contact that causes the destabilization of strands or the protein may exert a force on duplex and simply pry it apart (Pyle, 2008). Byrd and Raney deduced from avaliable crystallografic structures of SF2 proteins that a number of NA unwinding enzymes contain a specific beta-hairpin loop in a position, where it could separate dsNA; alternatively, a similarly positioned wedge made of two alpha-helices can play the same role. In contrast, crystal srtuctures of enzymes that have only translocase activity do not reveal such a structure (Byrd & Raney, 2012).

An alternative way of duplex base pair separation has been proposed for DEAD-box family of SF2 proteins. In contrast to previously described mechanisms of unwinding, where translocation on a single DNA strand is obligatory, the DEAD family proteins do not exhibit translocation activity. Proteins of this family of RNA helicases bind to dsRNA in an ATP-dependent manner and bend a single strand of the duplex thus causing local duplex separation. The resulting unwinding is sufficient for spontaneous melting of the remaining duplex region. In case of longer duplexes, enzyme dissociates and ds region reanneals back. After ATP degradation, conformational changes lead to protein dissociation from RNA and enzyme recycling (Chen *et al.*, 2008; Liu *et al.*, 2008; Yang *et al.*, 2007).

### 2.1.3. SF2 helicases involved in genome maintenance

Helicases and nucleic acid translocases are involved in all aspects of NA metabolism. Genome replication and maintenance of its functionality are key processes for every form of life. The most well-known example of helicase involvement in DNA maintenance is the replicative helicase that unwinds duplex DNA during replication. Classical replicative helicases are homo- or heterohexameric representatives of SF3-SF6 and therefore are out of the scope of this study.

SF2 helicases are a large group of diverse proteins involved in wide spectrum of processes such as transcription, repair, chromatin rearrangement and all aspects of RNA metabolism (Byrd & Raney, 2012). Based on sequence information, structural and mechanistic data SF2 was divided into 9 helicase families, 1 group and 2 subgroups. Not all members of SF2 are able to translocate on NA and perform duplex separation as some of them can only translocate on NA and others unwind NA without translocation (Table 1) (Fairman-Williams *et al.*, 2010).

**Table 1.** Mechanistic characteristics and enzymatic activities of SF2 protein families.

| Protein family | NA preference | | NTP preference | | | | Unwinding polarity | | Functional classi-fication[1] | Activities |
|---|---|---|---|---|---|---|---|---|---|---|
| | DNA | RNA | A | G | C | U/T | 3´5´ | 5´3´ | | |
| DEAD-box | | + | + | | | | + | + | n/a[2] | dsRNA unwinding |
| DEAH/RHA | + | + | + | + | + | + | + | +[3] | SF2Aα | ssRNA translocase dsRNA unwinding |
| NS-3/NPH-II | + | + | + | + | + | + | + | | SF2Aα | ssRNA translocase dsRNA unwinding |
| Ski2-like | + | + | + | | | | + | | SF2Aα | ssRNA translocase dsRNA/dsDNA[4] unwinding |
| RIG-I-like | + | + | + | | | | + | | SF2Aα/β | dsRNA translocase[5] branched DNA unwinding[6] |
| RecQ-like | + | | + | | | | + | | SF2Aα | ssDNA translocase dsDNA unwinding |
| RecG-like | + | | + | | | | + | | SF2Aβ | dsDNA translocase branched DNA unwinding |
| Swi/Snf | + | | + | | | | n/a | | SF2β | dsDNA translocase branched DNA unwinding [7] |
| T1R | + | | + | | | | n/a | | SF2β | dsDNA translocase |
| Rad/XPD | + | | + | | | | | + | SF2Bα | ssDNA translocase dsDNA unwinding |

[1] Functional classification according to system proposed by Wiley and co-workers (Singleton *et al.*, 2007). [2] DEAD-box proteins do not unwind RNA with defined polarity. [3] Several DEAH are able to unwind in both directions, but 3´–5´is more preferred. [4] Ski2-like family has Hel308 protein which unwinds branched DNA (Marini & Wood, 2002; Tafel *et al.*, 2011). [5] RIG-I-like family proteins can also unwind dsRNA. [6] RIG-I-like family proteins exhibit also branched DNA unwinding activity (Gari *et al.*, 2008a; Nishino *et al.*, 2005). [7] Some Swi/Snf family act on branched DNA (Betous *et al.*, 2012; Blastyak *et al.*, 2010). Modified from (Byrd & Raney, 2012; Fairman-Williams *et al.*, 2010).

Interestingly, the classification of the proteins in SF2 does not guaranty similar structural or mechanistic behavior and biological function of the different members of this superfamily. As the DEAD-box, DEAH/RHA and NS-3/NPH-II family enzymes are RNA helicases, they will not be reviewed further. I will also not discuss Type 1 restriction enzymes (T1R family), which defend bacteria from foreign DNA. The further review will be focused on SF2 DNA helicases involved in processing of branched molecules, recombination, recombination mediated repair and maintenance of genome integrity.

#### *2.1.3.1. Hel308 proteins of Ski2-like family*

Isolated as a mutation sensitive to DNA-cross-linking reagents in *Drosophila*, *mus308* gene (Boyd *et al.*, 1990) contains DNA polymerase domain in its C-terminus and a putative DNA helicase in the N-terminus. Search for homologs of the *Drosophila* mus308 helicase accessory domains resulted in the discovery of HEL308 (HELQ) helicase in mouse and human (Marini & Wood, 2002), HEL-308 in *C.elegans* (Muzzini *et al.*, 2008) and Hel308 in archaeon (Guy & Bolt, 2005). Surprisingly, no homologs were found in bacteria and yeasts.

In general, Hel308 ATPase activity is most efficiently stimulated by ssDNA and *in vitro* the protein binds to dsDNA with ss overhangs and to a variety of replication fork resembling structures (Figure 3A-E) (Guy & Bolt, 2005; Tafel *et al.*, 2011). Human and archaeal proteins exhibited moderate unwinding activities with dsDNA substrate having 3′ ss overhang and different forked substrates. Unwinding of forked structures containing only nascent lagging strand was the most effective (Guy & Bolt, 2005; Tafel *et al.*, 2011).

In addition, ssDNA binding protein RPA (Replication Protein A) stimulated the unwinding activities of human HEL308 (Marini & Wood, 2002; Tafel *et al.*, 2011). Physical interaction between archaeal RPA and the C-terminal domain of archaeal Hel308 was shown to modestly stimulate the helicase activity, giving rise to a proposal that ssDNA bound RPA may function as a loading platform for Hel308 (Woodman *et al.*, 2011). Archaeal and human Hel308 have shown different activities on replication fork-like substrates with nascent lagging strand only. Human HEL308 is primarily separating parental duplex and the ds region with 3′ overhang (parental strand with nascent lagging strand on it) as a secondary reaction. Also, human enzyme fails to process Holliday junction (HJ) resembling structures (Figure 3I) and the replication fork structure with both nascent strands also inhibits the activity of HEL308 (Tafel *et al.*, 2011). On the contrary, the archaeal Hel308 preferentially unwinds nascent lagging strand from replication fork like structure and is active on replication forks with both nascent strands present. Hel308 exhibits minor HJ processing activity (Guy & Bolt, 2005) but Hel308 homolog from another archaea, the Hjm protein, is much more potent on this type of substrate (Fujikane *et al.*, 2005).

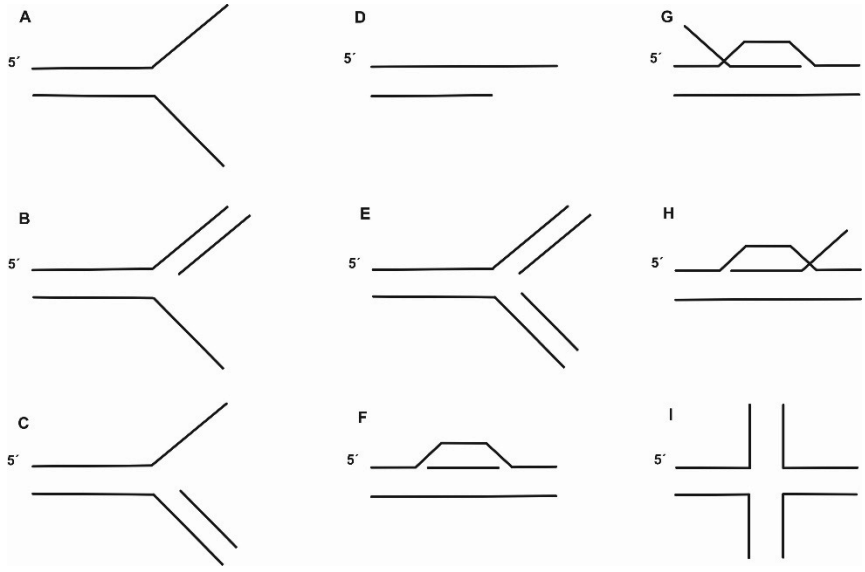


**Figure 3.** DNA substrates utilized by human Hel308 and archeal HEL308 proteins. (A) Flayed duplex. (B) Fork with nascent leading strand. (C) Fork with nascent lagging strand. (D) dsDNA with 3´ ss overhang. (E) Fork with both leading and lagging strand. (F) Displacement loop (D-loop), (G) D-loop with 5´ssDNA overhang and (H) D-loop with 3´ssDNA overhang. (I) Holliday junction.

Hel308 can unwind different invading strands of D-loop substrates (Figure 3F-H) (Guy & Bolt, 2005); however, such substrates have not been tested with human HEL308. Taken together, Hel308 proteins both from archaea and higher eukaryotes utilize their unwinding activities on stalled replication forks with nascent lagging strands to provide ssDNA template for loading proteins required for replication restart or for generating a signal for DNA damage checkpoints via unwinding parental strands or the nascent lagging stand of the replication fork (Guy & Bolt, 2005; Tafel *et al.*, 2011). Biochemical and genetic studies of HELQ-1, the homolog of Hel308 in *C.elegans*, revealed that HELQ-1 can disrupt the dsDNA-RAD51 filaments, supporting the idea that Hel308 is involved in replication mediated repair of damaged DNA (Ward *et al.*, 2010).

### *2.1.3.2. The RecQ family: watchmen of genome*

The first prototypical member of the RecQ family was identified in *E.coli* more than 30 years ago (Nakayama *et al.*, 1984). Since then, RecQ proteins have been found in all three kingdoms of life. Unicellular organisms have generally single RecQ helicase (RecQ in *E.coli* and Sgs1 in *S. cerevisiae*), while human have five: *BLM*, *WRN*, *RECQ4*, *RECQ1* and *RECQ5*. Typical RecQ protein consists of three domains: the helicase core, the RQC domain (RecQ carboxy-terminal) unique for RecQ family and the HRDC domain (Helicase and RNAse D

C-terminal domain) which is least conserved and could be found in other helicase families (Figure 4) (Larsen & Hickson, 2013). The helicase domain defines RecQ as a SF2 protein and confers the ability to couple ATP hydrolysis with strand separation activity (Fairman-Williams *et al.*, 2010). The RQC domain is composed of $Zn^{2+}$-binding subdomain and winged-helix subdomain (WH). The RQC domain is essential for stable binding of DNA, overall protein stability and is evolutionarily conserved (Guo *et al.*, 2005; Liu *et al.*, 2004). The crystal structure of RECQ1 protein and the RQC fragment of WRN showed that RQC domain is involved in DNA strand separation while the helicase core confers the translocase activity (Kitano *et al.*, 2010; Pike *et al.*, 2009). In addition, the RQC domain may also confer the ability to bind specific DNA structures to RecQ helicases (Huber *et al.*, 2006). The C-terminal HRDC domain is an auxiliary DNA binding domain. Its sequence and binding affinities for different DNA substrates vary between members of the RecQ family. This fact may explain the functional diversity of RecQ proteins (Larsen & Hickson, 2013).

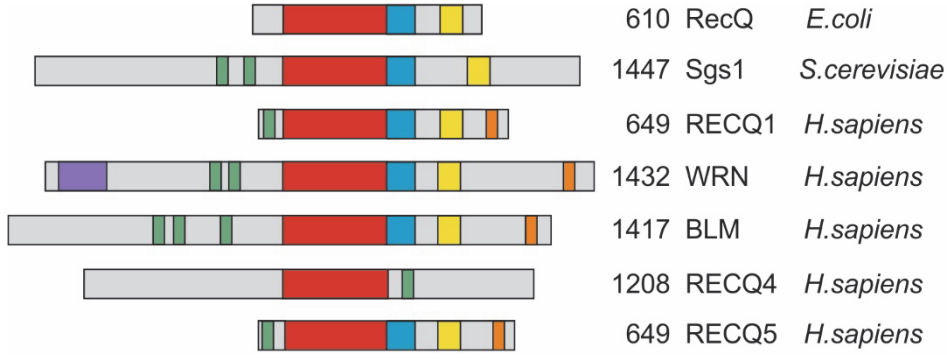


**Figure 4.** The structural features of RecQ helicases show high degree of conservation from bacteria through humans. The helicase core is depicted red. The RQC (blue) and HRDC (yellow) domains are present almost in every RecQ protein and are thought to mediate interactions with nucleic acid or other proteins. In addition to the most conserved domains, RecQ proteins have acidic regions (green) that are involved in protein-protein interactions and some of the RecQ proteins have nuclear localization sequences (orange). WRN protein has an exonuclease domain (purple). The number of amino acids (aa) for each protein and the names are indicated on the right. Modified from (Bernstein *et al.*, 2010).

The proteins of RecQ family function as complex with other proteins. *E. coli* RecQ activity is stimulated by the single-stranded DNA binding protein (SSB) via indirect trapping of ssDNA that prevents the formation of inactive ssDNA-RecQ complexes (Harmon & Kowalczykowski, 2001). After that, the direct interaction of SSB with WH subdomain of RecQ has been shown (Shereda *et al.*, 2009). Additional interaction with type IA topoisomerase Top3 is needed to release negatively supercoiled DNA resulted from RecQ unwinding activity (Harmon *et al.*, 1999). Finally, RecQ together with SSB and Recombination

protein A (RecA) act to promote RecA assembly in the homologous pairing reaction (Harmon & Kowalczykowski, 1998). All these interactions are highly conserved throughout evolution as Sgs1 in *S. cerevisiae* directly binds to Rad51 and interacts with Top3 and the oligonucleotid/oligosaccharide binding-fold (OB-fold) containing Rmi1 protein (Chang *et al.*, 2005; Gangloff *et al.*, 1994; Wu *et al.*, 2001). Among the human RecQ helicases, only BLM forms enzymatic complex with type IA topoisomease and a homolog of Rmi1. These findings strongly suggest that BLM is the functional homolog of *E.coli* RecG and Sgs1, at least in processes where the interaction with Top3 and Rmi1 is required.

RecQ helicases are acting on a wide variety of substrates *in vitro* and their preferences are largely overlapping. The RecQ proteins are 3′ –5′ DNA helicases that can unwind 3′ overhang dsDNA, forked DNA, bubble structures, D-loops, G-quadruplexes and Holliday junctions (Figure 5). These substrates were shown to be processed by BLM and WRN which are the two most extensively studied helicases of the family (Chu & Hickson, 2009). RECQ1 displays similar substrate preference except for the inactivity on G-guadruplexes which, in contrast, are the best substrates for WRN and BLM (Popuri *et al.*, 2008). All three proteins can also promote branch migration of HJ (Chu & Hickson, 2009).

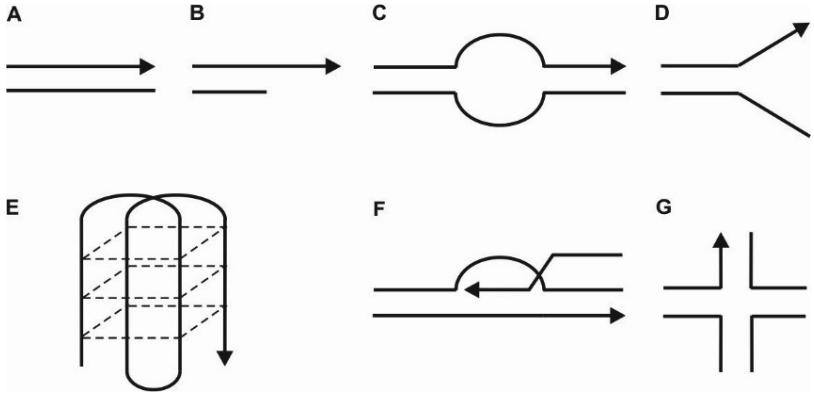


**Figure 5.** The common substrates of RecQ family enzymes. (A) Blunt-ended dsDNA substrate. The less preferable substrate and was shown to be unwound only by *E.coli* RecQ and RECQ4. (B) dsDNA with 3′ ss overhang. (C) Bubble in dsDNA. (D) Flayed duplex. (E) G-guadruplex DNA. (F) Displacement loop (D-loop), (G) Holliday junction. The displacement loop (D-loop) and the four-way Holliday junction are early and late intermediates in the homologous recombination process. Adapted from (Chu & Hickson, 2009).

Not all members of RecQ family can utilize such a wide variety of substrates. For example, RECQ5 prefers only forked DNA and has low activity on HJ and bubble DNA (Ozsoy *et al.*, 2003) and RECQ4 unwinds forked duplexes, bubbles and blunt dsDNA (Xu & Liu, 2009). The helicase activity of RECQ4 is believed to be “masked” by strong DNA annealing activity which was also

demonstrated for BLM helicase (Xu & Liu, 2009). Same authors found another DNA unwinding domain located in the N-terminus of RECQ4 that lacks any homology to known helicases (Xu & Liu, 2009).

The enzymes of RecQ family were shown to be involved in different stages of recombination and replication. HR is a desirable process needed for double-stranded break (DSB) repair but, on the other hand, excessive recombination is harmful. Depending on context, RecQ proteins were shown to have pro- and anti-recombinogenic roles. As pro-recombinases, Sgs1 and BLM defend the genome from excessive crossovers by different ways. First, they participate in additional (more extensive) resection of blunt DNA ends during the initial stage of HR (Mimitou & Symington, 2008; Nimonkar *et al.*, 2008; Zhu *et al.*, 2008). This activity may ensure the switch from non-homologous end joining (NHEJ) to the non crossing-over pathway of HR (Chu & Hickson, 2009). In case when DSB is resected to limited extent, the formed double HJ can be resolved by BLM-TOP3-RMI1 complex in an alternative way to conventional HJ dissolution known as decatenation which produces exclusively non-crossover products (Wu & Hickson, 2003). Finally, after extensive resection, the possibility to generate double HJ is lost and the break is repaired via the synthesis dependent strand annealing (SDSA) pathway, producing again only non-crossover products (Chu & Hickson, 2009). As anti-recombinase, BLM can displace invading strand from the D-loop and disrupts RAD51-ssDNA filaments, thus aborting HR in the initial stage (Bachrati *et al.*, 2006; Bugreev *et al.*, 2007). Human RECQ5 has also been shown to prevent the formation of D-loops by disrupting RAD51-ssDNA filaments (Hu *et al.*, 2007).

RecQ proteins were shown to be involved in the maintenance of DNA replication. Many enzymes of this family are able to unwind G-guadruplexes and hairpins which act as roadblocks for replication machinery (Chu & Hickson, 2009). In addition, when replication is blocked by a DNA lesion, RecQ helicases can catalyse regression of replication forks. The resulting HJ can be migrated back by RECQ1, BLM and WRN to overcome the lesion or can be resolved by HJ resolvase, leading to the replication fork collapse with subsequent restart of this fork by HR (Chu & Hickson, 2009).

#### *2.1.3.3. RecG family of proteins*

RecG was originally found in a screen for recombination deficient mutants in *E.coli* (Storm *et al.*, 1971). The recG deletion strain exhibited sensitivity to the crosslinking agent mitomycin C and mild sensitivity to UV and ionising radiation (Lloyd, 1991; Lloyd & Buckman, 1991). RecG is conserved in almost every bacterial species and there are probably no homologs of RecG in fungi or animals. Recently, a plant homolog of RecG was found to be targeted into plant organelles. The plant recG protein expressed in *E.coli* could partially complement the deletion of the bacterial homolog, showing at least partial functional conservation (Odahara *et al.*, 2015; Wallet *et al.*, 2015).

*E.coli* RecG is a monomeric protein with molecular mass of 76,5 kDa that consists of the N-terminal wedge domain and the classical helicase core that can translocate on DNA and utilize ATP as the energy source (Figure 6D). The wedge domain is connected to the helicase core via an α-helical linker. The wedge domain has a unique structure not found in any other helicase and is involved in binding and unwinding of branched DNA structures (Briggs *et al.*, 2005; Singleton *et al.*, 2001).

RecG can bind and unwind a wide spectra of DNA substrates like replication forks with nascent lagging and leading strand or both (McGlynn & Lloyd, 2001b), different D-loops, HJ (Figure 3, substrates B,C,E-I), (Lloyd & Sharples, 1993; McGlynn *et al.*, 1997) and RNA oligonucleotide containing R-loops (Vincent *et al.*, 1996). RecG prefers to unwind substrates with nascent lagging strand, catalyzes replication fork reversal and branch migrates resulting HJ (Figure 6A and 6B) (McGlynn & Lloyd, 2000; Whitby *et al.*, 1993).

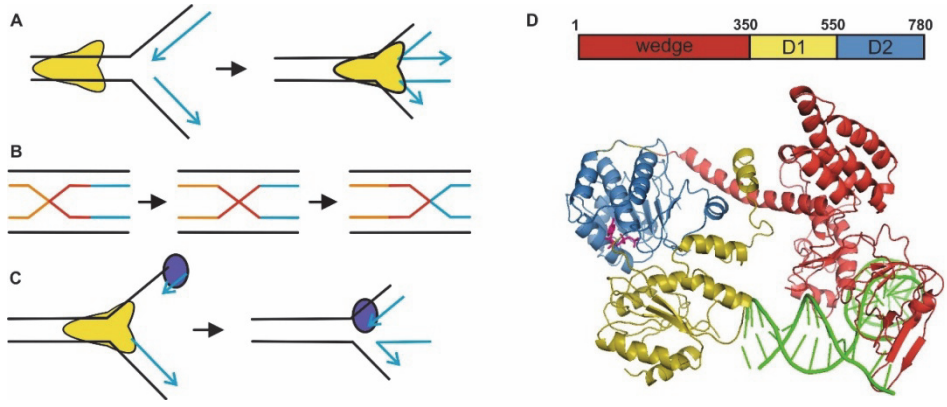


**Figure 6.** Schematic presentation of fork reversal and branch migration catalyzed by RecG. (A) Fork reversal catalyzed by RecG. Parental strands depicted as black lines. Nascent leading and lagging strands depicted as blue. The 3´ ends of nascent strands are indicated as arrows. RecG is a yellow shape. (B) Branch migration reaction. Homologous regions of two templates are exchanging strands. Migration proceeds through differently colored regions. (C) Replication fork stabilization by RecG (yellow) and PriA (purple). (D) Schematic representation of *Thermotoga maritima* RecG and RecG crystal structure bound to three-way DNA junction. Numbers correspond to amino acid residues. The wedge domain is red, helicase core domains are yellow (domain 1) and blue (domain 2), DNA is green and ATP bound to domain 2 is pink (Protein Data Bank code 1GM5).

As many other DNA repair proteins, RecG is specifically interacting with SSB (Buss *et al.*, 2008). The interaction is mediated via C-terminus of the SSB protein and resembles interactions with other DNA repair proteins (Shereda *et al.*, 2008). Recent computer modulations predict that RecG interacts with SSB via the wedge domain (Bianco & Lyubchenko, 2017) and this interaction assists the loading of RecG onto stalled replication fork (Bianco, 2015; Sun *et al.*, 2015).

As RecG is acting on many different substrates *in vitro*, a logical question arises on its role in the bacterial cell. Biochemical characterization of RecG and genetic studies with *recG ruv* double mutants lead to a common view that RecG is involved in DNA repair where it catalyzes replication fork reversal and the resulting HJ is a further substrate for branch migration for the multimeric enzyme Ruv complex that has a RuvC subunit capable of dissecting HJ (Lloyd, 1991; McGlynn & Lloyd, 2001a). Recent reviews centered on biological functions of RecG both pointed at lack of *in vivo* evidence supporting this conception (Azeroglu & Leach, 2017; Lloyd & Rudolph, 2016). Interestingly, it was found that RecG regresses partial replication fork until reaching the leading strand, whereupon another RecG family helicase PriA holds both strands in the fork stabilization model (Figure 6C) (Tanaka & Masai, 2006). Based on these and other findings, a novel explanation to genetic redundancy between RecG and RuvAB has recently emerged. According to this hypothesis, both previously mentioned enzymes are involved in the stabilization of joint DNA molecules during double-stranded DNA break repair pathway (DSBR). Such a joint molecule arises from RecA generated D-loop which at the side of HJ is branch migrated by RuvAB and stabilized by cooperative action of RecG and PriA from the other side. PriA next loads the bacterial replication helicase DnaB on the lagging strand of the generated replication fork and DNA synthesis can proceed (Azeroglu *et al.*, 2016).

Another function of RecG in *E. coli* DNA metabolism is to prevent DNA amplification at the terminal sites of bacterial chromosome. Normally, bacterial genome replication is strictly regulated. It starts from a single origin and the established replication forks proceed in bipolar manner until they meet in broadly defined termination area. Upon deletion of RecG, abnormal over-replication of DNA in this area occurs (Rudolph *et al.*, 2013). There are several hypotheses on how RecG could limit over-replication. When two replication forks converge, 3´flap structure on dsDNA is generated. The 3´ flap structure resembles a fork structure with the lagging strand and is normally degraded by a 3´exonuclease or converted into 5´ flap by RecG and degraded by a 5´ exonuclease (Lloyd & Rudolph, 2016). If the 3´flap structure is not removed, it could be targeted by the PriA protein to facilitate re-replication (Rudolph *et al.*, 2013). According to a second scenario, RecG directs the proper loading of PriA on an arrested or newly formed replication fork. As a result of RecG deletion, PriA is targeted to the replication fork in a wrong direction, leading to the over-replication of previously amplified DNA (Azeroglu & Leach, 2017). Further studies are needed to clarify the correctness of proposed hypotheses.

#### *2.1.3.4. SMARCAL1 protein of SWI/SNF family*

The SMARCAL1 protein (SWI/SNF related, matrix-associated, actin-dependent, regulator of chromatin, subfamily A-like 1), also known as HARP (HepA-related protein), is a SNF2-family protein of SF2. The orthologs of human

protein have also been found in mouse, rat and other multicellular eukaryotes including *C. elegans*, but not in yeast, suggesting a unique function in higher eukaryotes (Coleman *et al.*, 2000; Ghosal *et al.*, 2011). Mutations and deletions in the coding sequence of SMARCAL1 were shown to cause Schimke immuno-osseous dysplasia (SIOD) which is a multi-system disorder with complex phenotypes (Boerkoel *et al.*, 2002). The depletion of SMARCAL1 protein increases spontaneous DNA damage and hypersensitivity to DNA damaging agents, suggesting that the protein is involved in the repair of double-strand breaks (DSB), the repair of stalled replication forks or replication restart (Yusufzai 2009, Postow 2009, Ciccia 2009, Bansbach 2009, Yuan 2009).

Human SMARCAL1 is a 954 amino acids protein with molecular mass of 106 kDa. The protein contains a RPA-binding motif (RBM) and two highly conserved HARP domains in its N-terminus and a C-terminal helicase domain (Figure 7).


**Figure 7.** Schematic representation of the human SMARCAL1/HARP protein. RBM is a domain critical for the binding of RPA. Two tandem HARP domains confer the annealing function of the protein. The classical helicase core converts the energy of ATP hydrolysis into directional movement on NA lattice.

The first 30 N-terminal aa of SMARCAL1 exhibit a very high rate of conservation among different species and encode the RPA interacting motif. These findings led to assumption that SMARCAL1 may also interact with RPA and further studies revealed an interaction of this protein and the C-terminal part of RPA32, the subunit of the heterotrimeric RPA protein. This interaction is persistent and is critical for the function of SMARCAL1 in human cells as RPA recruits SMARCAL1 to stalled replication forks (Bansbach *et al.*, 2009; Ciccia *et al.*, 2009; Postow *et al.*, 2009; Xie *et al.*, 2014; Yuan *et al.*, 2009; Yusufzai *et al.*, 2009).

Another distinctive feature of SMARCAL1 is the twin HARP domain. The first clue to understand the biochemical role of SMARCAL1 protein was made when the DNA annealing activity of this protein was discovered (Yusufzai & Kadonaga, 2008). The annealing activity of SMARCAL1 was later shown to be dependent on the twin HARP domains (Ghosal *et al.*, 2011). Studies with different SMARCAL1 mutant versions showed the importance of the HARP domains *in vivo*: the deletion of the two HARP domains leads to the inactivation of the protein. In addition, chimeras of two SNF2 family proteins which lacked annealing helicase activity, were fused with the twin HARP domains and the chimeric proteins exhibited DNA annealing and ATPase activities *in vitro*, leading to assumption that the HARP domain is a distinct functional domain (Ghosal et al., 2011). Next study on HARP domains expanded this finding by

revealing that the dual HARP domain is able to weakly bind forked DNA and that the presence of only the second, C-terminal HARP (HARP2) domain is sufficient for DNA binding, annealing and other activities of SMARCAL1 *in vitro.* Furthermore, HARP2 together with the helicase domain form a structural and functional core which is sufficient for translocation (Betous *et al.*, 2012).

SMARCAL1 as an ATPase was shown to be stimulated by substrates which combined dsDNA with ssDNA regions (Betous *et al.*, 2012; Yusufzai & Kadonaga, 2008) and further studies showed that SMARCAL1 can bind, regress or restore replication forks and perform DNA branch migration in ATP dependent manner *in vitro* (Betous *et al.*, 2013; Betous *et al.*, 2012). These further experiments led to a model where the activity of SMARCAL1 on stalled replication fork is modulated by RPA. SMARCAL1 regresses stalled replication forks with a leading strand gap into an intermediate chicken foot structure. After the repair of the template and treatment of lagging strand with 5′–3′ exonuclease, SMARCAL1 restores replication fork in configuration where gap is already in the lagging strand. This modification is more suitable for replication restart (Betous *et al.*, 2013). Presented findings suggest that SMARCAL1 promotes the repair and restart of the damaged replication fork.

As SMARCAL1 activity is important for replication and genome integrity it should be properly regulated. The ATR (ataxia telangiectasia and Rad3-related protein) kinase, that regulates many proteins involved in replication stress response, was shown to regulate SMARCAL1 activity by phosphorylation (Bansbach *et al.*, 2009; Postow *et al.*, 2009). The phosphorylation of S652 by ATR kinase decreases SMARCAL1 activity on replication fork and therefore balances its activity. It is important to fine-tune SMARCAL1 activity as overexpression and suppression of this protein were shown to generate dsDNA breaks via various mechanisms (Bansbach *et al.*, 2009; Couch *et al.*, 2013).

Recently, SMARCAL1 was shown to be involved in the replication of telomeres. This activity was RPA independent leading to assumption that on telomeres SMARCAL1 resolves another type of replication-associated stress than on conventional replication forks, but studies are needed to understand the role of SMARCAL1 on telomeres (Poole & Cortez, 2016).

#### *2.1.3.5. FANCM of RIG-I like family*

Fanconi anemia (FA) is a rare genetic disease which leads to chromosomal instability, cancer predisposition, bone marrow failure and developmental abnormalities. The distinctive feature of the FA cells is their hypersensitivity to induced DNA interstrand crosslinks (ICL) suggesting that FA cells are defective in one or more steps in specific DNA repair pathway. FANCM is a component of the FA pathway consisting of at least 20 genes (FANCA-T) (Duxin & Walter, 2015).

FANCM was simultaneously found as the FAAP250 protein which co-immunoprecipitated together with several FA core complex components and as

vertebrate ortholog of archaeal Hef helicase-nuclease involved in replication fork processing. Mph1, the yeast homolog, is a helicase functioning in error-free recombination repair pathway (Meetei *et al.*, 2005; Mosedale *et al.*, 2005).

FA pathway is activated in response to ICL. In this pathway, FANCM together with its interacting partners FAAP24 and MHF1/MHF2 dimer binds to a replication fork structure and recruits other FA core complex subunits (FANC A, B, C, E, F G, L and FA associated proteins). The main purpose of this assembly is to monoubiquitinate FANCI-FANCD2 dimer which acts down-stream in the DNA repair pathway (Duxin & Walter, 2015).

In addition to the role in assembly of the FA core complex, FANCM was shown to be a DNA-dependent ATPase and translocase, preferentially binding the replication fork and Holliday junction resembling structures (Gari *et al.*, 2008b; Meetei *et al.*, 2005). Further investigation showed the fork reversal, branch migration and D-loop dissociation activities of FANCM *in vitro*, the latter was specifically denoted as branch point translocation to emphasize the lack of helicase activity in this processes (Gari *et al.*, 2008b; Xue *et al.*, 2008).

Sequence analysis of the 250 kDa FANCM revealed a N-terminal helicase domain and a degenerate endonuclease domain in its C-terminus (Meetei *et al.*, 2005). FANCM contains several proteins interacting domains. The first protein, shown to interact with FANCM, was FAAP24 that forms a heterodimer via interaction with C-terminus of FANCM (Ciccia *et al.*, 2007). FAAP24 was previously not described as a member of the FA core. FAAP24 confers ssDNA binding preference to the heterodimer and may play an important role in the re-cognition of blocked replication forks with ssDNA gaps and therefore be a sensor of a DNA damage for the FA pathway (Ciccia *et al.*, 2007). While FAAP24 changes the DNA binding preference of FANCM, the branch migra-tion and fork reversal activities of heterodimer are not influenced (Gari *et al.*, 2008b) .

The second interaction partner of FANCM is the MHF complex made of histone-fold proteins MHF1 and MHF2 that form a tetramer consisting of two MHF1-MHF2 dimers and has a dsDNA binding activity itself (Tao *et al.*, 2012; Yan *et al.*, 2010). MHF tetramer in complex with FANCM promotes its dsDNA binding, branch migration and fork reversal activities (Yan *et al.*, 2010). FANCM interaction via its N-terminal region with MHF is vital for FA complex recruit-ment to start the DNA repair cascade (Yan *et al.*, 2010).

Numerous studies show that FANCM protein exists in different complexes and the cellular role of FANCM in the cell depends on the interacting partners. As previously mentioned, the FAAP24-FANCM-MHF complex localizes to the site of ICL and acts as a scaffold for FA pathway proteins that will “unhook” the ICL, bypass it and finish the repair procedure by homologous recombination (Xue *et al.*, 2015).

In addition, there are several FA independent roles of the FANCM enzyme in the maintenance of DNA integrity. FANCM and MHF orthologs have been found in the yeast where they are engaged in DNA repair damage, while the FA pathway appears only in vertebrates (Yan *et al.*, 2010). Thus, the function of

FANCM-MHF complex independent of FA pathway is not surprising. It has been shown that the complex promotes replication traverse of ICL and this activity depends on the translocation of FANCM (Huang *et al.*, 2013). Recent finding that replication protein PCNA interacts with FANCM under replication stress, connects FANCM with replication machinery that is required for ICL traverse (Rohleder *et al.*, 2016).

Besides overcoming the ICL caused by DNA damage, FANCM probably together with MHF is required for DNA replication under normal or non-DNA damaging stress conditions. This function relies on FANCM ability to reverse replication forks, while MHF was shown to enhance this activity. The regressed fork is an intermediate for further pathways of the replication fork restart (Xue *et al.*, 2015).

Another FA-independent role of FANCM is to suppress excessive sister chromatid exchange (SCE) supported by its ability to displace D-loops (Gari *et al.*, 2008a). By removing the invaded strand from a D-loop, FANCM directs the DNA double-strand break repair to the synthesis dependent strand annealing pathway that generates only non-crossover products (Xue *et al.*, 2015).

The interaction of FANCM with another well-known and previously discussed BLM-TOP3-RMI1/2 complex bridges the two pathways (FA and Bloom's syndrome (BS)) on stalled replication forks for efficient DNA repair. BLM-TOP3-RMI1/2 has implications into all previously discussed FANCM functions: FA pathway stimulation, recombination outcome control, ICL traverse and SCE suppression (Deans & West, 2009; Ling *et al.*, 2016; Rosado *et al.*, 2009). Taking into account BLM-TOP3-RMI1/2 functions and enzymatic properties, it is possible to propose that this complex is capable to support FANCM in all previously noted activities.

Finally, FANCM together with FAAP24 are involved in checkpoint signaling mediated by kinases and FANCM recruitment was shown to be mediated by its Ser1045 phosphorylation in response to genotoxic stress (Xue *et al.*, 2015).

#### *2.1.3.6. UvsW an orphan of SF2*

T4 bacteriophage is a DNA virus that encodes 300 protein genes needed for various processes during the viral life cycle including the components of DNA replication, repair and recombination machineries. The replication of viral genome occurs by two separate mechanisms: origin-dependent replication during early stages of the viral life cycle and recombination-dependent replication (RDR) in the late stage. UvsW, one of the helicases encoded by the T4 genome, is associated with transition between these two modes of replication (Derr & Kreuzer, 1990).

UvsW (UV sensitivity W) is a 58 kDa protein which together with other two proteins UvsX (strand invasion protein) and UvsY (mediator protein) was referred to as the WXY system, first shown to be involved in the tolerance of UV damage probably via recombinational repair pathway (Conkling & Drake, 1984).

The 503 aa long protein UvsW contains two domains that are separated by a short linker. The small N-terminal domain which comprises first 83 aa was shown to be structurally similar to dsDNA binding motif of the T4 transcription factor MotA and to the HARP domain of SMARCAL1 protein (see section *2.1.3.4*) (Mason *et al.*, 2014; Sickmier *et al.*, 2004). The C-terminal domain is the SF2 helicase core containing two RecA domains (Kerr *et al.*, 2007).

Initial experiments with UvsW-GST fusion proteins revealed ssDNA dependent ATPase activity of UvsW and its ability to unwind Y-shaped substrates composed of all-dsDNA arms, but inability to unwind linear dsDNA duplex (CarlesKinch *et al.*, 1997). Further *in vitro* studies revealed wide spectra of activities including unwinding of DNA substrates such as partial and full replication forks, HJ, D-loop and RNA oligonucleotide containing R-loop, reversal of stalled replication forks and DNA strand branch migration (Dudas & Kreuzer, 2001; Long & Kreuzer, 2009; Nelson & Benkovic, 2007; Webb *et al.*, 2007). Additionally, UvsW is able to anneal complementary ssDNA in ATP dependent manner (Nelson & Benkovic, 2007).

The data obtained from biochemical and genetic studies of UvsW suggest that the enzyme has several roles in T4 bacteriophage life cycle including replication, repair and recombination. Transition between origin-dependent replication and RDR late replication is regulated by different pathways and involves UvsW dependent unwinding of R-loops that are vital for initiation of origin-dependent replication (Dudas & Kreuzer, 2001).

Recombination-dependent replication starts when a 3´ overhang of the replicated genome copy invades into homologous dsDNA and forms a D-loop which is further utilized by the viral replication machinery (Kreuzer & Brister, 2010). Strand exchange process is mediated by viral recombination proteins UvsX, UvsY and UvsW. UvsW promotes the UvsX catalyzed strand exchange reaction and, thus, is directly involved RDR (Gajewski *et al.*, 2011).

In addition, the branch migration activity of UvsW could also play a role in RDR via stabilization and migration of the formed HJ after strand exchange reaction (Webb *et al.*, 2007). The RDR and DSB repair are tightly interconnected as DSB repair pathways are based on the same principles as RDR. Therefore, the branch migration and strand exchange activities of UvsW could also be applied for DSB repair in the T4 genome. Interestingly, during SDSA pathway of DSB repair, the 3´ overhang of the inserted strand has to be slightly extended, removed from the D-loop and annealed to another complementary strand of DSB. The D-loop dissociation and ssDNA annealing activities of UvsW suggest that the protein is involved in aforementioned processes (Nelson & Benkovic, 2007; Webb *et al.*, 2007).

As previously noted, UvsW was shown to regress replication forks *in vivo* and *in vitro* (Long & Kreuzer, 2008, 2009). While regressed replication fork is an intermediate that could be processed in several ways, the biological role of this event needs further clarification. Nelson and Benkovic showed that when the T4 phage replication machinery encounters a lesion in the leading strand template it was capable to synthesize lagging strand further over the site of this

lesion before stalling (Figure 8A-C) (Nelson & Benkovic, 2010). Such a stalled replication fork with a gap in the leading strand is then reversed by UvsW and the lagging strand is used as the template to synthesize the leading strand (Figure 8D). Rewinding of the modified stalled fork by UvsW allows replication to overcome the lesion and to continue the genome replication (Figure 8E). In this process of the leading strand bypass, UvsW is able to switch randomly the strand and thereby catalyzes both the regression and restoration of the replication fork (Manosas *et al.*, 2012).

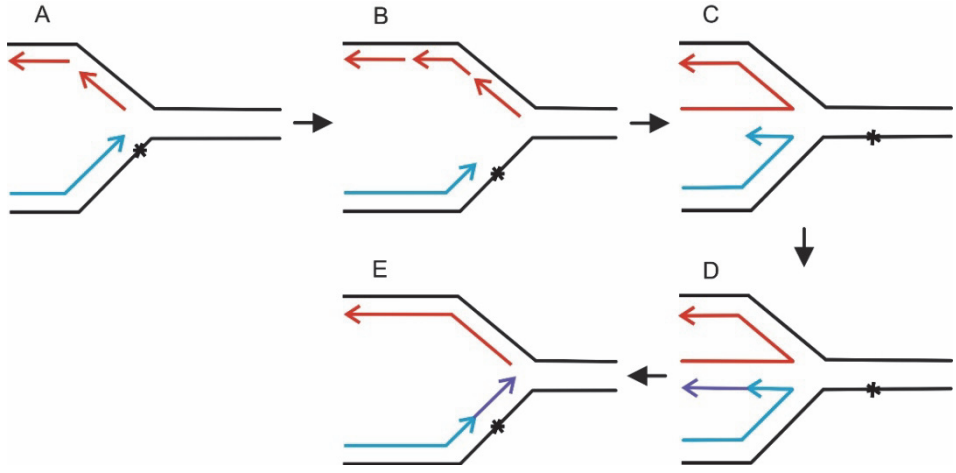


**Figure 8.** The lesion bypass mechanism in T4 bacteriophage. (A) Leading strand polymerase encounters a lesion in the template. (B) The lagging strand could be synthesized further before the replication machinery stalls. (C) The stalled fork is then regressed and (D) the 5´overhang used as a template for synthesis. (E) The rewinded leading strand bypasses the lesion and replication can be restarted. Leading strand – blue, lagging stand – red, lesion – asterisk. Synthesized leading strand – purple.

The fact that bacteriophage genome encodes for single DNA polymerase incapable to bypass the lesion region (not acting as translesion polymerase) and no reports of any host polymerase participating in T4 replication cycle also supports the view that this lesion bypass mechanism is the main pathway in the phage life cycle (Manosas *et al.*, 2012; Nelson & Benkovic, 2010).

Many helicases are regulated by their interaction partners and it seems likely that UvsW may also be regulated in a similar way. Therefore, further studies of potential interaction candidates such as the little-studied UvsW.1 together with the viral single stranded binding protein gp32 (Nelson & Benkovic, 2007), the recombination proteins UvsX and UvsY or some unknown protein could probably reveal new aspects of UvsW activities.

## 2.2. Mitochondrial genome of the yeast *Saccharomyces cerevisiae*

### 2.2.1. The many faces of mitochondrial DNA

Mitochondria are cellular organelles of eukaryotic organisms involved in energy production, biosynthesis of various molecules, cell signaling and apoptosis. Mitochondria originate from α-proteobacteria which was engulfed by the host cell following different scenarios (reviewed in (Roger *et al.*, 2017)) .

Mitochondria contain their own genome which is highly degenerated and has only a limited number of genes left. Most α-proteobacterial genes were relocated to nuclear genome during endosymbiotic gene transfer and are now functioning in other cellular compartments (Roger *et al.*, 2017). Mitochondrial gene content is very conserved among eukaryotes and contains the components of respiratory complexes I–V and mitochondrial translational machinery: rRNAs of the small and large subunits of mitoribosome and a full or partial set of mitochondrial tRNAs. In addition, mtDNA could encode ribosomal proteins (mainly in plants and protists), some subunits of the protein import and RNA maturation machineries and in one case multiple subunits of the mitochondrial RNA polymerase of *Reclinomonas americana* (Burger *et al.*, 2003; Gray *et al.*, 1999).

The size of mitochondrial genome varies significantly between different eukaryotic clades and even within them. The smallest known mt genome of *Plasmodium falciparum* (malaria parasite) has a size of 6 kb and contains only 5 genes. The largest genomes found in land plants could exceed 200 kb (f.e. 490 kb in rice) (Feagin, 2000; Notsu *et al.*, 2002). Interestingly, the coding capacity of mitochondrial genome does not correlate with size. The jacobid protozoan *R. americana* has a 70 kb mt genome with 97 genes encoded (Lang *et al.*, 1997), while aforementioned plant mt genomes carry 50–70 genes and animal genomes that are usually at least ten times smaller (15–20 kb) contain approximately 35 genes (Nosek & Tomaska, 2003). Such a disproportion between size and content is caused by the difference in the lengths of intergenic non-coding regions and introns (Burger *et al.*, 2003).

The observation of coding capacity and specificity of genes encoded by mtDNA reveals (1) that all the genes involved in biogenesis and other non-energy production are during the evolution transferred to the nucleus supporting, thus, the main evolutional specialization of mitochondria and (2) the full dependence of mitochondria from nuclear genetic library while mtDNA encoded proteins comprise roughly less than 1.5% of the whole mitochondrial proteome.

In most cases, mt genome is presented as many copies of a single chromosome. In contrast to such congeniality, mt genomes exhibit strong variation in their genetic organization. The first described mt genome architecture was the circular DNA from mitochondria of chick, mice and cows (Sinclair & Stevens, 1966; van Bruggen *et al.*, 1966). Taking into account the circularity of genome

in mitochondrial ancestor these findings seemed to be very reasonable and for many years it was assumed that mtDNA exists only as circular molecules even though, the first linear mt genome was discovered only a few years later (Suyama & Miura, 1968). By now, after accumulation of mtDNA studies in different species, it is clear that linear genomes represent a significant part if not the majority of mt genomes (Burger *et al.*, 2003). While animal mt genomes are mainly circular monomers and sometimes dimers, the linear genomes could appear as linear molecules with uniform genome length having different telomeric structures at their termini or polydisperse (heterogeneous length), linear head-to-tail molecules which are larger than genome unit size, contain no specific ends and, in addition, have a tiny population of circular molecules (reviewed in (Nosek & Tomaska, 2003). Both types of linear genomes were mainly found in different protist, fungal and plant species.

### 2.2.2. Mitochondrial DNA of *S. cerevisiae*

While mitochondria fulfill a variety of different functions, the mitochondrial genome itself encodes only a limited number of genes required for the proper functioning of this organelle. In the baker's yeast *S. cerevisiae,* at least 901 nuclear encoded proteins have been found to be transported into mitochondria and involved in various mitochondrial functions (Morgenstern *et al.*, 2017). The yeast mitochondrial genome (85,8-kb) is A+T rich with low gene density encoding for 7 protein components of respiratory chain complexes (*COX1*, *COX2*, *COX3*, *COB*, *ATP6*, *ATP8*, *ATP9*), one protein of the small ribosomal subunit (*VAR1*), 15S and 21S rRNAs, a complete set of 24 tRNAs and 9S RNA component of RNAse P (Foury *et al.*, 1998). The mitochondrial DNA is the only source of the previously mentioned respiratory chain proteins and therefore the functional mitochondrial genome is absolutely required for the proper work of respiratory chain.

The baker's yeast mitochondrial genome belongs to the class of linear mt genomes which are represented mainly by branched linear head-to-tail concatemers of various lengths (between 75–150 kb) (Bendich, 1996; Maleszka *et al.*, 1991).

In a yeast cell 50–100 copies of the mt genome are packaged into 10–40 protein-DNA complexes called nucleoids, where each nucleoid contains several copies of the mt genome. Nucleoids are anchored to the inner membrane of mitochondria (Chen & Butow, 2005; Westermann, 2014). The non-DNA component of nucleoid consists mainly of non-histone high mobility group packaging protein Abf2 (Diffley & Stillman, 1991) which protects mtDNA from damage and has an additional role in mtDNA recombination (MacAlpine *et al.*, 1998; Westermann, 2014). Other mtDNA packaging proteins are the bifunctional enzyme Ilv5, also implicated in branched amino acid synthesis and Aco1, an enzyme required for the Krebs cycle. These enzymes are involved in the remodeling of mt nucleoids in response to metabolic changes in the cell. The mitochondrial chaperonin Hsp60 binds to specific regions of mtDNA and is

required for proper nucleoid division. Finally, mt nucleoids contain many proteins required for DNA replication, transcription, recombination and repair (Chen & Butow, 2005; Westermann, 2014).

*S. cerevisiae* belongs to a relatively small group of petite-positive yeasts that can survive the loss of mtDNA and therefore exist without mitochondrially derived energy production. Yeast strains containing the functional or wt mitochondrial genome are designated as *rho*$^{+}$, mutants containing short noncoding direct or inverted repeats of mtDNA are *rho*$^{-}$ and mutants that totally lack mtDNA are *rho*$^{0}$. The simplicity of differentiation between the wt and *rho* mutants based on the inability of *rho*$^{0}$/*rho*$^{-}$ to grow on non-fermentable media has been a useful tool in search for novel mitochondrial proteins. The *rho*$^{0}$/*rho*$^{-}$ mutants appear in normal yeast populations with a frequency of 1–2% and their formation rate could remarkably be increased by the treatment of yeast culture with different chemicals or physical agents (Faugeron-Fonty *et al.*, 1979; Ferguson & von Borstel, 1992). In addition, the stability of wt mitochondrial genome depends of plethora of nuclear encoded genes that are directly involved in mtDNA metabolism (replication, recombination and repair), but also in mitochondrial gene expression, post-transcriptional modifications and translation. Surprisingly, many genes needed for processes seemingly unrelated to mtDNA such as maintenance of proper mt membrane structure and morphology, fatty acid metabolism, molecule transport and ion homeostasis also affect mt genome stability (Lipinski *et al.*, 2010). Connections between these pathways and mtDNA stability are not fully understood and require further elucidation.

### 2.2.3. Replication of *S. cerevisiae* mtDNA

The details of mtDNA replication in *S. cerevisiae* are not completely known and therefore the current state of our understanding will be summarized below. First, the synthesis of mtDNA is conducted by a single subunit mtDNA polymerase Mip1, which in addition to DNA polymerase activity has 3′–5′ proofreading exonuclease activity (Foury *et al.*, 2004). The yeast mitochondrial genome contains seven to eight (depending on the strain) conserved sequences regarded as replication origins designated as *ori*s or *rep*s (de Zamaroczy *et al.*, 1981; Foury *et al.*, 1998). Four of them containing functional mitochondrial promoter are thought to be active and are believed to act as initiation point for Rpo41 (mtRNA polymerase) primed bidirectional DNA replication (Figure 9A) (Baldacci *et al.*, 1984; Ramachandran *et al.*, 2016; Sanchez-Sandoval *et al.*, 2015), but the description of Rpo41 independent replication in *ori*-devoid *rho*$^{-}$ mutants (Fangman *et al.*, 1990) suggests that alternative mechanisms of mtDNA replication initiation must exist. The overturning of the all-circular mtDNA theory of the yeast mtDNA also suggested that the mode of genome replication could be rolling circle where linear tandem repeats of genomic units are produced from circular molecules (Figure 9C) (Maleszka *et al.*, 1991). In addition,

the alternative mode of replication initiation emerged after describing the ability of Mhr1 protein to catalyze homologous pairing and studies of its role in generation of mtDNA concatemers (Ling & Shibata, 2002). According to a proposed model the mt base excision repair (mtBER) protein Ntg1 produces dsDNA breaks in the *ori* region and the 5′ exonuclease Din7 generates the 3′ single-stranded DNA overhang that could be used by the recombinase Mhr1 for strand invasion into circular mt DNA molecule and to initiate the synthesis of mt genome concatemers by Mip1 (Figure 9B) (Ling *et al.*, 2007; Ling *et al.*, 2013) . In a few years later, Mgm101, another yeast mitochondrial Rad52-type recombinase capable to catalyze strand invasion was described (Mbantenkhu *et al.*, 2011) while the protein Mhr1 was found in mt ribosomes (Amunts *et al.*, 2014; Woellhaf *et al.*, 2016), and therefore further studies are required to clarify the exact roles of these proteins.

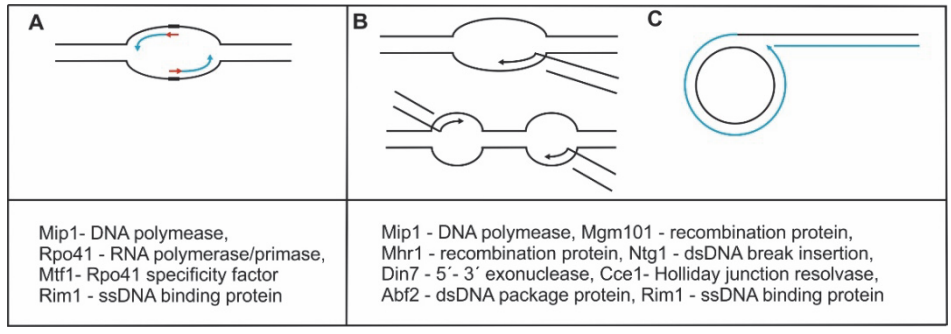


**Figure 9.** Proposed models of *S. cerevisiae* mtDNA replication and proteins associated with these processes. (A) *ori*-primed DNA replication model. Nascent leading strands produced by Mip1 are blue and RNA primers synthesized by Rpo41 are red. Rim1 covers free ssDNA and stimulates Mip1 primer utilization (Sanchez-Sandoval *et al.*, 2015). Mtf1 is a specificity factor of Rpo41p required for efficient and precise transcription initiation. The requirement of Mtf1 for replication initiation is a matter of discussion (Ramachandran *et al.*, 2016; Sanchez-Sandoval *et al.*, 2015). (B) RDR model. Double-stranded DNA break is produced by Ntg1 and further processed by Din7. Strand invasion which is catalyzed by Mgm101 or Mhr1, serves as primer for further amplification by Mip1. (C) Concatemeric DNA synthesis by the rolling circle mechanism. Nascent leading strands produced by Mip1 are depicted in blue.

Several studies of the cruciform cutting endonuclease Cce1 and the DNA packaging protein Abf2 have shown that these proteins are involved in the dynamics of recombination intermediates. Cce1 dissects Holliday junctions and, thus, it influences the segregation of mitochondrial genomes (Kleff *et al.*, 1992; Lockshon *et al.*, 1995). In addition, Cce1 was proposed to be implicated in concatemeric DNA formation pathway which acts in parallel to Mhr1-mediated pathway (Ling & Shibata, 2002). The level of Abf2 was shown to influence recombination in yeast mitochondria (MacAlpine *et al.*, 1998; Zelenaya-Troitskaya *et al.*, 1998). The fact that Abf2 preferentially binds to branched

DNA intermediates (HJ, replication forks) compared to linear dsDNA supports the previous findings (Bakkaiova *et al.*, 2016). It is tempting to speculate that both these proteins act in the same recombination/replication pathway, but direct evidence is still missing.

As previously mentioned, many questions concerning the replication in yeast mitochondria remain open. Interestingly, a hexameric replicative helicase, in contrast to metazoans, has not been found in yeast mitochondrial replication system. The DNA polymerase Mip1 can displace parental strands during DNA synthesis *in vitro* (Viikov *et al.*, 2011) rising a possibility that in yeast mitochondria the replicative helicase is not necessary. Another question remaining unclear is the synthesis of the lagging strand during mt replication. Biochemical experiments showing the potential of Rpo41 to promote RNA primed DNA synthesis on Rim1 coated substrates (Ramachandran *et al.*, 2016) support the model of bidirectional *ori*-primed replication and could explain the synthesis of the lagging strand during rolling circle replication. Moreover, similarly to metazoan system (reviewed by (Holt, 2009)) some protein factors that could act on maturation of Okazaki fragments are available in yeast mitochondria. These include the Rad27 flap endonuclease, Pif1 helicase, Cdc9 ligase and probably RNAse H1 (Donahue *et al.*, 2001; El Hage *et al.*, 2014; Kalifa *et al.*, 2009; Lahaye *et al.*, 1991).

As a conclusion, it should be noted that while the full understanding of the proportion between the transcription-dependent and recombination-dependent replication mechanisms is missing, a number of reports supporting the RDR as the leading mechanism in yeast mitochondria during last 15 years have emerged. Importantly, recent studies showed the extreme domination of RDR pathway in *rho*$^{+}$ genome (Prasai *et al.*, 2017).

### 2.2.4. Proteins involved in maintenance of mtDNA in *Saccharomyces cerevisiae*

Mitochondrial DNA is constantly exposed to various damaging agents of endogenous (f.e. reactive oxygen species (ROS) or exogenous (UV, toxins) origin. Therefore, cells have developed mitochondrial repair systems in addition to nuclear machineries. Enzymes known to be involved in mtDNA repair are summarized in Table 2. This table includes many proteins involved in other processes of mtDNA maintenance such as replication and recombination.

The simplest and best characterized repair system for the most abundant types of DNA lesions is mtBER. In the yeast mitochondria, four enzymes are involved in lesion recognition and excision: Rad27, Mip1, Cdc9 and Ntg1. Rad27p flap endonuclease participates in long-patched BER. During the final stages of repair, probably Mip1 polymerase incorporates the excised nucleotides and Cdc9 finishes the repair by ligation of the strand (reviewed in (Kaniak-Golik & Skoneczna, 2015)). A good example of multifunctionality in mitochondria are the mtBER enzyme Ntg1 that participates in mtDNA replication

(see previous chapter) and Rad27 recently shown to act on DSB repair in the yeast mitochondria (Nagarajan *et al.*, 2017).

Another canonical repair pathway, the mismatch repair in the yeast mitochondria is presented by Msh1 which has homology to the bacterial MutS protein and is a DNA binding ATPase recognizing mismatches (Chi & Kolodner, 1994; Reenan & Kolodner, 1992). The findings that the level of Msh1 influences the rate of HR in mtDNA support the idea that this protein has another role in mitochondria (Kaniak *et al.*, 2009; Mookerjee & Sia, 2006). It has been proposed that Mhr1 is using its ability to sense mismatches to conduct recombination surveillance, during which the protein promotes or rejects the HR, depending on the "quality" of invaded strands. This model seems to be very promising, while similar activities of Msh1 homologs were shown in other organisms. The involvement of Mhr1 in mtMMR and the importance of this pathway in *S. cerevisiae* mitochondria needs further clarification (Chen, 2013; Kaniak-Golik & Skoneczna, 2015).

Exonucleases are essential for the HR process during which they produce the 3′ invading strands from dsDNA breaks. In *S. cerevisiae* mitochondria, several proteins exhibiting exonuclease activity have been found (Table 2). MRX complex, Nuc1, Din7 all possess at least 5′–3′exonuclease activity and single deletions of each protein influence the rate of mtDNA recombination, but do not affect the stability of the mitochondrial genome. Probably, the biological functions of these exonucleases overlap or some of these proteins participate in more specific pathways of mtDNA repair (Chen, 2013; Kaniak-Golik & Skoneczna, 2015). Interestingly, Exo5 is an exception from this list, it has 5′–3′ exonuclease activity and can process only ssDNA. Furthermore, in contrast to other exonucleases, the deletion of Exo5 produces *rho*$^{-}$ mitochondrial genomes (Burgers *et al.*, 2010) suggesting a unique role for this protein in mtDNA maintenance. One possible scenario for Exo5 is that some mitochondrial helicase can unwind dsDNA duplex, thus, supporting the exonuclease with substrate to generate a recombinogenic 3′ end (Burgers *et al.*, 2010).

Interestingly, one report has indicated the involvement of NHEJ in mtDSBR. In this study, the Ku complex, that binds to broken dsDNA ends and initiates NHEJ, was shown to synergistically act with the MRX complex in mtDSBR. As the MRX exonuclease complex is associated with the initial steps in HR, the authors propose that both pathways are active in yeast mitochondria and are at least partially redundant (Kalifa *et al.*, 2012).

## 2.2.5. *S. cerevisiae* helicases involved in mtDNA maintenance

### *2.2.5.1. Pif1*

Helicases participate in different aspects of DNA metabolism, and therefore, it is reasonable to assume that some of them are found to be involved in mtDNA maintenance. The first DNA helicase discovered in *S. cerevisiae* mitochondria was the 5′–3′ distributive DNA helicase Pif1 that can unwind dsDNA regions with 5′ ssDNA overhangs or forked DNA structures and has similar activity with RNA-DNA duplexes (Boule & Zakian, 2007; Lahaye *et al.*, 1993). Pif1 was later shown to be a multifunctional protein implicated in various functions in the nucleus (reviewed in (Bochman *et al.*, 2010)). Initially, Pif1 was described as a protein required for mtDNA maintenance at higher temperatures and implicated in mtDNA maintenance via functioning in recombination and repair (Foury & Kolodynski, 1983; Foury & Lahaye, 1987). More specifically, Pif1 is involved in the repair or prevention of dsDNA breaks in specific regions of mtDNA (Cheng *et al.*, 2007). Pif1 could interact with various recombination factors such as Cce1 or Din7 to promote DNA repair via recombination or participate in the control of unwanted recombination events (Cheng *et al.*, 2007). Interestingly, Pif1 is thought to act on mtDNA maintenance also in recombination independent manner (O'Rourke *et al.*, 2002). The AT-rich mtDNA can potentially form many hazardous secondary structures at elevated temperatures and Pif1 may reduce the level of these structures to ensure proper replication (Cheng et al., 2007). This idea is supported by the findings that mtSSB Rim1p partially suppresses the deleterious effect of Δpif1 strain (Van Dyck et al., 1992). Pif1 can also unwind G-quadruplexes (Figure 5E) (Ribeyre et al., 2009) frequent in mtDNA (Capra et al., 2010). The biochemical activity of Pif1 on RNA:DNA hybrids also requires further analysis as these structures represent a potential threat for genome stability (reviewed in (Sollier & Cimprich, 2015)).

### *2.2.5.2. Hmi1*

The second mitochondrial DNA helicase in *S. cerevisiae* is Hmi1. This protein is a distributive 3′–5′ translocase that unwinds 3′overhang-containing structures such as partial dsDNA, forked and flap structures (Kuusk *et al.*, 2005). The analysis of Hmi1 deletion strain showed the importance of this enzyme for mtDNA maintenance (Sedman *et al.*, 2000). Interestingly, the Hmi1 mutant defective in ATP hydrolytic and helicase activities could partially restore the severe Δ*hmi1* phenotype. This finding lead to an assumption that Hmi1, in addition to its unwinding function, could have a structural role where its binding to mtDNA promotes the initiation of replication or replication restart after replication fork collapse (Monroe *et al.*, 2005; Sedman *et al.*, 2005). As a helicase,

Hmi1 can unwind specific structures in mtDNA to facilitate replisome progression or promote recombination (Kuusk *et al.*, 2005). The functional redundancy between Pif1 and Hmi1 also supports the role of Hmi1 in mitochondrial recombination (Sedman *et al.*, 2000).

#### *2.2.5.3. Irc3*

The third *S. cerevisiae* mitochondrial helicase known to date is Irc3. The protein is encoded by the intronless ORF YDR332w on the chromosome IV. This ORF was first mentioned during the screening for novel helicase-like proteins and was shown to be non-essential for cell survival, but exhibited slow growth phenotype (Shiratori *et al.*, 1999). The same protein emerged in another whole genome screen for genes involved in recombination-driven repair of nuclear DNA. During the screen, in addition to genes with known functions, the authors found 22 novel genes with no known biological function that influenced the number of recombination foci in the nucleus and therefore were called Increased Recombination Centers or IRC genes (Alvaro *et al.*, 2007). *In silico* analysis placed Irc3 in the helicase SF2 and a large-scale study showed its localization in mitochondria (Huh *et al.*, 2003).

Our group initiated a study to determine the biological function of Irc3. Using biochemical fractionation and different truncated versions of Irc3 we showed that the protein localizes into mitochondria. The Δ*irc3* strains were found to be respiratory inactive due to the loss of mtDNA pointing to a straight involvement of Irc3 in mitochondrial DNA metabolism (Sedman *et al.*, 2014). We took advantage of the possibility to examine the *rho*$^{+}$ mitochondrial DNA topology in *IRC3* deficient cells by applying selective growth conditions. The analysis revealed the appearance of specific spots interpreted as dsDNA breaks which are associated with highly transcribed regions and active *ori*5 in mitochondrial genome. The analysis of Δ*irc3* cells in *rho*$^{-}$ phenotype demonstrated the appearance of dsDNA breaks, but more importantly, the loss of different branched DNA structures that represent putative intermediates of recombination pathways. Interestingly, the deletion of mitochondrial RNA polymerase Rpo41 in Δ*irc3* restored the recombination intermediates supporting the role of Irc3 in avoiding instability caused by the mitochondrial transcription machinery (Sedman *et al.*, 2014). As differences in mtDNA topology in Δ*rpo41*, Δ*irc3* strain in comparison to wild-type are still detectable, another transcription independent function of Irc3 was suggested to be connected with some recombination based process (Sedman *et al.*, 2014).

Our *in vitro* biochemical studies showed that Irc3 translocates on dsDNA lattice (Sedman *et al.*, 2017) and exhibits ATPase and helicase activity on specific branched DNA structures. It is important to note that Irc3 is conserved in different yeast species and the closest homolog outside the yeasts is the bacteriophage T4 helicase UvsW (Gaidutsik *et al.*, 2016) that exhibits similar biochemical activities. Furthermore, another helicase, bacterial RecG, with

similar biochemical properties was shown to partially complement phenotype of Δ*irc3* strains (Gaidutsik *et al.*, 2016). Interestingly, a RecG homolog was found to be involved in the maintenance of mtDNA in plants, pointing to the importance of such activity in mitochondria with complex genome organization (Gaidutsik *et al.*, 2016). Our findings on Irc3 will be thoroughly described and discussed in the Results and Discussion section.

**Table 2**. Proteins involved in mtDNA repair and recombination pathways and their functions.

| Pathway | Protein | Function |
|---|---|---|
| BER | Ung1 | DNA glycosylase. Removal of Uracil base (Chatterjee & Singh, 2001). |
| | Apn1 | Apurinic/apyrimidinic (AP) endonuclease. Removal of phosphodiester backbone (Vongsamphanh *et al.*, 2001). |
| | Ogg1 | DNA glycosylase and AP endonuclease. Removal of 8-oxoG nucleotide (Singh *et al.*, 2001). |
| | Ntg1 | DNA glycosylase and AP endonuclease. Removal of Fapy-A and G nucleotides. Initiation of RDR of mtDNA (reviewed in (Kaniak-Golik & Skoneczna, 2015)) |
| | Rad27 | Flap endonuclease. Removal of 5′-flaps in long-patch BER. Potential maturation of Okazaki fragments. Involvement in DSB repair (Kalifa *et al.*, 2009; Lipinski *et al.*, 2010; Nagarajan *et al.*, 2017) |
| MMR | Msh1 | Detection of mismatches. Involved in homologous recombination (Mookerjee & Sia, 2006; Reenan & Kolodner, 1992) |
| Exo-nucleases | Nuc1 | 5′–3′ exo- and endonuclease. Involved in mtDNA recombination by generation of 3′ends on dsDNA (Chen, 2013) |
| | MRX | Mre11-Rad50-Xrs2 complex. 5′–3′ exonuclease. Involved in mtDNA recombination by generation of 3′ends on dsDNA (Kalifa *et al.*, 2012) |
| | Din7 | 5′–3′ exonuclease. Involved in mtDNA recombination by generation of 3′ends on dsDNA (Ling *et al.*, 2013). |
| | Exo5 | 5′–3′exonuclease activity on ssDNA (Burgers *et al.*, 2010). |
| NHEJ | Ku | Ku70-Ku80 complex, involved in mitochondrial DNA maintenance (Kalifa *et al.*, 2012). |
| DNA helicases | Pif1 | 5′–3′ DNA helicase. In addition unwinds DNA: RNA structures and G-quadruplexes. Involved in mtDNA maintenance via recombination and repair (Boule & Zakian, 2007; Cheng *et al.*, 2007; Lahaye *et al.*, 1993; Ribeyre *et al.*, 2009). |
| | Hmi1 | 3′–5′ DNA helicase. Involved in mtDNA maintenance as helicase and as structural protein. Has a role in recombination (Kuusk *et al.*, 2005; Sedman *et al.*, 2005). |
| | Irc3 | dsDNA translocase. Unwinds branched DNA structures. Involved in mtDNA maintenance (Gaidutsik *et al.*, 2016; Sedman *et al.*, 2014; Sedman *et al.*, 2017). |

# 3. AIM OF THE STUDY

Despite the many years of studies, the mechanism of mitochondrial genome maintenance is not fully understood. One of the reasons for that is the lack of full list of proteins involved in mtDNA metabolism. Therefore, when novel protein crucial for mDNA is found, its exact role should be determined using genetical approaches and by validating its exact biochemical parameters. Assesment of biochemical parameters is especially important in the case of helicases because the power of *in silico* analysis is still low and the important data concerning possible biological function of the enzyme can be generated only from *in vitro* experiments.

The experimental part of this work is concentrated on following issues:

1. Studies of Irc3 mitochondrial targeting signal

2. Assesment of Irc3 implication in mitochondrial nucleic acid metabolism

3. Purification of recombinant Irc3 protein and its N- and C-terminal variants

4. Assessment of biochemical properties of Irc3 enzyme and its N- and C-terminal versions

# 4. RESULTS AND DISCUSSION

## 4.1. N-terminus of Irc3 is required for transport into mitochondria (Ref. I)

The large-scale study of yeast protein localization showed that Irc3 is transported into mitochondria of *S. cerevisiae* (Huh *et al.*, 2003). First, we analyzed Irc3 sequence *in silico* and found that the N-terminal amino acids 1–28 are predicted to form a mitochondrial targeting signal or MTS (Fig. 1A in I). The peptide is characterized by the presence of positively charged and hydrophobic amino acids that form an amphipathic helix (Mossmann *et al.*, 2012). The MTS region of Irc3 was predicted to be required for protein transport into mitochondria and processed in matrix by the MPP protease (Mossmann *et al.*, 2012). To check the validity of the predictions, we initially performed a plasmid shuffling assay with a set of Irc3 mutants lacking 15 (NΔ15) and 28 (NΔ28) N-terminal residues (Fig. 1A, D in I). The laboratory *S. cerevisiae* strain W303 contains ADE2 gene mutation that causes the accumulation of red pigment in the cell during respiration. When respiration is abrogated cells turn white. The deletion of *IRC3* resulted in 90% of white colonies and 10% of colonies with red sectors, while the complementation of Δ*irc3* with wt *IRC3* restored the phenotype by giving more than 80% of red colonies (Fig. 1D in I). The shuffling of NΔ15 resulted in approximately 65% of red colonies while NΔ28 mutants had only 5% of red colonies. The NΔ15 retained the mitochondrial localization function. On the contrary, NΔ28 was almost fully inactive. To exclude the possibility of Irc3 inactivation due to removing important structural residues, we exchanged the first 28 amino acids of the protein with previously characterized targeting peptide of mitochondrial Cit1p. Such construct was able to restore the respiration of cells by giving rise to 75% of red colonies (Fig. 1D in I). This data indicates that the first 28 residues of Irc3 are MTS required for the protein transport into mitochondria. The results of biochemical fractionation experiments with HA (Human influenza hemagglutinin) tagged Irc3 suggested that the protein is localized into mitochondrial matrix and, thus, these results supported the results of our respiration activity test (Fig. 1C in I).

## 4.2. Irc3 protein is required for the stable propagation of mitochondrial genome (Ref. I)

The preliminary studies of *IRC3* showed that the gene encodes mitochondrial DExH/DEAD-box protein which belongs to SF2 of helicases (Alvaro *et al.*, 2007). Four SF2 helicases previously found in *S. cerevisiae* mitochondria – Mss166, Suv3 and Mhr4 – have different functions in gene expression. The SF2 enzymes are known to play various roles in NA metabolism. Some of them are RNA or DNA helicases and a subset of them has only the translocase activity

(Byrd & Raney, 2012). We first compared the Δ*irc3* phenotype with disruption strains of other SF2 mitochondrial helicases. We generated heterozygous diploid strains of Δ*mss116*/*MSS116*, Δ*suv3*/*SUV3*, Δ*mhr4*/*MHR4*, Δ*irc3*/*IRC3* and after sporulation, dissected spores of each strain on media containing glucose (YPD) or glycerol (YPG) as the carbon sources. *MSS166*, *SUV3* and *MHR4* are vital for growth on non-fermentable media such as YPG (Bifano *et al.*, 2010; De Silva *et al.*, 2013; Turk & Caprara, 2010) and, thus, produced only two viable spores on glycerol containing media. In contrast, Δ*irc3*/*IRC3* gave rise to four colonies on YPG but two of them exhibited a strong growth defect. When Δ*irc3*/*IRC3* spores were dissected on YPD containing media, two Δ*irc3* spores gave rise to ragged edge colonies (Fig. 1B in I) indicating that they have sectors of slowly growing respiratory-deficient cells. The experiments lead us to suggestion that Irc3 may function in some mtDNA maintenance pathway, but it is probably not an essential gene for mitochondrial gene expression.

Next, we decided to test the involvement of Irc3 in mtDNA maintenance. For this reason respiratory active Δ*irc3* and wt yeast cells were pre-selected on YPG liquid media and then released on glucose containing media where they were incubated for several days. After the release, an even amount of cells of both strains were plated out on glucose and glycerol containing media and the ratio between the number of colonies formed on both media was calculated. The glucose containing media is a non-selective environment for respiration activity as the cells that lose functional mtDNA due to the gene deletion can survive by producing energy from glycolysis. The cells that are able to stably propagate their mitochondrial genome will give rise to colonies on both glucose and glycerol containing media. If the functional mtDNA is absent, the colonies will form only on YPD media. The experiment conducted with wt and Δ*irc3* strains showed that already at zero time point the respiring fraction was only 15% in Δ*irc3* and 70% in case of wt (Fig. 2B in I). The percentage of wt respiring cells remained relatively stable during the cultivation in liquid YPD while the fraction of Δ*irc3* respiring cells rapidly decreased during the first 24h of incubation. Interestingly, a small fraction (~2–3%) of Δ*irc3* remained respiratory active even after 6 days of incubation on YPD media (Fig. 2B in I). We decided to monitor the level of mtDNA in Δ*irc3* strain and found that the dramatic decrease of respiratory active cells observed in Δ*irc3* background was accompanied with the loss of functional mt genomes (Fig. 2A in I). Direct comparison of mtDNA signal of Δ*irc3* and other yeast strains in southern blot analysis also supported these findings (Fig. 2C in I) and therefore we suggest that Irc3 may be involved in some process of mtDNA maintenance.

## 4.3. Purification of the recombinant Irc3 and Irc3 K65A mutant (Ref. I, II, III)

In order to study biochemical properties of Irc3, the enzyme lacking 1–28 N-terminal amino acids was expressed in *E.coli* as a glutathione-S-transferase (GST) fusion protein. Amino acids 1–28 comprise proteolytically cleaved MTS and, therefore, Δ28Irc3 is treated as the functional form of protein and will be further referred to as Irc3 (Figure 10B). The GST-Δ28Irc3 was next purified on glutathione sepharose matrix after which the GST part was cleaved off by thrombin. The final step of purification was conducted on S-Sepharose ion exchange matrix. The purified protein was aliquoted and stored at –80ºC for further use. The purification procedure resulted in approximately 90–95% pure fraction of Irc3 as determined by the SDS-polyacrylamide electrophoresis (Fig. 5A in I) with average yield approximately 0,15–0,2 mg from 1 liter of bacterial culture. The concentration of all purified proteins was measured by UV adsorption at 280 nm using their molecular weights and calculated molar extinction coefficients. The protocol of recombinant Irc3 production has been later improved by the addition of the third purification step on heparin agarose column (Ref. III).

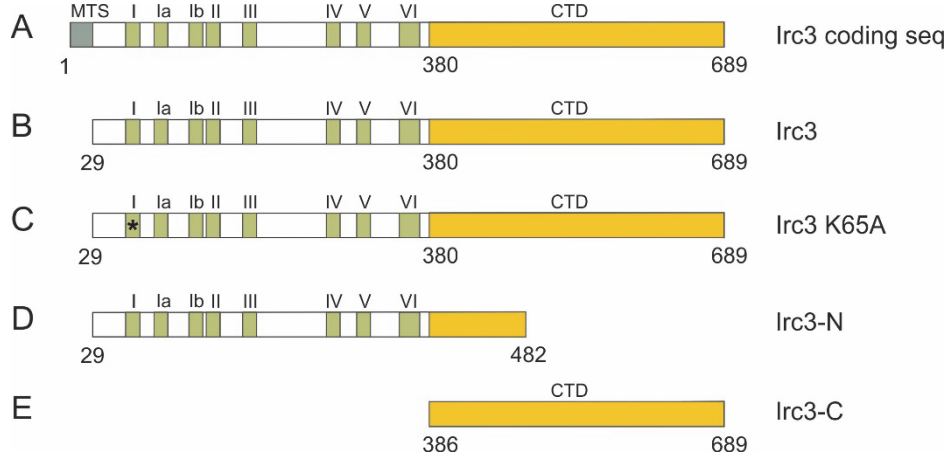


**Figure 10.** Schematic depiction of Irc3 coding sequence and different versions of Irc3 protein purified during this study. MTS is grey, conserved helicase motifs are green and C-terminal domain (CTD) is yellow. (A) *IRC3* ORF translated into amino acid sequence. (B) Irc3 without the 1–28 aa MTS which is used in the study as the wild-type protein. (C) ATPase negative Irc3 without the 1–28 aa MTS containing a mutation (K65A) depicted as asterisk in the motif I (D) Irc3 truncated version containing complete helicase domain. (E) Irc3 truncated version lacking the N-terminal helicase domain.

In addition to the wild-type Irc3, the initial purification protocol was followed to obtain the mutant version of Irc3 (Irc3-K65A), where the lysine (K) in the position 65 was substituted with alanine (Fig. 5A in I and Figure 10C). The

lysine 65 is a part of highly conserved helicase motif I (also known as Walker A motif) which is specifically involved in the binding of the polyphosphate residues of NTP and interaction of $Mg^{2+}$ ion (Caruthers & McKay, 2002). Therefore, the K65A mutation of the motif I is predicted to result in inactivation of Irc3 helicase, similarly to other helicases of SF1 and SF2 (Hishida *et al.*, 1999; Monroe *et al.*, 2005; Pause & Sonenberg, 1992; Zegeye *et al.*, 2014). Furthermore, our *in vivo* studies with different Irc3 mutant forms showed that Irc3-K65A mutant was not able to suppress the Δ*irc3* phenotype in plasmid shuffling assay supporting the idea that functional ATPase is essential for the Irc3 role in mitochondria (Fig. 1D in I). The yield of Irc3-K65A per 1 liter of bacterial culture was approximately the same as that of the wild-type Irc3 protein. Irc3-K65A was used as a control for the Irc3 purification procedure and for biochemical assays.

## 4.4. ATPase activity of Irc3 protein (Ref. I, II)

*In vivo* analysis and DNA topology analysis indicated that the biochemical role of Irc3 differs from the roles of other mitochondrial SF2 enzymes involved in mitochondrial RNA metabolism. However, the exact biochemical function remained elusive and, therefore, we continued with the biochemical characterization of purified recombinant protein to better understand its biological role. *In silico* analysis revealed that Irc3 contains the conserved helicase core that binds and hydrolyzes NTP (Gorbalenya & Koonin, 1993) leading us to the assumption that Irc3 could be ATPase acting on nucleic acid. We, therefore, applied an ATPase activity assay based on the ability of charcoal to bind and precipitate organic forms of phosphate from reaction solution leaving detectable inorganic phosphate in a soluble form detected as $^{32}P$ (Kuusk *et al.*, 2002). The reactions described here were performed at 30ºC under conditions where the concentrations of cofactor and nucleotide are not limiting for the activity of the enzyme.

It is often complicated to find a proper cofactor that stimulates the ATPase activity of purified protein and therefore we conducted the first assays in the presence of nucleic acid cofactors purified from yeast mitochondria. Our experiments showed that mtDNA was able to stimulate the ATPase of Irc3. In contrast, the addition of mtRNA did not stimulate any ATP hydrolysis (Fig. 5B in I). As expected, virtually no ATPase activity was detected when no NA cofactor was added to Irc3. Importantly, the mutant version Irc3 K65A was not active as an ATPase in the presence or absence of mtNA. Therefore, the activities detected with Irc3 are not produced by any other ATP consuming enzymes in the recombinant Irc3 preparations co-purified during the fractionation procedure (Fig. 5B in I).

Next, to more precisely define the cofactor specificity of Irc3 we tested a set of NA cofactors, including ssDNA, circular dsDNA, ssRNA and rRNA. Of those, circular plasmid dsDNA pRS316 stimulated the ATPase activity of the

Irc3 protein much more efficiently than the ssDNA of M13 bacteriophage and no ATPase with ssDNA or any type of RNA as a cofactor was detected (Fig. 5C in I). Finally, we conducted ATPase assays in the presence of two 46bp long oligonucleotides capable to form a dsDNA duplex of the same length adding them separately or in combination in annealed dsDNA form. The results showed that both oligonucleotides separately were incapable to stimulate Irc3 ATPase, while DNA duplex made of two oligonucleotides efficiently stimulated the ATPase activity of Irc3 (Fig. 5D in I). This set of experiments led us to the conclusions that Irc3 is a dsDNA-dependent ATPase and this supported our *in vivo* findings suggesting that Irc3 is a mitochondrial DNA maintenance factor involved directly in mtDNA maintenance.

The maintenance of mtDNA implicates different transient dynamic events such as replication, recombination and repair, when DNA is not exclusively double-stranded, but could fold into complicated branched secondary structures containing both double-stranded and single-stranded regions. Irc3 as an enzyme with potential helicase activity could have a role in the aforementioned processes. We next tested branched DNA cofactors in the ATPase activity assay with Irc3. A set of DNA cofactors resembling different replication forks and HJ were constructed from oligonucleotides and used in ATPase stimulation experiment (Fig. 2C in II). Our experiments showed that synthetic branched DNA cofactors generally stimulated the Irc3 ATPase activity better than linear dsDNA. Two model HJ cofactors had the best stimulatory activity, suggesting that Irc3 could preferentially act on these structures *in vivo* (Fig. 2C in II). This data is in agreement with our *in vivo* findings that point to involvement of Irc3 in generation or processing of branched mtDNA intermediates (Ref. I and II).

## 4.5. Helicase activity of Irc3 protein (Ref. I, II)

Interestingly, not all the proteins containing conserved helicase motifs and classified as helicases are able to unwind NA duplexes. Instead, they could act as translocases or have a role in NA remodeling (Pyle, 2008; Wu & Spies, 2013). However, our data obtained from ATPase assays strongly suggested that Irc3 is a DNA specific enzyme and not an RNA helicase as previously suggested (de la Cruz *et al.*, 1999). To understand, whether Irc3 is a DNA helicase or merely translocates along the nucleic acid lattice we set up unwinding assays where Irc3 helicase activity was tested in the presence of ATP and $Mg^{2+}$. Different short substrates were prepared of annealed oligonucleotides, where one of them was radioactively labelled for the detection of unwinding products on nondenaturing polyacrylamide gels (Figure 11).

Initial assays conducted with a set of simple linear substrate DNA molecules containing 3´ or 5´ overhangs did not show any DNA unwinding catalyzed in the presence of Irc3. Furthermore, Irc3 did not unwind forked DNA substrate (Fig. S5 in I). As the best stimulation of the Irc3 ATPase was observed with branched DNA cofactors, we decided to test a set of branched DNA substrates in the

helicase reaction (Fig. 3 in II). In contrast to the first set of substrates, we detected robust unwinding of structures resembling a replication fork (Fig. 3D,E in II). More specifically, Irc3 removed nascent leading and lagging strands from the fork-shaped substrates releasing >50% of the labelled oligonucleotides in 20 minutes. No significant preference for the leading or lagging strand was observed when the other fork branch was single-stranded (Fig. 3F in II). To better understand the nature of Irc3 action on replication forks, we next tested the three-way branched replication fork analogs where both the leading and lagging strand branches were double-stranded (Fig. 4A,B,E in II). The substrate HOM contained homologous nascent strands and therefore could undergo fork reversal or dissolution, generating, thus, dsDNA or ssDNA products. In contrast, the substrate HET with heterologous nascent strands can only undergo fork dissolution resulting in the appearance of ssDNA bands on gel. Our observations showed that the reaction with HOM produced only a dsDNA product which could be interpreted as the results of Irc3 fork reversal activity (Fig. 4A in II). As expected, the control substrate HET generated only a ssDNA product (Fig. 4B in II). The Irc3 catalyzed unwinding/remodelling reaction had significantly higher preference for the HOM substrate, as >50% of dsDNA product was released in 5 minutes in comparison to the reaction with the HET substrate, where the maximal amount of the released product was only 25% (Fig. 4E in II). Finally, we compared the activity of Irc3 on two HJ model substrates. The substrate X12 contains a movable core composed of complementary regions of oligonucleotides and is, thus, able to promote branch migration while X0 is composed from heterologous oligonucleotides and therefore represents an immovable structure difficult to separate (Fig. 4C,D in II). The ATP-dependent remodeling reactions with Irc3 resulted in the appearance of only two forked DNA products without any release of ssDNA and the reaction with X12 was much faster than with X0 (Fig. 4C,D,F in II). It is important to note that no product formation was observed in reactions with Irc3-K65A protein and, therefore, the remodeling reactions were Irc3-specific (Fig. S6 in II).

As expected, the activities of Irc3 in ATPase assays mostly mimicked the results of helicase assays (Fig. 2C; Fig 3, Fig.4 in II). The relatively high ATPase activity levels stimulated by dsDNA and inability of Irc3 to unwind this substrate could be explained by ATP-dependent translocation of Irc3 along dsDNA lattice (Ref. III). In the case of the forked substrate, Irc3 binds to the dsDNA region of the substrate and probably moves through consuming ATP during this action. The same mode of action, characteristic to branch migration enzymes, explains the indifference of Irc3 to the leading and lagging strand containing substrates (Fig. 3F in II).

Taken together, the experiments with branched substrates showed the ability of Irc3 to catalyze fork reversal and branch migration reactions. These biochemical characteristics of enzyme support and extend our previous *in vivo* findings and, thus, promote Irc3 into the complex field of mitochondrial DNA recombination (Ref. I and II).

| | ssDNA | dsDNA | 3´OH DNA | 5´OH DNA | forked | lead RF | lag RF | HOM | HET | X12 HJ | X0 HJ |
|---|---|---|---|---|---|---|---|---|---|---|---|
| substrate scheme |  |  |  |  |  |  |  |  |  |  |  |
| ATPase activity | − | + | ND | ND | + | + | + | ND | + | +++ | + + |
| Helicase activity | ND | − | − | − | − | + + | + + | + + | + | +++ | + + |
| DNA binding activity | − | +/− | ND | ND | + + | + + | + + | ND | + | + + | + + |

**Figure 11.** Summary of Irc3 biochemical activities discusses in this study. Schematic representation of substrate is depicted on top, "+" – activity is present, "–" – activity is missing, "ND" – not done. ssDNA – single stranded oligonucleotide, dsDNA – blunt ended dsDNA substrate, 3´OH DNA – substrate containing 3´overhang and double-stranded region, 5´OH DNA – substrate containing 5´overhang and double-stranded region, forked – substrate containing double-stranded region and unpaired ssDNA arms, lead RF – replication fork resembling structure with only leading strand oligonucleotide annealed, lag RF – replication fork resembling structure with only lagging strand oligonucleotide annealed, HOM – three-way branched replication fork analog with homologous nascent strands, HET – three-way branched replication fork analog with heterologous nascent strands, X12 HJ – contains a movable core composed of complementary regions of oligonucleotides and promotes branch migration, X0 HJ – contains an immovable core composed of non-complementary regions of oligonucleotides.

## 4.6. The role of the Irc3 C-terminal domain (Ref. III)

The N-terminal part of Irc3 contains conserved domains which are folded into SF2 motor core capable to hydrolyze ATP and potentially translocate along DNA lattice while about 300 residues in the C-terminus represent a region of unknown function with no homology to any described protein domain. The proteins of SF2 are known to often contain different accessory domains that modulate helicase substrate specificity or interact with other proteins and thereby specify the cellular role of the enzyme (Beyer *et al.*, 2013; Fairman-Williams *et al.*, 2010).

To elucidate the function of the C-terminal region, Irc3 mutants containing only the N-terminal or the C-terminal domain (Figure 10D,E) were expressed and purified similarly to the scheme described previously (see section 4.3.) (Fig. 6A;S1 in III). The N-terminal mutant (Irc3-N) contained residues 28–482 comprising mostly the motor core of Irc3 (Figure 10D); the C-terminal mutant (Irc3-C) covered the residues 386–689 (Figure 10E). Next, we tested the mutants for the ATPase and DNA binding activities. The analysis revealed that Irc3-N mutant retained the ability to hydrolyze ATP in the presence of dsDNA linear cofactor, but did not demonstrate stronger stimulation with the branched DNA cofactor X12 as in the case of the full-length Irc3 (Fig. 6E in III). The mutant Irc3-C did not have any ATPase activity (Fig. 6E in III) as the motor core required for nucleotide hydrolysis was missing. However, the C-terminal domain was able to bind branched DNA molecule with efficiency that was comparable to the full length Irc3 protein (Fig. 6H in III). Somewhat

surprisingly, Irc3-N was not able to act as a dsDNA translocase (Fig. 6D,S2 in III) and lacked DNA unwinding activities (Figure 12). All presented activities of Irc3 truncated mutants are summarized in Table 3.

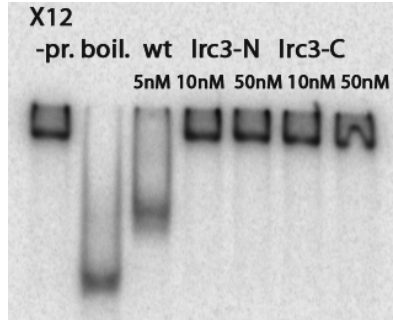


**Figure 12.** Unwinding activities of Irc3-N and Irc3-C mutants. Activities were tested on the X12 branched DNA substrate. The (–pr.) lane is the substrate only, (boil.) is the substrate heated at 95ºC for 3 minutes, (wt) lane is Irc3. Protein concentrations used in the experiment are indicated as well. The concentration of the X12 substrate was 0,25 nM (molecules). Both mutants were unable to unwind X12 substrate.

It is important to note that the ability of Irc3-N to hydrolyze ATP in the presence of dsDNA suggests that the purified protein was properly folded and to some extent it is able to bind DNA using conserved helicase core structure, but the inability to translocate on dsDNA and unwind branched substrate shows that the minimal core lacking CTD is insufficient to fulfill the biological role of Irc3 (Table 3). In turn, the C-terminal domain displays affinity for branched DNA structures and, thus, could direct Irc3 motor to specific structures (Table 3). Even though the sequence of CTD does not match any known DNA binding motifs, several functional analogs have been described in previous studies of branch migration proteins. This includes the wedge domain in the bacterial RecG (section 2.1.3.3.) and structurally similar MotA and HARP domains of the viral UvsW and the metazoan SMARCAL1 proteins (section 2.1.3.6. and section 2.1.3.4.). Therefore, the modular organization of Irc3 represents a typical architecture of SF2 helicases and branch migration proteins in particular.

**Table 3.** Biochemical activities of Irc3 in comparison to its truncated versions.

| Protein | ATPase activity | Helicase activity | DNA binding activity | Translocase activity |
|---|---|---|---|---|
| Irc3 | + | + | + | + |
| Irc3-N | + | − | ND | − |
| Irc3-C | − | − | + | − |

## 4.7. Possible functions of Irc3

The biochemical studies of Irc3 presented in this study together with *in vivo* data reviewed in sections 2.2.5.3 suggest several roles of this protein in mtDNA maintenance. Our biochemical studies establish that Irc3 is a branch migration and fork reversal enzyme in yeast mitochondria.

On the basis of its biochemical activities it is reasonable to conclude that Irc3 is involved in some recombinogenic pathway in mitochondria and several lines of evidence support this view. The phenotype, caused by the deletion of *IRC3* leads to relatively slow mtDNA loss. Similar effect was described for Abf2 which is the mitochondrial DNA packaging protein, also implicated in recombination (MacAlpine *et al.*, 1998). Direct evidence of Irc3 involvement in recombinative pathways can be deduced from 2D gel analysis of mitochondrial DNA topology that can visualize complex DNA structures produced during different processes of DNA metabolism. Such an analysis first demonstrated the appearance of specific dsDNA breaks in wt and *rho*$^-$ genomes that are at least partially localized in *ori* regions (Fig. 3,4 in I) which have shown to be recombination hot-spots (Zinn *et al.*, 1988) and were suggested then to be starting points for RDR pathway in mitochondria (Ling *et al.*, 2007; Prasai *et al.*, 2017). Furthermore, the mtDNA 2D analysis in Δ*irc3 rho*$^-$ strains revealed the dramatic decrease of typical branched DNA structures (Fig. 4 in I and Fig. 1 in II) that are thought to be recombination intermediates (Lockshon *et al.*, 1995) and accumulation of high order unresolved irregular structures with complex branched topology containing extensive ssDNA regions (Fig. 4 in I and Fig.1 in II). Finally, an *in vivo* assay demonstrated partial suppression of Δ*irc3* phenotype when mitochondrially targeted *E.coli* RecG was expressed in the yeast, suggesting that RecG could be a functional homolog of Irc3 (Fig. 5 in II). Furthermore, these results support the assumption that branch migration enzyme is required for the maintenance of mitochondrial genomes with complex topology (Ref. II).

Homologous recombination-related processes are presumably involved in several aspects of DNA metabolism. Therefore, several possible models can be proposed for the role of Irc3 in yeast mitochondria. First, the appearance of dsDNA breaks in Δ*irc3* background places the protein in classical HR pathway where broken DNA strands are treated with an exonuclease and a recombinase promotes invasion of homologous single DNA strands. Irc3 may catalyze branch migration of HJs that are next dissected by a resolvase. Similar activity was shown for Rad54 (Bugreev *et al.*, 2006), BLM-Top3-Rmi1 complex (Wu & Hickson, 2003), RuvAB (West, 1997) and has been proposed for RecG (Whitby *et al.*, 1993). Secondly, the aforementioned DNA breaks could form as a consequence of DNA damage that represents a block for replication fork movement. Therefore, Irc3 with its fork regression activity is a perfect candidate as a suppressor of this type of damage. The regression of stalled replication forks at the sites of lesions can lead to many outcomes such as template strand switching to overcome the lesion, repair of the lesion in front of the regressed fork or

cleavage of the chicken foot structure by a nuclease with further repair via HR (Meng & Zhao, 2017). Very likely, the fork regression process is a frequent event in the cellular DNA metabolism and there are many proteins exhibiting this type of activity (discussed in subsections of 2.1.3.). The process of mtDNA replication is not fully understood and the presence of classical replication forks with leading and lagging strands remains a matter of debate. However, we have detected Y-type mtDNA intermediates on 2D gels and, therefore, believe that fork regression events can take place in mitochondria as well. In this context, Irc3 is a perfect candidate to stimulate fork reversal *in vivo*. Third, 2D gel analysis of the mtDNA in Δ*irc3* strain reveals significant increase in partially single stranded unresolved branched structures (Fig. 4 in I and Fig. 1 in II). This could be a consequence of irregular ectopic recombination that occurs between not fully homologous regions of mtDNA or a result of aberrant recombination events between multiple copies of mt genomes that are also potentially deleterious for genome maintenance. It is possible that Irc3 could suppress ectopic recombination by acting on D-loop structures as the geometry of DNA in D-loops partially resembles HJs and replication forks. The plant RECG was recently suggested to suppress ectopic recombination between abundant repeats of plant mtDNA (Odahara *et al.*, 2015; Wallet *et al.*, 2015).

Finally, another set of *in vivo* findings demonstrate that mitochondrial transcription influences Δ*irc3* phenotype. Interestingly, all branched structures that were lost in Δ*irc3* background were restored by deletion of Rpo41 (Fig. 4D and 6B in I). It is known that improper regulation of replication and transcription machineries can cause dsDNA breaks via formation of R-loops or topological constraints (reviewed in (Helmrich *et al.*, 2013)). Our group has demonstrated that active transcription in *S. cerevisiae* mtDNA inhibits the synthesis of long concatemeric molecules (Fig. 4C in I and (Sedman *et al.*, 2005)) and therefore the regulation of transcription and replication interaction is essential for mitochondrial genome maintenance. Irc3 biochemical activities suggest that the enzyme might also unwind R-loops to regulate their level in mitochondria. In the nucleus, different molecular systems are involved in the removal of R-loops. In addition to RNA packaging complexes and RNA exosome, these include RNAse H1 and H2 together with the helicase Sen1 (El Hage *et al.*, 2014; Mischo *et al.*, 2011). The RNAse H1 involvement in the degradation of RNA in RNA:DNA hybrid was demonstrated to regulate the level of R-loops in mtDNA as the deletion of RNAse H1 resulted in a threefold increase in respiratory deficient cells. To what extent Irc3 and other protein factors are involved in the process of R-loop removal is the matter of further studies. It is noteworthy to mention that the closest homolog of Irc3 outside the yeasts, UvsW, exhibits similar biochemical activities including the removal of R-loops that has very important implication in viral lifecycle by switching between two different replication modes of the T4 bacteriophage (Derr & Kreuzer, 1990). Therefore, the R-loop processing by Irc3 may have a regulatory role in mitochondrial DNA replication.

Irc3 appears to be a multifunctional protein composed of the SF2 helicase core and the accessory domain in the C-terminus that is required for specific binding to branched DNA and translocation activity (Ref. III). The inability of Irc3 helicase core containing mutant to translocate on dsDNA indicates that CTD is required this activity. The translocation experiments have shown that Irc3 requires both DNA strands for directional movement while the gap introduced in each strand of dsDNA substrate blocked the ability of enzyme to displace the third strand of DNA triplex (Fig. 3 in III). Such a mode of action is uncommon for dsDNA translocases of SF2 that mainly make contacts with a single strand of dsDNA during the movement (Singleton *et al.*, 2007). Probably, in the process of Irc3 translocation some region(s) of CTD also interact with another strand of DNA lattice to ensure a proper grip. Interestingly, Irc3 still exhibit minor 3′–5′ polarity because the contacts with 3′–5′ strand only allow some translocation to happen (Fig. 3C in III). Although, the described mode of action is rare, it is not unique. The HLTF translocase involved in restart of replication forks was shown to have similar mode of translocation (Blastyak *et al.*, 2010).

In addition to the essential role in branched DNA recognition, CTD may also act as the interaction platform with other proteins. The main candidate for such interaction is the mitochondrial ssDNA binding protein Rim1 which was previously shown to interact with another mitochondrial helicase Pif1 and to stimulate, thus, the unwinding activity of the helicase (Ramanagoudr-Bhojappa *et al.*, 2013). Similar interactions have been demonstrated for Hel308, RecQ and SMARCAL1 with RPA (the nuclear analog of SSB in eukaryotes), for RecG and bacterial SSB (Bansbach *et al.*, 2009; Buss *et al.*, 2008; Shereda *et al.*, 2009; Woodman *et al.*, 2011). Moreover, several other interaction partners are needed to direct FANCM to distinct repair pathway (Ciccia *et al.*, 2007; Rohleder *et al.*, 2016; Yan *et al.*, 2010) and BLM-TOP3-RMI1 complex is required for the decatenation activity of BLM (Wu & Hickson, 2003). The examples presented here suggest the importance of accessory proteins in regulation of helicase activity. While various activities of Irc3 represent different roles of the enzyme, these functions could be regulated according to the functional status of mtDNA and accessory proteins are the most suitable candidates for the regulatory role.

# 5. CONCLUSIONS

Helicases are defined by the presence of conserved motifs and they can act on various NA substrates. This includes unwinding and remodeling of nucleic acids and translocation on single-stranded or double-stranded nucleic acid lattices. Currently, it is not possible to predict the properties of a putative helicase *in silico* and, therefore, the only reliable way to understand the activities of a helicase is to purify the protein and assay its activities. The biochemical activities of a purified protein combined with *in vivo* studies is the most effective strategy for dissection the biological role of a helicase. This thesis concentrates on the biochemical studies of Irc3 and the main conclusions of the study are:

1. Irc3 is a SF2 protein that contains an N-terminal targeting signal and is transported into yeast mitochondria.

2. Functional Irc3 protein is required for mtDNA maintenance.

3. In contrast to other mitochondrial SF2 enzymes, Irc3 is directly involved in DNA metabolism.

4. Irc3 is an ATPase that is stimulated by different types of dsDNA cofactors. The best effectors of Irc3 are branched DNA containing structures.

5. Irc3 unwinds nascent strands from replication fork resembling substrates and exhibits fork regression activity on fully double-stranded three-way branched replication fork substrates *in vitro.*

6. Irc3 catalyzes *in vitro* branch migration of synthetic Holliday junctions composed of oligonucleotides.

7. Irc3 has a modular structure; the C-terminal region comprises a domain implicated in binding of branched DNA structures. The C-terminal domain is indispensable for the helicase, translocase and DNA binding activities. Irc3 N-terminal domain is a SF2 motor core able to hydrolyze ATP in presence of a dsDNA cofactor

# SUMMARY IN ESTONIAN

## Mitokondriaalne DNA hargnemisi mobiliseeriv ensüüm Irc3

Mitokonder (mt) on rakuline organell, mis on seotud erinevate rakuliste protsessidega kuid enamasti asotsieeritud oksüdatiivse fosforüleerimisega ehk rakulise energia tootmisega ATP kujul. Mitokondris on säilinud redutseeritud genoom, mis on pärit mitokondri eellasest α-proteobakterist. Mitokondriaalne genoom kodeerib mõned oksüdatiivse fosforüleerimise komplekside valgulised komponendid ning on seetõttu hädavajalik enamiku eukarüootsete organismide ellujäämiseks. Valdav enamik mitokondriaalseid valke kodeeritakse tuumas ning transporditakse mitokondrisse. Mitokondris toimuvad protsessid ning nendega seotud valkude roll ja arv pole lõplikult teada. Seetõttu on uute valguliste faktorite leidmine ja kirjeldamine tähtis mitokondriaalsetest protsessidest arusaamiseks. Pagaripärm *S. cerevisiae* on eukarüootne mudelorganism, mille eripäraks on võime eksisteerida ilma mtDNA-ta, ehk ilma funktsionaalse hingamisahelata, kasutades energia saamiseks glükolüüsi. Antud omadus teeb pagaripärmi mugavaks mudelobjektiks mtDNA säilitamisega seotud protsesside uurimisel.

Helikaasid on valgud, mis võivad liikuda piki nukleiinhapet ning harutavad lahti selle ahelaid, kaksikheeliksi struktuure või remodellerivad nukleiinhape-valk komplekse. Lähtudest kirjeldusest võib järeldada, et helikaasid osalevad kõikides DNA-ga ja RNA-ga seotud võtmeprotsessides nagu replikatsioon, rekombinatsioon, DNA parandamine, kromatiini remodelleerimine, transkriptsioon ning valgu biosüntees. Iga helikaas koosneb konserveerunud motiive sisaldavast helikaasi tuumikstruktuurist, mille peamiseks funktsiooniks on nukleotiidi (enamasti ATP) lagundamisest sõltuv liikumine piki nukleiinhape ahelat. Lisaks eelpool kirjeldatud motoorsele tuumale sisaldavad helikaasid sageli ühte või mitut lisadomeeni, mis näiteks määravad ensüümi spetsiifika kindla substraadi suhtes, tagavad uusi ensümaatilisi funktsioone, reguleerivad helikaasi aktiivsust rakus või töötavad kui interaktsiooni platvormid teiste valkudega. Konseveerunud motiivide erinevus määrab helikaasi kuuluvuse superperekondadesse (SF), millest SF1 ja SF2 on kaks kõige suuremat. Suure komplekssuse tõttu ei seosta superperekonda kuulumine valku kindla valdkonna või protsessiga ning seetõttu tuleb funktsiooni mõistmiseks iga helikaas välja puhastada ja biokeemiliselt iseloomustada.

Pagaripärmi mitokondris on kirjeldatud mitu helikaasi, millest kolm on geenikspressiooniga seotud RNA helikaasid ning kaks on mtDNA säilitamisega seotud DNA helikaasid. Irc3 oli seni kirjeldamata valk, mis sisaldab konserveerunud helikaasseid motiive, tundmatu funktsiooniga C-terminaalset domeeni ning klassifitseerub helikaaside SF2 perekonda. Ülegenoomne uuring demonstreeris Irc3 kuuluvust mitokondriaalsete valkude hulka. Edasised katsed, mis olid teostatud meie rühma poolt, näitasid, et Irc3 tõepoolest lokaliseerub mitokondri maatriksisse ning esimesed 28 aminohapet Irc3 N-terminuses moodustavad mitokondrisse lokaliseerimise signaali. *IRC3* geeni deletsioon

põhjustab hingamisvõime kadumist, mis on tingitud mitokondriaalse DNA koopiaarvu langusest ja fragmenteerumisest, mis omakorda vihjab antud valgu seotusele mtDNA säilitamise protsessidega. DNA topoloogia analüüsimisel Δ*irc3* tüvedes ilmnesid kaksikahelalised mtDNA katked spetsiifilistes replikatsiooniga seotud regioonides ja/või alades kus on kõrge transkriptsiooniline aktiivsus. Samal ajal kõrgmolekulaarsete korrapäratute hargnenud struktuuride signaal intensiivistus. Δ*irc3* tüvedes kadusid homoloogilise rekombinantsiooni (HR) vaheproduktid nagu Holliday struktuurid ja DNA Y-struktuurid. Kirjeldatud struktuure sai taastada mt RNA polümeraasi Rpo41 deleteerimisega Δ*irc3* taustal, mis viitab Irc3 osalusele transkriptsiooni ja replikatsiooni omavahelises regulatsioonis.

Antud töö üks peamistest eesmärkidest oli Irc3 valgu biokeemiline iseloomustamine. Selleks ekspresseeriti Irc3 ja mitmed selle variandid rekombinantse liitvalguna *E.coli* tüves ning puhastati kahe kromatograafilise kolonni abil. Puhastatud valguga katsetes selgus, et:

1. Irc3 on ATP-d lagundav ensüüm, ehk ATPaas, mille aktiivsust saab stimuleerida erinevat tüüpi kaksikahelaliste DNA kofaktoritega ning paljud hargnenud ahelatega DNA struktuurid stimuleerivad Irc3 ATPaasset aktiivsust paremini kui lineaarsed
2. Irc3 harutab lahti replikatsioonikahvleid meenutavatel struktuuridel juhtiva ja mahajääva ahela ning demonstreerib üleni kaksikahelalistel replikatsioonikahvlitel ahelate regressiooni aktiivsust
3. Irc3 on võimeline katalüüsima Holliday ühendustes DNA harude migratsiooni
4. Irc3 on modulaarse struktuuriga ensüüm koosnedes N- ja C-terminaalsest domeenist. C-terminaalne domeen osaleb hargnenud struktuuride sidumises ning on hädavajalik helikaasse, translokaasse ja efektiivse DNA sidumise jaoks. Irc3 N-terminaalne domeen on SF2 motoorne tuumik, mis on võimeline ATP-d hüdrolüüsima kaksikahelalise DNA kofaktori juuresolekul

Töö käigus saadud tulemused ei anna lõplikku vastuse Irc3 bioloogilise rolli kohta, kuid annavad aluse pakkuda välja mõned selle valgu võimalikud funktsioonid pärmi mitokondris. Esiteks, DNA harude migreerimise võime paigutab Irc3 homoloogilise rekombinatsiooni teostajate hulka ning võttes arvesse dsDNA katkete teket Δ*irc3* tüvedes, võiks antud protsessi eesmärgiks nimetada DNA reparatsiooni. Teiseks, Irc3 saab osaleda DNA reparatsioonis kasutades võimet regresseerida täielikku või osalist replikatsioonikahvlit. Kirjeldatud aktiivsust saab kasutada juhtudel kui matriitsahel on kas blokeeritud või kui replikatsiooni protsess on pärsitud mõnel muul põhjusel. Edasised sammud võivad hõlmata kas matriitsahela parandamist, replikatsiooni taaskäivitamist või reparatsiooni HR abil. Kolmandaks, on väga tõenäoline, et Irc3 on suuteline harutada lahti D-loop ja R-loop struktuure nagu ka valdav enamik sellist tüüpi valke. D-loop struktuurid kujutavad endast HR algstaadiumi, kus homoloogiline 3´ üleulatuv DNA ahel invaseerub kahe doonor DNA ahela vahele. Irc3 võib invaseerunud

ahelat lahti harutades käituda kui antirekombinaas – valk, mis peatab üleliigse või ektoopilise (mitte täielikult identsete alade vahel) rekombinatsiooni, protsessi mis toimub mittetäielikult homoloogiliste alade vahel ning mis on ohtlik genoomi terviklikuse jaoks. Korrapäratute hargnenud struktuuride signaali intensiivistumine Δ*irc3* tüves on tingitud arvatavasti just sellist tüüpi rekombinatsiooni produktide kuhjumisest. Neljandaks, Irc3 võib olla seotud R-loop'ide ehk transkriptsiooni kõrvalproduktide deleteerimisega. Need struktuurid esinevad mtDNA-s arvatavasti sageli ning võivad põhjustada replikatsiooni peatumist ja dsDNA katkeid. Irc3 valgu biokeemilised aktiivsused ja modulaarne kompositsioon sarnaneb mitmete teiste genoomi stabiilsust tagavate helikaasidega. Ühe sellise valgu, bakterist pärit RecG ekspressioon pärmi mitokondris suutis osaliselt kompenseerida Irc3 deletsiooni. Praeguse seisuga on raske oletada, millised Irc3 aktiivsused *in vitro* leiavad aset rakus ning tagavad mtDNA säilitamise. Antud töö näitas aga veenvalt, et hargnenud DNA molekulide peal töötav helikaasse aktiivsusega valk on vajalik pärmi mitokondriaalse säilitamise DNA jaoks.

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