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**A cluster of pathogenic mutations in the 3'-5'
exonuclease domain of DNA polymerase gamma
defines a novel module coupling DNA synthesis
and degradation.**

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grade de Docteur en Sciences Agronomiques et Ingénierie Biologique.

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2011

I wish to express my gratitude to my supervisor Professor Françoise Foury for giving me the opportunity of doing my PhD in Belgium. I would like to address her special acknowledgements for all her support, help, indulgence, solicitude, invaluable knowledge and experience.

I would also like to thank my thesis reviewers for all their help and invaluable comments.

I would like to thank all members of my research group – especially Enrico, Daphné, Sébastien, and Frédéric – for creating a pleasant working atmosphere and for the productive discussions we shared.

My best gratitude also extends to the all present and past members of FYSA/FYMO for all their guidance and help in the lab and outside the work.

Szczególne podziękowania należą się całej mojej rodzinie - mamie Heni, Romkowi, siostrze Misi i Anieli – za ich wsparcie, troskę i wiarę we mnie.

Wszystkim moim polskim przyjaciołom w Belgii za ich bezinteresowną pomoc i miłe towarzystwo.

Moją pracę dedykuję mężowi Frankowi i synkowi Stasiowi. Dziękuję Wam za waszą miłość, cierpliwość i za to, że byliście przy mnie.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS	7
LIST OF FIGURES AND TABLES	10
ABSTRACT	15
1. INTRODUCTION	17
1.1. The importance of studies on mitochondrial DNA polymerase gamma mutations.	
<i>Main assumptions and aims of the research.</i>	18
1.2. Yeast and human mitochondrial genomes and their maintenance. <i>Similarities and differences.</i>	21
1.2.1. <i>Architecture of human and yeast mitochondrial genomes.</i>	23
1.2.2. <i>Replication of mitochondrial genomes in yeast and human.</i>	24
1.2.3. <i>Mitochondrial DNA maintenance actors in yeast and human.</i>	25
1.2.4. <i>Mitochondrial DNA repair.</i>	36
1.3. Mitochondrial DNA polymerase gamma.	39
1.3.1. <i>World of DNA polymerases.</i>	39
1.3.2. <i>DNA polymerisation as a universal mechanism common for all DNA polymerases.</i>	43
1.3.3. <i>Fidelity of DNA replication.</i>	48
1.3.3.1. <i>Fidelity of DNA polymerases.</i>	50
1.3.3.2. <i>Role of 3'-5' exonuclease activity in the fidelity of DNA polymerases.</i>	54
1.3.3.3. <i>Structural comparison of DNA polymerases</i>	

<i>harbouring 3'-5' exonuclease activity.</i>	55
1.3.3.4. <i>Balance between polymerase and 3'-5' exonuclease functions in DNA polymerases.</i>	70
1.3.4. <i>DNA polymerase gamma as the unique replicase of the mitochondrial genome.</i>	73
1.3.5. <i>DNA polymerase gamma forms a new subfamily of Pol A polymerases.</i>	74
1.3.6. <i>DNA polymerase gamma from yeast and human.</i>	83
1.3.6.1. <i>Functional insight into the linear representation and three-dimensional structure of pol g.</i>	83
1.3.6.2. <i>Yeast DNA polymerase gamma as a functional model for human pol g.</i>	91
1.3.6.3. <i>Systems generated for study on pol g structure and function.</i>	95
1.4. <i>Mutations in DNA polymerase gamma – from mutation to pathology.</i>	98
1.4.1. <i>Defects in nuclear genes associated with mitochondrial disorders.</i>	98
1.4.2. <i>Types of mutations existing in mitochondrial genome.</i>	100
1.4.3. <i>Pol g mutations affecting mitochondrial genome stability.</i>	101
1.4.4. <i>Mutator activity of pol g mutants.</i>	105
1.4.5. <i>Complexity of mitochondrial disorders.</i>	105
1.4.6. <i>Relationship between genotype and phenotype in mitochondrial disorders associated with</i>	

<i>pol g</i> mutations.	109
1.5. Yeast as a tool for studies on molecular mechanism in <i>pol g</i> -related mitochondrial disorders.	112
2. AIM OF THE STUDY	119
3. RESULTS	123
3.1. Selection and description of the disease-associated <i>pol g</i> mutations.	124
3.2. Construction of the <i>mip1</i> mutants.	129
3.3. Cellular respiration and stability of the mitochondrial genome.	129
3.3.1. <i>Cellular respiration.</i>	129
3.3.2. <i>Stability of the mitochondrial genome.</i>	130
3.4. Frequency of mtDNA point mutations.	133
3.5. <i>In vivo</i> phenotype of the heteroallelic F268R/R467W <i>mip1</i> mutant.	136
3.6. <i>In vivo</i> phenotype of the C261R, C261S and C261T <i>mip1</i> mutants.	138
3.7. Expression of wild-type and mutant Mip1.	140
3.7.1. <i>Wild-type and mutant Mip1 protein expression in mitochondrial extracts.</i>	140
3.7.2. <i>Wild-type and Mip1 protein purification and quantification.</i>	142
3.8. Gap-filling activity and processive DNA synthesis.	143
3.8.1. <i>Gap-filling activity.</i>	143
3.8.2. <i>Processive DNA synthesis.</i>	145
3.9. DNA binding activity.	149
3.10. 3'-5' exonuclease activity.	151

3.11. dNTP misincorporation.	156
3.12. Coupling of 3'-5' exonuclease and polymerase functions.	157
4. DISCUSSION AND CONCLUSIONS	163
4.1. Classification of yeast <i>mip1</i> mutations and correlation between disease severity and yeast phenotypes.	164
4.2. A novel module within the 3'-5' exonuclease domain, coupling DNA synthesis and degradation.	174
4.3. Model of a DNA primer strand switching mechanism between the exonuclease and polymerase active sites of pol γ .	176
4.4. Perspectives and conclusions.	185
5. MATERIAL AND METHODS	189
5.1. Growth media.	190
5.2. Strains and plasmids.	191
5.3. Construction of the <i>mip1</i> mutants.	193
5.4. Cytofusion.	196
5.5. <i>In vivo</i> techniques.	196
5.5.1. Cellular respiration.	196
5.5.2. Frequency of $\rho^{-/0}$ mutants.	197
5.5.3. DAPI staining.	197
5.5.4. Retention of mtDNA in $\rho^{-/0}$ clones.	198
5.5.5. Frequency of point mutations in mtDNA.	199
5.6. Preparation of the mitochondrial extracts.	200
5.7. Partial purification of Mip1 protein with DEAE chromatography.	201
5.8. SDS-PAGE, western blot analysis and quantification of Mip1.	201

5.9. Preparation of radiolabeled singly primed M13 (or PTZ18U) DNA substrate for <i>in vitro</i> assays.	202
5.10. DNA polymerase assays.	203
5.10.1. <i>Gap-filling activity</i> .	203
5.10.2. <i>Total DNA synthesis</i> .	204
5.10.3. <i>Processivity of DNA synthesis</i> .	204
5.11. 3'-5' exonuclease and misincorporation assays.	205
5.12. Gel electrophoretic mobility shift assay.	206
5.13. Polymerase/ 3'-5' exonuclease coupling assay.	207
5.14. Optimal conditions for 3'-5' exonuclease and polymerase assays.	208
5.15. Presentation of the structural data.	209
6. APPENDIX	211
7. REFERENCES	217

LIST OF ABBREVIATIONS:

ATP	adenosine-triphosphate
BER	base excision repair
BSA	bovine serum albumin
compound heterozygous	two different alleles of one gene in trans
DAPI	4'-6-diamidino-2-phenylindole
DEAE	diethylaminoethyl cellulose
DNA	deoxyribonucleic acid
ddNTP	dideoxynucleotide triphosphate
dNTP	deoxiribonucleotide triphosphate
dNMP	deoxiribonucleotide monophosphate
DTT	dithiotreitol
EDTA	ethylenediaminetetraacetic acid
EMSA	electrophoretic mobility shift assay
E^R	erythromycin resistant
G₀	minimal glucose media
HTH	helix-turn-helix
kDa	kilo Dalton
LB	Luria-Bertani
MIRAS	mitochondrial-associated ataxia syndrome
MMR	mismatch repair
MELAS	mitochondrial myopathy, encephalopathy, lactic acidosis and stroke
MNGIE	mitochondrial neurogastrointestinal

	encephalopathy syndrome
MOPS	3-(N-morpholino)propanesulfonic acid
<i>mit</i> ⁻	yeast mutant harboring point mutation in mitochondrial DNA
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial deoxyribonucleic acid
NER	nucleotide excision repair
OXPHOS	respiratory chain complexes and ATP synthase
PCR	polymerase chain reaction
PEO	progressive external ophtalmoplegia
PMSF	phenylmethanesulfonyl fluoride
Pol	polymerase
pol g	DNA polymerase gamma
Pol1k	Klenow fragment of DNA polymerase I
<i>rho</i> ⁺	yeast strain with wild-type mitochondrial DNA
<i>rho</i> ⁻	yeast mutant with deletion of mitochondrial DNA
<i>rho</i> ⁻⁰	yeast mutant with deletion or loss of mitochondrial DNA
<i>rho</i> ⁰	yeast mutant with loss of mitochondrial DNA
RITOLS	RNA incorporation throughout the lagging strand
RNA	ribonucleic acid
ROS	reactive oxygen species

rRNA	ribosomal ribonucleic acid
SANDO	ataxia-neuropathy with dysphagia and ophthalmoplegia
SCAE	spino-cerebral ataxia-epilepsy syndrome
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SNP	single nucleotide polymorphism
TLS	translesion synthesis
Tris	tris(hydroxymethyl)amino methane
tRNA	transfer ribonucleic acid
YD	yeast extract dextrose
YPD	yeast extract peptone dextrose

LIST OF FIGURES AND TABLES:

Figures:

INTRODUCTION:

Fig.1.1. Human and yeast mitochondrial genome.	22
Fig.1.2. Models of mtDNA replication.	27
Fig.1.3. Schematic organization of yeast and human replication forks.	35
Fig.1.4. The half-open right hand model of polymerase domain for the polynucleotide polymerase. General kinetics of the nucleotide incorporation for DNA polymerases.	44, 46
Fig.1.5. Two-metal ion mechanism of nucleotidyl transfer for the catalysis of the DNA polymerase reaction.	49
Fig.1.6. Crucial determinants of DNA synthesis fidelity of DNA polymerases with intrinsic 3'-5' exonuclease activity.	54
Fig.1.7. Comparison of crystal structures of five DNA polymerases with intrinsic 3'-5' exonuclease activity.	58
Fig.1.8. Comparison of the primary structure of five DNA polymerases with intrinsic 3'-5' exonuclease activity.	59
Fig.1.9. Conservation of the polymerase active site structural framework of five DNA polymerases with intrinsic 3'-5' exonuclease activity.	64
Fig.1.10. Spatial organization of the polymerase and exonuclease domain in human pol γ .	69
Fig.1.11. Polymerisation and editing mode of DNA polymerases with intrinsic 3'-5' exonuclease activity.	72
Fig.1.12. Comparison of crystal structures of three DNA	

polymerases belonging to Pol A family.	75
Fig.1.13. Comparison of the primary structure of three DNA polymerases belonging to Pol A family.	76
Fig.1.14. Coil and helix elements in the DNA binding channel of human pol g catalytic subunit.	82
Fig.1.15. Heterotrimer of the human DNA polymerase gamma.	84
Fig.1.16. Conservation of human pol g and yeast Mip1.	86
Fig.1.17. Amino acid sequence alignment of yeast and human pol g.	90
Fig.1.18. <i>mip1</i> mutations generated for pol g functional studies.	94
Fig.1.19. Possible pathways for repeat mediated DNA deletions in yeast and human mitochondria.	104
Fig.1.20. Pol g mutations associated with human disorders.	118

RESULTS:

Fig.3.1. Localization of the disease associated pol g mutations and their Mip1 equivalents.	126
Fig.3.2. Multiple amino acid sequence alignment of pol g fragments harbouring pol g mutations.	127
Fig.3.3. Determination of cellular respiration in mutant and wild-type strains.	130
Fig.3.4. Determination of mtDNA stability of wild-type and mutant strains at 28°C and 37°C.	132
Fig.3.5. Retention of mtDNA fragments in <i>rho</i> ⁻⁰ clones.	133
Fig.3.6. DAPI staining of wild-type and <i>mip1</i> -L210P cells.	133
Fig.3.7. Illustration of the mutator properties of <i>mip1</i> mutants in a qualitative test.	134

Fig.3.8. Determination of the frequency of point mutations in mtDNA.	135
Fig.3.9. Determination of the frequency of <i>rho</i> ⁻⁰ mutants present in the cultures spread erythromycin-containing plates in Fig.3.8.	135
Fig.3.10. Determination of mtDNA stability of wild-type (<i>MIP1</i> and <i>MIP1/MIP1</i>) and mutant (<i>mip1</i> -F268R, <i>mip1</i> -R467W, <i>MIP1/mip1</i> -F268R, <i>MIP1/mip1</i> -R467W, <i>mip1</i> -F268R/ <i>mip1</i> -R467W) strains at 28°C and 37°C.	136
Fig.3.11. Determination of point mutation frequency in mtDNA from wild-type (<i>MIP1</i> and <i>MIP1/MIP1</i>) and mutant (<i>mip1</i> -F268R, <i>mip1</i> -R467W, <i>MIP1/mip1</i> -F268R, <i>MIP1/mip1</i> -R467W, <i>mip1</i> -F268R/ <i>mip1</i> -R467W) strains.	137
Fig.3.12. Determination of mtDNA stability in wild-type and <i>mip1</i> -C261S, <i>mip1</i> -C261R, and <i>mip1</i> -C261T mutant strains at 28°C and 37°C.	139
Fig.3.13. Determination of point mutation frequency in mtDNA from wild-type and <i>mip1</i> -C261S, <i>mip1</i> -C261R, <i>mip1</i> -C261T mutants.	139
Fig.3.14. Wild-type and mutant Mip1 protein expression.	141
Fig.3.15. DEAE chromatography-purified fractions of wild-type and Mip1-L260R.	142
Fig.3.16. Quantification of wild-type and mutant Mip1.	143
Fig.3.17. Gap-filling activity.	144
Fig.3.18. Total DNA synthesis.	146
Fig.3.19. Processivity of wild-type and mutant Mip1.	146
Fig.3.20. Kinetics of DNA binding activity of wild-type Mip1	

in the absence and presence of 5 mM MgCl ₂ .	150
Fig.3.21. DNA binding activity of wild-type and mutant Mip1.	151
Fig.3.22. DNA binding activity of wild type and mutant Mip1.	152
Fig.3.23. 3'-5' exonuclease activity of wild-type and mutant Mip1.	154
Fig.3.24. MgCl ₂ dependent 3'-5' exonuclease activity of wild-type and R467W Mip1.	155
Fig.3.25. dGTP misincorporation by wild-type and L260R and R265H Mip1.	156
Fig.3.26. Exonuclease/polymerase (exo/pol) ratios for wild-type and mutant Mip1.	158
Fig.3.27. Polymerase/exonuclease-coupled assay.	160
DISCUSSION AND CONCLUSIONS:	
Fig.4.1. Correlation between disease severity and yeast phenotype.	165
Fig.4.2. Localization of the disease associated pol g mutations.	169
Fig.4.3. Structure of the 3'-5' exonuclease and fingers module in human pol g docked with DNA.	177
Fig.4.4. Comparison of the B-loop (residues 652-672) / plug (residues 170-202) and 'stabilizer' (residues 1049-1095)/ 'orienter' (residues 293-316) modules located in the DNA-binding channels of bacteriophage N4 virion RNA polymerase and human pol g, respectively.	180
Fig.4.5. Structural representation of wild-type and disease-associated mutated residues.	183
Fig.4.6. Structural comparison and amino acid sequence	

alignment of helix-turn-helix motif from several DNA-binding proteins and ‘orienter’ module from DNA polymerase gamma.	184
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Tables:

Table 1. Enzymes involved in mtDNA maintenance.	34
Table 2. Comparison of β -sheet panels from the 3’-5’ exonuclease and polymerase active sites of five DNA polymerases characterized by intrinsic 3’-5’ exonuclease activity.	70
Table 3. List of the <i>mip1</i> mutations generated as an equivalence of human disease associated mutations.	114
Table 4. Disease-associated pol g mutations, clinical presentations of the patient and yeast Mip1 equivalents.	128
Table 5. <i>In vivo</i> and <i>in vitro</i> phenotype data.	161
Table 6. Residues in the polymerase domain that are close neighbors of the 3’-5’ exonuclease module (residues 295-316).	178
Table 7. Fingers subdomain residues (1049-1095) that are close to docked DNA.	181
Table 8. Strains and plasmids.	191
Table 9. Oligonucleotides used for site-directed mutagenesis, DNA binding and other <i>in vitro</i> assays.	194

ABSTRACT:

Mitochondrial DNA (mtDNA) encodes a small set of components of the respiratory complexes. As these are strictly required for respiratory function, it is essential that integrity of the mitochondrial genome be maintained. DNA polymerase gamma (pol g in human, Mip1 in yeast), the unique DNA polymerase identified in mitochondria, bears sole responsibility for all DNA synthesis events associated with replication, recombination and repair of the mitochondrial genome. Pol g plays a key role in fidelity of mtDNA replication through accurate selection of nucleotides and 3'-5' exonuclease proofreading activity. It is well documented that defective maintenance of mtDNA leads to mitochondrial dysfunction and disease. More than 150 *POLG* mutations associated with a broad spectrum of clinical presentations have been identified in human.

Based on the good conservation of the pol g catalytic subunits we have used Mip1 as a model enzyme to generate yeast mutations equivalent to seven human pol g mutations localizing to the 3'-5' exonuclease and linker domains and associated with PEO, various neuropathies and Alpers syndrome. Five mutations clustered in a novel subdomain of the 3'-5' exonuclease, which is highly conserved in pol g family but absent in prokaryotic polA family, are localized in the DNA-binding channel in close vicinity to the polymerase domain (Lee, 2009). This novel subdomain is referred to as the 3'-5' exonuclease module throughout this thesis.

We have determined the *in vivo* phenotypes of the yeast mutants and biochemical properties of mutant polymerases. The *mip1* mutations increase the frequency of mtDNA point mutations (mutators) and deletions with generally a good correlation between mtDNA instability in yeast and disease severity in human. Mutations clustered in the 3'-5' exonuclease module result in increased frequency of point mutations and high instability of the mitochondrial DNA (mtDNA) in yeast cells, and unexpectedly for mutator mutations in the exonuclease domain, they favour exonucleolysis versus polymerization. This trait is associated with highly decreased DNA binding activity and poorly processive DNA synthesis. Our data show for the first time that a 3'-5' exonuclease module of DNA polymerase gamma plays a crucial role in the coordination of the polymerase and exonuclease functions and they strongly suggest that in patients the disease is not caused by defective proofreading but results from poor mtDNA replication generated by a severe imbalance between DNA synthesis and degradation.

1. INTRODUCTION

1.1.The importance of studies on mitochondrial DNA polymerase gamma mutations. *Main assumptions and aims of the research.*

Mitochondria contain their own DNA (mtDNA) which encodes a small number of components that are absolutely required for respiration and ATP synthesis. Correct replication of the mitochondrial genome is therefore crucial to mitochondrial function and health. DNA polymerase gamma (pol g) is the unique replicase of the mitochondrial genome (Kaguni, 2004). Studies on pol g were initiated at the beginning of 1970s by several groups (Temin and Mizutani, 1970; Baltimore, 1970; Wintersberger and Wintersberger, 1970). The first evidence supporting a role for pol g as a replicase of the mitochondrial genome was obtained in 1979 (Hubscher *et al*, 1979). Nowadays, it is almost sure that pol g is the only DNA polymerase found in animal and yeast mitochondria and thus bears sole responsibility for all DNA synthesis events in replication, recombination and repair of the mitochondrial genome (Kaguni, 2004; Graziewicz *et al*, 2006).

Mutations in the human nuclear *POLG1* gene which encodes the catalytic subunit of pol g are an important cause of mitochondrial disorders. Since 2001, when the first disease mutations of pol g were described (Van Goethem, 2001), more than 150 pathogenic *POLG1* alleles have been identified in human (Chan and Copeland, 2009; Human DNA Polymerase Gamma Database, <http://tools.niehs.nih.gov/polg/>). Progressive external ophthalmoplegia (PEO) is a frequent pol g pathology characterized by progressive weakness of the external eye muscles and exercise intolerance, and associated with multiple deletions of mtDNA coexisting with the

intact mtDNA molecules. Nevertheless, many other severe diseases such as myopathy, mental disorders, various neuropathies, premature menopause or Parkinsonism symptoms have also been associated with *POLG1* mutations (Graziewicz *et al*, 2006). Several mutations associated with mtDNA depletion result in the Alpers syndrome, a fatal disease in young children characterized by brain deterioration and hepatic failure (Naviaux *et al*, 1999). Some *POLG1* mutations are dominant or found at the homozygous state in the patients; however, the majority of the patients are compound heterozygous, harboring one or several mutations on each chromosome.

This shows that pol g mutations result in a large variety of complex clinical presentations that are presently not precisely understood. Therefore, it was proposed by many investigators working on human pol g disorders to develop quick and faithful *in vivo* and *in vitro* models which could help evaluate the results obtained from clinical studies (among others: Lamantea *et al*, 2002; Ferrari *et al*, 2005; Horvath *et al*, 2006; Copeland, 2008). In response to that idea we used yeast as a tool to determine the function of several amino acids of pol g involved in those pathologies and to investigate the impact of amino acid substitutions on the structure and function of the protein. The catalytic subunit of *Saccharomyces cerevisiae* pol g encoded by the *MIP1* gene (Foury, 1989) displays about 40% amino acid sequence identity with its human homologue in their shared regions (Ropp and Copeland, 1996; Lecrenier *et al*, 1997). Based on the hypothesis that conserved residues in conserved regions of different pol g should play a similar role, Mip1 has previously been used as a tool to determine the *in vivo* phenotypes conferred in yeast by several pathogenic pol g

mutations (Baruffini *et al*, 2006; Baruffini *et al*, 2007; Stuart *et al*, 2006; Stumpf *et al*, 2010; Baruffini *et al*, 2010). Additionally, yeast possesses peculiar genetic and molecular biology properties which are particularly well appropriate for studies on mitochondrial metabolism. In this work, the impact of six mutations in the 3'-5' exonuclease domain and one mutation in the linker region have been investigated. Most of the human pathogenic polg mutations are localized in the polymerase domain. They are thought to affect polymerisation activity, processivity of DNA synthesis, DNA or dNTP binding, and thus result in the most severe disease presentations characterized by early onset and high mortality. On the contrary, the less frequent disease mutations in the 3'-5' exonuclease domain are more commonly found in patients presenting milder disorders in teenage or adult life and are believed to affect most likely the 3'-5' exonuclease function of pol g rather than DNA synthesis (Horvath *et al*, 2006). It has generally been assumed that the pathogenicity of 3'-5' exonuclease mutations is associated with proofreading defects. However, in contrast with studies on the 3'-5' exonuclease mutations residing at the active site (Foury and Vanderstraeten, 1992; Vanderstraeten *et al*, 1998), we found that several of these exonuclease mutations result in severe impairment of the polymerase function and DNA binding without any effect on the 3'-5' exonuclease activity and thus escape the simplified domain-based classifications commonly used for disease pol g mutations. We believe that determination of the effects of pol g mutations on the biochemical properties of the mutated enzyme should help classifying the mutations and thus give light on the molecular defects responsible for

some mitochondrial disorders at the cellular level. Moreover, the yeast pol g model has led us to identify novel 3'-5' exonuclease modules which play important roles in the pol g function and disease onset. These results can potentially broaden our knowledge of the genetical and biochemical bases of the diseases, and be helpful in the design of mitochondrial pathology treatment methods in the distant future.

1.2. Yeast and human mitochondrial genomes and their maintenance.

Similarities and differences.

According to the endosymbiotic theory (Lang *et al*, 1999), mitochondria became an element of the eukaryotic cell approximately 1.8 billion years ago as a small endocysted α -proteobacterium transformed into intracellular generator of ATP. However, the detailed scenario of this transformation and exact relationship between the host and resident bacteria species remain unclear. The existence of DNA inside the mitochondria was confirmed in the early 1960s (Nass and Nass, 1963) and since then it appeared that mitochondrial genomes from different organisms can vary strongly in structure, size and coding capacity (Nosek and Tomaska, 2003; Bullerwell and Lang, 2005; Gray *et al*, 2004; Gissi *et al*, 2008). Extensive reduction of the mitochondrial genome coupled with progressive loss of metabolic autonomy of mitochondria occurred in the course of evolution as a result of either loss or transfer of genes between mitochondrial and nuclear DNAs (Andersson and Kurland, 1998). Thus the overwhelming majority of mitochondrial proteins are nuclearly encoded (Dimmer and Rapaport, 2008). However, in all cases mtDNA still encodes a small subset of components of the respiratory chain

complexes and ATP synthase (OXPHOS) from the mitochondrial inner membrane (Lodish *et al*, 2008).

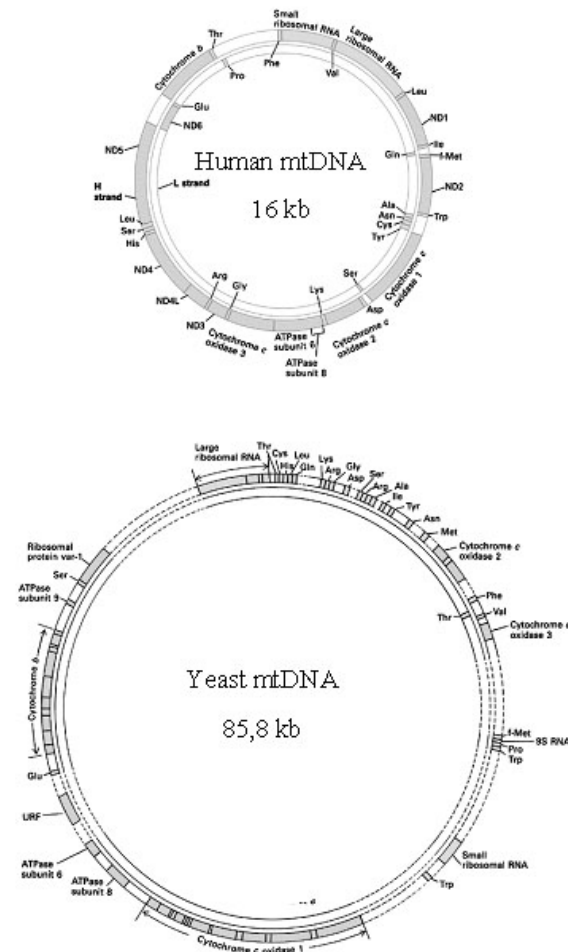


Figure 1.1. Human and yeast mitochondrial genome.

Schematic organization of the human and yeast *Saccharomyces cerevisiae* mitochondrial DNA. Mitochondrial DNA from human (at the top) is a 16kb intron-free circular molecule and encodes: 2 mt-rRNAs, 22 mt-tRNAs and 13 OXPHOS polypeptides (7 genes for complex I, 1 gene for complex III, 3 genes for complex IV and 2 genes for complex V) (Palmer, 1997). Yeast mitochondrial genome (at the bottom) is characterized by an intron-rich 85.8 kb sequence harboring genes for 7 OXPHOS-related mRNAs (1 gene for complex III, 3 genes for complex IV and 3 genes for complex V), 1 ribosomal protein (*var1*) and 27 RNA molecules (3 mt-rRNAs and 24 mt-tRNAs) (Palmer, 1997).

1.2.1. Architecture of human and yeast mitochondrial genomes:

Diversity in unity is the common attribute of organelle genomes from various organisms. Recent decades of studies suggest that this is also the case of human and yeast *Saccharomyces cerevisiae* mitochondrial genomes. Despite fundamental similarities, several notable differences in mtDNA structure and maintenance exist between both species (Fig.1.1.). Both mitochondrial genomes are characterized by coexistence of numerous identical DNA molecules. However, the mtDNA copy number in human cells fluctuates from 10^2 to 10^4 per cell, depending strongly on the cell type, and constitutes up to ~1% of total DNA cell mass, while in yeast cells the mtDNA copy number is reduced to 20~100 molecules per cell and represents up to 5-20% of total cellular DNA (Maleszka *et al*, 1991; Shadel, 1999; Holt *et al*, 2007). Interestingly, the human and *S. cerevisiae* mitochondrial genomes are quite different in size (16.6 kb to 85.8 kb, respectively) (Fig.1.1.; Anderson *et al*, 1981; Foury *et al*, 1998). Furthermore, it was shown that human mtDNA is a supercoiled, circular molecule characterized by a high gene density and the absence of introns (Anderson *et al*, 1981). In contrast, yeast mitochondrial genome is characterized by its low gene density and the presence of numerous introns. In addition, it is highly heterogeneous in base composition with long almost AT pure sequences separated by short GC clusters. Finally, despite the circular representation of its genetic map, the mtDNA of *S. cerevisiae* occurs mostly in the form of linear tandemly-arrayed molecules characterized (Maleszka *et al*, 1991; Shadel, 1999; Lecrenier and Foury, 2000). There are also several differences in the

genetic information harbored by both genomes. Yeast mtDNA bears 35 genes encoding 8 protein subunits, 24 tRNAs and 3 rRNAs, while human mitochondrial genome keeps 37 genes for 13 OXPHOS-related mRNAs, 22 tRNAs and 2 rRNAs (Fig.1.1.; Palmer, 1997; Foury, 1998). Among all differences in coding properties of both genomes, the most significant is the absence of genes for complex I subunits in *S. cerevisiae* mtDNA.

1.2.2. Replication of mitochondrial genomes in yeast and human:

The last three decades of studies on mitochondrial genome maintenance brought several models trying to explain the mechanism of mtDNA replication. These models were established based on the biochemical characterization of mtDNA and mtRNA species, as well as electron microscopic observations of mtDNA molecules and analysis of mtDNA synthesis intermediates and products by 2-D gel electrophoresis. Despite the vast pool of evidence collected by researchers and high variety of suggested mechanisms, the exact mode of mtDNA replication remains unclear. Generally, for all vertebrates as well as for lower eukaryotes, two basic models of mitochondrial genome replication were accepted (Fig.1.2.): the asymmetric strand-displacement model and conventional leading and lagging strand-coupled model (Tapper and Clayton, 1981; Brown *et al*, 2005). Both models were originally characterized by continuous synthesis of the DNA leading strand and involved unique initiation sites on each strand (Fig.1.2.). However, it has been proposed more recently that multiple replication origins are present in the lagging strand

(Bowmaker *et al*, 2003; Brown *et al*, 2005; Yasukawa *et al*, 2006). This hypothesis was supported by the observation that human mtDNA replication can be initiated at multiple sites through generation of Okazaki fragments and massive ribonucleotide formation (Bowmaker *et al*, 2003). Indeed, numerous mitochondrial RNA-DNA hybrids were recently reported in mice (Brown *et al*, 2008). Although some RNA-DNA hybrids (R-loops) are involved in priming DNA replication, others seem to fulfill another function and appear to be a general feature of the mitochondrial genome (Brown *et al*, 2008). Noteworthy, mitochondria contain all tools indispensable for Okazaki fragments formation and maturation (Holt, 2009). Both models have been proposed for the synthesis of human mtDNA; however, the strand-displacement model seems to occur more frequently in nature and is assumed to play a key role in the replication of the human mitochondrial genome (Holt, 2009). Recent studies revealed a third type of mtDNA synthesis in human, so-called RITOLS replication (termed for RNA incorporation throughout the lagging strand) (Yasukawa *et al*, 2006). RITOLS model, closely related to the asymmetric model, assumed that delayed lagging-strand DNA synthesis is accompanied by incorporation of RNA on the lagging strand (instead of mtSSB recruitment) and has been proposed to supersede the strand-displacement mechanism of DNA replication in human mitochondria (Fig.1.2.; Holt, 2009). RITOLS model has recently been modified based on the formation of R-loops in mouse mtDNA molecules (Brown *et al*, 2008). For many years the mitochondrial genome of budding yeast was believed to be circular similarly to animal mtDNA or to DNA of bacterial ancestors.

However, studies performed by Maleszka and colleagues showed that only a small fraction of DNA in mitochondria of *S. cerevisiae* is circular, while the majority is present in the form of heterogeneous linear tandem arrays of genome fragments (Skelly and Maleszka, 1989; Maleszka *et al*, 1991). These observations led them to propose a rolling-circle mechanism for yeast mtDNA replication, which perfectly supports the results obtained by 2-D gel electrophoresis and genetic investigations (Maleszka, 1991; Shibata and Ling, 2007; Ling *et al*, 2007). Although several evidences support a rolling-circle model of mtDNA synthesis, the replication of the yeast mitochondrial genome seems to be still more complex and requires further investigations.

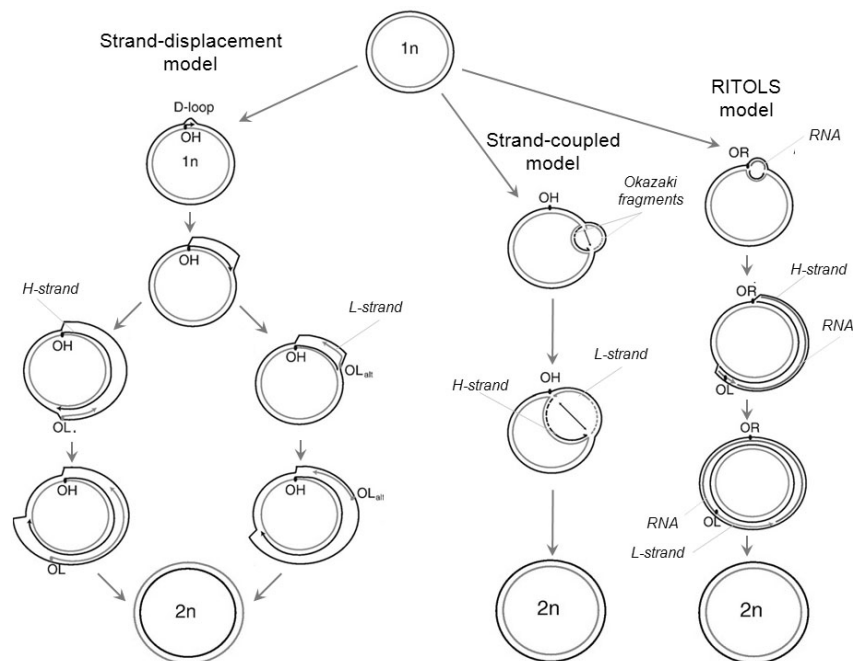


Figure 1.2. Models of mtDNA replication.

Strand-displacement replication model (on the left part of the scheme) assumes asymmetric synthesis of leading (H-strand) and lagging (L-strand) strands. Replication of the leading strand is unidirectional and initiates at the OH site. Replication of lagging strand initiates at the OL site as soon as it is exposed and proceeds in opposite direction. **Strand-coupled replication model** (in the middle part of the scheme) assumed bidirectional synthesis of leading and lagging strands in the form of the double-stranded replication fork. Synthesis of both strands is initiated from multiple origins localized downstream the OH site and involves formation of Okazaki fragments. **RITOLS model**. Ribonucleotide incorporation throughout the lagging strand (RITOLS) model of replication (on the right part of the schema) is closely related to the asymmetric mode of mtDNA synthesis and involves incorporation of short RNA fragments during L-strand synthesis. Unidirectional replication of the leading strand is initiated at the OH site, while the lagging strand is initially synthesized as RNA using leading strand as a template. RNA fragment is subsequently replaced by DNA lagging strand. However the exact mechanism of this replacement remains unclear. Adapted from Wanrooij and Falkenberg, 2010.

1.2.3. Actors of mitochondrial DNA maintenance in yeast and human:

Replication of the mitochondrial genome requires a unique enzymatic machinery, composed of a set of factors encoded by nuclear DNA and

recruited by mitochondria. Although several of the basic components of this system have been identified and characterized, some have not been reported up to now (as an example, the primase for lagging-strand DNA synthesis). *In vitro* studies showed that the basic replication fork of human mitochondria can be reconstituted using singly primed single-stranded DNA substrate and at least five highly purified recombinant proteins (Fig.1.3.): catalytic and accessory subunits of DNA polymerase gamma (pol gA and pol gB), mitochondrial RNA polymerase (POLRMT), replicative DNA helicase (TWINKLE) and the mitochondrial single-stranded DNA binding protein (mtSSB) (Korhonen *et al*, 2004; Wanrooij *et al*, 2008; Wanrooij and Falkenberg, 2010). Although this simple replisome is sufficient to duplicate a DNA substrate *in vitro*, proper maintenance and effective replication of the mitochondrial genome involve a much longer and still growing list of proteins and factors which will not be fully presented here. A brief characterization of the key components of the replicative machinery in yeast and human mitochondria is given below (Fig.1.3.). Mitochondrial DNA polymerase gamma, as the subject of this thesis, will be presented in great details in the following chapters.

Mitochondrial RNA polymerase: Mitochondria recruit their own nuclearly encoded RNA polymerase, which is essential not only for expression of the mitochondrial genome in the transcription process but also generates the RNA primers which are necessary for initiation of DNA duplication (Chang *et al*, 1985). In mammals, initiation of the leading-strand DNA synthesis occurs at the replication origin of the

heavy strand (O_H) by priming with approximately 100 nt RNA oligonucleotide fragments generated at the so-called light strand promoter site (LPS) by transcription initiation (Chang and Clayton, 1985). Recent findings suggest an essential role of the mitochondrial RNA polymerase in priming of lagging DNA strand synthesis and R-loop formation (Bowmaker *et al*, 2003; Yasukawa *et al*, 2006; Brown *et al*, 2006; Brown *et al*, 2008). Coding sequences of the mitochondrial RNA polymerases, which show high homology to T7 RNA polymerase, were cloned both in *S. cerevisiae* (*RPO41*) and in human (h-mtRPOL) (Masters *et al*, 1987; Tiranti *et al*, 1997).

Replicative DNA helicase: The DNA replication process requires separation of DNA strands. Unwinding of the DNA strands is normally catalyzed by a group of enzymes called DNA helicases. The replicative helicase of human mitochondria remained elusive up to 2001, when the TWINKLE gene was identified in a screen for human patients bearing mtDNA deletions (Spelbrink *et al*, 2001). The name TWINKLE refers to the T7 gp4-like protein with intramitochondrial nucleoids localization. TWINKLE is a 5'-3'-directed DNA helicase (Korhonen *et al*, 2003). Two DNA helicases were identified in yeast mitochondria. Hmi1 is specific to fungi and is essential for mtDNA maintenance (Sedman *et al*, 2005; Kuusk *et al*, 2005; Monroe *et al*, 2005). Pif1 belongs to a family of helicases conserved in eukaryotes including human. In yeast Pif1 is found in mitochondria and in nucleus where it removes telomerase from the telomere. It is not essential for mtDNA replication but plays an important role in recombination and repair of mitochondrial genome (Foury and

Lahaye, 1987; Lahaye *et al*, 1991; Schulz and Zakian, 1994; Bessler *et al*, 2001; Boule and Zakian, 2006; Paeschke *et al*, 2010).

Mitochondrial single-stranded DNA-binding protein: The mitochondrial single-stranded DNA-binding protein (SSB) plays a key role in mtDNA replication and repair. This small protein of ~120 amino acids is conserved in eukaryotic mitochondria and shows homology to bacterial SSB. Through its ability to coat single-stranded DNA and thus maintain the DNA strands of the double helix separated, it stimulates TWINKLE and pol γ activity in human (Korhonen *et al* 2003; Korhonen *et al* 2004; Pestryakov and Lavrik, 2008). The *RIM1* gene which encodes the 13-kDa yeast mitochondrial SSB was isolated in our laboratory as a multi-copy suppressor of the growth temperature-sensitive defect displayed by null *pif1* mutants on the respiratory substrate glycerol (Van Dyck *et al*, 1992). This suppressor activity supports a cooperative role between Pif1 and Rim1 in maintaining DNA in an unwound form, as demonstrated in an *in vitro* assay (Van Dyck *et al*, 1992). The yeast *RIM1* gene is essential for maintenance of mitochondrial genome. The gene encoding human mtSSB was cloned in 1993 (Tiranti *et al*, 1993).

Mitochondrial nucleases: Phosphodiesterase activities are easily detected in mitochondria. Among others, endonuclease activity is essential for formation and maturation of the RNA primers required for initiation of mtDNA replication. This role is executed by the Rnase mitochondrial-RNA-processing (RNase MRP), a site-specific endonuclease, which cleaves LSP transcript *in vitro* in regions

corresponding to the major initiation sites of leading-strand mtDNA synthesis (Bennett and Clayton, 1990; Lee and Clayton, 1997, Graves *et al*, 1998). RNase MRP was found both in nucleus and mitochondria of yeast and human cells (Topper and Clayton, 1990; Schmitt and Clayton, 1992; Kiss and Filipowicz, 1992; Stohl and Clayton, 1992; Li *et al*, 1994; Mosig *et al*, 1995).

Primers for mtDNA synthesis may be also processed by endonuclease G (Endo G), which cleaves nascent RNA transcripts mostly in mitochondria of mammalian cells (Cote and Ruiz-Carrillo, 1993; Prats *et al*, 1997). Although the enzymatic activity is different, Endo G shares some sequence identity with the potent yeast mitochondrial nuclease, Nuc1 (Lecrenier and Foury, 2000). Moreover, Endo G and Nuc1 are able to cleave efficiently both RNA and DNA substrates, and in a yeast *Nuc1Δ* strain mtDNA recombination is reduced.

Endonucleases also participate in mtDNA repair and recombination. Apurinic/apyrimidinic endonucleases (AP-endonucleases), which catalyze incision of the DNA phosphate backbone at the AP site, are involved in base excision repair in mitochondria (Tomkinson *et al*, 1988). AP-endonuclease activity appears to increase in mitochondria of aging mouse cells (Szczesny *et al*, 2003). The Cce1 resolvase, which acts as a cruciform cutting endonuclease resolving Holiday junctions, is possibly involved in intramolecular recombination in yeast mitochondria (Ezekiel *et al*, 1993; Sembongi *et al*, 2007). The human homologue of Cce1p remains unknown.

Other factors: TFAM: This mitochondrial transcription factor (also known as TFAM or mtTFA) is one of the factors required by

mitochondrial RNA polymerase to maintain a high level of initiation specificity. It belongs to the high-mobility-group (HMG) box proteins which play an important role in DNA replication. This specific factor is able to recruit other replication factors to the D-loop of mtDNA molecule through its binding to conserved regulatory sequences localized in the D-loop fragment (Ghivizzani *et al*, 1994). TFAM is believed to act in wrapping, bending and unwinding of mitochondrial DNA (Fisher *et al*, 1992). Moreover, it was also postulated to provide protection to mtDNA in a histone-like fashion (Shen and Bogenhagen, 2001; Takamatsu *et al*, 2002). TFAM is not unique to higher eukaryotes since the yeast Abf2 protein is homolog to human TFAM (Parisi *et al*, 1993). *TFB2M*: A second important mitochondrial transcription factor present both in yeast and human cells and involved in mtDNA maintenance is TFB2M (termed also as mtTFB or MTF1) (Schinkel *et al*, 1987; Falkenberg *et al*, 2002). Mitochondrial RNA polymerase is unable to initiate transcription, and thus initial DNA priming, on its own. It requires assistance of TFB2M which promotes specific binding to the catalytic subunit of RNA polymerase (Schinkel *et al*, 1987; Xu and Clayton, 1992). *Topoisomerases*: Topoisomerases is a class of enzymes able to control topology of DNA by winding and unwinding of DNA strands in order to facilitate DNA replication. Generally, topoisomerases cut the phosphate backbone of either double-stranded or single-stranded DNA substrates, reducing potential tensions, and thus stabilize the proper topology of DNA molecule. Up to now several topoisomerase activities were identified in mitochondria of various organisms. Top1mt and Top3 α were proposed to be the major topoisomerases of human mitochondria (Zhang *et al*,

2007). An unconfirmed report suggests that topoisomerase activities are also present in the mitochondria of budding yeast (Tua *et al*, 1997). Among other factors involved in mtDNA maintenance we can distinguish also ligases, RNases and glycosylases which are present both in yeast and human mitochondria and play important roles in replication, repair and recombination of mitochondrial genome (Foury *et al*, 2004; de Souza-Pinto *et al*, 2008; Liu and Demple, 2010).

Table 1. Enzymes involved in mtDNA maintenance.			
Enzyme	Yeast	Man	Function
DNA polymerase			
gamma			
Catalytic subunit	Mip1	Pol gA	5'-3' polymerase; 3'-5' exonuclease
Accessory subunit	Not identified	Pol gB	Processivity factor
Single-stranded DNA binding protein	Rim1	mtSSB	Single-stranded DNA stabilization
Helicase	Hmi1 Pif1	TWINKLE	Unwinding of DNA strands
Nuclease	RNase MRP	RNase MRP	Cleavage of RNA primers
	Nuc1	Endo G	Maturation of RNA primers/ DNA cleavage
	Cce1	Not identified	Resolvase of Holiday junctions
RNA polymerase	Rpo41	mtRPOL	5'-3' RNA synthesis
Topoisomerase	Not identified	Topo I Topo III α	Winding and unwinding DNA strands
Ligase	Cdc9	DNA ligase III	Ligation of DNA molecules
Other factors			
TFAM	Abf2	h-mtTFA	Transcription factor; wrapping, bending and unwinding of mtDNA
TFB2M	Sc-mtTFB	h-mtTFB	Transcription factor; initiation of priming

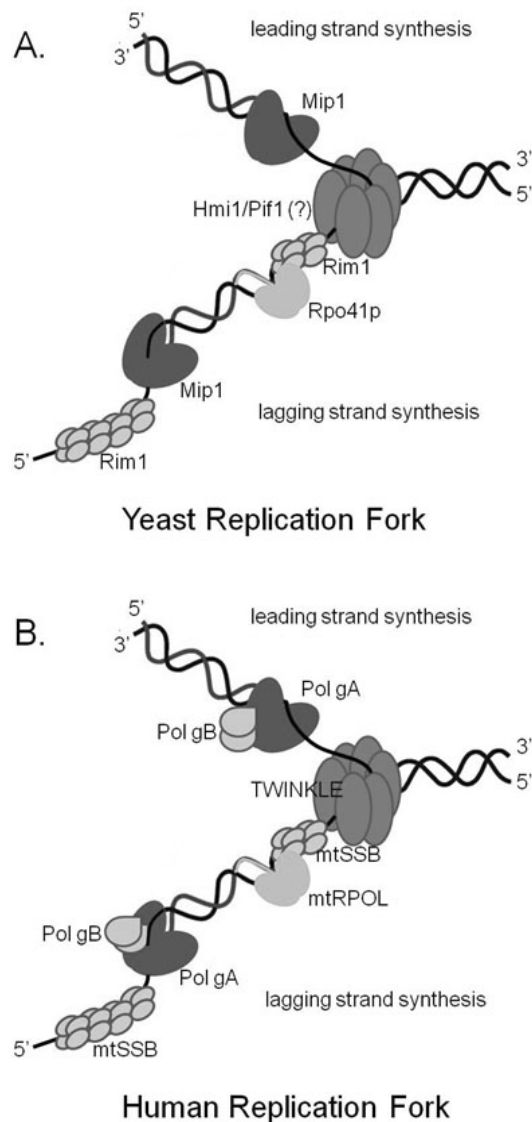


Figure 1.3. Schematic organization of yeast and human replication fork.

A. Simplified representation of the yeast *Saccharomyces cerevisiae* mtDNA replication machinery. Mip1: yeast DNA polymerase gamma; Hmi1/Pif1: two potential mitochondrial DNA helicases; Rim1: yeast mitochondrial single-stranded DNA-binding protein (SSB); Rpo41: yeast mitochondrial RNA polymerase. **B.** DNA replication machinery in human mitochondria. Pol gA: human DNA polymerase gamma catalytic subunit; Pol gB: human DNA polymerase gamma accessory subunit; TWINKLE: human mitochondrial DNA helicase; mtSSB: human mitochondrial SSB protein; mtRPOL: human mitochondrial RNA polymerase. Partially adapted from Wanrooij and Falkenberg, 2010.

1.2.4. Mitochondrial DNA repair:

Studies on mitochondrial physiology showed that the mitochondrial genome is more exposed to damage than nuclear DNA. The point mutation frequency and the level of damages measured in human mtDNA exceed over 10 times those observed for nuclear DNA (Brown *et al*, 1979; Yakes and Van Houten, 1997; Santos *et al*, 2002). Two main factors are believed to account for these damages. First, mtDNA is near the mitochondrial inner membrane which is the site of extensive generation of reactive oxygen species (ROS) through the respiratory chain electron transfer. Secondly, contrary to nuclear DNA, and despite its association with TFAM/abf2 (Diffley and Stillman, 1991; Sia *et al*, 2009), mtDNA is deprived of the effective protection guaranteed by histones. Several DNA repair pathways were identified in mitochondria in the last decades of research and DNA polymerase gamma is a key player in most of them, since it is the only enzyme synthesizing DNA in mitochondria. Four repair pathways will be briefly reported below.

Base excision repair:

Base excision repair (BER) is a major DNA repair pathway in human mitochondria which deals with small DNA lesions generated as a result of oxidation, alkylation or hydrolysis. Thus it mediates removal of damaged bases which can lead to mutations or DNA breaks. The sequence of events involved in BER is as follows: recognition and removal of damaged base by DNA glycosylases resulting in formation of an abasic (AP) site, cleavage of the AP site by AP endonuclease,

filling-in the short-patch (SP) or long-patch (LP) gapped nucleotide sequences by DNA polymerase gamma (for SP-BER or LP-BER respectively) and finally sealing of the DNA fragments by DNA ligase (Liu *et al*, 2007). The BER pathway and its enzymatic machinery are present and rather well conserved both in yeast and human mitochondria (Mandavilli *et al*, 2002; de Souza-Pinto *et al*, 2008).

Nucleotide excision repair:

Nucleotide excision repair (NER) is the DNA repair pathway involved in removing of bulky adducts from DNA strands. It is grounded on the excision and resynthesis of large fragments of the DNA sequence, in contrast to small lesions repaired by the BER machinery. NER is a common repair pathway in bacterial cells and in the nucleus of eukaryotes, however this pathway does not exist in human or yeast mitochondria (Shuck *et al*, 2008). Moreover, there is evidence that in mitochondria bulky lesions in DNA are not repaired but extensively degraded (Clayton *et al*, 1975).

Mismatch repair:

Base mismatches, short deletions or insertions are removed by the mismatch repair (MMR) machinery. It is a strand-specific repair mainly involved in removing mistakes introduced by the polymerase during DNA processing. The MMR machinery involves several protein components which firstly recognize the mismatched nucleotide, then remove the incorrect base, and finally resynthesize the missing fragment of DNA strand (Marsischky *et al*, 1996; Kolodner and Marsischky, 1999; Iyer *et al*, 2006). MMR has widely

been described in human and yeast nuclear DNA. Initiation of this process is carried by two protein complexes: MutS α (MSH2-MSH6 proteins) and MutS β (MSH2-MSH3 proteins). The presence of an MMR pathway has been established in yeast mitochondria (Chi and Kolodner, 1994; Marsischky *et al*, 1996; Sia *et al*, 2000). Based on the homology with the *MutS* gene involved in MMR in *E. coli* the yeast *MSH1* gene was isolated (Reenan and Kolodner, 1993). Deletion of the *MSH1* gene results in loss of mtDNA, while mutants show high mtDNA instability and elevated point mutation frequency (Reenan and Kolodner, 1992; Koprowski *et al*, 2001). There is no *MSH1* equivalent in human mitochondria. However, there is evidence for mismatch repair activities in human mitochondria, although a role in removal of point mutations has not been reported (Buermeier *et al*, 1999; Mason *et al*, 2003; de Souza-Pinto *et al*, 2009).

MtDNA recombination:

Recombination mechanisms play an important role, not only during genetic recombination, but also in the repair of broken DNA ends generated for instance, at sites of double strand DNA breaks. Two major mechanisms have been proposed; homologous recombination and non-homologous-end-joining. Homologous recombination was shown to occur extensively in yeast mitochondria (Dujon *et al*, 1981; Sena *et al*, 1986; Kleff *et al*, 1992). Although for many years this was strongly debated, recent *in vitro* experiments have lent the support to the existence of both homologous recombination and non-homologous end-joining in human mitochondria (Srivastava and Moraes, 2005; Bacman *et al*, 2009; Fukui and Moraes, 2009). Moreover, studies on

the human patients revealed the presence of recombination intermediates in tissue samples from the patients with multiple mtDNA deletions, and tissue biopsies from human heart muscle (Poulton *et al*, 1993; Kajander *et al*, 2001; Kraytsberg *et al*, 2004). However, these recombination events are very rare, and much remains to be learned in that field.

1.3. Mitochondrial DNA polymerase gamma:

1.3.1. World of DNA polymerases:

The information about the structure and function of all living organisms is recorded, stored and transmitted in the form of nucleic acids, mainly as DNA. However, to ensure the adequate manner and conditions of maintenance of this crucial information, organisms had to develop a specialized machinery in which DNA polymerases constitute the crucial point. The major function of DNA polymerases is to efficiently and accurately replicate the genetic information. This role is fulfilled by catalysis of the deoxyribonucleotides polymerization into a DNA strand based on the reading of a template. This process can be executed only in the 5'-3' direction of the nascent DNA strand. Moreover, as no polymerase is able to synthesize DNA *de novo*, all DNA polymerases require a primer which can provide the free 3'-OH group required for formation of the phosphodiester bond between the DNA core and incoming dNTP.

Copying of the DNA has to be accurate to ensure the faithful transmission of the genetic information through generations.

Additionally, DNA is continuously exposed to environmental damaging conditions, which can result in generation of errors in the DNA sequence. Therefore, the second, though not less important role of DNA polymerases, is DNA repair in order to assure the integrity of the genome. Numerous DNA repair pathways have been described in prokaryotic and eukaryotic cells that involve various DNA polymerases and often require additional catalytic activities.

Altogether, the complexity of living organisms and the variety of tasks requiring DNA have led to numerous and highly specialized DNA polymerases. As an illustration of the wealth of the world of DNA polymerases, until now, five different DNA polymerases have been described in *Escherichia coli*, eight in the yeast *Saccharomyces cerevisiae*, twelve in plant *Arabidopsis thaliana* and sixteen in humans (Garcia-Diaz and Bebenek, 2007). Moreover, recent studies have reported new types of DNA polymerases and the total number increases every year. Comparison of the primary features of DNA polymerase sequences has led to a classification into four basic families of DNA polymerases: A, B, C and X (Ito and Braithwaite, 1991; Braithwaite and Ito, 1993). Further studies allowed classifying the C family polymerases as a subfamily of X family, and revealed the Y family as a completely new type of DNA polymerases (Ohmori *et al*, 2001, Burgers *et al*, 2001). More recently, several archaeal DNA polymerases have been incorporated into the D family (Cann and Ishino, 1999; Autexier and Lue, 2006; Hizi and Herschhorn, 2008). A brief specification of DNA polymerase families with special emphasis on PolA family is presented below.

Family A:

The A family contains both replicative and repair polymerases derived from phages, archaeobacteria, eubacteria and eukaryotes. The prototype member of this family and also the first DNA polymerase with solved crystal structure was Pol I from *E. coli* (Lehman *et al*, 1958, Ollis *et al*, 1985). Beside DNA polymerization, Pol I is characterized by 3'-5' and 5'-3' exonuclease activities, supporting its key role in Okazaki fragments maturation generated during DNA lagging strand synthesis and DNA repair in *E. coli* (Kornberg and Baker, 1992). Among other polymerases specialized in DNA repair the KlenTaq pol I from *Thermus aquaticus* and large fragment of DNA polymerase I from *Bacillus stearothermophilus* can be mentioned (Korolev *et al*, 1995; Kiefer *et al*, 1998). Two main replicative polymerases belonging to Pol A family have also been extensively described: DNA polymerase from bacteriophage T7 (Doublet and Ellenberger, 1998) and eukaryotic mitochondrial DNA polymerase gamma (pol g) which, as the subject of this work, will be fully described in the following chapters. Beside pol g, two other eukaryotic pol A polymerases were found, pol ν and pol θ , that were proposed to participate in translesion synthesis (Marini *et al*, 2003; Seki *et al*, 2003; Seki *et al* 2004; Takata *et al*, 2006).

Family B:

This group of polymerases includes the most important replicative, faithful and multisubunit polymerases isolated from replicative forks of higher organisms: pol α , pol δ and pol ϵ (Garg and Burgers, 2005). With the exception of pol α which is involved in the Okazaki

fragments formation in eukaryotes, all these polymerases (in the form of holoenzyme with processivity factors) are highly processive and possess 3'-5' exonuclease activity. In contrast, the other eukaryotic Pol B member, pol ζ , is rather unfaithful (Lawrence, 2004). Several polymerases derived from bacteria, phages and viruses also belong to Pol B family, including the extensively studied polymerases from T4, Phi29, and RB69 bacteriophages (Wang *et al*, 1997; Shamoo and Steitz, 1999; Berman *et al*, 2007).

Family C: As mentioned above, the α subunit of the bacterial multisubunit DNA polymerase III was originally classified in the Pol C family, however further investigations led to place it in the Pol X family.

Family X:

The best characterized member of Pol X family is eukaryotic polymerase β involved in short-patches base excision repair (Wilson *et al*, 2000; Garcia-Diaz and Bebenek, 2007). More recently, other Pol X eukaryotic polymerases, such as pol σ , pol λ and pol μ , were identified. All are rather small and monomeric enzymes involved in DNA repair pathways (Ramadan *et al*, 2004). Besides BER, pol λ and pol μ participate in nonhomologous end-joining and V(D)J recombination (terminal deoxynucleotidyl transferase) (Dominguez *et al*, 2000; Bertocci *et al*, 2006), while in *S. cerevisiae* pol σ is involved in sister chromatid cohesion (Wang *et al*, 2000; Wang and Christman, 2001).

Family Y:

Polymerases belonging to Pol Y family are characterized by their low fidelity and ability to process damaged DNA templates and the lack of 3'-5' exonuclease activity (Kunkel, 2004). Moreover, these polymerases have no typical DNA-binding pocket, a property which ensures them flexibility, so that the polymerase structure can adjust to the incoming DNA template, allowing the synthesis of damaged DNA (Ling *et al*, 2001). Through these specific properties Pol Y polymerases are mainly involved in translesion synthesis repair pathways (TLS), acting as a bypass in the replication of the lesion-rich DNA and providing DNA lesion tolerance. Among Pol Y family members, two proteins called UmuD'2C and DinB were originally described in *E. coli* (Tang *et al*, 1999; Wagner *et al*, 1999). However, the first eukaryotic Pol Y polymerase, pol η was discovered almost simultaneously (Johnson *et al*, 1999; Masutani *et al*, 1999).

Family D:

Pol D family includes a weakly characterized group of archaeal polymerases whose function remains unclear (Cann and Ishino, 1999).

1.3.2. DNA polymerization as a universal mechanism common for all DNA polymerases:

Canonical right-hand configuration of DNA polymerases:

Nucleic acid polymerization, as probably one of the earliest and most primitive catalytic processes carried out by living organisms, had to be

not only simple but also faithful. Nowadays, we have a number of crystal structures of DNA polymerases derived from various families at our disposal. Based on the three-dimensional structure similarities in the overall architecture features shared by various polynucleotide synthases including RNA polymerases, the mechanism of DNA synthesis is likely universal for all DNA polymerases. The typical structure of DNA polymerases has been compared to an open right hand in which the fingers, thumb and palm (Fig.1.4.A. and B.) form a large cleft containing the DNA substrate and receiving the nucleotides (Kohlstaedt *et al*, 1992). The palm at the bottom of the cleft corresponds to the catalytic site of the DNA polymerase, a crucial site for the phosphoryl transfer reaction. The major function of fingers subdomain is recognition and positioning of incoming nucleoside triphosphates towards the DNA template. Finally, the thumb is mainly involved in positioning of the DNA duplex. It is also involved in the translocation of the primer strand during DNA processing and thus it can influence the processivity of the enzyme (Kohlstaedt *et al*, 1992; Steitz, 1999).

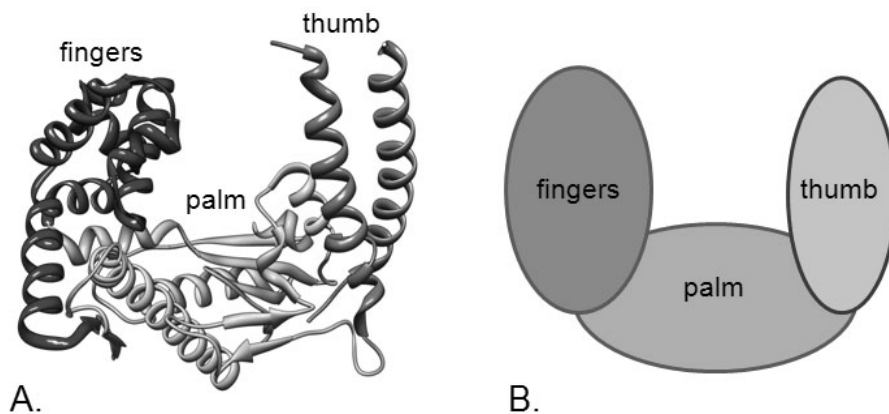


Figure 1.4.A. and B. The half-open right hand model of polymerase domain for the polynucleotide polymerase.

The canonical right-hand configuration of polymerase domain with “palm”, “fingers” and “thumb” subdomains forming catalytic site for polymerase activity was proposed by Kohlstaedt *et al* in 1992. **A.** Three dimensional representation of the polymerase domain from the *E. coli* DNA polymerase I Klenow fragment (PolIk) crystal structure (pdb ID: 1KFD). Figure prepared by using the UCSF Chimera software. **B.** Schematic organization of the polynucleotide polymerase domain.

Kinetics of nucleotide incorporation:

At the beginning of the 1990s a general kinetic reaction pathway for dNTP incorporation was proposed (Fig.1.4.) based on the crystal structures of PolIk from *E. coli* and bacteriophage T7 DNA polymerase (Mizrahi *et al*, 1985; Polesky *et al*, 1990; Patel *et al*, 1991; Wong *et al*, 1991). All DNA polymerases catalyze the addition of dNTP to the DNA nascent chain in a step-by-step reaction, which includes a conformational change of the enzyme (Fig.1.4.C.). A change in the enzyme conformation from “open” (“relaxed”) to “closed” (“tight”) was proposed to be one of the most crucial rate-limiting steps occurring during the DNA synthesis process (Mizrahi *et al*, 1985; Johnson, 1993). Despite an overall unity in architecture, no extensive similarities in amino acid sequences between the various DNA polymerases is observed. Whereas the palm subdomain shares high structural homology in different DNA polymerases, the finger and thumb subdomains show rather distant architecture properties in polymerases belonging to various families (Steitz, 1999). Noteworthy, correct orientation of the dNTP in the ternary complex with DNA polymerase and DNA primer-template seems to be crucial for correct and faithful DNA processing. In a first step, the primer-template complexed with polymerase binds the incoming dNTP in a non-template-dependent fashion (step 1 in Fig.1.4.C.). This non-rigorous

step is followed by a rate-limiting change in the conformation of the DNA polymerase which allows matching and tight binding of the dNTP to the DNA template sequence (step 2 in Fig.1.4.C.). This key conformational change can be achieved by rotation of the fingers subdomain and is limited by correct Watson Crick base pairing (Bryant *et al*, 1983; Sawaya *et al*, 1997; Steitz, 1999). The change in DNA polymerase conformation occurring under the impact of ternary complex formation should be strongly dependent on the architecture of the DNA binding pocket, making the reciprocal internal fitness of the ternary complex a flashpoint of DNA processing and enzyme fidelity. Moreover, DNA polymerases harboring 3'-5' exonuclease activity are forced to conciliate the cooperating polymerase and exonuclease functions with the positioning/switching of the DNA substrate and with the conformational changes occurring during these two coexisting catalytical processes.

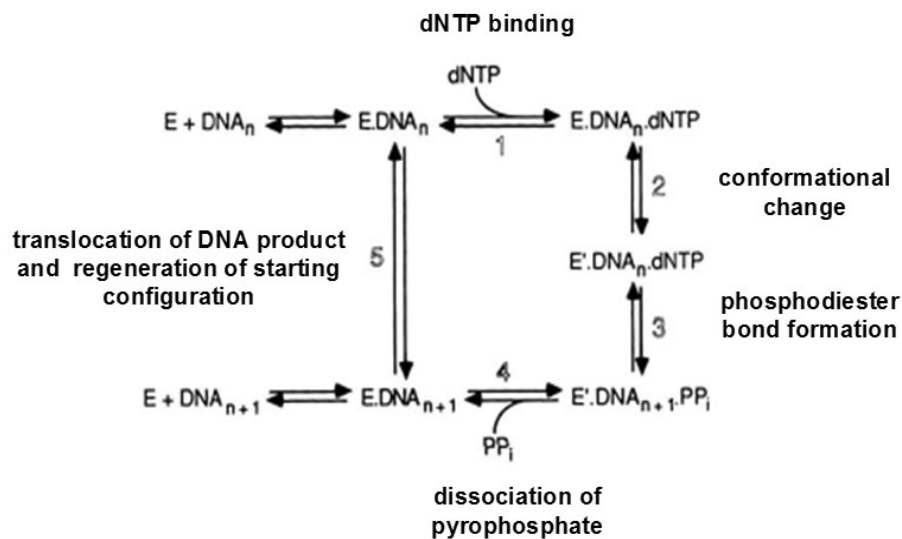


Figure 1.4. C. General kinetics of the nucleotide incorporation for DNA polymerases.

The reaction of DNA synthesis starts when the DNA polymerase (E) binds to the DNA substrate (DNA_n). Following steps are included: (1) binding of the dNTP to the enzyme-DNA primer-template complex; (2) conformational change of the DNA polymerase (E') complexed with dNTP and DNA primer-template ($\text{E}'\cdot\text{DNA}_n\cdot\text{dNTP}$); (3) formation of the phosphodiester bond between dNTP and 3' end of DNA primer strand ($\text{E}\cdot\text{DNA}_{n+1}\cdot\text{PP}_i$); (4) dissociation of the pyrophosphate (PP_i) from the DNA polymerase complexed with DNA primer-template ($\text{E}\cdot\text{DNA}_{n+1}$); (5) polymerase cycle and with translocation of the DNA product (DNA_{n+1}) to regenerate the starting configuration of DNA polymerase (E). Adapted from Polesky *et al*, 1990.

Two-metal ion mechanism for catalysis of DNA polymerization:

Similarities in the crystal structure and amino acid sequence of various DNA polymerases and, in particular, the presence within the palm subdomain, of at least two acidic residues and two metallic ions crucial for DNA polymerization, have led to the suggestion that in all cases the reaction of phosphoryl transfer is catalyzed by the so-called two-metal ion mechanism (Fig.1.5.) (Steitz, 1999; Garcia-Diaz and Bebenek, 2007). The metal ions catalyze, through their coordination with several amino acid residues, and in particular two aspartates, dNTP incorporation into the newly synthesized DNA chain (Fig.1.5.). Noteworthy, these aspartic acid residues are strictly conserved among DNA polymerases belonging to different families (Steitz, 1999). The metal ion A activates the 3'-OH terminus of the DNA primer strand for attack on the α -phosphate of the dNTP through a reduction of hydroxyl pK_a (Fig.1.5.). Metal ion B plays a double role, firstly, by stabilization of the negative charge that builds up during catalysis and secondly, by chelating of the β - and γ -phosphates to facilitate their leaving (Fig.1.5.). Moreover, both metal ions were proposed to participate in stabilization of the structure and charge of the pentacovalent transition state (Fig.1.5.) formed during the catalytic

process (Steitz and Steitz, 1993; Brautigam and Steitz, 1998; Steitz, 1999).

All the features of DNA polymerases presented above as well as clear differences in structure, sequence and basic functional properties of DNA polymerases derived from the same family, suggest that studying one representative model enzyme for each DNA polymerase family is not sufficient to understand the function of each enzyme precisely (Garcia-Diaz and Bebenek, 2007).

1.3.3. Fidelity of DNA replication:

Fidelity of DNA replication constitutes a major determinant of the genetic information stability. Proper balance between correct and incorrect DNA synthesis must be maintained by organisms to avoid mutations which can lead to development of disease, cancer and even death. On the other hand, low DNA synthesis fidelity provides diversity of genetic information and thus allows flexibility of organisms that have to stay in contact with variable environmental conditions. Fidelity of DNA replication strongly depends not only on DNA polymerase, the key enzyme responsible for DNA synthesis fidelity, but also on other enzymes and factors involved in DNA maintenance, as well as on the type of damage in DNA and local DNA sequence environment (Kunkel and Bebenek, 2000; Kunkel, 2004).

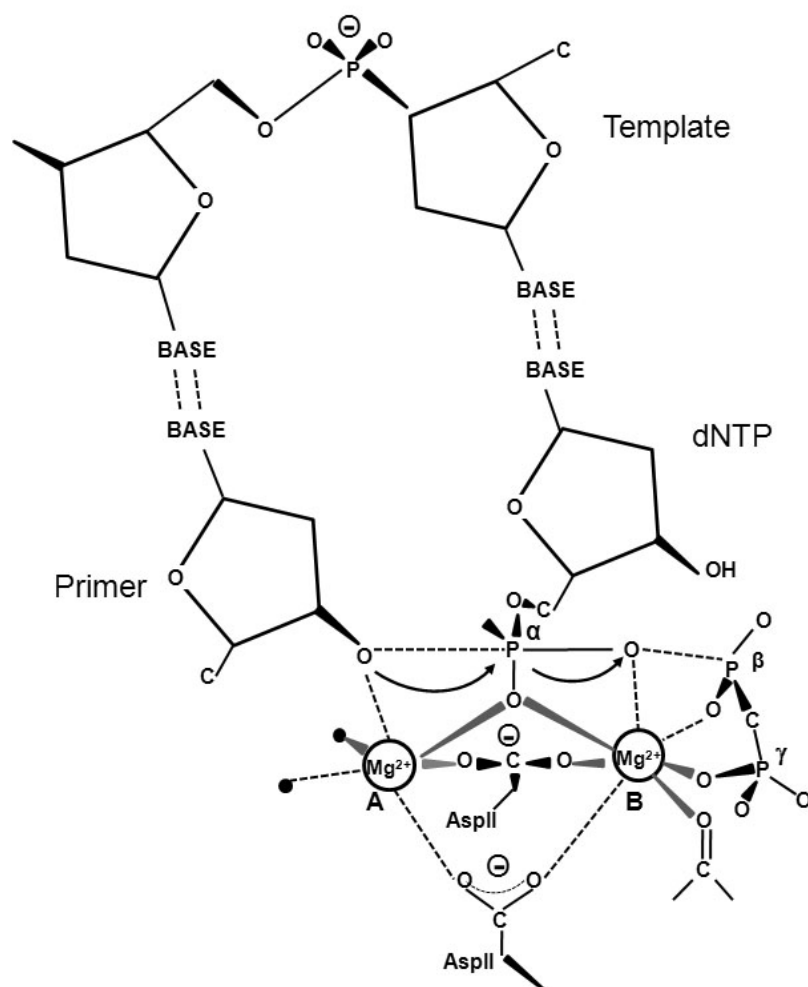


Figure 1.5. Two-metal ion mechanism of the nucleotidyl transfer for the catalysis of the DNA polymerase reaction.

The metal ion A (Mg^{2+} A) activates the 3'-OH terminus of the DNA primer strand (**Primer**) for nucleophilic attack on the α -phosphate ($P\alpha$) of the incoming dNTP (**dNTP**). Metal ion B (Mg^{2+} B) chelates β and γ phosphates ($P\beta$ and $P\gamma$) and stabilizes the negative charge generated during catalysis. Moreover, both metal ions participate in the stabilization of the pentacovalent transition state formed during catalytic process. Adapted from Brautigam and Steitz, 1998.

1.3.3.1 Fidelity of DNA polymerases:

DNA polymerases, as key enzymes involved in DNA synthesis, are the main determinants of faithful DNA replication. Generally, error rates for single base substitutions obtained by proofreading-deficient DNA polymerase range from 10^{-3} to $>10^{-6}$ (Kunkel and Bebenek, 1988; Kunkel and Bebenek, 2000; Kunkel, 2004; Garcia-Diaz and Bebenek, 2007). The major proofreading proficient polymerases from Pol A, Pol B and Pol C families display *in vitro* base substitution error rates that vary from 10^{-6} to 10^{-8} (Schaaper, 1993; Kunkel, 2004). Generally, the high accuracy in DNA copying observed with polymerases possessing intrinsic proofreading exonuclease activity results from the combination of three main factors (Fig.1.6.): (i) selection of correct nucleotide during DNA synthesis, (ii) selective extension of well-paired primer 3' terminus and (iii) preferential excision of incorrect nucleotide during 3'-5' exonuclease reaction (Echols and Goodman, 1991; Johnson, 1993; Joyce and Steitz, 1994). The most crucial determinant of DNA polymerase accuracy consists in proper selectivity for incorporated nucleotides (Fig.1.6.), a property that mainly depends on appropriate hydrogen bonding, base pair geometry and conformational changes in polymerase structure induced by dNTP binding (Kunkel and Bebenek, 2000; Kunkel, 2004).

Hydrogen bonding:

At the beginning of the 1950s Watson and Crick established that proper hydrogen bond base pairing (between A-T and G-C bases)

contributes basic specificity for DNA synthesis (Watson and Crick, 1953). Based on principles of thermodynamics hydrogen bonding should depend on differences in free energy between correctly and incorrectly paired base-pairs that facilitate preferential incorporation of complementary bases (Loeb and Kunkel, 1982; Kunkel, 2004). This principle was additionally supplemented by an hypothesis assuming that partial removal of water molecules from the polymerase active site elevates the differences in free energy between complementary and non-complementary base-pairing (Petruska *et al*, 1988).

Base pair geometry and minor groove recognition in DNA binding pocket:

The double helix of ordinary B-form DNA molecule is generally characterized by two unequally sized grooves, the minor (12 Å wide) and major grooves (24 Å wide) (Wing *et al*, 1980). The major groove is generally a site of interaction with sequence-specific DNA binding proteins such as transcriptional factors, whereas the minor groove forms a sequence-unspecific site of DNA substrate recognition for DNA polymerases (Pabo and Sauer, 1984). In addition to hydrogen bonding, proper geometry of the complementary base pair also provides correct dNTPs incorporation. Active sites of DNA polymerases display a specific architecture that only accepts well-paired nucleotides, while mismatches are preferentially rejected (Echols and Goodman, 1991; Goodman, 1997; Kunkel and Bebenek, 2000). It is believed that abnormal geometry within the active site and DNA-binding pocket of DNA polymerases generates steric clashes which interrupt catalytic efficiency of DNA synthesis (Kunkel, 2004).

Moreover, amino acid substitutions within the active site and the DNA-binding pocket can also influence proper architecture of the catalytic area of DNA polymerase, resulting in decreased fidelity of DNA synthesis (Kool, 2002; Kunkel, 2004).

Conformational changes and induced-fit of DNA polymerase:

Similarly to other enzymes, DNA polymerases are not rigid but exhibit remarkable conformational changes that are absolutely required for faithful and efficient catalytic activity. First of all, positioning of dNTPs for DNA synthesis requires at least one conformational change of DNA polymerase. Furthermore, the final checkpoint in dNTPs selectivity that takes place in the area of the polymerase active site and occurs during formation of the phosphodiester bond also involves a conformational transition of the DNA polymerase. Thus, each step of the catalytical process, from dNTP recognition to translocation of the elongated DNA fragment, exerts architectonic modifications in the three dimensional structure of polymerase. The induced-fit hypothesis predicts that binding of incorrect dNTP results in lack of, or inefficient induction of the required conformational changes in the structure of DNA polymerases, leading to catalytic perturbations (Wong *et al.*, 1991; Post and Ray, 1995; Kunkel and Bebenek, 2000; Purohit *et al.*, 2003). Finally, conformational changes occurring during catalysis of DNA synthesis are thought to be a major rate-limiting determinant for kinetics of DNA polymerases (Doublie and Ellenberger, 1998). Illustrating the great importance of correct protein conformation, fidelity level of DNA polymerases can be altered by amino acid

substitutions which affect directly (modification of immediate contact with DNA substrate), or indirectly (influencing the position of residues involved in direct contact with DNA substrate), the geometry of the DNA binding pocket (Kunkel and Bebenek, 2000).

Preferential extension of correctly paired DNA product:

The important interaction with the minor groove of DNA substrate and polymerase, which requires correct geometry of Watson-Crick base pairing, occurs in the DNA-binding pocket of DNA polymerases (Kiefer *et al*, 1998; Doublet *et al*, 1998). Generally, in the area of the polymerase active site the DNA-binding pocket forms a cleft that accommodates several base pairs of template-primer duplex DNA. Their positioning is controlled by the conformation of the pocket. Furthermore, terminal mismatches were observed to stall extension of DNA primer strand, reducing the elongation efficiency and processivity of DNA polymerase. The presence of terminal mismatches also reduces selectivity of the next incorporated dNTPs (reviewed by Kunkel and Bebenek, 2000). Misincorporated bases in newly synthesized DNA product can become mutations only if the 3' terminus of DNA primer strand is further extended. Thus, proper recognition and fitness of DNA minor groove in the DNA binding pocket constitutes another fidelity checkpoint for DNA synthesis.

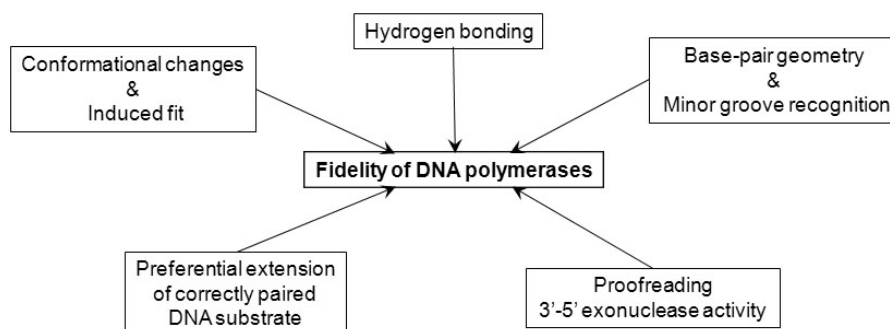


Figure 1.6. Crucial determinants of DNA synthesis fidelity of DNA polymerases with intrinsic 3'-5' exonuclease activity.

1.3.3.2. Role of 3'-5' exonuclease activity in the fidelity of DNA polymerases:

The 3'-5' exonuclease catalytic activity allows to remove nucleotides from 3' terminus of a DNA strand with simultaneous release of deoxyribonucleotide monophosphates (dNMPs). Since this activity bestows proofreading editing feature on enzymes, important replicative and repair DNA polymerases generally possess an intrinsic, or associated, 3'-5' exonuclease function. Moreover, the 3'-5' exonuclease function can also be harbored by other more specialized enzymes involved in maintenance of genetic information, suggesting high importance of exonuclease activity for fidelity of DNA synthesis and repair (Fig.1.6.; Kunkel and Bebenek, 2000). The 3'-5' exonuclease active site in DNA polymerases characterized by intrinsic proofreading activity, such as DNA polymerase from bacteriophage T4 or DNA polymerase I from *E. coli*, is situated within

a specific domain characterized by conserved catalytic blocks that contain essential aspartate and glutamate residues participating in catalysis of the exonuclease reaction.

Two-metal ion mechanism for catalysis of 3'-5' exonuclease reaction:

The 3'-5' exonucleolytic reaction catalyzed by DNA polymerases with intrinsic 3'-5' exonuclease activity takes place in the active site of the exonuclease domain which is characterized by a general architecture similar to that of the polymerase active site. As previously described for DNA polymerization, the exonuclease reaction involves essential aspartate and glutamate residues and is mediated by two divalent metal ions according to the two-metal ion mechanism (Fig.1.5.) of catalysis (Joyce and Steitz, 1994; Brautigam and Steitz, 1998).

It has been suggested that in the presence of dNTPs and divalent metal ions, the duplex DNA substrate is first bound to the polymerase active site, followed by submission of the nascent DNA strand for hydrolysis of potential mispaired product at the exonuclease active site (Derbyshire *et al*, 1995).

1.3.3.3. Structural comparison of DNA polymerases harboring 3'-5' exonuclease activity:

DNA polymerases possessing intrinsic 3'-5' exonuclease activity are comprehensive enzymes involved in replication and repair of prokaryotic, archaeal and viral genomes. Out of 16 well characterized eukaryotic polymerases only the three replicative eukaryotic

polymerases that deal with DNA elongation (pol δ , pol ϵ and pol γ) exhibit 3'-5' exonuclease activity. Here, a brief structural comparison of the catalytic subunits of five different DNA polymerases harboring 3'-5' exonuclease function will be presented (Fig.1.7. and Fig.1.8.): two viral DNA polymerases (from bacteriophages RB69 and phi29) belonging to Pol B family and three Pol A family members, firstly, eubacterial Klenow fragment of DNA polymerase I from *E. coli* (PolIk), secondly, DNA polymerase from phage T7 (T7 pol) and thirdly, human mitochondrial DNA polymerase gamma (Wang *et al*, 1997; Kamtekar *et al*, 2004; Beese *et al*, 1993; Doublie *et al*, 1998; Lee *et al*, 2009).

It must be noted that T7 pol and human pol γ contain an accessory subunit conferring processivity to the enzyme: thioredoxin from *E. coli* for T7 pol and pol γ B for human pol γ (for details see section 1.3.5.). Although the crystal structure of pol δ , the eukaryotic high-fidelity Pol B DNA polymerase that replicates leading strand, was recently solved, it will not be included in this comparison as its three dimensional structure shares strong structural homology with that of RB69 DNA polymerase (Swan *et al*, 2009).

General architecture:

All five DNA polymerases exhibit a clear structural distribution of their functions into at least two components, polymerase and 3'-5' exonuclease domains (Fig.1.7.A.-E). These domains are clearly spatially separated in T7 pol and PolIk (Fig.1.7.C. and D.), while in RB69, phi29 and pol γ they present more merged structural profiles (Fig.1.7.A., B. and E.). Moreover, spatial arrangement of these two

domains presents (i) a rather coherent and disk-like character in the two Pol B polymerases; (ii) half-closed, falcate shape in pol g; (iii) and a rather linear organization in T7 pol and Polk. Interestingly, this linear arrangement of the exonuclease and polymerase domains, that is also characteristic for other Pol A polymerases, such as KlenTaq from *Thermus aquaticus* and *Bacillus stearothermophilus* (Korolev *et al*, 1995; Kiefer *et al*, 1998), succumbs to bending, flattening and partial mutual superimposition in human pol g (Fig.1.7.E). However, in T7 pol slight and discrete creasing and overlapping of both domains can also be observed (Fig.1.7.D.).

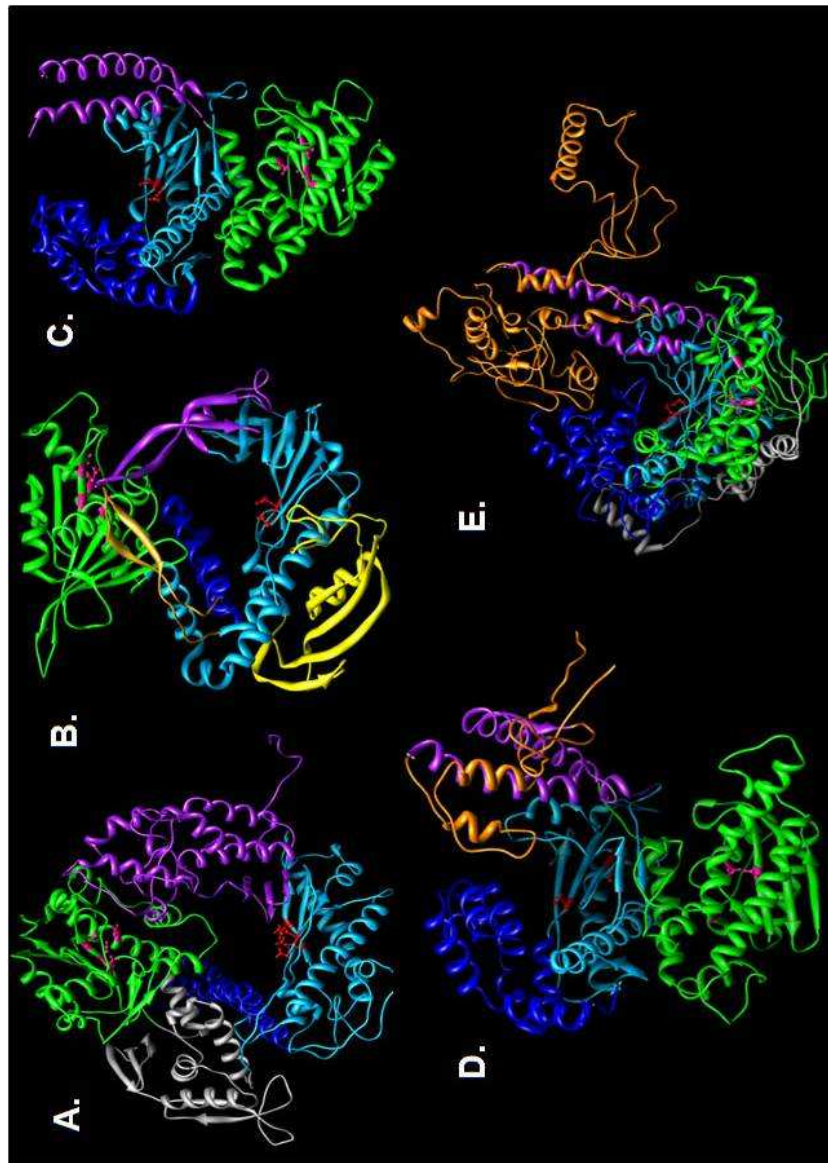


Figure 1.7. Comparison of crystal structures of five DNA polymerases with intrinsic 3'-5' exonuclease activity.

A. DNA polymerase from the bacteriophage RB69 (pdb ID: 1WAJ); **B.** DNA polymerase from the bacteriophage Phi29 (pdb ID: 2PYL); **C.** Klenow Fragment of the DNA polymerase I from *E. coli* (pdb ID: 1KFD); **D.** DNA polymerase from the bacteriophage T7 (pdb ID: 1T7P); **E.** Catalytic subunit of the human mitochondrial DNA polymerase gamma (pdb ID: 3IKM). Domains and subdomains of DNA polymerases are indicated as follows: N-terminal extension (grey), 3'-5' exonuclease domain (green), TPR1 (yellow) and TPR2 (dark yellow) domains, linker region (orange), palm (light blue), fingers (dark blue) and thumb (violet). Essential catalytic residues of the 3'-5' exonuclease (pink) and polymerase (red) active sites are displayed as ball & sticks atom representation. Figure prepared by using the UCSF Chimera software.

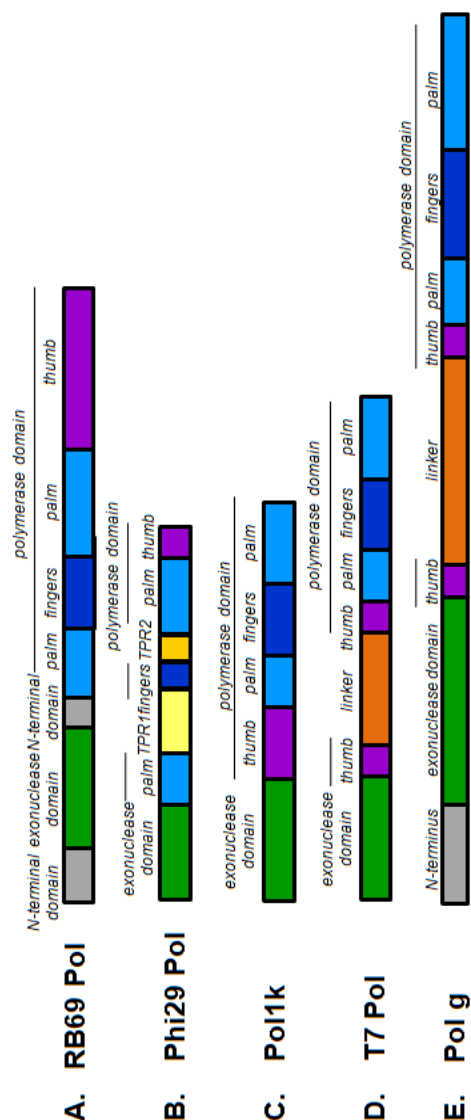


Figure 1.8. Comparison of the primary structure of five DNA polymerases with intrinsic 3'-5' exonuclease activity.

All diagrams represent linear organization of five selected DNA polymerases based on the comparison of their three dimensional structures. **A.** DNA polymerase from the bacteriophage RB69 (Wang *et al*, 1997). **B.** DNA polymerase from the bacteriophage Phi29 (Berman *et al*, 2007). **C.** Klenow Fragment of the DNA polymerase I from *E. coli* (Beese *et al*, 1993). **D.** DNA polymerase from the bacteriophage T7 (Doublié *et al*, 1998). **E.** Catalytic subunit of the human mitochondrial DNA polymerase gamma (Lee *et al*, 2009). Domains and subdomains of DNA polymerases are indicated as follows: N-terminal extension and N-terminal domains (grey); 3'-5' exonuclease domain (green); TPR1 (yellow) and TPR2 (dark yellow) domains; linker region (orange); palm (light blue); fingers (dark blue); thumb (violet).

Supplementary domains:

Beside the 3'-5' exonuclease and polymerase domains, all analyzed DNA polymerases except PolIk possess additional domains involved in various functions (Fig.1.7.A., B., D. and E., Fig.1.8.). The function of some of these domains is well established, whereas for others it remains unclear.

N-terminal extension: Both RB69 polymerase and pol γ are characterized by specific N-terminal extensions (Fig.1.7.A. and E.; Fig.1.8.A. and E.). The N-terminal domain of RB69 pol shares high structural homology with the N-terminal domain from T4 gp43 polymerase and makes contacts with both the exonuclease domain and the fingers subdomain (Wang *et al*, 1996; Wang *et al*, 1997). It therefore participates in formation of the central tunnel for DNA substrate that consists of three clefts in a disk-like structure. In most of the Pol B family polymerases this N-terminal extension has been postulated to display a regulatory function (Wang *et al* 1997). The exact role of the N-terminal extension of human pol γ remains unclear (see further chapters).

Insertions: Although phi29 polymerase does not possess a large N-terminal extension, it includes two insertions (Fig.1.7.B. and Fig.1.8.B.), termed as terminal protein regions 1 and 2 (TPR1 and TPR2) that generate discontinuity in the polymerase domain since they interrupt the linear sequence of the polymerase domain. These subdomains form a site of interaction with terminal proteins (TP) (Blasco *et al*, 1990; Dufour *et al*, 2000), the latter being used as primers for DNA synthesis initiation. These specific and discrete TPR subdomains are unique to protein-primed DNA polymerases

(Kamtekar, 2006). TPR1 defines a completely new type of subdomain, whereas TPR2 shares structural similarity to the specificity loop of T7 RNA polymerase (Cheetham *et al*, 1999; Kamtekar *et al*, 2004). It was observed that truncation of the TPR2 subdomain affects processivity and strand displacement of phi29 polymerase (Rodriguez *et al*, 2005). Generally, both domains harbor a regulatory function for phi29 DNA polymerase (Kamtekar, 2004; Kamtekar, 2006).

Both T7 pol and human pol g display a well shaped and clearly separated linker region which mainly acts as a site of interaction with their processivity accessory factor (Yakubovskaya *et al*, 2006; Lee *et al*, 2009; Modrich and Richardson, 1975). Both linker regions are localized between the first and second helix of thumb subdomain, disrupting the continuity of the linear sequence of the polymerase domain (Fig.1.7.D. and E.; Fig.1.8.D. and E.). Noteworthy, the linker region of human pol g is much longer and complex (see details in section 1.3.5.) than that of T7 pol (Fig.1.7.D. and E.; Fig.1.8.D. and E.). An explanation might be that the function of the linker region of pol g is not restricted to its specific interaction with pol gB; indeed, the linker region also contains a processivity module (IP) and moreover, forms the upstream part of the DNA-binding channel (Lee *et al*, 2009). Consistent with the absence of an accessory processivity factor, bacterial PolIk does not exhibit any distinct linker domain (Fig.1.7.C. and Fig.1.8.C.).

Polymerase domain:

In agreement with theoretical assumptions, the polymerase domain of all analyzed DNA polymerases presents the canonical right hand

configuration, characterized by palm, thumb and fingers subdomains (Kohlstaedt *et al*, 1992). However, the general spatial architecture and linear position of each subdomain vary significantly between polymerases. This variation is observed not only amongst members of different families but also within the same family (Fig.1.7. and Fig.1.8.). We report below a comparative analysis that we have performed on the architecture of RB69 pol, phi29 pol, PolIk, T7 pol and pol g polymerase domains.

Palm:

The palm subdomain shares the highest level of structural conservation. In the polymerases studied here the primary structure of palm subdomain is divided in two parts separated from each other by the fingers subdomain (Fig.1.8.A.-E.). All palms consist of at least two helices forming a framework for a β -sheet panel which is stretched above them in the central part of the subdomain.

The β -sheet panel contains essential aspartate and glutamate residues involved in the catalysis of DNA synthesis (residues presented in red in Fig.1.7.). Therefore, this part of the palm forms the polymerase active site. The number, length and localization of the helices present within the palm subdomain vary between polymerases. The most conserved is the helix localized at the base of the palm, corresponding to Q-helix (Fig.1.9.) according to PolIk and T7 pol numbering system (Beese *et al*, 1993; Doublie *et al*, 1998). The structural equivalent of the Q-helix has been identified in polymerases belonging both to Pol A and Pol B families (Fig.1.9.A.-E.). Other helices of the palm from RB69 and phi29 polymerases do not share structural similarities

(Fig.1.9.A. and B.). In Pol A polymerases, Q-helix conservation is accompanied by the presence of rear R-helix (Fig.1.9.C.-E.) based on PolIk and T7 numbering system (Beese *et al*, 1993; Doublet *et al*, 1998). J-helix, which is at the border of the thumb subdomain (Fig.1.9.C.) and is important for proper DNA binding in Klenow fragment, is absent in the palm of T7 pol and pol g (Fig.1.9.D. and E.) (Tuske *et al*, 2000). The general architecture of the β -sheet panel is well conserved between PolIk and T7 pol (Fig.1.7.C. and D.; Table 2.), whereas in human pol g this β -sheet panel is reduced and depressed in lower parts of the palm, placing the catalytic site more deeply in the cleft (Fig.1.7.E.; Table 2.). Moreover, the upper part of the cleft in pol g is surrounded by multiple coils, an unusual feature compared to other DNA polymerases (Fig.1.7.E. and Fig.1.14.).

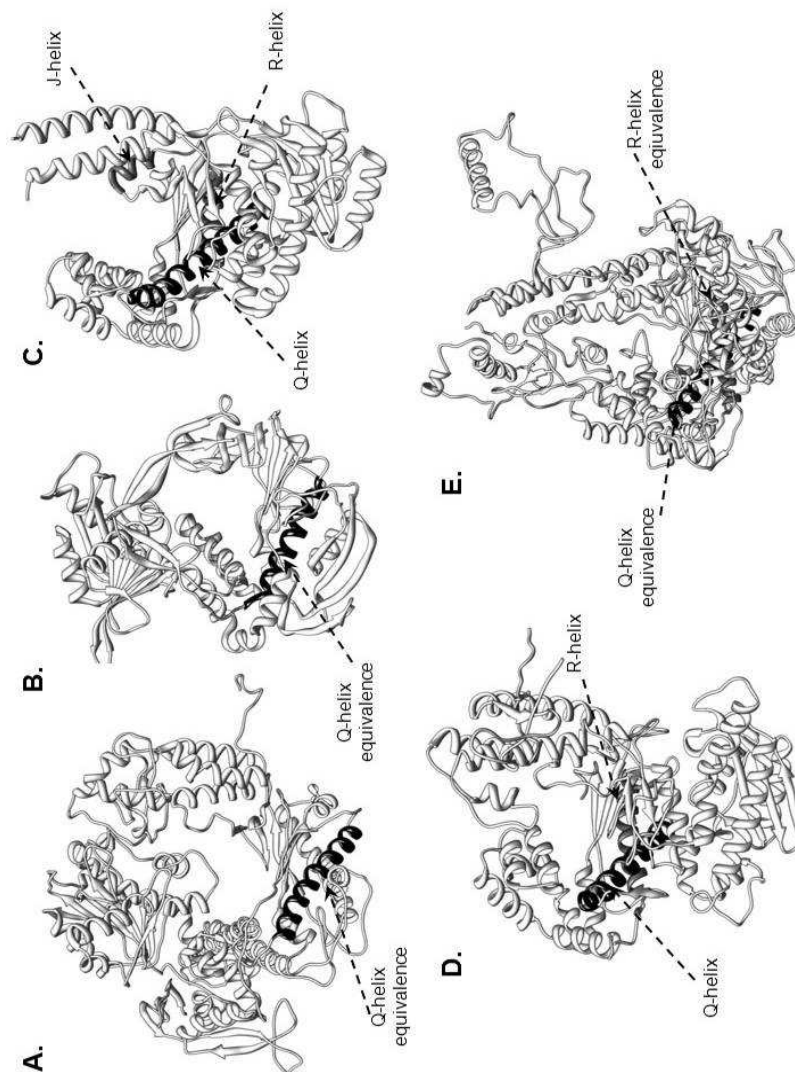


Figure 1.9. Conservation of the polymerase active site structural framework of five DNA polymerases with intrinsic 3'-5' exonuclease activity.

A. DNA polymerase from the bacteriophage RB69 (pdb ID: 1WAJ); **B.** DNA polymerase from the bacteriophage Phi29 (pdb ID: 2PYL); **C.** Klenow Fragment of the DNA polymerase I (PolIk) from *E. coli* (pdb ID: 1KFD); **D.** DNA polymerase from the bacteriophage T7 (pdb ID: 1T7P); **E.** Catalytic subunit of the human mitochondrial DNA polymerase gamma (pdb ID: 3IKM). Q-helix (based on PolIk and T7 DNA polymerase numbering system) and Q-helix equivalents are shown in black and indicated with arrowheads. R-helix (based on PolIk and T7 DNA polymerase numbering system) and R-helix equivalents as well as J-helix unique for PolIk are shown in grey and indicated with arrowheads. Figure prepared by using the UCSF Chimera software.

Thumb:

The thumb subdomain is well conserved in DNA polymerases belonging to Pol A family and generally consists of two opposite and adjacent helices (Fig.1.7.C., D. and E.). In the linear representation of PolI κ the thumb is localized in the N-terminal part of the polymerase domain (Fig.1.7.C. and Fig.1.8.C.). As reported above, the thumbs of T7 pol and pol γ are interrupted by the linker region and are thus linearly discontinuous (Fig.1.7.D. and E.; Fig.1.8.D. and E.). In strong contrast with Pol A family, the thumbs in Pol B polymerases are localized in the C-terminal part of the polymerase domain (Fig.1.8.A. and B.). The thumb of RB69 polymerase consists of multiple helices, whereas the thumb of phi29 is characterized by a specific β -sheet panel (Fig.1.7.A. and B.).

Fingers:

The fingers subdomain also displays different topologies depending on the family of polymerases. Generally, fingers consist of a single continuous sequence that is localized in the middle of the palm primary structure (Fig.1.8.A.-E.). The fingers of Pol B polymerases are formed by two adjacent helices gently sloping towards the polymerase active site (Fig.1.7.A. and B.). The fingers from Pol A family show a more complex architecture composed of several tightly packed helices (Fig.1.7.C.-E.). Noteworthy, human pol γ exhibits within its fingers subdomain an additional coil-loop motif fencing the cleft over the palm subdomain (Fig.1.7.E.).

3'-5' exonuclease domain:

The 3'-5' exonuclease domains of the DNA polymerases show various levels of complexity and the structural conservation is only partially maintained. Similarly to the palm subdomain, the general architecture of all exonuclease domains is characterized by a basic framework of helices which forms a deep cleft for a β -sheet panel (Fig.1.7.A.-E., Table 2.). This β -sheet panel, which is the most conserved part of the exonuclease domain, contains crucial aspartates and glutamates and is the catalytic site for 3'-5' exonuclease reaction (Bernad *et al*, 1989; Freemont *et al*, 1988; Beese and Steitz, 1991).

Despite a few differences in the number, shape and length of helices, the general topology of the exonuclease domain from T7 and PolIk is well conserved (Fig.1.7.C. and D.). Similarly, the 3'-5' exonuclease domains of the RB69 and phi29 DNA polymerases from Pol B family exhibit approximately similar shapes and organization. Compared to Pol A family their exonuclease active site is diametrically opposite to the polymerase active site on the disk-like structure of the DNA polymerase and is separated from the polymerase domain by the central tunnel containing the DNA substrate (Fig.1.7.A. and B.). In these four polymerases the β -sheet panel comprises 5 to 8 strands that contain from 2 to 10 residues.

The 3'-5' exonuclease domain of pol g can be distinguished by several features. Firstly, and surprisingly, the β -sheet panel of human pol g exonuclease active site is considerably reduced, consisting of only two very short β -strands surrounded by several coils (Table 2.). Nevertheless, the essential catalytic residues are localized within the limits of the β -sheet center in the 3'-5' exonuclease cleft (Fig.1.7.E.).

Secondly, the topology of the helix frames and general flattening of exonuclease domain significantly vary from those presented by other Pol A polymerases. Thirdly, part of the exonuclease domain of pol g consists of several coil-helix turns (residues 293-316 according to pol g numbering system) extends in the direction of the polymerase domain and appears as an outpost fencing the DNA-binding channel (Fig.1.14.). This kind of structure is not present in the other DNA polymerases and implies the existence of a novel module (residues 293-316 according to pol g numbering system) which interrupts the continuity of the exonuclease domain.

DNA-binding pocket:

The pocket that binds the DNA substrate is variously organized in DNA polymerases belonging to different families (Fig.1.7.A-E.). In Pol B polymerases the DNA-binding pocket can be visualized as a tunnel in the central part of the enzyme and consists of several clefts (Wang *et al*, 1997; Kamtekar *et al*, 2004). Limits of this tunnel are constituted by the centripetal walls of the 3'-5' exonuclease and polymerase domains, a structure that allows DNA substrate to accede both active sites (Fig.1.7.A.-B.). The DNA-binding pocket of Pol B polymerases is always deep, tight and spatially limited, while the DNA-binding pocket of Pol A polymerases is more shallow, spacious and largely opened (Fig.1.7.C. and D.). Clefts containing each of the active site (polymerase and 3-5' exonuclease) are easily accessible for DNA primer-template. The shallowest DNA-binding pocket is presented by PolI κ since both active sites are positioned on one axis generated by a straight linear organization of the polymerase and 3'-5'

exonuclease domains (Fig.1.7.C.). Compared to PolI κ the DNA-binding pocket of T7 pol is deeper because the inclination angle between exonuclease and polymerase domains is more acute (Fig.1.7.D.). The DNA-binding pocket of pol g presents an intermediate state between the tight tunnel observed in Pol B polymerases and the open DNA-binding pocket characteristic of prokaryotic Pol A polymerases. In the case of pol g, the discrete pocket is replaced by an immense, complex, deep and partially closed DNA-binding channel (Lee *et al*, 2009; Fig.1.7.E.). The general topology of this channel is an outcome of the inclination of the exonuclease domain at a slightly acute angle with respect to the polymerase domain (Fig.1.10.). This angle generates a deep and tight cleft hiding the access to exonuclease and polymerase active sites and shortening the distance between both catalytic sites (Fig.1.10.). Moreover, sinking of the catalytic β -sheet panels inside each domain, as it takes place in human pol g, makes the channel even deeper. The DNA-binding channel of pol g is rather spread out, since its boundaries are formed by the centripetal walls of 3'-5' exonuclease domain, fingers, palm, thumb subdomains and linker region (Fig.1.7.E.). Finally, coiled and helical structures are present in the luminal space of the channel that could be assumed to play a role in the regulation of DNA binding (Fig.1.14.). These unique structural elements are not observed in other Pol A polymerases (Fig.1.7.C.-D.).

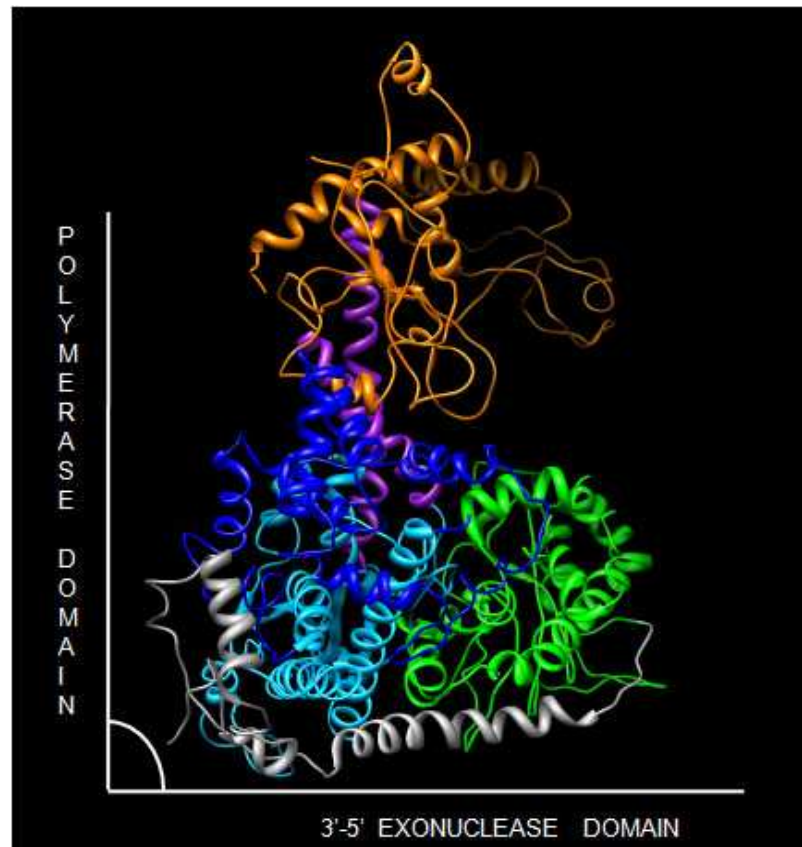


Figure 1.10. Spatial organization of the polymerase and exonuclease domain in human pol g.

The slightly acute angle between polymerase and exonuclease domains generates a deep and tight cleft which forms an internal part of the DNA binding channel. Domains and subdomains of pol g are indicated as follows: N-terminal extension (grey), 3'-5' exonuclease domain (green), linker region (orange), palm (light blue), fingers (dark blue) and thumb (violet). Pdb ID: 13KM. Figure prepared by using the UCSF Chimera software.

Table 2. Comparison of β -sheet panels from the 3'-5' exonuclease and polymerase active sites of five DNA polymerases characterized by intrinsic 3'-5' exonuclease activity.

DNA polymerase	β -sheet panel of polymerase active site		β -sheet panel of 3'-5' exonuclease active site	
	Number of β -strands	Range of amino acid residues per β -strand	Number of β -strands	Range of amino acid residues per β -strand
RB69 pol	4	5 - 7	7	3 - 8
Phi29 pol	7	2 - 14	8	2 - 10
PolIk	6	3 - 8	6	2 - 9
T7	5	3 - 7	5	3 - 8
Pol gA	3	3 - 7	2	3

1.3.3.4. Balance between polymerase and 3'-5' exonuclease functions in DNA polymerases:

The contribution of 3'-5' exonuclease excision to fidelity of DNA synthesis depends on the proofreading efficiency generated by the balance between mismatch extension and excision (Fig.1.11) (Kunkel and Bebenek, 2000). Undisturbed contact between polymerase and exonuclease active sites is crucial for proper function of polymerases possessing intrinsic 3'-5' exonuclease activity. Generally, kinetic partitioning between polymerase and exonuclease functions favors excision and DNA binding at the exonuclease site when the DNA substrate is single-stranded and contains a mismatch at its 3' terminus. In contrast, correctly paired double-stranded DNA is preferentially bound at the polymerase active site. The distance between polymerase and exonuclease active sites varies from 30Å to 40Å and can be

significantly reduced when a mismatch occurs at the 3' end of the primer strand (Kunkel and Bebenek, 2000). Moreover, some investigations suggest that during nucleotide incorporation at the polymerase site, the exonuclease active site can display a “stressed” conformation, preventing the access of DNA primer strand (Li *et al*, 1998; Kunkel and Bebenek, 2000). Finally, the subtle balance between both functions can be affected not only by mutations in each of the active sites but even by mutations localized elsewhere in the protein (Kunkel and Bebenek, 2000).

The DNA primer strand can move between polymerase and exonuclease active sites (Fig.1.11), via intermolecular or intramolecular mechanisms. In several processive DNA polymerases, as T4 pol, T7 pol or PolI, the DNA substrate is allowed to move between both active sites with an intramolecular mode, which means without dissociation from the enzyme (Joyce, 1989; Donlin *et al*, 1991; Johnson, 1993; Capson *et al*, 1992; Reddy *et al*, 1992). Mechanisms of intramolecular switching between both active sites involve various structural elements depending on the type of DNA polymerase. For instance, PolI utilizes J-helix from the palm subdomain to shuttle the primer DNA strand between exonuclease and polymerase domains, whereas in some Pol B polymerases translocation of DNA substrate involves a specific β -hairpin loop localized within the 3'-5' exonuclease domain (Tuske *et al*, 2000; Kuroita *et al*, 2005; Hogg *et al*, 2007; Kim *et al*, 2008; Subuddhi *et al*, 2008; Trzemecka *et al*, 2009). The thumb subdomain can also participate in coordination of the switching mechanism, as in phi29 polymerase (Perez-Arnaiz *et al*, 2006). Some polymerases use an

intermolecular mechanism. They dissociate from DNA after switching to the editing mode and rebind another DNA molecule (Joyce, 1989; Johnson, 1993; Reddy *et al*, 1992).

These observations suggest that mechanisms ensuring proper balance between polymerase and exonuclease function are complex, depend significantly on protein architecture and can vary strongly between different types of DNA polymerases.

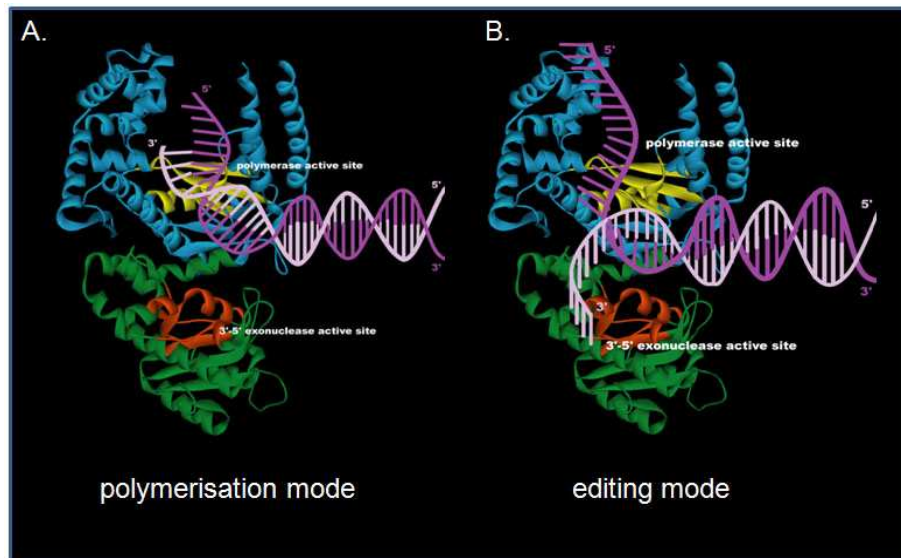


Figure 1.11. Polymerization and editing mode of DNA polymerases with intrinsic 3'-5' exonuclease activity. Shifting of the DNA primer strand between the polymerase and 3'-5' exonuclease active sites. Diagram prepared using crystal structure of PolIk from *E. coli* (pdb ID: 1KFD). **A.** Polymerization mode. **B.** Editing mode. Legend: polymerase domain (blue), area of polymerase active site (yellow), 3'-5' exonuclease domain (green), area of 3'-5' exonuclease active site (orange), DNA primer strand (white), DNA template (violet). Figure prepared by using the Accelrys DS Visualizer software.

1.3.4. DNA polymerase gamma as the unique replicase of the mitochondrial genome:

Among the 16 highly specialized eukaryotic DNA polymerases, 15 are involved in maintenance of nuclear genetic information (Kaguni, 2004). Nonetheless, pol g is the only DNA polymerase found in animal and yeast mitochondria, mediating all DNA synthesis events inside these crucial organelles. Although the mitochondrial DNA accounts for approximately 1% of cellular DNA and pol g activity comprises merely 1-5% of the total cellular DNA polymerase activity, the nuclearly encoded pol g plays a key role in the integrity and viability of eukaryotic cells (Graziewicz *et al*, 2006).

Pol g was identified in the early 1970s as an RNA-dependent DNA polymerase in human HeLa cells and almost simultaneously described for fungi and animal mitochondria (Temin and Mizutani, 1970; Baltimore, 1970; Wintersberger and Wintersberger, 1970; Fridlender *et al*, 1972). Studies on the pol g from *S. cerevisiae* were initiated in 1970 (Wintersberger and Wintersberger, 1970). Pol g was firstly isolated and purified to homogeneity from *Drosophila* in the middle 1980s (Wernette and Kaguni, 1986). The nuclear *MIP1* gene encoding *S. cerevisiae* mitochondrial DNA polymerase was cloned and sequenced by the group of Foury in 1989 (Foury, 1989). The *POLG1* gene encoding the catalytic subunit of human pol g was only isolated seven years later (Ropp and Copeland, 1996).

Pol g is characterized by several biochemical properties that distinguish it from nuclear polymerases. In particular, this enzyme exhibits a reverse transcriptase activity *in vitro* that can utilize

ribohomopolymeric templates. It is resistant to aphidicolin and extremely sensitive to dideoxynucleosides triphosphates (ddNTPs) and ethylmaleimide (Lecrenier *et al*, 1997; Kaguni, 2004; Graziewicz *et al*, 2006).

As a major mitochondrial enzyme responsible for DNA maintenance pol g possesses two basic catalytic activities: (i) a DNA polymerase activity which synthesizes DNA and (ii) a 3'-5' exonuclease activity that proofreads nascent DNA (Foury and Vanderstraeten, 1992). More recently, a third enzymatic property was described for pol g: 5'-deoxyribose phosphate (dRP) lyase activity which catalyzes the release of the 5'-terminal dRP sugar moiety from incised apurinic/apyrimidinic sites during postreplicational base excision repair (Pinz and Bogenhagen, 2000).

Human pol g is a heterotrimer composed of the catalytic pol gA subunit and an asymmetrical dimer of the pol gB subunit (Fig.1.15.) (Lee *et al*, 2009). Pol gB shows homology to class II aminoacyl-tRNA synthases and confers processivity to the holoenzyme (Fan *et al*, 1999; Carrodeguas *et al*, 1999).

1.3.5. DNA polymerase gamma forms a new subfamily of Pol A polymerases:

To determine if pol g constitutes a new subfamily of Pol A polymerases, we have performed a comparison of the linear representations and crystal structures of the catalytic subunit of pol g and two Pol A family members.

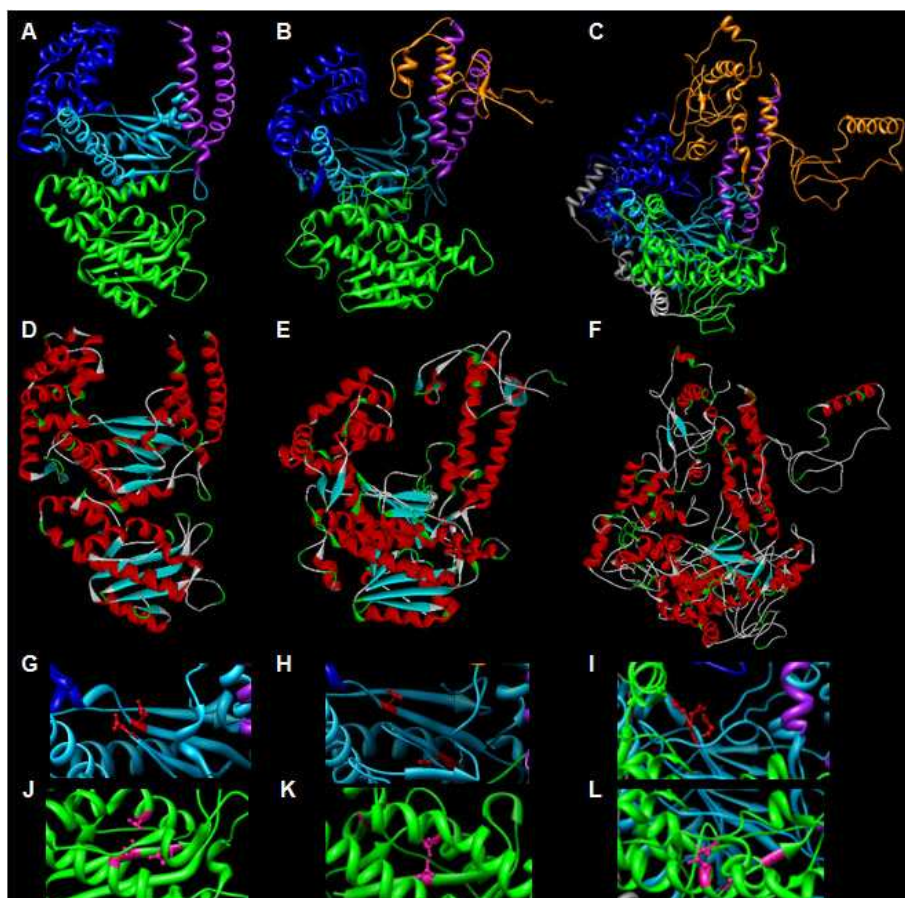


Figure 1.12. Comparison of crystal structures of three DNA polymerases belonging to Pol A family.

A.-C. Spatial organization of domains and subdomains in: **A.** Pol1k (pdb ID: 1KFD); **B.** T7pol (pdb ID: 1T7P); **C.** Pol g (pdb ID: 3IKM). Domains and subdomains of DNA polymerases are indicated as follows: N-terminal extension (grey), 3'-5' exonuclease domain (green), linker region (orange), palm (light blue), fingers (dark blue) and thumb (violet).

D.-F. Secondary structure representation of: **D.** Pol1k; **E.** T7 pol; **F.** pol g. For secondary structure representation on **D.-E.**: helices are red, β -strands are cyan, coils are white and turns are green.

G.-I. Polymerase active site of: **G.** Pol1k; **H.** T7 pol; **I.** pol g. Essential catalytic residues of the polymerase active site are indicated in red.

J.-L. 3'-5' exonuclease active site of: **J.** Pol1k; **K.** T7 pol; **L.** pol g. Essential catalytic residues of the 3'-5' exonuclease active site are indicated in pink.

Figure prepared by using the UCSF Chimera (**A.-C.** and **G.-L.**) and Accelrys DS Visualizer software (**D.-E.**).

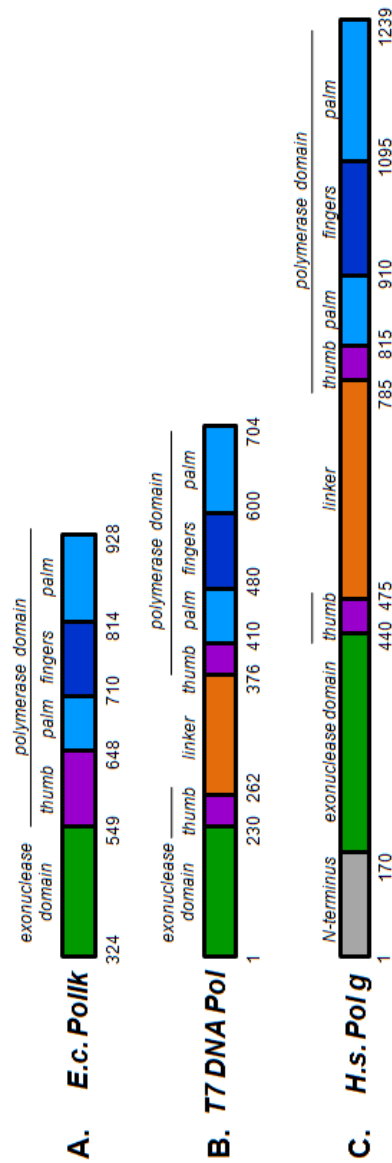


Figure 1.13. Comparison of the primary structure of three DNA polymerases belonging to Pol A family.

Diagrams represent linear DNA polymerases organization based on comparison of their three dimensional structures and amino acid sequences alignment. **A.** Klenow Fragment of the DNA polymerase I (*PolI*) from *E. coli* (Beese *et al*, 1993). **B.** DNA polymerase from the bacteriophage T7 (*T7 DNA Pol*) (Doublet *et al*, 1998). **C.** Catalytic subunit of the human mitochondrial DNA polymerase gamma (*H.s. Pol gamma*) (Lee *et al*, 2009). Domains and subdomains of DNA polymerases are indicated as follows: N-terminal extension and N-terminal domains (grey); 3'-5' exonuclease domain (green); linker region (orange); palm (light blue); fingers (dark blue); thumb (violet).

The most important features of eubacterial Klenow fragment from *E. coli*, DNA polymerase from bacteriophage T7 and human mitochondrial DNA polymerase gamma are shown in Fig.1.12. and Fig.1.13.. Based on the crystal structures and multiple alignments of amino acid sequences obtained for pol g and prokaryotic DNA polymerases belonging to Pol A family, several conclusions can be drawn.

First of all, a simple comparison of amino acid sequences shows that the catalytic subunits of yeast and human pol g are much larger (1254 and 1239 amino acid residues, respectively) (Fig.2.1.) than that of other Pol A family members (as examples, lengths of PolI κ and T7 pol are 605 and 704 amino acid residues, respectively). This feature is imprinted in the three dimensional structure of these polymerases (Fig.1.12.). Pol g partially shares the linear organization of PolI κ and T7 DNA polymerases, particularly in the catalytic blocks which are well conserved; however, sequence similarities are merely limited to small regions of the proteins (Fig.1.13.).

Processivity factors:

Several DNA polymerases belonging to PolA family are natively associated with accessory subunits which serve as processivity factors increasing the ability of polymerase for efficient DNA synthesis and improving its affinity to DNA substrate. Klenow fragment of DNA polymerase I from *E. coli* has no processivity factor. T7 DNA polymerase annexes, during the phage growth, thioredoxin of *E. coli* as its processivity factor forming a tight heterodimeric complex (Modrich and Richardson, 1975). This complex is able to polymerize

thousands of nucleotides before dissociating from the DNA primer-template (Tabor *et al*, 1987; Doublie *et al*. 1998). Human pol g has a dimeric accessory subunit, so-called pol gB, that forms a structural and functional heterotrimer with the pol gA catalytic subunit (Fig.1.15.) (Wang *et al*, 1997; Lee *et al*, 2009). Human pol gB bears homology to the anticodon-binding domain of class IIa aminoacyl-tRNA synthetases (Fan *et al*, 1999; Carrodegua *et al*, 1999) and some structural similarity with *E. coli* thioredoxin and the δ' subunit of the γ complex in DNA polymerase III from *E. coli*. While a single contact site (at the tip of thumb subdomain) exists between T7 DNA polymerase and thioredoxin (Doublie *et al*, 1998), the human pol g heterotrimer shows multiple contact sites between pol gA and pol gB (Lee *et al*, 2009). Unlike other processivity factors which only increase enzyme affinity to DNA template, pol gB also enhances pol gA processivity by simultaneously accelerating polymerization rate and decreasing exonuclease activity (Johnson and Johnson, 2001; Lee *et al*, 2009). Whereas all known animal pol g require accessory subunits to replicate mtDNA efficiently, yeast pol g does not possess any accessory subunit (Lucas *et al*, 2004; Viikov *et al*, 2010). Despite this lack of a processivity factor, yeast Mip1 is a highly processive enzyme (Foury and Vanderstraeten, 1992).

Polymerase domain:

The strongest conservation of the structural architecture between pol g and other Pol A polymerases can be observed for the polymerase domain. The canonical right-hand configuration, with its specific architecture of fingers and thumb, is properly maintained (Fig.1.12.A.-

C.). Interestingly, the central part of the palm subdomain of human pol g including the polymerase active site is significantly different from that of PolI κ or T7 DNA polymerase (Fig.1.12.A.-I.). Whereas the catalytic site of the latter polymerases is characterized by a large β -sheet panel, the β -sheet panel of pol g is dramatically reduced, containing only short β -strands, and is replaced by multiple coils (Fig.1.12.D.-I.; Table 2.). It must be mentioned that contrary to the vast majority of polymerases the pol g crystal structure was solved without any additional cofactor or substrate (Mg^{2+} ions, dNTP, DNA primer strand or DNA duplex). Interestingly, although the three dimensional structure of DNA polymerase from *Bacillus stearothermophilus* has also been determined in the absence of cofactors a clear β -sheet pattern is observed in the central part of the polymerase active site (Kiefer *et al*, 1998). Therefore, although it cannot be excluded that the pol g active site structure has been modified by the conditions of protein crystallization in the absence of co-factors, the presence of coils instead of β -sheet could point to specific properties in the DNA polymerization mechanism of pol g.

3'-5' exonuclease domain:

The overall architecture of the 3'-5' exonuclease domain is rather poorly conserved between pol g and other Pol A polymerases. Weak conservation is also observed among prokaryotic members of Pol A family, with the exception of the well conserved β -sheet panel which characterizes all prokaryotic 3'-5' exonuclease active sites (Fig.1.12.D.-F. and J.-L). Similarly to polymerase domain the β -sheet panel of pol g is extremely reduced, comprising only two strands of

three residues, and is replaced by multiple coils (Fig.1.12.D.-F. and J.-L.; Table 2.). Nevertheless, the main structural differences mostly concern the peripheral parts of the 3'-5' exonuclease domain (see chapter 1.3.3.3) (Fig.1.12.A.-F.).

Linker region:

The most significant structural discrepancies among Pol A polymerases are observed for the so-called “linker” region (Fig.1.12.A.-C. and Fig.1.13.). As previously mentioned, in Polk and DNA polymerase from *Bacillus stearothermophilus* the linker region is almost completely absent, whereas in T7 DNA polymerase it is limited to a 71-amino acid loop which is able to interact with its accessory subunit, the thioredoxin from *E. coli* (Doublié *et al*, 1998).

Human pol g linker region is 428-amino acid long and is flanked on each side by one helix of the thumb subdomain (Fig.1.12.C. and F.; Fig.1.13.C.). Apart a contact with the thumb, the linker region is spatially far away from the bulk of pol g core (Fig.1.12.C. and F.). As discussed in section 1.3.6.1. the linker region is composed of two main domains, an extended accessory-interacting determinant (AID) subdomain (residues 511-570) that forms an interface with the proximal pol gB subunit and a globular intrinsic processivity (IP) subdomain (residues 475-510 and 571-785) that plays a role in enzyme processivity (Lee *et al*, 2009). As the linker region is spatially quite distinct from the other parts of the enzyme, (Fig.1.12.C. and F.) it could be expected that it modulates, rather than strongly modifies, the basic properties of pol g. Nevertheless, the linker region plays an important role in processivity and DNA binding (it forms the upstream

part of the DNA binding channel). Its proper folding is thus important to ensure correct biochemical properties to pol g.

Active sites:

The essential aspartate and glutamate residues from catalytic blocks are highly conserved between pol g and PolIk or T7 DNA polymerase (Fig.1.12.G-I.). Therefore, the two-metal ion mechanism for catalysis of polymerase and 3'-5' exonuclease reactions is also certainly used by pol g (Esteban *et al*, 1994). Finally, the selectivity and binding of dNTPs exhibited by pol g seem to be grounded on mechanisms similar to those presented by other Pol A polymerases. Amino acid sequence alignments reveal that PolIk, T7 DNA polymerase and pol g have a tyrosine at the same position (Y766 in PolIk, Y530 in T7 DNA polymerase, Y757 in yeast and Y955 in human), which plays an important role in the positioning of dNTP towards the DNA primer-template at the active site (Graziewicz *et al*, 2006).

DNA-binding pocket:

As it was described in the section 1.3.3.3., compared to the discrete DNA-binding pocket of PolIk and T7 pol the DNA-binding channel of pol g is more spread out and complex and DNA seems to be packed more tightly in the channel of pol g (Fig.1.12.A.-F.). Moreover, thanks to additional helices and coils supplied by exonuclease and polymerase domains the downstream part of this channel adopts a bottleneck-like shape which could confer new features to pol g (Fig.1.14.). Nevertheless, the mechanisms that are associated with this unique structural architecture that distinguishes the DNA-binding

channel of pol g from its prokaryotic prototypes have still to be elucidated.

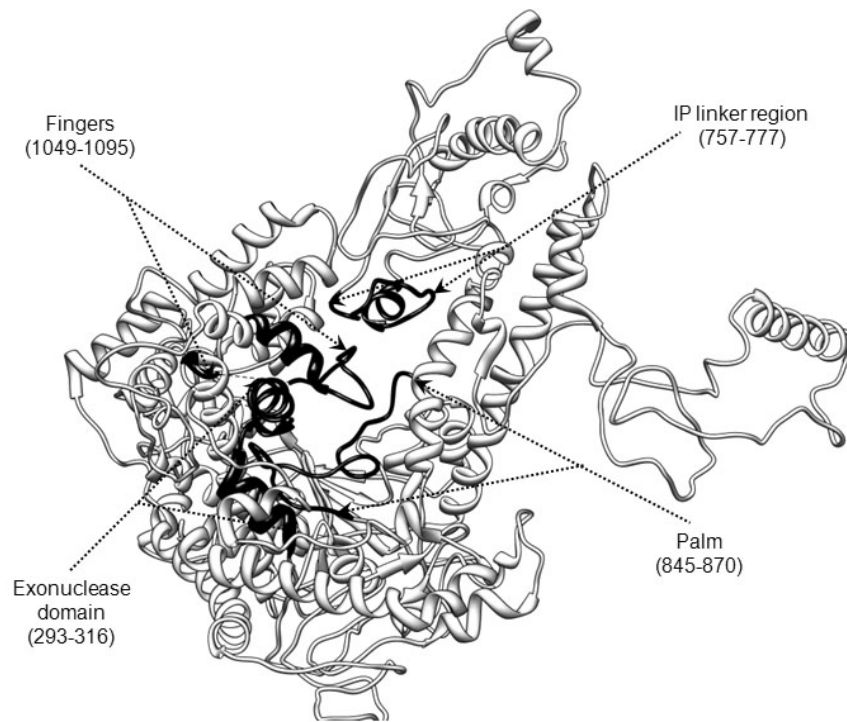


Figure 1.14. Coil and helix elements in the DNA binding channel of human pol g catalytic subunit.

Unique structural architecture of DNA binding channel of human pol g is provided by several coil and helix elements (indicated in black) supplied by 3'-5' exonuclease (residues 293-316), polymerase (residues 845-870 and 1049-1095) and linker (757-777) domains. Pdb ID: 13KM. Figure prepared by using the UCSF Chimera software.

To summarize, beside many clear but basic similarities shared by three dimensional structures of pol g and other Pol A polymerases, several significant and important differences do exist. As a consequence, pol g may have evolved specific mechanisms with respect to DNA binding, processive DNA synthesis, substrate specificity or functional coupling between polymerase and

exonuclease functions. All these observations suggest that mitochondrial DNA polymerases gamma form a new subfamily that is distantly related to other members of Pol A family.

1.3.6. DNA polymerase gamma from yeast and human:

1.3.6.1 Functional insight into the linear representation and three-dimensional structure of pol g:

As previously mentioned human pol g is composed of one pol gA catalytic subunit and two asymmetrical pol gB subunits. The proximal pol gB subunit makes extensive interaction with hydrophobic residues of the L-helix of the AID subdomain and hydrophilic residues of the thumb subdomain (Lee *et al*, 2009). With the exception of an interaction with Arg232 in the 3'-5' exonuclease domain (Lee *et al*, 2010), the distal pol gB subunit has no interface with pol gA. There is no homologue of pol gB in yeast and moreover, no evidence for an accessory subunit (Lucas *et al*, 2004; Viikov *et al*, 2010). Despite this remarkable difference between human and yeast mitochondrial DNA polymerases, their catalytic subunit is quite similar, with 40% amino acid identity (Fig.1.17.) observed in the shared regions (Ropp *et al*, 1996; Lecrenier *et al*, 1997).

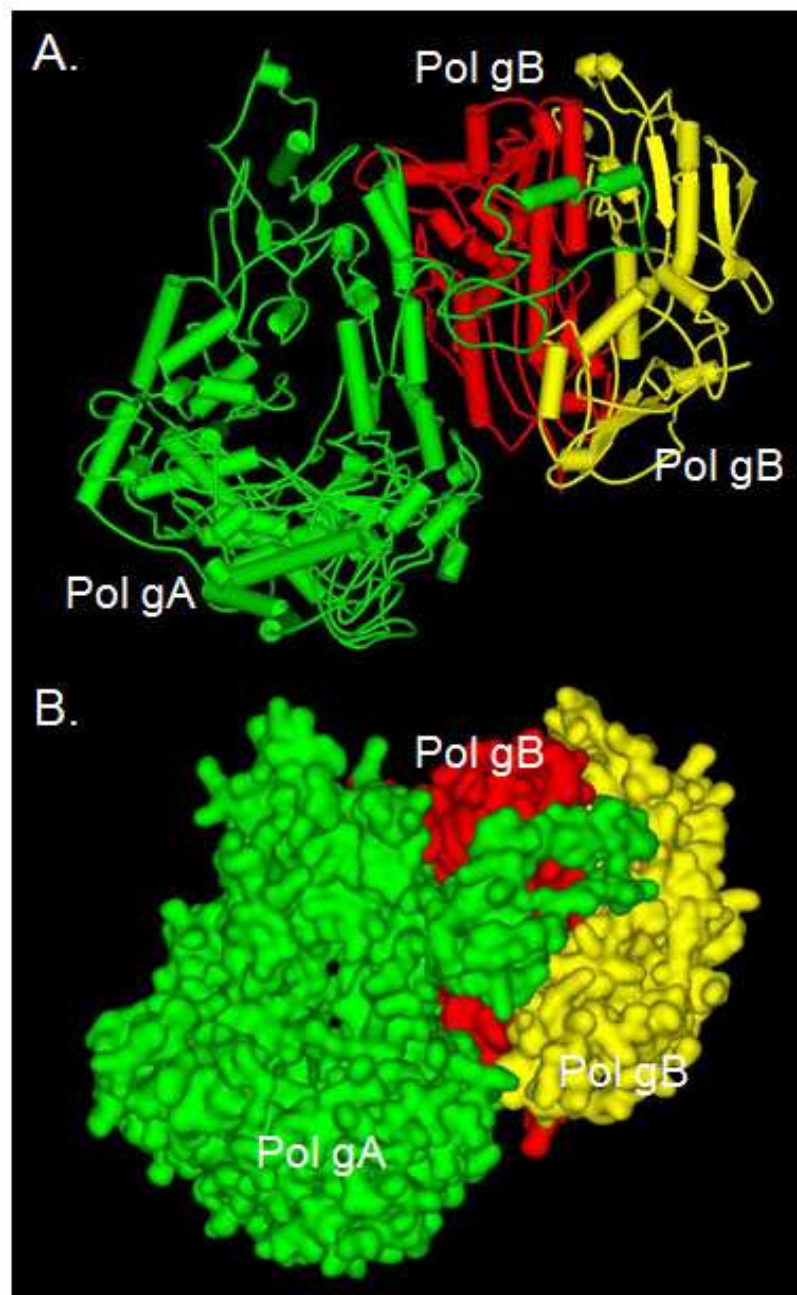


Figure 1.15. Heterotrimer of the human DNA polymerase gamma.

A. Schematic and **B.** surface representations of the crystal structure of heterotrimeric pol g holoenzyme containing one catalytic subunit pol gA (green) and proximal (red) and distal (yellow) monomers of pol gB accessory subunit. Pdb ID: 13KM. Figure prepared by using the Accelrys DS Visualizer software.

General architecture:

The amino acid sequence alignment of human and yeast pol γ shows that their linear organization is similar (Fig.1.17. and Fig.2.1.A.). Both proteins contain a mitochondrial targeting presequence at their very N-terminus, and the N-terminal 3'-5' exonuclease and C-terminal polymerase domains are connected by a linker region (Fig.1.17. and Fig.2.1.A.). Exonuclease and polymerase domains are well conserved. The catalytic aspartate and glutamate residues characterizing the active sites of the exonuclease domain (D171 in ExoI, D230 in ExoII, D347 in ExoIII for Mip1, that are equivalent to D198, D274 and D399 in human pol γ , respectively) and the polymerase domain (D693 in motif A and D892 in motif C for Mip1 that are equivalent to D890 and D1135 in human pol γ , respectively) are strictly conserved (Fig.1.17.). Moreover, homology is not restricted to catalytic blocks and extends all along polymerase and exonuclease domains. It includes tyrosines 753 and 757 in motif B of Mip1 fingers subdomain that are equivalent to human pol γ Y951 and Y955 (Fig.1.17.) which are directly acting in dNTP recognition and positioning with respect to DNA primer-template and catalytic site (Graziewicz *et al*, 2006). The only notable differences are that a few residues present between ExoI and ExoII in human are lacking in yeast, and a finger segment between residues 992 and 1030 (human pol γ numbering) is truncated from Mip1 (Fig.1.16. and Fig.1.17.).

The major differences between yeast and human pol γ consist in a polyglutamine stretch present in the N-terminal part of human pol γ , a 240-residue extension at the C-terminal part of Mip1 and the linker region (Fig.2.1.A.).

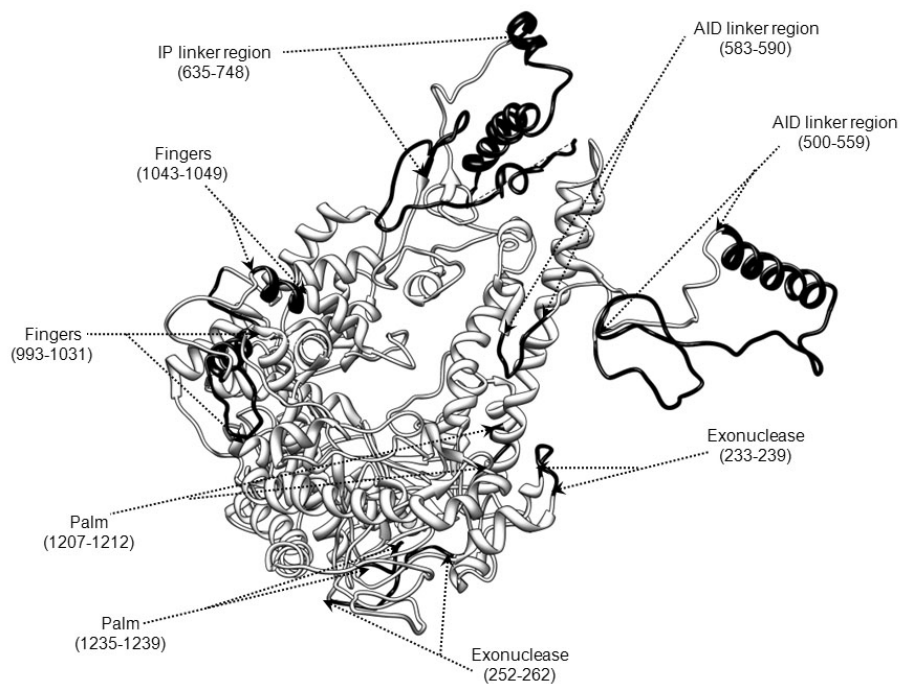


Figure 1.16. Conservation of human pol g and yeast Mip1.

Structural elements of human pol g catalytic subunit which are absent in yeast Mip1 are indicated in black: two elements of 3'-5' exonuclease domain (residues 233-239 and 252-262), two fragments of AID linker region subdomain (residues 500-559 and 583-590), large fragment of IP linker region subdomain (residues 635-748), two elements of fingers (residues 993-1031 and 1043-1049) and two elements of palm (residues 1207-1212 and 1235-1239). Pdb ID: 13KM. Figure prepared by using the UCSF Chimera software.

N-terminal extension:

The N-terminal part of human pol g is characterized by a stretch of glutamines that immediately follows the mitochondrial targeting presequence and is generated by repetitions of the CAG triplet in the *POLG1* gene (Lecrenier *et al*, 1997). This expansion is reminiscent of the polyglutamine stretches associated with several neurodegenerative disorders such as Huntington's disease (OMIM ID: #143100). In most individuals, the repeat contains 10 contiguous glutamines. It was initially reported that an alteration in the size of this expansion (less than 10, or 11-14 glutamines) is associated with loss of sperm quality

in human and can even lead to 5-10% of male infertility cases in the European population (Rovio *et al*, 2001; Jensen *et al*, 2004). However, further studies did not confirm any relationship between male infertility and disturbance of the polyglutamine extension (Krausz *et al*, 2004; Akinin-Seifer *et al*, 2005). Finally, more recent studies showed that the size of the expansion can also be associated with cases of idiopathic sporadic Parkinson's disease and testicular cancer and even possibly, when associated with other mutations, with Alpers syndrome (Nowak *et al*, 2005; Luoma *et al*, 2007; Blomberg-Jensen *et al*, 2008; Baruffini *et al*, 2010). However, the function of this glutamine stretch which is absolutely specific to human and is not even found in monkeys remains unknown. Moreover, it has not been demonstrated that the polyglutamine stretch is present in the mature protein.

C-terminal extension:

The long C-terminal extension (~280 residues, residues 279-1254) present in Mip1 characterizes all *Saccharomycetales* members but is absent from other organisms including human (Fig.2.1.A.). Deletion of the last 351 or 279 amino acid residues from the carboxyl end of Mip1 (Mip1p Δ 279 or Mip1p Δ 351) result in loss of the mitochondrial genome. However, yeast cells expressing a less severely truncated version of pol g (Mip1p Δ 175) are respiratory competent, leading to the conclusion that the last 175 residues of the C-terminal tail are not required for pol g function (Young *et al*, 2006).

Linker region:

The most divergent part between Mip1 and human pol g is the linker region that is much shorter in yeast (Fig.2.1.A.). This is not really surprising since in human pol g the linker region is an important interface for physical interactions with pol gB and the latter is absent from yeast pol g (Fig.1.15.). Consequently, large parts of accessory-interacting determinant (AID) subdomain and several segments of the intrinsic processivity (IP) subdomain are lacking in Mip1 (Fig.1.16.). Extensive interactions between the catalytic and proximal pol gB subunits substantially increase the polymerase activity and processivity of human pol g. Notably, the L-helix of the AID subdomain which interacts with pol gA, Arg232 which provides a salt bridge to E394 residue in distal pol gB, and a strongly positively charged stretch (456KQKKAKKVKK505) that binds the duplex DNA are absent in the yeast linker region (Lee *et al*, 2009; Lee *et al*, 2010). Therefore, it can be expected that the mechanisms that direct processive DNA synthesis in Mip1 are partially different from those of human pol g. However, a helix-coil fragment (residues ~760-772 in human pol g) from IP subdomain which provides a binding site for the DNA duplex is conserved in Mip1 (Lee *et al*, 2009). From the observation that, when alone, the catalytic subunit of human pol g still exhibits significant processivity, it can be expected that processivity mechanisms are partially conserved in yeast and human enzymes (Viikow *et al*, 2010). Finally, it must be mentioned that the amino acid sequence of the linker region fragment encompassing residues 559-581 in human pol g is strongly conserved in Mip1 (residues 452-474)

(Fig.1.17.). Noteworthy, this module is localized in the upstream part of the DNA-binding channel.

DNA-binding channel:

The architecture of the downstream part of the DNA-binding channel - downstream of the active site in the DNA-binding pocket- should be well conserved (Fig.1.17.). Indeed, the limits of the channel lumen are constituted by the palm and thumbs (with several highly conserved residues) on one side and the most conserved parts of the 3'-5' exonuclease domain on the other side (Fig.1.16.). Moreover, the very peculiar bottleneck-like module that closes the channel in human pol γ is formed by highly conserved segments of 3'-5' exonuclease (residues ~290-320 in human pol γ equivalent to residues 246-275 in Mip1), palm (residues ~840-866 equivalent to residues 643-669 in Mip1) and fingers (residues ~1050-1100 equivalent to residues 805-855 in Mip1) (Fig.1.14.; Fig.1.16.; Fig.1.17). However, the more external and upstream part of the DNA-binding channel (with respect to the catalytic site in the DNA-binding pocket) is constituted by less conserved regions of pol γ in the periphery of the 3'-5' exonuclease domain and in the linker region (Fig.1.16.). Thus there is no evidence that the architecture of the upstream part of the DNA-binding channel is well conserved between yeast and human pol γ , and this could also differently influence processive DNA synthesis.

homo	70	RHNPLDIQMLSRGLHEQIFGQ-GGEMPGERR--VRRSVEHLQKHGLWGQPAVFLPDVEL	
cerevisiae	40	RINPVGIQYLGEQLRQVFGSCGGKDEVEQSDKLMELSHKSLKDHGLWGKKTITDPISF	
		* * * * *	
homo	126	RLPFLYGDNLQHFRLLAQKQSLPYLAANLLLQAQLPFPKPPAWAEGWTRYGPEGEAV	
cerevisiae	100	ELPFLQGRSLDEHFQKIGRFNSEPYKSPCEDKF-TEMVARPAEWLRKPGWVKYVPGMAPV	
		* * * * *	
homo	186	FVAIPERERALVFDVEVCLAEGTCTPLAVAISSAWYSWCSQRLVEERYSWTSQLSFADLI	244
cerevisiae	159	EVAYPDEELVVFDETLVNSDYPTLATALSSAWYLSWCSFFICGGD-----DPAALI	
		* * * * *	*210*
homo	244	PLEVPTGASSPTQRDNQELVVGHNVSFIRAHIREQYLIQGSRRFLDTMSMHMAISGLS	304-305
cerevisiae	210	PLNTLN-----KEQVITIGHNVAYDRARVLEEYNFRDSKAPFLDTQSLHIASFGLC	
		* * * * *	*260-261
homo	306	SFQRSLWIAAQGHKVPPTKQGGQSKRKARSGPAISSWDNLDISSVNSLAUVHRLVVG	309 312
cerevisiae	262	SRQRPMFMENNR-KKEAEVESEVHPEISIEDYDDP-----WLVNSALNSLKDVAKFHCK	
		* * * * *	
homo	366	GPPELEPRELFVKGTMKDIRNFQDLNQYCAQDVNAHEVQQQLPLFLERCPHFVTLA	
cerevisiae	315	IDLDKTDRTDFFASTDKSTIENFQKLVNYCATDVATATSQVDEIFPVFLKKCPHFVSPA	
		* * * * *	
homo	426	GMLEMGVSYLFWNQW-WERYLAEAQGTVEELQREMKKSLMDLANDACQLLGG-ERYWEDP	
cerevisiae	374	GLKSLSKCILPTKLNWDNDYLNSSSLYQQSKVQIESATVQIKIDIVLLKDKPDFYLRDP	
		* * * * *	
homo	484	WLWDLWDLQEFQKQKAKKVKKEPATASKLPIEGAGAPGDMQDEDLGPCSEEEFQQDV	
cerevisiae	434	WLSQLDWITKFLRLTK-----	
		* * * * *	
homo	544	MARACLQKLGTELLPWRPQHLPGHFGWYKLCPLRDPANTPGPSLLSLQMRVTPKLM	574
cerevisiae	450	-----KG-----VPANCKLPGFPENYRQLFPSSKD-----TVEPKI-TIKSAIPIILF	
		* * * * *	*467*
homo	604	ALTWDGFLHYSERHGWGYLVPGRDNLAKLP TGT TLESAGVVCPIYRAIESLYKHCLQ	
cerevisiae	492	KLWENSFVWNSKESGWCENVEHEQVEYTK-----	
		* * * * *	
homo	664	GWQQLMPEAGLAEEFLITDNSAINQTVEELDYLEVEAEAHMENLRAAVGQPLALTARG	
cerevisiae	522	-----AKNYVLADSVS-----QEEETHTNHLGLQCTGV-----	
		* * * * *	
homo	724	GPEDTQPSYHHNGFYNVDVIPGCWFFKLPKDGNSCNVGSFPKDFLEKMEGSTLQAGP	748
cerevisiae	551	-----LFKVPHPNGPTFNCTNLLTKSYNHFFEGVGLKSES	
		* * * * *	
homo	784	GGASGFRALEINWMSFWNAHKRISSQMVV--WLPSALPRAVIRHPDYDEEGLYGA	
cerevisiae	586	ELAH--QALQINSSGSYWMSARERISQSFVVPSCKFPNEFQSLAKSSLNNEKTNDLAI	
		* * * * *	
homo	841	LPQVVVAGTITTRAVEPTWLTASNARPDVGS ELKAMVQAPPGYTLVGADVDSQELWIAA	A
cerevisiae	644	IPKIVPMGTITTRAVENAWLTASNAKANRIGS ELKTQVKAAPPGYCVGADVDSEELWIAS	
		* * * * *	
homo	901	VLGDAHFAGMHGCTAFGWMTLQGRKSGTDLH SKTATTVGSISREHAKIFNVGRIYGAGQP	B
cerevisiae	704	LVGDSIF-NVHGGAIGWMCLEGIKNEGTDLH TKTATLGCSANEAKIPNVGRIYGAGAK	
		* * * * *	
homo	961	FAERLIMQFNHRLTQQAEEKAQQMYAATKGLRWYRLSDEGEWLVRLELNLVDRTEGGWI	
cerevisiae	763	FASQLLKRFPPLTDEETKKIANKLYENTKG-----	
		* * * * *	
homo	1021	SLQDLKVVQRETARKSQWKKNEVVAERANKGGTESEMFNKLESIATSDIPRTFVLGCCIS	
cerevisiae	794	-----KTRSKLPKKF-----WYGGSESILFNKLESIAEQETPKTFVLGCGIT	
		* * * * *	
homo	1081	RALEPSAVQ-EEFMTSRVNVVQSSAVDYLHMLVAMKGLFEFAIDGRFCISIHDEVRY	C
cerevisiae	837	YSLMKHNLRLANSFLPSRINWAIQSSGVVDYLHLCCSMEYIKHYNLEARLCISIHDEIRF	
		* * * * *	
homo	1140	LVRDEDRYRALALQITNLLTRCMFAYKLGINDLPQSVAFFSAVDIDRCLRKEVTMDCKT	
cerevisiae	897	LVSERDKYRAAMALQISNIWTRAMFCQMGINELPQNCAPFSQVDIDSVIRKEVNMDICIT	
		* * * * *	
homo	1200	PSNPTGMERRYGIPQGEALDIYQIIELTWGSLK	
cerevisiae	957	PSNKTAE-----IPHGEALDIQQLDKSNSKLGK	
		* * * * *	

Figure 1.17. Amino acid sequence alignment of yeast and human pol g.

Alignment of the yeast Mip1 and human Pol gA amino acid sequence was performed with the Clustal W program and adjusted with eye. Identical residues were indicated with an asterisk. The ExoI, ExoII, ExoIII, PolA, PolB and PolC conserved blocks containing the invariant aspartates and glutamates are overlined. Residues analyzed in this study are shown in red. Domains and subdomains of pol g are indicated with color frames as follows: N-terminal extension in grey, 3'-5' exonuclease domain in green, thumb in violet, linker region in orange, palm in light blue, fingers in dark blue. The C-terminal extension of yeast pol g is not included into the figure.

1.3.6.2. Yeast DNA polymerase gamma as a functional model for human pol g:

MIP1, the gene encoding yeast pol g, has been cloned and sequenced more than twenty years ago (Foury, 1989). The *MIP1* gene was cloned by genetic complementation of a yeast mutant (ts71) exhibiting low levels of mtDNA synthesis and high accumulation of *rho*⁻ mutants in a temperature-sensitive manner (Genga et al, 1986; Foury, 1989). A single substitution was present in the *MIP1* gene producing a TAG codon resulting in truncation of pol g at position 1026 of the amino acid sequence (Foury, unpublished data). Deletion of the *MIP1* gene results in total loss of mtDNA suggesting that Mip1 plays a crucial role in the replication of the mitochondrial genome. Nowadays, we have good evidence that Mip1 is the unique mtDNA replicase.

Later on, several *mip1* mutator mutants were isolated by site-directed and *in vitro* random mutagenesis. The singly substituted D171G, E173K, D230A and D347A mutants in essential residues of the 3'-5' exonuclease active site (Fig.1.18.) were exhibiting a 10⁴ fold decrease in the 3'-5' exonuclease activity *in vitro* and a 100-200 fold increase in the frequency of mtDNA point mutations, while the

stability of mitochondrial genome was only slightly affected (Foury and Vanderstraeten, 1992; Hu *et al*, 1995; Vanderstraeten *et al*, 1998). Moreover, a *mip1* mutant harboring the double D171G/D230A substitution was totally devoid of 3'-5' exonuclease activity *in vitro*, and showed a 1500-fold increased frequency of point mutations in mtDNA and low stability of mitochondrial genome *in vivo* compared to the wild-type. However, no important effect of these mutations on polymerase activity was observed *in vitro* (Vanderstraeten, 1992; Vanderstraeten *et al*, 1998).

A systematic screen for mutator and/or temperature-sensitive *mip1* clones revealed several novel amino acid substitutions in the 3'-5' exonuclease domain, G224D and H225D immediately upstream of ExoII site, T351I just downstream of ExoIII site, S198L and S304L in the periphery of the exonuclease domain between ExoI and ExoII, and ExoII and ExoIII sites, respectively (Fig.1.18.). Mutations in these residues, which are conserved in human pol g, gave a strong mutator phenotype, and, with the exception of S198L, did not greatly increase mtDNA instability (Hu *et al*, 1995; Foury *et al*, 2004). The S198L mutation gave a hypermutator phenotype with a ~5000-fold increase in the frequency of mtDNA point mutations, and showed temperature-sensitive accumulation of *rho*⁻ mutants. Mip1-S198L was exhibiting wild-type polymerase activity and about 10% of the wild-type 3'-5' exonuclease activity *in vitro*. The very low levels of Mip1-S198L protein present in mitochondria and the temperature-sensitive phenotype *in vivo* suggest that proper folding of Mip1 is affected (Hu *et al*, 1995). Several *mip1* mutator mutations localized in the polymerase domain were also identified in this screen. While the

moderately mutator P851L and R1001I mutations (50-fold point mutation increase) are localized in non conserved residues, a peripheric segment of the fingers subdomain that is highly conserved in human pol γ was found to be a hot spot of temperature-sensitive weak mutator mutants (G114S, G115D, M716I, M721I and E724K; Fig.1.18.) (Hu *et al*, 1995; Foury *et al*, 2004). Processive DNA synthesis was strongly decreased in the mutant polymerases while the 3'-5' exonuclease to polymerase activity ratio was dramatically increased. Data obtained in our laboratory suggest that these residues play a crucial role in proper folding of Mip1. Tyrosine 757, which is localized in the fingers subdomain (Fig.1.18.) and is strictly conserved in polA family (helix O in PolIk) is believed to be involved in recognition and positioning of dNTP during catalysis of the polymerization reaction (Y955 in human pol γ , Graziewicz *et al*, 2006). This residue has been changed to phenylalanine, cysteine and serine. While Y757F substitution elicits a mild mutator phenotype (5-fold point mutation increase *in vivo*) and does not significantly increase mtDNA instability, Y757S and Y757C elicit total loss of mtDNA (Baruffini *et al*, 2006). Moreover, the Y757C mutation has a dominant phenotype resulting in an increased frequency of mtDNA point mutations and deletions in heterozygous cells. It has been shown that *in vitro* MipY757F increases the K_m (dNTP), in agreement with the data obtained with the human pol γ -Y955C mutation that elicits a 45-fold decrease in affinity for incoming dNTPs (Wallace, 2005; Baruffini, 2006).

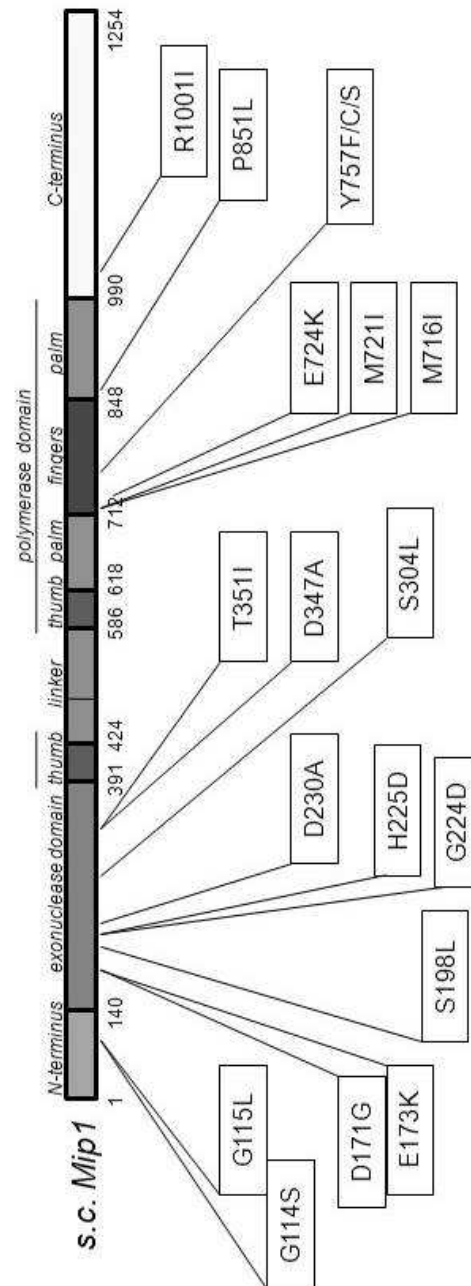


Fig.1.18. *mip1* mutations generated for pol g functional studies.

Localization of *MIP1* mutations generated for pol g functional studies is indicated by arrowheads. Domains and subdomains of pol g are indicated in boxes. N-end indicates N-terminal extension of pol g; T indicates thumb subdomain.

In conclusion, while *Drosophila* pol g was the first mitochondrial polymerase that was purified to homogeneity as a two-subunit enzyme and biochemically characterized as soon as 1986 (Warnette and Kaguni, 1986), yeast Mip1, which was sequenced many years before other pol g, has been the earliest model combining both *in vivo* and *in vitro* approaches and providing insights in pol g mechanisms and biological function. Yeast studies have shown that pol g is a faithful replicase through high precision in selection of the correct nucleotides and through the 3'-5' exonuclease activity which enables it to remove mispaired bases during DNA synthesis. Moreover, from the middle of last decade, yeast Mip1 has successfully been used as a model for studies on human pathogenic mutations of pol g. The last chapter of this introduction will be fully devoted to that issue.

1.3.6.3. Systems generated for studies on pol g structure and function.

The first native form of pol g was isolated from chicken (Yamaguchi *et al*, 1980). Later on, various groups isolated and purified pol g from *Drosophila melanogaster*, *Xenopus laevis*, pig and human cells (Warnette and Kaguni, 1986; Insdorf and Bogenhagen, 1989; Mosbaugh, 1988; Kunkel and Mosbaugh, 1989; Gray and Wong, 1992). The gene encoding the pol g catalytic subunit was initially cloned from *Saccharomyces cerevisiae* (Foury, 1989), and by the mid of 1990, pol g coding sequences were also determined for human, mouse, chicken, frog, fruit fly, *Schizosaccharomyces pombe* and

Pichia pastoris (Ropp and Copeland, 1995; Ropp and Copeland, 1996; Ye *et al*, 1996; Lewis *et al*, 1996).

Several systems were used to characterize pol g in vitro and to determine its role in mitochondrial mutagenesis and dysfunction in vivo.

The first systems were devised to purify and characterize the pol g protein. Pol g was purified to near homogeneity for the first time in 1986 from *Drosophila* embryos, and in 1992, from human HeLa cells (Warnette and Kaguni, 1986; Gray and Wong, 1992). However, expression of the catalytic subunit from animals is now routinely obtained in baculovirus, while the accessory subunit is expressed in *E. coli*. The holoenzyme is efficiently reconstituted in vitro from the two purified proteins. The baculovirus system has allowed the biochemical characterization of many human and *Drosophila* mutated forms of pol g (Lim *et al*, 1999; Wang and Kaguni, 1999; Farr *et al*, 1999; Kaguni, 2004; Luo and Kaguni, 2005; Luoma *et al*, 2005; Palin *et al*, 2010). The yeast Mip1 pol g has been purified in low amounts from yeast using the strong inducible *GAL1* promoter (Vanderstraeten *et al*, 1998). Recently, high yields of pure Mip1 were obtained through expression in *E. coli* (Viikov *et al*, 2010).

In vivo studies were performed with HeLa cell cultures, fly, mouse and yeast models. A few mutant studies were performed using transgenic HeLa cell cultures. While cell lines harboring a deficient human pol g enzyme were characterized by a strong negative dominant phenotype, defects in 3'-5' exonuclease proofreading activity led to only mild impairment of mtDNA maintenance, and long term cultures resulted in cell growth arrest and reduced mutational

load (Spelbrink *et al*, 2000). Cell cultures were however of limited interest as they were considered as non physiologically relevant. *Drosophila* was used in a genetic screen that revealed various behavioral, development and morphological abnormalities associated with pol g mutations (Iyengar *et al*, 1999). It was also found that overexpression of pol g in transgenic flies result in significant mtDNA depletion at the cellular level and various phenotypic effects, as pupal lethality or morphological abnormalities in adults (Lefai *et al*, 2000). Genetically modified mouse models were also obtained. Transgenic mice harboring a mutation in an essential catalytic aspartate residue (Asp181) of the 3'-5' exonuclease showed congestive heart failure associated with high point mutation and deletion rates in mtDNA (Mott *et al*, 1999; Zhang *et al*, 2000; Zhang *et al*, 2003). However, the significance of the symptoms is debated as pol g was overexpressed. Two groups have produced homozygous knockin mice expressing an error-prone version of pol g carrying a mutation in the essential Asp257 of the 3'-5' exonuclease (Trifunovic *et al*, 2004; Kujoth *et al*, 2005). These mice exhibited significantly increased mtDNA point mutation and deletion rates. The mutator properties were associated with reduced life span and premature aging characterized by osteoporosis, hair loss and graying, weight reduction and decrease fertility (Trifunovic *et al*, 2004; Kujoth *et al*, 2005; Trifunovic and Larsson, 2008).

As developed in this thesis the yeast model is of great importance for pol g studies by combining in vivo and in vitro approaches (see chapter 1.3.6.2. and 1.5.)

1.4. Mutations in DNA polymerase gamma – from mutation to pathology:

Mitochondrial diseases are a highly heterogeneous group of disorders resulting from defects in mitochondrial function, mainly in the oxidative phosphorylation. The idea of mitochondrial disorders was born in the middle of the last century (Luft *et al*, 1962; DiMauro, 2004). However, the first clear evidences supporting a relationship between primary mitochondrial defects and a wide spectrum of human diseases appeared only twenty years ago (Holt *et al*, 1988; Wallace *et al*, 1988). Dozens of various disease presentations have currently been observed and described in human. It is now admitted that the prevalence of mitochondrial disorders is much higher than originally expected and reaches 1 per 8500 individuals (Chinnery, 2006, NIH Database, Gene Review, Mitochondrial Disorders Overview). More than 100 mtDNA point mutations have been associated with a broad spectrum of diseases in human (McFarland *et al*, 2010; Wong, 2010). Here, we will only mention those nuclear genes that at the mutated state lead to mtDNA defects and are associated with disease in human.

1.4.1. Defects in nuclear genes associated with mitochondrial disorders:

Since the end of last century more than a dozen of nuclear genes involved in maintenance of the mitochondrial genome have been discovered. Mutations in human genes directly involved in mtDNA transactions at the replication fork, such as *POLG1* (pol g catalytic

subunit), *POLG2* (pol g accessory subunit), and *TWINKLE* (mtDNA helicase) were associated with point mutations, multiple deletions or depletion of mtDNA (Van Goethem *et al*, 2001; Spelbrink *et al*, 2001; Longley *et al*, 2006). Despite well documented impairment of mtDNA maintenance in human mutant cell lines (as well as yeast, fruit fly or mouse mutants) leading to deletions or depletion of the mitochondrial genome, no disease alleles in mtSSB or TFAM have been identified in human patients up to now (Maier *et al*, 2001; van Dyck *et al*, 1992; Ruhanen *et al*, 2010; Larsson *et al*, 1998; Mao and Holt, 2009). A distinct category is formed by genes that encode proteins participating in mtDNA maintenance in a more indirect manner, as for instance, by supplying mitochondria with deoxyribonucleosides triphosphates pools. Mutations in *TK2* (mitochondrial thymidine kinase), *dGK* (deoxyguanosine kinase), *ANT1* (adenine nucleotide translocator-1), *RMM2B* (ribonucleotide reductase inducible p532B), *SUCLA2* (succinyl-CoA synthetase), *DNC* (deoxynucleotide transporter) and *TP* (*ECGF1* thymidine phosphorylase) can lead to deletion or total loss of the mitochondrial genome (Gotz *et al*, 2008; Wang and Eriksson, 2003; Fontanesi *et al*, 2004; Lewis *et al*, 2005, Lopez *et al*, 2009, Copeland, 2008; Mao and Holt, 2009). Mutations in these genes were found to be a cause of autosomal dominant PEO (adPEO) or *mtDNA depletion syndrome* (MDS).

In 2001, the group of van Goethem found that *POLG1* gene is a locus for PEO (Van Goethem *et al*, 2001). This observation led to rapid discovery of other human mitochondrial disorders resulting from pol g mutations. Currently, more than 150 pathogenic alleles of *POLG1* (Fig.1.20.) have been identified in human (Chan and Copeland, 2009;

Human DNA Polymerase Gamma Mutations Database). Although several molecular mechanisms through which deficient pol g can cause mitochondrial defects have been described, it seems that each mutated state of pol g should be analyzed individually. This is due to the large complexity of the pol g disorders, difficulties in simple classification of disease presentations of the patients and lack of universal rules and patterns for pathogenicity of *POLG* mutations.

1.4.2. Types of mutations existing in mitochondrial genome:

The proper architecture and function of the mitochondrial genome can be affected by two types of mutational changes: point mutations and small or large scale deletions. Studies on yeast mitochondrial genetics provide an excellent basis to approaching mtDNA abnormalities.

MtDNA deletions and depletion: Yeast strains harboring an intact mitochondrial genome are designed as ρ^+ . Commonly used laboratory yeast strains lose their mitochondrial genome at the frequency of 1-2 %, or more (Sherman, 1991). Respiratory-deficient cells harboring a defective mitochondrial genome (frequently due to mtDNA deletions) are termed as ρ^- , whereas strains characterized by complete loss of mitochondrial DNA (mtDNA depletion) are termed as ρ^0 . Both ρ^- and ρ^0 mutants are respiratory deficient and thus are not able to grow on respiratory carbon sources such as glycerol or ethanol. However, as a facultative anaerobic organism, yeast is able to utilize glucose as a carbon source, in the absence of respiration, through the fermentation process.

MtDNA point mutations: Punctual mutations are less commonly spread in the yeast mitochondrial genome than deletions. However, in yeast numerous so-called *mit⁻* point mutations leading to respiratory deficiency were identified and well characterized (Tzagoloff *et al*, 1975). *Ant^R* mutations conferring resistance to specific antibiotics, such as erythromycin, oligomycin or chloramphenicol, were also identified in genes encoding subunits of the respiratory complexes (OXPHOS) or mitochondrial protein synthesis machinery (tRNAs or rRNAs). They are localized either in the 21S rRNA gene (*Ery^R*, *Cap^R*) or in the membrane subunits (*atp6*, *atp9*) of the ATP synthase (*Oli^R*) (Dujon, 1981; Sor and Fukuhara, 1982).

1.4.3. Pol g mutations affecting mitochondrial genome stability:

Many pol g mutations result in rearrangement of mtDNA molecules, which appear as an accumulation of multiple deletions, or in depletion of the mitochondrial genome. Mechanisms involved in mtDNA instability are not precisely understood. Progressive accumulation of mtDNA deletions is a common feature for pol g mutants both in polymerase, 3'-5' exonuclease and linker region domains. Noteworthy, the exonuclease deficient forms of pol g in human cell cultures and mice are mostly associated with an increased rate of point mutations and deletions in mtDNA, while the polymerase-deficient forms of pol g result in complete (or almost complete) inhibition of mtDNA replication leading to loss of the mitochondrial genome (Spelbrink *et al*, 2000; Trifunovic *et al*, 2004).

It is believed that in polymerase deficient pol γ mutants low processivity of the enzyme can determine stalling and premature interruption of replication resulting in formation of incomplete nascent DNA strands. Subsequently, presence of fragmental DNA strands could promote intermolecular recombination obtained via branch-migration and strand invasion mechanisms (Kajander *et al*, 2000). Interestingly, several studies on yeast respiratory-deficient cells and on muscles biopsies obtained from patients suffering from mitochondrial myopathies have demonstrated that deleted fragments of mtDNA are frequently flanked by specific directly repeated sequences (Dujon, 1981; Zeviani *et al*, 1989; Burgart *et al*, 1995). Approximately 66% of all rearrangements and deletions identified in prokaryotic and eukaryotic genomes are associated with repeated sequences (Bianchi, 2001). Therefore, it has been proposed that mtDNA rearrangements could be mediated by repeat-dependent deletion events (Fig.1.19.). The mechanisms by which these deletions arise could be (i) formation of a hairpin structure and polymerase slippage, (ii) homologous recombination obtained by a double-strand break in the repeat sequence and/or (iii) non-homologous end joining generated by a double-strand break in the intervening sequence (Fig.1.19.) placed between repeats (reviewed by Phadnis *et al*, 2005 and Krishnan *et al*, 2008). Polymerase slippage involving formation of secondary DNA structure and leading to small deletions or additions (Fig.1.19.) was characterized both in yeast and human mitochondria (Shoffner *et al*, 1989; Tran *et al*, 1995; Gordenin *et al*, 1997; Phadnis *et al*, 2005). Homologous and non-homologous recombination involving double-strand breaks might be an important mechanism for

formation of large-scale deletions (Fig.1.19.) both in nuclear and mitochondrial genomes (Prado *et al*, 2003; Chomyn and Attardi, 2003; Phadnis *et al*, 2005; O'Driscoll and Jeggo, 2006; Krishnan *et al*, 2008; Fukui and Moraes, 2009).

Given to the crucial role of DNA polymerase gamma in maintenance of the mitochondrial genome, pathogenic mutations in pol g resulting in impairment of structure or function of polymerase can favor formation of deletions and depletion in mtDNA. On one hand, structural defects of pol g can lead to its proteolytic degradation by the mitochondrial protein quality control machinery impeding mtDNA replication and consequently resulting in total loss of the mitochondrial genome. On the other hand, disease mutations affecting functional features of pol g can influence proper DNA synthesis reaction, enzyme processivity and/or DNA binding affinity, facilitating formation of mtDNA deletions and depletion.

Multiple mtDNA deletions and depletion could also result from reduced interactions of the mutant pol g with other factors at the mtDNA replication fork, such as pol g accessory subunit or mtSSB protein.

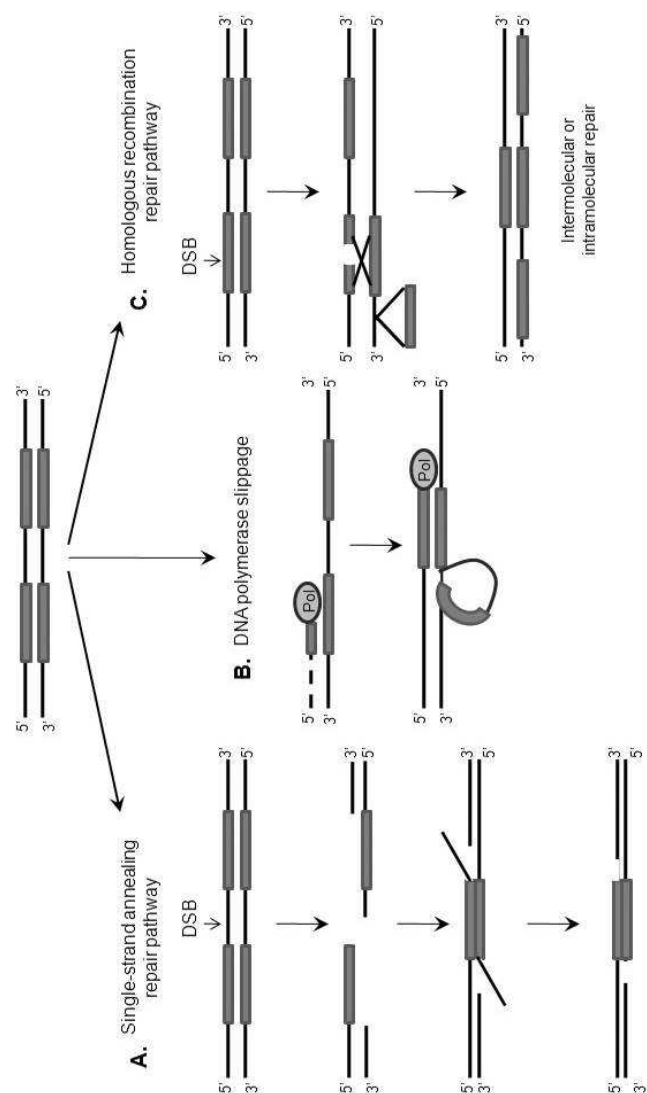


Figure 1.19. Possible pathways for repeat mediated DNA deletions in yeast and human mitochondria.

A. Single-strand annealing (SSA) repair pathway. This pathway is initiated by double-strand break (DSB) in the intervening sequence between the directly repeated sequences. SSA pathway involves non-homologous end joining and it always occurs within the same DNA molecule. **B. DNA polymerase slippage.** Slippage of DNA polymerase can occur between two directly repeated sequences leading to small deletions of mtDNA. During DNA replication polymerase can easily dissociate from the DNA template on the first repeated sequence and reanneals at the second repeated sequence, losing one of the repeated sequence accompanied by an intervening sequence. **C. Homologous recombination repair pathway.** This repair pathway is initiated by a DSB event occurring within the repeated sequence and involves homologous recombination which can lead to deletion of repaired DNA strand. Homologous recombination pathway can occur within the same DNA molecule or between two different copies of mtDNA. Adapted from Phadnis *et al*, 2005.

1.4.4. Mutator activity of pol g mutants:

As described in previous sections, the pathological state of mitochondria in human mitochondrial disorders could result not only from large-scale rearrangements of mtDNA, but also from increased generation of point mutations in the mitochondrial genome. Beside mutagenic environmental conditions, point mutations in mtDNA may also result from DNA replication errors, insufficient post-replicative error correction or error-prone repair of damaged DNA (Foury *et al*, 2004). Therefore, disease mutations in pol g amino acid sequence affecting the structure or function of this enzyme might lead to generation of point mutations in mtDNA through deficiency in selection of correct dNTPs during mtDNA synthesis and through insufficient or erroneous proofreading editing during 3'-5' exonuclease reaction (Vanderstraeten *et al*, 1998; Foury *et al*, 2004).

To conclude, mutations in polymerase, exonuclease and linker region domains of pol g can result in structural or functional alteration of the enzyme, leading to decreased stability of the mitochondrial genome and/or increased frequency of point mutations in mtDNA. Moreover, pol g mutations might influence important interactions between DNA polymerase and other replication factors.

1.4.5. Complexity of mitochondrial disorders:

Accurate maintenance of mitochondrial genome is vital for living cells. Unfortunately, two major factors exist in mitochondria that

make mitochondrial genetic information more prone to damage than its nuclear counterpart. First, although it is associated with the HMG-box containing protein abf2, mtDNA is not complexed with histones, which have a documented role in protecting nuclear DNA against damage (Friddle *et al*, 2004; Sia *et al*, 2009). Second, there is a strong and chronic exposure to highly mutagenic reactive oxygen species (ROS). Indeed, the mtDNA point mutation frequency of human cells exceeds by 10-fold that of the nuclear genome (Brown *et al*, 1979). Since it was found that human mitochondrial genome is prone to mutate at much higher level than nuclear DNA, mtDNA mutations and rearrangements have become a center of interest for researchers. For the last two decades a wide spectrum of human mitochondrial disorders was identified. Despite extensive characterization of many various disease presentations, the relationship between the phenotype and genotype of affected individuals as well as the underlying molecular mechanisms of disease development remain poorly understood. The most problematic aspect appears to be the complexity of mitochondrial disorders, which is itself illustrated by diversity of mtDNA changes, from seldom point mutations to large-scale deletions and depletion. Moreover, multiple additional factors are believed to be involved in development of mitochondrial disorders in human, such as the tissue distribution of mutated DNA, mtDNA copy number per cell, proportion of mutated mtDNA (heteroplasmy), mtDNA haplotype and nuclear genetic background. The homoplasmic or heteroplasmic states of mtDNA molecules in affected cells and type of inheritance (maternal or nuclear) is of particular importance. Finally, mitochondrial disorders can equally be caused by mutations in the

mitochondrial or nuclear genomes (Pulkes and Hanna, 2001; Taylor and Turnbull, 2005; Mao and Holt, 2009). The most fundamental aspects of human mitochondrial genetics will be summarized below.

Maternal inheritance:

In practice, human ovum is the only actual donor of mitochondria and mitochondrial genome in the process of fertilization, thus the mitochondrial genetic information in human is maternally inherited (Giles *et al*, 1980; Baasner *et al*, 1998). Although the overwhelming majority of mtDNA point mutations is maternally transmitted, large-scale rearrangements are not inherited (Egger and Wilson, 1983; Zeviani *et al*, 1990; Chen *et al*, 1995; Pulkes and Hanna, 2001). Noteworthy, undergoing to maternal inheritance does not mean that disease will be developed by all progeny, since disease development strongly depends on numerous coexisting factors (Pulkes and Hanna, 2001).

Homoplasmy and heteroplasmy:

Mitochondrial genome is characterized by polyploidy, meaning that genetic information in mitochondria can reach several thousand copies per cell. All copies can bear an identical genetic information and this state is referred to as homoplasmy, or the mitochondrial genetic information is an heterogeneous mixture of two or more genotypes and this state is referred to as heteroplasmy (Taylor and Turnbull, 2005). The majority of mtDNA mutations (with several exceptions) are present in the heteroplasmic state (Pulkes and Hanna, 2000). However, even if the mutation by itself is deleterious, heteroplasmic

state is not always pathogenic, as it depends on mtDNA segregation, tissue specificity and threshold level of mutations (Pulkes and Hanna, 2000).

Threshold effect:

It was frequently noted that a certain proportion of the mutant mtDNA in the total cell pool is required to observe a clear reduction in respiratory function. This phenomenon was defined as threshold effect (Chomyn *et al*, 1992; Bourgeron *et al*, 1993; Pulkes and Hanna, 2001). Noteworthy, threshold effect seems to depend significantly on mtDNA mutation type and is different for point mutations and large-scale deletions (Hanna *et al*, 1995; Tully *et al*, 2000; Chomyn *et al*, 1992; Bourgeron *et al*, 1993; Pulkes and Hanna, 2001).

Tissue specificity and mtDNA segregation:

An heteroplasmic cell is believed to randomly transmit variable fractions of mtDNA to daughter cells during the cell division (Jenuth *et al*, 1996). However, some studies have shown that this rule might be not appropriate for all cell types, suggesting that mtDNA segregation could be highly tissue specific (Pulkes and Hanna, 2001). MtDNA segregation seems to depend also on the type of mutation, nuclear genetics and environmental factors (Battersby *et al*, 2003; Taylor and Turnbull, 2005). Finally, some evidence suggests the existence of a genetic bottleneck phenomenon in the mtDNA segregation during its transmission to daughter cells in the oocyte maturation process (Brown *et al*, 2001).

1.4.6. Relationship between genotype and phenotype in mitochondrial disorders associated with pol g mutations:

Despite the high complexity of mitochondrial disorders associated with pol g mutations and the absence of strict genotype-phenotype relationship, attempts to classify pol g diseases have been undertaken by several groups (Longley *et al*, 2005; Horvath *et al*, 2006; Copeland, 2008; Milone and Massie, 2010). According to Horvath and colleagues individuals with mitochondrial disorders resulting from pol g mutations can be divided in three main groups (i) patients with sporadic and familial PEO characterized by multiple mtDNA deletions; (ii) patients with late-onset ataxia characterized by mtDNA deletions in skeletal muscles; (iii) patients with Alpers syndrome. Moreover, an additional fourth group was constituted for patients who escape this basic classification (Horvath *et al*, 2006). Generally, pol g mutations were identified in patients suffering from PEO, Alpers syndrome, other hepatocerebral syndromes, ataxia-neuropathy syndromes, Charcot-Marie-Tooth disease, idiopathic Parkinsonism, nucleoside reverse-transcriptase inhibitor (NRTI) toxicity, premature menopause, male infertility and testicular cancer (Graziewicz *et al*, 2006; Hudson and Chinnery, 2006; Copeland, 2008; Chan and Copeland, 2009). A brief presentation of the basic pol g-related disorders is given below.

Progressive External Ophthalmoplegia: The most common clinical features of progressive external ophthalmoplegia (PEO) are adult onset of disease, weakness of the external eye muscles and exercise

intolerance. Additional symptoms may include cataract, hearing loss, sensory axonal neuropathy, ataxia, depression, hypogonadism and Parkinsonism. Both autosomal dominant and autosomal recessive mode of inheritance can occur; however, autosomal recessive PEO cases are associated with more severe phenotypes. Furthermore, sporadic cases of PEO devoid of familial history are also quite frequent (OMIM # 157640).

Ataxia-Neuropathy Syndrome: Clinical manifestations of ataxia-neuropathy resulting from pol g mutations are highly complex with disease onset in early teens to late thirties. Several types of mitochondrial ataxia-neuropathies and encephalopathies were distinguished, among others, mitochondria-associated ataxia-syndrome (MIRAS), mitochondrial neurogastrointestinal encephalopathy syndrome (MNGIE), spino-cerebral ataxia-epilepsy syndrome (SCAE) or SANDO characterized by dysarthria and ophthalmoparesis as coexisting symptoms (OMIM # 174763; OMIM # 603041; OMIM # 607459). Symptoms of these ataxic syndromes include peripheral neuropathy, dysarthria, mild cognitive impairment, involuntary movements, psychiatric symptoms, myoclonus, hypotonia, hearing loss, gastrointestinal dysmotility, cardiomyopathy and epileptic seizures.

Alpers syndrome: The onset of Alpers syndrome usually falls to an early age, often within the first year of life. Alpers syndrome can be described generally as a rare fatal rapidly progressive neurodegenerative disorder characterized by failure to thrive,

myoclonus, psychomotor regression, refractory seizures, cortical blindness and spasticity. Additional liver failure gives an even more severe phenotype of disease termed as Alpers-Huttenlocher syndrome. The disease is characterized by a recessive mode of inheritance (OMIM # 203700).

To recapitulate, mitochondrial disorders associated with pol g mutations affect a large variety of organs and are characterized by variable age of onset. It is believed that specific manifestations of the disease depend on the type of mutation and its localization in amino acid sequence of pol g (Horvath, 2006; Copeland 2008). In compound heterozygous patients most cases with severe and early disease onset result from at least one mutation in the linker region and another one in the polymerase domain. Contrary, late onset of the disease is more commonly found in association with mutations in the 3'-5' exonuclease domain of pol g. However, more recent studies, including results presented in this work, provide many exceptions to these rules. Furthermore, simple classifications of pol g-related disease are complicated by the fact that a pol g mutation can frequently be found in association with at least one or two additional amino acid substitutions in pol g. This multiplicity of mutations makes the molecular mechanism of pathogenesis still more complex.

1.5. Yeast as a tool for studies on molecular mechanisms in pol g-related mitochondrial disorders:

Based on the hypothesis that conserved residues localized in conserved regions of pol g derived from various organisms should play a similar role, yeast Mip1 has repeatedly been used as a tool to determine the *in vivo* phenotypes conferred in yeast by several pathogenic pol g mutations (Baruffini *et al*, 2006; Baruffini *et al*, 2007; Stuart *et al*, 2006; Stumpf *et al*, 2010; Baruffini *et al*, 2010). The assumption that Mip1 could be used as a model enzyme was based on the observation that pol g catalytic subunit of *S. cerevisiae* encoded by the *MIPI* gene displays approximately 40% amino acid sequence identity with human pol gA in their shared regions (Ropp and Copeland, 1996; Lecrenier *et al*, 1997). Moreover, baker's yeast as a facultative anaerobic eukaryote seems to be a great tool to evaluate the molecular mechanisms that are altered in human pol g mutants. Large flexibility, well characterized genetics of yeast mitochondria, easy availability of various strains for *in vivo* studies and specific tools for molecular biology, relatively low labor cost and high speed of research make yeast an excellent model organism for studies on human pol g disease related mutations. *In vivo* studies on *mip1* mutants could provide light on the severity of individual pol g mutations and on the type of changes which affect the mitochondrial genome, while *in vitro* studies might help determine the specific defects exhibited by each mutated pol g version.

Utilization of yeast as a model system for disease-related mutations of human pol g started five years ago and up to now, more than 40 out of

150 pathogenic alleles were partially or fully characterized (Baruffini *et al*, 2006; Stuart *et al*, 2006; Baruffini *et al*, 2007; Stumpf *et al*, 2010; Cochonneau and Foury, unpublished data; Baruffini *et al*, 2010; see Table 3.). These studies also include two single nucleotide polymorphisms (SNPs) of human pol gA, Y831C and E1143G, which are single nucleotide changes found in at least 1% individuals of the population. Beside *in vivo* studies, characterization of biochemical properties of several mutant polymerases was also performed (Stuart *et al*, 2006; Stumpf *et al*, 2010; data not shown). The affected pol g residues were localized in polymerase, exonuclease and linker domains, mostly within functionally important regions, and exhibited either weak or strong conservation between yeast and human. However, a correlation between yeast phenotypes and severity of human disease presentations was only observed for a fraction of pol g mutations. Curiously, some of the mutations that were studied by different groups gave contradictory results (see Table 3.). Discrepancies could be explained in part by the different backgrounds of the yeast laboratory strains and cell culture conditions methodology used by each group. The strain used by Copeland's group contains the *mip1*-T661A mutation which has been shown to enhance mtDNA instability and acts synergistically with many *mip1* mutations (Baruffini *et al*, 2007).

Table 3. List of the *mip1* mutations generated as an equivalence of human disease associated mutations.

Human mutation	Yeast mutation	Associated disease	Localization	mtDNA instability [% <i>rho</i>⁻⁰]^a	Point mutations frequency^b
H110Y	H84Y	hypotonia	Periphery of exonuclease domain	10 ^c	0.4 ^c
L244P	L210P	Alpers	Periphery of exonuclease domain	10 ^c	16 ^c
G268A	G224A	PEO; parkinsonism/ neuropathy	Close to 3'-5' exonuclease active site	20 ^d	10 ^d
G303R	G259R	Alpers	DNA binding channel (downstream ExoII site)	100 ^h	nd ^h
L304R	L260R	PEO; ataxia-neuropathy; Alpers	DNA binding channel (downstream ExoII site)	40 ^c / >99 ^e	0.6 ^c / nd ^e
S305R	C261R	Alpers; ataxia-neuropathy	DNA binding channel (downstream ExoII site)	84 ^h	14 ^h
Q308H	Q264H	PEO	DNA binding channel (downstream ExoII site)	100 ^c / 84 ^f	nd ^c / nd ^f
R309L	R265L	PEO	DNA binding channel (downstream ExoII site)	35 ^c	0.2 ^c

R309H	R265H	Alpers	DNA binding channel (downstream ExoII site)	8.5 ^c	0.7 ^c
R386H	I334H	Alpers	Periphery of exonuclease domain	6 ^h	6 ^h
A467T	I416T	PEO; ataxia- neuropathy; Alpers	Subunit interface (thumb)	wt ^c	nd ^c
R574W	R467W	PEO; polyneuropath y; Alpers	DNA binding channel (periphery of linker region)	6.1 ^c /60 ^h	0.6 ^c /23 ^h
P625R	P513R	Alpers	IP subdomain of linker region	4 ^h	2 ^h
R807C	R607C	SANDO	DNA binding channel (thumb)	100 ^c /88 ^f	nd ^c /1 ^f
R807P	R607P	PEO	DNA binding channel (thumb)	67 ^c /100 ^f	13 ^c /nd ^f
G848S	G651S	PEO; Alpers; MELAS	DNA binding channel (palm)	100 ^c /46 ^g	nd ^c /nd ^g
T851A	T654A	Alpers	DNA binding channel (palm)	100 ^c	nd ^c
R852C	R655C	Alpers	DNA binding channel (palm)	10 ^c	0.2 ^c
R853W	R656W	PEO; ptosis	DNA binding channel (palm)	100 ^c	nd ^c
R853Q	R656Q	Alpers	DNA binding channel (palm)	100 ^c	nd ^c
N864S	N667S	PEO	DNA binding channel (palm)	100 ^c	nd ^c
G888S	G691S	Alpers	Close to the polymerase active site (palm)	18 ^c	1.7 ^c
A889T	A692T	PEO;	Close to the	80 ^c	3.7 ^c

		neuropathy	polymerase active site (palm)		
G923D	G725D	PEO	fingers	100 ^c	nd ^e
H932Y	H734Y	PEO; SANDO	fingers	100 ^c /98 ^g	1.3 ^c /nd ^g
T914P	T716P	Alpers; PEO/ myopathy	fingers	nd ^f	nd ^f
D930N	D732N	Alpers	DNA binding channel (fingers)	100 ^h	nd ^h
R943H	R745H	PEO	Polymerase active site (fingers)	>99 ^e	nd ^e
K947R	K749R	PEO; ovarian failure	DNA binding channel (fingers)	21 ^h	3.5 ^h
R953C	R755C	PEO	DNA binding channel (fingers)	9.1 ^c	0.5 ^c
Y955C	Y757C	PEO; Parkinsonism, premature menopause	Polymerase active site (fingers)	>99 ^d	nd ^d
A957S	A759S	PEO	Polymerase active site (fingers)	13 ^c /32.5 ^e	0.2 ^c /nd ^e
A957P	A759P	Alpers	Polymerase active site (fingers)	100 ^c	nd ^c
G1051R	G807R	PEO; SANDO	DNA binding channel (fingers)	5.9 ^c /25 ^g	0.9 ^c /10.2 ^g
P1073L	P829L	Alpers	DNA binding channel (fingers)	70 ^h	23 ^h
G1076V	G832V	PEO	DNA binding channel (fingers)	100 ^c	nd ^c
R1096C	R853C	PEO; Alpers	DNA binding channel (fingers)	100 ^c	nd ^c
R1096H	R853H	Alpers	DNA binding channel (fingers)	100 ^c	nd ^c
S1104C	S861C	PEO	fingers	100 ^c	nd ^c
V1106I	V863I	PEO	fingers	100 ^c	nd ^c
E1143G	E900G	SNP	Periphery of palm	1.3 ^g	1.9 ^g
Y831C	K903C	SNP	Periphery of palm	12 ^f	19 ^f

M1163R	M920R	Alpers	Periphery of palm	8.6 ^c / 100 ^f	0.8 ^c / nd ^f
F1164I	F921I	PEO	Periphery of palm	2.8 ^c / 12 ^f	0.6 ^c / 19 ^f
D1184N	D941N	PEO	Subunit interface (palm)	33 ^c	3.5 ^c
K1191N	K948N	Alpers	DNA binding channel (palm)	11 ^c	1.7 ^c

Table 3. List of the *mipI* mutations generated as an equivalence of human disease associated mutations.

^a % of *rho*⁻⁰ cells in haploid strain; ^b point mutation frequency was determined as a E^R mutant frequency expressed as a fraction of the wild-type frequency arbitrarily set to 1; ^c Stumpf *et al*, 2010; ^d Baruffini *et al*, 2006; ^e Stuart *et al*, 2006; ^f Cochonneau and Foury, unpublished data; ^g Baruffini *et al*, 2007; ^h Baruffini *et al*, 2010

2. AIMS OF THE STUDY

2. Aims of the study

Our goal was to better understand the molecular bases of human pol g disease-associated mutations that are localized in regions of the 3'-5' exonuclease and linker domains that have not been explored so far. We have used *Saccharomyces cerevisiae* as a model system to analyze the *in vivo* and *in vitro* phenotypes of mip1 mutants harboring the yeast counterparts of human L244P, L304R, S305R, R309H(L) and W312R mutations in the exonuclease domain, and the R574W mutation in the linker region.

We have estimated the impact of *mip1* mutations (L240P, L260R, C261R(S,T), R265H(L), F268R, R467W) on global mitochondrial functions by measuring cellular oxygen consumption, and on the stability of the mitochondrial genome by determining the production of *rho*⁻ and *rho*⁰ mutants. The fidelity of mtDNA replication was determined by measuring the frequency of mtDNA mutants that have acquired resistance to erythromycin. Estimation of Mip1 protein expression levels in mitochondrial extracts has allowed us to distinguish mutations that affect primarily pol g function from those that alter the structure and stability of the protein. Using a partially purified Mip1 enzyme we have measured processivity of DNA synthesis, DNA binding activity, and 3'-5' exonuclease proofreading activity, and analyzed the coupling between DNA synthesis and degradation.

This work has led us to identify a novel module of the 3'-5' exonuclease domain that is important for coupling the polymerase and 3'-5' exonuclease functions of the enzyme, and propose a model for the function of this module. Finally, we have analyzed whether there was a good correlation between the severity of the symptoms observed in patients and the phenotypes conferred by *mip1* mutations.

3. RESULTS

Large parts of the Results section have been published:

Szczepanowska K. and Foury F. (2010) A cluster of pathogenic mutations in the 3'–5' exonuclease domain of DNA polymerase gamma defines a novel module coupling DNA synthesis and degradation. *Hum. Mol. Genet.* 19, 3516–3529.

3.1. Selection and description of the disease-associated pol g mutations:

The first group of pol g mutations (Table 4.) is represented by L244P which was identified in two brothers presenting with fatal hepatoenkephalopathy (Simonati *et al*, 2003; Ferrari *et al*, 2005; Spinazzola *et al*, 2009). Leu244 is a conserved pol g residue between the ExoI and ExoII sites in a coil localized at the periphery of the 3'–5' exonuclease domain (Fig.3.1.A. and B.; Fig.3.2.).

The second group comprises four mutations (L304R, S305R, R309H(L) and W312R) that are clustered downstream of the ExoII site (Fig.3.1.A.). These mutations are often associated with PEO, myopathy and a broad spectrum of neuropathies (Table 3). Moreover, the L304R, S305R and R309H substitutions were fatal in several patients as homozygous (L304R), or in association with other mutations in trans, leading to severe encephalopathies or even Alpers syndrome (Table 4.) (Van Goethem *et al*, 2001; Lamantea *et al*, 2002; Agostino *et al*, Del Bo *et al*, 2003; Di Fonzo *et al*, 2003; Van Goethem *et al*, 2003; Horvath *et al*, 2006; Naimi *et al*, 2006; Ashley *et al*, 2008; Wong *et al*, 2008; Blok *et al*, 2009; Baruffini *et al*, 2010). Based on the pol g three dimensional structure this cluster of

mutations is localized in a coil- α -helix segment (Fig.3.1.B. and C.) which is not present in the prokaryotic PolA family but is strongly conserved in all pol γ (Fig.3.2.). This area forms part of the DNA-binding channel. Arg309 is a strictly conserved residue (Fig.3.2.) localized in the α -helix, whereas Ser305 which is localized at the border of the coil and α -helix, is replaced by cysteine in several yeasts including *S. cerevisiae* and threonine in *Drosophila* and *Xenopus*. Both residues point towards the lumen of the DNA-binding channel. In contrast, Leu304, a highly conserved residue localized in the coil, and Trp312, which is localized in the distal part of the helix, do not face the channel (Fig.3.1.C.).

The third group of mutation is represented by R574W which was identified as compound heterozygous in patients showing symptoms of variable severity. PEO, myopathy and dysphagia were observed in an adult patient harboring the W312R mutation in trans and Alpers syndrome was diagnosed in three infants harboring the A467T mutation (Table 4.) (Kollberg *et al*, 2006; Spinazzola *et al*, 2009). Arg574 is a highly conserved residue residing in a region of the intrinsic processivity (IP) subdomain which is shared by Mip1 and points towards the lumen of the DNA-binding channel (Fig.3.1.A. and B.).

This brief overview gives an idea of the extreme complexity and variability of the disease traits, which are the result of the impact of each mutation, alone and in combination with other mutations, as well as of the genetic and environmental parameters specific to each patient. Moreover, studies are complicated by the small number of

patients bearing the same mutations and by the absence of family history for several cases.

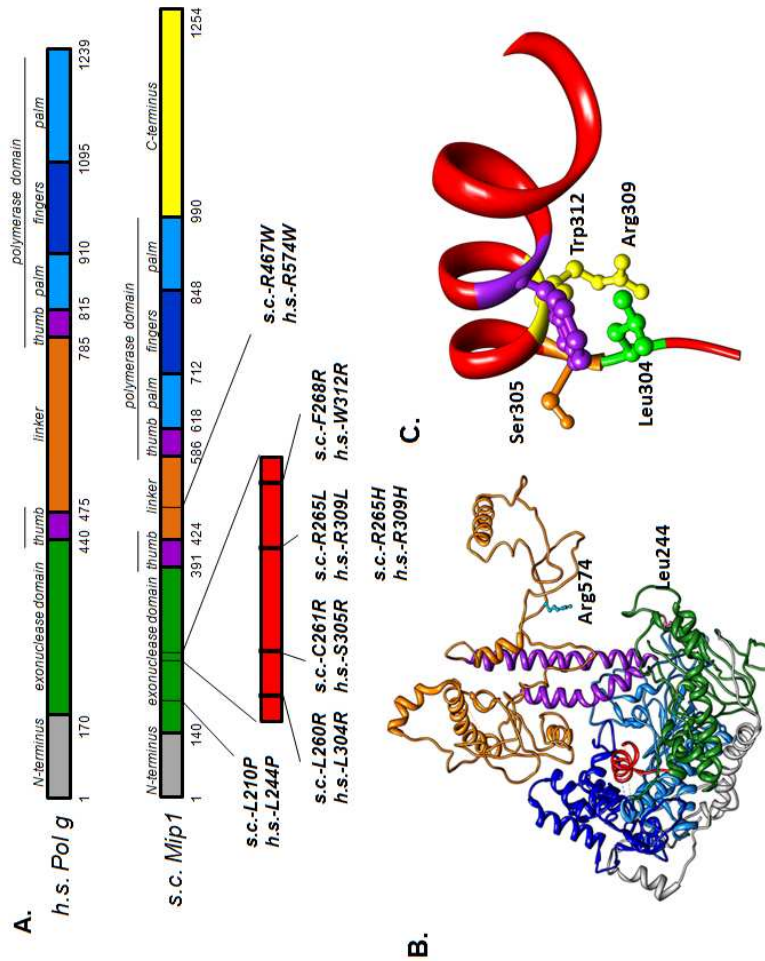


Figure 3.1. Localization of the disease associated pol g mutations and their Mip1 equivalents.

A. Linear representation of the functionally relevant domains of human pol g and Mip1. The N-terminal part including the mitochondrial targeting sequence is in grey, the 3'-5' exonuclease domain is in green, the linker region is in orange, the thumb is in violet, the fingers are in dark blue, the palm is in light blue and the Mip1 C-terminal extension is in light pink. The position of the five mutations clustered downstream the *ExoII* site is shown in the red box. **B. Three-dimensional structure of human pol g** (pdb ID: 31KM). Colours are the same as in (A.). Leu244 and Arg574 are shown in pink and blue, respectively. The coil-helix fragment corresponding to the red box in (A.) is in red. **C. Zoom in on the coil-helix fragment** showing Leu304 (green), Ser305 (orange), Arg309 (yellow) and Trp312 (violet). This figure was prepared by using UCSF Chimera software.

P			
207	P-----AA- L IPLNTLNK-	218	<i>S.cerevisiae</i>
182	T-----NDH L IPLNTFET-	194	<i>P.pastoris</i>
220	-----LSVEK L EPLNTFET-	233	<i>D.melanogaster</i>
208	TWSKELLSD L FPLETSMNC	227	<i>X.laevis</i>
217	SWTSQLSPAD L TPLGGSTSA	236	<i>M.musculus</i>
234	SWTSQLSPAD L IPLLEVPTGA	253	<i>H.sapiens</i>
	* * *		
R H/L R			
254	HIASFGLCSRQ R PM F MKNNK	273	<i>S.cerevisiae</i>
230	HVSVSG M CTRQ R GT W IKHNC	250	<i>P.pastoris</i>
287	HMCVSG V TSYQ R AM L --KSK	304	<i>D.melanogaster</i>
272	HMAISGLTG F Q R TL W MAS-K	290	<i>X.laevis</i>
281	HMAISGLSS F Q R SL W MGA-K	299	<i>M.musculus</i>
298	HMAISGLSS F Q R SL W IAA-K	316	<i>H.sapiens</i>
	* * * *		
W			
457	QKLPGFPEWY R QLFPSKDTV	476	<i>S.cerevisiae</i>
450	QKLPGYPNWY K QLIVKNEL-	468	<i>P.pastoris</i>
509	PLLPGYPLWY R KLCRKPPAK	528	<i>D.melanogaster</i>
536	QHLP ^g HPGWY R KLCPKLE--	553	<i>X.laevis</i>
545	QHLP ^g HPGWY R KLCPRLD--	562	<i>M.musculus</i>
564	QHLP ^g HPGWY R KLCPRLD--	581	<i>H.sapiens</i>
	*** * ** *		

Figure 3.2. Multiple amino acid sequence alignment of pol g fragments harboring pol g mutations.

The residues studied here are in bold letters and the amino acid modifications are shown above the residues of interest. Conserved residues are marked with an asterisk. Alignments were performed with the Pileup software from GCG package and Clustal program.

Table 4. Disease-associated pol g mutations, clinical presentations of the patients and yeast Mip1 equivalents.											
Pat.	Mutation	Yeast equivalent	Associated mutation(s)	Disease group	Disease onset	Age of LI Age of D.	Sex	Family history	Primary clinical features	Other features	References
1.	L244P	L210P	W748S-(E1143G)	Encephalopathy	10 m	15 y†	M	patient 2.	Encephalopathy, liver failure, ataxia, spasticity, myoclonus (NVT)	Epilepsy	Simonati et al, 2003 Ferrari et al, 2005 Spinazzola et al, 2009
2.	L244P	L210P	W748S-(E1143G)	Liver + encephalopathy	5 m	13 m†	M	patient 1.	Alpers syndrome (VT)	Epilepsy	Simonati et al, 2003 Ferrari et al, 2005 Spinazzola et al, 2009
3.	L304R	L260R	T251L-P687L	PEO +/- other features	45 y	74 y	F	patient 4.	PEO, myopathy, ataxia	-	Horvath et al, 2006
4.	L304R	L260R	T251L-P687L	PEO +/- other features	60 y	68 y	M	patient 3.	PEO, neuropathy	-	Horvath et al, 2006
5.	L304R	L260R	homozygous	PEO +/- other features	8 y	15 y†	M	sporadic	PEO, SANDO	-	Naimi et al, 2006
6.	L304R	L260R	W748S-(E1143G)	PEO +/- other features	20 y	27 y	F	sporadic	PEO, SCAE	-	Naimi et al, 2006
7.	L304R	L260R	A467T	PEO +/- other features	18 y	37 y†	M	patient 8. and 9.	PEO, myopathy	Depression, dysaesthesia	Van Goethem et al, 2006
8.	L304R	L260R	A467T	PEO +/- other features	25 y	50 y	F	patient 7. and 9.	PEO, SANDO	Depression, dysaesthesia	Van Goethem et al, 2006
9.	L304R	L260R	A467T	PEO +/- other features	24 y	38 y	F	patient 7. and 8.	PEO, SANDO, myopathy	Depression, dysaesthesia	Van Goethem et al, 2006
10.	L304R	L260R	homozygous	Encephalopathy	10 y	NG	NG	NG	Encephalopathy	-	Ashley et al, 2008
11.	S305R	C261R	ND	Liver + encephalopathy	1 y	NG	M	NG	Alpers syndrome	Development delay, seizures	Wong et al, 2008
12.	S305R	C261R	A467T	Liver + encephalopathy	NG	4 y†	M	sister (Alpers)	Alpers syndrome	-	Blok et al, 2009
13.	S305R	C261R	R627Q	Neuropathy	5 y	25 y	M	sporadic	Ataxia, sensory-axonal peripheral neuropathy	Seizures	Baruffini et al, 2010
14.	S305R	C261R	P1073L	Liver + encephalopathy	9 m	2 y†	F	sporadic*	Alpers syndrome	Hypotonia, seizures, epilepsy	Baruffini et al, 2010
15.	R309L	R265L	T251L	PEO +/- other features	NG	NG	M	patient 16	PEO, myopathy	-	Lamantea et al, 2002
16.	R309L	R265L	T251L	PEO +/- other features	NG	NG†	M	patient 15	PEO, myopathy	-	Lamantea et al, 2002
17.	R309H	R265H	R627Q	Liver + encephalopathy	6 m	16 m†	M	sporadic	Alpers syndrome	-	Horvath et al, 2006
18.	W312R/ R574W	F268R/ R467W	-	PEO +/- other features	37 y	62 y	M	sporadic	PEO, myopathy, dysphagia	-	Horvath et al, 2006
19.	W312R	F268R	dominant	PEO +/- other features	60 y	62 y	F	father (ptosis)	PEO	-	Horvath et al, 2006
20.	W312R	F268R	homozygous	PEO +/- other features	52 y	57 y	M	sporadic	PEO, polyneuropathy, dysphagia	-	Di Fonzo et al, 2003 Del Bo et al, 2003
21.	R574W	R467W	A467T	Liver + encephalopathy	12 m	4 y†	F	patient 22	Alpers-Huttenlocher syndrome (NVT)	-	Kolberg et al, 2006
22.	R574W	R467W	A467T	Liver + encephalopathy	7 m	14 m†	F	patient 21	Alpers-Huttenlocher syndrome (NVT)	-	Kolberg et al, 2006
23.	R574W	R467W	A467T	Liver + encephalopathy	3 y	10 y	F	sporadic	Alpers syndrome	-	Spinazzola et al, 2009

Table 4. Disease-associated pol g mutations, clinical presentations of the patients and yeast Mip1 equivalents. Legend: L.I. – last investigation; D. – death; NG - data not given; F – female; M – male; † - age of death; m – months; y – years; * - mutation identified in other members of family giving no disease phenotype; VT – sodium valproate treatment; NVT – no sodium valproate treatment; PEO – progressive external ophtalmoplegia; SANDO – sensory ataxic neuropathy, dysarthria and ophthalmoparesis; SCAE – spino-cerebral ataxia-epilepsy syndrome

3.2. Construction of the *mip1* mutants:

The L210P, L260R, C261R, R265H(L), F268R and R467W *mip1* mutations equivalent to the human L244P, L304R, S305R, R309H(L), W312R and R574W *POLG1* mutations were obtained by site-directed mutagenesis of the *MIP1*[Σ] allele (Baruffini *et al*, 2007) harbored by a low copy centromeric plasmid (pFL39*MIP1* or pFL38*MIP1*). Since the Mip1 equivalent residue of Ser305 is Cys261, *mip1*-C261S as well as *mip1*-C261T were also constructed for comparison. The plasmid-borne *mip1* alleles were cloned into the haploid W303-1B Δ *mip1 rho*⁰ strain (Foury and Vanderstraeten, 1992) and mtDNA was introduced into *rho*⁰ cells by the cytoduction technique (Baruffini *et al*, 2010).

3.3. Cellular respiration and stability of the mitochondrial genome:

3.3.1. Cellular respiration:

Defects in either polymerase or exonuclease proofreading functions can impair mitochondrial biogenesis, leading to production of poorly expressed or functionally defective subunits in the respiratory complexes. Thus, the rate of cellular oxygen consumption is an indicator of the impact of pol g mutations on the mitochondrial function and reflects the state of the mitochondrial genome. All mutants showed, to various extents, impaired respiratory function compared to the wild type ($8.5 \pm 2.1 \mu\text{l O}_2/\text{h/mg}$) (Fig.3.3.). The *mip1*-L260R, *mip1*-R265H and *mip1*-F268R mutations conferred low

respiration levels ($1.1 \mu\text{l O}_2/\text{h}/\text{mg}$; $2.2 \pm 0.2 \mu\text{l O}_2/\text{h}/\text{mg}$; $2.3 \pm 0.2 \mu\text{l O}_2/\text{h}/\text{mg}$; respectively), whereas the *mip1*-R265L and *mip1*-R467W mutations had only a slight effect (7.5 ± 1.1 and 6.5 ± 0.5 , respectively). Cellular respiration of *mip1*-L210P, *mip1*-C261R, *mip1*-C261S, *mip1*-C261T and *mip1*-F268R/*mip1*-R467W mutants was not determined.

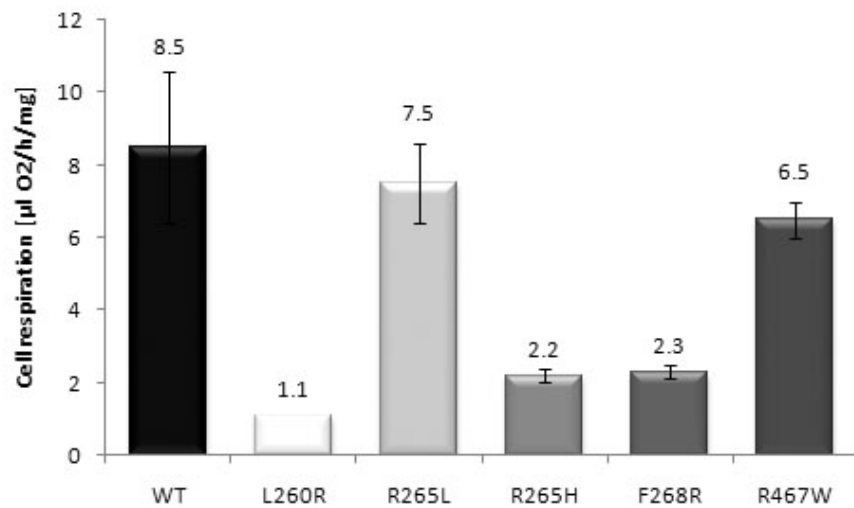


Figure 3.3. Determination of cellular respiration in mutant and wild-type strains. The uptake of dioxygen was measured on cell suspensions with a Clark electrode. Cellular respiration was estimated as a quantity of oxygen consumed per mg of yeast cells during one hour ($\mu\text{l O}_2/\text{h}/\text{mg}$ cells).

3.3.2. Stability of the mitochondrial genome:

In *Saccharomyces cerevisiae* mtDNA replication defects result in mtDNA instability characterized by accumulation of respiratory-deficient cells that either harbor large mtDNA deletions (*rho*⁻) or have lost mtDNA (*rho*⁰). Background levels of *rho*^{-/0} mutant frequency for

wild-type *Mip1* were < 1 % at physiological temperature and < 1.5 % at 37°C.

The *mip1*-L260R, *mip1*-C261R, *mip1*-R265H, and *mip1*-F268R mutants grown at 28°C produced $\rho^{-/0}$ (ρ^{-} + ρ^0) mutants at high frequency and the majority of the respiratory-deficient clones were ρ^0 (Fig.3.4. and Fig.3.5.). Only 20-25% of the colonies were $\rho^{-/0}$ in the *mip1*-R265L and *mip1*-R467W mutants (Fig.3.4.). In these six mutants the percentage of $\rho^{-/0}$ was not increased when cells were grown at 37°C (Fig.3.4.).

In contrast, whereas the percentage of $\rho^{-/0}$ mutants produced by the *mip1*-L210P mutant at 28°C was below 10% (Fig.3.4.), the cell population was almost completely converted to $\rho^{-/0}$ mutants (>99%) at 37°C (Fig.3.4.). Moreover, 4'-6-diamino-2-phenylidole (DAPI) staining showed that while most *mip1*-L210P cells grown at 28°C contained mtDNA, cells grown at 37°C were completely (or almost completely) devoid of mtDNA (Fig.3.6.). These data show that in the *mip1*-L210P mutant mtDNA replication defects are temperature-sensitive.

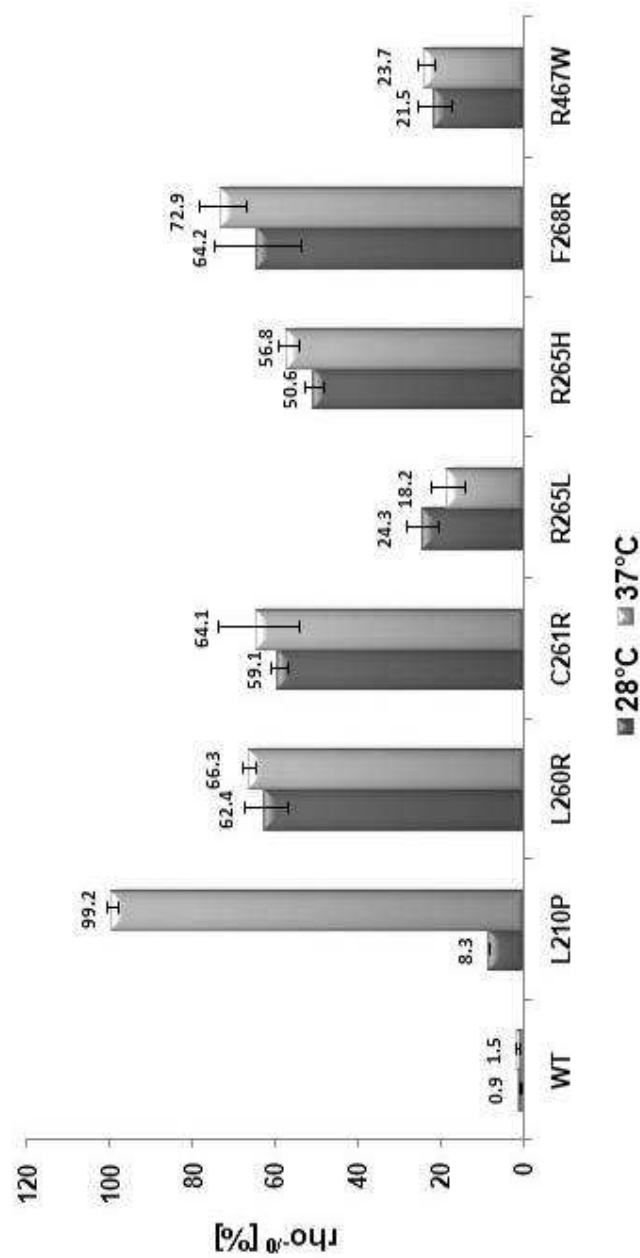


Figure 3.4. Determination of mtDNA stability of wild-type and mutant strains at 28°C and 37°C.

The stability of mtDNA was determined as the percentage of respiratory deficient ($\rho^{-/0}$) colonies on the Petri dishes. The experiment was carried out on three independent clones of each strain with several repetitions and at least 20 000 colonies from each mutant were examined. Dark grey blocks represents results obtained at 28°C. Light gray blocks represents results obtained at 37°C.

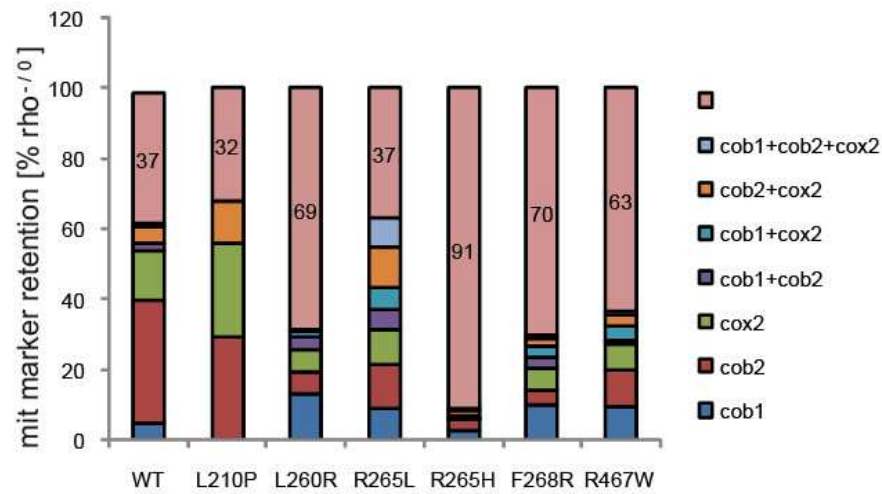


Figure 3.5. Retention of mtDNA fragments in $\rho^{-/0}$ clones.

The test which is based on the high efficiency of homologous mtDNA recombination in diploids exploits the capacity of ρ^{-} genomes to restore *mit*⁻ mutations to wild-type in crosses between strains of opposite mating type. The three *mit*⁻ markers tested (*cob1*, *cob2* and *cox2*) correspond to the most frequently retained mtDNA fragments in ρ^{-} strains, and thus the clones that did not retain any of these markers have little chance to contain another mtDNA fragment and are most likely ρ^0 clones (Baruffini et al, 2010). At least 250 $\rho^{-/0}$ colonies from each mutant and wild-type strain (from three independent transformants were examined).

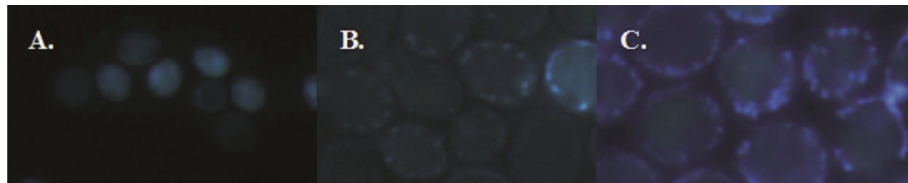


Figure 3.6. DAPI staining of wild-type and *mip1*-L210P cells.

Cell suspensions incubated with DAPI were observed under an optic microscope as reported (Baruffini et al, 2010). Under our conditions, mtDNA was preferentially stained by DAPI and appeared as spots in wild type (C) and L210P (B) strains grown at 28°C, whereas only the nucleus was stained in *mip1*-L210P grown at 37°C (A).

3.4. Frequency of mtDNA point mutations

Accumulation of point mutations reflects the fidelity of mtDNA replication. The mutator properties of mtDNA were estimated by the

frequency of erythromycin resistant (E^R) mutants. It has previously been shown that E^R mutants are only produced by mutations in the mitochondrial 21S rRNA gene. All types of substitutions can affect any of the three adjacent GAA nucleotides at positions 1951-1953 of the gene (Vanderstraeten *et al*, 1998).

In all mutants the frequency of E^R mutations was increased (Fig.3.7., Fig.3.8.), from 4.5-fold in *mip1*-R265L to 18-fold in *mip1*-R467W, and more than 45-fold in *mip1*-L260R, *mip1*-C261R, *mip1*-R265H, *mip1*-F268R and *mip1*-L210P mutants (51, 45-, 58.5-, 85- and 83.7-fold increase, respectively). Furthermore, the actual accumulation of point mutations in mtDNA was probably underestimated due to the high percentage of $\rho\text{-}^{0}$ mutants coexisting with $\rho\text{-}^{+}$ cells in the cultures (Fig.3.9.).

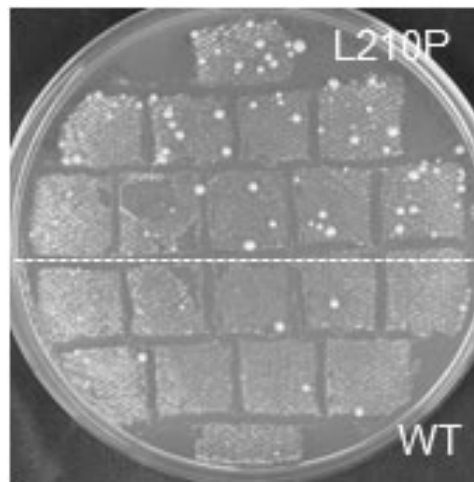


Figure 3.7. Illustration of the mutator properties of *mip1* mutants in a qualitative test.

To get a rough estimation of the frequency of E^R point mutations in the mtDNA of *mip1* mutants, 10 independent colonies of wild-type (lower panel) and mutant (upper panel) strains were spread on standardized squares in Petri dishes containing synthetic glucose medium and after 2 days of growth they were successively replica-plated on rich glycerol and glucose containing media and ERY medium. The pictures were done after 8 days of incubation at 28°C. Image illustrates mutator properties of *mip1*-L210P as an example.

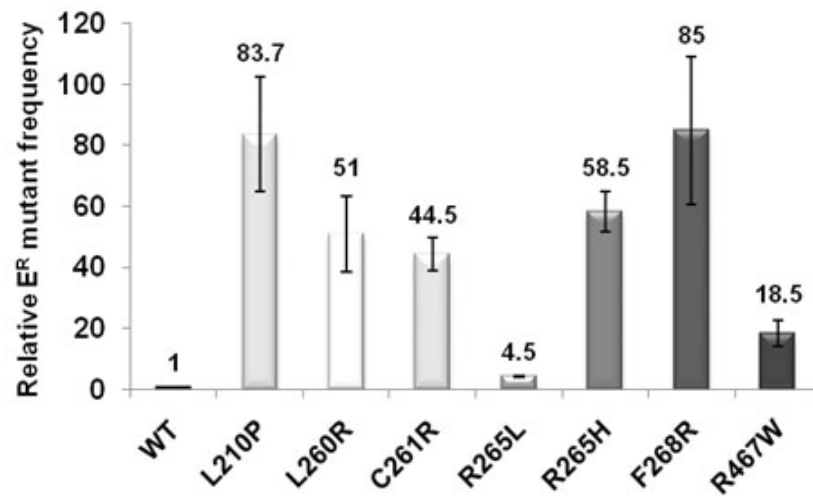


Figure 3.8. Determination of the frequency of point mutation in mtDNA.

The mutator activity of *mip1* mutants was determined as the average number of E^R mutants in a defined volume of liquid cultures. E^R mutant frequency was expressed as a fraction of the wild-type frequency arbitrarily set to 1.

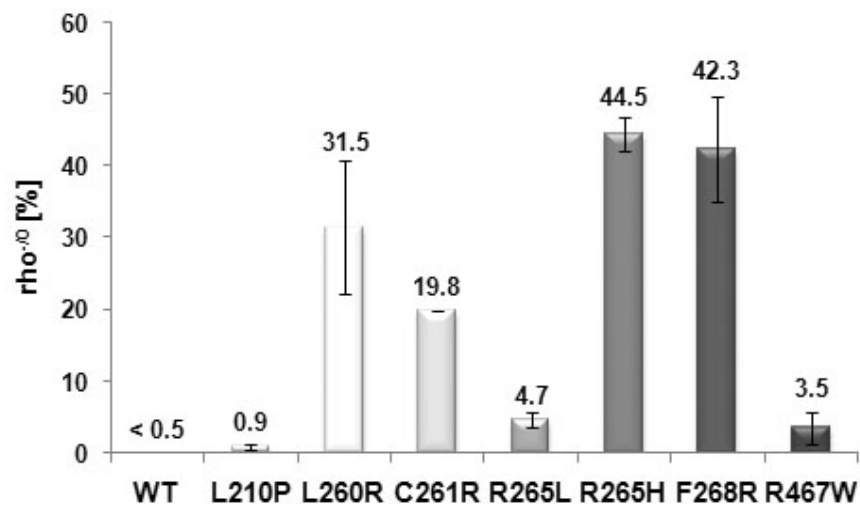


Figure 3.9. Determination of the frequency of ρ^{-0} mutants present in the cultures spread on erythromycin-containing plates in Fig.3.8.

ρ^{-0} frequency was estimated as the percentage of respiratory deficient colonies in a defined volume of liquid cultures.

3.5. In vivo phenotype of the heteroallelic F268R/R467W *mip1* mutant.

The heteroallelic F268R/R467W *mip1* mutant, equivalent to the W312R/R574W human combination (Horvath *et al*, 2006), was also analyzed. Haploid heteroallelic strains carrying a combination of two *MIP1* (or *mip1*) alleles were obtained by transformation with two centromeric plasmids, each harboring one *MIP1* (or *mip1*) allele and a different auxotrophic marker (*TRP1* or *URA3*). Frequencies of $\rho\text{ho}^{-/0}$ and E^{R} mutants were quite similar in *MIP1/MIP1*, *MIP1/mip1*-F268R and *MIP1/mip1*-R467W strains suggesting that F268R and R467W alleles are recessive (Fig.3.10. and 3.11.). Interestingly, the percentage of $\rho\text{ho}^{-/0}$ clones as well as the frequency E^{R} mutants from the heteroallelic strain *mip1*-F268R/*mip1*-R467W were similar to those of the singly substituted *mip1*-R467W mutant (Fig.3.10. and 3.11.), indicating that the deleterious phenotype conferred by the F268R mutation was attenuated in the presence of the R467W mutation.

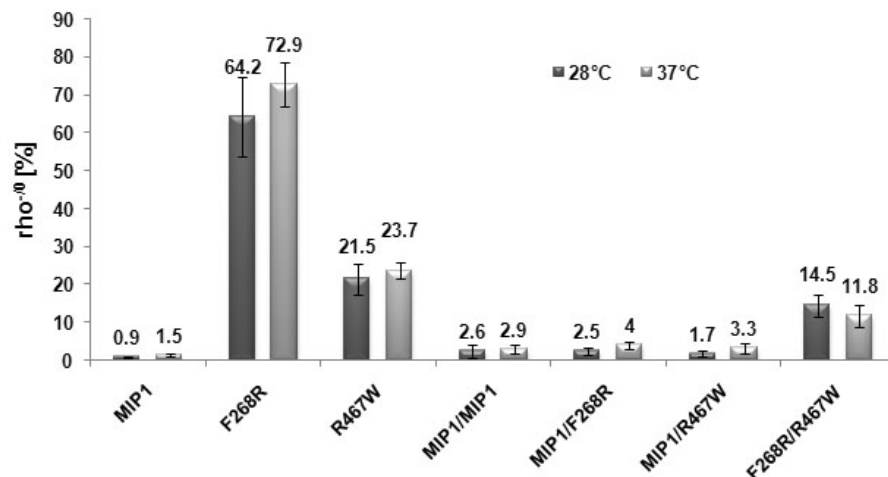


Figure 3.10. Determination of mtDNA stability of wild-type (*MIP1* and *MIP1/MIP1*) and mutant (*mip1*-F268R, *mip1*-R467W, *MIP1/mip1*-F268R, *MIP1/mip1*-R467W, *mip1*-F268R/*mip1*-R467W) strains at 28°C and 37°C.

The stability of mtDNA was determined as the percentage of respiratory deficient (*rho*⁻⁰) colonies on the Petri dishes. The experiment was carried out on three independent clones from each strain with several repetitions and at least 20 000 colonies from each mutant were examined.

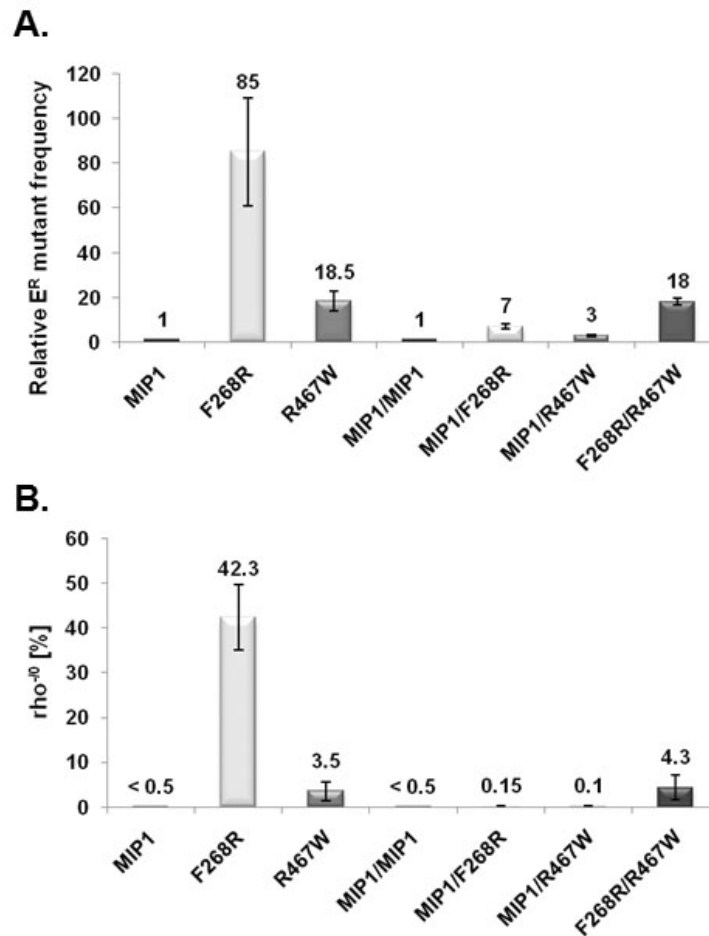


Figure 3.11. Determination of point mutation frequency in mtDNA from wild-type (*MIP1* and *MIP1/MIP1*) and mutant (*mip1*-F268R, *mip1*-R467W, *MIP1/mip1*-F268R, *MIP1/mip1*-R467W, *mip1*-F268R/*mip1*-R467W) strains. A. The mutator activity of *mip1* mutants was determined as the average number of E^R mutants in a defined volume of liquid cultures. E^R mutant frequency was expressed as a fraction of the wild-type frequency arbitrarily set to 1. **B.** Determination of the *rho*⁻⁰ mutant frequency in wild-type and *mip1* mutant liquid cultures spread on erythromycin- containing plates as reported in panel A. *Rho*⁻⁰ frequency was estimated as the percentage of respiratory deficient colonies in a defined volume of liquid cultures.

3.6. In vivo phenotype of the C261R, C261S and C261T mip1 mutants.

The experiments reported below were performed by Frédéric Toussaint under my supervision for his Master thesis (Toussaint, 2010).

Serine 305 in human pol g is substituted by a cysteine at position 261 in yeast Mip1 and a threonine in pol g from fruit fly and frog (Fig.3.2.). Therefore, in addition to the C261R mutation, equivalent to the human pathogenic S305R substitution, the C261S and C261T mutations were also generated in yeast.

In agreement with the low mtDNA stability observed for other mutants clustered downstream of the ExoII site (see section 3.3.2. and 3.4.), the *mip1*-C261R mutant grown at 28°C produced *rho*⁻⁰ mutants at high frequency (59.1 ± 2.2 %) and exhibited high point mutation frequency (45-fold increased compare to the wild-type) (Fig.3.12. and Fig.3.13.). Moreover, the actual accumulation of point mutations in mtDNA was probably underestimated due to the high percentage of *rho*⁻⁰ mutants coexisting with *rho*⁺ cells in the cultures (Fig.3.13.). In contrast, in *mip1*-C261S and *mip1*-C261T strains, no significant differences in mtDNA stability, neither at 28°C nor at 37°C, (Fig.3.12.) were observed by comparison to the wild-type. However, an 18-fold increase in mtDNA point mutation frequency was observed in the *mip1*-C261T mutant (Fig.3.13.).

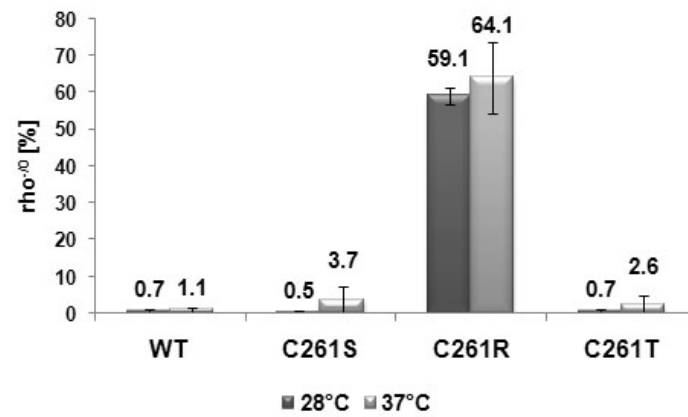


Figure 3.12. Determination of mtDNA stability in wild-type and *mip1*-C261S, *mip1*-C261R and *mip1*-C261T mutant strains at 28°C and 37°C.

The stability of mtDNA was determined as the percentage of respiratory deficient (*rho*⁻⁰) colonies on the Petri dishes. The experiment was carried out on three independent clones from each strain.

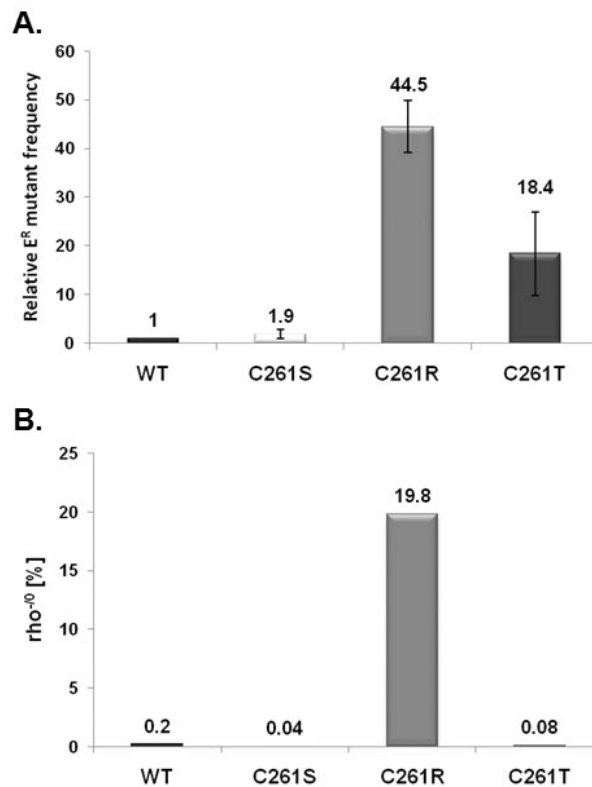


Figure 3.13. Determination of point mutation frequency in mtDNA from wild-type and *mip1*-C261S, *mip1*-C261R, *mip1*-C261T mutants.

A. The mutator activity of *mip1* mutants was determined as the average number of E^R mutants in a defined volume of liquid cultures. E^R mutant frequency was expressed as a fraction of the wild-type frequency arbitrarily set to 1. **B.** Determination of the percentage of *rho*⁻⁰ mutants present in wild-type and *mip1* mutant liquid cultures spread on erythromycin-containing plates as reported in panel A. *Rho*⁻⁰ frequency was estimated as the percentage of respiratory deficient colonies in a defined volume of liquid cultures.

3.7. Expression of wild-type and mutant Mip1.

3.7.1. Wild-type and mutant Mip1 protein expression in mitochondrial extracts.

The biochemical properties of wild-type and mutant Mip1 proteins were determined in a strain that was devoid of the potent mitochondrial Nuc1 nuclease (Zassenhaus, 1988; Lahaye *et al*, 1991) and harbored the *MIP1* (*mip1*) gene under the control of the strong inducible *GALI* promoter on a high-copy number plasmid (Foury and Vanderstraeten, 1992). Mip1 levels were estimated in soluble mitochondrial extracts from cells grown at 28°C by Western blotting using a polyclonal Mip1-antibody. With the exception of Mip1-L210P, the expression levels of wild-type and mutant Mip1 were rather similar (Fig.3.14.A. and C.), suggesting that polymerases are stable and properly folded. In contrast, Mip1-L210P was almost undetectable in the extracts (Fig.3.14.B.).

Since in *mip1*-L210P cells grown at 28°C mtDNA is stable (8% *rho*⁻⁰ only) it must be admitted that the amount of Mip1-L210P protein is sufficient in mitochondria to replicate mtDNA. A likely cause to the loss of Mip1-L210P in the mitochondrial extracts is the high

temperature (37°C) required for cell wall digestion during spheroplast preparation. Indeed, as suggested by the temperature-sensitive phenotype of the *mip1*-L210P mutant, the folding defect of the polymerase is certainly enhanced at this temperature and leads to degradation of the protein by the mitochondrial quality control machinery (Tatsuta *et al*, 2009; Voos *et al*, 2009).

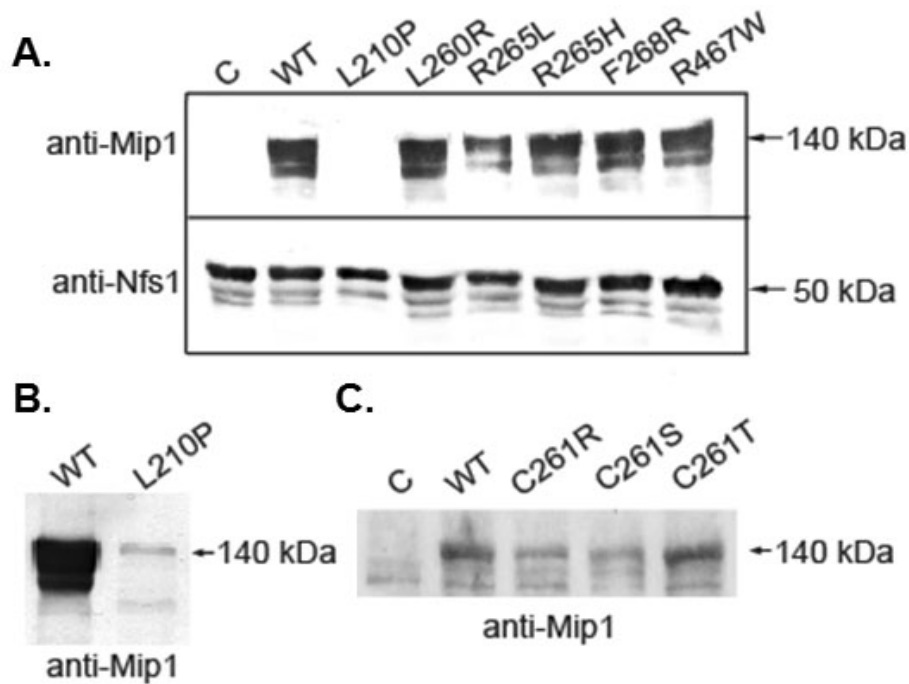


Figure 3.14. Wild-type and mutant Mip1 protein expression.

A. Mip1 levels in mitochondrial extracts (120 µg protein in each lane). After 7% SDS-PAGE Mip1 and Nfs1 proteins were detected by western blot analysis using polyclonal anti-Mip1 (1:750) and anti-Nfs1 (1:1000) antibodies. The mitochondrial Nfs1 protein was used as a loading control. Three Mip1 bands were observed as a result of C-terminal proteolysis. **B.** Detection of Mip1-L210P after immunoblot overexposure. **C.** Mip1, Mip1-C261R, Mip1-C261S and Mip1-C261T levels in mitochondrial extracts. 15 µl of each mitochondrial extract was loaded on SDS-PAGE. Protein concentration, from left: negative control, 3.9, mg/ml; Mip1, 2.6 mg/ml; Mip1-C261R, 3.3 mg/ml; Mip1-C261S, 3.4 mg/ml; Mip1-C261T, 3.5 mg/ml. After 7% SDS-PAGE Mip1 was detected by western blot analysis using a polyclonal anti-Mip1 (1:500) antibody. Fig.3.14.C. was obtained by Toussaint, 2010. The western blot should be repeated to determine whether expression levels are significantly different. The arrows indicate the Mip1 protein.

3.7.2. Wild-type and mutant Mip1 protein purification and quantification.

Nucleic acids and many contaminating proteins were eliminated from the Mip1-containing fraction by DEAE chromatography (Fig.3.15.). The purification was not pursued further to avoid potential inactivation of the mutant enzymes. As previously shown in studies of proofreading deficient mutants (Foury and Vanderstraeten, 1992), the purity of the MIP1 preparation after the DEAE step and the presence of only negligible contaminating nuclease activities allow a reliable of its biochemical properties (Fig.3.16.).

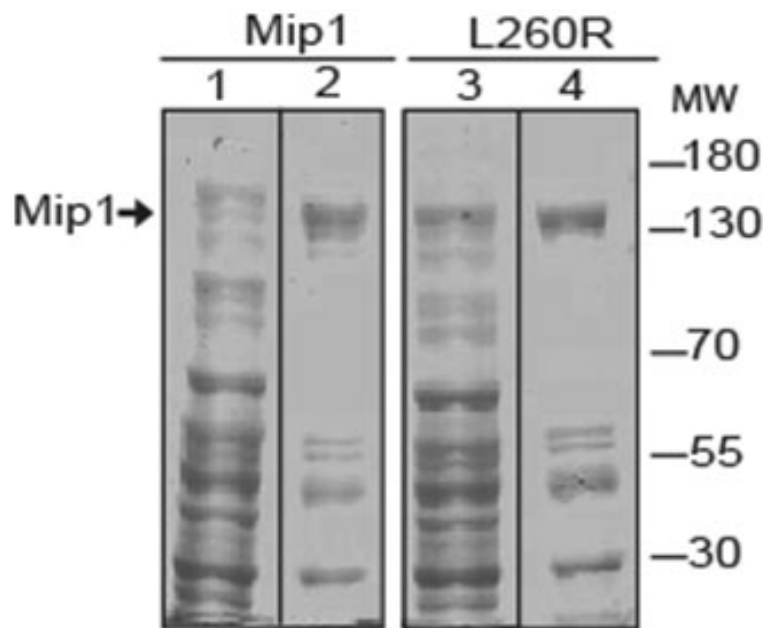


Figure 3.15. DEAE chromatography-purified fractions of wild-type and Mip1-L260R. After 7% SDS-PAGE proteins were detected by Coomassie blue staining of the gel. Mitochondrial extracts were loaded in lanes 1 (wild-type, 105µg protein) and 3 (L260R, 108 µg protein). DEAE-purified fractions were 2-fold concentrated by chloroform-methanol precipitation and loaded in lanes 2 (wild-type, 8.7 µg protein) and 4 (L260R, 7.8 µg protein). The arrow indicates the Mip1 protein.

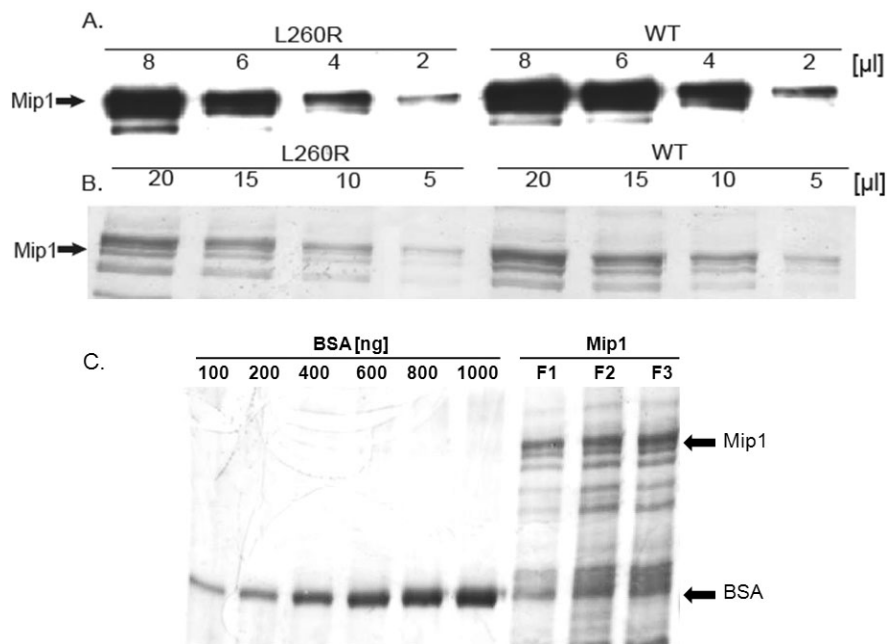


Figure 3.16. Quantification of wild-type and mutant Mip1.

A. and **B.** Estimation of the relative amounts of wild-type and mutant Mip1 in DEAE chromatography-purified fractions. Serial dilution of proteins were prepared and subjected to 7% SDS-PAGE. The numbers above the lanes correspond to the volume (μl) loaded on the gel. Mip1 bands were detected by western blotting (**A**) and colloidal Coomassie blue staining (**B**). **C.** Estimation of Mip1 concentration in DEAE chromatography-purified fractions. Equal volumes from 3 different Mip1 preparations (lanes F1, F2, F3) were loaded on the gel. The concentration (ng/μl) of wild-type Mip1 was estimated after 7% SDS-PAGE and colloidal Coomassie blue staining of the gel using known amounts of bovine serum albumin (BSA, Pierce) as reference. Quantifications were performed using the Kodak Image Station 4000 R software.

3.8. Gap-filling activity and processive DNA synthesis.

3.8.1. Gap-filling activity.

Non-processive gap-filling activity measures the capacity of a polymerase to fill in short single-stranded DNA (ssDNA) gaps in a double-stranded DNA (Fig.3.17.B.) (Wernette and Kaguni, 1986; Wernette *et al*, 1988; Longley *et al*, 1998 ; Wang and Kaguni, 1999;

Farr *et al*, 1999; Luoma *et al*, 2005). A decrease in this robust activity often indicates a catalytic defect or low affinity for dNTP or DNA. However, an excess exonuclease activity may also lead to decreased gap-filling activity. Gap-filling activity was significantly affected in the three mutants that had the highest mtDNA instability *in vivo*, with only 35% of the wild-type activity for Mip1-L260R, and 63% and 70% of the wild-type activity for Mip1-R265H and Mip1-F268R, respectively (Fig.3.17.). Activity was similar to wild-type for Mip1-R265L and Mip1-R467W (Fig.3.17.).

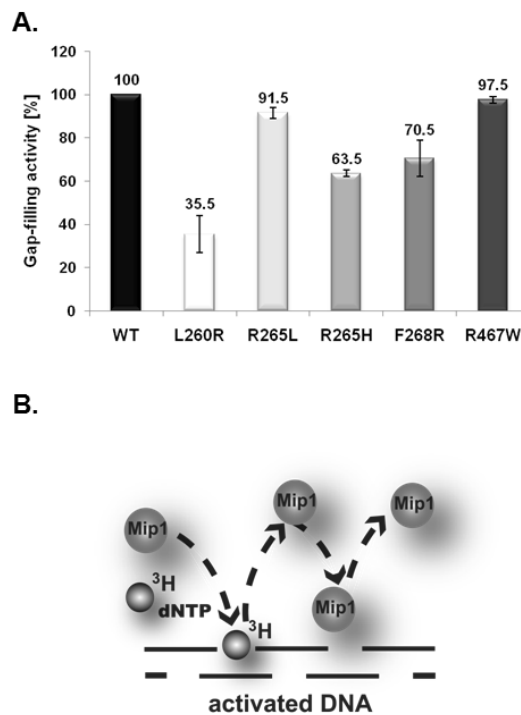


Figure 3.17. Gap-filling activity.

A. Assays were performed at 28°C on DEAE chromatography-purified fractions using the same amounts of wild-type and mutant Mip1. For each strain, gap-filling activity was expressed as the average percentage of the wild-type activity (100%) estimated from three independent experiments. **B.** Schematic presentation of the substrate used in the assay. Non-processive gap-filling activity measures the capacity of a polymerase to fill in short single-stranded DNA (ssDNA) gaps in a double-stranded DNA.

3.8.2. Processive DNA synthesis.

Processive DNA synthesis represents the capacity of a polymerase to elongate a nascent chain to a full-length product without dissociation from the DNA substrate. Using an excess of single-stranded DNA template paired with an oligonucleotide primer as substrate, we carried out two types of assay to test the processivity of the mutant Mip1 preparations.

Total DNA synthesis:

The amount of dNTPs incorporated into the nascent DNA chain (total DNA synthesis) was measured by using an unlabeled primer and [α - ^{32}P] dATP in the assay (Fig.3.18.C.). Total DNA synthesis was decreased to less than 5% of the wild-type level for Mip1-L260R, Mip1-R265H and Mip1-F268R mutants, while it reached 35% and 59% of the wild-type level for Mip1-R265L and Mip1-R467W, respectively (Fig.3.18.).

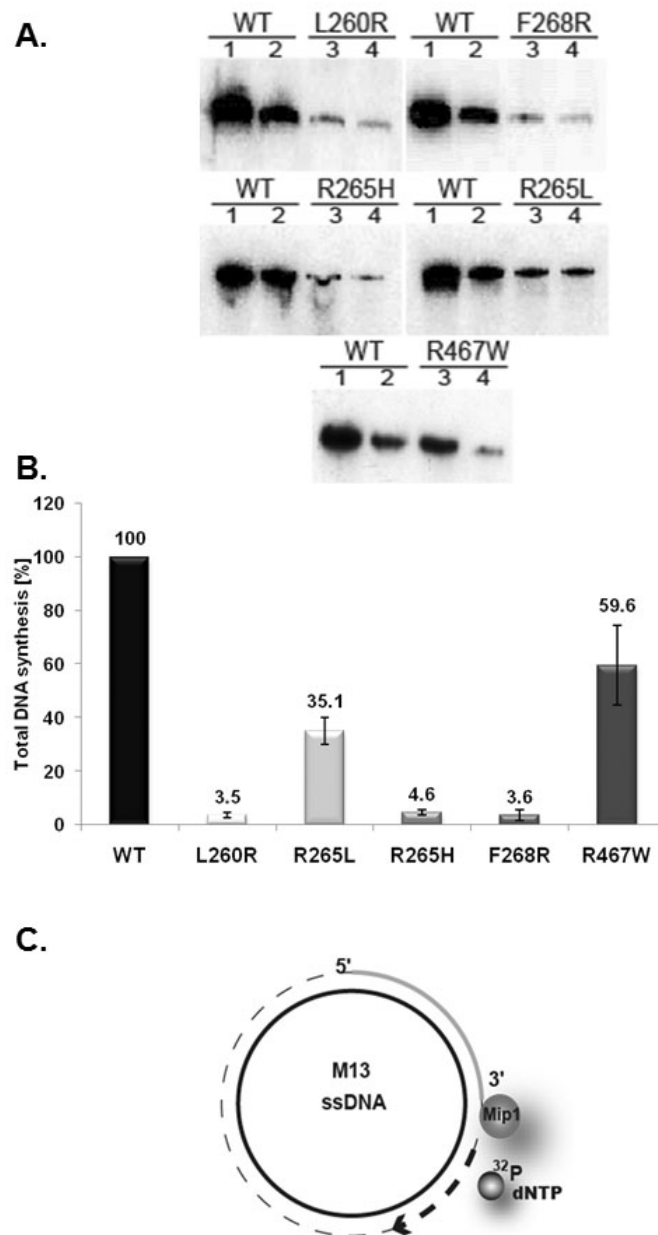


Figure 3.18. Total DNA synthesis.

A. Assays were performed at 28°C on DEAE chromatography-purified fractions using same amounts of wild-type and mutant Mip1 (2.5 ng in lanes 1 and 3 and 1.25 ng in lanes 2 and 4). Assays were carried out for 2 minutes. **B.** For each strain, total DNA synthesis activity was the average of six independent experiments and expressed as a percentage of the wild-type Mip1 value (100%). **C.** Schematic presentation of the substrate used in the assay. The amount of dNTPs incorporated into the nascent DNA chain (total DNA synthesis) was measured by using an unlabeled primer and [α - ^{32}P] dATP in the assay

Processivity of DNA synthesis:

The fraction of full-length DNA molecules was determined by using a 5'-end [^{32}P]-labeled oligonucleotide primer (Fig.3.19.C.). Consistent with the observed defects in total DNA synthesis, full-length products could barely be detected for Mip1-L260R, Mip1-C261R, Mip1-R265H and Mip1-F268R (Fig.3.19.). These mutant Mip1 enzymes were not processive, suggesting frequent arrests of the polymerase *in vivo* followed by dissociation of the enzyme from the mtDNA. This defect was less pronounced for Mip1-R265L and Mip1-R467W (Fig.3.19.).

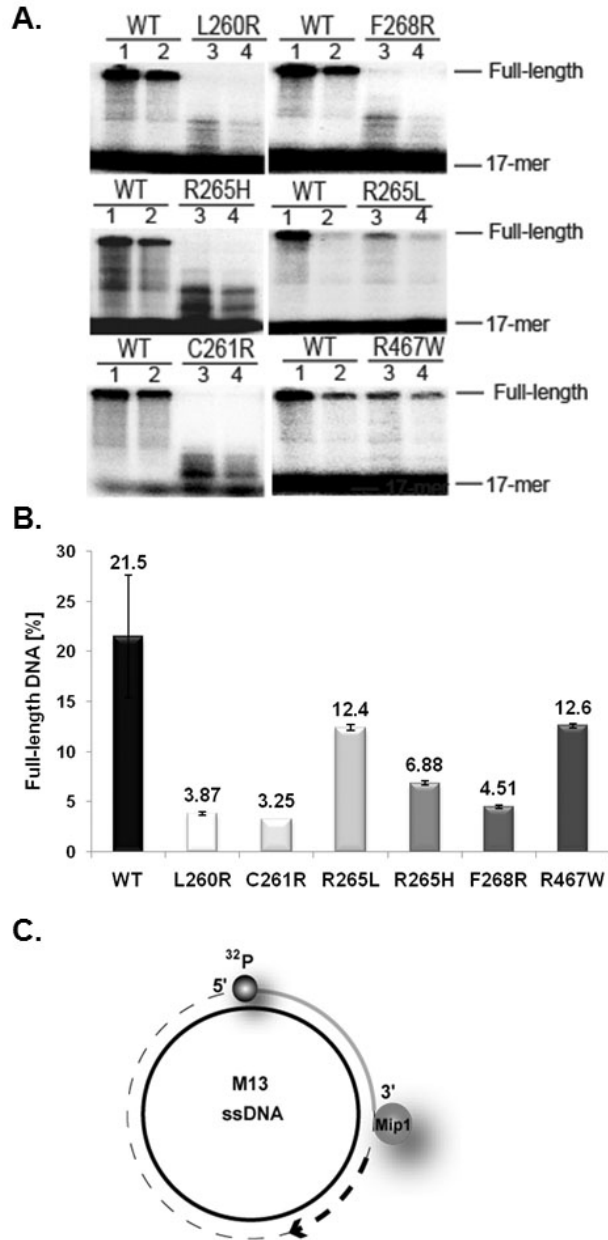


Figure 3.19. Processivity of wild-type and mutant Mip1.

A. Assays were performed at 28°C on DEAE chromatography-purified fractions using same amounts of wild-type and mutant Mip1 (2.5 ng in lanes 1 and 3 and 1.25 ng in lanes 2 and 4). Assays were carried out for 5 minutes. **B.** For each strain processivity was expressed as the ratio of the intensity of the signal give by full-length DNA to the intensity of the signal given by all DNA primers that have been initiated (100%). **C.** Schematic presentation of the substrate used in the assay. The fraction of full-length DNA molecules was determined by using a 5'-end [^{32}P]-labeled oligonucleotide primer.

3.9. DNA binding activity.

Based on the crystal structure of human pol γ , the mutated residues were all predicted to be in the vicinity of the DNA-binding channel (Lee *et al*, 2009). Therefore, an electrophoretic mobility shift assay (EMSA) was used to analyze the DNA binding activity of wild-type and mutant Mip1 polymerases (Fig.3.20.C.).

First, we performed kinetics of the DNA binding activity of wild-type Mip1 on a ^{32}P -labelled 21/45-mer primer-template substrate in the absence and in the presence of 5 mM MgCl_2 (Fig.3.20.A.). In addition to the free DNA probe, for retarded bands were observed by EMSA. The highly-retarded band corresponds to the Mip1-DNA complex (Foury, unpublished data) while the three other bands (U1-U3) correspond to complexes with unknown factors co-purifying with Mip1 in DEAE chromatography. Mip1 DNA binding activity was remarkably increased in the presence of magnesium (Fig.3.20.A.) suggesting that Mip1 adopts a more biologically relevant conformation in the presence of this cation. We decided to use 3 mM MgCl_2 and an incubation time of 3 minutes to avoid as much as possible degradation of the substrate by the 3'-5' exonuclease activity and also because these conditions decreased contaminant DNA binding activities. Under these conditions the DNA binding activity of wild-type Mip1 was ~3-4 fold stimulated by MgCl_2 (Fig.3.20.B.).

DNA binding activities measured in the presence of increasing Mip1 concentrations and 3 mM MgCl_2 were strongly impaired for Mip1-L260R, Mip1-R265H and Mip1-F268R and partially decreased for Mip1-R265L and Mip1-R467W (Fig.3.21.). The DNA binding activities of wild-type and mutant Mip1 were also compared in the

absence and presence of magnesium (Fig.3.22.). Compared to wild-type the DNA binding activity of Mip1-R265L was not deeply modified in the absence of MgCl_2 , whereas that of L260R, R265H, F268R and R467W was only ~2 fold decreased. Remarkably however, the DNA binding activity of the mutants clustered downstream of the ExoII site was not stimulated by magnesium. In the presence of MgCl_2 this activity was 3 fold lower in Mip1-R265L and ~10 fold lower in the other mutants. The DNA binding activity of the linker region Mip1-R467W polymerase in the presence of MgCl_2 was ~4 times lower than in wild-type (Fig.3.22.).

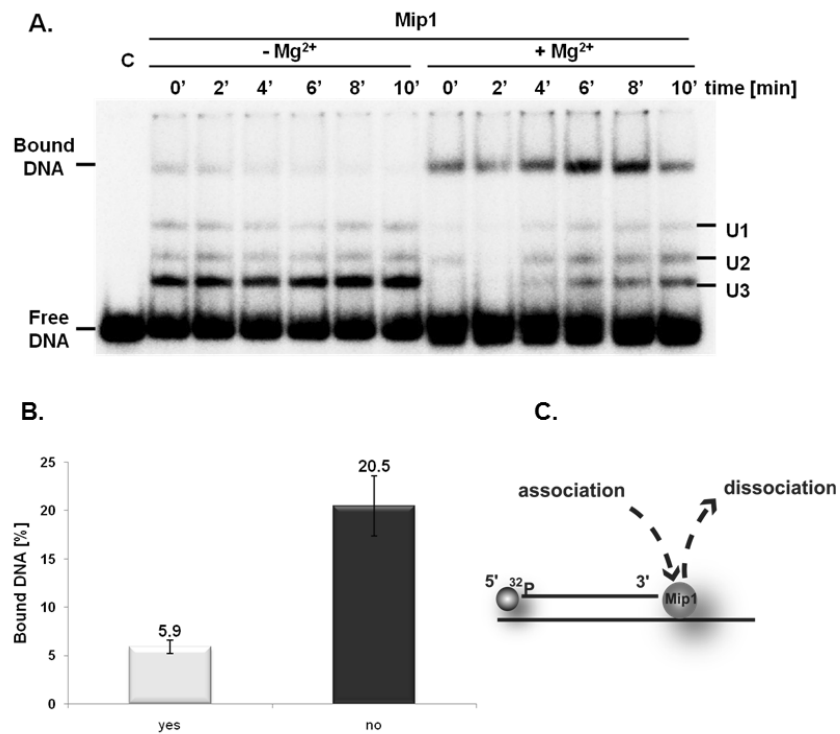


Figure 3.20. Kinetics of DNA binding activity of wild-type Mip1 in the absence and presence of 5 mM MgCl₂.

Assays were performed at 28°C in the absence or presence of 5 mM MgCl₂ using ~6 ng of wild-type Mip1 from DEAE chromatography-purified fractions. DNA binding was measured after 0, 2, 4, 6, 8 and 10 minutes of incubation followed by sample transfer to ice. **A.** EMSA image. The first lane on the left (C) represents the DNA substrate without protein. Bound DNA corresponds to Mip1-DNA complex. Lanes U1, U2 and U3 represent DNA substrate bound to unknown factors co-purifying with Mip1 in DEAE chromatography. **B.** Quantification of DNA bound to wild-type Mip1. Incubation time was 2-3 minutes and MgCl₂ concentration was 3 mM. The percentage of bound DNA (bound/bound + free) is the average of 50 independent assays. **C.** Schematic presentation of the substrate used in the assay. The DNA binding activity was determined on a ³²P-labelled 21/45-mer primer-template substrate.

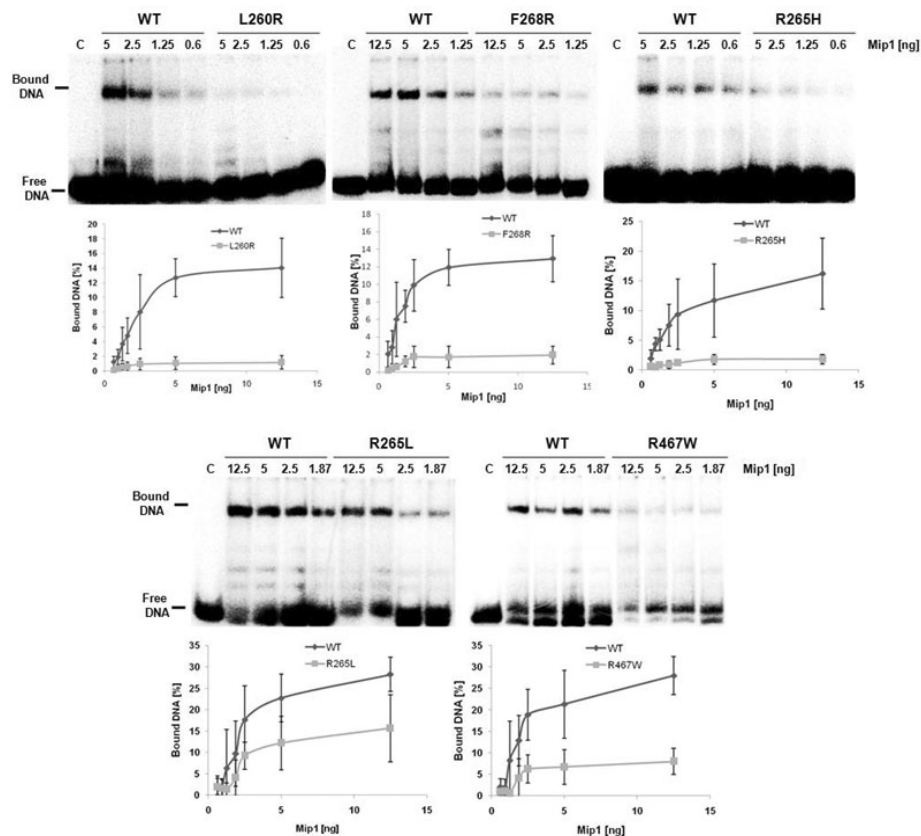


Figure 3.21. DNA binding activity of wild-type and mutant Mip1.

Assays were performed at 28°C in the presence of 3 mM MgCl₂ using increasing concentrations of wild-type and mutant Mip1 from DEAE-purified fractions. Assays were carried out for 2-3 minutes. The top panel of each section represents EMSA images. In each top panel, the first lane on the left (C) represents the DNA substrate without protein. The bottom panel of each section represents quantification of bound DNA for increasing concentrations of wild-type and mutant Mip1. The curves are averages of 8-12 independent assays.

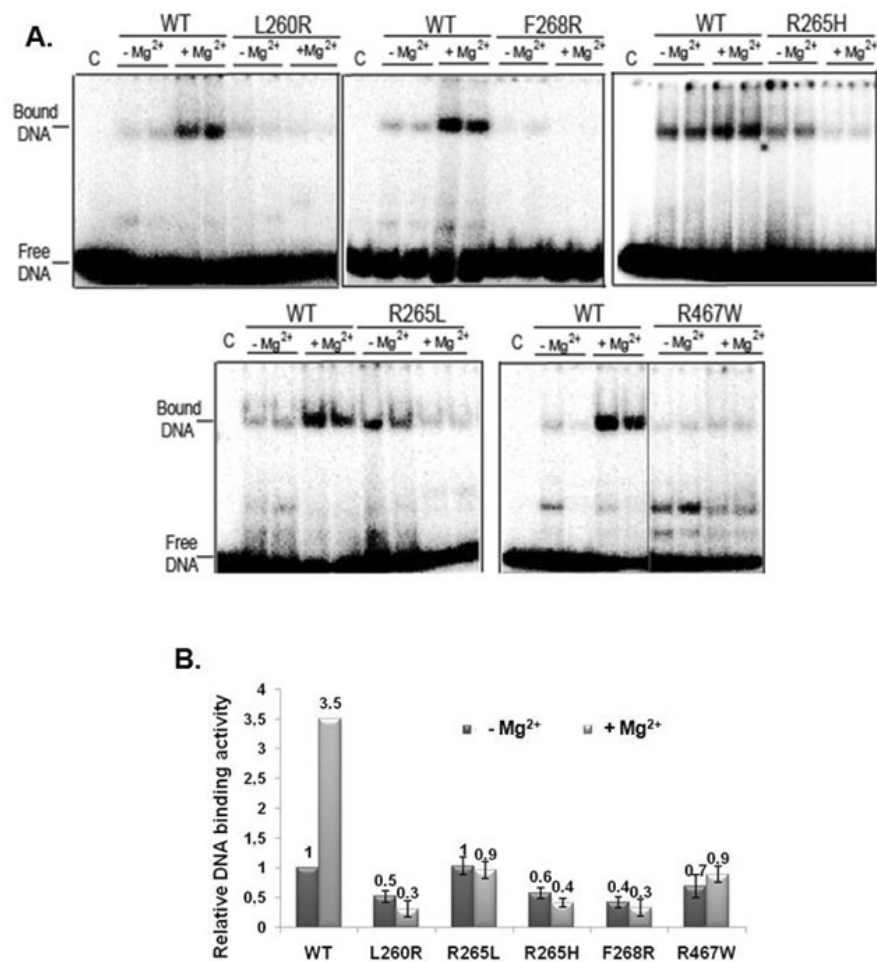


Figure 3.22. DNA binding activity of wild-type and mutant Mip1.

Assays were performed at 28°C in the absence and presence of 3 mM MgCl₂ using ~6 ng of wild-type and mutant Mip1 from DEAE chromatography-purified fractions. Assays were carried out for 2-3 minutes. **A.** EMSA images. In each panel, the first lane on the left (C) represents the DNA substrate without protein. For each condition, two samples were run in parallel. **B.** Quantification of bound DNA in mutant Mip1. The relative DNA-binding activities of wild-type and mutant Mip1 were expressed as a fraction of the wild-type Mip1 value which was arbitrarily set to 1 for the assay performed in the absence of MgCl₂. The values are averages of 8-10 independent assays.

3.10. 3'-5' exonuclease activity.

Most mutations mapping to the 3'-5' exonuclease domain confer mutator properties resulting from defects in the 3'-5' exonuclease function (Goodman and Fyngenson, 1998; Foury *et al*, 2004). Therefore, the mutator properties exhibited *in vivo* by the 3'-5' exonuclease mutants studied here suggested that the 3'-5' exonuclease activity could be impaired.

The 3'-5' exonuclease activity of wild-type and mutant Mip1 was determined in the presence of 50 mM MgCl₂ (optimal concentration) using a template primed with a 17-mer oligonucleotide which was either complementary to the DNA template or contained a single nucleotide A:A mispair at its 3'-end (Fig.3.20.H.). Unexpectedly, none of the mutants in the 3'-5' exonuclease domain showed substantially decreased 3'-5' exonuclease activity on correctly paired or mispaired substrates (Fig.3.23.A.-E. and G.). The exonuclease activity was similar to wild-type for Mip1-R265L and Mip1-F268R, reached 76% of the wild-type activity for Mip1-R265H and was even two times higher for Mip1-C261R and Mip1-L260R. In contrast, Mip1-R467W showed defective exonuclease activity at 50 mM MgCl₂ (27% of wild-type) (Fig.3.23.F. and G.). Whereas the excision rate of wild-type Mip1 affected by the presence of Mg⁺⁺ (being highest at 50 mM MgCl₂), the cation had no effect on the activities of Mip1-R467W (Fig.3.24.), suggesting that the Mip1-R467W properties might be dependent on the conditions prevailing inside mitochondria.

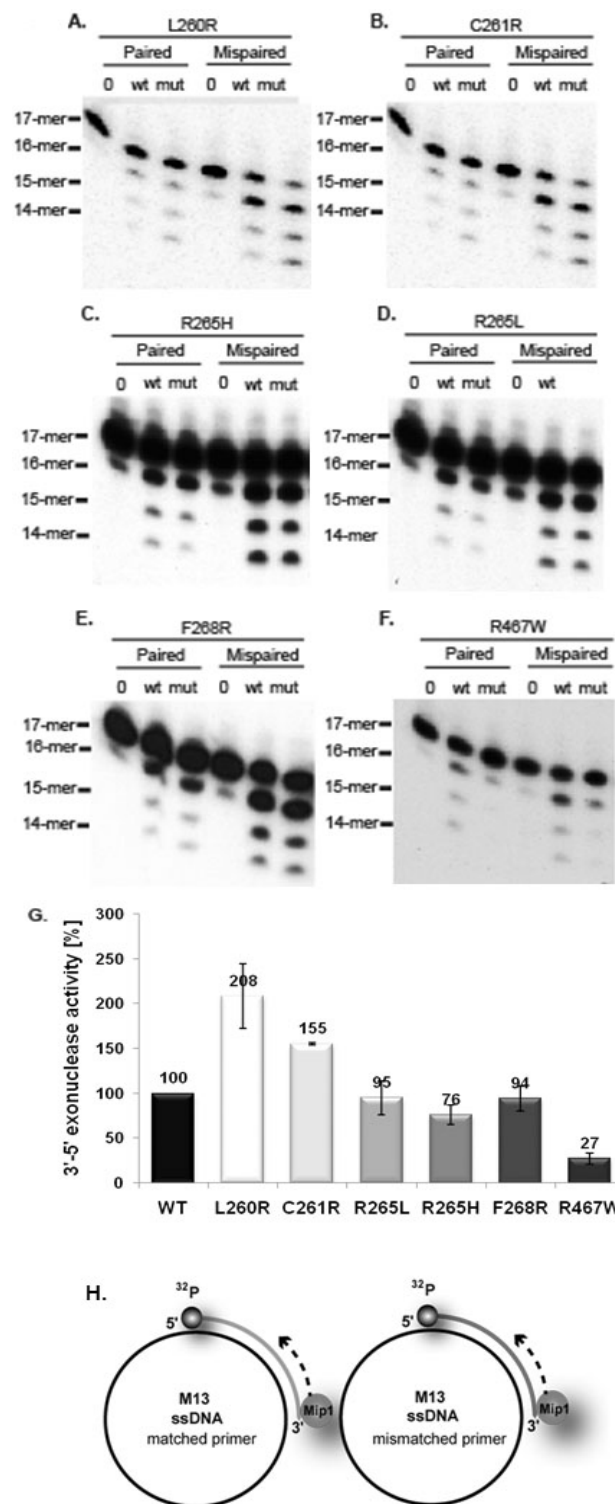


Figure 3.23. 3'-5' exonuclease activity of wild-type and mutant Mip1.

Assays were performed at 25°C on paired and mispaired DNA substrates using 2.5 ng wild-type and mutant Mip1 from DEAE chromatography-purified fractions. **A.-F.** PhosphorImager images of the degradation products after a 2 min incubation for wild-type and mutant Mip1 in the presence of 50 mM MgCl₂. The lanes labeled (0) contain the DNA substrate without protein. The 16 mer product that contaminates the original 17 mer oligonucleotide is visible. **G.** Quantification of the 3'-5' exonuclease activity on correctly paired substrate. For each strain (with the exception of Mip1-C261R (B.); Toussaint, 2010) values are the average of at least four independent assays and are expressed as percentage of the wild-type Mip1 activity (100%). **H.** Schematic presentation of the substrate used in the assay. The 3'-5' exonuclease activity of wild-type and mutant Mip1 was determined using a template primed with a 17-mer oligonucleotide which was either complementary to the DNA template or contained a single nucleotide A:A mispair at its 3'-end.

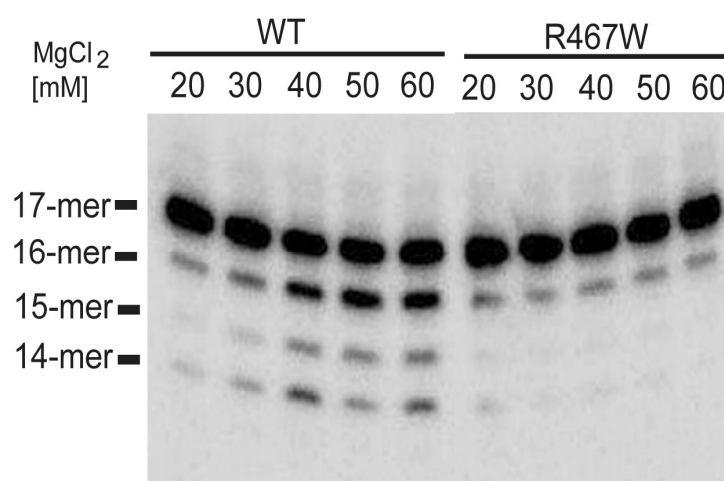


Figure 3.24. MgCl₂ dependent 3'-5' exonuclease activity of wild-type and R467W Mip1.

Assay was performed at 25°C on correctly paired DNA substrate using 2.5 ng wild-type and R467W Mip1 from DEAE chromatography-purified fractions. Assays were carried out for 4 minutes. Image shows the degradation products for wild-type and R467W Mip1 in the presence of increasing MgCl₂ concentrations.

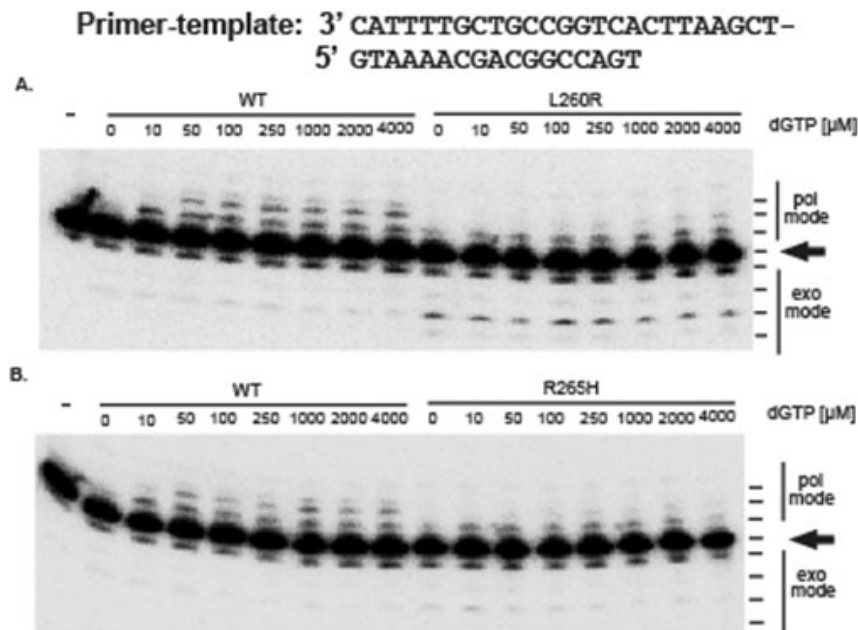


Figure 3.25. dGTP misincorporation by wild-type and A. L260R and B. R265H Mip1. Assays were performed at 28°C using ~2.5 ng Mip1 from DEAE chromatography-purified fractions in the presence of increasing dGTP concentrations. Assays were carried out for 3 minutes. The 17mer product is indicated by an arrowhead.

3.11. dNTP misincorporation.

Since the *mip1*-L260R, *mip1*-C261R, *mip1*-R265H and *mip1*-F268R mutants showed mutator properties *in vivo* and robust 3'-5' exonuclease activity *in vitro*, it could not be excluded that the high point mutation rate *in vivo* was produced by increased dNTP misincorporation during DNA synthesis. Misincorporation assays were performed using a 25-mer ssDNA substrate primed with a 17-mer oligonucleotide, as depicted in Fig.3.25., in the presence of increasing concentrations of dGTP. Whereas this nucleotide correctly

incorporated opposite to C at position +18, it would lead to G:T mispairing at position +19 (Fig.3.25.). The assay was performed for Mip1-L260R, which shows a two-fold increase in 3'-5' exonuclease activity, and Mip1-R265H in which exonuclease activity is slightly decreased (Fig.3.23.). Whereas wild-type Mip1 readily formed the G:T mispair, neither Mip1-L260R nor Mip1-R265H displayed misincorporation (Fig.3.25.A. and B.), excluding that the mutator trait is caused by increased dNTP misincorporation.

3.12. Coupling of 3'-5' exonuclease and polymerase functions

Since pol g has both polymerase and 3'-5' exonuclease activities, the DNA primer strand can be localized at either of the two active sites and the balance between these two localizations is an important parameter of the replicative properties (Kunkel and Bebenek, 2000). The data obtained for the total DNA synthesis (pol, measured in the presence of 20 mM MgCl₂, the optimal concentration) and 3'-5' exonuclease activity (exo, measured in the absence of dNTP and in the presence of 50 mM MgCl₂, the optimal concentration) provide an estimate of the relative polymerization and degradation capacities of a given Mip1 preparation. For an exo/pol ratio set to one for wild-type Mip1, all exonuclease mutants exhibited ratios significantly higher than one, from 2.7 for Mip1-R265L to 16 and 26 for Mip1-R265H and Mip1-F268R, respectively, and up to 59 for Mip1-L260R (Fig.3.26.).

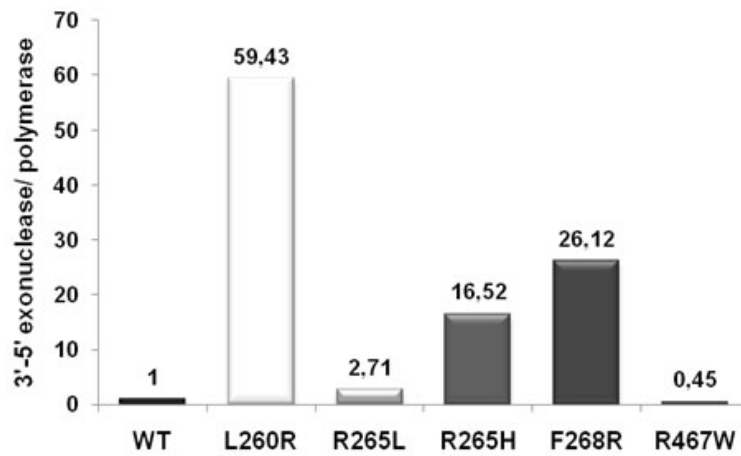


Figure 3.26. Exonuclease/ polymerase (exo/pol) ratios for wild-type and mutant Mip1. Estimation of the 3'-5' exonuclease to polymerase ratio. For each strain, the ratio of the value obtained in Fig.3.23.G. for exonuclease activity to the value obtained in Fig.3.18.B. for total DNA synthesis was reported to the exo/pol ratio of the wild-type Mip1 arbitrarily set to 1.

To corroborate these data, we carried out polymerase/exonuclease coupling experiments (Garmendia *et al*, 1992; Truniger *et al*, 1996). DNA synthesis versus degradation was analyzed using a PTZ18U DNA template paired with a 5'-end [^{32} P]-labeled oligonucleotide, in the presence of increasing dNTP concentrations (Fig.3.27.G.). In the absence of dNTP only degradation can occur. As the concentration of dNTP is increased there is a progressive competition between synthesis, degradation, and preferential positioning of the primer strand at the polymerase site. When wild-type Mip1 was examined, efficient elongation of the nascent chain was initiated already in the presence of nanomolar range dNTP concentrations, with concomitant decrease in the 3'-5' exonuclease activity. In contrast, 10 to 20 times higher dNTP concentrations were required to elongate the primer strand in the Mip1-L260R, Mip1-C261R, Mip1-R265H and Mip1-

F268R mutants (Fig.3.27.A.-D.). Elongation efficiency was also notably decreased for Mip1-R265L (Fig.3.27.E.). In contrast, the elongation efficiencies were quite similar for wild-type and Mip1-R467W (Fig.3.27.F.). These data show that all mutant Mip1 polymerases except Mip1-R467W favoured excision over polymerization as a result of preferential positioning of the primer strand at the exonuclease site.

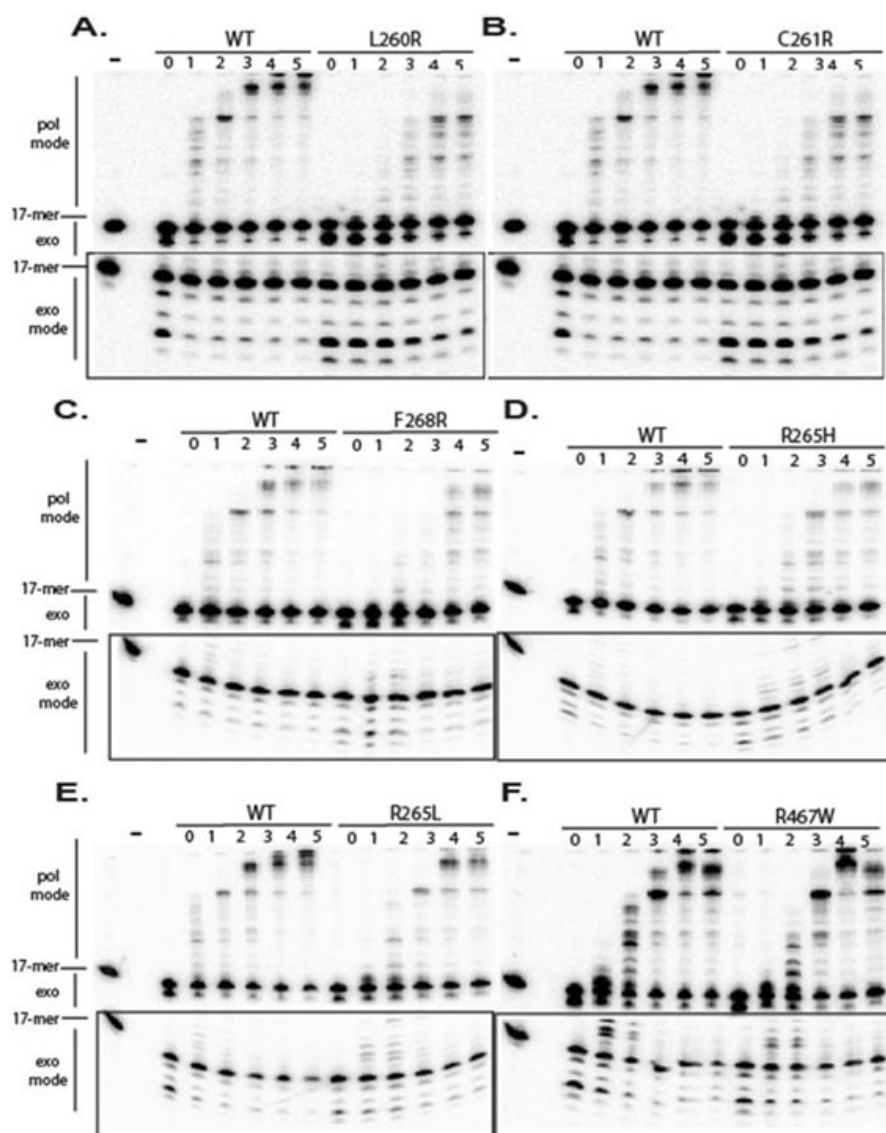


Figure 3.27. Polymerase/exonuclease-coupled assay.

A.-F. Images of the dried gels. Assays were performed at 28°C using ~1.5 ng wild-type and mutant Mip1 from DEAE chromatography-purified fractions and increasing dNTP concentrations (lane 0, no dNTP; lane 1, 25 nM; lane 2, 100 nM; lane 3, 500 nM; lane 4, 5 µM; lane 5, 25 µM). The lanes (-) on the left contain the DNA substrate alone. Assays were carried out for 3 minutes. The products were separated by electrophoresis in denaturated 15% polyacrylamide/7 M urea gels. The top panels show the products of higher molecular weight than the 17-mer oligonucleotide after an 8 cm Bromophenol blue migration (polymerase activity). The bottom panels show the products of lower molecular weight than the 17mer oligonucleotide after a 30cm Bromophenol blue migration (exonuclease activity).

MIP1 mutation	POLG1 mutation	mtDNA stability (% rho ⁰)	ER frequency ^a	Cell respiration ^b	Mip1 expression ^c	Gap-filling activity ^d (%)	Total DNA synthesis ^e (%)	Processivity ^f (% full-length DNA)	DNA binding activity ^g - Mg ²⁺	DNA binding activity ^g + Mg ²⁺	3'-5' exonuclease activity ^h (%)	Exonuclease / polymerase ⁱ
MIP1	POLG1	0.9 ± 0.3	1	8.5 ± 2.1	+	100	100	21.5 ± 6.13	1	3.5	100	1
L210P	L244P	8.33 ± 0.01	83.7	ND ^j	-	ND	ND	ND	ND	ND	ND	ND
L260R	L304R	62.4 ± 10.5	51	1.1	+	35.5 ± 8.5	3.5 ± 1	3.87 ± 0.17	0.52 ± 0.1	0.31 ± 0.13	208 ± 36	59.43
C261R	S305R	59.1 ± 2.2	44.5	ND	+	ND	ND	3.25	ND	ND	155	ND
R265L	R309L	24.3 ± 3.9	4.5	7.5 ± 1.1	+	91.1 ± 2.5	35.1 ± 5	12.4 ± 0.3	1.03 ± 0.15	0.96 ± 0.14	95 ± 19	2.71
R265H	R309H	50.6 ± 2.4	58.5	2.2 ± 0.2	+	63.5 ± 2.5	4.6 ± 1	6.88 ± 0.19	0.58 ± 0.09	0.41 ± 0.07	76 ± 11	16.52
F268R	W312R	64.2 ± 10.5	85	2.3 ± 0.2	+	70.5 ± 8.5	3.6 ± 2	4.51 ± 0.17	0.42 ± 0.09	0.33 ± 0.14	94 ± 14	26.12
R467W	R574W	21.5 ± 4.1	18.5	6.5 ± 0.5	+	97.5 ± 1.5	59.6 ± 15	12.6 ± 0.2	0.69 ± 0.19	0.89 ± 0.14	27 ± 6	0.45
MIP1/ MIP1	POLG1/ POLG1	2.6 ± 1.7	1	ND	ND	ND	ND	ND	ND	ND	ND	ND
F268R/ R467W	W312R/ R574W	14.5 ± 3.4	18	ND	ND	ND	ND	ND	ND	ND	ND	ND

Table 4. *In vivo* and *in vitro* phenotype data.

^a E^R mutant frequency was expressed as a fraction of the wild-type frequency arbitrarily set to 1;

^b Microliters of O₂ per milligram of yeast cells (wet weight) per hour;

^c (+) well expressed, (-) low Mip1 levels;

^d Gap-filling activity was expressed as the average percentage of the wild-type activity (100%);

^e Total DNA synthesis was expressed as the average percentage of the wild-type activity (100%);

^f Processivity was expressed as the ratio of the intensity of the signal given by full-length DNA to the intensity of the signal given by all DNA primers that have been initiated;

^g The relative DNA binding activities of wild-type and mutant Mip1 were expressed as a fraction of the wild-type Mip1 value which was arbitrarily set to 1 for the assay performed in the absence of MgCl₂. -Mg²⁺, no MgCl₂ added; +Mg²⁺, +3 mM MgCl₂;

^h 3'-5' exonuclease activity measured at 50 mM MgCl₂ was expressed as the average percentage of the wild-type activity (100%);

ⁱ The ratio of the value obtained for 3'-5' exonuclease activity to the value obtained for total DNA synthesis was reported to the exo/pol ratio of the wild-type Mip1 arbitrarily set to 1;

^j ND, not determined;

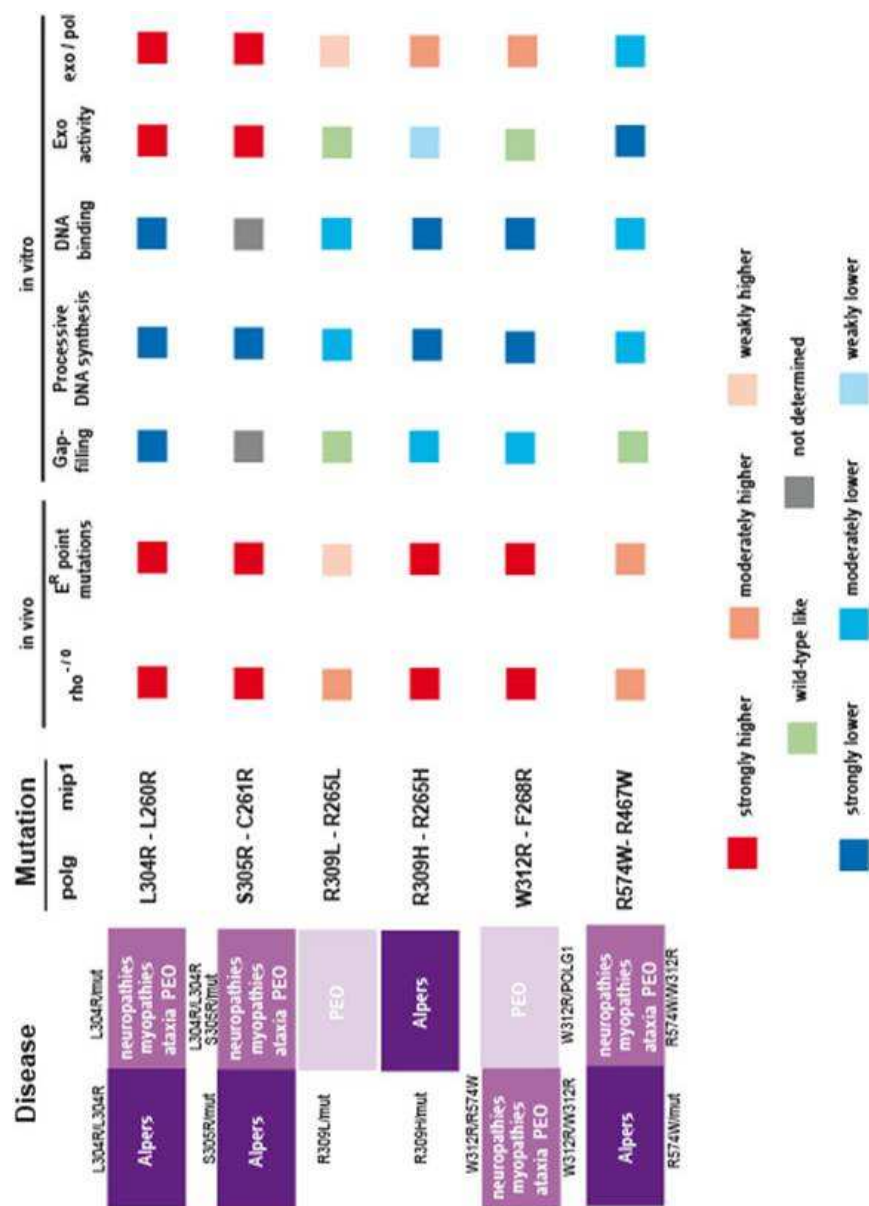
4.
DISCUSSION
AND
CONCLUSIONS

4.1. Classification of yeast *mip1* mutations and correlation between disease severity and yeast phenotypes.

This work had two goals: the first was to shed light on the dysfunction caused by mutations localized in hitherto unexplored regions of pol g; the second was to determine to which extent the yeast model was valid. To this end we have generated and characterized the *mip1* equivalents of six disease-associated *POLG1* mutations. As discussed below, all Mip1 mutations impaired mtDNA replication *in vivo* and polymerase function *in vitro* (Table 5.), implying that their human counterparts play a crucial role in the disease onset. Despite a few exceptions, there is generally a good correlation between yeast phenotypes and disease severity (Fig.4.1.). Whereas the symptoms observed in the patients did not allow classification of the pol g mutations, the yeast model has allowed us to range them into three categories.

Figure 4.1. Correlation between disease severity and yeast phenotype.

Disease are indicated in the violet rectangles on the left site, from intense violet for the most severe disease (Alpers) to violet for severe disease (neuropathies/ataxia) and light violet for mild PEO. The patient genotypes are also given. The *in vivo* and *in vitro* phenotypes are indicated by squares. These squares are red for *in vivo* frequencies or *in vitro* activities that are higher than in wild-type, from the highest in intense red (strongly higher) to the lowest in light pink (weakly higher). Blue squares represent frequencies or activities that are lower than in wild-type, from the lowest in intense blue (strongly lower) to the highest in light blue (weakly lower). Green squares represent wild-type like phenotypes. Grey squares represent not determined data. Severe diseases are generally associated with squares of intense colour, while milder diseases are associated with squares of light colour. For example, *mip1*-I260R phenotypes and L304R-associated diseases are mostly represented by squares of intense colour, whereas *mip1*-R265L phenotypes and R309L-associated diseases are mostly represented by squares of light colour.



The first category results in insufficient pol g levels and is represented by the human L244P mutation localized between the ExoI and ExoII sites in a conserved residue of a peripheral coil (Fig.4.2.A.). At 28°C

the yeast *mip1*-L210P mutant does not lose mtDNA but exhibits an 80-fold increase in mtDNA point mutation frequency. Recently, a mutator phenotype was also reported for this mutant (Stumpf *et al*, 2010). The authors did not mention the intense loss of mtDNA at 37°C revealing that mtDNA replication is defective at elevated temperature. This remarkable *in vivo* temperature-sensitive phenotype is associated with very low levels of Mip1-L210P protein in the mitochondrial extracts from cells grown at 28°C. Based on these data we propose that the change of Leu210 to proline results in a protein folding defect that is mild enough in cells grown at 28°C to maintain a sufficient amount of Mip1-L210P to replicate mtDNA. However, the Mip1 protein is unstable and degraded during preparation of the mitochondrial extracts. In addition, the 3'-5' exonuclease activity is probably not optimal and this results in a mutator phenotype *in vivo*. In cells grown at 37°C the folding defect is probably more pronounced, Mip1-L210P is not expressed and mtDNA is lost. These data strongly suggest that in human L244P mutant form is poorly expressed at 37°C, the physiological body temperature. We cannot also rule out in human a possible chaperone effect provided by pol gB subunits.

In patients the L244P mutation is associated in trans with W748S, a mutation localized in the linker IP subdomain which is very common in the Northern population (Hakonen *et al*, 2005) and always found in cis with the SNP E1143G that is localized downstream of motif C of the polymerase domain (Chan *et al*, 2006; Horvath *et al*, 2006; Kollberg *et al*, 2006; Sarzi *et al*, 2007). While severe impairment of polymerase function, DNA binding and processivity of polg harboring

the W748S-E1143G mutation was reported (Chan, 2006), another research group failed to reproduce these data and did not find substantial defects in the purified mutant pol g (Palin *et al.*, 2010). Nevertheless, the pathogenicity of W748S is undoubtful. Most patients, as compound heterozygous, suffer from the mitochondria recessive ataxia syndrome (MIRAS); however, phenotypes range from late-onset polyneuropathy to severe childhood encephalopathy and juvenile Alpers syndrome (Van Goethem, 2003; Van Goethem, 2004; Hakonen *et al.*, 2005; Naviaux, 2004; Nguyen, 2005). If human L244P, like its yeast counterpart, is poorly expressed, then it is likely that presentation of the patients heterozygous for L244P/W748S results from the defects conferred by W748S, which must be exacerbated due the haploinsufficiency resulting from the L244P mutation. This results in acute infantile Alpers syndrome (Table 4.; Ferrari *et al.*, 2005; Spinazzola *et al.*, 2009).

The second category is represented by the human R574W mutation. Arg574 is a highly conserved residue residing in a segment of the IP domain which forms a tangent plane with respect to the DNA-binding channel (Fig.4.2.A.). This segment is shared by Mip1 and thus should play a similar function in both organisms. However, the proximity of one accessory pol gB subunit (Arg574 is 7Å distant from the pol gB Lys477 residue) and the increased length of the human IP domain suggest that the R574W mutation might elicit more complex effects than *mip1*-R467W. Indeed, the data obtained in yeast only partially correlate with the clinical presentations. Even though the studies with Mip1-R467W validate the expected impact of this mutation on the

DNA binding activity, the moderately severe defects observed in yeast both *in vivo* and *in vitro* do not really explain the fatal Alpers syndrome presented by three infants harboring the R574W mutation combined with the A467T mutation in trans (Table 4.; Kollberg *et al*, 2006; Spinazzola *et al*, 2009). It must be noted that in fact most pol g mutations are found as compound heterozygous with A467T which is the most common pol g mutation in the Northern population (Horvath *et al*, 2006). The biochemical data show that A467T is a very severe mutation reducing the polymerase activity of polg to 4% (Chan *et al*, 2005). Although usually less severe at the homozygous state than as compound heterozygous, A467T is often associated with Alpers syndrome (Horvath *et al*, 2006). The discrepancy between yeast and human phenotypes points to a potential weakness in the yeast model for mutations in the linker region, even for those in highly conserved residues.

The third category is represented by the human L304R, S305R, R309H, R309L and W312R mutations downstream of the ExoII motif (Fig.4.2.A. and B.). They define a novel subdomain of the 3'-5' exonuclease that is not present in prokaryotic family A, but is strongly conserved in all pol g. This region which is distant from the exonuclease active site and in close proximity to the polymerase domain is localized in the DNA-binding channel and characterized by a coil-helix motif (Fig.4.2.A.).

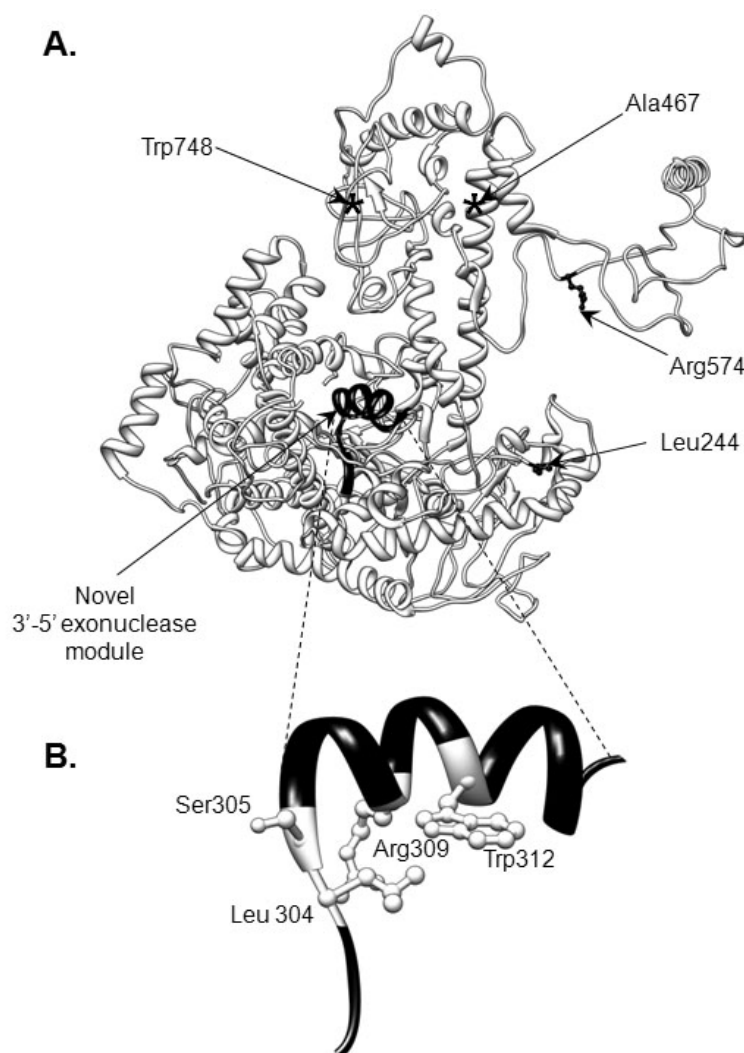


Figure 4.2. Localization of the disease associated pol g mutations.

A. Three-dimensional structure of human pol g (pdb ID: 31KM). Leu244 and Arg574 are displayed as ball & sticks atomic representations and indicated with arrowheads. Localization of Ala467 and Trp748 was indicated with asterisk and arrowheads. The coil-helix fragment of the novel 3'-5' exonuclease module (residues 302-318) is in black. **B. Zoom in on the coil-helix fragment of the novel 3'-5' exonuclease subdomain.** Leu304, Ser305, Arg309 and Trp312 are displayed as white ball & sticks atomic representations. This figure was prepared by using UCSF Chimera software.

The *mip1*-L260R, *mip1*-C261R, *mip1*-R265H and *mip1*-F268R mutants are characterized by low respiration, high mtDNA instability

and a more than 50-fold increased frequency of mtDNA point mutations (Table 5.). It must be noted that the *mip1*-G259R and *mip1*-Q264H mutations, which are the equivalents of the human disease-associated G303R and Q308H mutations, also confer high mtDNA instability (see Table 3.; Baruffini *et al*, 2010; Cochonneau and Foury, unpublished data). Thus, despite the milder phenotype of the *mip1*-R265L mutant, these data suggest that the Mip1 fragment encompassing residues 260-268 is of particular importance in mtDNA maintenance. The *in vivo* genetic properties of the *mip1*-L260R, and *mip1*-R265H(L) mutants have also been studied by another group (Stumpf *et al*, 2010). They did not find a mutator phenotype and reported higher mtDNA instability in *mip1*-R265L than in *mip1*-R265H mutant. However, our *in vivo* data support our *in vitro* data (Fig.4.1.). While all mutants showed decreased polymerase activity associated with low processivity and deeply modified DNA binding activity with no stimulation by MgCl₂, the defects were more pronounced for Mip1-R265H than for Mip1-R265L (Table 5.).

Unexpectedly for mutator strains, the 3'-5' exonuclease activity was only slightly decreased in Mip1-R265H, unchanged in Mip1-R265L and Mip1-F268R and even increased in Mip1-C261R and Mip1-L260R (Table 5.). Moreover, exonuclease/polymerase coupling experiments showed that the balance between exonuclease and polymerase functions favours the exonuclease activity. These data suggest that increased exonuclease activity combined with poor polymerization activity is not an advantage *in vivo* to efficiently remove the replication mistakes. As neither Mip1-L260R nor Mip1-R265H polymerases displayed tendency to misincorporate

nucleotides, it is unlikely that the mutator trait could be explained by an increased incorporation of incorrect nucleotides into mtDNA. However, mtDNA synthesis in yeast is highly dependent on the mitochondrial Msh1-dependent mismatch-repair pathway (Vanderstraeten *et al*, 1998; Chi and Kolodner, 1994). A plausible explanation of the mutator trait of these mutants is that their low DNA polymerase activity is a rate-limiting factor for the mitochondrial mismatch-repair reactions that require efficient DNA synthesis (Vanderstraeten *et al*, 1998). It must be noted that there is no Msh1 equivalent pathway in human mitochondria.

The hypotheses proposed above to explain the mutator activity of pol g mutants are based on several different mechanisms.

Firstly, mutators in the 3'-5' exonuclease domain may have a proofreading defect. Mutations leading to 3'-5' exonuclease deficiency should be localized in close neighborhood to the exonuclease active site, altering directly or indirectly the two-metal ion mechanism of 3'-5' exonuclease catalysis. In principle, pol g mutations conferring a proofreading could also reside in the DNA binding pocket, affecting the proper entrance of DNA substrate into the exonuclease active site area. However, decreased 3'-5' exonuclease activity was observed up to now only for mutations residing in essential catalytic blocks of the 3'-5' exonuclease domain (Foury and Vanderstraeten, 1992; Hu *et al*, 1995; Vanderstraeten *et al*, 1998). While the strong mutator properties of *mip1*-L210P could be explained by decreased exonuclease activity, the low expression of the Mip1-L210P protein prevents further investigations in vitro.

Secondly, mutators may have reduced dNTP selectivity. Incorrect selectivity toward incoming dNTPs can result from mutations localized in the fingers subdomain or in the area of polymerase active site. A good example is residue Mip1-Y757 which is strongly conserved among all polA family members and responsible for proper recognition and positioning of dNTP toward DNA primer-template. If mutated in the heterozygous state (Y757S) or in the homozygous state (Y757F) mtDNA point mutation frequency is increased *in vivo* and Km (dNTP) is increased *in vitro* (Wallace, 2005; Baruffini *et al*, 2006). However, none of the mutations presented in this thesis fulfills such a condition.

Thirdly, mutators may exhibit defects in correcting post-replicative errors in mtDNA. Pol g, as the sole DNA polymerase in mitochondria, participates in all mtDNA repair events, as base excision repair or mismatch repair. BER requires pol g to fill short or longer patches in DNA substrate appearing after excision of incorrect nucleotides and thus involves non-processive gap-filling activity, whereas MMR requires efficient mtDNA synthesis during repair of nucleotide mismatches or small deletions/ insertions of mtDNA. Although difficult to prove *in vivo*, this function might be altered in the *mip1* mutants clustered in the 3'-5' exonuclease module studied in this work.

Finally, a fourth possible cause for a mutator phenotype is a low mtDNA copy number, resulting from severe mtDNA replication defects. Indeed, as only a small number of mtDNA molecules segregate into the daughter cell, the probability to fix a mtDNA molecule harboring a mutation is increased when the number of

mtDNA copies is low. This situation could also explain the mutator activity of the mutants in the 3'-5' exonuclease module and could easily be verified by measuring mtDNA content using Southern blot or RT-PCR analysis.

The phenotypes of the yeast *mip1*-L260R, *mip1*-C261R, *mip1*-R265H and *mip1*-R265L mutants fit well with the clinical presentations conferred by their human equivalents (Table 4., Table 5. and Fig.4.1.). Young adults harboring the L304R mutation combined with A467T or W748S in trans developed severe neuropathies leading to premature death for two of them (Van Goethem *et al*, 2001; Van Goethem *et al*, 2003; Naimi *et al*, 2006). Moreover, early disease onset below 10 years of age was observed for two L304R homozygous patients and was followed by a fatal issue in one subject (Naimi *et al*, 2006; Ashley *et al*, 2008). Severe neuropathy characterized by an early onset or even Alpers syndrome were fatal for patients harboring the S305R mutation associated in trans with A467T, R627Q or P1073L mutations (Wong *et al*, 2008; Blok *et al*, 2009; Baruffini *et al*, 2010).

The severe *mip1*-R265H phenotype is in accordance with the fatal Alpers syndrome developed by a six-month old infant harboring the R309H substitution (Horvath *et al*, 2006), whereas the mild phenotype conferred by the *mip1*-R265L mutation agrees with the less severe myopathy/PEO symptoms observed in two patients harboring the R309L substitution (Lamantea *et al*, 2002). However, the in trans mutations harbored by the compound heterozygous patients should also be considered. The T251I mutation, which was found to be associated with R309L, is believed to be a mild mutation, whereas the

R627Q mutation associated with R309H seems more severe (Horvath, 2006).

The validity of the yeast model is more questionable when considering the human W312R mutation and its *mipI*-F268R counterpart. The W312R substitution, identified in patients as dominant heterozygous, compound heterozygous or homozygous, results in PEO, PEO with myopathy and sensori-motor polyneuropathy with dysphagia and PEO, respectively (Horvath, 2006; Di Fonzo, 2006; Del Bo, 2006). The compound heterozygous patient (W312R/R574W) showed first symptoms at an earlier age than homozygous patient; however the severity and complexity of the clinical presentation of the homozygous patient were higher. The severe *in vivo* and *in vitro* phenotypes conferred by the *mipI*-F268R mutation do not fit well with the late disease onset in patients harboring the W312R mutation at the homozygous state or combined with severe mutations such as R574W in trans (Horvath *et al*, 2006). The discrepancy between yeast and human might be imputable in part to the different properties conferred by Trp312 and Phe268 residues.

4.2. A novel module within the 3'-5' exonuclease domain, coupling DNA synthesis and degradation:

Altogether the data obtained in yeast show for the first time that a module within of the 3'-5' exonuclease domain plays a crucial role in the DNA polymerase function with no major effect on the exonucleolytic activity. In other words, defective proofreading is certainly not the cause of the disease in patients harboring the L304R,

S305R, R309H(L) and W312R mutations. Indeed, patients homozygous for L304R did not show any sign of the premature ageing syndrome displayed by proofreading deficient mutator mice (Trifunovic *et al*, 2004). Our studies suggest that in these patients mtDNA replication dysfunction is generated through decreased DNA binding activity and a severe imbalance in the coupling between the exonuclease and polymerase functions that favours the exonucleolytic process. Therefore, the diseases associated with these mutations are caused by poor mtDNA replication.

We would like to propose a function for the 3'-5' exonuclease module encompassing residues 304-312 in human pol γ . All DNA polymerases which can proofread the errors introduced during DNA synthesis possess two distinct catalytic sites and thus must direct the DNA primer strand from one active site to another (Kunkel and Bebenek, 2000). A subtle balance in positioning of the primer strand at either the polymerase or exonuclease site is particularly important in replicative DNA polymerases such as pol γ which require both fast and faithful DNA replication. The transfer of the 3' end of the primer strand between polymerase and exonuclease active sites requires precise mechanisms that are not perfectly understood and differ from one polymerase to another. Whereas in *E. coli* DNA polymerase I the J-helix seems to play a role in the primer strand shuttling between the polymerase and exonuclease sites (Tuske *et al*, 2000), in several family B polymerases a β -hairpin loop structure localized in the 3'-5' exonuclease domain plays an important role in DNA binding during the transfer of the primer end to the exonuclease site (Kuroita *et al*, 2005; Hogg *et al*, 2007; Kim *et al*, 2008; Subuddhi *et al*, 2008;

Trzemecka *et al*, 2009). Yet another mechanism operates in Phi29 DNA polymerase, where the thumb is mainly responsible for the coordination of primer strand switching (Pérez-Arnaiz *et al*, 2006). No switching mechanism has been described so far for pol g. Our data strongly suggest that the 3'-5' exonuclease segment comprising residues 304-312 plays a crucial role in the regulation of the primer strand switching between the polymerase and exonuclease active sites.

4.3. Model of a DNA primer strand switching mechanism between the exonuclease and polymerase active sites of pol g.

The 3'-5' exonuclease segment studied here belongs to the DNA-binding channel, is at an equivalent distance from the polymerase and exonuclease active sites (26-27Å), and is part of a larger region (residues 293-316) that is closer to the polymerase domain than any other exonuclease region. This region which comprises two α -helices (residues 295-301 and 306-318) connected by a very short 4-residue coil is in close proximity to segments localized in a loop of the palm subdomain (residues 845-852) and in the fingers subdomain (residues 1022-1027 and 1047-1091, respectively) (Fig.4.3.A.-C. and Table 6.).

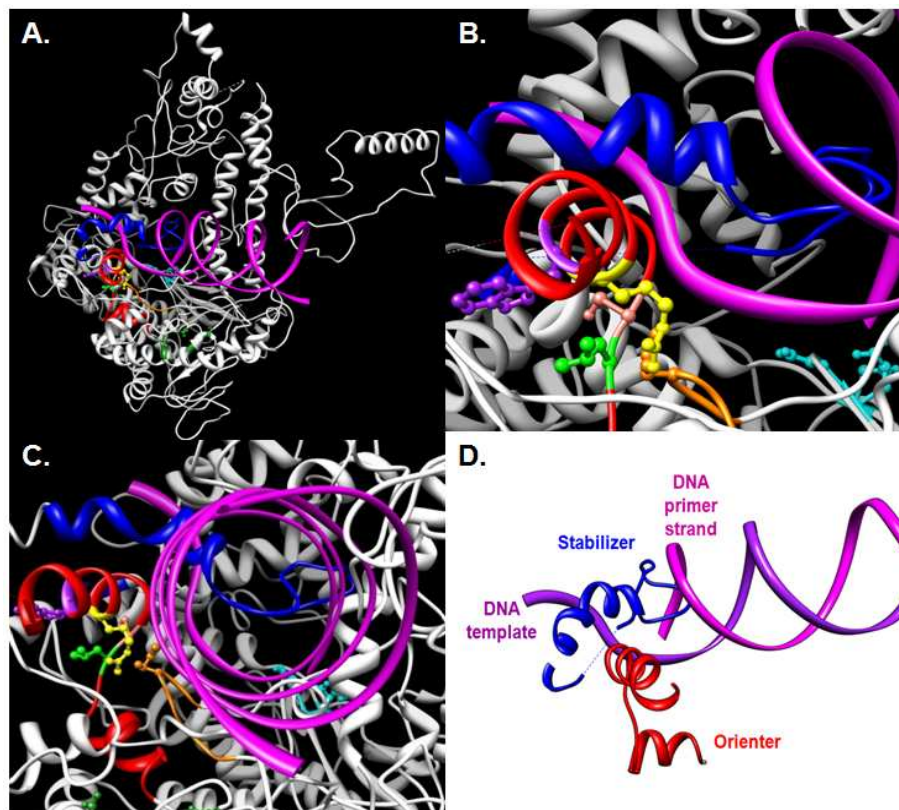


Figure 4.3. Structure of the 3'-5' exonuclease and fingers module in human pol g docked with DNA.

A. Localization of the exonuclease (residues 293-316, red), fingers (residues 1049-1095, dark blue) and loop (residues 845-852, orange) domains. The active sites of the polymerase (Asp890, Asp1135, Glu1136, cyan) and 3'-5' exonuclease (Asp198, Glu200, Asp274, Asp399, forest green) domains and docked DNA (ribbons, magenta) are also shown (**B.** and **C.**) Enlarged views of the region containing the exonuclease and fingers modules. Positions of Leu304 (green), Ser305 (salmon), Arg309 (yellow), Trp312 (violet) and Ile850 (orange) are shown. **D.** Model of a primer strand switching mechanism between the exonuclease and polymerase active sites. Modelled pol g-DNA complex was obtained by the superimposition of bacteriophage T7 DNA polymerase and pol g structures using the UCSF Chimera software. The fingers subdomain (residues 1049-1095, blue), referred to as 'stabilizer', forms a loop that tightly grabs the DNA template strand (violet), and the 3'-5' exonuclease module (residues 293-316, red) referred to as 'orienter', could play a role in the orientation of the primer strand (pink) towards the active site in response to pol g conformational changes.

Table 6.		
Exonuclease^a	Palm	Fingers
Met295	Val845 ^b	
Ser296	Val845; <u>Thr846^c</u> ; <u>Ala847</u> ; Gly848	
Met297	Thr846; Ala847	
Met299	Ala847; Gly848; <u>Thr849</u> ; Ile850	
Ala300	Ala847; Thr849; Arg852	Leu1022; Asp1024
Ile301		<u>Asp1024</u>
Ser302		Asp1024
Gly303	Ile850	Asp1024; Leu1025
Leu304	Ile850	Leu1025
Ser305	Ile850	Lys1027
Phe307		Ala1048; Gly1051; Gly1052; Ser1055
Gln308		<u>Glu1090</u> ; <u>Glu1091</u>
Leu311		Arg1047; Glu1090; Glu1091
Trp312		<u>Glu1090</u> ; Glu1091

Table 6. Residues in the polymerase domain that are close neighbors of the 3'-5' exonuclease region encompassing residues 293-316.

^a The distances were estimated with the Swiss-PdbViewer software. ^b Distance is less than 5 Å (not underlined) ^c Distance is less than 3.5 Å (underlined).

Lee *et al*, (2009) have pointed out that residues 1051-1095 partially block the DNA-binding channel, a very unusual feature that has never

been reported for a DNA polymerase but is reminiscent of a structural element described for the RNA polymerase from the bacteriophage N4 virion (vRNAP) (Murakami *et al*, 2008; Gleghorn *et al*, 2008). In the vRNAP apo-form the DNA template-binding channel is blocked by the cooperating action of two domains, the B-loop and the plug localized in the fingers and in the N-terminal subdomains respectively, whereas in the binary DNA-vRNAP complex a conformational change associated with the expulsion of the B-loop opens the channel. Based on similarities displayed by the plug /B-loop modules of vRNAP and the 3'-5' exonuclease /fingers segments of pol g with respect to positioning in the linear amino acid sequence, secondary structures and localization in the DNA-binding channel, we first thought that the pol g elements could fulfill plug-like and B-loop-like functions in the switching mechanism between the exonuclease and polymerase active sites. However, alignment of the entire protein structures shows that pol g and vRNAP modules cannot be superimposed, displaying instead a different spatial organization (Fig.4.4.). In addition, the mechanisms that are involved in the two polymerases are different. The vRNAP requires a mechanism that blocks the entrance of unspecific DNA substrates to the unique polymerase active site, whereas pol g, as an enzyme possessing two distinct but cooperating active sites, requires a switching mechanism that blocks the access of the DNA primer strand to one active site while facilitating its transfer to the other site.

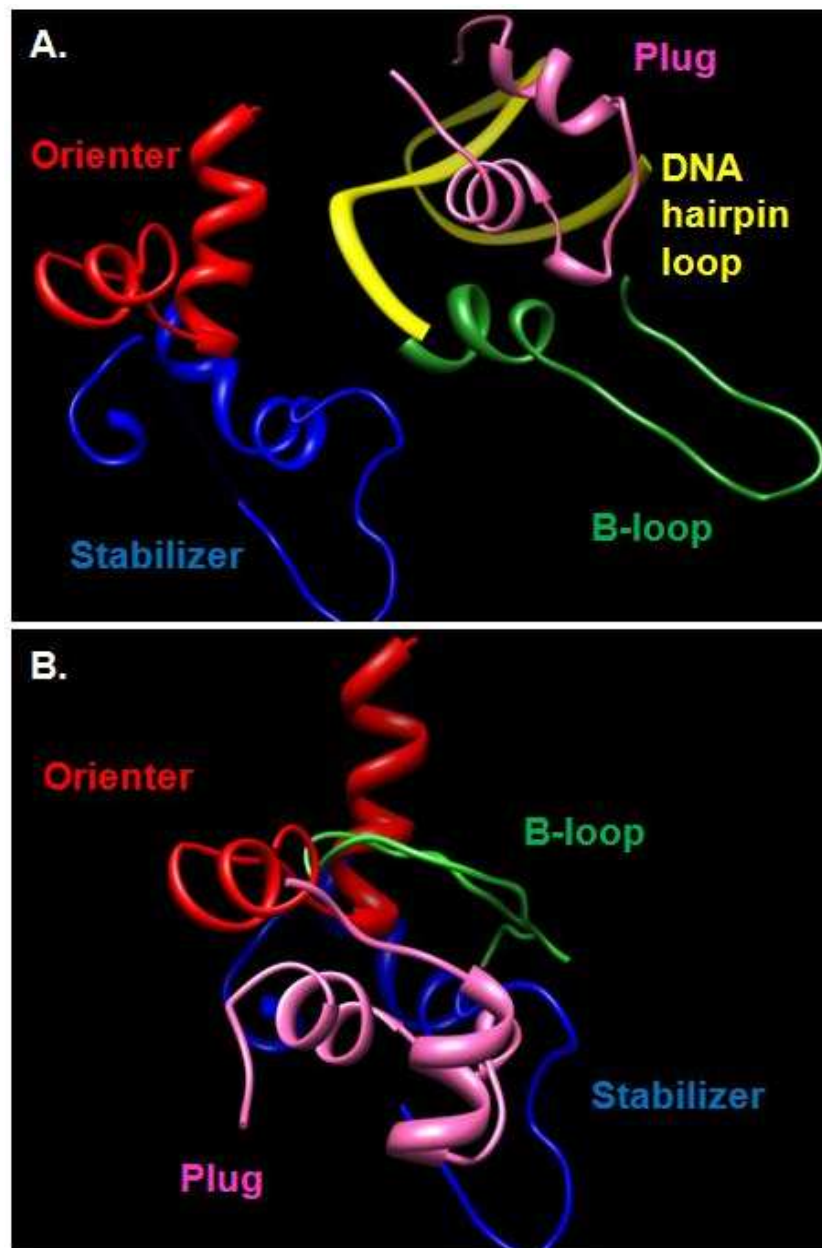


Figure 4.4. Comparison of the B-loop (residues 652-672) / plug (residues 170-202) and ‘stabilizer’ (residues 1049-1095)/ ‘orienter’ (residues 293-316) modules located in the DNA-binding channels of bacteriophage N4 virion RNA polymerase and human pol g respectively.

The structures of the modules were superimposed based on alignment of the structures of the complete protein sequences. **A.** Superimposition of the modules of pol g and vRNAP bound to DNA promoter (DNA hairpin loop shown as a yellow ribbon) (pdb ID: 3CL3L). **B.** Superimposition of pol g and apo-vRNAP modules (pdb ID: 2PO4).

Table 7.	
Nucleotide	Pol g residue
Template strand^a	
1	Trp1049<1 Å
2	Gly1051<1Å; Gly1052<1Å
3	Glu1056<1Å
4	Glu1056<1Å; Val1074<1Å
5	Val1074<2Å; Asn1059<2Å
6	Lys1060<1Å
7	Glu1062<1Å
8	Leu1061<1Å
9	Leu1061<3Å
Primer strand^a	
22	Lys1060<1Å
21	Lys1060<2Å; Asn1059<2Å
20	Ser1063<1Å
19	Ser1063<1Å; Glu1062<1Å
18	Leu1061<2Å

Table 7. Fingers subdomain residues (1049-1095) that are close to docked DNA.

^a Nucleotides of the template and primer strand are numbered from 5' end (nucleotide 1). Distances were estimated with the UCSF Chimera software. All amino acid residues but Glu1056 are conserved in Mip1.

Therefore, we turned towards a switching mechanism which could fulfill such a function (Fig.4.3.D.). As the pol g structure has been obtained without bound DNA, we have superimposed the structures of pol g and bacteriophage T7 DNA polymerase complexed with DNA to provide a rough model of the pol g-DNA complex (Lee *et al*, 2009). Such modelling with docked DNA shows that residues 1049-1095 of the finger form a loop that tightly grabs the template strand through several residues localized at less than 1 Å from the DNA (Fig.4.3.B. and C.; Table 7.). This region of the finger which could have a DNA anchoring function will be referred to as the 'stabilizer' subdomain. A second element of this model is the 3'-5' exonuclease module (residues 293-316) which could play a role in the orientation of the

primer strand towards the active sites in response to pol g conformational changes. This module will be referred to as the 'orienter'. Ser305, Ser306 and Phe307 residues are less than 5Å distant from the template DNA strand. In our model, the 'orienter' module could play a double role. Firstly, through a pressure exerted by residues proximal to the template strand, it could create spatial constraints to destabilize of the template strand and modify in the arrangement of the primer strand. Secondly, this module could operate as a mobile barrier that prevents the access of the primer strand to the exonuclease site during the transition from the open to the closed conformation, and reciprocally, allows access to the exonuclease site in the event of base mispairing. The disease-associated mutations located in the 'orienter' may affect the architecture of this module by a more tightly packed and less flexible structure with additional hydrogen bonds (Fig.4.5.). As a result, the exonuclease site would remain widely accessible to the primer strand. Thus the 'stabilizer' and 'orienter' may form a bottleneck in the DNA-binding channel to ensure an optimal environment for the positioning of the primer and template strands. It must be pointed out, however, that our model relies on a modelled pol g-DNA complex based on the structure of bacteriophage T7 DNA polymerase which does not contain these modules. It is clear that a crystal structure of pol g bound to DNA is required to test our model.

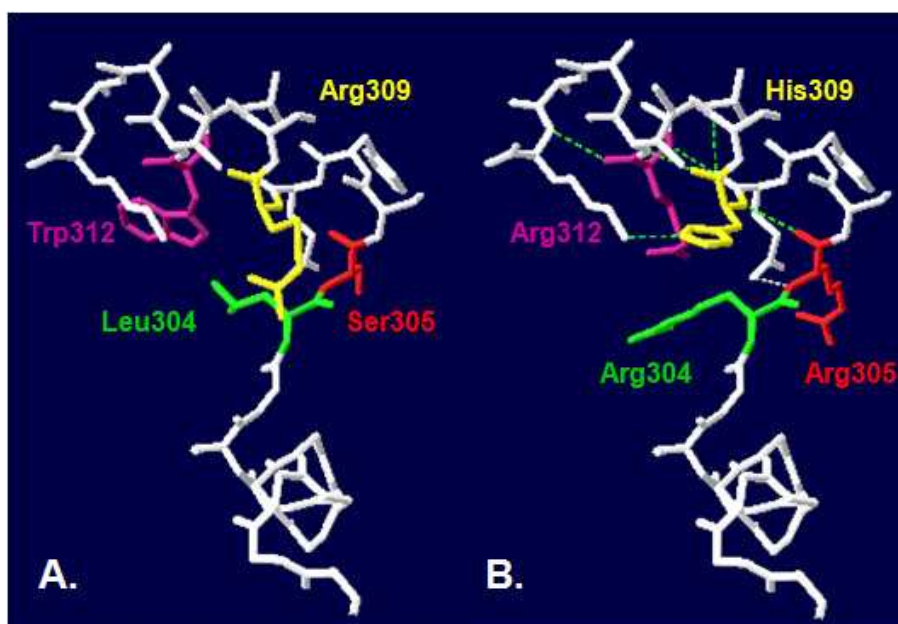


Figure 4.5. Structural representation of A. wild-type and B. disease-associated mutated residues.

Mutagenesis *in silico* was obtained with the Swiss-PdbViewer software. Newly formed hydrogen bonds were indicated with dotted line.

Finally, it is interesting to note that the ‘orienter’ module of human pol g displays several basic features associated with the helix-turn-helix (HTH) type of DNA binding motif (summarized in Fig.4.6). Although the ‘orienter’ module described in this work is suspected to modulate the fate of the single-stranded DNA primer, whereas DNA substrates bound by HTH-proteins are usually double stranded in nature (Fig.4.6), it is therefore tempting to speculate that the orienter module operates at least partially through direct interactions with DNA.

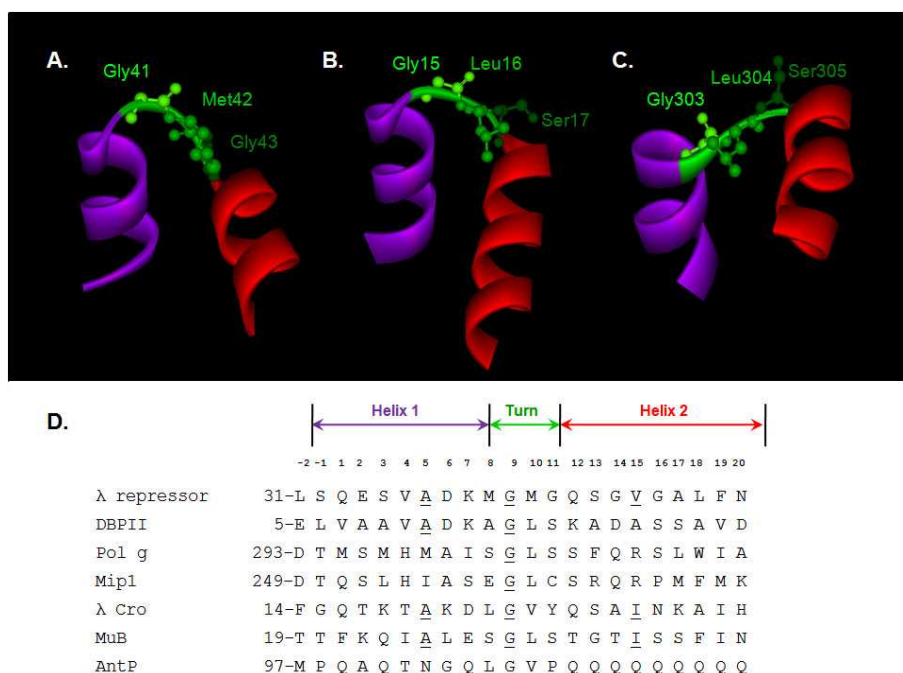


Figure 4.6. A putative HTH motif in the exonuclease module of polg? Structural comparison and amino acid sequence alignment of helix-turn-helix motif from several DNA-binding proteins and ‘orienter’ module from DNA polymerase gamma.

Architecture of the helix-turn-helix motif from **A.** λ repressor (pdb ID: 1LMB), **B.** DNA-binding protein II (prokaryotic protein Hu) from *Bacillus stearothermophilus* (pdb ID: 1HUU) and **C.** HTH-like ‘orienter’ module of human pol g (pdb ID: 3IKM). N-terminal helix (violet), turn (green) and C-terminal helix (red). The three amino acid residues of the turn element are indicated as ball & sticks atom representation. It must be noted that the angle of the two helices of human pol g module is more widely open than in the HTH motif of the two other proteins. Figures A.-B. were prepared by using the Accelrys DS Visualizer software. **D.** Multiple alignment of amino acid sequences of the HTH motif from five DNA-binding proteins and HTH-like ‘orienter’ module from yeast and human pol g. From the top: λ repressor (Pabo and Lewis, 1982), DNA-binding protein II (DBPII) from *Bacillus stearothermophilus* (Tanaka et al, 1984), human pol g catalytic subunit, yeast Mip1, Cro repressor (λ Cro) from bacteriophage λ (Anderson et al, 1981), Mu B transposition protein (Hung et al, 2000) and *Antennapedia* homeodomain (AntP) prokaryotic repressor (Qian et al, 1989), successively. Numbering at the top of alignment indicates position of amino acid residues.

This motif is a major DNA binding structural element found in hundreds of proteins, mainly in transcriptional factors from both prokaryotic and eukaryotic genomes, but also in proteins involved in DNA repair, DNA replication, RNA metabolism and in protein-protein interactions (Aravind *et al.*, 2005). The HTH motif is 20-25 amino acids long and is formed of two short α-helices (N-terminal and C-terminal) joined by a short amino acid segment (turn) which results in a sharp turn of the two helices. DNA sequence recognition is generally achieved by a network of hydrogen bonds and van der Waals contacts between the side chains of the protein, principally from the C-terminal helix of HTH motif, and a set of base pairs exposed within the major groove of the DNA (Brennan and Matthews, 1989). Thus the C-terminal helix, referred to as the ‘recognition helix’, protrudes from the protein surface adjusting to the DNA major groove, when it is complexed with DNA (Brennan and Matthews, 1989). The N-terminal helix stabilizes the structure.

Curiously, DNA does not interact with HTH motifs in the same manner in all DNA-protein complexes. Indeed, although the general structural topology is well maintained, HTH motifs derived from various DNA-binding proteins do not show strict conservation of their amino acid sequences and display distinct binding geometry (Anderson *et al*, 1987; Aggarwal *et al*, 1988; Jordan and Pabo, 1988; Wolberger *et al*, 1988; Otwinowski *et al*, 1988; Brennan and Matthews, 1989). (Fig.4.6.). However, some general rules in HTH motif amino acid sequence conservation can be observed (Brennan and Matthews, 1989): (i) residue 9 localized in the 'turn' should be a glycine; (ii) residues 4 and 15 should not be charged (iii) residues 3-8 and 15-20 should not be proline and (iv) residue 5 should not be a β -branched residue (Fig.4.6.).

The structural architecture and length of the 'orienter' module of pol g follow the definition of HTH motif (Fig.4.6.). Glycine is present at position 9 both in human pol g and yeast Mip1 (Fig.4.5.D.). Moreover, the amino acid sequence of pol g 'turn' element (GLS) is perfectly conserved with that of several proteins such as Mu phage DNA transposition protein B (MuB) and DNA-binding protein II (DBPII) from *Bacillus stearothermophilus* (Fig.4.6.D.). Noteworthy, all three residues of the turn element from human pol g, when substituted by a positively charged arginine, are associated with severe mitochondrial disorders (see Tables 2. and 4.). The second condition is fulfilled neither for human pol g nor for yeast Mip1, since both proteins have positively charged and strictly conserved histidine (position 4) and arginine (position 15) residues (Fig.4.6.D.). Moreover, we have shown that mutation of this arginine (R309 in human pol g) results in defective polymerase function. The third and fourth rules are fulfilled for human pol g but not abided by Mip1 or other fungi pol g homologs (proline at position 16 and isoleucine at position 5) (Fig.4.6.D.). However, these two rules are not followed in all HTH motifs and several exceptions can be observed (Brennan and Matthews, 1989).

4.4. Perspectives and conclusions.

Although, at the beginning of this work closely related projects (involving yeast genetics and biochemical analysis of pol g disease-associated mutations) were progressing, progressively we were able to discover new perspectives for our studies.

First of all, we have established a new model of coupling of polymerase and 3'-5' exonuclease functions of pol g. However, our model requires extensive structural and experimental testing. Evidence supporting a cooperation between the 'stabilizer' and 'orienter' modules might be obtained by biochemical analysis of pol g, truncated or mutagenized in those two structural elements of the DNA binding

channel, with special focus on those residues of the exonuclease domain that are in close proximity to the palm and fingers subdomains. Our model is based on *in silico* modeling of pol g structure with docked DNA substrate, using bacteriophage T7 DNA polymerase as a model. Therefore, there is a need for a crystal structure of human pol g complexed with DNA-primer template, dNTPs and metal ions to verify the structural bases of the mechanism of the DNA primer strand switching between the polymerase and 3'-5' exonuclease active sites. The three dimensional model of human pol g ternary complex could also explain some questionable differences in structure of polymerase and 3'-5' exonuclease active sites observed between pol g and other polA family polymerases. It may also give some light on structure of DNA-binding channel in closed, catalytically active pol g conformation. Moreover, the crystal structure of yeast Mip1 should be solved to provide information on possible differences in functional mechanisms between the yeast and human pol g. The three dimensional structure of Mip1 could also give more support for using yeast Mip1 as a structural and functional model for human pol g and its disease-associated mutations. Finally, a better knowledge of the protein members of the yeast mtDNA replisome could help understanding the impact of pol g mutations on mtDNA replication. Mass spectral analysis of pol g complexes isolated from blue native gels could bring information on composition of the yeast mitochondrial replisome. This approach is however difficult in yeast, due to the low abundance of endogenous Mip1.

It would also be interesting to determine whether defects in DNA replication produced by mutant Mip1 can be compensated by overexpression of genes involved in the nucleic acids function or in another function of the

mitochondrial metabolism. Increased temperature-sensitivity, presented in this work by *mip1*-L210P mutant, is a common trait of mutants involved in mitochondrial metabolism. The *mip1*-L210P mutant, transformed with a high copy genomic library, could be used to search for genes that in high copy number restore growth on glycerol at elevated temperature (37°C).

Finally, each year several novel disease-associated pol g alleles and compound heterozygous states are reported in patients suffering from pol g-related mitochondrial disorders. For many of these mutations, the genetic and biochemical analyses performed in the yeast model have proved to bring valuable information with respect to the structural and functional defects affecting pol g. However, as many residues are not strictly conserved between yeast and human and moreover, several fragments are not or poorly conserved, the yeast model has limits and it is necessary to develop animal models. These animal models are also essential to explain the specific role of pol g mutations with regards to tissue specificity, threshold effect in disease development, mitochondria-induced apoptosis and aging processes.

In conclusion, this work performed with yeast as a model system has revealed that dysfunction in the coordination of the coupling between polymerase and 3'-5' exonuclease functions is an important cause of human pol g-associated diseases. A 3'-5' exonuclease module localized in the DNA-binding channel and in close vicinity to a small fingers subdomain that partially blocks the DNA-binding channel plays a crucial regulatory role in this functional coupling

5.
M A T E R I A L S
A N D
M E T H O D S

5.1. Growth media:

Solid media were supplemented with 2% agar Aldrich.

Media for bacterial strains:

LB: 1% bacto-tryptone Difco, 0.5% yeast extract Kat, 0.5% NaCl

LB_{amp}: **LB** + ampicillin (75 mg/l)

Media for yeast strains:

YD: 2% yeast extract Kat, 2% glucose

YPD-ade: 2% peptone Difco, 1% yeast extract Difco, 2% glucose

GLY: 2% yeast extract Kat, 3% glycerol

G₀: 0.7% yeast nitrogen base without amino acids Difco, 2% glucose

G₀+HAL: G₀ supplemented with histidine (30 mg/l), adenine (40 mg/l), leucine (40 mg/l)

G₀+HAUL: G₀ supplemented with histidine (30 mg/l), adenine (40 mg/l), uracil (30 mg/l) and leucine (30 mg/l)

G₀+HAWL: G₀ supplemented with histidine (30 mg/l), adenine (40 mg/l), tryptophan (60 mg/l) and leucine (40 mg/l)

G₀+DROP OUT : G₀ supplemented with adenine (20 mg/l), alanine (20 mg/l), arginine (40 mg/l), asparagine (40 mg/l), aspartic acid (40 mg/l), glutamine (10 mg/l), glutamic acid (10 mg/l), glycine (40 mg/l), histidine (40 mg/ml), inositol (40 mg/ml), isoleucine (40 mg/ml), leucine (20 mg/l), lysine (40 mg/l), methionine (40 mg/l), phenylalanine (40 mg/l), proline (20 mg/l), serine (40 mg/l), threonine (40 mg/l), tyrosine (40 mg/l), valine (20 mg/ml) and tryptophan (40 mg/l) or uracil (20 mg/l).

ERY: 3% glycerol, 1% yeast extract difco, 2% peptone Difco, adenine 40mg/l, 50mM KH₂PO₄ pH 6.5, erythromycin 3g/l solubilized in 30 ml ethanol.

Raffinose drop-out medium: 2% raffinose, 0,7% yeast nitrogen base without amino acids Difco, 0.5% ammonium sulphate, and a mixture of amino acids: arginine (10 mg/l), methionine (10 mg/l), tyrosine (15 mg/l), lysine (15 mg/l), phenylalanine (25 mg/l), leucine (30 mg/l), aspartic acid (50 mg/l), glutamic acid (50 mg/l), valine (75 mg/l), threonine (100 mg/l), serine (187 mg/l), adenine (30 mg/l), tryptophan (30 mg/l) and histidine (40 mg/l).

Galactose drop-out medium: the composition is the same as raffinose drop-out medium but raffinose is replaced by galactose.

5.2. Strains and plasmids:

Table 8. Strains and plasmids

Strain	Genotype and characteristics	References
<i>E. coli</i>		
JM109	<i>RecA1 endA1 gyrA96 thi hadR17 sup44 relA1 Δ(lac-proAB F'[traΔ36 proAB lac19 lacZ M15]</i>	Yannish-Perron <i>et al.</i>
<i>S. cerevisiae</i>		
W303-1B/ <i>mip1Δ1-01</i>	<i>MATα ade2-1 his3-11,15 trp1-1 leu2-3,112 ura3-1 can1-100 bud4 rad5-535 mip1::URA3 rho⁰</i>	Foury, 1989
W303-1BΔ <i>MIPI::KAN^R</i>	as above but <i>MIPI::KAN^R, rho⁰</i>	Cochonneau, 2010

A25	<i>MATa ade2 leu2 ura3 trp1 nuc1 ::LEU2</i>	Lahaye <i>et al</i> , 1991
M17-162-4D	<i>MATa ade1 [mit^(cob2)]</i>	Slonimski and Tzagoloff, 1976
M17-40-5B	<i>MATa ade1 [mit^(cob1)]</i>	Slonimski and Tzagoloff, 1976
M9-94-4B	<i>MATa ade1 [mit^(cox2)]</i>	Slonimski and Tzagoloff, 1976
JC7	<i>MATa leu1 kar1-1 rho⁺</i>	Conde and Fink, 1976

Plasmid	Genotype and characteristics	References
pFL39	Low copy number centromeric plasmid derived from pUC19. It contains <i>TRP1</i> gene as selectable marker.	Lacroute
pFL39MIP1	As above but <i>MIP1</i> gene (3.8 kb) was inserted.	Foury
pFL38	Low copy number centromeric plasmid derived from pUC19. It contain <i>URA3</i> gene as selectable marker.	Lacroute
pFL38MIP1	As above but <i>MIP1</i> gene (3.8 kb) was inserted.	Cochonneau, 2010
pSK	High copy number bacterial plasmid. It contains <i>LacZ</i> , <i>MCS</i> , <i>ColE1 ori</i> and <i>AMP</i> .	Bluescript
pSKMIP1	As above but <i>MIP1</i> gene (3.8 kb) was inserted.	Foury
pGALZ3	High copy number yeast plasmid. It contains selectable	Lahaye <i>et al</i> , 1991

	marker <i>URA3</i> and <i>LacZ'</i> gene under the control of <i>GAL1</i> promoter.	
pGALMIP1	As above but <i>LacZ'</i> was replaced by <i>MIP1</i> gene.	Foury and Vanderstraeten, 1992

5.3. Construction of the *mip1* mutants:

The L210P, L260R, C261R, C261S, C261T, R265H(L), F268R and R467W *mip1* mutations were obtained by site-directed mutagenesis of the *MIP1*[Σ] allele (Baruffini *et al*, 2007) harbored by a low copy centromeric plasmid (pFL39*MIP1* or pFL38*MIP1*). The oligonucleotides used for site-directed mutagenesis are listed in Table 9.

For mutations L210P, L260R, R265L, R265H and F268R the mutagenized PCR-amplified fragment was digested with *NotI* and *BglII* and substituted for the wild-type fragment in the *MIP1* gene borne by the pSK*MIP1* plasmid. The *NotI*-*ApaI* fragment of the *mip1* gene harbored by pSK*MIP1* was subcloned into the centromeric pFL39*MIP1* plasmid (*TRP1* selectable marker).

For mutations C261R, C261T and C261S the mutagenized PCR-amplified fragment was digested with *NotI* and *BglII* and substituted for the wild-type fragment in the *MIP1* gene borne by the pGAL*MIP1* plasmid. The *NotI*-*NgoMIV* fragment of the *mip1* gene harbored by pGAL*MIP1* was subcloned into the centromeric pFL39*MIP1* plasmid.

For mutation R467W, the mutagenized PCR-amplified fragment was digested with *BglII*-*ApaI* and substituted for the wild-type fragment in

the *MIP1* gene borne by the pSK*MIP1* plasmid. The *NotI*-*ApaI* fragment of the *mip1*-R467W gene harbored by pSK*MIP1* was subcloned into the centromeric pFL39*MIP1* plasmid. The entire *mip1*-R467W gene was isolated by *SacI*-*SalI* restriction and subcloned into the centromeric pFL38*MIP1* plasmid (*URA3* selectable marker).

Plasmids containing *MIP1(mip1)* genes were used to transform the yeast haploid W303-1BΔ*MIP1 rho*⁰ strain. The heteroallelic *mip1*-F268R/*mip1*-R467W mutant was obtained by transforming the W303-1BΔ*MIP1* strain which was harboring the pFL38*mip1*-R467W plasmid and was *rho*⁺ with the pFL39*mip1*-F268R plasmid.

All mutagenized PCR-amplified *mip1* fragments were verified by DNA sequencing at each step of the constructs and also after introduction of the *mip1* alleles into yeast strains. The *pGALmip1* plasmids were obtained by subcloning the *NotI*-*BglII* fragment for L210P, L260R, R265L, R265H and F268R mutations and by subcloning the *NgoMIV*-*NotI* fragment for R467W mutation. After DNA sequence verification the A25 strain was transformed with the constructs.

Table 9. Oligonucleotides used for site-directed mutagenesis and DNA binding and other *in vitro* assays.

Primer	Sequence ^a	Orientation
L210Pa^b	GATCCTGCTGCGCCGATACCCCTAAACACATTG	Forward
L210Pb^b	GTTTAGGGGTATCGGCGCAGCAGGATCATCACC	Reverse
L260Ra	GCATCTTTCGGGCGGTGTTCAAGACAGCGT	Forward
L260Rb	CTTGAACACCGCCCGAAAGATGCAATATGC	Reverse
C261Ra	GCATCTTTCGGGCTGCGTTCAAGACAGCGT	Forward

C261Rb	TCTTGAACGCAGCCCGAAAGATGCAATATGC	Reverse
C261Sa	GCATCTTTCGGGCTGTCTTCAAGACAGCGT	Forward
C261Sb	TCTTGAAGACAGCCCGAAAGATGCAATATGC	Reverse
C261Ta	GCATCTTTCGGGCTGACTTCAAGACAGCGT	Forward
C261Tb	TCTTGAAGTCAGCCCGAAAGATGCAATATGC	Reverse
R265La	TGTTCAAGACAGCTTCCGATGTTTCATGAGA	Forward
R265Lb	ACATCGGAAGCTGTCTTGAACACAGCCC	Reverse
R265Ha	TGTTCAAGACAGCATCCGATGTTTCATGAAGA	Forward
R265Hb	GAACATCGGATGCTGTCTTGAACACAGCCCGAA	Reverse
F268Ra	CAGCGTCCGATGCGCATGAAGAATAACAAGAAA	Forward
F268Rb	ACAAGTTCTGTGCGCAGGCTACGCGTACTTCTTATTG	Reverse
R467Wa	CCAGAATGGTATTGGCAACTTTTTCCCTCAAAG	Forward
R467Wb	GAAAAAGTTGCCAATACCATTCTGGAAAGCCGGGCA	Reverse
mip1- 101^c	ATTGATGGTAGATTTGAATGCAT	Forward
mip1- 102^c	GAAATCCTATCAGTCTTATCAAG	Reverse
mip1- 106^d	TGTAGCGAATTTCACTGCAA	Forward
mip1- 107^d	ACGATTTGGTTAAAAGATTAGTAC	Reverse
A1^e	GACCCGATCTGATCCGATTCTG	
B1^e	ATCCAACCTCGCGTCGTATCGAATCGGATCAGATCGGG TCGTCAA	DNA binding
AB353^f	GTAAAACGACGGCCAGT	
AB354^g	GTAAAACGACGGCCAGA	

Table 8. Oligonucleotides used for site-directed mutagenesis and DNA binding.

^a Mutated nucleotides are in bold letters. ^b Overlapping primers used for mutagenesis. ^c Primers used for construction of the L210P, L260R, R265L, R265H and F268R mutations using the overlap extension technique. ^d Primers used for construction of the R467W mutation using the overlap extension technique. ^e Primers used for DNA binding activity assays. ^f Primer used for DNA polymerase assays, 3'-5' exonuclease assay, dNTP misincorporation assay and polymerase/ exonuclease coupling assay. ^g Primer containing a single mismatch at the 3' terminus used for 3'-5' exonuclease assay.

5.4. Cytoaduction.

Since the *Δmip1* strain cannot replicate mtDNA, it is devoid of mtDNA (*rho*⁰) and remains *rho*⁰ after transformation. It is therefore necessary to introduce mtDNA into cells by cytoaduction using a *kar1-1* strain. The cytoaduction procedure was as follows. JC7 strain was refreshed on glycerol rich plate (GLY) for two days at 28°C. Three independent *rho*⁰ transformants of W303-1BΔ*MIP1* containing plasmid pFL39*MIP1/mip1* (or pFL38 *MIP1/mip1*) and JC7 strain (from GLY) were refreshed on glucose rich plates (YD) for one day at 28°C. The two strains were crossed on YD plates and incubated 20 hours at 28°C. Subsequently, crossed strains were spread for single colonies on glucose rich low-adenine media (YD-ade) and incubated 3 days at 28°C. Colonies of different size and colour were produced. Average size, pink colonies were W303-1BΔ*MIP1* [pFL39(8) *MIP1/mip1*] *rho*⁺ haploid cytoductants. Three independent cytoductants of each mutant were used for phenotype analysis *in vivo*.

5.5. *In vivo* techniques.

5.5.1. Cellular respiration.

Three independent clones of the mutant and wild-type strains were refreshed on the G₀+HAUL/G₀+HAWL/G₀+HAL plates for 2 days at 28°C and then

on GLY plates for 2 days at 28°C. Refreshed cells were inoculated in liquid glucose rich precultures and incubated overnight at 28°C. Then, liquid glucose rich cultures were inoculated with defined volumes of precultures and run overnight at 28°C. Cells were collected and wet weight was determined. The uptake of oxygen was measured on cell suspensions with a Clark electrode. The level of cell respiration was estimated as a quantity of oxygen consumed by mg of yeast cell mass (dried weight) and by hour.

5.5.2. Frequency of $\rho^{-/0}$ mutants.

The frequency of ρ^{-} mutants was estimated as follows. Cells were grown for 2 days on GLY plates at 28°C. Subsequently, cells were replica-plated on glucose minimal synthetic (G_{0+HAUL} / G_{0+HAWL} / G_{0+HAL}) medium for 2 days at 28°C. Then, cells were replica-plated twice on YD and incubated for 1 day at 28°C, or 37°C. Suspensions from cells grown at each of temperature were spread on glucose minimal low adenine synthetic medium plates ($G_{0+DROP OUT}$) and incubated for 3-4 days at 28°C. The stability of mtDNA was determined by the percentage of $\rho^{-/0}$ colonies on the Petri dishes. The $\rho^{-/0}$ mutants were easily identified by the small size of their colonies and their white color in low adenine media. The respiratory proficient colonies were big and pink in contrast with $\rho^{-/0}$ mutants (Vanderstraeten *et al.*, 1998).

5.5.3. DAPI staining.

Selected yeast strains were refreshed on G_{0+HAUL} plates. After two days at 28°C, yeast cells were suspended in 50mM Tris-HCl pH 7.5 buffer and

sonicated (time 5s, amplitude 30%). After sonication yeast cells were treated with DAPI (4'-6-diamidino-2-phenylindole) and incubated on ice for 15 minutes. Several drops of cell suspension were deposited on microscope slides covered with 1.5% low melting point agarose. Samples were observed under the optic microscope using a DMR (Leica) microscope and a 100x oil immersion objective. Excitation was obtained with a mercury HBO 103 w/2 lamp (OSRAM) and observation was performed using a DAPI filter (Leica). The images were taken with a DC200 camera (Leica) and analysed with the Leica IM1000 software.

5.5.4. Retention of mtDNA in $\rho^{-/0}$ clones.

The test which is based on the high efficiency of homologous mtDNA recombination in diploids exploits the capacity of ρ^{-} genomes to restore *mit*⁻ mutations to wild-type in crosses between strains of opposite mating type (Baruffini *et al*, 2010). At least 250 $\rho^{-/0}$ colonies from each mutant and wild-type strain (from three independent transformants) were gridded on rich glucose medium-containing plates (YD) and cross-replica plated on lawns of *mit*⁻ strains harboring point mtDNA mutations at different loci (aM17-162-4D, aM7-40-5B and aM9-94-4B mutants in *cob2*, *cob1* and *cox2*, respectively). After 3-4 days at 28°C, restoration of wild-type phenotype in the diploids was determined by replica-plating on glycerol medium (GLY). The three markers tested correspond to the most frequently retained mtDNA fragments in ρ^{-} strains, and thus the clones that did not retain any of these markers have little chance to contain another mtDNA fragment and are most likely ρ^0 clones (Baruffini *et al*, 2010).

5.5.5. Frequency of point mutations in mtDNA.

Qualitative test:

To get a rough estimation of the frequency of E^R point mutations in the mtDNA of *mip1* mutants, 10 independent colonies of wild-type and mutant strains were spread on standardized squares in a Petri dish containing synthetic glucose medium (G_{0+HAUL}/G_{0+HAWL}/G_{0+HAL}) and after 2 days of growth they were successively replica-plated on rich glycerol (GLY) and glucose-containing (YD) media and ERY medium. Results were analyzed after 8 days of incubation at 28°C.

Quantitative test:

Three independent clones of mutant and wild-type strains were spread for single colonies on synthetic glucose-containing plates (G_{0+HAUL}/G_{0+HAWL}/G_{0+HAL}) for 2 days at 28°C. Ten independent colonies from each clone were inoculated in 2.5 ml of liquid glycerol medium and incubated for 48 hours at 28°C. Cells from 1.5 ml of the liquid cultures were harvested by centrifugation, spread on plates containing erythromycin (concentration: 3mg/ml) and incubated for 8 days at 28°C. The number of E^R colonies on each plate was determined and the mutator activity was estimated as the average number of E^R mutants in 1.5 ml of culture. To measure *rho*⁻⁰ frequency in the liquid cultures, 100 µl of the cell suspensions were serially diluted, spread for single colonies on G_{0+DROP OUT} plates and incubated for 3-4 days at 28°C. The fraction of white colonies in the total colony population was determined.

5.6. Preparation of the mitochondrial extracts.

A25 strains harboring the multicopy plasmid-borne *MIPI* or *mipI* gene under the control of *GALI* promoter were first grown on raffinose drop-out medium for 20 hours at 28°C. 50 ml of this preculture was used to inoculate 1 liter of galactose drop-out medium and cells were grown for 15 hours at 28°C. Approximately 8-10 g cells were obtained from 1 liter of culture. These cells were successively incubated for 30 min at 28°C in 100 ml of a solution containing 25 mM EDTA pH 8.0 and 1% β -mercaptoethanol, and for 60 min at 37°C in 100 ml of a solution containing 1.2 M sorbitol, 50 mM sodium phosphate pH 7.5 and 50 mg Zymolyase 20T (Seikagaku Corp.) to obtain spheroplasts. After 30 and 45 min, 0.75 ml of 200 mM phenyl methyl sulfonyl fluoride (PMSF) solubilized in dimethyl sulfoxide were added to decrease proteolysis. The spheroplasts were collected by centrifugation and washed twice in 1.2 M sorbitol. The spheroplasts were suspended in 80 ml of an ice-cold lysis medium containing 0.6 M sorbitol, 50 mM imidazole pH 7.0, 0.1% BSA (fatty acid free) and 2 mM PMSF and they were lysed using the maximum speed of a Waring blender for 45 s. After several low and high speed centrifugations the mitochondrial pellet was obtained and resuspended in an equal volume of an ice-cold solution containing 20 mM Tris-HCl pH 8.5, 10 mM dithiotreitol (DTT) and 2 mM PMSF. After a 1-min vortex treatment 0.125 ml of 4 M NaCl was added to 1 ml of suspension and suspensions were vortexed for 2 additional min. The soluble extracts were obtained by centrifugation at 100 000 x g for 60 min, frozen in liquid nitrogen and stored at -70°C.

5.7. Partial purification of Mip1 protein with DEAE chromatography.

The mitochondrial extract was 10-fold diluted in a solution containing 25 mM MOPS pH 7.0, 80 mM NaCl, 5 mM DTT, 1 mM PMSF, and 1 ml of this diluted extract was applied to a DEAE-silica gel column (Nucleobond AX-100, Marcherey-Nagel). The column was washed with 10 ml of 180-mM NaCl-containing buffer and the Mip1 protein was eluted in the presence of 400 mM NaCl. This fraction contains negligible nuclease activities as shown by the absence of DNA degradation in extracts of 3'-5' exonuclease deficient *mip1* mutants (Foury and Vanderstraeten, 1992). After galactose induction Mip1 is 50-100 fold overexpressed. It has previously been shown that in a mitochondrial extract containing a 30-40 fold overexpressed Mip1 protein the contamination by nuclear polymerases is negligible (Foury, 1989). All fractions were collected and the presence of Mip1 was verified by silver nitrate and Coomassie blue staining. Protein concentration was determined with Bradford assay (Bradford, 1976) using BSA (Pierce, 2 mg/ml) as a standard.

5.8. SDS-PAGE, western blot analysis and quantification of Mip1.

The mitochondrial extracts or DEAE chromatography-purified fractions were solubilized in loading buffer (0.24 M Tris, 6% SDS, 30% glycerol, 3%DTT, 0.005% bromophenol blue, pH 6.8) in proportion 2:3 and incubated 15 min at 56°C before loading on the gel and electrophoresis in 7% SDS polyacrylamide gel. SDS-PAGE was used for western blot analysis (see below), silver-nitrate (Wray *et al.*, 1981), Coomassie blue or colloidal Coomassie blue staining.

After SDS-PAGE proteins were transferred to a nitrocellulose membrane. Blots were saturated and incubated 1 hour at room temperature and then overnight at 4°C with a rabbit polyclonal antibody (750x diluted) raised

against Mip1. Subsequently, blots were incubated for 1 hour with an anti-rabbit secondary antibody conjugated to peroxidase (14700 x dilution). Mip1 protein was detected using BM Chemiluminescence Blotting Substrate kit (POD) from Roche.

Polyclonal Mip1-antibodies (Vanderstraeten and Foury, unpublished data) were obtained in rabbit using a fusion protein prepared as follows. The C-terminal part of Mip1 (residues 432-1254) was fused behind the glutathione-*S*-transferase and the fusion protein was expressed in *E. coli*, isolated in inclusion bodies and after SDS solubilization and separation of the proteins in 7% SDS-PAGE, a Mip1-containing acrylamide slice was excised from the gel, soaked in water, carefully crushed in 0.9% NaCl and injected to a rabbit.

To compare the relative wild-type and mutant Mip1 levels, increasing amounts of protein were subjected to 7% SDS-PAGE and the signals were detected by western blotting using a polyclonal antibody raised against Mip1 or by colloidal Coomassie blue staining. Mip1 protein levels were determined using Kodak Image Station 4000 R software. The concentration (ng/μl) of reference wild-type Mip1 was estimated after SDS-PAGE and colloidal blue staining of the gel using known amounts of bovine serum albumin (Pierce) as a reference.

5.9. Preparation of radiolabeled singly primed M13 (or PTZ18U) DNA substrate for *in vitro* assays.

To obtain PTZ18U DNA, the JM109 *E. coli* strain harboring PTZ18U plasmid was induced with IR408 helper phage. The PTZ18U (or M13) DNA was isolated and purified with a DEAE-silica gel column (Nucleobond AX-500, Marcherey-Nagel). The AB353 and AB354 oligonucleotide primers

(200 ng) (Table 9.) were labeled at their 5'-end with [γ - ^{32}P]ATP (300 Ci/mmol) and T4 polynucleotide kinase in a 25- μl reaction mix (final volume) at 37°C for 40 min. After heat inactivation of the T4 polynucleotide kinase the [^{32}P]-labeled primers were hybridized with M13 (or PTZA18U) single-stranded DNA (3-4 μg) in a 50- μl hybridization mix (final volume) containing 10 mM Tris-HCl, pH 7.5/100 mM KCl (5 min at 75°C) and the solution was slowly cooled to room temperature. Hybridization medium was then loaded on a 1-ml CL-4B sepharose column equilibrated with 20 mM Tris-HCl pH 7.5/ 80 mM NaCl. Fractions of two drops were collected. The first two highly radioactive fractions were selected (~200 μl). Singly-primed single-stranded M13 (or PTZ18U) substrate was stored at 4°C.

5.10. DNA polymerase assays.

5.10.1. Gap-filling activity.

To determine gap-filling activity the reaction medium (100 μl) contained 20 mM Tris-HCl pH 8.0, 2 mM DTT, ~200 $\mu\text{g}/\text{ml}$ DNase I-activated calf-thymus DNA as the substrate, 25 μM of each dATP, dGTP, dCTP and 5 μM [^3H]-dTTP (3000 cpm/pmol). The reactions performed at 28°C were initiated by adding equal amounts of wild-type and mutant Mip1 and aliquots of 45 μl were removed after 4 and 8 min. Aliquots were put on Whatman DE81 filters that were dipped in 7% cold trichloroacetic acid (TCA), washed three times with TCA and twice with Norvanol (83.5% ethanol and 2.5% ether). The radioactivity present in the acid-precipitated material was counted in a liquid scintillation counter. Values were expressed as a percentage of the wild-type Mip1 activity (100%).

5.10.2. Total DNA synthesis.

Total DNA synthesis was determined on ssDNA primed with the AB353 17-mer oligonucleotide (see Table 9.). An excess DNA was used to prevent as much as possible the reassociation of the polymerase with the same DNA molecule after a dissociation event. The reaction mixture contained 20 mM Tris-HCl pH 7.5, 5 mM DTT, 20 mM MgCl₂, 25 μM of each dGTP, dCTP and dTTP, 5 μM [α -³²P]dATP (3000 Ci/mmol) and ~10 μM M13 DNA primed with the AB353 oligonucleotide. Reactions were initiated by adding equal amounts (1.25 and 2.5 ng) of wild-type and mutant Mip1 diluted in ice-cold buffer containing 20 mM Tris-HCl pH 7.5/ 80 mM NaCl. Reactions were stopped with 80% formamide/ 1 % sodium dodecylsulfate (SDS)/ 10 mM EDTA, pH 8.0 and the products were analyzed by electrophoresis in 15% polyacrylamide/7M urea gel. Migration was performed in 30 cm long gels at 1300 V until bromophenol blue reached 8 cm. Gels were fixed with 40% Norvanol/ 7% acetic acid and dried. At this high acrylamide concentration and after 8-cm bromophenol blue migration, DNA was visualized as a single band whose intensity can be quantified with a PhosphorImager.

5.10.3. Processivity of DNA synthesis.

Processivity was determined on ssDNA primed with the 17-mer AB353 oligonucleotide. An excess DNA was used to prevent as much as possible the reassociation of the polymerase with the same DNA molecule after a dissociation event. The reaction mixture contained 20 mM Tris-HCl pH 7.5, 5 mM DTT, 20 mM MgCl₂, 25 μM of each dATP, dGTP, dCTP and dTTP and ~10 μM PTZ18U ssDNA (2.8 kb) primed with a 5'-end [³²P]-labeled AB353 oligonucleotide as the substrate. The reactions performed at 28°C were initiated by adding equal amounts (1.25 and 2.5 ng) of wild-type and

mutant Mip1 diluted in ice-cold buffer containing 20 mM Tris-HCl pH 7.5/ 80 mM NaCl and carried on for 5 and 10 minutes. Reactions were quenched in a 1/5 volume of a buffer containing 1 mg/ml Proteinase K/ 5% SDS/ 100 mM EDTA, pH 8.0. DNA was extracted from the samples with phenol equilibrated with 50 mM Tris-HCl pH 8.0 and denaturated in a buffer containing 100 mM NaOH/ 4 mM EDTA and 8% glycerol. Samples were electrophoresed for 4-5 hours in 1% alkaline agarose gel in a buffer containing 50 mM NaOH/ 1 mM EDTA pH 8.0. After electrophoresis gels were fixed in 7% TCA and dried between thick paper towels. The radioactivity from the full-length DNA product and from all DNA primers that have been initiated were visualized and quantified with PhosphorImager.

5.11. 3'-5' exonuclease and misincorporation assays.

3'-5' exonuclease activity was determined using PTZU18 DNA primed with a 5'-end [³²P]-labeled oligonucleotide which was either complementary to PTZ18U (AB353) or contained a single A:A mispairing at its 3' end (AB354) (Table 9.). The assay mixture contained 20 mM Tris-HCl pH 7.5, 5 mM DTT, ~10 μM DNA substrate and unless otherwise indicated 50 mM MgCl₂. The reactions were initiated by adding equal amounts (2.5 ng) of wild-type and mutant Mip1 diluted in ice-cold buffer containing 20 mM Tris-HCl pH 7.5/ 80 mM NaCl. Reactions were quenched after 2, 4, 8 and 12 min at 25°C with an equal volume of 80% formamide/ 1 % SDS/ 10 mM EDTA, pH 8.0 and the samples were electrophoresed in 15% polyacrylamide/7 M urea gels. Migration was performed in 30 cm long gels at 1300 V until the bromophenol blue reached the bottom of

the gel. Gels were fixed with 40% Norvanol/ 7% acetic acid and dried. The degradation products were visualized and quantified using a PhosphorImager. The intensity (I) of each band (I_{16} for the 16-mer, I_{15} for the 15-mer, I_{14} for the 14-mer, and I_{13} for the 13-mer) was determined and the total degradation was calculated using the following algorithm: $I_{16} \times 1 + I_{15} \times 2 + I_{14} \times 3 + I_{13} \times 4 + \dots + I_x \times n$, where $n=1$, $n=2$, $n=3$ and $n=4$ are the number of excision events giving rise to the 16, 15, 14 and 13 mers, respectively. Activities were expressed as a percentage of the wild-type activity (100%).

The misincorporation assay was performed as above using the 5'-end [^{32}P]-labeled AB353 oligonucleotide, 10 mM MgCl_2 and increasing dGTP concentrations with a 3-min incubation time.

5.12. Gel electrophoretic mobility shift assay.

DNA binding activity of wild-type and mutant Mip1 was determined by gel electrophoretic mobility shift assay (EMSA). The substrate was a [^{32}P]-labeled 21/45 mer (A1/B1) primer-template (Table 9.). The A1 oligonucleotide (30 pmol in a final volume of 25 μl) was labeled at its 5'-end with [γ - ^{32}P]ATP (3000 Ci/mmol) and T4 polynucleotide kinase at 37°C for 40 min and T4 polynucleotide kinase was inactivated for 5 minutes at 65°C. Free [γ - ^{32}P]ATP was removed from the labeled A1 oligonucleotide with Bio-spin P-6 chromatography columns (Biorad) by a 4-min centrifugation at 1000 x g. Oligonucleotide recovery from Bio-spin P-6 column was >90%. The [^{32}P]-labeled A1 oligonucleotide was hybridized with 180 pmol of B1 oligonucleotide in a buffer containing 10 mM Tris-HCl pH 8.0/ 150 mM NaCl and 30 mM

sodium citrate (final volume of 75 μ l) at 80-85°C for 3 min and the solution was slowly cooled to room temperature. The A1/B1 substrate was stored at 4°C. The binding mixture (9 μ l) contained 10 mM Tris-HCl pH 8.0, 5 mM DTT, 90 mM NaCl, 0.05 pmol primer-template DNA and when indicated 3 mM MgCl₂. The assay performed at 28°C was initiated by adding 3 μ l of wild-type or mutant Mip1 (~6 ng protein for DNA binding activity in the presence and absence of 3 mM MgCl₂, and 0.6, 0.9, 1.25, 1.8, 2.5, 5 and 12.5 ng for DNA binding activity as a function of protein concentration). Mip1 was diluted 25 times in an ice-cold buffer containing 10 mM Tris-HCl pH 8.0 and 5 mM DTT. After 2 or 3 min, 3 μ l of loading buffer (0.1 % bromophenol blue, 50% glycerol) was added to the samples which were placed on ice and immediately electrophoresed at 4°C in a 6% native (45 mM Tris-borate pH 8.3, 5 mM DTT and 1 mM EDTA) polyacrylamide (1:37.5) gel (20 x 30 x 0.1 cm) in a buffer containing 45 mM Tris-borate pH 8.3, 0.5 mM DTT and 1 mM EDTA. Gels and buffers were precooled at 4°C. A pre-run was performed at 100 V for 30 minutes. The buffer was changed after the pre-run before loading the samples. The products of the dried gel were visualized and quantified with a PhosphorImager.

5.13. Polymerase/ 3'-5' exonuclease coupling assay.

The reaction mixture contained 20 mM Tris-HCl pH 8.0, 5 mM DTT, 20 mM MgCl₂, ~10 μ M PTZ18U ssDNA primed with a 5'-end [³²P]-labeled AB353 oligonucleotide and increasing dNTP concentrations. All assay and electrophoresis conditions were those of the 3'-5'

exonuclease assay. Samples were electrophoresed in two independent gels to observe the polymerization mode after an 8-cm migration of bromophenol blue and the exonuclease mode after a 28 cm migration of the dye. The products of the reactions were visualized with a PhosphorImager.

5.14. Optimal conditions for 3'-5' exonuclease and polymerase assays.

The optimal salt conditions were selected from activity curves obtained by increasing MgCl_2 or NaCl concentrations (Foury, unpublished data). In brief, optimal exonuclease and gap-filling activities are obtained in the presence of 50 mM MgCl_2 , or 10 mM MgCl_2 plus 100 mM NaCl , and the optimal DNA synthesis activity on a primer-template substrate is obtained in the presence of 20 mM MgCl_2 plus 10-30 mM NaCl .

5.15. Presentation of the structural data.

The bacteriophage T7 DNA polymerase (pdb ID: 18TE) and pol g (pdb ID: 3IKM) structures were superimposed by using the UCSF Chimera software. Distances were measured using Swiss-Pdb Viewer and UCSF Chimera softwares. Figures were prepared with the UCSF Chimera, Accelrys DS Visualizer or Swiss-Pdb Viewer softwares. The Swiss-Pdb Viewer software was used to estimate the distance between each residue of the exonuclease module and the polymerase or exonuclease active site. The distance corresponds to the average distance of Leu304, Arg309 and Trp312 from each of Asp890,

Asp1135 and Glu1136 (polymerase) and Asp198, Glu200, Asp274 and Asp399 (3'-5' exonuclease) residues.

6. APPENDIX

App. 1. *MIP1* sequence and translation.

MIP1 *S.cerevisiae*

Accession number of nucleotide seq: J105117.1 april 1994

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1      atgacgaaattgatggtagatttgaatgcatgctgcgaatggcgcgcgccgctg
1      M T K L M V R F E C M L R M V R R R P L

61     cgtgtgcagttttgtgctcgatgggttctccacaaagaagaatgccgcagaagcaccagg
21     R V Q F C A R W F S T K K N A A E A P R

121    attaatcctgtggggatacagtatttaggtgagtccttgcaaagacaggtatttgtagt
41     I N P V G I Q Y L G E S L Q R Q V F G S

181    tgcggtggtaaagatgaggtggagcaaagcgacaaactgatggagtatcgaaaaagtcg
61     C G G K D E V E Q S D K L M E L S K K S

241    ctaaaggaccatgggttgtgggggaagaagacgctcatcacggatccaatatcgtttcct
81     L K D H G L W G K K T L I T D P I S F P

301    ctgccgccactacaaggcaggtcgctagatgagcatttccagaagattggcggtttcaat
101    L P P L Q G R S L D E H F Q K I G R F N

361    tctgaaccgtacaagagtttttgcgaggacaagttcacggagatggtggcacgaccggcg
121    S E P Y K S F C E D K F T E M V A R P A

421    gaatggctgcggaagccaggtgggttaaatacgtacctggaatggcacccgctcgaagtg
141    E W L R K P G W V K Y V P G M A P V E V

481    gcatacccagacgaagagctgggtggtgtttgatgtagaaacactctataacgtctctgac
161    A Y P D E E L V V F D V E T L Y N V S D

541    tatccgactttggccacggccttgtcatcgacggcggttacctttggtgttcgccgttc
181    Y P T L A T A L S S T A W Y L W C S P F

601    atatgtggtggtgatgatcctgctgcgctgatacccctaacaacattgaataaagagcaa
201    I C G G D D P A A L I P L N T L N K E Q

661    gtagtaattggtcacaatgttgcgtatgatagggcacgggttctcgaagaatacaacttc
221    V V I G H N V A Y D R A R V L E E Y N F

721    agggactcgaaggcggtttttcctcgacactcaatctctgcatattgcatctttcgggctg
241    R D S K A F F L D T Q S L H I A S F G L

781    tgttcaagacagcggtccgatgttcatgaagaataacaagaaaaagaggcagaagtggaa
261    C S R Q R P M F M K N N K K K E A E V E

841    tcggaagtacatccagaaatttccatagaagattatgacgacccttggttaaatgtgtct
281    S E V H P E I S I E D Y D D P W L N V S

901    gctttaaactcgctaaggatgtagcgaaaatttctactgcaagatagatcttgataagact
301    A L N S L K D V A K F H C K I D L D K T

961    gatagagatttctttgcttccacagataaatcaacgataatagaaaatttccaaaaactg
321    D R D F F A S T D K S T I I E N F Q K L

1021   gtcaactactgcgccactgacgtaacggccacaagccaagtgtttgataagattttccct
341    V N Y C A T D V T A T S Q V F D K I F P

1081   gttttcctgaaaaagtgctcctcatccagtttctggttgagggttaaaagtccttaagcaaa
361    V F L K K C P H P V S F A G L K S L S K
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1141 tgcattctgccgacgaagcttaacgactggaatgattacttgaacagctctgagtcctttg
 381 C I L P T K L N D W N D Y L N S S E S L

 1201 taccaacagtccaaagtccaaatagaatccaaaattgttcaaatcataaaagatatcgtc
 401 Y Q Q S K V Q I E S K I V Q I I K D I V

 1261 ctgttgaaggacaaaccagatTTTTtctcaaggatccttggttatcacaattggattgg
 421 L L K D K P D F Y L K D P W L S Q L D W

 1321 accaccaaacttttaagattgacaaagaaagggtgttcctgcaaagtgtaaagctgccc
 441 T T K P L R L T K K G V P A K C Q K L P

 1381 ggctttccagaatggtatagacaactTTTTccctcaaaggacactgtagaacctaaagatc
 461 G F P E W Y R Q L F P S K D T V E P K I

 1441 actattaaatcaagaataattccgattttgttcaagttatcggtgggagaattccccgta
 481 T I K S R I I P I L F K L S W E N S P V

 1501 atatggtcgaaggagctctggatggtgcttcaacgtgccccatgaacaagtagaaacatac
 501 I W S K E S G W C F N V P H E Q V E T Y

 1561 aaggctaaaaattatgttttggcagatagtgtatctcaagaggaagaggaaataaggatg
 521 K A K N Y V L A D S V S Q E E E E I R M

 1621 aataacctaggattgcaatgcacaggtgttcttttcaaagtacctcatcctaattgggccc
 541 N N L G L Q C T G V L F K V P H P N G P

 1681 acttttaattgtactaatcttttaaccaaactcgataaccatttttttgaaggcgta
 561 T F N C T N L L T K S Y N H F F E K G V

 1741 ctaaaatctgagtcagaattggctcatcaggctttacaaattaactcttcaggttcttat
 581 L K S E S E L A H Q A L Q I N S S G S Y

 1801 tggatgtcagcaagggaagaatccaatcccaatttgtagtccccaattgtaaattccct
 601 W M S A R E R I Q S Q F V V P N C K F P

 1861 aatgagttccagtcctttgtccgcaaaatcgctgttaaataatgagaagacaaatgatctt
 621 N E F Q S L S A K S S L N N E K T N D L

 1921 gccataattataccgaaaatcgctcccatgggcacaatcactagaagagctgtggaaaat
 641 A I I I P K I V P M G T I T R R A V E N

 1981 acgtggtaaccgcatccaatgcaaaagcaaataggataggttccgaattgaaaactcaa
 661 T W L T A S N A K A N R I G S E L K T Q

 2041 gtcaaagctcctccaggttattgttttgttggggcgagcgtggatagtgaagaattgtgg
 681 V K A P P G Y C F V G A D V D S E E L W

 2101 atagcatcttttagtcggtgactccattttcaatgttcatggtggtaccgccattggttg
 701 I A S L V G D S I F N V H G G T A I G W

 2161 atgtgcttagaaggcactaaaaacgaaggtacagatttgcacacgaagactgctcaaatt
 721 M C L E G T K N E G T D L H T K T A Q I

 2221 ttgggggtttctcgtaatgaggcgaaaatttttaattatggtagaatttacggcgctgg
 741 L G C S R N E A K I F N Y G R I Y G A G

 2281 gctaaatttgcgagtcagttactttaaagggttaaccatctctaactgatgaagagaca
 761 A K F A S Q L L K R F N P S L T D E E T

 2341 aaaaaaattgcgaataagttatatgaaaatacgaaaggtaaaacgaaaagatcaaaatta

781 K K I A N K L Y E N T K G K T K R S K L
 2401 ttcaaaaaatTTTggtatggtggatctgagTcaattctattcaataaattagaaagcatc
 801 F K K F W Y G G S E S I L F N K L E S I
 2461 gcggaacaagaaactccaaagacaccggtactgggatgtggtattacctattcacttatg
 821 A E Q E T P K T P V L G C G I T Y S L M
 2521 aagaaaaatctgagggcgaattcctttctaccttcaagaatcaattgggccatccagtca
 841 K K N L R A N S F L P S R I N W A I Q S
 2581 tctggagtggattatctacatcttctttgttgcTccatggaatatattattaagaaatat
 861 S G V D Y L H L L C C S M E Y I I K K Y
 2641 aaccttgaggaagcctttgcatttccatccatgatgagattagatttttggTgagcgaa
 881 N L E A R L C I S I H D E I R F L V S E
 2701 aaggacaaatacagagctgctatggcttttgcaaTcagcaacatatggacgagagcaatg
 901 K D K Y R A A M A L Q I S N I W T R A M
 2761 ttttgccagcaaTggggataaatgaattaccgcaaaactgtgcctttttctcgcaagta
 921 F C Q Q M G I N E L P Q N C A F F S Q V
 2821 gatattgattctgtcatacgtaaagaagtcaatatggactgcataacccctcgaacaaa
 941 D I D S V I R K E V N M D C I T P S N K
 2881 accgccattcctcatggggaggcgcttgatatcaatcaactgctagacaaacccaatagT
 961 T A I P H G E A L D I N Q L L D K P N S
 2941 aaattgggtaaaccaagtctcgatatcgacagcaaagtatcacaatatgcctataactac
 981 K L G K P S L D I D S K V S Q Y A Y N Y
 3001 agagaacctgtatttgaagaatataataaatcttatactccagagtTcttaaaatatttt
 1001 R E P V F E E Y N K S Y T P E F L K Y F
 3061 cttgcgatgcaagTccagtcagataagcgcgatgtgaatcggtagaagatgagtatctg
 1021 L A M Q V Q S D K R D V N R L E D E Y L
 3121 cgggagtgtacatccaaagaatacgctagagatgggaacactgcagattacagcctcta
 1041 R E C T S K E Y A R D G N T A E Y S L L
 3181 gactatataaaggatgtcgagaaggcTaaaaggactaaagtacgtattatgggatccaat
 1061 D Y I K D V E K G K R T K V R I M G S N
 3241 tttttagatggtaccaaaaatgccaaagctgatcaacgaatcagattacctgtcaatatg
 1081 F L D G T K N A K A D Q R I R L P V N M
 3301 ccagattatcccacacttcataaaattgctaacgattcagcaattccagaaaagcaattg
 1101 P D Y P T L H K I A N D S A I P E K Q L
 3361 ctggagaacaggagaaagaaggagaaacaggatagatgacgaaaataagaaaaattaacc
 1121 L E N R R K K E N R I D D E N K K K L T
 3421 agaaaaaagaataccacgcctatggaaaggaagtacaaaagagtgtatggtggcaggaag
 1141 R K K N T T P M E R K Y K R V Y G G R K
 3481 gcatttgaagcgttctatgaatgtgcaaataaaccattagactatactttgggagacagaa
 1161 A F E A F Y E C A N K P L D Y T L E T E
 3541 aaacaattctttaatattcctatagatggagtgattgacgacgttctgaacgacaaatct
 1181 K Q F F N I P I D G V I D D V L N D K S

3601 aattataaaaagaaaccatcacaaagcaagaacggcatcatcatcgccaataagaaagact
 1201 N Y K K K P S Q A R T A S S S P I R K T

 3661 gcgaaagcagtacattccaaaaaattgccggcaaggaagtcaagcactacaaatagaaat
 1221 A K A V H S K K L P A R K S S T T N R N

 3721 ttggttgagctggaaaggacattactatcttagagagtactag
 1241 L V E L E R D I T I S R E Y -

7. REFERENCES

Aggarwal AK, Rodgers DW, Drottar M, Ptashne M, Harrison SC. (1988) Recognition of a DNA operator by the repressor of phage 434: a view at high resolution. *Science*. **242**: 899-907

Agostino A, Valletta L, Chinnery PF, Ferrari G, Carrara F, Taylor RW, Schaefer AM, Turnbull DM, Tiranti V, Zeviani M (2003) Mutations of ANT1, Twinkle, and POLG1 in sporadic progressive external ophthalmoplegia (PEO). *Neurology* **60**: 1354-1356

Aknin-Seifer IE, Touraine RL, Lejeune H, Jimenez C, Chouteau J, Siffroi JP, McElreavey K, Bienvenu T, Patrat C, Levy R. (2005) Is the CAG repeat of mitochondrial DNA polymerase gamma (POLG) associated with male infertility? A multi-centre French study. *Hum. Reprod.* **20**: 736-740.

Anderson WF, Ohlendorf DH, Takeda Y, Matthews BW. (1981) Structure of the cro repressor from bacteriophage lambda and its interaction with DNA. *Nature*. **290**: 754-758.

Anderson JE, Ptashne M, Harrison SC. (1987) Structure of the repressor-operator complex of bacteriophage 434. *Nature*. **326**: 846-852.

Andersson SG, Kurland CG. (1998) Reductive evolution of resident genomes. *Trends. Microbiol.* **6**: 263-268

Anderson S, Bankier AT, Barell BG, de Bruijn MH, Coulson AR, Drouin J, Eperon IC, Nierlich DP, Roe BA, Sanger F, Schreier HP, Smith AJ, Staden R, Young IG, (1981) Sequence and organization of the human mitochondrial genome. *Nature*. **290**: 457-465

Aravind L, Anantharaman V, Balaji S, Babu MM, Iyer LM. (2005) The many faces of the helix-turn-helix domain: transcription regulation and beyond. *FEMS Microbiol. Rev.* **29**: 231-262.

Ashley N, O'Rourke A, Smith C, Adams S, Gowda V, Zeviani M, Brown GK, Fratter C, Poulton J (2008) Depletion of mitochondrial DNA in fibroblast cultures from patients with POLG1 mutations is a consequence of catalytic mutations. *Hum. Mol. Genet.* **17**: 2496-2506

Autexier C, Lue NF. (2006) The structure and function of telomerase reverse transcriptase. *Annu. Rev. Biochem.* **75**: 493-517.

Baasner A, Schäfer C, Junge A, Madea B. (1998) Polymorphic sites in human mitochondrial DNA control region sequences: population data and maternal inheritance. *Forensic. Sci. Int.* **98**: 169-178.

Bacman SR, Williams SL, Moraes CT. (2009) Intra- and inter-molecular recombination of mitochondrial DNA after in vivo induction of multiple double-strand breaks. *Nucleic. Acids. Res.* **37**: 4218-4226.

Baltimore D (1970) RNA-dependent DNA polymerase in virions of RNA tumor viruses. *Nature.* **226**: 1209-1211.

Baruffini E, Lodi T, Dallabona C, Puglisi A, Zeviani M, Ferrero I (2006) Genetic and chemical rescue of the *Saccharomyces cerevisiae* phenotype induced by mitochondrial DNA polymerase mutations associated with progressive external ophthalmoplegia in humans. *Hum. Mol. Genet.* **15**: 2846-2855

Baruffini E, Ferrero I, Foury F (2007 A) Mitochondrial DNA defects in *Saccharomyces cerevisiae* caused by functional interactions between DNA polymerase gamma mutations associated with disease in human. *Biochim. Biophys. Acta.* **1772**: 1225-1235

Baruffini E, Lodi T, Dallabona C, Foury F (2007 B) A single nucleotide polymorphism in the DNA polymerase gamma gene of *Saccharomyces cerevisiae* laboratory strains is responsible for increased mitochondrial DNA mutability. *Genetics* **177**: 1227-1231

Baruffini E, Ferrero I, Foury F (2010 A) In vivo analysis of mtDNA replication defects in yeast. *Methods* **51**: 426-436

Baruffini E, Horvath R, Dallabona C, Czermin B, Lamantea E, Bindoff L, Invernizzi F, Ferrero I, Zeviani M, Lodi T. (2010 B) Predicting the contribution of novel POLG mutations to human disease through analysis in yeast model. *Mitochondrion*. Sep 29 doi:10.1016/j.mito.2010.09.007

Battersby BJ, Loredó-Osti JC, Shoubridge EA. (2003) Nuclear genetic control of mitochondrial DNA segregation. *Nat. Genet.* **33**: 183-186.

Bendich AJ. (2010) The end of the circle for yeast mitochondrial DNA. *Mol. Cell.* **39**: 831-832

Berman AJ, Kamtekar S, Goodman JL, Lázaro JM, de Vega M, Blanco L, Salas M, Steitz TA. (2007) Structures of phi29 DNA polymerase complexed with substrate: the mechanism of translocation in B-family polymerases. *EMBO J.* **26**: 3494-3505.

Bernad A, Blanco L, Lázaro JM, Martín G, Salas M. (1989) A conserved 3'--5' exonuclease active site in prokaryotic and eukaryotic DNA polymerases. *Cell*. **59**: 219-228.

Bertocci B, De Smet A, Weill JC, Reynaud CA. (2006) Nonoverlapping functions of DNA polymerases mu, lambda, and terminal deoxynucleotidyltransferase during immunoglobulin V(D)J recombination in vivo. *Immunity*. **25**: 31-41.

Beese LS, Steitz TA. (1991) Structural basis for the 3'-5' exonuclease activity of Escherichia coli DNA polymerase I: a two metal ion mechanism. *EMBO J*. **10**: 25-33.

Beese LS, Friedman JM, Steitz TA. (1993 A) Crystal structures of the Klenow fragment of DNA polymerase I complexed with deoxynucleoside triphosphate and pyrophosphate. *Biochemistry*. **32**: 14095-14101

Beese LS, Derbyshire V, Steitz TA. (1993 B) Structure of DNA polymerase I Klenow fragment bound to duplex DNA. *Science*. **260**: 352-355.

Bessler JB, Torredagger JZ, Zakian VA. (2001) The Piflp subfamily of helicases: region-specific DNA helicases? *Trends. Cell. Biol*. **11**: 60-65.

Bianchi NO, Bianchi MS, Richard SM. (2001) Mitochondrial genome instability in human cancers. *Mutat. Res*. **488**: 9-23.

Blasco MA, Blanco L, Parés E, Salas M, Bernad A. (1990) Structural and functional analysis of temperature-sensitive mutants of the phage phi 29 DNA polymerase. *Nucleic. Acids. Res*. **18**: 4763-4770.

Blok MJ, van den Bosch BJ, Jongen E, Hendrickx A, de Die-Smulders CE, Hoogendijk JE, Brusse E, de Visser M, Poll-The BT, Bierau J, de Coo IF, Smeets HJ. (2009) The unfolding clinical spectrum of POLG mutations. *J. Med. Genet.* **46**: 776-785.

Blomberg Jensen M, Leffers H, Petersen JH, Daugaard G, Skakkebaek NE, Rajpert-De Meyts E. (2008) Association of the polymorphism of the CAG repeat in the mitochondrial DNA polymerase gamma gene (POLG) with testicular germ-cell cancer. *Ann. Oncol.* **19**: 1910-1914.

Boulé JB, Zakian VA. (2006) Roles of Pif1-like helicases in the maintenance of genomic stability. *Nucleic. Acids. Res.* **34**: 4147-4153.

Bourgeron T, Chretien D, Rötig A, Munnich A, Rustin P. (1993) Fate and expression of the deleted mitochondrial DNA differ between human heteroplasmic skin fibroblast and Epstein-Barr virus-transformed lymphocyte cultures. *J. Biol. Chem.* **268**: 19369-19376.

Bowmaker M, Yang MY, Yasukawa T, Reyes A, Jacobs HT, Huberman JA, Holt IJ (2003) Mammalian mitochondrial DNA replicates bidirectionally from an initiation zone. *J. Biol. Chem.* **278**: 50961-50969

Braithwaite DK, Ito J. (1993) Compilation, alignment, and phylogenetic relationships of DNA polymerases. *Nucleic. Acids. Res.* **21**: 787-802

Brautigam CA, Steitz TA. (1998 A) Structural and functional insights provided by crystal structures of DNA polymerases and their substrate complexes. *Curr. Opin. Struct. Biol.* **8**: 54-63.

Brautigam CA, Steitz TA. (1998 B) Structural principles for the inhibition of the 3'-5' exonuclease activity of Escherichia coli DNA polymerase I by phosphorothioates. *J. Mol. Biol.* **277**: 363-377.

Brennan RG, Matthews BW. (1989) The helix-turn-helix DNA binding motif. *J. Biol. Chem.* **264**: 1903-1906.

Brown WM, George M Jr, Wilson AC (1979) Rapid evolution of animal mitochondrial DNA. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 1967-1971.

Brown DT, Samuels DC, Michael EM, Turnbull DM, Chinnery PF. (2001) Random genetic drift determines the level of mutant mtDNA in human primary oocytes. *Am. J. Hum. Genet.* **68**: 533-536.

Brown TA, Cecconi C, Tkachuk AN, Bustamante C, Clayton DA (2005) Replication of mitochondrial DNA occurs by strand displacement with alternative light-strand origins, not via a strand-coupled mechanism. *Genes. Dev.* **19**: 2466-2476.

Brown TA, Tkachuk AN, Clayton DA (2008) Native R-loops persist throughout the mouse mitochondrial DNA genome. *J. Biol. Chem.* **283**: 36743-36751.

Bryant FR, Johnson KA, Benkovic SJ. (1983) Elementary steps in the DNA polymerase I reaction pathway. *Biochemistry.* **22**: 3537-3546.

Buermeyer AB, Deschênes SM, Baker SM, Liskay RM. (1999) Mammalian DNA mismatch repair. *Annu. Rev. Genet.* **33**: 533-564.

Bullerwell CE, Lang BF. (2005) Fungal evolution: the case of the vanishing mitochondrion. *Curr. Opin. Microbiol.* **8**: 362-369.

Burgart LJ, Zheng J, Shu Q, Strickler JG, Shibata D. Somatic mitochondrial mutation in gastric cancer. *Am. J. Pathol.* **147**: 1105-1111.

Burgers PM, Koonin EV, Bruford E, Blanco L, Burtis KC, Christman MF, Copeland WC, Friedberg EC, Hanaoka F, Hinkle DC, Lawrence CW, Nakanishi M, Ohmori H, Prakash L, Prakash S, Reynaud CA, Sugino A, Todo T, Wang Z, Weill JC, Woodgate R. (2001) Eukaryotic DNA polymerases: proposal for a revised nomenclature. *J. Biol. Chem.* **276**: 43487-43490.

Cann IK, Ishino Y. (1999) Archaeal DNA replication: identifying the pieces to solve a puzzle. *Genetics.* **152**: 1249-1267.

Capson TL, Peliska JA, Kaboord BF, Frey MW, Lively C, Dahlberg M, Benkovic SJ. (1992) Kinetic characterization of the polymerase and exonuclease activities of the gene 43 protein of bacteriophage T4. *Biochemistry.* **31**: 10984-10994.

Carrodegua JA, Kobayashi R, Lim SE, Copeland WC, Bogenhagen DF. (1999) The accessory subunit of *Xenopus laevis* mitochondrial DNA polymerase gamma increases processivity of the catalytic subunit of human DNA polymerase gamma and is related to class II aminoacyl-tRNA synthetases. *Mol. Cell. Biol.* **19**: 4039-4046.

Chan SS, Longley MJ, Copeland WC. (2005) The common A467T mutation in the human mitochondrial DNA polymerase (POLG) compromises catalytic efficiency and interaction with the accessory subunit. *J. Biol. Chem.* **280**: 31341-31346.

Chan SS, Longley MJ, Copeland WC. (2006) Modulation of the W748S mutation in DNA polymerase gamma by the E1143G polymorphism in mitochondrial disorders. *Hum. Mol. Genet.* **15**: 3473-3483.

Chan SS, Copeland WC (2009) DNA polymerase gamma and mitochondrial disease: understanding the consequence of POLG mutations. *Biochim. Biophys. Acta.* **1787**: 312-319

Chang DD, Clayton DA. (1985) Priming of human mitochondrial DNA replication occurs at the light-strand promoter. *Proc. Natl. Acad. Sci. U. S. A.* **82**: 351-355.

Chang DD, Hauswirth WW, Clayton DA. (1985) Replication priming and transcription initiate from precisely the same site in mouse mitochondrial DNA. *EMBO J.* **4**: 1559-1567.

Chen X, Prosser R, Simonetti S, Sadlock J, Jagiello G, Schon EA. (1995) Rearranged mitochondrial genomes are present in human oocytes. *Am. J. Hum. Genet.* **57**: 239-247.

Cheetham GM, Jeruzalmi D, Steitz TA. (1999) Structural basis for initiation of transcription from an RNA polymerase-promoter complex. *Nature.* **399**: 80-83.

Chi NW, Kolodner RD (1994) Purification and characterization of MSH1, a yeast mitochondrial protein that binds to DNA mismatches. *J. Biol. Chem.* **269**: 29984-29992

Chomyn A, Martinuzzi A, Yoneda M, Daga A, Hurko O, Johns D, Lai ST, Nonaka I, Angelini C, Attardi G. (1992) MELAS mutation in mtDNA binding site for transcription termination factor causes defects in protein synthesis and in respiration but no change in levels of upstream and downstream mature transcripts. *Proc. Natl. Acad. Sci. U. S. A.* **89**: 4221-4225.

Chomyn A, Attardi G. (2003) MtDNA mutations in aging and apoptosis. *Biochem. Biophys. Res. Commun.* **304**: 519-529.

Clayton DA, Doda JN, Friedberg EC. (1975) Absence of a pyrimidine dimer repair mechanism for mitochondrial DNA in mouse and human cells. *Basic. Life. Sci.* **5B**: 589-591.

Cochonneau D. (2010) Characterization of yeast polymerase gamma mutations associated with human disorders. Université Catholique de Louvain, Faculté d'ingénierie biologique, agronomique et environnementale, PhD thesis.

Conde J, Fink GR. (1976) A mutant of *Saccharomyces cerevisiae* defective for nuclear fusion. *Proc. Natl. Acad. Sci. U. S. A.* **73**: 3651-3655.

Contamine V, Picard M. (2000) Maintenance and integrity of the mitochondrial genome: a plethora of nuclear genes in the budding yeast. *Microbiol. Mol. Biol. Rev.* **64**: 281-315.

Copeland WC (2008) Inherited mitochondrial diseases of DNA replication. *Annu. Rev. Med.* **59**: 131-146.

Del Bo R, Bordini A, Sciacco M, Di Fonzo A, Galbiati S, Crimi M, Bresolin N, Comi GP (2003) Remarkable infidelity of polymerase gammaA associated with mutations in POLG1 exonuclease domain. *Neurology* **61**: 903-908

Derbyshire V, Pinsonneault JK, Joyce CM. (1995) Structure-function analysis of 3'-->5'-exonuclease of DNA polymerases. *Methods. Enzymol.* **262**: 363-385.

de Souza-Pinto NC, Wilson DM 3rd, Stevensner TV, Bohr VA. (2008) Mitochondrial DNA, base excision repair and neurodegeneration. *DNA Repair.* **7**: 1098-1109.

de Souza-Pinto NC, Mason PA, Hashiguchi K, Weissman L, Tian J, Guay D, Lebel M, Stevensner TV, Rasmussen LJ, Bohr VA. (2009) Novel DNA mismatch-repair activity involving YB-1 in human mitochondria. *DNA Repair* **8**: 704-719.

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

Di Fonzo A, Bordini A, Crimi M, Sara G, Bo RD, Bresolin N, Comi GP (2003) POLG mutations in sporadic mitochondrial disorders with multiple mtDNA deletions. *Hum. Mutat.* **22**: 498-499

DiMauro S. (2004) Mitochondrial diseases. *Biochim. Biophys. Acta.* **1658**: 80-88.

DiMauro S. (2004) The many faces of mitochondrial diseases. *Mitochondrion.* **4**: 799-807.

Dimmer KS, Rapaport D. (2008) Proteomic view of mitochondrial function. *Genome. Biol.* **9**: 209

Domínguez O, Ruiz JF, Laín de Lera T, García-Díaz M, González MA, Kirchhoff T, Martínez-A C, Bernad A, Blanco L. (2000) DNA polymerase mu (Pol mu), homologous to TdT, could act as a DNA mutator in eukaryotic cells. *EMBO J.* **19**: 1731-1742.

Donlin MJ, Patel SS, Johnson KA. (1991) Kinetic partitioning between the exonuclease and polymerase sites in DNA error correction. *Biochemistry.* **30**: 538-546.

Doublé S, Ellenberger T. (1998) The mechanism of action of T7 DNA polymerase. *Curr. Opin. Struct. Biol.* **8**: 704-712.

Doublé S, Tabor S, Long AM, Richardson CC, Ellenberger T. (1998) Crystal structure of a bacteriophage T7 DNA replication complex at 2.2 Å resolution. *Nature.* **391**: 251-258.

Dufour E, Méndez J, Lázaro JM, de Vega M, Blanco L, Salas M. (2000) An aspartic acid residue in TPR-1, a specific region of protein-priming DNA polymerases, is required for the functional interaction with primer terminal protein. *J. Mol. Biol.* **304**: 289-300.

Dujon B, Slonimski PP, Weill L. (1974) Mitochondrial genetics IX: A model for recombination and segregation of mitochondrial genomes in *Saccharomyces cerevisiae*. *Genetics*. **78**: 415-437.

Dujon B. Mitochondrial genetics and functions, In “The Molecular Biology of Yeast *Saccharomyces*: Life Cycle and Inheritance”, Tome 2, Cold Spring Harbor Laboratory, Cold Spring Harbor, 1981, 505-592.

Echols H, Goodman MF. (1991) Fidelity mechanisms in DNA replication. *Annu. Rev. Biochem.* **60**: 477-511.

Egger J, Wilson J. (1983) Mitochondrial inheritance in a mitochondrially mediated disease. *N. Engl. J. Med.* **309**: 142-146.

Esteban JA, Soengas MS, Salas M, Blanco L. (1994) 3'→5' exonuclease active site of phi 29 DNA polymerase. Evidence favoring a metal ion-assisted reaction mechanism. *J. Biol. Chem.* **269**: 31946-31954.

Falkenberg M, Gaspari M, Rantanen A, Trifunovic A, Larsson NG, Gustafsson CM. (2002) Mitochondrial transcription factors B1 and B2 activate transcription of human mtDNA. *Nat. Genet.* **31**: 289-294.

Fan L, Sanschagrin PC, Kaguni LS, Kuhn LA. (1999) The accessory subunit of mtDNA polymerase shares structural homology with aminoacyl-tRNA synthetases: implications for a dual role as a primer recognition factor and processivity clamp. *Proc. Natl. Acad. Sci. U. S. A.* **96**: 9527-9532.

Farr CL, Wang Y, Kaguni LS. (1999) Functional interaction of mitochondrial DNA polymerase and single-stranded DNA-binding protein.

Template-primer DNA binding and initiation and elongation of DNA strand synthesis. *J. Biol. Chem.* **274**: 14779-14785.

Ferrari G, Lamantea E, Donati A, Filosto M, Briem E, Carrara F, Parini R, Simonati A, Santer R, Zeviani M (2005) Infantile hepatocerebral syndromes associated with mutations in the mitochondrial DNA polymerase γ . *Brain* **128**: 723-731

Fisher RP, Lisowsky T, Parisi MA, Clayton DA. (1992) DNA wrapping and bending by a mitochondrial high mobility group-like transcriptional activator protein. *J. Biol. Chem.* **267**: 3358-3367.

Fontanesi F, Palmieri L, Scarcia P, Lodi T, Donnini C, Limongelli A, Tiranti V, Zeviani M, Ferrero I, Viola AM. (2004) Mutations in AAC2, equivalent to human adPEO-associated ANT1 mutations, lead to defective oxidative phosphorylation in *Saccharomyces cerevisiae* and affect mitochondrial DNA stability. *Hum. Mol. Genet.* **13**: 923-934.

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

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

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

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

Foury F, Hu J, Vanderstraeten S (2004) Mitochondrial DNA mutators. *Cell. Mol. Life. Sci.* **61**: 2799-2811

Freemont PS, Friedman JM, Beese LS, Sanderson MR, Steitz TA. (1988) Cocystal structure of an editing complex of Klenow fragment with DNA. *Proc. Natl. Acad. Sci. U. S. A.* **85**: 8924-8928.

Friddle RW, Klare JE, Martin SS, Corzett M, Balhorn R, Baldwin EP, Baskin RJ, Noy A. (2004) Mechanism of DNA compaction by yeast mitochondrial protein Abf2p. *Biophys. J.* **86**: 1632-1639.

Fridlender B, Fry M, Bolden A, Weissbach A. (1972) A new synthetic RNA-dependent DNA polymerase from human tissue culture cells (HeLa-fibroblast-synthetic oligonucleotides-template-purified enzymes). *Proc. Natl. Acad. Sci. U. S. A.* **69**: 452-455.

Fukui H, Moraes CT. (2009) Mechanisms of formation and accumulation of mitochondrial DNA deletions in aging neurons. *Hum. Mol. Genet.* **18**: 1028-1036.

Garcia-Diaz M, Bebenek K. (2007) Multiple functions of DNA polymerases. *CRC Crit. Rev. Plant. Sci.* **26**: 105-122.

Garg P, Burgers PM. (2005) DNA polymerases that propagate the eukaryotic DNA replication fork. *Crit. Rev. Biochem. Mol. Biol.* **40**: 115-128.

Garmendia C, Bernad A, Esteban JA, Blanco L, Salas M. (1992) The bacteriophage phi 29 DNA polymerase, a proofreading enzyme. *J. Biol. Chem.* **267**: 2594-2599.

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

Gerhold JM, Aun A, Sedman T, Jöers P, Sedman J. (2010) Strand invasion structures in the inverted repeat of *Candida albicans* mitochondrial DNA reveal a role for homologous recombination in replication. *Mol. Cell.* 2010 **39**: 851-861

Ghivizzani SC, Madsen CS, Nelen MR, Ammini CV, Hauswirth WW. (1994) In organello footprint analysis of human mitochondrial DNA: human mitochondrial transcription factor A interactions at the origin of replication. *Mol. Cell. Biol.* **14**: 7717-7730.

Giles RE, Blanc H, Cann HM, Wallace DC. (1980) Maternal inheritance of human mitochondrial DNA. *Proc. Natl. Acad. Sci. U. S. A.* **77**: 6715-6719.

Gissi C, Iannelli F, Pesole G. (2008) Evolution of the mitochondrial genome of Metazoa as exemplified by comparison of congeneric species. *Heredity.* **101**: 301-320

Gleghorn ML, Davydova EK, Rothman-Denes LB, Murakami (2008) Structural basis for DNA-hairpin promoter recognition by the bacteriophage N4 virion RNA polymerase. *Mol. Cell.* **32**: 707-717

Goodman MF. (1997) Hydrogen bonding revisited: geometric selection as a principal determinant of DNA replication fidelity. *Proc. Natl. Acad. Sci. U. S. A.* **94**: 10493-10495.

Goodman MF, Fygenon KD (1998) DNA polymerase fidelity: from genetics toward a biochemical understanding. *Genetics.* **148**: 1475-1482

Gordenin DA, Kunkel TA, Resnick MA. (1997) Repeat expansion--all in a flap? *Nat. Genet.* **16**: 116-118.

Götz A, Isohanni P, Pihko H, Paetau A, Herva R, Saarenpää-Heikkilä O, Valanne L, Marjavaara S, Suomalainen A. (2008) Thymidine kinase 2 defects can cause multi-tissue mtDNA depletion syndrome. *Brain.* **131**: 2841-2850.

Gray H, Wong TW. (1992) Purification and identification of subunit structure of the human mitochondrial DNA polymerase gamma. *J. Biol. Chem.* **267**: 5835-5841.

Gray MW, Lang BF, Burger G. (2004) Mitochondria of protists. *Annu. Rev. Genet.* **38**: 477-524.

Graziewicz MA, Longley MJ, Copeland WC (2006) DNA polymerase gamma in Mitochondrial DNA Replication and Repair. *Chem. Rev.* **106**: 383-405

Hakonen AH, Heiskanen S, Juvonen V, Lappalainen I, Luoma PT, Rantamaki M, Goethem GV, Lofgren A, Hackman P, Paetau A, Kaakkola S, Majama K, Varilo T, Udd B, Kaariainen H, Bindoff LA, Suomalainen A (2005) Mitochondrial DNA polymerase W748S mutation: a common cause

of autosomal recessive ataxia with ancient European origin. *Am. J. Hum. Genet.* **77**: 430-441.

Hanna MG, Nelson IP, Morgan-Hughes JA, Harding AE. (1995) Impaired mitochondrial translation in human myoblasts harbouring the mitochondrial DNA tRNA lysine 8344 A-->G (MERRF) mutation: relationship to proportion of mutant mitochondrial DNA. *J. Neurol. Sci.* **130**: 154-160.

Hizi A, Herschhorn A. (2008) Retroviral reverse transcriptases (other than those of HIV-1 and murine leukemia virus): a comparison of their molecular and biochemical properties. *Virus. Res.* **134**: 203-220.

Holt IJ, Harding AE, Morgan-Hughes JA. (1988) Deletions of muscle mitochondrial DNA in patients with mitochondrial myopathies. *Nature.* **331**: 717-719.

Holt IJ, He J, Mao CC, Boyd-Kirkup JD, Martinsson P, Sembongi H, Reyes A, Spelbrink JN. (2007) Mammalian mitochondrial nucleoids: organizing an independently minded genome. *Mitochondrion.* **7**: 311-321.

Holt IJ. (2009) Mitochondrial DNA replication and repair: all a flap. *Trends. Biochem. Sci.* **34**: 358-365

Hogg M, Aller P, Konigsberg W, Wallace SS, Doublié S (2007) Structural and biochemical investigation of the role in proofreading of a beta hairpin loop found in the exonuclease domain of a replicative DNA polymerase of the B family. *J. Biol. Chem.* **282**: 1432-1444

Horvath R, Hudson G, Ferrari G, Futterer N, Ahola S, Lamantea E, Prokisch H, Lochmuller H, McFarland R, Ramesh V, Klopstock T, Freisinger P, Salvi

F, Mayr JA, Santer R, Tesarova M, Zeman J, Udd B, Taylor RW, Turnbull D, Hanna M, Fialho D, Suomalainen A, Zeviani M, Chinnery PF (2006) Phenotypic spectrum associated with mutations of the mitochondrial polymerase {gamma} gene. *Brain* **129**: 1674-1684

Hu JP, Vanderstraeten S, Foury F (1995) Isolation and characterization of ten mutator alleles of the mitochondrial DNA polymerase-encoding MIP1 gene from *Saccharomyces cerevisiae*. *Gene* **160**: 105-110

Hubscher U, Kuenzle CC, Spadari S (1979) Functional roles of DNA polymerases beta and gamma. *Proc. Natl. Acad. Sci. U. S. A.* **76**: 2316-2320.

Hudson G, Chinnery PF. (2006) Mitochondrial DNA polymerase-gamma and human disease. *Hum. Mol. Genet.* **15 Spec No 2**: R244-252.

Hung LH, Chaconas G, Shaw GS. The solution structure of the C-terminal domain of the Mu B transposition protein. *EMBO J.* **19**: 5625-5634.

Insdorf NF, Bogenhagen DF. (1989) DNA polymerase gamma from *Xenopus laevis*. I The identification of a high molecular weight catalytic subunit by a novel DNA polymerase photolabeling procedure. *J. Biol. Chem.* **264**: 21491-21497.

Iyengar B, Roote J, Campos AR. (1999) The *tamas* gene, identified as a mutation that disrupts larval behavior in *Drosophila melanogaster*, codes for the mitochondrial DNA polymerase catalytic subunit (DNApol-gamma125). *Genetics.* **153**: 1809-1824.

Iyer RR, Pluciennik A, Burdett V, Modrich PL. (2006) DNA mismatch repair: functions and mechanisms. *Chem. Rev.* **106**: 302-323.

Ito J, Braithwaite DK. (1991) Compilation and alignment of DNA polymerase sequences. *Nucleic. Acids. Res.* **19**: 4045-4057.

Jensen M, Leffers H, Petersen JH, Nyboe Andersen A, Jørgensen N, Carlsen E, Jensen TK, Skakkebaek NE, Rajpert-De Meyts E. (2004) Frequent polymorphism of the mitochondrial DNA polymerase gamma gene (POLG) in patients with normal spermiograms and unexplained subfertility. *Hum. Reprod.* **19**: 65-70.

Jenuth JP, Peterson AC, Fu K, Shoubridge EA. (1996) Random genetic drift in the female germline explains the rapid segregation of mammalian mitochondrial DNA. *Nat. Genet.* **14**: 146-151.

Johnson KA (1993) Conformational coupling in DNA polymerase fidelity. *Annu. Rev. Biochem.* **62**: 685-713.

Johnson RE, Prakash S, Prakash L. (1999) Efficient bypass of a thymine-thymine dimer by yeast DNA polymerase, Poleta. *Science.* **283**: 1001-1004.

Johnson AA, Johnson KA. (2001) Exonuclease proofreading by human mitochondrial DNA polymerase. *J. Biol. Chem.* **276**: 38097-38107

Jordan SR, Pabo CO. (1988) Structure of the lambda complex at 2.5 Å resolution: details of the repressor-operator interactions. *Science.* **242**: 893-899.

Joyce CM. (1989) How DNA travels between the separate polymerase and 3'-5'-exonuclease sites of DNA polymerase I (Klenow fragment). *J. Biol. Chem.* **264**: 10858-10866.

Joyce CM, Steitz TA. (1994) Function and structure relationships in DNA polymerases. *Annu. Rev. Biochem.* **63**: 777-822.

Kaguni LS (2004) DNA polymerase gamma, the mitochondrial replicase. *Annu. Rev. Biochem.* **73**: 293-320

Kajander OA, Rovio AT, Majamaa K, Poulton J, Spelbrink JN, Holt IJ, Karhunen PJ, Jacobs HT. (2000) Human mtDNA sublimons resemble rearranged mitochondrial genomes found in pathological states. *Hum. Mol. Genet.* **9**: 2821-2835.

Kajander OA, Karhunen PJ, Holt IJ, Jacobs HT. (2001) Prominent mitochondrial DNA recombination intermediates in human heart muscle. *EMBO Rep.* **2**: 1007-1012.

Kaiser C, Michaelis S, Mitchell A (1994) *Methods in Yeast Genetics: a Laboratory Course Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Kamtekar S, Berman AJ, Wang J, Lázaro JM, de Vega M, Blanco L, Salas M, Steitz TA. (2004) Insights into strand displacement and processivity from the crystal structure of the protein-primed DNA polymerase of bacteriophage phi29. *Mol. Cell.* **16**: 609-618.

Kamtekar S, Berman AJ, Wang J, Lázaro JM, de Vega M, Blanco L, Salas M, Steitz TA. (2006) The phi29 DNA polymerase: protein-primer structure suggests a model for the initiation to elongation transition. *EMBO J.* **25**: 1335-13343.

Kiefer JR, Mao C, Braman JC, Beese LS. (1998) Visualizing DNA replication in a catalytically active Bacillus DNA polymerase crystal. *Nature.* **391**: 304-307.

Kim SW, Kim DU, Kim JK, Kang LW, Cho HS (2008) Crystal structure of Pfu, the high fidelity DNA polymerase from *Pyrococcus furiosus*. *Int. J. Biol. Macromol.* **42**: 356-361

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

Kohlstaedt LA, Wang J, Friedman JM, Rice PA, Steitz TA (1992) Crystal structure at 3.5 Å resolution of HIV-1 reverse transcriptase complexed with an inhibitor. *Science* **256**: 1783-1790

Kollberg G, Moslemi AR, Darin N, Nennesmo I, Bjarnadottir I, Uvebrant P, Holme E, Melberg A, Tulinius M, Oldfors A (2006) POLG1 Mutations Associated With Progressive Encephalopathy in Childhood. *J. Neuropathol. Exp. Neurol.* **65**: 758-768

Kolodner RD, Marsischky GT. (1999) Eukaryotic DNA mismatch repair. *Curr. Opin. Genet. Dev.* **9**: 89-96.

Kool ET. (2002) Active site tightness and substrate fit in DNA replication. *Annu. Rev. Biochem.* **71**: 191-219.

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

Korhonen JA, Gaspari M, Falkenberg M. (2003) TWINKLE Has 5' -> 3' DNA helicase activity and is specifically stimulated by mitochondrial single-stranded DNA-binding protein. *J. Biol. Chem.* **278**: 48627-48632.

Korhonen JA, Pham XH, Pellegrini M, Falkenberg M. (2004) Reconstitution of a minimal mtDNA replisome in vitro. *EMBO J.* **23**: 2423-2429.

Kornberg A, Baker T. (1992) DNA replication. Freeman; New York.

Korolev S, Nayal M, Barnes WM, Di Cera E, Waksman G. (1995) Crystal structure of the large fragment of *Thermus aquaticus* DNA polymerase I at 2.5-Å resolution: structural basis for thermostability. *Proc. Natl. Acad. Sci. U. S. A.* **92**: 9264-9268.

Krausz C, Guarducci E, Becherini L, Degl'Innocenti S, Gerace L, Balercia G, Forti G. (2004) The clinical significance of the POLG gene polymorphism in male infertility. *J. Clin. Endocrinol. Metab.* **89**: 4292-4297.

Kraytsberg Y, Schwartz M, Brown TA, Ebrald K, Kunz WS, Clayton DA, Vissing J, Khrapko K. (2004) Recombination of human mitochondrial DNA. *Science*. **304**: 981.

Krishnan KJ, Reeve AK, Samuels DC, Chinnery PF, Blackwood JK, Taylor RW, Wanrooij S, Spelbrink JN, Lightowlers RN, Turnbull DM. (2008) What causes mitochondrial DNA deletions in human cells? *Nat. Genet.* **40**: 275-279.

Kujoth GC, Hiona A, Pugh TD, Someya S, Panzer K, Wohlgemuth SE, Hofer T, Seo AY, Sullivan R, Jobling WA, Morrow JD, Van Remmen H, Sedivy JM, Yamasoba T, Tanokura M, Weindruch R, Leeuwenburgh C, Prolla TA. (2005) Mitochondrial DNA mutations , oxidative stress, and apoptosis in mammalian ageing. *Science*. **309**: 481-484.

Kunkel TA, Bebenek K. (1988) Recent studies of the fidelity of DNA synthesis. *Biochim. Biophys. Acta*. **951**: 1-15.

Kunkel TA, Mosbaugh DW. (1989) Exonucleolytic proofreading by a mammalian DNA polymerase. *Biochemistry*. **28**:988-995.

Kunkel TA, Bebenek K (2000) DNA replication fidelity. *Annu. Rev. Biochem.* **69**: 497-529

Kunkel TA. (2004) DNA replication fidelity. *J. Biol. Chem.* **279**: 16895-16898.

Kuroita T, Matsumura H, Yokota N, Kitabavashi M, Hashimoto H, Inoue T, Imanaka T, Kai Y (2005) Structural mechanism for coordination of

proofreading and polymerase activities in archaeal DNA polymerases. *J. Mol. Biol.* **351**: 291-298

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

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

Lamantea E, Tiranti V, Bordoni A, Toscano A, Bono F, Servidei S, Papadimitriou A, Spelbrink H, Silvestri L, Casari G, Comi GP, Zeviani M (2002) Mutations of mitochondrial DNA polymerase gamma are a frequent cause of autosomal dominant or recessive Progressive External Ophthalmoplegia. *Ann. Neurol.* **52**: 211-219

Lang BF, Gray MW, Burger G (1999) Mitochondrial genome evolution and the origin of eukaryotes. *Annu. Rev. Genet.* **33**: 351-397

Larsson NG, Wang J, Wilhelmsson H, Oldfors A, Rustin P, Lewandoski M, Barsh GS, Clayton DA. (1998) Mitochondrial transcription factor A is necessary for mtDNA maintenance and embryogenesis in mice. *Nat. Genet.* **18**: 231-236.

Lawrence CW. (2004) Cellular functions of DNA polymerase zeta and Rev1 protein. *Adv. Protein. Chem.* **69**: 167-203.

Lecrenier N, Van Der Bruggen P, Foury F (1997) Mitochondrial DNA polymerases from yeast to man: a new family of polymerases. *Gene* **185**: 147-152

Lecrenier N, Foury F. (2000) New features of mitochondrial DNA replication system in yeast and man. *Gene*. **246**: 37-48.

Lee Y-S, Kennedy WD, Yin YW (2009) Structural insight into processive human mitochondrial DNA synthesis and disease-related polymerase mutations. *Cell* **139**: 312-324

Lee YS, Lee S, Demeler B, Molineux JJ, Johnson KA, Yin YW. (2010) Each monomer of the dimeric accessory protein for human mitochondrial DNA polymerase has a distinct role in conferring processivity. *J. Biol. Chem.* **285**: 1490-1499.

Lefai E, Calleja M, Ruiz de Mena I, Lagina AT 3rd, Kaguni LS, Garesse R. (2000) Overexpression of the catalytic subunit of DNA polymerase gamma results in depletion of mitochondrial DNA in *Drosophila melanogaster*. *Mol. Gen. Genet.* **264**: 37-46.

Lehman IR, Bessman MJ, Simms ES, Kornberg A. (1958) Enzymatic synthesis of deoxyribonucleic acid. I. Preparation of substrates and partial purification of an enzyme from *Escherichia coli*. *J. Biol. Chem.* **233**: 163-170.

Lewis W, Haase CP, Miller YK, Ferguson B, Stuart T, Ludaway T, McNaught J, Russ R, Steltzer J, Santoianni R, Long R, Fiermonte G, Palmieri F. (2005) Transgenic expression of the deoxynucleotide carrier causes mitochondrial damage that is enhanced by NRTIs for AIDS. *Lab. Invest.* **85**: 972-981.

Li Y, Korolev S, Waksman G. (1998) Crystal structures of open and closed forms of binary and ternary complexes of the large fragment of *Thermus aquaticus* DNA polymerase I: structural basis for nucleotide incorporation. *EMBO J.* **17**: 7514-7525.

Lim SE, Longley MJ, Copeland WC. (1999) The mitochondrial p55 accessory subunit of human DNA polymerase gamma enhances DNA binding, promotes processive DNA synthesis, and confers N-ethylmaleimide resistance. *J. Biol. Chem.* **274**: 38197-38203.

Ling H, Boudsocq F, Woodgate R, Yang W. (2001) Crystal structure of a Y-family DNA polymerase in action: a mechanism for error-prone and lesion-bypass replication. *Cell.* **107**: 91-102.

Ling F, Hori A, Shibata T. (2007) DNA recombination-initiation plays a role in the extremely biased inheritance of yeast [rho-] mitochondrial DNA that contains the replication origin ori5. *Mol. Cell. Biol.* **27**: 1133-1145.

Lipinski KA, Kaniak-Golik A, Golik P. (2010) Maintenance and expression of the *S. cerevisiae* mitochondrial genome-From genetics to evolution and systems biology. *Biochim. Biophys. Acta.* **1797**: 1086-1098

Liu Y, Prasad R, Beard WA, Kedar PS, Hou EW, Shock DD, Wilson SH. (2007) Coordination of steps in single-nucleotide base excision repair mediated by apurinic/apyrimidinic endonuclease 1 and DNA polymerase beta. *J. Biol. Chem.* **282**: 13532-13541

Liu P, Demple B. (2010) DNA repair in mammalian mitochondria: Much more than we thought? *Environ. Mol. Mutagen.* **51**: 417-426.

Lodish HF, Berk A, Kaiser CA, Krieger M, Scott MP, Bretscher A, Ploegh H, Matsudaira P (2008) *Molecular Cell Biology* 6th edition, WH Freeman

Loeb LA, Kunkel TA. (1982) Fidelity of DNA synthesis. *Annu. Rev. Biochem.* **51**: 429-457.

Longley MJ, Clark S, Yu Wai Man C, Hudson G, Durham SE, Taylor RW, Nightingale S, Turnbull DM, Copeland WC, Chinnery PF. (2006) Mutant POLG2 disrupts DNA polymerase gamma subunits and causes progressive external ophthalmoplegia. *Am. J. Hum. Genet.* **78**: 1026-1034

Longley MJ, Ropp PA, Lim SE, Copeland WC. (1998) Characterization of the native and recombinant catalytic subunit of human DNA polymerase gamma: identification of residues critical for exonuclease activity and dideoxynucleotide sensitivity. *Biochemistry.* **37**: 10529-10539.

López LC, Akman HO, García-Cazorla A, Dorado B, Martí R, Nishino I, Tadesse S, Pizzorno G, Shungu D, Bonilla E, Tanji K, Hirano M. (2009) Unbalanced deoxynucleotide pools cause mitochondrial DNA instability in thymidine phosphorylase-deficient mice. *Hum. Mol. Genet.* **18**: 714-722.

Lucas P, Lasserre JP, Plissonneau J, Castroviejo M (2004) Absence of accessory subunit in the DNA polymerase gamma purified from yeast mitochondria. *Mitochondrion* **4**: 13-20

Luft R, Ikkos D, Palmieri G, Ernster L, Afzelius B. (1962) A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: a correlated clinical, biochemical, and morphological study. *J. Clin. Invest.* **41**: 1776-1804.

Luo N, Kaguni LS (2005) Mutations in the spacer region of *Drosophila* mitochondrial DNA polymerase affect DNA binding, processivity, and the balance between Pol and Exo function. *J. Biol. Chem.* **280**: 2491-2497

Luoma PT, Eerola J, Ahola S, Hakonen AH, Hellström O, Kivistö KT, Tienari PJ, Suomalainen A. (2007) Mitochondrial DNA polymerase gamma variants in idiopathic sporadic Parkinson disease. *Neurology.* **69**: 1152-1159.

Maier D, Farr CL, Poeck B, Alahari A, Vogel M, Fischer S, Kaguni LS, Schneuwly S. (2001) Mitochondrial single-stranded DNA-binding protein is required for mitochondrial DNA replication and development in *Drosophila melanogaster*. *Mol. Biol. Cell.* **12**: 821-830.

Maleszka R, Skelly PJ, Clark-Walker (1991) GD Rolling circle replication of DNA in yeast mitochondria. *EMBO J.* **10**: 3923-3929.

Mandavilli BS, Santos JH, Van Houten B. (2002) Mitochondrial DNA repair and aging. *Mutat. Res.* **509**: 127-151.

Mao CC, Holt IJ. (2009) Clinical and molecular aspects of diseases of mitochondrial DNA instability. *Chang. Gung. Med. J.* **32**: 354-369.

Marini F, Kim N, Schuffert A, Wood RD. (2003) POLN, a nuclear PolA family DNA polymerase homologous to the DNA cross-link sensitivity protein Mus308. *J. Biol. Chem.* **278**: 32014-32019.

Marsischky GT, Filosi N, Kane MF, Kolodner R. (1996) Redundancy of *Saccharomyces cerevisiae* MSH3 and MSH6 in MSH2-dependent mismatch repair. *Genes. Dev.* **10**: 407-420.

Mason PA, Matheson EC, Hall AG, Lightowlers RN. (2003) Mismatch repair activity in mammalian mitochondria. *Nucleic. Acids. Res.* **31**: 1052-1058.

Masters BS, Stohl LL, Clayton DA. (1987) Yeast mitochondrial RNA polymerase is homologous to those encoded by bacteriophages T3 and T7. *Cell.* **51**: 89-99.

Masutani C, Kusumoto R, Yamada A, Dohmae N, Yokoi M, Yuasa M, Araki M, Iwai S, Takio K, Hanaoka F. (1999) The XPV (xeroderma pigmentosum variant) gene encodes human DNA polymerase eta. *Nature.* **399**: 700-704.

McFarland R, Taylor RW, Turnbull DM. (2010) A neurological perspective on mitochondrial disease. *Lancet. Neurol.* **9**: 829-840.

Milone M, Massie R. (2010) Polymerase gamma 1 mutations: clinical correlations. *Neurologist.* **16**: 84-91.

Mizrahi V, Henrie RN, Marlier JF, Johnson KA, Benkovic SJ. (1985) Rate-limiting steps in the DNA polymerase I reaction pathway. *Biochemistry.* **24**: 4010-4018.

Modrich P, Richardson CC. (1975) Bacteriophage T7 Deoxyribonucleic acid replication in vitro. A protein of Escherichia coli required for bacteriophage T7 DNA polymerase activity. *J. Biol. Chem.* **250**: 5508-5514.

Monroe DS Jr, Leitzel AK, Klein HL, Matson SW. (2005) Biochemical and genetic characterization of Hmi1p, a yeast DNA helicase involved in the maintenance of mitochondrial DNA. *Yeast*. **22**: 1269-86

Mosbaugh DW. (1988) Purification and characterization of the porcine liver DNA polymerase gamma: utilization of dUTP and dTTP during in vitro DNA synthesis. *Nucleic. Acids. Res.* **16**: 5645-5659.

Mott JL, Zhang D, Farrar PL, Chang SW, Zassenhaus HP. (1999) Low frequencies of mitochondrial DNA mutations cause cardiac disease in the mouse. *Ann. N. Y. Acad. Sci.* **893**: 353-357.

Murakami KS, Davydova EK, Rothman-Denes LB (2008) X-ray crystal structure of the polymerase domain of the bacteriophage N4 virion RNA polymerase. *Proc. Natl. Acad. Sci. U.S.A.* **105**: 5046-5051

Naimi M, Bannwarth S, Procaccio V, Pouget J, Desnuelle C, Pellissier JF, Rotig A, Munnich A, Calvas P, Richelme C, Jonveaux P, Castelnovo G, Simon M, Clanet M, Wallace D, Paquis-Flucklinger V (2006) Molecular analysis of ANT1, TWINKLE and POLG in patients with multiple deletions or depletion of mitochondrial DNA by a dHPLC-based assay. *Eur. J. Hum. Genet.* **14**: 917-922

Nass MM, Nass S (1963) Intramitochondrial fibers with DNA characteristics. *J. Cell. Biol.* **19**: 593-611.

Naviaux RK, Nyhan WL, Barshop BA, Poulton J, Markusic D, Karpinski NC, Haas RH. (1999) Mitochondrial DNA polymerase gamma deficiency and mtDNA depletion in a child with Alpers' syndrome. *Ann. Neurol.* **45**: 54-58.

Naviaux RK and Nguyen KV (2004) POLG Mutations associated with Alpers' Syndrome and Mitochondrial DNA Depletion. *Ann. Neurol.* **55**: 706-712

Nguyen KV, Ostergaard E, Ravn SH, Balslev T, Danielsen ER, Vardag A, McKiernan PJ, Gray G, Naviaux RK (2005) POLG Mutations in Alpers Syndrome. *Neurology* **65**: 1493-1495

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

Nowak R, Zub R, Skoneczna I, Sikora K, Ligaj M. (2005) CAG repeat polymorphism in the DNA polymerase gamma gene in a Polish population: an association with testicular cancer risk. *Ann. Oncol.* **16**: 1211-1212.

O'Driscoll M, Jeggo PA. (2006) The role of double-strand break repair - insights from human genetics. *Nat. Rev. Genet.* **7**: 45-54.

Ohmori H, Friedberg EC, Fuchs RP, Goodman MF, Hanaoka F, Hinkle D, Kunkel TA, Lawrence CW, Livneh Z, Nohmi T, Prakash L, Prakash S, Todo T, Walker GC, Wang Z, Woodgate R. (2001) The Y-family of DNA polymerases. *Mol. Cell.* **8**: 7-8.

Ollis DL, Brick P, Hamlin R, Xuong NG, Steitz TA. (1985) Structure of large fragment of *Escherichia coli* DNA polymerase I complexed with dTMP. *Nature.* **313**: 762-766.

Otwinowski Z, Schevitz RW, Zhang RG, Lawson CL, Joachimiak A, Marmorstein RQ, Luisi BF, Sigler PB. (1988) Crystal structure of trp repressor/operator complex at atomic resolution. *Nature*. **335**: 321-329.

Pabo CO, Lewis M. (1982) The operator-binding domain of lambda repressor: structure and DNA recognition. *Nature*. **298**: 443-447.

Pabo CO, Sauer RT. (1984) Protein-DNA recognition. *Annu. Rev. Biochem.* **53**: 293-321.

Paeschke K, McDonald KR, Zakian VA. (2010) Telomeres: structures in need of unwinding. *FEBS Lett.* **584**: 3760-3772.

Palin EJ, Lesonen A, Farr CL, Euro L, Suomalainen A, Kaguni LS. (2010) Functional analysis of H. sapiens DNA polymerase gamma spacer mutation W748S with and without common variant E1143G. *Biochim. Biophys. Acta*. **1802**: 545-551.

Palmer JD (1997) Genome evolution. The mitochondrion that time forgot. *Nature*. **387**: 454-455.

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

Patel SS, Wong I, Johnson KA. (1991) Pre-steady-state kinetic analysis of processive DNA replication including complete characterization of an exonuclease-deficient mutant. *Biochemistry*. **30**: 511-525.

Pérez-Arnaiz P, Lázaro JM, Salas M, de Vega M. (2006) Involvement of phi29 DNA polymerase thumb subdomain in the proper coordination of synthesis and degradation during DNA replication. *Nucleic. Acids. Res.* **34**: 3107-3115.

Pestryakov PE, Lavrik OI. (2008) Mechanisms of single-stranded DNA-binding protein functioning in cellular DNA metabolism. *Biochemistry* **73**: 1388-13404.

Petruska J, Goodman MF, Boosalis MS, Sowers LC, Cheong C, Tinoco I Jr. (1988) Comparison between DNA melting thermodynamics and DNA polymerase fidelity. *Proc. Natl. Acad. Sci. U. S. A.* **85**: 6252-6256.

Phadnis N, Sia RA, Sia EA. (2005) Analysis of repeat-mediated deletions in the mitochondrial genome of *Saccharomyces cerevisiae*. *Genetics*. **171**: 1549-1559.

Pinz KG, Bogenhagen DF. (2000) Characterization of a catalytically slow AP lyase activity in DNA polymerase gamma and other family A DNA polymerases. *J. Biol. Chem.* **275**: 12509-12514

Polesky AH, Steitz TA, Grindley ND, Joyce CM. (1990) Identification of residues critical for the polymerase activity of the Klenow fragment of DNA polymerase I from *Escherichia coli*. *J. Biol. Chem.* **265**: 14579-14591.

Post CB, Ray WJ Jr. (1995) Reexamination of induced fit as a determinant of substrate specificity in enzymatic reactions. *Biochemistry*. **34**: 15881-15885.

Poulton J, Deadman ME, Bindoff L, Morten K, Land J, Brown G. (1993) Families of mtDNA re-arrangements can be detected in patients with mtDNA deletions: duplications may be a transient intermediate form. *Hum. Mol. Genet.* **2**: 23-30.

Prado F, Cortés-Ledesma F, Huertas P, Aguilera A. (2003) Mitotic recombination in *Saccharomyces cerevisiae*. *Curr Genet.* **42**: 185-198.

Pulkes T, Hanna MG. (2001) Human mitochondrial DNA diseases. *Adv. Drug. Deliv. Rev.* **49**: 27-43

Purohit V, Grindley ND, Joyce CM. (2003) Use of 2-aminopurine fluorescence to examine conformational changes during nucleotide incorporation by DNA polymerase I (Klenow fragment). *Biochemistry.* **42**: 10200-10211.

Qian YQ, Billeter M, Otting G, Müller M, Gehring WJ, Wüthrich K. (1989) The structure of the Antennapedia homeodomain determined by NMR spectroscopy in solution: comparison with prokaryotic repressors. *Cell.* **59**: 573-580.

Ramadan K, Shevelev I, Hübscher U. (2004) The DNA-polymerase-X family: controllers of DNA quality? *Nat. Rev. Mol. Cell. Biol.* **5**: 1038-1043.

Reddy MK, Weitzel SE, von Hippel PH. (1992) Processive proofreading is intrinsic to T4 DNA polymerase. *J. Biol. Chem.* **267**: 14157-14166.

Reenan RA, Kolodner RD. (1992) Characterization of insertion mutations in the *Saccharomyces cerevisiae* MSH1 and MSH2 genes: evidence for separate mitochondrial and nuclear functions. *Genetics*. **132**: 975-985.

Rodriguez I, Lázaro JM, Blanco L, Kamtekar S, Berman AJ, Wang J, Steitz TA, Salas M, de Vega M. (2005) A specific subdomain in phi29 DNA polymerase confers both processivity and strand-displacement capacity. *Proc. Natl. Acad. Sci. U. S. A.* **102**: 6407-6412.

Ropp PA, Copeland WC (1996) Cloning and characterization of the human mitochondrial DNA polymerase, DNA polymerase gamma. *Genomics*. **36**: 449-58

Rovio AT, Marchington DR, Donat S, Schuppe HC, Abel J, Fritsche E, Elliott DJ, Laippala P, Ahola AL, McNay D, Harrison RF, Hughes B, Barrett T, Bailey DM, Mehmet D, Jequier AM, Hargreave TB, Kao SH, Cummins JM, Barton DE, Cooke HJ, Wei YH, Wichmann L, Poulton J, Jacobs HT. (2001) Mutations at the mitochondrial DNA polymerase (POLG) locus associated with male infertility. *Nat. Genet.* **29**: 261-262.

Ruhanen H, Borrie S, Szabadkai G, Tyynismaa H, Jones AW, Kang D, Taanman JW, Yasukawa T. (2010) Mitochondrial single-stranded DNA binding protein is required for maintenance of mitochondrial DNA and 7S DNA but is not required for mitochondrial nucleoid organisation. *Biochim. Biophys. Acta*. **1803**: 931-939.

Santos JH, Mandavilli BS, Van Houten B. (2002) Measuring oxidative mtDNA damage and repair using quantitative PCR. *Methods. Mol. Biol.* **197**: 159-176.

Sarzi E, Bourdon A, Chrétien D, Zarhrate M, Corcos J, Slama A, Cormier-Daire V, de Lonlay P, Munnich A, Rötig A. (2007) Mitochondrial DNA depletion is a prevalent cause of multiple respiratory chain deficiency in childhood. *J. Pediatr.* **150**: 531-534, 534.e1-6.

Sawaya MR, Prasad R, Wilson SH, Kraut J, Pelletier H. (1997) Crystal structures of human DNA polymerase beta complexed with gapped and nicked DNA: evidence for an induced fit mechanism. *Biochemistry.* **36**: 11205-11215.

Schaaper RM. (1993) Base selection, proofreading, and mismatch repair during DNA replication in *Escherichia coli*. *J. Biol. Chem.* **268**: 23762-23765.

Schinkel AH, Groot Koerkamp MJ, Stuiver MH, Van der Horst GT, Tabak HF. (1987) Effect of point mutations on in vitro transcription from the promoter for the large ribosomal RNA gene of yeast mitochondria. *Nucleic. Acids. Res.* **15**: 5597-5612.

Schulz VP, Zakian VA. (1994) The *Saccharomyces* PIF1 DNA helicase inhibits telomere elongation and de novo telomere formation. *Cell.* **76**: 145-155

Sedman T, Jöers P, Kuusk S, Sedman J. (2005) Helicase Hmi1 stimulates the synthesis of concatemeric mitochondrial DNA molecules in yeast *Saccharomyces cerevisiae*. *Curr. Genet.* **47**: 213-22.

Seki M, Marini F, Wood RD. (2003) POLQ (Pol theta), a DNA polymerase and DNA-dependent ATPase in human cells. *Nucleic. Acids. Res.* **31**: 6117-6126.

Seki M, Masutani C, Yang LW, Schuffert A, Iwai S, Bahar I, Wood RD. (2004) High-efficiency bypass of DNA damage by human DNA polymerase Q. *EMBO J.* **23**: 4484-4494.

Sena EP, Revet B, Moustacchi E. (1986) In vivo homologous recombination intermediates of yeast mitochondrial DNA analyzed by electron microscopy. *Mol. Gen. Genet.* **202**: 421-428.

Shadel GS (1999) Yeast as a model for human mtDNA replication. *Am. J. Hum. Genet.* **65**: 1230-1237.

Shamoo Y, Steitz TA. (1999) Building a replisome from interacting pieces: sliding clamp complexed to a peptide from DNA polymerase and a polymerase editing complex. *Cell.* **99**: 155-166.

Shen EL, Bogenhagen DF. (2001) Developmentally-regulated packaging of mitochondrial DNA by the HMG-box protein mtTFA during *Xenopus* oogenesis. *Nucleic. Acids. Res.* **29**: 2822-2828.

Shibata T, Ling F. (2007) DNA recombination protein-dependent mechanism of homoplasmy and its proposed functions. *Mitochondrion*. **7**: 17-23.

Shoffner JM, Lott MT, Voljavec AS, Soueidan SA, Costigan DA, Wallace DC. (1989) Spontaneous Kearns-Sayre/chronic external ophthalmoplegia plus syndrome associated with a mitochondrial DNA deletion: a slip-replication model and metabolic therapy. *Proc. Natl. Acad. Sci. U. S. A.* **86**: 7952-7956.

Sia RA, Carrol S, Kalifa L, Hochmuth C, Sia EA. (2009) Loss of the mitochondrial nucleoid protein, Abf2p, destabilizes repetitive DNA in the yeast mitochondrial genome. *Genetics*. **181**: 331-334.

Simonati A, Filosto M, Savio C, Tomellieri G, Tonin P, Dalla Bernardina B, Rizzuto N (2003) Features of cell death in brain and liver, the target tissues of progressive neuronal degeneration of childhood with liver disease (Alpers–Huttenlocher disease). *Act. Neuropathol.* **106**: 57–65

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

Slonimski PP, Tzagoloff A. Localization in yeast mitochondrial DNA of mutations expressed in a deficiency of cytochrome oxidase and/or coenzyme QH2-cytochrome c reductase. *Eur J Biochem.* **61**: 27-41.

Sor F, Fukuhara H. (1982) Identification of two erythromycin resistance mutations in the mitochondrial gene coding for the large ribosomal RNA in yeast. *Nucleic. Acids. Res.* **10**: 6571-6577.

Sor F, Fukuhara H. (1984) Erythromycin and spiramycin resistance mutations of yeast mitochondria: nature of the rib2 locus in the large ribosomal RNA gene. *Nucleic. Acids. Res.* **12**: 8313-8318.

Spelbrink JN, Toivonen JM, Hakkaart GA, Kurkela JM, Cooper HM, Lehtinen SK, Lecrenier N, Back JW, Speijer D, Foury F, Jacobs HT. (2000) In vivo functional analysis of the human mitochondrial DNA polymerase POLG expressed in cultured human cells. *J. Biol. Chem.* **275**: 24818-24828.

Spelbrink JN, Li FY, Tiranti V, Nikali K, Yuan QP, Tariq M, Wanroij S, Garrido N, Comi G, Morandi L, Santoro L, Toscano A, Fabrizi GM, Somer H, Croxen R, Beeson D, Poulton J, Soumolainen A, Jacobs HT, Zeviani M, Larrson C (2001) Human mitochondrial DNA deletions associated with mutations in the gene encoding Twinkle, a phage T7 gene 4-like protein localized in mitochondria. *Nat. Genet.* **28**: 223-231.

Sherman F. (1991) Getting started with yeast. *Methods. Enzymol.* **194**: 3-21.

Spinazzola A, Invernizzi F, Carrara F, Lamantea E, Donati A, DiRocco M, Giordano I, Meznaric-Petrusa M, Baruffini E, Ferrero I, Zeviani M (2009) Clinical and molecular features of mitochondrial DNA depletion syndromes. *J. Inher. Metab. Dis.* **32**: 143-158

Srivastava S, Moraes CT. (2005) Double-strand breaks of mouse muscle mtDNA promote large deletions similar to multiple mtDNA deletions in humans. *Hum. Mol. Genet.* **14**: 893-902.

Steitz TA, Steitz JA. (1993) A general two-metal-ion mechanism for catalytic RNA. *Proc. Natl. Acad. Sci. U. S. A.* **90**: 6498-6502.

Steitz TA. (1999) DNA polymerases: structural diversity and common mechanisms. *J. Biol. Chem.* **274**: 17395-17398.

Stumpf JD, Bailey CM, Spell D, Stillwagon M, Anderson KS, Copeland WC (2010) mip1 containing mutations associated with mitochondrial disease causes mutagenesis and depletion of mtDNA in *Saccharomyces cerevisiae*. *Hum. Mol. Genet.* **19**: 2123-2133

Stuart GR, Santos JH, Strand MK, Van Houten B, Copeland WC (2006) Mitochondrial and nuclear DNA defects in *Saccharomyces cerevisiae* with mutations in DNA polymerase gamma associated with progressive external ophthalmoplegia. *Hum. Mol. Genet.* **15**: 363-374

Subuddhi U, Hogg M, Reha-Krantz LJ (2008) Use of 2-aminopurine fluorescence to study the role of the beta hairpin in the proofreading pathway catalyzed by the phage T4 and RB69 DNA polymerases. *Biochemistry.* **47**: 6130-6137

Swan MK, Johnson RE, Prakash L, Prakash S, Aggarwal AK. (2009) Structural basis of high-fidelity DNA synthesis by yeast DNA polymerase delta. *Nat. Struct. Mol. Biol.* **16**: 979-986.

Tabor S, Huber HE, Richardson CC. (1987) Escherichia coli thioredoxin confers processivity on the DNA polymerase activity of the gene 5 protein of bacteriophage T7. *J. Biol. Chem.* **262**: 16212-16223.

Takamatsu C, Umeda S, Ohsato T, Ohno T, Abe Y, Fukuoh A, Shinagawa H, Hamasaki N, Kang D. (2002) Regulation of mitochondrial D-loops by transcription factor A and single-stranded DNA-binding protein. *EMBO Rep.* **3**: 451-456.

Takata K, Shimizu T, Iwai S, Wood RD. Human DNA polymerase η (POLN) is a low fidelity enzyme capable of error-free bypass of 5S-thymine glycol. *J. Biol. Chem.* **281**: 23445-23455.

Tanaka I, Appelt K, Dijk J, White SW, Wilson KS. (1984) 3-A resolution structure of a protein with histone-like properties in prokaryotes. *Nature.* **310**: 376-381.

Tang M, Shen X, Frank EG, O'Donnell M, Woodgate R, Goodman MF. (1999) UmuD'(2)C is an error-prone DNA polymerase, Escherichia coli pol V. *Proc. Natl. Acad. Sci. U. S. A.* **96**: 8919-8924.

Tapper DP, Clayton DA (1981) Mechanism of replication of human mitochondrial DNA. Localization of the 5' ends of nascent daughter strands. *J. Biol. Chem.* **256**: 5109-5115.

Tatsuta T (2009) Protein quality control in mitochondria. *J. Biochem.* **146**: 455-61

Taylor RW, Turnbull DM. (2005) Mitochondrial DNA mutations in human disease. *Nat. Rev. Genet.* **6**: 389-402.

Temin HM, Mizutani S (1970) RNA-dependent DNA polymerase in virions of Rous sarcoma virus. *Nature.* **226**: 1211-1213.

Tiranti V, Rocchi M, DiDonato S, Zeviani M. (1993) Cloning of human and rat cDNAs encoding the mitochondrial single-stranded DNA-binding protein (SSB). *Gene.* **126**: 219-225.

Tiranti V, Savoia A, Forti F, D'Apolito MF, Centra M, Rocchi M, Zeviani M. (1997) Identification of the gene encoding the human mitochondrial RNA polymerase (h-mtRPOL) by cyberscreening of the Expressed Sequence Tags database. *Hum. Mol. Genet.* **6**: 615-625.

Tran HT, Degtyareva NP, Koloteva NN, Sugino A, Masumoto H, Gordenin DA, Resnick MA. (1995) Replication slippage between distant short repeats in *Saccharomyces cerevisiae* depends on the direction of replication and the RAD50 and RAD52 genes. *Mol. Cell. Biol.* **15**: 5607-5617.

Trifunovic A, Wredenberg A, Falkenberg M, Spelbrink JN, Rovio AT, Bruder CE, Bohlooly-Y M, Gidlöf S, Oldfors A, Wibom R, Törnell J, Jacobs HT, Larsson NG. (2004) Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature.* **429**: 417-423.

Trifunovic A, Larsson NG. (2008) Mitochondrial dysfunction as a cause of ageing. *J. Intern. Med.* **263**: 167-178.

Truniger V, Lázaro JM, Salas M, Blanco L. (1996) A DNA binding motif coordinating synthesis and degradation in proofreading DNA polymerases. *EMBO J.* **15**: 3430-3441.

Trzemecka A, Płochocka D, Bebenek A (2009) Different behaviors in vivo of mutations in the beta hairpin loop of the DNA polymerases of the closely related phages T4 and RB69. *J. Mol. Biol.* **389**: 797-807

Tua A, Wang J, Kulpa V, Wernette CM. (1997) Mitochondrial DNA topoisomerase I of *Saccharomyces cerevisiae*. *Biochimie.* **79**: 341-350.

Toussaint F. (2010) Caractérisation de la mutation C261R dans l'ADN polymérase mitochondriale (pol g) de *S. Cerevisiae*, équivalente à la mutation pathogène S305R chez l'homme. Université Catholique de Louvain, master thesis.

Tully LA, Parsons TJ, Steighner RJ, Holland MM, Marino MA, Prenger VL. (2000) A sensitive denaturing gradient-Gel electrophoresis assay reveals a high frequency of heteroplasmy in hypervariable region 1 of the human mtDNA control region. *Am. J. Hum. Genet.* **67**: 432-443.

Tuske S, Singh K, Kaushik N, Modak MJ. (2000) The J-helix of *Escherichia coli* DNA polymerase I (Klenow fragment) regulates polymerase and 3'- 5'-exonuclease functions. *J. Biol. Chem.* **275**: 23759-23768.

Tzagoloff A, Akai A, Needleman RB, Zulch G. (1975) Assembly of the mitochondrial membrane system. Cytoplasmic mutants of *Saccharomyces cerevisiae* with lesions in enzymes of the respiratory chain and in the mitochondrial ATPase. *J. Biol. Chem.* **250**: 8236-8242.

Vanderstraeten S, Van den Brule S, Hu J, Foury F (1998) The role of 3'-5' exonucleolytic proofreading and mismatch repair in yeast mitochondrial DNA error avoidance. *J. Biol. Chem.* **273**: 23690-23697

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

Van Goethem G, Dermaut B, Lofgren A, Martin JJ, Van Broeckhoven C (2001) Mutation of POLG is associated with progressive external ophthalmoplegia characterized by mtDNA deletions. *Nat. Genet.* **28**: 211-212

Van Goethem G, Martin JJ, Dermaut B, Lofgren A, Wibail A, Ververken D, Tack P, Dehaene I, Van Zandijcke M, Moonen M, Deuterick C, De Jonghe P, Van Broeckhoven C (2003) Recessive POLG mutations presenting with sensory and ataxic neuropathy in compound heterozygote patients with progressive external ophthalmoplegia. *Neuromuscul. Disord.* **13**: 133-142

Van Goethem G, Luoma P, Rantamaki M, Al Memar A, Kaakkola S, Hackman P, Krahe R, Lofgren A, Martin JJ, De Jonghe P, Suomalainen A, Udd B, Van Broeckhoven C (2004) POLG mutations in neurodegenerative disorders with ataxia but no muscle involvement. *Neurology* **63**: 1251-1257

Viikov K, Våljamäe P, Sedman J. (2010) Yeast mitochondrial DNA polymerase is a highly processive single-subunit enzyme. *Mitochondrion*. 2010 Aug 31. Doi:10.1016/j.mito.2010.08.007

Voos W (2009) Mitochondrial protein homeostasis: the cooperative roles of chaperones and proteases. *Res. Microbiol.* **160**: 718-725

Wagner J, Gruz P, Kim SR, Yamada M, Matsui K, Fuchs RP, Nohmi T. (1999) The *dinB* gene encodes a novel *E. coli* DNA polymerase, DNA pol IV, involved in mutagenesis. *Mol. Cell.* **4**: 281-286.

Wallace DC, Singh G, Lott MT, Hodge JA, Schurr TG, Lezza AM, Elsas LJ 2nd, Nikoskelainen EK. (1988) Mitochondrial DNA mutation associated with Leber's hereditary optic neuropathy. *Science.* **242**: 1427-1430.

Wallace DC, Zheng XX, Lott MT, Shoffner JM, Hodge JA, Kelley RI, Epstein CM, Hopkins LC. (1988) Familial mitochondrial encephalomyopathy (MERRF): genetic, pathophysiological, and biochemical characterization of a mitochondrial DNA disease. *Cell.* **55**: 601-610.

Wallace DC. (2005) A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. *Annu. Rev. Genet.* **39**: 359-407

Wang J, Yu P, Lin TC, Konigsberg WH, Steitz TA. (1996) Crystal structures of an NH₂-terminal fragment of T4 DNA polymerase and its complexes with single-stranded DNA and with divalent metal ions. *Biochemistry.* **35**: 8110-8119.

Wang J, Sattar AK, Wang CC, Karam JD, Konigsberg WH, Steitz TA. (1997) Crystal structure of a pol alpha family replication DNA polymerase from bacteriophage RB69. *Cell.* **89**: 1087-1099.

- Wang Y, Kaguni LS. (1999) Baculovirus expression reconstitutes *Drosophila* mitochondrial DNA polymerase. *J. Biol. Chem.* **274**: 28972-28977.
- Wang Z, Castaño IB, De Las Peñas A, Adams C, Christman MF. (2000) Pol kappa: A DNA polymerase required for sister chromatid cohesion. *Science.* **289**: 774-779.
- Wang Z, Christman MF. (2001) Replication-related activities establish cohesion between sister chromatids. *Cell. Biochem. Biophys.* **35**: 289-301.
- Wang L, Eriksson S. (2003) Mitochondrial deoxyguanosine kinase mutations and mitochondrial DNA depletion syndrome. *FEBS Lett.* **554**: 319-322.
- Wanrooij S, Fusté JM, Farge G, Shi Y, Gustafsson CM, Falkenberg M. (2008) Human mitochondrial RNA polymerase primes lagging-strand DNA synthesis in vitro. *Proc. Natl. Acad. Sci. U. S. A.* **105**: 11122-11127
- Wanrooij S, Falkenberg M. (2010) The human mitochondrial replication fork in health and disease. *Biochim. Biophys. Acta.* **1797**: 1378-1388.
- Watson JD, Crick FH. (1953) Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid. *Nature.* **171**: 737-738.
- Wernette CM, Kaguni LS. (1986) A mitochondrial DNA polymerase from embryos of *Drosophila melanogaster*. Purification, subunit structure, and partial characterization. *J. Biol. Chem.* **261**: 14764-14770.

Wernette CM, Conway MC, Kaguni LS. (1988) Mitochondrial DNA polymerase from *Drosophila melanogaster* embryos: kinetics, processivity, and fidelity of DNA polymerization. *Biochemistry*. **27**: 6046-6054.

Wilson SH, Sobol RW, Beard WA, Horton JK, Prasad R, Vande Berg BJ. (2000) DNA polymerase beta and mammalian base excision repair. *Cold. Spring. Harb. Symp. Quant. Biol.* **65**: 143-155.

Wing R, Drew H, Takano T, Broka C, Tanaka S, Itakura K, Dickerson RE. (1980) Crystal structure analysis of a complete turn of B-DNA. *Nature*. **287**: 755-758.

Wintersberger U, Wintersberger E (1970) Studies on deoxyribonucleic acid polymerases from yeast. 1. Partial purification and properties of two DNA polymerases from mitochondria-free cell extracts. *Eur. J. Biochem.* **13**: 11-19.

Wolberger C, Dong YC, Ptashne M, Harrison SC. (1988) Structure of a phage 434 Cro/DNA complex. *Nature*. **335**: 789-795.

Wong I, Patel SS, Johnson KA. (1991) An induced-fit kinetic mechanism for DNA replication fidelity: direct measurement by single-turnover kinetics. *Biochemistry*. **30**: 526-537.

Wong LJ. (2010) Molecular genetics of mitochondrial disorders. *Dev. Disabil. Res. Rev.* **16**: 154-162.

Xu B, Clayton DA. (1992) Assignment of a yeast protein necessary for mitochondrial transcription initiation. *Nucleic. Acids. Res.* **20**: 1053-1059.

Yakes FM, Van Houten B. (1997) Mitochondrial DNA damage is more extensive and persists longer than nuclear DNA damage in human cells following oxidative stress. *Proc. Natl. Acad. Sci. U. S. A.* **94**: 514-519.

Yakubovskaya E, Chen Z, Carrodegua JA, Kisker C, Bogenhagen DF (2006) Functional human mitochondrial DNA polymerase gamma forms a heterotrimer. *J. Biol. Chem.* **281**: 374-382

Yamaguchi M, Matsukage A, Takahashi T. (1980) Chick embryo DNA polymerase gamma. Purification and structural analysis of nearly homogenous enzyme. *J. Biol. Chem.* **255**: 7002-7009.

Yasukawa T, Reyes A, Cluett TJ, Yang MY, Bowmaker M, Jacobs HT, Holt IJ. (2006) Replication of vertebrate mitochondrial DNA entails transient ribonucleotide incorporation throughout the lagging strand. *EMBO J.* **25**: 5358-5371

Young MJ, Theriault SS, Li M, Court DA. (2006) The carboxyl-terminal extension on fungal mitochondrial DNA polymerases: identification of a critical region of the enzyme from *Saccharomyces cerevisiae*. *Yeast.* **23**: 101-116.

Zassenhaus HP, Hofmann TJ, Uthayashanker R, Vincent RD, Zona M. (1988) Construction of a yeast mutant lacking the mitochondrial nuclease. *Nucleic. Acids. Res.* **16**: 3283-3296.

Zeviani M, Bresolin N, Gellera C, Bordini A, Pannacci M, Amati P, Moggio M, Servidei S, Scarlato G, DiDonato S. (1990) Nucleus-driven multiple large-scale deletions of the human mitochondrial genome: a new autosomal dominant disease. *Am. J. Hum. Genet.* **47**: 904-914.

Zhang D, Mott JL, Chang SW, Denniger G, Feng Z, Zassenhaus HP. (2000) Construction of transgenic mice with tissue-specific acceleration of mitochondrial DNA mutagenesis. *Genomics.* **69**: 151-161.

Zhang D, Mott JL, Farrar P, Ryerse JS, Chang SW, Stevens M, Denniger G, Zassenhaus HP. (2003) Mitochondrial DNA mutations activate the mitochondrial apoptotic pathway and cause dilated cardiomyopathy. *Cardiovasc. Res.* **57**: 147-157.

Zhang H, Meng LH, Pommier Y. (2007) Mitochondrial topoisomerases and alternative splicing of the human TOP1mt gene. *Biochimie.* **89**: 474-481.