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Review

DNA polymerase gamma and mitochondrial disease: Understanding the consequence of *POLG* mutations

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ABSTRACT

DNA polymerase γ is the only known DNA polymerase in human mitochondria and is essential for mitochondrial DNA replication and repair. It is well established that defects in mtDNA replication lead to mitochondrial dysfunction and disease. Over 160 coding variations in the gene encoding the catalytic subunit of DNA polymerase γ (*POLG*) have been identified. Our group and others have characterized a number of the more common and interesting mutations, as well as those disease mutations in the DNA polymerase γ accessory subunit. We review the results of these studies, which provide clues to the mechanisms leading to the disease state.

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1. Introduction

Mitochondria have their own small 16.5 kb circular double-stranded DNA that encodes 22 tRNAs, 2 rRNAs and 13 polypeptides that are absolutely essential for electron transport and oxidative phosphorylation. Nuclear genes encode the other 1000–1500 proteins that are imported into the mitochondria [1]. These include the proteins involved in mitochondrial DNA (mtDNA) replication, which if defective can produce mtDNA mutations leading to mitochondrial dysfunction and disease [2] (Fig. 1).

MtDNA is replicated by an assembly of proteins and enzymes including DNA polymerase γ (pol γ) and its accessory protein, single-stranded DNA binding protein (mtSSB), mtDNA helicase (Twinkle), and a number of accessory proteins and transcription factors (reviewed in [3]). The minimal proteins needed for in vitro mtDNA replication include the two subunit pol γ, the mitochondrial helicase and mtSSB [4]. Two modes of DNA replication have been proposed, an asynchronous strand displacement model and a strand-coupled bidirectional replication model (reviewed in [5]). In the asynchronous strand displacement model mtDNA is replicated in an asymmetric fashion where DNA synthesis is primed by transcription through the H strand origin within the D-loop [6]. After two-thirds of the nascent H strand is replicated, the L strand origin is exposed, allowing initiation of nascent L strand synthesis. In the strand-coupled model bidirectional replication is initiated from a zone near OriH followed by progression of the two forks around the mtDNA circle [7]. In both models the DNA polymerization reaction is performed by pol γ.

Of the 16 DNA polymerases in the eukaryotic cell, only pol γ is known to function in the mitochondria [8–10]. The holoenzyme of human pol γ consists of a catalytic subunit (encoded by *POLG* at chromosomal locus 15q25) and a homodimeric form of its accessory subunit (encoded by *POLG2* at chromosomal locus 17q24.1) [11]. The catalytic subunit is a 140 kDa enzyme (p140) that has DNA polymerase, 3′–5′ exonuclease and 5′ dRP lyase activities. Alignments reveal that the catalytic subunit sequence is well conserved across species, with all genes containing conserved sequence motifs for DNA polymerase and 3′–5′ exonuclease functions (Fig. 2). The accessory subunit is a 55 kDa protein (p55) required for tight DNA binding and processive DNA synthesis [12]. In 2001, the first disease mutations were identified in *POLG* [13]. These mutations were associated with progressive external ophthalmoplegia (PEO). Subsequently, mutations in *POLG* were identified in patients with Alpers syndrome and other infantile hepatocerebral syndromes, ataxia–neuropathy syndromes, Charcot–Marie–Tooth disease, idiopathic parkinsonism, nucleoside reverse-transcriptase inhibitor (NRTI) toxicity, among others (for review see [2,3,14]). These diseases are characterized by mtDNA deletions or depletion in symptomatic tissues. To date, approximately 150 disease mutations in *POLG* have been identified, which places *POLG* as a major locus for mitochondrial disease (http://tools.niehs.nih.gov/polg/) (Fig. 3). *POLG2* mutations associated with

Abbreviations: mtDNA, mitochondrial DNA; PEO, progressive external ophthalmoplegia; pol γ, DNA polymerase γ; NRTI, nucleoside reverse-transcriptase inhibitor; ad, autosomal dominant; WT, wild-type; ROS, reactive oxygen species; 8-oxo-dG, 8-oxo-7,8-dihydro-2′-deoxyguanosine; PTC, premature termination codon; NMD, nonsense-mediated mRNA decay pathway; NAS, nonsense-associated alternative splicing pathway

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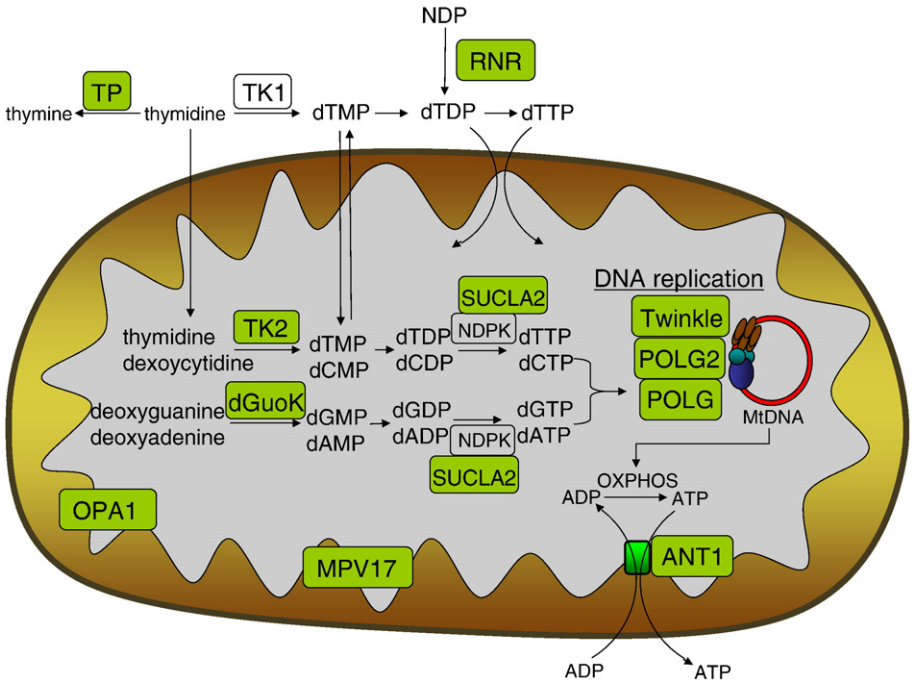

Fig. 1. The mitochondrion and some of the enzymes involved in mtDNA replication or nucleotide metabolism. Mutations in some of these genes (green boxes) are associated with mtDNA depletion or mutations in humans.

PEO have also been discovered (Fig. 1) [15]. Thus, our laboratory and others have been motivated to determine how mutations in both subunits of pol γ cause disease. Here is a comprehensive review of what is currently understood from experimental studies.

2. *POLG* mutations associated with autosomal dominant mitochondrial disease

PEO is the only mitochondrial disease that has cosegregated with autosomal dominant (ad) mutations in *POLG*. Nearly all of the adPEO mutations in *POLG* are located in the polymerase domain of pol γ (Fig. 4). One of the first adPEO mutations to be discovered was the Y955C mutation [13], and this was also the first to be biochemically characterized [16]. We found the Y955C p140 to be a mutator, causing a 10- to 100-fold increase in misinsertion errors, most likely as a consequence of a 45-fold decrease in binding affinity for the incoming nucleoside triphosphate. This enhanced mutagenesis is mitigated by a functional intrinsic exonuclease activity resulting in only a 2-fold mutator effect for base pair substitutions by the exonuclease-proficient Y955C enzyme. In a subsequent study of four adPEO mutant p140 variants, the Y955C and R943H substitutions were predicted to interact directly with the incoming dNTP by analysis of a structural model of the polymerase active site based upon the solved crystal structure of T7 DNA polymerase [17]. Recombinant proteins carrying these substitutions retain less than 1% of the wild-type (WT) polymerase activity and display a severe decrease in processivity. The significant stalling of DNA synthesis and extremely low catalytic activities of both enzymes are the two most likely causes of the severe clinical presentation in R943H and Y955C heterozygotes [17]. The G923D and A957S p140 proteins retained less than 30% WT polymerase activity. This is consistent with the reduced clinical severity of PEO in individuals heterozygous for the G923D and A957S mutations [17].

In a genetic model developed to evaluate the homologous PEO mutations in the yeast *MIP1* gene, we studied Y757C (Y955C in human *POLG*), as well as L260R (human L304R), I416T (human A467T), G725D (human G923D), R745H (human R943H), and A759S (human A957S) in *Saccharomyces cerevisiae* [18]. The amino acid codon changes were made directly into the chromosomal *MIP1* gene by *delitto perfetto* site-directed mutagenesis. The L260R, G725D, R745H and

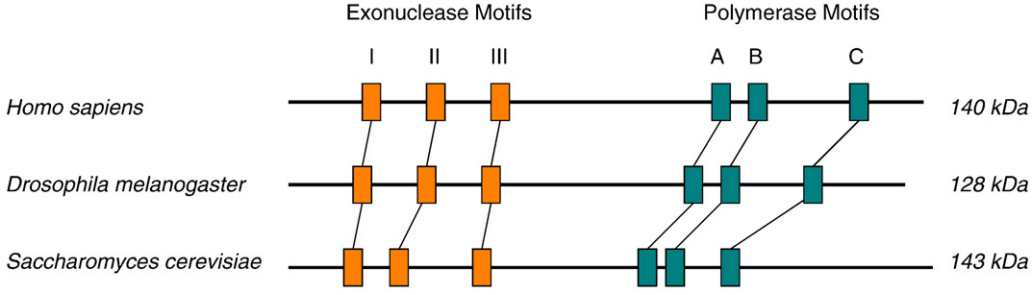

Fig. 2. Schematic linear organization of the pol γ catalytic subunit with conserved domains. The conserved 3′–5′ exonuclease domains (orange) are encoded by the three motifs I, II, and III while the DNA polymerase domains (green) are encoded by the three ABC motifs.

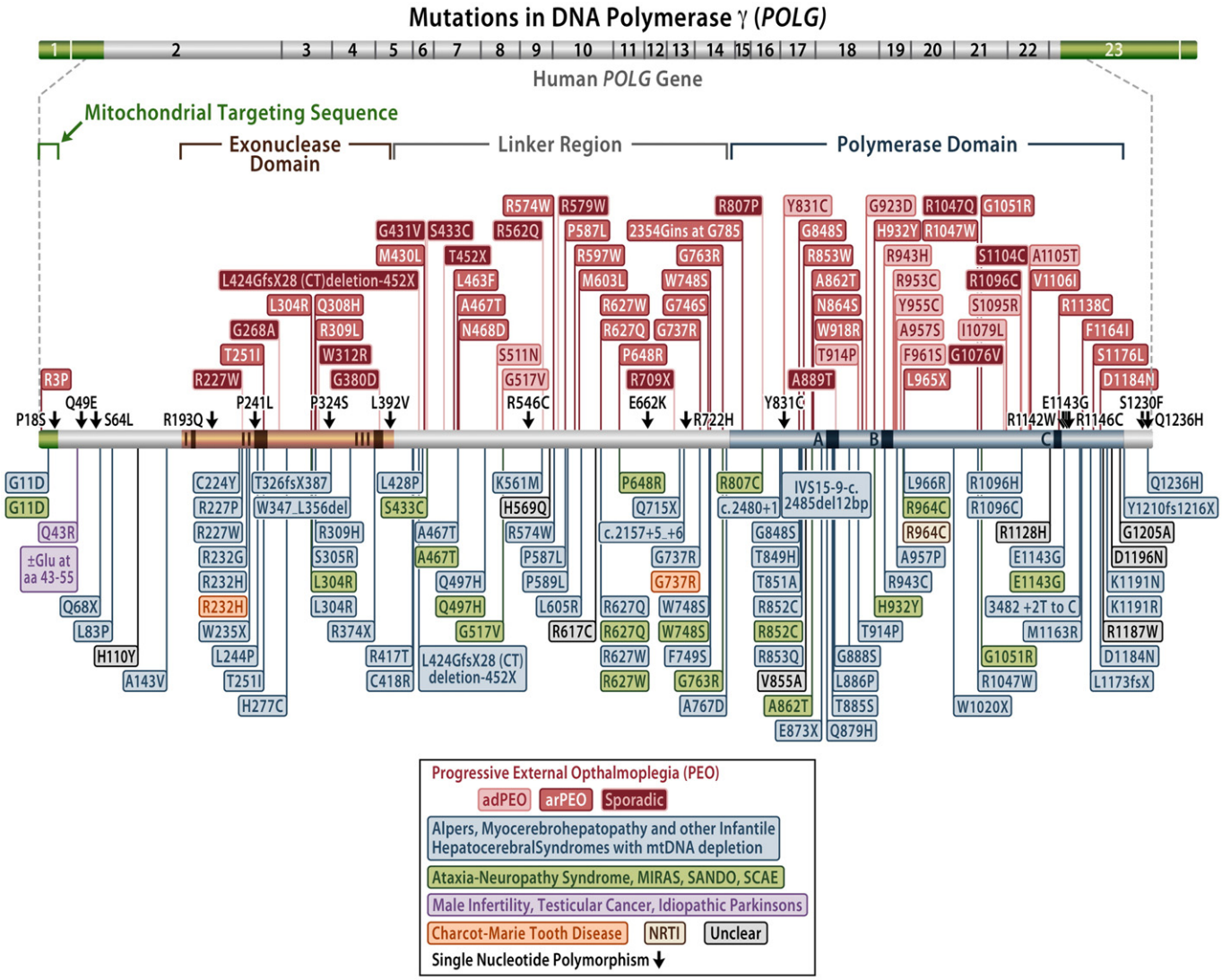

Fig. 3. *POLG* disease mutations and polymorphisms (modified from http://tools.niehs.nih.gov/polg/).

Y757C mutations in Mip1 produced 100% petites in haploid cells as a result of mtDNA depletion while only the R745H and Y757C mutations showed dominant petite frequency in diploid cells. The A759S mutation produced 35% petites in haploid yeast with no appreciable increases in diploid cells, which suggests this mutation is not dominant in yeast. Interestingly, the Y757C mutation demonstrated enhanced mtDNA damage indicative of oxidative damage and over 80% petites in the diploid strain [18]. In a related study, Baruffini et al. reported that this high petite frequency could be rescued by treatment with a reactive oxygen species (ROS) scavenger (dihydrolipoic acid) or up-regulation of ribonucleotide reductase (*RNR1*, another mitochondrial disease locus, Fig. 1) [19]. The reduction of petite formation by up-regulation of dNTP pools via RNR overexpression is predicted based on the lower affinity for dNTPs by this enzyme. However, the reversal of the high petite phenotype by dihydrolipoic acid and the enhanced mtDNA oxidative damage suggests a role of oxidative stress with the Y955C mutation.

An increase in mitochondrial ROS, as shown by an increase in the marker, 8-oxo-7,8-dihydro-2′-deoxyguanosine (8-oxo-dG) in mtDNA, was also seen in a transgenic mouse study, the first mouse study involving a bona fide *POLG* disease mutation [20]. Y955C *POLG* was targeted to mouse hearts, which lead to multiple markers of mitochondrial dysfunction, such as mtDNA depletion, and ultrastructural changes in mitochondria with damaged cristae. Increased ROS and mitochondrial dysfunction gave rise to a number of cardiac defects, such as cardiomegaly, biventricular dilation, increased left ventricular mass and cardiac rhythm disturbances, ultimately causing premature death. These results indicate that oxidative damage to mtDNA may be part of the pathological mechanism associated with the Y955C mutation.

The association of oxidative damage and the Y955C enzyme was investigated biochemically and structurally by assaying the ability of WT and Y955C p140 to incorporate 8-oxo-dGTP or replicate past an 8-oxo-dG lesion in DNA [21]. The 8-oxo-dG base can form two conformations in DNA, the *anti*-conformation, which favors Watson–Crick base pairing with dCMP, and the *syn*-conformation, which allows mutagenic Hoogsteen base pairing with dAMP. Pol γ normally prevents mutagenesis by 8-oxo-dG through interactions from Tyr955 and Phe961, which limit the *syn*-conformation of the 8-oxo-dG template base during translesion DNA synthesis. However substitution of Tyr955 with Cys allows significantly more mutagenic *syn*-conformation and causes the polymerase to display relaxed discrimination when either incorporating 8-oxo-dGTP onto the primer-terminus or translesion synthesis opposite 8-oxo-dG in the template strand [21]. Collectively, these studies suggest that patients harboring the Y955C mutation may have elevated

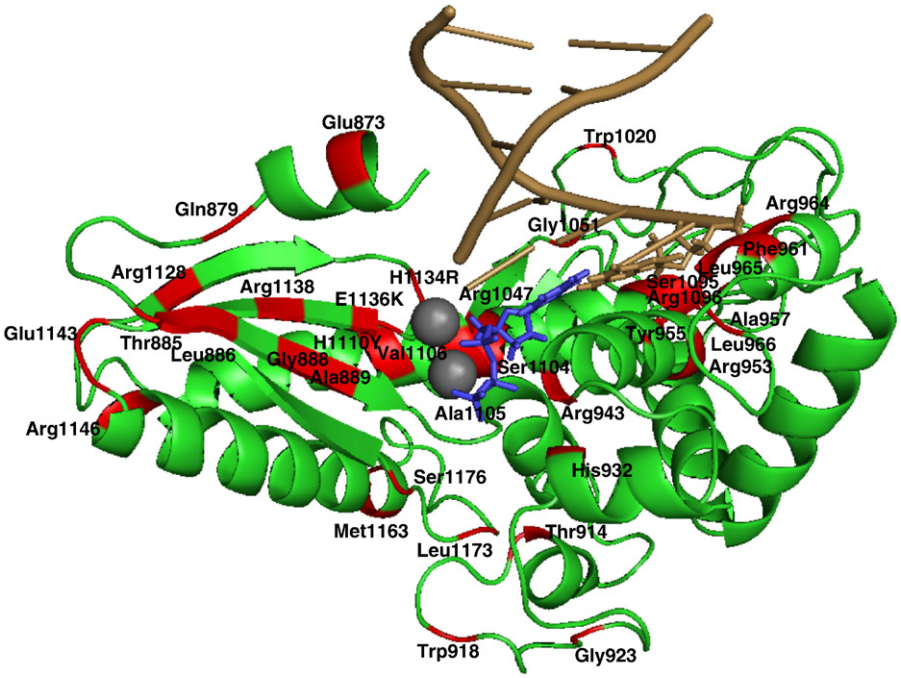

Fig. 4. *POLG* disease mutations and their location within the structural homology model of the polymerase domain of pol γ against the solved crystal structure of T7 DNA polymerase [17].

oxidative damage and that antioxidant therapy may delay progression of disease.

3. The most common *POLG* disease mutation

The A467T mutation has been found in all of the major *POLG*-related diseases, i.e. Alpers syndrome, ataxia–neuropathy syndromes and PEO. The frequency of the A467T mutation is approximately 0.6% in the Belgian control population [13], 0.69% in the British control population [22], 1% in the Norwegian control population [23], and less than 0.2% in 380 Finnish controls [24]. However, in mitochondrial disease populations, the A467T allele is estimated to occur in 36% of all alleles associated with *POLG* disease, as shown by five large mitochondrial disease population studies identifying *POLG* mutations [22, 25–28]. These frequencies make A467T the most common disease mutation in *POLG*.

We characterized the recombinant pol γ containing the A467T mutation purified from baculovirus-infected insect cells. A467T p140 had poor polymerase activity, with only 4% activity compared to WT enzyme. This was due to a greater than 5-fold decrease in k_{cat} and a 4.7-fold increase in $K_{m(dNTP)}$. Additionally, exonuclease activity was reduced 2-fold compared to WT [29]. The A467T p140 showed poor DNA binding in an electrophoretic mobility shift assay, with a 5-fold increase in K_d compared to WT. Strikingly, the A467T mutation rendered the p140 enzyme poorly able to bind to the p55 accessory subunit, as shown by immunoprecipitation experiments, processivity and primer extension assays. Furthermore, the p55 accessory subunit was unable to protect the A467T p140 catalytic subunit from N-ethylmaleimide [29]. Thermolysin digestion experiments of WT and A467T p140 revealed quite different digestion profiles. The p55 accessory subunit protected an 80 kDa fragment of WT p140 that was degraded in the absence of the p55 accessory subunit, whereas there were no significant differences in the A467T p140 digestion profiles, either with or without p55. Collectively, these results demonstrate the poor interaction of A467T p140 with the accessory subunit. Luoma and colleagues also characterized recombinant p140 containing the A467T substitution [24]. This A467T mutant enzyme showed decreased polymerase activity, poor DNA binding and poor primer extension, however stimulation by p55 was observed.

A467T mutation is generally reported as a recessive mutation, however it was suggested that A467T may give rise to a mild dominant phenotype causing late-onset ptosis [24]. Solid-phase mini-sequencing was used to determine gene expression levels of the two alleles. A467T *POLG* gene expression was slightly lower (40%) than that of the WT *POLG* allele (60%), suggesting that there was no expression bias for the A467T allele [24]. Yeast models of mitochondrial disease can help to determine the contribution of both alleles to disease phenotype, and are especially helpful for verifying dominant or recessive mutations of interest. Although this region is not well conserved in *S. cerevisiae*, studies revealed that the yeast homolog of the human A467T pol γ mutation, I416T in Mip1, is recessive [18]. Interestingly, I416T Mip1 had significantly elevated levels of both mitochondrial and nuclear DNA damage, as measured by the QPCR method [18].

4. Nonsense mediated decay of transcripts containing a premature termination codon

There are more than 10 mutations in *POLG* that produce a premature termination codon (PTC), a frameshift or an alternative spliced transcript (Fig. 2). The first *POLG* mutations (A467T and E873X) associated with Alpers syndrome were identified in 2004 in two unrelated pedigrees [30, 31]. We studied the skin fibroblasts of one patient whose *POLG* genotype was E873X/A467T [32]. The allele containing the E873X mutation in exon 17 was expected to produce a truncated protein. However, only full-length p140 protein was detected by Western blot analysis. Sequence analysis of the cDNA from the pre-spliced message showed that both alleles were represented. However, upon sequencing the cDNA from the mature message, only transcripts containing the A467T mutation were found. Thus, full-length p140 arose from the allele containing the A467T mutation. Further analysis revealed that message arising from the E873X allele were degraded by the nonsense-mediated mRNA decay pathway (NMD) [32]. The NMD pathway degrades transcripts containing PTCs that are at least 50–55 nucleotides

upstream from at least one intron. Treatment with NMD inhibitors showed an increase in transcripts containing the PTC. Amplification of the region flanking exon 17 revealed a smaller, faint band in patient cells, but not in normal foreskin fibroblasts. Sequencing showed that exon 17 had been skipped by the nonsense-associated alternative splicing pathway (NAS), which produces a frameshift leading to another PTC. Thus, both the NMD and NAS pathways were involved. Virtually all mRNAs produced from the allele containing the PTC were destroyed by the NMD and NAS mechanisms. Thus, the severity of disease for this patient is most likely due to mono-allelic expression of A467T p140 [32]. Extrapolating to other mutations of this nature, NMD and NAS are expected to remove those *POLG* mRNAs containing PTCs.

5. The contribution of *POLG* polymorphisms to mitochondrial disease

Luoma and colleagues studied the contribution of the Q1236H polymorphism to the disease in patients with the *POLG* genotype A467T/R627Q-Q1236H [24]. Q1236H occurs in Caucasian populations at a frequency of 3.7%, however a much higher frequency (14.8%) in Finnish controls was observed [24]. Luoma et al. characterized pol γ p140 proteins containing each of these three mutations, as well as R627W. R627W has been associated with recessive mitochondrial disease, whereas R627Q may be a dominant *POLG* mutation. The R627Q and R627W p140 proteins had similar DNA binding properties compared to WT and were equally stimulated by the accessory subunit. R627Q had a slightly lower polymerase activity (77%). Interestingly, both R627Q-Q1236H p140 and Q1236H p140 had greater polymerase activity than WT (151% and 117% respectively), which may be a consequence of their significantly higher DNA binding.

The E1143G polymorphism is found in 4% of the general population and is also linked with many alleles containing known disease mutations. Thus, it has been unclear as to whether E1143G is a neutral polymorphism. The W748S mutation is the most common mutation in ataxia–neuropathy spectrum disorders and has also been associated with Alpers syndrome [33]. The W748S mutation is generally found *in cis* with E1143G. To investigate the contributions of both mutations, we biochemically characterized pol γ p140 proteins containing W748S, E1143G, or both [34]. W748S p140 exhibited low DNA polymerase activity, low processivity and a severe DNA-binding defect. Interactions between the W748S p140 and the p55 accessory subunit were normal, but did not rescue the catalytic defect. Surprisingly, E1143G p140 was 1.4-fold more active than WT. The E1143G substitution partially rescued the deleterious effects of the W748S mutation, as DNA binding, catalytic activity and fidelity values were greater for W748S-E1143G p140 than W748S p140. However, W748S-E1143G p140 had a notably lower change in enthalpy for protein folding than W748S alone, suggesting that E1143G does not confer a purely beneficial effect to the double mutant. In light of these results, E1143G and Q1236H when *in cis* with pathogenic mutations may modulate the effects of the pathogenic mutations.

Baruffini and colleagues studied the A692T Mip1 polymerase domain mutation (A889T in human *POLG*) in *S. cerevisiae* with and without Mip1 E900G (E1143G in human *POLG*) [35]. In yeast, A692T Mip1 had low specific gap-filling activity (29–30% compared to WT) and significantly increased mtDNA instability. Similar to what was observed with the recombinant E1143G pol

Table 1
Summary of experimental data on DNA polymerase γ disease variants[#]

Human mutation	Yeast mutation	Human disease	Biochemical data	Genetic data
(CAG)n	n.h.[a]	Male infertility, testicular cancer, Parkinson's disease		No defect in mtDNA detected in human cells transfected with *POLG*-deleted CAG repeat.
G268A	G244A	arPEO		2.5-fold increase in petite frequency in yeast. 10-fold increase in point mutations (haploid yeast)
L304R	L260R	arPEO		100% petites in haploid yeast, 7.9% in diploid
A467T	I416T	Alpers syndrome, ANS, PEO	Defect in accessory subunit binding. 4% catalytic activity due to low DNA binding, low k_{cat}, high K_m.	Causes mtDNA and nuclear DNA damage in yeast, 9% petites as haploid yeast.
R627Q	n.h.	PEO, ANS	77% polymerase activity	
W748S	n.h.	Alpers syndrome, ANS, PEO	Defect in catalysis, DNA binding, and enzyme stability.	Nearly always occurs with the E1143G mutation.
G848S	G651S	Alpers syndrome, PEO	Reduced gap-filling activity	46% petites in haploid yeast and high mutation frequency.
E873X	E676	Alpers syndrome		Stop codon leads to nonsense mediated decay of message, and pseudo monoallelic expression of other *POLG* allele
A889T	A692T	PEO	Reduced gap-filling activity	17% petite frequency in haploid yeast
G923D	G725D	adPEO	21% polymerase activity	100% petites in haploid yeast, only 7.6% in diploid.
H932Y	H734Y	PEO, ataxia	Reduced gap-filling activity	Nearly 100% petites in haploid yeast
R943H	R745H	adPEO	0.2% polymerase activity due to high K_m(dNTP) and lower k_{cat}.	100% petites in haploid yeast, 37% in diploid yeast. Loss of mtDNA in yeast.
Y955C	Y757C	adPEO, parkinsonism	0.03% polymerase catalytic activity. Mutator polymerase	Loss of mtDNA in transgenic mice, oxidative stress, cardiomyopathy. High petite frequency in yeast (100% in haploid, 80% in diploid).
A957S	A759S	adPEO	23% polymerase activity	35% petites in haploid yeast, only 2.5% in diploid.
R964C	n.h.	NRTI toxicity	Reduced catalytic activity, low k_{cat}	
G1051R	G807R	PEO, ataxia	Slightly reduced gap-filling activity	Mild increase of petites in haploid yeast
E1143G	E900G	Polymorphism, 4% in population	1.4-fold more active than WT	Temperature sensitive mutant in yeast, high petite frequency at 36 °C.
Q1236H	n.h.	Polymorphism, 3.7% in population	Higher polymerase activity than WT	

ANS = ataxia–neuropathy syndromes; arPEO = autosomal recessive PEO.
[#]References for data contained in this table can be found in the text.
[a] Not homologous in yeast.

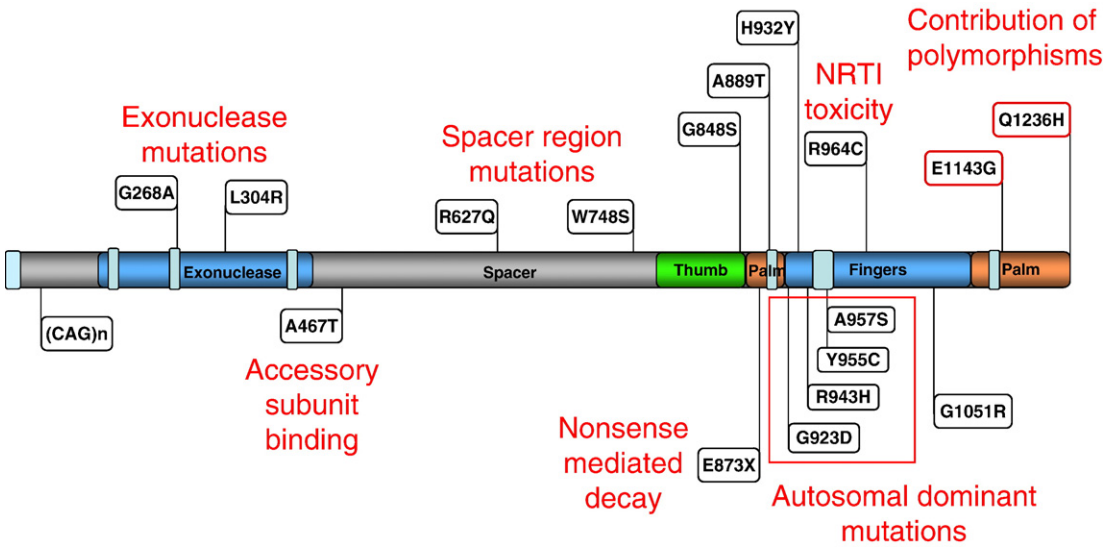

Fig. 5. An overview of what is currently known about *POLG* mutations from experimental studies.

γ, the E900G Mip1 overexpressed in yeast had a higher specific gap-filling activity (107–139% compared to WT). E900G also demonstrated a modifying effect, as the A692T-E900G Mip1 double mutant had a slightly higher gap-filling activity than A692T (30–40% compared to WT) [35]. However, E900G increased petite frequency approximately 2-fold, and presented a temperature-sensitive phenotype with a higher petite frequency at 36 °C as compared to 28 °C.

6. *POLG* mutations associated with susceptibility to NRTI toxicity

In 2007, the first *POLG* mutation associated with mitochondrial toxicity as a result of NRTI therapy was reported [36]. NRTIs are chain terminators that are used to inhibit replication of viruses such as HIV-1. Mitochondrial toxicity has been associated with the use of NRTIs, both in treated human patient populations, as well as in animal and human studies of toxicity [37, 38]. In vitro studies by our group showed that NRTIs can inhibit the enzymatic activities of DNA polymerase γ, but at different levels [39].

Yamanaka and colleagues identified an HIV-1 infected patient with a history of hyperlactatemia induced by d4T treatment [36]. Sequencing analysis of the *POLG* gene in this patient identified a homozygous R964C mutation. The R964C mutation was recently identified *in trans* with A862C in a patient with ataxia–neuropathy syndrome [28]. Yamanaka and colleagues also overexpressed and purified R964C p140 from baculovirus-infected silkworms [36]. The recombinant R964C p140 had only 14% polymerase activity compared to WT [36]. Ratios of mtDNA to nuclear DNA were measured in order to determine mtDNA copy number in lymphoblastoid cells from the index patient, the index patient's unaffected heterozygous niece, and three patients who had long term d4T use as controls (two out of three patient controls had normal lactate levels). Lymphoblastoid cells from the index patient and her niece showed the most severe decrease in mtDNA copy number after treatment with 10 μM d4T. This suggests that pol γ containing the R964C mutation is not normally deleterious, but certain conditions (such as NRTI treatment), may push mitochondrial function below the clinical threshold, causing mitochondrial disease.

7. Analysis of other *POLG* mutations in yeast

Baruffini et al. recently analyzed several other mutations in the *MIP1* gene that correspond to disease mutations in human *POLG* [19, 35]. These mutations were assayed for petite formation, mtDNA mutation frequency and polymerase activity from mitochondrial extracts. The yeast *mip1*-G224A allele, equivalent to the human *POLG* G268A substitution associated with autosomal recessive PEO, produced a 2.5-fold increase in petite frequency and a 10-fold increase in point mutations as measured by erythromycin resistant mutants in haploid cells [19]. However, this effect was repressed to WT levels in heterozygous diploid cells indicative of the recessive nature of this mutation. Similar to their observation with the *mip1*-Y757C mutation, the petite frequency by the *mip1*-G224A allele decreased with either over-expression of *RNR1* or treatment with dihydrolipoic acid [19].

In haploid yeast strains, the G651S, A692T and H734Y *Mip1* alleles (corresponding to G848S, A889T and H932Y in human *POLG*, respectively) produced petite frequencies of 46%, 17% and 98%, respectively, at 28 °C, which increased at 36 °C [35]. A fourth mutation, G807R (corresponding to G1051R in human *POLG*) caused only a mild increase in petite frequency compared to the other mutations, and displayed a 4 to 5-fold increase in petites at 36 °C. Interestingly, G651S had high mtDNA instability and significantly increased mutation frequency as measured in the erythromycin resistance assay [35]. Gap-filling activities in mitochondrial extracts from yeast strains overexpressing these proteins were measured and all showed reduced levels of gap-filling activity in the range of 30–85% of WT Mip1 protein [35].

8. The *POLG* CAG trinucleotide repeat

Near the N-terminus of the mature human DNA polymerase is a stretch of 13 glutamine residues encoded by ten CAG codons followed by one CAA codon and two additional CAG codons [9]. This CAG trinucleotide stretch is not found in other eukaryotic *POLG* genes with the exception of the African great apes, where the length of the CAG repeat is species-specific [40]. *POLG* CAG repeat variants are associated with idiopathic sporadic Parkinson's disease [41] and testicular cancer [42, 43]. *POLG* CAG repeat variants have also been associated with male infertility [44, 45], although some studies reported alterations in the CAG trinucleotide tract of *POLG* at the same frequency in both normal and infertile men [46–49]. However, studies using human cells with a deletion of the CAG repeat showed no detectable effect on mitochondrial function in tissue culture cells [50]. The role of the

CAG trinucleotide repeat in *POLG* has been controversial and more studies are needed.

9. The accessory subunit of DNA polymerase γ

The first *POLG2* mutation associated with disease was discovered in 2006 [15]. The heterozygous G451E mutation was identified in a patient presenting with adPEO with multiple mtDNA deletions and cytochrome *c* oxidase-deficient muscle fibers. This mutation was not seen in 100 PEO patient samples or in 288 control chromosomes. Biochemical characterization of purified, recombinant G451E-substituted accessory subunit protein in vitro revealed incomplete stimulation of the catalytic subunit due to weak subunit interaction, as shown by immunoprecipitation experiments, N-ethylmaleimide protection assays, processivity, and primer extension assays. The G451E accessory subunit protein retained normal DNA binding function as measured by single-stranded DNA cellulose chromatography, but failed to enhance DNA-binding strength of the p140–p55 holoenzyme complex. In vivo, the disease most likely arises through haplotype insufficiency or heterodimerization of the mutated and WT proteins, which promote mtDNA deletions by stalling the DNA replication fork. The progressive accumulation of mtDNA deletions produces cytochrome *c* oxidase deficiency in muscle fibers and results in the clinical phenotype.

G416A is the second *POLG2* variation associated with a mitochondrial disease patient [51]. This patient presented with hearing loss, PEO, loss of central vision, macrocytic anemia, and hypogonadism. Analysis of the G416A protein revealed WT-like p55 function as displayed by normal chromatographic properties, stimulation of processive DNA synthesis for pol γ, and normal protection of the catalytic subunit by N-ethylmaleimide. The G416A accessory subunit protein did not have significantly impaired enzyme function, suggesting that it was unlikely to be the cause of pathogenesis. Further sequencing of mitochondrial disease loci revealed a novel Y582C mutation in the *OPA1* gene (encoding a dynamin-related GTPase, mutations in which are more commonly associated with optic atrophy, Fig. 1). The authors concluded that the mutation in *OPA1* may better explain the patient's causative features of disease. The latest data at NCBI now identifies G416A in *POLG2* as a polymorphism (http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?rs=17850455), suggesting it is more likely that the novel *OPA1* mutation in this patient could be contributing to their mitochondrial disease status.

10. Closing remarks

Sequencing the *POLG* gene in patients with mitochondrial disease is helpful for confirming the diagnosis of mitochondrial disease, especially in the case of Alpers syndrome. *POLG* sequencing offers the added benefits of carrier testing, prenatal diagnosis, postnatal pre-symptomatic diagnosis of siblings and optimized clinical management from the early stages of disease. In a recent study by Wong et al., the majority (92.5%) of mutated alleles in various populations were missense mutations, while frameshift and nonsense mutations accounted for a smaller fraction (7.5%) of the overall mutated alleles [28]. However, several newly identified mutations in mitochondrial disease patients may be neutral polymorphisms. For example, in the Wong et al. study, 44% (27/61) of the mitochondrial disease patients with a *POLG* mutation had only one allele affected that was not consistent with autosomal dominant inheritance [28]. As a result, the molecular diagnosis of these 27 patients was unclear, either suggesting locus heterogeneity (Fig. 1), undetected mutations outside of the *POLG* coding region (such as the intronic or promoter regions), or environmental factors. Digenic effects have been identified previously, for example, in a PEO patient with recessive mutations in both *POLG* and the mtDNA helicase [52].

Despite the increasing level of knowledge on pol γ mutations in disease (Table 1; Fig. 5), there is still much to learn. A three-dimensional structure of the pol γ holoenzyme will be very instructive for identifying more contacts between the subunits of pol γ, and will provide a better understanding of these disease mutations. Additionally, knowledge of the holoenzyme structure will be essential for the rational development of new NRTIs, to determine whether the new drug causes inhibition of the holoenzyme. We still have much to learn about the role of *POLG* mutations in parkinsonism, Charcot–Marie–Tooth disease, and male infertility. Why do some mutations cause mtDNA deletions, whereas others cause depletion or point mutations in mtDNA? Furthermore, factors such as tissue specificity, age of onset, and the effect of environmental factors have not yet been resolved. Analysis of pol γ should enable a greater understanding of mtDNA replication and the role of defective mtDNA replication proteins in disease.

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