Cell. Mol. Life Sci. (2011) 68:219–233
DOI 10.1007/s00018-010-0530-4

Cellular and Molecular Life Sciences

REVIEW

Mitochondrial DNA replication and disease: insights from DNA polymerase γ mutations

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Received: 21 July 2010 / Revised: 31 August 2010 / Accepted: 2 September 2010 / Published online: 8 October 2010

Abstract DNA polymerase γ (pol γ), encoded by *POLG*, is responsible for replicating human mitochondrial DNA. About 150 mutations in the human *POLG* have been identified in patients with mitochondrial diseases such as Alpers syndrome, progressive external ophthalmoplegia, and ataxia-neuropathy syndromes. Because many of the mutations are described in single citations with no genotypic family history, it is important to ascertain which mutations cause or contribute to mitochondrial disease. The vast majority of data about *POLG* mutations has been generated from biochemical characterizations of recombinant pol γ. However, recently, the study of mitochondrial dysfunction in *Saccharomyces cerevisiae* and mouse models provides important in vivo evidence for the role of *POLG* mutations in disease. Also, the published 3D-structure of the human pol γ assists in explaining some of the biochemical and genetic properties of the mutants. This review summarizes the current evidence that identifies and explains disease-causing *POLG* mutations.

Keywords Mitochondria · mtDNA replication · DNA polymerase gamma · *POLG* · DNA repair

Mitochondrial replication and repair by DNA polymerase γ

Mitochondria produce the majority of ATP in eukaryotic cells as a result of oxidative phosphorylation. Although nuclear genes encode most mitochondrial proteins, 11 genes that encode necessary proteins for oxidative phosphorylation reside in the mitochondrial genome. Mitochondrial DNA (mtDNA) is organized into 16,569 base pair circular double-stranded DNA genomes (Fig. 1). In humans, there are several mtDNA copies per mitochondria and hundreds of mitochondria per cell, accounting for thousands of copies of mtDNA per cell. Depletion of mtDNA leads to dysfunctional mitochondria, which can ultimately lead to cell death. Additionally, mtDNA point mutations and deletions can disrupt mitochondrial function if the percentage of mutant mtDNA per cell, referred to as heteroplasmy, reaches a threshold where not enough functional mitochondria remain to maintain proper ATP levels. Therefore, effective and faithful mtDNA replication is necessary for cell survival.

mtDNA is replicated and repaired by the pol γ complex, which is the only polymerase of the cellular 16 DNA polymerases that is known to function in the mitochondria [1–3]. The pol γ holoenzyme is a heterotrimer consisting of a single 140 kDa catalytic subunit (encoded by *POLG* at chromosomal locus 15q25) and a 55 kDa accessory subunit that forms a tight dimer (encoded by *POLG2* at chromosomal locus 17q24.1). The catalytic subunit has DNA polymerase, 3′–5′ exonuclease and 5′ dRP lyase activities [4]. The accessory subunit is required for tight DNA binding and processive DNA synthesis [5]. The pol γ holoenzyme functions in conjunction with the mitochondrial DNA helicase, c10orf2, and the mtSSB to form the minimal replication apparatus [6].

Two mechanisms of mtDNA replication

Two modes of DNA replication have been proposed to copy the mitochondrial genome, an asynchronous strand

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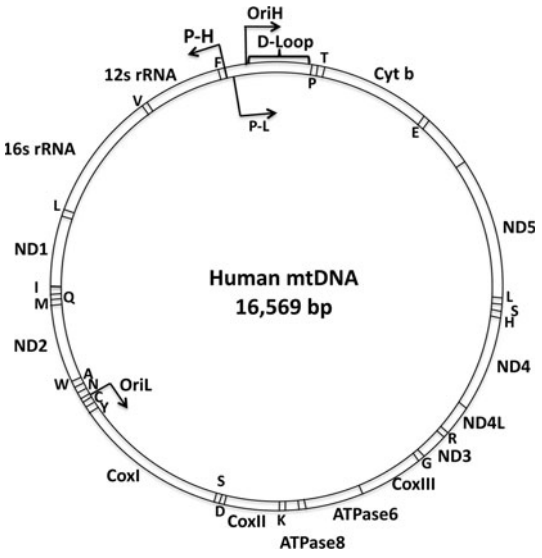

Fig. 1 Schematic of the mitochondrial genome. *Single letters* represent the amino acid code for the specific mitochondrial tRNA. The *H* strand is replicated clockwise beginning at *OriH*. The *L* strand is replicated counter-clockwise beginning at *OriL*

displacement model and a strand-coupled bidirectional replication model (reviewed in [7]). These models have been aggressively defended by their authors in several published arguments [8–10]. 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 [11]. 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 [12]. In both models, the DNA polymerization reaction is performed by pol γ.

In the asynchronous strand displacement model, transcription initiates replication of mtDNA at OriH within the D-loop at the light-strand promoter (P-L) [13]. The primer for initiation of mtDNA replication at OriH is generated by processing the transcript starting at P-L [11, 14]. Pol γ initiates H-strand synthesis by extending the RNA primer [11, 15, 16]. When nascent H-strand synthesis is $\sim$70% complete, the replication fork exposes the major origin for L-strand synthesis (OriL), allowing initiation of L-strand synthesis on the displaced H-strand to proceed in the opposite direction [17–19]. L-strand replication is initiated near the WANCY tRNA coding region that in a single-strand form is postulated to assume a stable stem loop structure, and DNA synthesis proceeds along the entire length of the mitochondrial DNA strand and terminates after H-strand replication is completed [20]. Like H-strand synthesis, L-strand replication has also been shown to be initiated in vitro by the mitochondrial RNA polymerase [21].

The coupled replication model of mtDNA replication is based on the ribonucleotide substitution pattern in mtDNA and analysis of replication intermediates by 2D-gel electrophoresis [12, 22, 23]. The 2D-gel electrophoresis revealed two types of replication intermediates [22]: one type is resistant to nucleases that digest single-stranded DNA consistent with conventional duplex replication intermediates from symmetric, semi-discontinuous DNA replication with coupled leading and lagging strand DNA synthesis. A second class of replication intermediates, presumably derived from the strand-asynchronous mechanism of mtDNA replication, was sensitive to single-strand nuclease and was most abundant in cultured cells not treated with ethidium bromide. Although this initial report suggested coexistence of both the asynchronous and strand-coupled modes of mtDNA replication [22], later findings by the same authors indicate that mammalian mtDNA replication proceeds mainly, if not exclusively, by a strand-coupled mechanism [12, 23]. Later, replication intermediates from highly purified mitochondria were demonstrated to be essentially duplex throughout their length, although they contain RNA/DNA hybrid regions, which result from the infrequent incorporation of ribonucleotides [23]. The authors suggested that in vitro RNaseH treatment or the process of extracting mtDNA from crude mitochondria leads to degradation of these ribonucleotide-rich regions and produces the partially single-stranded molecules previously assumed to arise by the asynchronous mechanism [22]. Analysis of mitochondrial DNA from rats, mice and humans revealed that mtDNA replication initiates at multiple origins that are distributed across a 4-kb fragment downstream from the 3′ end of the displacement loop, and that DNA replication is restricted to one direction after fork arrest near OriH [12]. Further mapping of prominent free 5′ ends identified two regions of start sites, one corresponding to OriH for the strand-asynchronous model, and the other several hundred nucleotides toward the non-coding D-loop region corresponding to a possible bidirectional replication origin [24].

Analysis of two-dimensional agarose gel electrophoresis also shows that mtDNA contains ribonucleotide incorporation throughout the lagging strand (RITOLS). Yasukawa et al. [25], has demonstrated that RITOLS maps to the major non-coding region of birds and mammals and is unidirectional. One of the more prominent RITOLS that is utilized for DNA replication starts in the region of OriL [25]. Transmission EM and antibodies specific for RNA/DNA duplex shows that duplex DNA intermediates can be found throughout mtDNA and contain extensive RNA tracts on one strand, indicative of a strand coupled model [26]. However, Brown et al. [27], observe that many

mitochondrial transcripts also form stable R-loops, similar to the well-documented R-loop at the leading strand replication origin (OriH). These stable but nonreplicative, partially hybridized RNA/DNA duplexes raise doubts concerning the function of RITOLS. There are certain elements in both models that are well supported by experimentation, but it is clear that further experimentation is needed to illuminate whether both models predominate in nature or are products of experimental artifacts.

Pol γ in mitochondrial DNA repair

DNA repair in mitochondria is mostly limited to base excision repair (BER) which is well suited with a host of glycosylases to recognize base damage, as would occur during oxidative stress. Mitochondrial base excision repair can proceed via two pathways, single-nucleotide-BER (SN-BER) or long-patch BER (LP-BER) (Fig. 2) [28]. With either repair pathway, an oxidized or damaged base is recognized and cleaved by a specific glycosylase, leaving an abasic site that is cleaved on the 5′ end by AP endonuclease (APE) to generate a nick with a 5′ deoxyribose phosphate (dRP) flap. During SN-BER, the mitochondrial DNA polymerase, pol γ, fills the gap and cleaves the 5′ dRP moiety prior to ligation by ligase III [29].

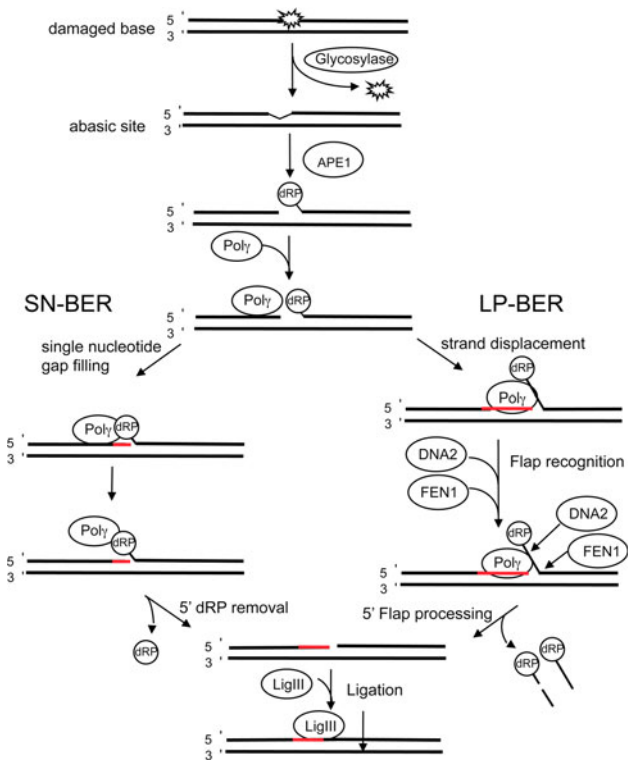

Fig. 2 Schematic of the base excision repair pathways present in mitochondria. The damaged base is excised by a *glycosylase* to create an abasic site. *APE1* endonuclease generates a strand break leaving a 5′ deoxyribose phosphate (5′ *dRP*) downstream of the lesion. *Pol γ* participates in either the single-nucleotide (*SN-BER*) or the long patch (*LP-BER*) base excision repair pathway. In SN-BER, Pol γ fills the nucleotide gap and removes the 5′ dRP, allowing for ligation by Ligase III. In LP-BER, Pol γ performs strand-displacement synthesis producing a 5′ flap that is excised by *DNA2* and *FEN1*. The resulting substrate is ligated by Ligase III

A LP-BER activity in mitochondrial extracts has recently been described, and several new proteins required for LP-BER in mitochondria have recently been described [30–32]. LP-BER requires an activity to remove the displaced 5′ DNA commonly known as a 5′-flap structure, and Liu et al. found FEN-1, a flap endonuclease, in their mitochondrial preparations that could carry out this activity [31]. In addition to FEN-1, DNA2, originally identified as a yeast nuclear DNA helicase with endonuclease activity, has also been implicated in mitochondrial LP-BER, as well as having a possible role in mtDNA replication [33]. Furthermore, overproduction of mutant forms of the mitochondrial DNA helicase, C10orf2, causes the accumulation of DNA2 in mitochondrial nucleoids [34]. In this capacity, DNA2 is believed to function with FEN-1 to process 5′ protruding flaps due to strand displacement synthesis during LP-BER prior to ligation by ligase III.

Mitochondrial diseases associated with pol γ

Several mitochondrial diseases have been linked to ineffective mtDNA replication by pol γ. Mutations in *POLG*, are associated with Alpers syndrome (and Alpers-like encephalopathy), childhood Myocerebrohepatopathy spectrum disorders, ataxia-neuropathy syndromes, myoclonus epilepsy myopathy sensory ataxia, and dominant and recessive forms of progressive external ophthalmalplegia (PEO). Detailed clinical features of pol γ-related diseases have recently been reviewed [35, 36]. Alpers syndrome is diagnosed in infants and young children, and most die during their first several years of life. Patients with Alpers commonly have psychomotor retardation, epilepsy, and liver failure, symptoms that are expedited following treatment of sodium valproate [37, 38]. mtDNA in muscle and liver samples of Alpers syndrome patients is depleted. PEO manifests during adulthood and is characterized by muscle weakness, exercise intolerance, sensory ataxic neuropathy, and respiratory failure due to muscle fatigue. In muscle tissue of PEO patients, cells with dysfunctional mitochondria and mtDNA deletions are prevalent. Ataxia-neuropathy syndromes include SANDO (sensory ataxic neuropathy, dysarthria, and ophthalmoparesis), MIRAS (mitochondrial recessive ataxia syndrome), and SCAE (spinocerebellar ataxia with epilepsy). Like PEO, ataxia-neuropathy syndromes are late-onset diseases, ranging from early teens to late thirties. MtDNA deletions are common in patients with these ataxia-neuropathy

syndromes. Especially with the different ataxia-neuropathy syndromes and PEO, the manifestations are similar enough that distinguishing among the syndromes is difficult.

The Human DNA Polymerase Gamma Mutation Database lists 147 mutations in *POLG* that were discovered in patients with mitochondrial disease or symptoms that suggest mitochondrial disease (http://tools.niehs.nih.gov/polg). The disease-associated mutations are located in all domains of *POLG* (Fig. 3). There is great interest in determining which *POLG* mutations cause mitochondrial disease and the symptoms of the disease. The lack of *POLG* genotypic data of families of several mitochondrial disease patients makes the determination of dominance problematic. Most of the mutations have been identified in compound heterozygotes where each *POLG* allele has one or more different mutations. In these instances, it is difficult to know the relative contribution of the two alleles. Thirty-two of the mutations have been identified only in *cis* with another *POLG* mutation or heterozygous with wild-type. Without genetic or biochemical data, it is impossible to predict whether any of these 32 mutations cause disease. The remaining 115 mutations have been identified either as dominant heterozyogotes (with evidence from adequate family history), in *trans* with one or more *POLG* mutations, or as homozygotes. Many times, the mutations are in *trans* with a *POLG* mutation that is known to encode a defective polymerase, suggesting that both mutations are defective in mitochondrial replication. However, only 43 mutations have been identified in multiple publications describing non-related families. The database contains mutations that were identified in publications. Therefore, the fact that most mutations have been found in single cases is probably due to an information bias because novel mutations are more likely to be published. A future goal of this and future databases would be to determine allelic frequencies of the myriad of *POLG* disease-associated mutations using unpublished observations from clinicians who primarily treat mitochondrial patients.

It has become an important focus to provide evidence of the disease-causing nature of these mutations. Biochemical evidence has been provided through purification of human pol γ and measuring various polymerase activities, DNA binding, and binding to its accessory subunit. Yeast and mouse model systems have shown mitochondrial defects in organisms with mutations in amino acids of the mitochondrial polymerase that are strongly conserved with disease-associated mutations of pol γ. The sum of these studies has determined DNA replication defects in 30 mutations, strongly supporting a model that these mutations contribute to the manifestation of disease.

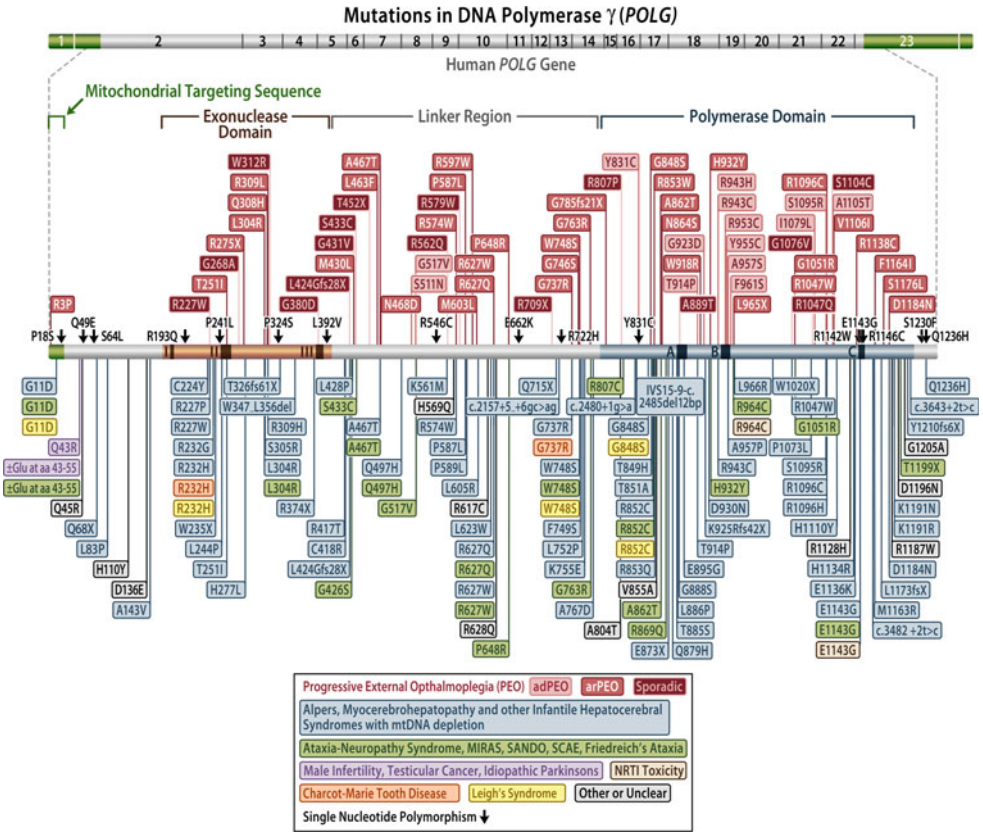

Fig. 3 Schematic diagram of human pol γ gene (*top*) and protein (*bottom*) showing the location of amino acid substitutions resulting from mutations associated with disease (in the *boxes*) and neutral polymorphisms found in unaffected populations (*arrows*). The *top line* displays the relative positions of the exons. The gene is separated into the mitochondrial targeting sequence (*green*), exonuclease domain (*brown*), linker domain, and the polymerase domain (*blue*)

Saccharomyces cerevisiae identifies mutations that cause mtDNA defects and suppressors that alleviate these defects

Yeast is an ideal genetic model system to study mtDNA replication mutants

For many years, budding yeast has been a model for studying mtDNA replication. Similar to humans, the yeast pol γ ortholog, Mip1, is responsible for mtDNA replication [39, 40]. Two reports suggest the involvement of pol ζ in yeast mtDNA replication, but the significance of these findings is not clear [41, 42]. Pol γ is 43% identical to the yeast mitochondrial protein, Mip1, but most of the polymerase domains of the two proteins are well conserved along with critical motifs of the exonuclease domain [2]. Mutations in *mip1* that are orthologous to disease-associated mutations in Pol γ have assisted in determining the effects of mutations in vivo and with few exceptions are the only evidence of the consequences of these mutations in a tractable genetic system.

In addition to simple but powerful genetic techniques used with yeast, mtDNA stability is easily assayed in *mip1* mutants (recently reviewed in [43]). Yeast without mtDNA (rho^0) or with mtDNA but unable to perform oxidative phosphorylation (rho^-) can live as long as fermentable carbon sources are present. Therefore, petite cells, or yeast that no longer have mitochondrial functions, are easily isolated. Although cells can lose the ability to perform oxidative phosphorylation for a number reasons, increased frequency of petite cells in *mip1* mutants correlates most often with decrease in mtDNA stability and depletion. Point mutagenesis can be assayed in rho^+ cells by measuring the frequency of cells becoming resistant to either erythromycin or oligomycin. Resistance to either antibiotic is conferred by one of several possible base substitutions in the small ribosomal RNA subunit encoded in mtDNA. Frameshift mutagenesis can be measured in yeast strains where *ARG8* with a +1 frameshift is inserted into mtDNA [44, 45]. Reversion to Arg^+ reflects the frequency of −1 frameshifts in the mtDNA. Finally, deletions between long direct repeats are measured in a particular strain where the *ARG8* gene, flanked by 96-mer repeats, is inserted into the *COX2* [46]. The *ARG8* insertion causes a rho^- phenotype but reverts to rho^+ when the deletion occurs. Deletions between long direct repeats are not affected by *mip1* mutations but are increased by *din7* and *msh1* mutants, which are implicated in mtDNA recombination and nucleoid assembly [47, 48].

Recently, it has been shown that yeast can be used to study the inhibition of mtDNA replication by nucleoside analogs that are important in antiretroviral therapy [49]. D4T in particular inhibits mtDNA replication after phosphorylation by thymidine kinase. Human thymidine kinase was expressed from a *GAL1* promoter to allow the yeast cell to activate the nucleotide analog and measure its effects on petite frequency. This will prove to be an important assay to learn about genetic predispositions that affect the severity of mitochondrial toxicity in response to nucleotide analogs.

Conserved polymerase domain mutations cause dominant and recessive phenotypes

Of the nearly 150 disease associated mutations in pol γ (Fig. 3), 55 mutations change amino acids that are identical to amino acids in conserved stretches of yeast *mip1*, while 29 other mutations change amino acids that are similar but in conserved regions of *mip1* [49]. Most of these conserved mutations that can be studied in yeast are located in the polymerase domain. Many of these mutations cause increased petite colony formation frequency due to mtDNA instability and depletion providing evidence for their role in the mtDNA depletion found in the diseased individuals.

Autosomal dominant PEO mutations have been identified in families, and the orthologous yeast mutations have also been studied. Similar to human disease presentation, mutant strains with mutations orthologous to autosomal dominant PEO mutations G923D, R943H, and Y955C exhibited increased petite colony formation frequency in diploid heterozygotes and lost mtDNA in haploids [50]. Similarly, orthologs of human R853Q, R853W, T851A, H932Y, and A957P also exhibited dominant increase of petite frequency and mutagenesis [51]. These data suggest that the mutant polymerase binds to mtDNA and not only is unable to replicate but either directly or indirectly inhibits the wild-type polymerase. Interestingly, human R853Q and T851A do not incorporate the incorrect nucleotide at a higher frequency than wild-type, indicating that these mutations do not affect fidelity [52]. These mutations are severely compromised for polymerase activity and so it is unlikely that they are participating in any mtDNA replication. However, the heteroallelic orthologs to R853Q and T851A increased mutation frequency 30- and 22-fold, respectively [51]. Therefore, it is possible that stalling by catalytically inactive polymerases leads to damaged template mtDNA that increases the likelihood of a mispairing by the wild-type polymerase. In support of this hypothesis, mtDNA damage accumulates in the Y955C yeast ortholog, and the increase in petite frequency in these heterozygotes is suppressed by exposure to the antioxidant dihydrolipoic acid and presumably decreasing ROS [50, 53].

Some of the disease-associated mutations were strictly recessive in yeast, only affecting mtDNA stability in a monoallelic strain [51]. Orthologs to human G848S, N864S, and G1076V were unable to replicate mtDNA but

did not affect the wild-type polymerase. G848S has an extremely low rate of catalysis like the dominant mutations and is also defective for binding DNA [52]. Its reduced DNA binding is consistent with strictly recessive nature. It is still unknown whether N864S and G1076V also have DNA binding defects or are unstable or not expressed.

Exonuclease domain disease mutations retain proofreading activity

The exonuclease domain is responsible for degrading the newly incorporated dNTP into the nascent strand of DNA. This occurs at replication forks that are stalled because of a misincorporated nucleotide and results in proofreading the original mismatched base pair. Mip1 exonuclease activity is dependent on at least two catalytic aspartates as evidenced by the 10^4-fold decrease in activity in D171G and even larger decrease in a D171G/D230A [54]. Correspondingly, mtDNA mutant frequencies in heteroallelic D171G and D230A *mip1* mutants are 110-fold and 219-fold higher than wild-type, respectively, while the double mutant is 1,440-fold higher than wild-type [55]. These double mutants were proficient at extending mismatches and incorporating incorrect nucleotides. Another exonuclease deficient *mip1*, a double D171A/G173A, was designed based on homology to the orthologous human pol γ exonuclease domain mutations that destroy exonuclease activity [56]. These mutants exhibited a 550-fold increase in mitochondrial DNA mutant frequency [51]. In any of these cases, the petite colony formation frequency of the double mutants at 30°C or lower is between 31 and 45%.

Many disease-associated mutations have been identified in the exonuclease domain. It has been assumed that any of these mutations disrupt exonuclease function; however, to date, no disease-associated mutation in *POLG* that were identified in disease patients has been shown to inactivate or decrease exonuclease activity. G268A is an exonuclease mutation associated with PEO and various neurological conditions but its ortholog in yeast (G224A) only causes a ninefold increase in mtDNA mutant frequency in haploids [53]. The Gly224 is an important residue for exonuclease function based on the 268-fold increase in mutant frequency caused by G224D substitution in haploid yeast, which was isolated in a random screen of *mip1* mutants for mutator phenotypes [57]. Because of the relatively mild increase of mutagenesis, it is logical to assume that G224A and possibly human G268A retain most of its exonuclease function. Several other exonuclease domain amino acid substitutions that are orthologous to disease-associated mutations only increase mtDNA mutant frequency to less than 12% of the mutant frequency of exonuclease-deficient *mip1* strains [51]. Heterozygous exonuclease-deficient mice exhibit a 500-fold increase in mutation frequency from wild-type and have no obvious defects, suggesting that such a small increase in mitochondrial mutagenesis do not decrease viability [58]. Interestingly, while exonuclease-deficient monoallelic strains cause a 31–45% petite frequency, Q264H monoallelic mutants were 100% petite suggesting that this mutation somehow inactivated polymerase function despite being in the exonuclease domain. Biochemical characterizations suggest that DNA synthesis by these exonuclease domain *mip1* mutants is severely reduced while the exonuclease activity is enhanced or unaffected [59]. In addition, L210P (homologous to human L244P) was unstable during extract preparation, and the mutant exhibited a temperature-sensitive petite phenotype. Therefore, this leucine to proline alteration may contribute to mitochondrial disease by causing protein instability. It is still possible that a mild increase in mutagenesis is sufficient to cause mitochondrial disease, but there are still no examples of disease-associated mutations whose exonuclease activity is inhibited.

Suppression of mitochondrial disease by increased nucleotide concentration and antioxidants

The obvious challenge to treating pol γ-related mitochondrial diseases is that there is no gene therapy available to be able to allow the translation of wild-type pol γ in these patients. However, the fact that similar genotypes can vastly change the age of onset and tissue specificity of the disease suggests that environmental factors could suppress or exacerbate the disease state. Most evidence of environmental triggers of mitochondrial disease is anecdotal, and little information has been useful for treating disease.

Using the yeast model for pol γ disease, suppressors of mitochondrial dysfunction have been identified. Overexpression of *RNR1*, which encodes ribonucleotide reductase, suppresses increased petite frequency in some *mip1* mutants without affecting point mutagenesis [51, 53, 60]. Ribonucleotide reductase catalyzes the rate-limiting step of de novo dNTP synthesis, and overexpression of *RNR1* has been shown to increase nucleotide concentrations [61]. This indicates that increased nucleotide concentration can allow at least some pol γ mutants to replicate mtDNA effectively. The *mip1* mutants that showed the greatest suppressive phenotypes were those that showed decreased affinity to the incoming dNTP but similar catalytic rate as wild-type, such as H932Y [51]. Therefore, it is possible to predict biochemical defects based on the suppressive effects of overexpression of *RNR1*. If these results are recapitulated in higher eukaryotes, it will be necessary to identify dNTP binding affinities of mutant pol γ to determine the potential efficacy of increasing nucleotide concentration to improve mitochondrial replication and delay the effects of the disease.

Mitochondria are a major source of oxidative damage in the cell. There are numerous pathways that describe how superoxides can damage mitochondria and mtDNA replication [62]. The reactive oxygen species scavenger dihydrolipoic acid (at 30 μM) was found to reduce the petite frequency in *mip1* Y757C and G224A mutants [53]. Although, the mechanism for this suppression is unknown, it would be interesting to test the therapeutic potential of similar antioxidants in mouse models or tissue culture systems.

Molecular model of the pol γ active site suggests mechanisms for aberrant mtDNA replication by disease-associated mutants

Molecular model of the active site of pol γ identifies potential functions of disease-associated amino acids

Pol γ is a Family A DNA polymerase whose polymerase active site is similar to the active site in T7 polymerase. Determination of the three-dimensional structure of the T7 polymerase resulted in a molecular model of the pol γ active site [63]. The closed conformation of the pol γ molecular model revealed possible contacts between Arg943, Lys947, and Tyr951 and the sugar and incoming nucleotide phosphates. Although mutations in Tyr951 have not been discovered in disease patients, Tyr951 has been shown to be primarily responsible for selection against the incorporation of chain-terminating dideoxynucleotides and D4T-TP that are used as antiviral agents to treat HIV infection [64, 65]. R943H is associated with autosomal dominant PEO [66] and not only binds to the pyrophosphate group of the incoming dNTP but is also expected to interfere with the nucleotidyl-transfer reaction, reducing both the nucleotide binding affinity and catalysis [63].

In addition to R943H, other autosomal dominant PEO mutations, G923D and A957S, alter amino acids in the active site [66]. G923D introduces a negative charge into a region of positively charged residues at the edge of the active site, resulting in a fivefold reduction of polymerase activity of wild-type [63]. Ala957 is located 2.26 Å from the phosphate group of the template. The A957S mutant causes increased DNA binding affinity, and the proximity of the serine to the template DNA suggests hydrogen bonding. However, A957S results in a fourfold reduction in catalytic activity compared to wild-type. H932Y is thought to be a recessive active site mutation that severely compromises binding affinity to the incoming nucleotide [51, 67, 68]. His932 is 2.6 Å away from the β-phosphoryl oxygen atom of the incoming nucleotide, suggesting a possible hydrogen bond. It is also possible that the elimination of the positive charge by substitution of histidine to tyrosine disrupts dNTP binding.

The commonly found autosomal dominant PEO mutation causes the Y955C amino acid substitution [66, 69]. Tyr955 is also located in the active site at the C terminus of the O-helix [63]. Together with Glu895 and Tyr951, Tyr955 forms a hydrophobic pocket for the incoming dNTP. Disruption of the hydrogen bonding of Tyr955, Tyr951, and Glu895 decreases DNA replication fidelity [70] and the discrimination against antiviral nucleotide analogs [56, 64]. In addition, Tyr955 with Phe961 allows for either error-free bypass or translesion synthesis of 8-oxo-dG by keeping 8-oxo-dG in the anti-conformation and favoring binding to dCTP. In contrast, the cysteine substitution at position 955 allows for the *syn*-conformation of 8-oxo-dG which can accommodate pairing with dATP via Hoogsteen hydrogen bonding [71]. Because there is an increase in mitochondrial oxidative damage caused by Y955C in yeast and transgenically targeted mouse models [50, 72], error-prone bypass or misincorporation opposite 8-oxo-dG may contribute to the detected increased mitochondrial mutagenesis caused by Y955C [53].

Crystal structure of human pol γ holoenzyme helps to explain biochemical and genetics phenotypes of disease-causing mutations

The crystal structure of human pol γ holoenzyme was determined to within 3.2 Å, confirming biochemical evidence that the holoenzyme is a heterotrimer comprised of two pol γ-β (accessory) subunits and one pol γ-α (catalytic) subunit [73, 74]. Holoenzyme used for crystallization was comprised of an exonuclease-deficient pol γ-α and pol γ-β with a deleted four-helix bundle that is distal to contacts with pol γ-α and does not affect polymerase activity [74].

The contact between the pol γ-α subunit and the β subunit homodimer is asymmetric with the pol γ-α subunit primarily contacting the proximal β monomer [74]. Each β monomer contributes to the overall processivity of the holoenzyme. The distal monomer enhances the polymerization rate, and the proximal monomer increases DNA binding affinity of the holoenzyme [75]. Pol γ-α amino acids Arg232 (Fig. 4a) and Glu540 comprise the main contact with the distal monomer [74]. Mutations that change Arg232 to glycine and histidine have been identified in several unrelated compound heterozygous patients afflicted with Alpers or Alpers-like infantile hepatocerebral diseases [37, 76–80]. Although mutations at Arg232 do not affect pol γ-α alone, the mutant holoenzyme displays decreased polymerase activity and exonuclease selectivity for mismatched base pairs [81]. Contact with the proximal subunit is much more substantial, involving hydrophilic interactions of amino acids in the N-terminal thumb domain area and positively charged residues in the palm domain (Lys1198, Arg1208, and Arg1209) [74].

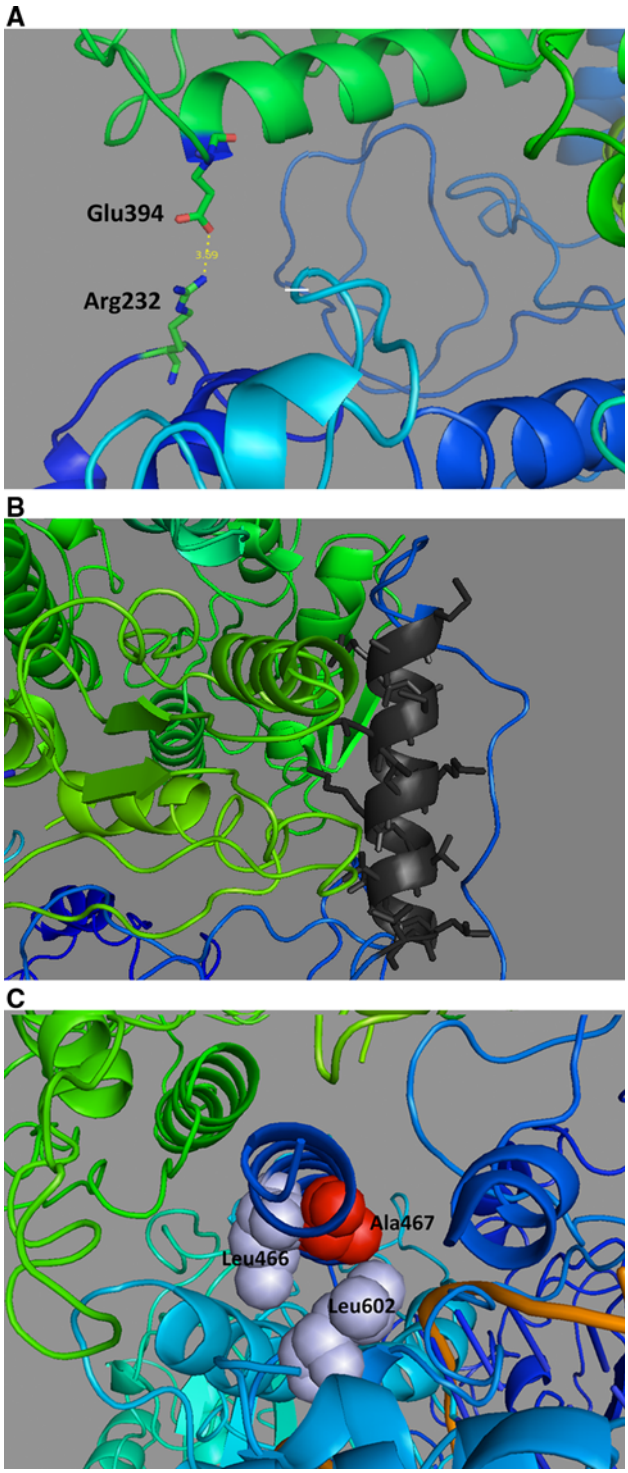

Fig. 4 The 3-dimensional structure of the human DNA polymerase γ holoenzyme depicting several residues involved in disease and important for interaction between the catalytic pol γ-α subunit (*blue*) and the accessory pol γ-β subunit (*green*). These illustrations were derived using PDB 3IKM [74] in the program PyMOL (http://www.pymol.org/). **a** The salt bridge between Arg232 of the pol gamma catalytic subunit and Glu394 of the distal p55 accessory subunit. **b** Pol γ- α amino acids 543–558 (shown in *black*) form a helix of hydrophobic residues that stabilize interactions with the proximal pol γ- β subunit. **c** Ala467 is located near Leu466 and Leu602, which is postulated to comprise an important hydrophobic environment for subunit interaction

Hydrophobic interactions involving a helix comprised of amino acids 543–558 in the spacer region predominantly stabilizes subunit interaction (Fig. 4b). In pol γ-α mutants that disrupt these hydrophobic interactions, the processivity is not enhanced by the accessory subunit. Ala467, which resides in the DNA-interacting thumb domain, is also important for pol γ subunit interaction. The most common disease-causing pol γ mutation is A467T, which causes decreased binding to pol γ-β subunit along with overall decreased polymerase activity [82, 83]. The Ala467 is located in the thumb domain which interacts with the pol γ-β subunit and substitution to threonine is thought to interrupt the hydrophobic area formed by nearby leucine residues causing a shift of this region (Fig. 4c) [74].

Crystal structure provides new glimpse at non-active site polymerase domain mutations

Mutation W748S is the second most common pol γ disease mutation and associates with Alpers and ataxia [84, 85]. W748S causes low polymerase activity and processivity along with defective DNA binding [86]. In patients, W748S is always associated with the neutral polymorphism E1143G, and the double mutant improves DNA binding and polymerase activity compared to the single W748S. The crystal structure shows that Trp748 associates with other aromatic residues Phe750 and His733 that must be stabilized to bind effectively to template DNA [74]. Trp748 faces away from the interface of the monomer of pol γ-β and has no effect on binding to pol γ-β [74, 86].

Mutations G848S, T851A, R852C, and R853Q are located in a conserved region of the thumb domain and are all associated with Alpers syndrome [66, 79, 87, 88] (Fig. 5a). In each case, the mutations were shown to nearly eliminate polymerase activity [52]. In addition, G848S and R852C also showed a four- to fivefold decrease in binding affinity to DNA [52]. Fig. 5a shows that Arg853 is located very close to and may provide electrostatic interaction with one of the magnesium-chelating aspartic acid residues [74]. A change to glutamine from Arg853 would cause a repulsive electrostatic interaction and could explain the dramatic decrease in catalytic activity. Interestingly, R853W has been associated with PEO [89, 90], and genetic data of the yeast homolog suggest that polymerase activity is not as severely compromised [51]. The tryptophan substitution is not expected to repel the nearby aspartic acid, but the bulky side chain may cause steric clashes.

Mutations in Arg807 residue to proline and cysteine have been associated with PEO and SANDO, respectively

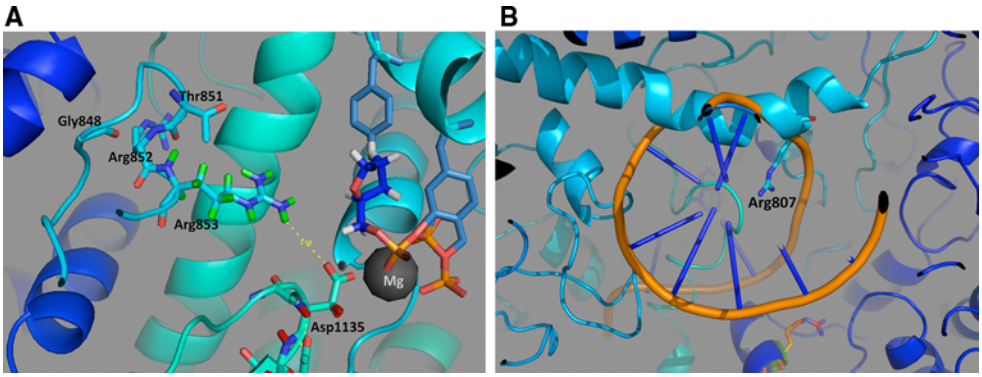

Fig. 5 The 3-dimensional structure of the human DNA polymerase γ holoenzyme depicting several residues involved in disease. These illustrations were derived using PDB 3IKM [74] in the program PyMOL (http://www.pymol.org/). **a** Active site of the human DNA polymerase gamma illuminating residues 848, 851, 852, and 853 that are altered in Alpers patients. The side chain of Arg853 is in a position to provide electrostatic interaction with Asp1135, which along with Glu1136, chelate the two active site Mg^{2+} ions. **b** The side chain of Arg807 is shown here to protrude into the DNA binding cleft in the open structure. Although the polymerase is in the 'closed' conformation, DNA is modeled into its predicted location in an 'open' conformation

[68, 91, 92]. Yeast homologs to R807P and R807C cause mitochondrial dysfunction suggesting that this conserved arginine is important for polymerase activity [51]. Though no biochemistry has been published about mutations of Arg807, it is predicted to be defective in DNA binding because the arginine side chain protrudes into the DNA binding cleft and may be necessary to the overall positive charge environment needed for DNA binding (Fig. 5b).

Mammalian pol γ mouse models implicates a role of mtDNA mutations in aging

Reduction in pol γ activity inhibits mtDNA replication and causes mtDNA depletion

Although pol γ was widely established as a key polymerase in mtDNA replication, the homozygous knockout mouse confirmed that pol γ is necessary for mtDNA replication [93]. Homozygous pol γ knockouts die between embryonic days 7.5–8.5 with no cytochrome *c* oxidase activity and a complete loss of mtDNA [93]. Interestingly, heterozygotes develop indistinguishably from wild-type, and the mtDNA content is identical despite the fact that *POLG* transcript level is half of wild-type [93]. This suggests that pol γ amount is not a limiting factor that determines mtDNA copy number.

Despite several years of research implicating the importance of a myriad of *POLG* mutations in mitochondrial disease, only Y955C has been developed into a mouse model. *POLG* Y955C mutation was transgenically targeted to cardiac tissue causing 10- to 20-fold overexpression of the mutant polymerase [72]. Median survival was decreased in the transgenic mice as compared to wild-type, which was attributed to massive cardiomegaly with bilateral atrial enlargement [72]. mtDNA depletion and oxidative stress occurred in the transgenic mice cardiac tissue, causing morphological mitochondrial defects. Although the Y955C transgenic mouse is important in establishing a model to study pol γ disease, there are two caveats that will hopefully motivate future research. First, the nature of transgenic overexpression does not allow the observations of dominant phenotypes as would a heterozygote model. Because Y955C is clinically established as a dominant mutation, a heterozygote would be informative. Second, it is unknown whether the effect of a 10- to 20-fold increase in wild-type pol γ in mice cardiac tissue is detrimental like the mtDNA depletion phenotype that occurs upon constitutive overexpression in neural tissues in *Drosophila* [94].

Exonuclease-deficient pol γ mouse links mtDNA mutations and deletions to aging

mtDNA point mutations and deletions accumulate in the course of the lifetime of a human. It has been proposed for many years that the increase of mtDNA mutations leads to defective oxidative phosphorylation, which increases reactive oxygen species and oxidative damage. To test the effect of mtDNA mutations on aging, mouse models have been created that increase mtDNA mutagenesis by substituting the catalytic aspartates for alanines in the exonuclease domain of pol γ. Mutations in the catalytic aspartates disrupt exonuclease activity increasing base substitutions 20-fold in vitro [95] and 500-fold in *S. cerevisiae* mtDNA in vivo [51, 54, 55, 57]. In mice, transgenic expression of exonuclease-deficient pol γ in cardiac tissue increased mtDNA point mutagenesis over 23-fold and resulted in detection of large mtDNA deletions [96]. Increased mtDNA mutagenesis in cardiac tissue resulted in extremely enlarged hearts without any detectable decrease

in mitochondrial respiratory function or increase in oxidative stress [96, 97]. Increased apoptosis occurred by opening of the permeability transition pore causing heart disease; however, the disease is limited by upregulation of Bcl-2 which inhibits pore opening [98–100]. Similarly, transgenic expression of exonuclease-deficient pol γ targeted to pancreatic islet cells led to impaired glucose tolerance, diabetes, and increased apoptosis in β-cells [101, 102].

A major link between aging and mitochondrial DNA mutations by pol γ was revealed with two other pol γ mouse models. Knock-in mice of exonuclease-deficient pol γ were created by two independent groups [103, 104]. In both models, elevated mutation frequencies caused premature aging in homozygous mutants but not in heterozygous mice. Symptoms of premature aging, occurring between 6 and 9 months, consisted of hair graying and loss, hearing loss, curvature of the spine, and decrease in body weight and bone density [103–106]. As in the transgenic heart model, these mice had enlarged hearts and an accumulation of abnormal mitochondria in the cardiomyocytes [103]. Homozygous exonuclease-deficient knock-in mice, like older mice, exhibit increased production of caspase-3 levels causing apoptosis [104]. Interestingly, these mutant mice showed no substantial increase of oxidative stress or ROS-induced damage [104, 107], arguing against the model that mtDNA mutations disrupts respiratory chain function and increases ROS production [108]. Evidence from all the exonuclease-deficient mouse models supports the model that increased mutagenesis by a error-prone pol γ causes rare critical mutations that causes mitochondrial dysfunction in a cell, targeting it for apoptosis [109]. The increased frequency of apoptosis leads to the loss of important and irreplaceable cells, tissue damage, and eventually organ failure [105].

Recent studies have shown interesting evidence of suppression of premature aging by mutations caused by an exonuclease-deficient pol γ. Although several reports showed no significant increase in ROS as a result of increased mtDNA mutagenesis, overexpression of mitochondrial catalase suppressed cardiomyopathy in these mutant mice [110]. Therefore, the role of ROS in the premature aging phenotype may need to be further elucidated. Interestingly, there is increasing evidence that resistance training reduces the mtDNA mutational load in muscles of older patients and those with mitochondrial cytopathies by a fusion event of mitochondrial proficient muscles cells with mitochondrial-deficient cells, a process called mtDNA shifting [111]. It would be extremely valuable to determine whether resistance exercise will suppress at least muscular phenotypes caused by increased mtDNA mutations. Finally, caloric restriction has been shown to reduce mtDNA damage and reduce age-related hearing loss in mice [112, 113], and could be successful in inhibiting premature aging in the mutator mice.

mtDNA molecular mechanisms of premature aging in exonuclease-deficient pol γ mice

The exonuclease-deficient pol γ mouse models demonstrate that faithful mtDNA replication is important for survival. However, accurate quantitation of mtDNA mutation frequencies has caused controversial interpretations of the link between mtDNA mutagenesis and normal aging. MtDNA mutation frequency in homozygous mutants was originally observed to be increased 3- to 8-fold over wild-type and heterozygotes [103, 104], which is consistent with an approximately 3- to 11-fold increase in mutation frequency from young mice (1–10 months old) to older mice (24–40 months old) [58, 107]. The mutation frequency in both knock-in mouse studies was determined by PCR cloning and sequencing, a method that is limited by the error frequency of the PCR polymerase, which was 1.3×10^{-4} mutations per base pair [104]. The "random capture method," which measures the altered frequency of restriction enzyme cleavage by a four base cutter in single molecules [114], estimated wild-type mutation frequency of young mice at 7.1×10^{-7} mutations per base pair and heterozygous mutation frequency of young mice at 1.6×10^{-4} mutations per base pair [58]. More importantly, the mutation frequency of older wild-type mice (5.4×10^{-6} mutations per base pair) did not approach that of the asymptomatic heterozygotes, suggesting that the frequency of mtDNA point mutations in normal aging mice is not sufficient to cause aging phenotypes [58].

One disadvantage of the random capture method of calculating mtDNA mutation frequency is that it only measures one of several restriction sites within the mitochondrial genome. Therefore, it is possible that the mutation frequency of these sites are not representative of the entire genome even though the frequencies were consistent among several different sites [58]. It is possible that hotspots can occur that would not be accounted for in the random capture method. Amplification of a single molecule by PCR before sequencing is a promising and accurate method of quantifying mtDNA mutation frequency as long as enough bases are sequenced so that more than one mutation has been detected [115]. This method eliminates the problem of the intrinsic error rate of the PCR polymerase and allows for the investigation of mutations in any sequence context.

A variation of the random capture assay demonstrated a controversial finding that random mtDNA deletions accumulated during the lifetime of homozygous pol γ exonuclease-deficient mice at a 90-fold increased frequency than the mtDNA deletions in heterozygotes or wild-type

[116]. The majority of mtDNA deletions in wild-type and heterozygous mice occur between direct repeats of six or more bases, whereas homozygous mutant mice accumulate deletions regardless of the presence of direct repeats [116]. However, the role of mtDNA deletion accumulation in the premature aging phenotype is unclear for at least two reasons. First, mtDNA deletions and point mutations both occur in prematurely aged mice, and the molecular phenotypes are not easily separated. Interestingly, transgenic overexpression of the Twinkle (c10orf2) mitochondrial helicase in mice, which caused a late onset mitochondrial disease that mimics PEO, resulted in increased mtDNA deletions but not mutations [117]. Identifying an exonuclease-proficient pol γ mutator mutant may suggest a mouse model that could distinguish between mtDNA deletions and point mutations. Second, the absolute number of mtDNA deletions in homozygous mutant prematurely aging mice is unknown. Southern blots and long-range PCR failed to detect deletions in the pol γ exonuclease-deficient mice [118]. However, it was argued that the random nature of the deletions makes these methods insufficient for quantifying deletions [119]. Amplification of large sections of single mtDNA molecules demonstrated that mtDNA deletions were identified in 2- to 3-year-old wild-type mice in only 0.07–0.2% of molecules screened [120]. The low percentage of deleted mtDNA molecules argues against the model that mtDNA deletions primarily causes normal aging in wild-type mice. Similar accurate and sensitive quantitation of deleted mtDNA per mtDNA molecule will be necessary to interpret the role of increased mtDNA deletions on premature aging in the exonuclease-deficient mice.

Although most studies have interrogated large populations of mtDNA in homogenized tissue, the measurement of individual cells may be the most illuminating to understand how mtDNA dynamics cause mitochondrial dysfunction. Cells in a tissue can be stained for loss of cytochrome oxidase (COX) activity, which directly measures mitochondrial function. In the duodenum (but not heart or brain), 20% of the cells in pol γ exonuclease-deficient heterozygous 15 month-old mice were negative for COX activity [116]. In all the COX-negative cells, the vast majority of mtDNA molecules contained a deleterious point mutation, but none contained deletions [116]. However, heterozygotes do not exhibit premature aging possibly because the number of mitochondrial-deficient cells is insufficient to elicit the phenotype. In contrast, there were many more COX-negative cells in brain, liver and duodenum in prematurely aging homozygous mutant mice [116]. The most important evidence to resolve this controversy is to determine the proportion of COX-negative cells with deleted mtDNA to COX-negative cells with mtDNA point mutations in the homozygous pol γ exonuclease-deficient mouse.

Conclusions and future directions

Three approaches have been utilized classically to understand and to diagnose mitochondrial disease due to mutations in pol γ. First, pol γ mutations have been identified in the clinic and comprehensively cataloged in an open-access database (http://tools.niehs.nih.gov/polg). Second, purification of recombinant human pol γ and standardization of enzyme kinetic measurements have permitted accurate measurements of polymerase activity. Finally, mutations in genetic model systems have provided in vivo measurements of mitochondrial dysfunction and mutagenesis. The use of each of these approaches will greatly expand in the future. There are now laboratories and companies devoted to sequencing mtDNA replication genes in mitochondrial patients to find novel mutations. The recently solved crystal structure of pol γ is becoming instrumental in explaining defects caused by the disease-causing mutations. The combination of all approaches will determine the replication efficiency and pol γ activity of disease-associated mutants. However, the field is only beginning to understand the process by which cells become devoid of mitochondrial function. How do pol γ mutations cause brain and liver damage in infants (with Alpers syndrome) and neuromuscular tissues in adults (with PEO)? What determines the percent of mutated mtDNA (heteroplasmy) in a cell? What selective forces drive mtDNA depletion or homoplastic mutations? Are these selective forces during embryonic development similar to those during adulthood? The role of mitochondrial turnover in removing mtDNA mutations are only beginning to be explored [121]. Next generation sequencing is being used to determine the causes for variability of heteroplasmic mutations in different tissues [122]. Uncovering the mechanisms by which pol γ-mediated mtDNA mutations and depletion are manifested in tissues is the next step in truly understanding causes for mtDNA-related diseases.

Acknowledgments We thank Margaret Humble for maintaining and updating the Pol Gamma mutation database (http://tools.niehs.nih.gov/polg) and for her help in generating Fig. 3 of this review. We thank Dr. Rachelle Bienstock for help with the structural images in this review. We also thank Drs. Matthew Young and Rajesh Kasiviswanathan for the comments and suggestions from their critical reading of this manuscript. This work was supported by the Intramural Research Program of the NIH, National Institute of Environmental Health Sciences (ES 065078).

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