

The *Saccharomyces cerevisiae* mitochondrial DNA polymerase and its contribution to the knowledge about human POLG-related disorders

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Abstract

Most eukaryotes possess a mitochondrial genome, called mtDNA. In animals and fungi, the replication of mtDNA is entrusted by the DNA polymerase γ , or Pol γ . The yeast Pol γ is composed only of a catalytic subunit encoded by *MIP1*. In humans, Pol γ is a heterotrimer composed of a catalytic subunit homolog to Mip1, encoded by *POLG*, and two accessory subunits. In the last 25 years, more than 300 pathological mutations in *POLG* have been identified as the cause of several mitochondrial diseases, called POLG-related disorders, which are characterized by multiple mtDNA deletions and/or depletion in affected tissues. In this review, at first, we summarize the biochemical properties of yeast Mip1, and how mutations, especially those introduced recently in the N-terminal and C-terminal regions of the enzyme, affect the in vitro activity of the enzyme and the in vivo phenotype connected to the mtDNA stability and to the mtDNA extended and point mutability. Then, we focus on the use of yeast harboring Mip1 mutations equivalent to the human ones to confirm their pathogenicity, identify the phenotypic defects caused by these mutations, and find both mechanisms and molecular compounds able to rescue the detrimental phenotype. A closing chapter will be dedicated to other polymerases found in yeast mitochondria, namely Pol ζ , Rev1 and Pol η , and to their

Abbreviations: AAF, N-2 acetyl-amino-fluorene; adPEO, progressive external ophthalmoplegia, autosomal dominant; AID, accessory-interacting determinant; AR, autosomal recessive; arPEO, progressive external ophthalmoplegia, autosomal recessive; ANS, ataxia-neuropathy syndrome; CPDs, cyclobutene pyrimidine dimers; d4T, 2',3'-didehydro-2',3'-dideoxythymidine; ddC, 2',3'-dideoxycytidine; Ery^R, erythromycin resistant; Exo, exonuclease; IP, intrinsic processivity; MEMSA, myoclonic epilepsy myopathy sensory ataxia; MNGIE, mitochondrial neurogastrointestinal encephalopathy syndrome; mtDNA, mitochondrial DNA; MTS, mitochondrial targeting signal; mt-rRNA, mitochondrial rRNA; mt-tRNA, mitochondrial tRNA; NGS, next-generation sequencing; NRTI, Nucleoside Reverse Transcriptase Inhibitors; NTE, N-terminal extension; PAD, polymerase-associated domains; pCTE, palm C-terminal extension; Pol, polymerase; Pol γ , DNA polymerase γ ; Pol ζ , DNA polymerase ζ ; Pol η , DNA polymerase η ; qPCR, quantitative polymerase chain reaction; R7B, Rev7-binding domain; RD, respiratory deficient; RDR, recombination-driven replication; RER, ribonucleotide excision repair; ROS, reactive oxygen species; RP, respiratory proficient; SANDO, sensory ataxia neuropathy dysarthria and ophthalmoplegia; SCAE, mitochondrial spinocerebellar ataxia with epilepsy; TLS, translesion synthesis; wt, wild-type; yCTE, yeast C-terminal extension.

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genetic interactions with Mip1 necessary to maintain mtDNA stability and to avoid the accumulation of spontaneous or induced point mutations.

KEYWORDS

DNA polymerase γ , DNA polymerase ζ -Rev1, DNA polymerase η , Mip1, POLG-related disorders, yeast

1 | YEAST MITOCHONDRIAL DNA AND MITOCHONDRIAL GENETICS

Most eukaryotes possess mitochondria, which have their own genome, called mitochondrial DNA or mtDNA. In most organisms, the mtDNA encodes for specific subunits of the respiratory complexes and of the ATP synthase as well as specific tRNAs and rRNAs involved in the mitochondrial translation. However, most of the mitochondrial proteome is composed of proteins that are encoded by nuclear genes, synthesized in the cytoplasm, and imported into mitochondria.

Depending on the strain, the length of the mtDNA of the yeast *Saccharomyces cerevisiae* can range from 68 kb in the so-called “short strains” to 86 kb in the “long strains”. The reference mtDNA sequence derives from strain FY1679, isogenic to the reference strain S288c.¹ The yeast mitochondrial genome contains seven genes encoding for subunits of the respiratory complexes, *COB*, *COX1*, *COX2*, *COX3*, *ATP6*, *ATP8* and *ATP9*, one gene, *VARI*, encoding for a subunit of the mitochondrial ribosome, two genes encoding for the 15S mt-rRNA and for the 21S mt-rRNA, 24 genes encoding for the mt-tRNAs and one gene, *RPM1*, encoding for an RNA subunit of the mitochondrial RNase P. In addition, several genes, most of which are located inside the introns of *COX1*, *COB* and 21S mt-rRNA genes, encode for maturases, endonucleases, and a reverse transcriptase.²

Like most eukaryotes, yeast cells contain several copies of mtDNA molecules. It has been estimated that each cell contains 10–50 to 50–200 mtDNA copies per nuclear genome depending on the strain and the growth conditions.^{3–5} mtDNA interacts with a set of specific proteins, forming discrete structures called nucleoids. A variable number of nucleoids are present in each mitochondrion and each nucleoid contains one or more copies of mtDNA. Most of the proteins of the nucleoid are directly or indirectly involved in maintaining the integrity of the mtDNA and are responsible for its replication, recombination, repair, and transmission to the daughter cells as well as transcription and gene regulation.^{6,7}

Whereas the mechanism of mtDNA replication in mammals is well-known, several mechanisms have been

hypothesized to explain the synthesis of mtDNA in *S. cerevisiae*. Although yeast mtDNA can be represented as a circular molecule, based on the genetic mapping and the sequencing data, most mtDNA molecules are not circular in vivo, but form head-to-tail DNA concatemers composed of several linear genome units.^{8,9} The replication could start at several sites called Ori sites, and it is RNA-primed and bidirectional like that of chromosomal DNA, as reviewed in.¹⁰ Alternatively, the replication can occur via a “rolling circle” mechanism, which produces long repeats of linear mtDNA molecules, which stay as concatemers or are cut and ligated into circular monomers.^{11,12} According to more recent findings, homologous recombination and strand invasion could account for the initiation of the replication, indicating a recombination-driven replication (RDR) mechanism.^{11,13}

Yeast mtDNA is highly recombinogenic. In particular, there are 2.3–3 recombination events per kb of mtDNA in diploids deriving from the crosses of two haploid clones with different mtDNA sequences.¹⁴ However, recombination is not randomly distributed along the mtDNA sequence, but hotspots, occurring mainly in intergenic regions and inside introns, are present.

Contrary to what happens in most eukaryotes, including several fungi, the mtDNA is dispensable for the viability of *S. cerevisiae*, due to its *petite* positivity. In fact, *S. cerevisiae* is a facultative anaerobe yeast able to survive in the absence of oxidative phosphorylation and of the mitochondrial genome, but only if a fermentable carbon source is added to the medium. Such respiratory deficient (RD) cells generate ATP by alcoholic fermentation. However, on media containing fermentable carbon sources, RD cells form smaller colonies compared to wild type respiratory proficient (RP) cells and are thus called *petites*. The *petite* colonies can derive from mutations either in nuclear genes encoding mitochondrial functions (*pet* mutants¹⁵) or in mitochondrial genes (cytoplasmic *petite* mutants, or simply *petites*¹⁶). The latter mutants are completely devoid of mtDNA (*rho*⁰ cells) or carry extensive deletions of mtDNA and regular repetitions of the conserved sequences (*rho*⁻ cells), contrarywise to cells harboring functional mtDNA, which are respiratory-proficient and termed *rho*⁺, as reviewed in detail in.² Several genes are involved in the maintenance of the integrity of the

mtDNA, and mutations in such genes affect the onset and the frequency of *petite* cells in a clonal population, referred to as mtDNA extended mutability, as reviewed in.^{17,18} mtDNA is also subjected to point mutations, which arise spontaneously at low frequencies (around 10^{-9} to 10^{-7}). Based on the phenotype, two principal groups of mtDNA mutants can be selected. The first class includes the *mit*⁻ mutants, which are cells showing an RD phenotype due to null mutations in mtDNA genes. The second class includes the antibiotic-resistant mutants, which are cells harboring specific mutations which confer resistance to antibiotics or chemicals that inhibit mitochondrial translation, such as erythromycin, chloramphenicol and paromomycin, ATP synthase activity, such as oligomycin, and cytochrome b activity, such as diuron (reviewed in²). Mutations in genes involved in mtDNA replication and repair affect the onset and thus the frequency of such antibiotic-resistant mutants, referred to as mtDNA point mutability.

2 | MIP1 STRUCTURE AND BIOCHEMICAL PROPERTIES

In all opisthokonta (fungi, protozoa and metazoan/animals), the replication is entrusted to the DNA polymerase γ , hereafter Pol γ , which has been considered for a long time the only DNA polymerase present in mitochondria. Although other DNA polymerase activities were identified in *S. cerevisiae* and animals' mitochondria, as described below, Pol γ is the only enzyme able to fully replicate mtDNA and can be considered the unique replicase: indeed, the lack of Pol γ makes the strain *rho*⁰.

Pol γ belongs to the subclass γ of the family A polymerases to which several bacterial and viral DNA polymerases also belong. In animals, Pol γ is composed of a catalytic subunit, encoded by POLG, and one or more accessory subunits. In humans, Pol γ consists of a catalytic subunit, encoded by *POLG*, and two accessory subunits, encoded by *POLG2*.¹⁹ On the contrary, fungal Pol γ is a monomeric protein, which in yeast is encoded by *MIP1*.^{20,21} Mip1 is a 1,254 amino acid-long protein of approximately 140 kDa and, like most mitochondrial proteins, is synthesized in the cytoplasm and imported into the mitochondria. The import into the mitochondrial matrix is allowed by the presence of a mitochondrial targeting sequence, which, based on a prediction by MitoFates,²² is 29 amino acids long. As its human counterpart, Mip1 contains an N-terminal exonuclease domain, a spacer, or linker, domain, a C-terminal polymerase domain, plus an N-terminal extension and a C-terminal extension (Figure 1). Recently, the structural organization of Mip1 was predicted through homology-based bioinformatic modeling using the crystal structure of human POLG as template, allowing to better define the role of the N-terminal and C-terminal extensions.^{23,24}

Like in most polymerases, the polymerase domain (pol domain, amino acids ~388–424 and ~585–948) is divided into three subdomains: thumb (388–424 and 585–618 stretches), palm (618–712 and 852–948 stretches) and fingers (712–852 stretch). Three motifs are highly conserved in all eukaryotic Pol γ and are called *polA*, *polB* and *polC*. The catalytic domain of Mip1 has a K_m for dNTPs lower than $1 \mu\text{M}$,²⁵ the k_{pol} is about 60 nt/s and the processivity is about 500 nt per one binding event.²¹ Mip1 can use as substrate poly [dA-dT] and poly[dA]-

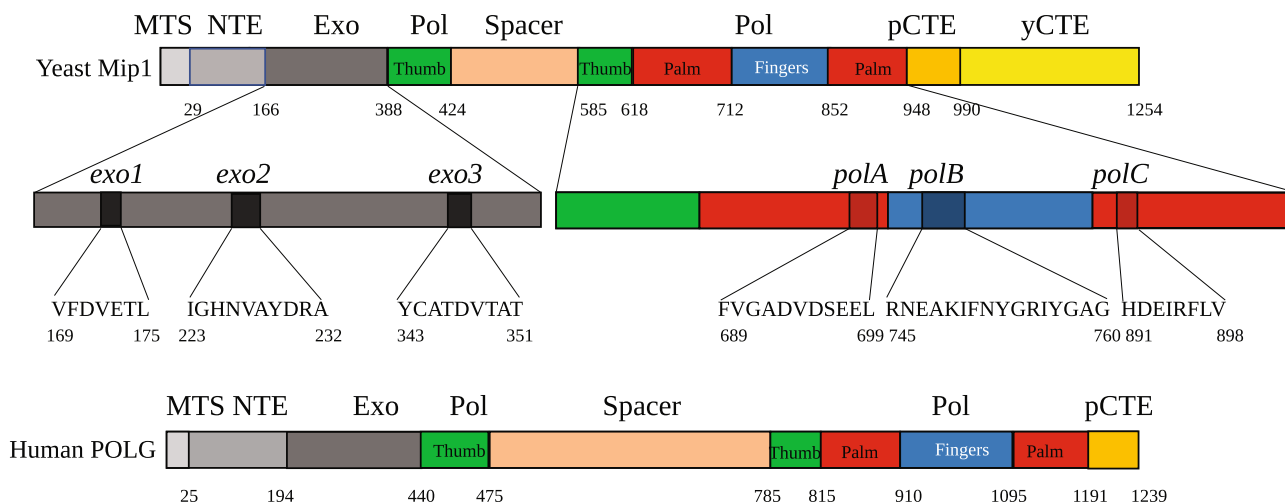


FIGURE 1 Schematic representation of yeast Mip1 and human POLG according to the structure of POLG³² and the structural prediction of Mip1.²³ Exo, exonuclease domain; MTS, mitochondrial targeting signal, as predicted with MitoFates; NTE, N-terminal extension; pCTE, palm C-terminal extension; Pol, polymerase domain; Spacer, spacer or linker region; yCTE, yeast C-terminal extension.

oligo[dT], but, contrary to human Pol γ , is unable to extend the poly [rA]-oligo [dT]. Furthermore, Mip1, unlike animal Pol γ and like family A prokaryotic polymerases, is also able to perform strand displacement with a rate of about 30 nt/s. Specific mutations in the *pol* motifs negatively affect the polymerase activity, and this increases both the mtDNA point mutability and the mtDNA extended mutability, measured as the frequency of *petite* clones in the population, indicating that the catalytic domain is involved both in the mtDNA polymerization and in replication fidelity.²⁶ A detailed abstract of the effects of single mutations in the *pol* domain has previously been reported.²⁷

The 3′–5′ exonuclease domain (exo domain, amino acids ~166–388) has a proofreading activity that, in human Pol γ , increases the fidelity of replication by at least 20-fold.²⁸ Exo domain contains three highly conserved motifs, called *exo1*, *exo2* and *exo3*. Specific mutations in one of these motifs strongly reduce the exonuclease activity.^{26,29,30} Mip1 proofreading activity corrects mainly transversions, whereas the protein Msh1, involved in the post-replication mismatch repair, corrects mainly transitions. Some of these mutations also promote the extension from a mismatched extremity, affecting further the replication fidelity. In addition, many mutations in the exo domain affect the stability of the mtDNA, suggesting that the activity of the exo domain is deeply connected to that of the *pol* domain and that such mutations also decrease the polymerase activity. The effects of several mutations in the exo domain have also been reported.²⁷ Interestingly, by random mutagenesis, it was identified a *MIP1* mutation, p.Ala256Thr, which shows an opposite effect. Indeed, it behaves as an antimutator allele, able to decrease the frequency of mtDNA point mutations, probably due to an increase in the exonuclease activity.³¹

The spacer region (amino acids ~424–585) connects the exo and the *pol* domains, and in fungi it is shorter than in animal counterparts. Indeed, in human Pol γ the spacer domain is divided into two subdomains: an intrinsic processivity (IP) subdomain and a long accessory-interacting determinant (AID) subdomain.³² The IP subdomain is responsible for the intrinsic processivity of the catalytic subunit, whereas the AID subdomain interacts with the accessory subunits, which enhanced further the processivity. Since the accessory subunit is absent in fungi, most of the sequence corresponding to the AID subdomain is absent in Mip1. However, also IP subdomain has a shorter sequence than the human counterpart. Mip1 is highly processive, suggesting that the mechanism responsible for processivity in Mip1 is different, at least partially, from that of the human Pol γ .²¹

The N-terminal extension (amino acids 29 to ~166) has been recently characterized through the combination of the predicted structure of Mip1 with *in vivo* and *in vitro* assays, showing an active role in the exonucleolytic and in the polymerization activity.²³ The N-terminal extension of Mip1, like its human counterpart, and despite the low degree of conservation, interacts with the palm and the fingers subdomain and with the C-terminal extension, including the long C-terminal extension typical of fungi and absent in animal Pol γ , as reported below. Deletion of fragments of different lengths of the N-terminal extension has consequences on the biochemical properties and on the *in vivo* phenotype. Deletion of the first 11 amino acids, from amino acids 31 to 41 (excluding the MTS necessary for the import in mitochondria and the first amino acid found in the mature enzyme) increased by 2.7-fold the binding affinity for the dsDNA *in vitro*. In a primer degradation assay, the variant was still able to remove nucleotides, though to a lesser extent than the wild-type Mip1 and producing longer fragments, indicating a reduction of the exonucleolytic activity. In addition, in a primer-template extension reaction, the variant extended only 11% of the primers compared to wild-type Mip1, demonstrating a direct effect on the polymerase activity. Although a polymerase activity is still present, when this variant was introduced in yeast, the strain was devoid of mtDNA, indicating that the variant is unable to fully replicate mtDNA. A deletion of 71 amino acids increased further the binding affinity (4.3-fold) and showed a stronger decrease of the exonucleolytic activity; the polymerase activity was completely abolished (Table 1).

Based on the structural prediction, the C-terminal extension of Mip1 has two subregions: the “palm” C-terminal extension (amino acids ~948–990), which is present in the catalytic subunit of all Pol γ , and a long positively-charged “yeast” C-terminal extension (amino acids ~990–1,254), which is typical of fungal Pol γ and whose length is different among species, reaching the maximum length in Saccharomycetales. In the predicted structure of Mip1, not only the “palm” C-terminal extension, as its human counterpart, but also the “yeast” C-terminal extension is oriented and interacts with the N-terminal extension and with the exo domain. The role of the C-terminal extension has been analyzed by evaluating the *in vitro* biochemical properties and the *in vivo* phenotypes of Mip1 variants with C-terminal deletions (Table 1).^{23,24,33,34} The last 175 amino acids (1,079–1,254) are dispensable for the *pol* activity and do not affect the maintenance of *rho*⁺ mtDNA: quite surprisingly, lack of this stretch slightly enhanced the exonuclease activity, resulting in a three-fold decrease of the mtDNA point mutability, and strongly increased the processivity, likely

TABLE 1 In vivo phenotypes and in vitro biochemical properties of Mip1 variants with truncation of either the N-terminal or the C-terminal extensions.

Mip1 deletion	Truncated fragment	In vivo phenotype				In vitro biochemical properties						Ref
		RP or RD	mtDNA extended mutability	mtDNA point mutability	Protein levels	Pol activity	Exo activity	Exo/pol ratio	DNA binding affinity	Processivity		
ΔN11	31–41	RD	NT	NT	ND	↓↓↓	↓	↑↑↑	↑	ND	23	
ΔN71	31–101	RD	NT	NT	ND	Absent	↓↓	↑↑↑	↑↑	ND	23	
ΔN91	31–121	RD	NT	NT	ND	ND	ND	ND	ND	ND	23	
ΔC175	1,079–1,254	RP	= or ↑	↓	=	=	↑	=	= or ↑	↑↑	23,33,34	
ΔC196	1,058–1,254	RP	↑↑	↑	ND	↓	=	↑	=	ND	23	
ΔC205	1,049–1,254	RP	↑	↑	ND	↓↓	ND	ND	ND	ND	24	
ΔC216	1,038–1,254	RP	↑↑↑	↑↑	=	Absent or ↓↓↓	↓	↑	↑↑	↑	24,34	
ΔC222	1,032–1,254	RD	NT	NT	ND	Absent	ND	ND	ND	ND	24	
ΔC227	1,027–1,254	RD	NT	NT	ND	↓↓↓	↓	↑	↑	ND	23	
ΔC263	991–1,254	RD	NT	NT	ND	↓↓↓	↓	↑	↑	ND	23	
ΔC279	975–1,254	RD	NT	NT	↓	Absent	↓↓	↑↑	ND	ND	34	
ΔC298	956–1,254	RD	NT	NT	ND	Absent	↓↓↓	↑↑↑	↑	ND	23	
ΔC351	903–1,254	RD	NT	NT	↓	ND	ND	ND	ND	ND	33	

Note: Arrows indicate a decrease, or an increase, compared to the wild type Mip1: one arrow indicates a slight variation, two arrows a moderate variation, three arrows a strong variation, while four arrows indicate that the activity is almost abolished. mtDNA extended mutability is measured as *petite* frequency; mtDNA point mutability is measured as the frequency of Ery^R mutants. Abbreviations: ND, not determined; NT, not testable due to respiratory deficient phenotype; RD, respiratory deficient and likely *rho*⁰; RP, respiratory proficient.

due to a two-fold increase in the DNA binding affinity. The following stretch of 21 amino acids (1,058–1,078) contributes to the full polymerase activity but has a minor effect on the exonuclease activity; therefore, the lack of this stretch (together with the last 175 amino acids) strongly increased the *petite* frequency and slightly the frequency of point mutants resistant to erythromycin (Ery^R mutant frequency). The latter phenotype may be due to a decrease in the fidelity of nucleotide incorporation by the pol domain. The stretch encompassing amino acids 1,038 to 1,057 is critical for the polymerase activity, which is almost abolished in the absence of this fragment, and for the balance between the exo activity and the pol activity; consequently, the deletion of a region encompassing also this region made the strain *rho*⁰ after a few generations on medium supplemented with glucose. However, when maintained in a medium supplemented with a non-fermentable carbon source, the mtDNA is maintained, but the Ery^R point mutability is 10 to 100-fold higher. The lack of 1,028–1,037 stretch and of the whole yeast C-terminal extension makes the strain RD and *rho*⁰, indicating that, although a minimal pol activity is maintained in vitro in a variant without this extension, the activity is not sufficient to replicate a full mtDNA molecule. Besides abolishing any residual polymerase activity in vitro, the lack of the last 16 amino acids of the palm C-terminal extension (975–990) partially reduced the exonuclease activity, indicating that the palm C-terminal extension influences the exonuclease domain. Finally, the removal of a further 19 amino acid-long stretch (956–974) abolished the exonuclease activity too, confirming the previous observation.

Altogether, the results show that the five regions/domains of Mip1 interacts and influence each other; this can explain why pathological mutations in the catalytic subunit of Pol γ associated with depletion or multiple deletions of the mtDNA are distributed all along the *POLG1* gene sequence, as reported in the following section.

The levels of the dNTP pool in the mitochondria affect the polymerase activity of Mip1. Pol γ , as some nuclear DNA polymerases, can incorporate rNMPs instead of dNMPs in the nascent strand, due to the higher concentrations of the rNTPs relative to the dNTPs, and this results in genomic instability.^{35,36} Whereas in nuclear DNA the rNMPs are removed by the ribonucleotide excision repair (RER), this activity is absent in mitochondria so that yeast mtDNA, as its human counterpart, contains rNMPs.^{37,38} It was recently demonstrated, by creating mutant strains in which the dNTP pools are unbalanced in favor of one dNTP relative to the others,

that increasing the concentration of a single dNTP decreases the incorporation of the corresponding rNMP compared to the other three rNMPs in mtDNA, and vice versa, indicating that the relative frequencies of the four rNMPs incorporated in mtDNA are dependent on the balance of the cellular dNTP pools.³⁹ Most intriguingly, an increase of all four dNTPs proportionally decreased the incorporation of the four rNMPs in the mtDNA, indicating that a reduction of the mtDNA instability obtained by increasing the dNTP pools, as reported below, may be due also to lower levels of rNMPs incorporated into the mtDNA. In vitro analysis, performed using physiological levels of dNTPs and rNTPs, confirmed that the purified Mip1 incorporated rNMPs at high frequency ($\sim 1/600$), both if the exonuclease domain is active or inactivated, indicating that the exo domain is unable to remove rNMPs. In addition, Mip1 showed a different degree of selectivity for each dNTP/rNTP pair.³⁹

In the absence of autophagy, the shortage of the dNTP pools induced by starvation negatively influenced the mtDNA stability, resulting in a decrease in the mtDNA copy number. Indeed, increasing the dNTPs levels in a mutant unable to perform autophagy increased the mtDNA levels. In the presence of limiting dNTP pools, the in vivo mtDNA instability is due to the shifting of Mip1 from the polymerase activity to the exonuclease activity, resulting in the degradation of mtDNA. In mutants devoid of the Mip1 exonucleolytic activity, the decrease in the mtDNA levels is limited, both in the absence of autophagy and, to a lesser extent, when autophagy is present, indicating that the exo domain of Mip1 decreased mtDNA stability in nondividing cells.⁴⁰

The biochemical properties of Mip1 are also influenced by polymorphisms. *MIP1* is a highly polymorphic gene, and more than 60 single nucleotide polymorphisms (SNPs) are present among different strains. Most of these SNPs are silent, whereas 28 SNPs result in amino acid substitutions.²⁷ Among the laboratory strains, the reference S288C strain, whose allele is indicated as *MIP1*[S], and the strain Sigma1278b, which possesses the *MIP1*[\Sigma] allele, are the most divergent strains.⁴¹ *MIP1*[S] allele is present also in the BY474X strains, isogenic to S288C and used for the construction of the original deletant strains collections, and in the W303-derived strains, often used for the analysis of mitochondrial phenotypes. Most of the substitutions are semi-conservative and/or map in positions that are poorly conserved in Pol γ of most opisthokonta, suggesting that these substitutions are neutral and do not affect the biochemical properties of the enzyme. A noteworthy exception concerns amino acid 661, which is Ala in S288C strains and Thr in Sigma1278b but also in

almost all opisthokontal Pol γ . S288C inherited Ala661 from its ancestor strain EM93, a heterozygous diploid strain that contains a Thr661 allele and an Ala661 allele. The Mip1[S] variant affects the in vivo phenotype and the biochemical properties, and the differences can be ascribed principally to the presence of Ala at position 661. Ala661 results both in a higher mtDNA extended mutability (2%–2.5% *petite* frequency compared to 0.5%–1% for an isogenic strain with Thr661) and in a thermo-sensitive phenotype (5%–20% *petite* frequency at 36°C, 25%–40% at 37°C, 60%–70% at 38°C).^{42,43} This substitution is also responsible for about 25% of the *petite* mutability in strains BY474X.⁴⁴ The Ala661 variant showed 50%–70% of the in vitro polymerase activity relative to the Thr661 variant.²⁴ Structurally, the amino acid 661 is in a linker region typical of the palm subdomain of the subfamily γ of the Family A polymerases. The structural prediction shows that Thr661 interacts with Arg607 through a hydrogen bond, and this interaction contributes to the positioning of the primer/template into the pol domain; the substitution of Thr661 with Ala abolishes this interaction and may alter the position of the DNA.²⁴

3 | YEAST AS A MODEL FOR STUDYING POLG-RELATED DISORDERS

Since the discovery that mitochondrial diseases are caused also by mutations in nuclear genes encoding for mitochondrial proteins, yeast has been extensively used as a model for studying the phenotypic consequences on the mitochondrial metabolism of such mutations. Yeast is an excellent model to study mutations associated with mitochondrial pathologies, since several genes are conserved from yeasts to mammals and yeast can survive and grow even if the oxidative phosphorylation is impaired. *S. cerevisiae* is useful for the validation of novel mutations in genes already known to be associated with mitochondrial disorders or in novel genes. Taking advantage of the techniques of next-generation sequencing (NGS), and especially of whole exome sequencing, the number of mutations identified as putatively associated with pathology has increased exponentially and, since often the familiar history is unknown, validation is critical to discriminate between pathological mutations and neutral mutations/polymorphisms. Yeast has been used to model hundreds of mutations in more than 80 genes.⁴⁵ Among these genes, yeast is particularly useful for studying mutations in genes associated with mtDNA instability, due to its *petite* positivity, as reported above. Indeed, mutations in six genes associated with deletions and/or depletion of mtDNA have been characterized in yeast,⁴⁶ among which *MIP1*.

3.1 | POLG-related disorders

To date, more than 300 pathological mutations in *POLG* have been identified. They are associated with a heterogeneous group of mitochondrial pathologies called POLG-related disorders (<https://tools.niehs.nih.gov/polg/>).⁴⁷ POLG-related disorders can be classified into different groups based on mtDNA defect, age of the onset, affected tissues and organs and kind of inheritance. Interestingly, POLG mutations are present in all the five regions/domains of the Pol γ catalytic subunit, suggesting, as reported previously, that also in animal Pol γ each region interacts with the other regions, affecting thus the polymerase activity.

POLG pathological mutations are associated with mtDNA instability, which, depending on the pathology, could consist in mtDNA depletion, that is, reduction of the mtDNA levels, or multiple mtDNA deletions. As primary mutations in mtDNA, secondary mutations in mtDNA caused by POLG mutations are typically heteroplasmic in patients. When two mtDNA molecules are present inside a cell (for example, a wild-type molecule and a mutant molecule), two alternative conditions can occur: homoplasmy and heteroplasmy. In the former case, after a few cell divisions, two populations of cells co-exist: cells with only wild type mtDNA and cells with only mutated mtDNA. In the latter case, even after several generations, each cell contains both the mtDNA species. A mitochondrial pathology associated with mtDNA mutations occurs when, in a tissue, the percentage of mutant mtDNA molecules exceeds a threshold, whose value depends on the specific kind of mutation and on the tissue. Since the percentage of mtDNA mutant molecules tends to increase with time, mitochondrial pathologies associated with such mutations are typically progressive.

POLG-related disorders can be classified also based on the age of onset. Some pathologies have a perinatal, infancy or childhood onset: these disorders are typically severe or very severe, often lethal, are associated, in most cases, with mtDNA depletion, and are multisystemic. Other pathologies have an adolescence, adulthood or old age onset: these disorders are mild, rarely lethal, affect a single or few tissues/organs and are associated, in most cases, with multiple mtDNA deletions. Depending on the pathology, the affected tissues are typically those which need a high energetic metabolism, especially neurons, muscles and liver. Finally, most POLG-related disorders are autosomal recessive, whereas a specific disorder is autosomal dominant. The POLG-related disorders have been extensively reviewed in⁴⁸ and reported in Table 2.

Interestingly, all the recessive pathological POLG mutations, considered together, have a frequency in the

TABLE 2 POLG-related disorders and their characteristics, including pathologies induced by external factors.

Disease	OMIM number	Onset	Inheritance	mtDNA alteration	Pathological phenotype
Childhood myocerebrohepatopathy spectrum disorders	-	Perinatal–infancy	AR	Depletion	Hypotonia, hepatopathy, developmental delay, faltering growth, renal dysfunction, and cataracts; death from liver failure
mtDNA depletion syndrome 4A or Alpers-Huttenlocher syndrome or Alpers syndrome	203,700	Early-childhood	AR	Depletion	Encephalopathy, neuropathy, and hepatopathy with neurodevelopmental regression, intractable seizures, and liver failure: death from epilepsy partialis continua and refractory status
mtDNA depletion syndrome 4B or MNGIE-like syndrome	613,662	Childhood to early adulthood	AR	Depletion and multiple deletions	Gastrointestinal dysmotility, myopathy and neuropathy
Epilepsy syndromes, including MEMSA	-	Childhood–adolescence	AR	Multiple deletions	Epilepsy with or without myoclonus, myopathy, and sensory ataxia
Mitochondrial recessive ataxia syndrome, including ANS and SANDO/SCAE	607,459	Childhood to adulthood	AR	Multiple deletions	Ataxia, neuropathy, encephalopathy, epilepsy and/or myopathy
Progressive external ophthalmoplegia, autosomal dominant 1, or adPEO	157,640	Adulthood	AD	Multiple deletions	Ophthalmoplegia and myopathy: rarely, ataxia, Parkinsonism, depression, sensorineural hearing loss, cataracts and premature ovarian failure
Progressive external ophthalmoplegia, autosomal recessive, or arPEO	258,450	Adolescence–adulthood	AR	Multiple deletions	
Parkinsonism	-	Adulthood–old age	AR or AD	Multiple deletions	Parkinsonism, movement disorders and in some cases restless legs syndrome
Other diseases	-	Variable	AR or AD	Multiple deletions and/or mtDNA point mutations	Ovarian failure, premature menopause, cataracts, or major depression
NRTIs-induced mitochondrial toxicity	-	-	Presence of SNPs or neutral mutations	mtDNA depletion	NRTIs-induced peripheral neuropathy and/or myopathy, reversible
Valproic acid-induced mitochondrial toxicity	-	-	Presence of SNPs or mutations	Not known	Hepatotoxicity, liver failure, reversible or lethal
Alkylating agents?	-	-	Presence of SNPs and/or mutations	Not known	Not known

Abbreviations: AD, autosomal dominant; ANS, ataxia-neuropathy syndrome; AR, autosomal recessive; MEMSA, myoclonic epilepsy myopathy sensory ataxia; MNGIE, mitochondrial neurogastrointestinal encephalopathy syndrome; SANDO, sensory ataxia neuropathy dysarthria and ophthalmoplegia; SCAE, mitochondrial spinocerebellar ataxia with epilepsy.

population higher than 1%. For this reason, it is expected that more than 1 in 10,000 subjects is affected by an autosomal recessive (AR) POLG-related disorder.⁴⁸ However, the number of patients with such diseases is lower, ranging from 1 to 3 in 100,000. This difference can be due to several reasons, among which abortion or misdiagnosis of a POLG-related disorder. Furthermore, some combinations of mutations are not pathological. In this regard, it must be underlined that the mutations identified in POLG may contribute differently to the development of the disorder. Some mutations are associated with a single pathology, whereas other mutations are associated with different pathologies, based on the second mutation in compound. Sometimes, the same combination of mutations leads to different pathologies with different severity. In other cases, more than two mutations are present in a patient, and it is not clear whether all the mutations participate in the phenotype and whether there is an interaction between two mutations present in the same allele. Finally, some mutations are pathological only when in compound heterozygosity with a severe mutation, but not when in homozygosity or in compound heterozygosity with a mild mutation. Overall, POLG-related disorders have an incomplete penetrance and variable expressivity. Nevertheless, these disorders are the most frequent mitochondrial diseases caused by mutations in nuclear genes, reaching 10% of adult mitochondrial pathologies.⁴⁹

Besides pathological mutations, *POLG* is a highly polymorphic gene that presents both SNPs with a frequency higher than 1% and neutral mutations with a frequency lower than 1%. Although these SNPs/mutations are unable to cause pathologies, some of them can behave as phenotypic modifiers, affecting the severity or the penetrance of POLG-related disorders, or can affect the toxicity of some drugs or environmental pollutants (Table 2). Several Nucleoside Reverse Transcriptase Inhibitors (NRTIs) used against HIV infection, once triphosphorylated, can determine mitochondrial toxicity due to the ability to inhibit Pol γ resulting in mtDNA deletions or depletion^{50,51}; some neutral SNPs or mutations have been demonstrated to increase the NRTIs-induced mitochondrial toxicity.^{52,53} Also, the liver toxicity induced by the antiepileptic valproic acid has been demonstrated to be influenced by common SNPs in *POLG*, although the mechanism is not fully understood.⁵⁴

3.2 | Analysis of human pathological mutations in a yeast model

To date, almost 60 mutations associated with POLG-related disorders have been modeled in yeast, as reported in Table 3 and references therein. Yeast Mip1 and human

POLG share a 45% similarity. However, the similarity is not homogeneously distributed, but it is higher in the exo and in the pol domains. Since most pathological mutations are located in one of these domains, the mutations have been introduced through site-directed mutagenesis in the corresponding position of Mip1, provided that the amino acid is conserved. When the mutated amino acid is not conserved, but localizes in a conserved stretch, the “humanized” allele was also constructed, in which the yeast amino acid was substituted with the amino acid found in wild type human POLG. In most haploid models, the genomic copy of *MIP1* was removed by gene disruption and substituted with a mutant *mip1* allele, either at the genomic locus or cloned in a centromeric plasmid, as previously described.⁴⁵ A few mutations have been modeled through heterologous complementation, in which *MIP1* was replaced by human *POLG* and *POLG2* cDNA: in most cases, the results were similar to those obtained by introducing the same mutations in *MIP1*.^{27,55,56} Most of the mutations found in patients were identified in compound heterozygosity, that is, a mutation was present in the maternal allele and a different mutation in the paternal allele, suggesting a recessive inheritance. However, several patients carry two mutations in one or both alleles. In these cases, it is unclear how the two substitutions in the same allele contribute to the pathological phenotype, especially if one is pathological and the other is neutral, or both contribute to the disease. In yeast, each mutation can be studied singularly or together with a second mutation, thus allowing the determination of the pathogenicity of each mutation.

Since POLG mutations are associated with multiple deletions or depletion, the mtDNA instability was evaluated in yeast by measuring the frequency of *petite* mutants, which is a direct index of alteration of the integrity of the mtDNA, as reviewed in.⁴⁶ Indeed, contrary to what happens in humans, heteroplasmy of mtDNA is just transient in yeast and, after a few cell divisions, cells harboring a single mtDNA are present in the clonal population.² However, as the *petite* frequency in a population recapitulates what happens in single human cells, the yeast experiments give direct information on mtDNA stability in human cells. In addition, through different techniques as reported in,⁵⁷ it is possible to measure, among the *petite* cells, how many clones are *rho*⁰. The frequency of *rho*⁰ mutants indicates if the *mip1* mutant allele induces primarily mtDNA deletions or the loss of the mtDNA. The mtDNA levels were also quantified through quantitative PCR (qPCR) in some studies.^{55,56,58,59} The copy number of mitochondrial genomes was determined by qPCR of short mtDNA fragments, especially inside *COX1*; no detectable PCR products indicate the absence of mtDNA. The integrity of mtDNA was also determined

TABLE 3 POLG mutations modeled in yeast by introducing the mutation in the equivalent position of yeast Mip1.

Human mutation	Domain	D/R in human ^a	Yeast mutation	Fold increase of <i>petite</i> frequency		Fold increase of Ery ^R frequency		Pathogenicity and dominance after analysis in yeast ^f	References
				Haploid ^b	Heteroallelic ^c	Haploid ^d	Heteroallelic ^e		
H110Y	N-terminal	R?	H84Y	= wt	= homoallelic	= wt	= wt	SNP?	62
L244P	Exo	R?	L210P	= wt 9.2	2.5	16-84	6.3	Pathogenic, recessive	62,70
G268A	Exo	R?	G224A	2.5	=homoallelic	9.0	= wt	Clinical variant	60
G303R	Exo	R?	G259R	100%	2.5 (= hemiallelic)	-	2	Pathogenic, recessive	76
L304R	Exo	R	L260R	100% 4.1 71.3	6.3	= wt 51	= wt	Pathogenic, recessive	58,62,70
S305R	Exo	R?	C261R	38.4	2.6 (= hemiallelic)	1.9	= wt	Pathogenic, recessive	76
Q308H	Exo	R?	Q264H	100%	5.5	-	7.6	Pathogenic, recessive	62
R309C	Exo	R	R265C	100%	16.2 (> hemiallelic)	-	= wt	Pathogenic, dominant?	73
R309H	Exo	R?	R265H	As wt 56.2	= homoallelic	= wt 58	= wt	Clinical variant? Pathogenic, recessive?	62,70
R309L	Exo	R	R265L	3.6 27	3	0.2 4.5	= wt	Pathogenic, recessive	62,70
W312R	Exo	R?	F268W	71.3	= homoallelic	85	= wt	Pathogenic, recessive?	70
R386H	Exo	?	I334H	1.5	= homoallelic	1.4	= wt	Clinical variant? Pathogenic, recessive?	76
A467T	Linker	R	I416T	= wt	= homoallelic	= wt	= wt	Pathogenic, recessive?	58
R574W	Linker	R	R467W	24.8 = wt 23.9	1.5 (< hemiallelic) = homoallelic	3.8 = wt 18	= wt	Pathogenic, recessive	62,70,76
P625R	Linker	R?	P513R	1.7	= homoallelic	= wt	= wt	Clinical variant? Pathogenic, recessive?	76
R807C	Pol	R?	R607C	100%	3.6	-	2.6	Pathogenic, recessive	62
R807P	Pol	R?	R607P	7	4	13	= wt	Pathogenic, recessive	62
G848S	Pol	R	G651S	100% 57.1 100%	2.2 (= hemiallelic) 2.0	-	= wt = wt	Pathogenic, recessive	61,62
T851A	Pol	D?	T654A	100%	60	-	22	Pathogenic, dominant	62
R852C	Pol	R?	R655C	= wt	= wt	0.2	= wt	SNP?	62
R853Q	Pol	R?	R656Q	100%	88	-	30	Pathogenic, dominant	62

TABLE 3 (Continued)

Human mutation	Domain	D/R in human ^a	Yeast mutation	Fold increase of <i>petite</i> frequency		Fold increase of Ery ^R frequency		Pathogenicity and dominance after analysis in yeast ^f	References
				Haploid ^b	Heteroallelic ^c	Haploid ^d	Heteroallelic ^e		
R853W	Pol	R?	R656W	100%	12	-	7.3	Pathogenic, dominant	62
A862T	Pol	R?	A665T	100%		-		Pathogenic, recessive	69
N864S	Pol	R?	N667S	100%	= homoallelic		= wt	Pathogenic, recessive	62
R869*	Pol	R	R672*	100%	15.9 (> hemiallelic)	-		Pathogenic, dominant?	73
G888S	Pol	R?	G691S	1.9	= homoallelic	= wt	2.7	Pathogenic, recessive	62
A889T	Pol	D?	A692T	29.7, 21.4	3.2 (= hemiallelic)	3.7	= wt	Pathogenic, recessive	61,62
				1.5	= homoallelic	= wt	= wt		
E895G	Pol	D?	E698G	100%	12.9 (> hemiallelic)	-		Pathogenic, dominant	109
G923D	Pol	D?	G725D	100%	8.4	-		Pathogenic, dominant	58
D930N	Pol	R?	D732N	100%	5.8 (> hemiallelic)	-	2.7	Pathogenic, dominant	76
H932Y	Pol	?	H734Y	>99%	9.2 (> hemiallelic)	-	= wt	Pathogenic, dominant	61,62
				100%	49		10		
R943H	Pol	D	R745H	100%	40.7	-		Pathogenic, dominant	58
K947R	Pol	?	K749R	100%	23.7 (> hemiallelic)	-	5.7	Pathogenic, dominant	76
R953C	Pol	D	R755C	= wt	= wt	= wt	= wt	SNP?	62
Y955C	Pol	D	Y757C	100%	21.3 (> hemiallelic)	-	11.4	Pathogenic, dominant	58,60
				100%	90.9				
A957P	Pol	R?	A759P	100%	24	-	12	Pathogenic, dominant	62
A957S	Pol	D	A759S	5.3	= homoallelic	0.2	= wt	Pathogenic, recessive	58,62
				= wt	2.3				
R964C	Pol	R	Q766C	1.7	= homoallelic	2.4	= wt	Clinical variant	69
Q968E	Pol	R	R770E	4	2.7 (= hemiallelic)	15.0		Pathogenic, recessive	73
G1051R	Pol	R	G807R	9.4, 2.7	2 (< hemiallelic)	10.2	= wt	Pathogenic, recessive	61,62
				= wt	= homoallelic	= wt	= wt		
T1053*	Pol	R	T809*	100%	13.9 (> hemiallelic)	-		Pathogenic, dominant	73
P1073L	Pol	R?	P829L	29.0	2.2 (= hemiallelic)	13.4	3.3	Pathogenic, recessive	76
G1076V	Pol	?	G832V	100%	= homoallelic	-	= wt	Pathogenic, recessive	62
R1096C	Pol	R	R853C	100%	4.9	-	3.9	Pathogenic, recessive	62
R1096H	Pol	R?	R853H	100%	4.3	-	11	Pathogenic, recessive	62
S1104C	Pol	?	S861C	100%	= homoallelic	-	9.4	Pathogenic, recessive	62

(Continues)

TABLE 3 (Continued)

Human mutation	Domain	D/R in human ^a	Yeast mutation	Fold increase of <i>petite</i> frequency		Fold increase of Ery ^R frequency		Pathogenicity and dominance after analysis in yeast ^f	References
				Haploid ^b	Heteroallelic ^c	Haploid ^d	Heteroallelic ^e		
V1106A	Pol	R	V863A	3.8	3.4 (= hemiallelic)	6.8		Pathogenic, recessive	73
V1106I	Pol	R	V863I	100%	5.9	-	4.3	Pathogenic, recessive	62
E1143G	Pol	R/SNP	E900G	2.2, 1.7	= homoallelic	1.9	= wt	Phenotypic modifier	61
M1163R	Pol	R?	M920R	= wt	= homoallelic	= wt	= wt	SNP?	62
F1164I	Pol	R	F921I	= wt	= homoallelic	= wt	= wt	SNP?	62
D1184N	Pol	R	D941N	3.4	1.9	3.5	1.2	Pathogenic, recessive	62,63
				24.4	1.8 (< hemiallelic)	2.1			
I1185T	Pol	R	I942T	16	1.5 (< hemiallelic)	13.5		Pathogenic, recessive	63
C1188R	Pol	R	V945R	22.4	1.5 (< hemiallelic)	5.5		Pathogenic, recessive	63
K1191N	Pol	R	K948N	= wt	= homoallelic	= wt	= wt	Clinical variant? Pathogenic, recessive?	62,63
				23.2	1.8 (< hemiallelic)	6.8			
K1191R	Pol	R	K948R	36.8	2.3 (= hemiallelic)	8.2		Pathogenic, recessive	63
D1196N	C-terminal	R	D953N	2.2	= homoallelic	= wt		SNP? Clinical variant?	63
A889T-E1143G	Pol/pol	D?	A692T/E900G	35.8, 44.4	3.1 (= hemiallelic)	3.4		Pathogenic, recessive	61
	<i>in cis</i>								
S305R/P1073L	Exo/pol		C261R/P829L	-	65.1 (> hemiallelic)	-	28.8	Pathogenic <i>in trans</i>	76
W312R/R574W	Exo/linker		F268R/R467W	-	5.6 (> hemiallelic)	-	18	Pathogenic <i>in trans</i>	70
G848S/E1143G	Pol/pol		G651S/E900G	-	8.6 (> hemiallelic)	-		Pathogenic <i>in trans</i>	61
A862T/R964C	Pol/pol		A665T/Q766C	-	5.2 (> hemiallelic)	-	11.3	Pathogenic <i>in trans</i>	69
H932Y/G1051R	Pol/pol		H734Y/G807R	-	70.7 (> hemiallelic)	-	18.7	Pathogenic <i>in trans</i>	61

Abbreviation: SNP, single nucleotide polymorphism.

^a"D/R in humans" means dominant mutation or recessive mutation based on the pedigree, when available. "D?" and "R?" mean that mutation is likely dominant or recessive, respectively. "?" means that the status of the mutation is ambiguous.

^b"= wt" means that the *petite* frequency is not significantly different from that of the wild type strain. If the *petite* frequency is higher than 99% or 100%, these percentages are reported. 100% means that the mutant strain is respiratory deficient.

^c"= homoallelic" means that the *petite* frequency of the heteroallelic strain is not significantly different from that of the wild type strain. If the *petite* frequency is higher than 99% or 100%, these percentages are reported. 100% means that the mutant heteroallelic strain is lower, equal, or higher than that of the hemiallelic strain harboring a single copy of *MIP1*.

^d"= wt" means that the Ery^R frequency is not significantly different from that of the wild type strain.

^e"= wt" means that the Ery^R frequency is not significantly different from that of the homoallelic strain.

^f"SNP" indicates that the analysis in yeast suggests that the mutation is neutral. "Pathogenic" means that the analysis in yeast suggests that the mutation is the cause of the pathology, and can be either recessive or dominant.

by qPCR of long mitochondrial targets in *petite* cells, providing information about the presence of damaged DNA that blocks the synthesis of the DNA.⁵⁸ It is also possible to evaluate the mtDNA point mutability by measuring, through a fluctuation test, the frequency of mutant resistant to a specific antibiotic active in mitochondria; in most studies, the Ery^R mutant frequency was measured as an index of the mtDNA point mutability.

Yeast demonstrated to be a useful model to evaluate also whether the mutation is likely dominant or recessive in humans, which is sometimes difficult due to the presence of several mutations in patients. Indeed, a moderate increase in the Mip1 levels is associated with a decrease in the mtDNA extended mutability. For this reason, either in haploid or in diploid strains, the presence of two copies of the *MIP1* allele (hereafter, homoallelic strain) is associated with a two- to three-fold decrease of the *petite* frequency compared to a strain with a single copy of *MIP1* (hemiallelic strain).⁶⁰ Heteroallelic strains, expressing a wild type *MIP1* allele and a mutant *mip1* allele, can behave in three ways. If the *petite* frequency of the heteroallelic strain is like that of the homoallelic strain, it suggests that the mutant *mip1* allele is still functional, and that the mutation is likely a neutral polymorphism. If the *petite* frequency is intermediate or similar to that of the hemiallelic strain, it means that the Mip1 variant does not replace the second copy of the wild type isoform, and the mutation is pathological and recessive in humans. If the *petite* frequency is higher than that of the hemiallelic strains, it means that the Mip1 variant interferes with the activity of the wild type isoform, and that the mutation is likely negative dominant in humans. Besides, two mutations found *in trans* in patients can be studied alone or together, mimicking the situation found in patients, and, in the latter case, the phenotype can be compared to that of the homoallelic and of the hemiallelic strains.

Validation results obtained by different authors are in general coherent, except for a few cases. Considering only mutations for which a pathological role has been demonstrated or postulated based on several observations, the prediction capability of the yeast model systems fluctuates from 70% to 100%, depending on the study. The pathogenicity of approximately 75% of the mutations found in humans was confirmed; for 15% of them, the results were ambiguous, and for 10% of the mutations the behavior in yeast suggests that they are neutral mutations. The ambiguity is often due to the use of a different *MIP1* background in different studies. When the *MIP1[S]* background, harboring Ala661, is used, most of the mutations showed a detrimental effect on the *petite* frequency, whereas the same mutations introduced in the *MIP1[Σ]* background, harboring Thr661, showed slight or no effect

on the mtDNA extended mutability, suggesting that the former background allows to highlight also slight defects in polymerase activity. For example, a group of mutations in the polymerase domain showed highly detrimental effects on mtDNA maintenance when introduced in the Ala661 background, whereas these effects were milder when the same mutations were introduced in the Thr661: however, in both backgrounds, all the mutations confirmed their pathogenicity.⁶¹ Another source of ambiguity can result from the temperature at which the analyses have been performed. Most mutations increased the mtDNA extended mutability at 28–30°C, the optimal growth temperature of *S. cerevisiae*, whereas a few mutant alleles behave as the wild type *MIP1* allele at these temperatures. However, if the experiments were performed at 37°C, a temperature at which the mitochondrial metabolism is subjected to stress, some mutant alleles which did not show any defect at 28–30°C, showed a mild to strong increase in the mtDNA extended mutability, indicating that the mutations are thermosensitive and likely pathogenic in humans.

Although the effects on mtDNA extended and point mutability, on the mtDNA loss, on the mtDNA lesions incorporation and on the mtDNA levels are specific for each mutation modeled in yeast, several general conclusions can be drawn.

- i. Mutations located in all the domains increased the *petite* frequency, indicating that also mutations in the exo domain and in the linker domain affect the ability of the polymerase to fully synthesize mtDNA. Interestingly, several mutations in the exonuclease domain strongly increased the *petite* frequency or result in the total loss of mtDNA, indicating that the exonuclease domain is fundamental for a proper replication process and to avoid deletions/depletion. The exceptions are the mutations located in the N-terminal extension and in the palm C-terminal extension, which behave as neutral polymorphisms; however, just two mutations have been introduced in these regions, due to the low degree of conservation between human Pol γ and Mip1.^{62,63} In addition, human p.Ala467Thr mutation, the most frequent variant found in humans and recognized as pathological,⁶⁴ behaved like a neutral mutation in yeast; this discrepancy could be explained by the fact that Ala467 is involved in the interaction with the accessory subunit, which is absent in yeast, suggesting another role for the corresponding amino acid Ile 416.⁵⁸
- ii. All the pathological mutations increased the *petite* frequency. However, the higher the *petite* frequency associated with a *MIP1* mutation is, the higher the

ρ^0 frequency among the *petite* colonies is, indicating that the most severe mutations result primarily in the loss of mtDNA. The most severe mutations also affected the mtDNA copy number.^{55,62} Anyway, different mutations showed a different severity, and the phenotypic defect on mtDNA stability induced by a mutation grossly parallelized with the severity of the pathology, especially for recessive mutations associated with Alpers syndrome, making yeast a suitable model for predicting the severity of the disease.

- iii. Some mutations, including those in the linker and in the pol domain, also increased the frequency of mtDNA point mutations, but this increase did not correlate with the increase in the *petite* frequency, indicating that the mechanisms at the basis of the point mutability are different from those at the basis of the extended mutability. The increase of the point mutability does not seem to be involved in the development of the pathology. However, a decrease in the replication fidelity could affect the progression of the disease.⁶⁰
- iv. As reported below, Mip1 mutant variant showed a defect in one or more biochemical properties, being these defects the main reason for the mtDNA instability. However, oxidized bases, which are produced in the mitochondria mainly by the presence of ROS, may play a role in the increase in mtDNA instability. In fact, some mutations, among which the dominant and severe human mutation p.Tyr955Cys, increased the frequency of mtDNA lesions which cannot be repaired by Mip1 polymerase or other polymerases present in mitochondria.⁵⁸ Indeed, in several *mip1* mutant strains, but not all, the *petite* frequency was decreased by the treatment with antioxidant molecules such as lipoic acid and MitoQ.^{60,65}
- v. Some mutations believed to be pathological behaved in yeast as neutral polymorphism, resulting in a mtDNA instability similar to that of wild type polymerase; other mutations slightly increased the *petite* frequency, and for this reason, cannot be considered neither as neutral polymorphism nor as pathological mutations. For example, human mutation p.Gly268Ala is now considered as an unclassified clinical variant also thanks to studies in yeast.^{60,66} Some of these unclassified variants, such as p.Glu1143Gly, the most common mutation considered as neutral found in humans and associated with a two-fold increase in *petite* mutability in yeast, and p.Arg964Cys, can alter the phenotypes of a second mutation *in cis* or *in trans*. Indeed, although human mutation p.Ala889Thr, when modeled in yeast, increased moderately the *petite* frequency, when this mutation was studied is *in cis* with the mutation equivalent to p.Glu1143Gly, as found in patients, the *petite* frequency reached almost 100%, indicating that p.Glu1143Gly behaves as a phenotypic modifier.^{61,67} p.Arg964Cys was not associated with pathologies when in homozygosis, and when modeled in yeast resulted in a two-fold increase of the *petite* frequency. However, it behaved as pathological when in compound heterozygosis with a severe mutation.^{66,68,69}
- vi. *in vitro* biochemical studies performed on mutant Mip1 variant purified from yeast or other organisms such as *E. coli* showed that mutations can affect the function of Mip1 in several ways: some mutations reduced protein stability, especially at higher temperatures, other mutations reduce polymerase activity, processivity, DNA binding affinity, affinity for the incoming dNTPs or the specific constant, whereas other mutations cause an increase in the *exo/pol* ratio. In most cases, the exonuclease activity was not or slightly decreased, even in the case of mutations in the exonuclease domain, indicating that the defects of mtDNA replication are not due to defects of the proofreading activity.^{61,63,70}
- vii. Several mutations located in the palm and in the fingers subdomains are dominant when modeled in yeast, as their human counterparts. Taking advantage of the information obtained studying yeast Mip1, two hypotheses have been proposed to explain such dominance. Some mutant alleles encode for a mutant variant that binds the DNA with the same affinity but is unable to synthesize DNA, thus stalling and blocking the replication, and preventing at the same time the binding of the wild-type enzyme, thus behaving as a dominant negative allele.⁷¹ Alternatively, some mutant variants may directly introduce lesions to mtDNA, such as oxidized bases, with a higher frequency, as it seems for mutation p.Tyr955Cys.^{58,72} Interestingly enough, a recent finding showed that two mutations, p.Arg869Ter and p.Thr1053Argfs*6, both in compound heterozygosis with the common missense hypomorphic mutation p.Trp748Ser and resulting in a truncated form of Mip1, behaved as partially dominant when modeled in yeast, with a *petite* frequency in the heteroallelic strain higher than that of the hemiallelic strain.⁷³ This phenomenon can be due to the fact that in yeast the nonsense-mediated mRNA decay is much less active so that the truncated variant is present and could interfere with the activity of the wild type variant, as also observed previously for mutations

in other genes involved in mtDNA stability, such as yeast *MGM1*/human *OPA1* (⁷⁴). Although these truncated variants in *POLG* are non-pathological in humans when in heterozygosis with a wild type allele, and are thus recessive, the analysis in yeast suggests that residual levels of these variants might interfere with the activity of hypomorphic recessive alleles and worsen the phenotype.

- viii. Search for multicopy suppressors, which are genes that, when overexpressed, decrease the detrimental effect of a mutation, identified the increase of the cell dNTP pools concentration, and likely of the mitochondrial dNTP pools concentration, as beneficial for the mtDNA instability induced by several, if not all, *Mip1* mutations. The increase of the dNTP pools can be obtained by overexpressing *RNR1*, encoding for the large subunit of the ribonucleotide reductase, or by deleting *SML1*, encoding for an inhibitor of Rnr1. The increase of the dNTP pool was associated with a decrease of the *petite* frequency in all *mip1* mutants which retain mtDNA, but the lower the basal *petite* frequency is, the greater the rescue activity induced by overexpression of *RNR1* is, suggesting that mutant variants that retain most of their catalytic activity benefit more by this rescue mechanism.^{60,62,65,75,76} Based on the observation reported above that *Mip1* can incorporate rNMPs in the nascent strand, and that the increase of the dNTP pools limits such incorporation, it can be speculated that the reduction in the *petite* frequency may be due not only to an increase of the *Mip1* substrates, but also to a decrease in mtDNA instability resulting from a lower rNMPs incorporation. Overexpression of *REV3* and *REV7*, encoding the two subunits of the DNA polymerase ζ (or Pol ζ), decreased the mtDNA extended mutability induced by mutations in *MIP1*. As described in detail below, Pol ζ is involved in the error-prone translesion synthesis and is present also in mitochondria both in yeast and, at least *Rev3*, in humans. Interestingly, overexpression of Pol ζ rescued the detrimental effects due to specific mutations which are not recovered by the treatment with antioxidant molecules, and vice versa, suggesting two different pathogenic mechanisms. The decrease in *petite* frequency induced by overexpression of Pol ζ could be due to its capacity to partially replace *Mip1* variants which showed a decreased polymerase activity.
- ix. Yeast has been used also to identify drugs to be used as a potential therapeutic treatment for *POLG*-related disorders. The first drug found was clofilium tosylate, an anti-arrhythmic drug, able to decrease

the *petite* frequency in several *mip1* mutant strains, to increase the protein levels of wild-type and mutant *Mip1* variants and to partially restore the respiratory activity of the mutant strains. Clofilium tosylate demonstrated to rescue the phenotypes caused by mutations/deletions in *POLG* also in the worm *Caenorhabditis elegans*, in zebrafish and in patients' fibroblasts, making it suitable as a potential treatment for *POLG*-associated diseases.^{77,78} More recently, a plethora of drugs have been identified for their capability of reducing the *petite* frequency in strains mutated in *MIP1* and in other genes associated with mtDNA deletions or depletion, among which the antimycotic posaconazole, fenticonazole and enilconazole, the antiseptic benzethonium chloride, the antipsychotic haloperidol, the antidepressant sertraline. In all cases, these drugs increased the concentration of at least one mitochondrial dNTP, indicating that the rescue could be due to an increase in the substrates of *Mip1*.⁷⁹ Experiments are ongoing to understand the molecular targets of these drugs, the activity of the drugs on higher eucaryotes harboring mutations in either *POLG* or other genes associated with mtDNA deletions or depletion and to find novel compounds.

Based on all information obtained from yeast, from other models mutated in *POLG*, from the biochemical properties of mutant Pol γ variants and from structural prediction, most mutations associated with *POLG*-related disorders have been included in five clusters, depending on the localization of the mutant amino acid and on defect associated with the polymerase.^{80,81} In addition, a database which works also for predicting the pathogenicity of a novel mutation in *POLG*, is available.⁴⁷

Besides *POLG*-related disorders, yeast has been used to evaluate whether neutral SNPs or mutations, as well as unclassified variants or pathological mutations, can modulate the mitochondrial toxicity induced by the treatment with drugs or mutagens. It was demonstrated that the mtDNA point and extended mutability induced by NRTIs used against HIV, especially stavudine (2',3'-dideohydro-2',3'-dideoxythymidine, or d4T) and/or zalcitabine (2',3'-dideoxycytidine, or ddC), is higher when the common SNPs/uncertain variants p.Pro241Leu, p.Gly268Ala, p.Leu392Val, p.Arg964Cys, p.Glu1143Gly are present in the equivalent position of *MIP1*, even in heterozygosis with a wild type allele; that valproic acid is more toxic for subjects harboring the p.Glu1143Gly variant and the common p.Gln1236Hys SNP; that the alkylating agent methyl methanesulfonate (MMS) is able to increase the mtDNA point mutability if *mip1* alleles harboring

substitutions equivalent to pathological ones are in heterozygosis with a wild type *MIP1* allele.^{54,59,82,83}

4 | OTHER DNA POLYMERASES FOUND IN YEAST MITOCHONDRIA

Although Mip1 is the only polymerase able to fully replicate mtDNA, in the last 15 years other DNA polymerases have been found in yeast and human mitochondria.⁸⁴ Here we describe the information relative to other DNA polymerases found in yeast mitochondria, in particular Pol ζ , Rev1 and Pol η : a single report identified also a subunit of Pol α in yeast mitochondria, without further evidence of its function.⁸⁴

4.1 | Pol ζ and Rev1

DNA polymerase ζ (Pol ζ) is a eukaryotic polymerase belonging to the B family and is involved in error-prone translesion synthesis (TLS). TLS is an important bypass system that enables the cells to bypass replication blocking due to oxidized bases and other lesions occurring in the nuclear DNA. Pol ζ is formed by a catalytic subunit encoded by *REV3* and an accessory subunit encoded by *REV7*. The purification of human Pol ζ holoenzyme also identified the presence of two other accessory subunits, called PolD2 and PolD3 (Pol31 and Pol32 in yeast), that increase the efficiency and the processivity of the holoenzyme.⁸⁵ The mammalian catalytic subunit is more than 3,000 amino acids long and is composed of three highly conserved domains: an N-terminal domain, the Rev7-binding domain (R7B), and the C-terminal polymerase domain. Yeast Rev3 is 1,504 amino acids long and shares a 65% similarity with other B-family DNA polymerases.⁸⁶ Unlike most of the DNA polymerases belonging to the B family, Pol ζ has no proofreading activity due to the lack of 3'-5' exonuclease domain.⁸⁷ However, it can replicate with an efficiency as high as 10% past a thymine-thymine dimer.^{88,89} In addition, it can extend terminally mismatched primers increasing the propensity to generate mutations. The characteristics of Pol ζ and other polymerases involved in TLS have been reviewed in.⁹⁰

In yeast and human, Pol ζ interacts with Rev1, also involved in the error-prone TLS. Rev1 is considered a deoxycytidyl transferase and represents the most divergent member of the eucaryotic Y-family polymerases.⁹¹ Besides incorporation opposite to G, Rev1 is specialized in the incorporation of C opposite to an apurinic site, but can incorporate also the three other nucleotides, despite at a frequency which is 10^3 to 10^5 lower relative to C. It

can also incorporate a C opposite an N2-adducted G, and opposite to A, T, and C with an efficiency of 10^{-2} to 10^{-3} . Structurally, Rev1 is composed of palm, fingers, thumb and polymerase-associated domains (PAD). The palm is involved in the catalytic activity, the fingers domain interacts with the incoming dCTP and the 5' end of the template and the thumb and the PAD domains approach the template-primer from opposite sides thanks to the connection of a long linker region.⁹²

In yeast, the presence of both Pol ζ and Rev1 has been identified also in the mitochondria.⁹³ Rev3 has been identified also in human mitochondria.⁹⁴ To determine if these proteins affect the mutability of mtDNA, the frequency of mtDNA point mutations was measured in strains devoid of one or more of these enzymes.⁹³ Spontaneous and UV-induced mtDNA frameshift point mutability was reduced in *rev1 Δ* , *rev3 Δ* , and *rev7 Δ* single mutants, as occurs in the nucleus. The combination of the deletion in each of these genes with a mutation in *MIP1* showed that Pol ζ and Mip1 belong to the same epistatic group, while Rev1p belongs to a different epistatic group. On the contrary, quite surprisingly, the Ery^R mutant frequency due to point substitutions was increased in the absence of Pol ζ and Rev1.⁹⁵ This observation suggests that in the absence of the TLS by Rev1 and/or Pol ζ , Mip1 could insert mutations in an effort to bypass lesions that block the replication fork. Therefore, Pol ζ and Rev1 function in a less mutagenic pathway in the mitochondrial compartment compared to the nucleus.

As reported above, the overexpression of whole Pol ζ or just the catalytic subunit Rev3, but not of Rev1, reduced the mtDNA extended mutability caused by some pathological mutations in Mip1.⁶⁵ The effect was synergic with the increase of dNTP pools obtained by overexpressing *RNR1*, probably because dNTPs are the substrate of both polymerases. The rescue mechanisms, which occur only for mutations in Mip1 that are not rescued by antioxidant treatment, suggest that Pol ζ may participate directly in the replication of undamaged mtDNA at the replicative fork partially playing the role of Mip1 variants which have a reduced polymerase activity or processivity and/or that Pol ζ may substitute Mip1 variants defective of TLS, either during the incorporation opposite to a lesion or the extension from mismatched terminal nucleotides.⁶⁵

4.2 | Pol η

The human DNA polymerase η (Pol η) is a TLS DNA polymerase that belongs to the Y-type family and is encoded by the *POLH* gene. Mutations in this gene are

associated with a xeroderma pigmentosum variant⁹⁶ Patients affected by this disease are sensitive to UV radiation and develop sunlight-induced skin cancers with a high incidence.^{96,97} Despite not being essential for DNA replication in mice, germline loss of Pol η function determines UV sensitivity.^{98,99} Pol η presents high fidelity when replicating on damaged DNA characterized by different types of lesions, like cyclobutene pyrimidine dimers (CPDs) and T-T dimers induced by UV, cis-platinum adduct, N-2 acetyl-amino-fluorene (AAF) adduct and O6-methylguanine formed by alkylating agents.^{100–103} However, it is devoid of a proofreading activity.¹⁰⁴ For this reason, Pol η performs both error-prone and error-free TLS.

Yeast Pol η is encoded by Rad30. A single study provides evidence that Rad30 localizes also in mitochondria.¹⁰⁵ Deletion of *RAD30* strongly increased the frequency of Ery^R mutants following treatment with UV. When both *RAD30* and *REV3* were deleted, the increase of the Ery^R mutant frequency was similar, indicating that the two genes are in the same epistatic group. This observation suggests that *Pol η* and Pol ζ may work together to promote lesion bypass in a less mutagenic way as Mip1 could do.

5 | CONCLUDING REMARKS

Despite the differences existing between yeast and humans, numerous information now available about human Pol γ derive from pivotal studies performed in yeast, where Pol γ was first identified,¹⁰⁶ allowing the subsequent identification in humans.¹⁰⁷ The replication of mtDNA seems to be sufficiently conserved in yeast and mammals, allowing to make yeast a useful model to study human mutations associated with POLG-related disorders. A model which can explain the pathogenicity of the mutations found in POLG, despite being far from being complete, has been elaborated also using information deriving from studies in yeast.

Besides Pol γ , other polymerases have been identified in yeast mitochondria, although, for two of them, only a single report is available. The roles of these other polymerases in replication, repair and recombination as well as their interactions with Mip1 should be determined in order to have a better knowledge on mtDNA integrity and maintenance.¹⁰⁸

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