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Human mitochondrial DNA replication machinery and disease

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Abstract

The human mitochondrial genome is replicated by DNA polymerase γ in concert with key components of the mitochondrial DNA (mtDNA) replication machinery. Defects in mtDNA replication or nucleotide metabolism cause deletions, point mutations, or depletion of mtDNA. The resulting loss of cellular respiration ultimately induces mitochondrial genetic diseases, including mtDNA depletion syndromes such as Alpers or early infantile hepatocerebral syndromes, and mtDNA deletion disorders such as progressive external ophthalmoplegia, ataxia-neuropathy, or mitochondrial neurogastrointestinal encephalomyopathy. Here we review the current literature regarding human mtDNA replication and heritable disorders caused by genetic changes of the *POLG, POLG2, Twinkle, RNASEH1, DNA2* and *MGME1* genes.

Key Words: *POLG*, *POLG2*, DNA polymerase γ, mitochondrial DNA replication, mitochondrial DNA depletion syndrome, Alpers syndrome, progressive external ophthalmoplegia, ataxia-neuropathy.

Introduction

Human mitochondrial DNA (mtDNA) occurs as a double stranded negatively supercoiled circular genome of 16,569 base pairs (bp) that encodes 37 genes required for energy production (Figure 1). Thirteen genes encode proteins required for the mitochondrial electron transport chain or oxidative phosphorylation (OXPHOS). The remaining 24 genes encode 22 transfer RNAs and 2 ribosomal RNAs required for synthesis of the 13-mitochondrial polypeptides. A cell can contain several thousand copies of mtDNA distributed within hundreds of individual mitochondria [1] or within an elaborate intracellular network of reticular mitochondria Several proteins associate with mtDNA at distinct nucleoid structures on the matrix-side of the inner membrane [2], and such protein-mtDNA nucleoids can be visualized as foci or puncta via immunocytochemistry or live-cell fluorescence microscopy [3,4].

Mitochondrial disorders can be caused by genetic defects in mtDNA or in nuclear genes that encode proteins that function within mitochondria [5]. A class of genes specifically linked to instability of mtDNA has emerged over the last fifteen years (Table 1). Disorders associated with multiple mtDNA deletions and point mutations comprise commonly known disorders such as progressive external ophthalmoplegia (PEO) and ataxia-neuropathy syndromes but also some very rare disorders of TCA cycle abnormalities [6]. MtDNA depletion syndromes (MDS) include early childhood disorders such as Alpers-Huttenlocher syndrome (AHS), hepatocerebral syndromes, myocerebrohepatopathy spectrum (MCHS), and fatal myopathies [7,8]. Mutations in genes required for nucleotide biosynthesis and mitochondrial homeostasis are also linked to MDS and deletion syndrome (Table 1), although a comprehensive review is beyond the scope of this paper. Here we review the known enzymes and proteins comprising the human mtDNA replication machinery and briefly discuss the current models of mtDNA replication. Attention is focused on mtDNA maintenance disorders associated with mutation of genes encoding components of the mtDNA replication and repair machinery: *POLG*, *POLG2*, *Twinkle, RNASEH1, DNA2*, and *MGME1* genes.

The mtDNA replisome

MtDNA is replicated and repaired by the mtDNA polymerase γ (pol γ). Human pol γ is a heterotrimer consisting of one 140-kDa catalytic subunit (p140 encoded by *POLG*) and a 110-kDa homodimeric processivity subunit (p55 accessory subunit encoded by *POLG2*), Figure 1 and Figure 2. The p140 catalytic subunit harbors active sites for 5'-3' DNA polymerase, 3'-5' exonuclease, and 5' dRP lyase activities [9,10]. The p55 imparts high processivity onto the holoenzyme by increasing the binding affinity to DNA [4,11]. The majority of intermolecular contacts occur between the C-terminal region of the 'proximal' p55 monomer (purple in Figure 2) and the AID subdomain (Accessory-Interacting Determinant subdomain that extends an an 'arm' around p55) of the p140 catalytic subunit [12-15]. Pol γ functions in conjunction with a number of additional replisome components including: 1) topoisomerase, 2) Twinkle mtDNA helicase, 3) mitochondrial RNA polymerase (mtRNAP), 4) RNaseH1, 5) mitochondrial single-stranded DNA-binding protein (mtSSB), and 6) mitochondrial DNA ligase III, (Figure 1). Other factors critical for maintenance of the mitochondrial genome include: the multifunctional mitochondrial transcription factor A (TFAM) with important roles in mtDNA replication and packaging, the RecB-type mitochondrial genome maintenance 5'-3' exonuclease 1 (MGME1), the RNA and DNA 5' flap endonuclease (FEN1), and the helicase/nuclease, DNA2 [16-18]. MGME1, FEN1, and DNA2 have all been implicated in the mtDNA base excision repair (BER) pathways [19]. Interestingly, DNA2 has also been demonstrated to stimulate pol γ activity and co-localizes with Twinkle in the mitochondrial nucleoid, suggesting an important role in the replisome [20,21]. Most all DNA polymerases start DNA synthesis by extension of an RNA primer that is synthesized by a primase. In mitochondria primase function is afforded by the mitochondrial RNA polymerase (mtRNAP) [22]. Recently the translesion DNA *pol*ymerase-primase, PrimPol, was identified in mitochondria isolated from a human embryonic kidney cell line [23]. Translesion DNA polymerases are specialized enzymes that pass through DNA

damage. However, PrimPol is likely required for mtDNA repair and not for mtDNA replication, as *PRIMPOL*$^{-/-}$ knockout mice are viable. Of note to human genetic disease, mutation of *PRIMPOL* is associated with the ocular disorder high myopia [24,25].

Overview of human mtDNA replication

Replication of animal cell mtDNA is complex and slow, taking approximately one hour to synthesize both daughter strands [26]. An asymmetric mode of replicating animal mtDNA daughter strands was proposed in the 1970s [27]. In this strand displacement model of mtDNA replication, two origins of replication direct the replisome to initiate continuous DNA synthesis but initiation is temporally regulated at these locations [26]. First, daughter heavy (H) strand synthesis is initiated at the H-strand origin of replication (O_H) located within the control region (Figure 1). The two mtDNA strands are named heavy and light (L) based on the ability to separate them on alkaline cesium chloride buoyant density gradients [28]. To initiate nascent H-strand synthesis pol γ must add nucleotides to the 3'-end of an existing RNA primer and in human mitochondria these RNA primers occur at very low frequency [26]. This low frequency implies that either primers are removed very quickly or another initiation mechanism takes place. Evidence supporting the role of human mtRNAP as the mtDNA primase comes from the identification of primers located adjacent to nascent displacement-loop (D-loop) H-strands isolated from human KB cell mitochondria [29] and from *in vitro* experiments demonstrating that mtRNAP has primase activity [22]. MtRNAP directs polycistronic transcription from H- and L-strand promoters located in the mtDNA control region (Figure 1). The 5'-end of RNA primers have been mapped to the L-strand promoter and, therefore, likely serve to initiate mtDNA replication at O_H [29]. Support for RNA priming of mtDNA synthesis comes from observations that replicating mtDNA obtained from mouse embryonic fibroblasts, and lacking RNase H1, retain unprocessed primers at replication [30].

According to the strand displacement model when H-strand synthesis is two-thirds of the way complete L-strand synthesis is initiated at O_L, the L-strand origin of replication. The template H-strand O_L sequence is predicted to adopt a stem-loop structure that is recognized by mtRNAP [31]. O_L-dependent initiation has been faithfully reconstituted *in vitro* and mtRNAP initiates primer synthesis from a poly-dT stretch located within the single-stranded region of the stem-loop [31]. Recent experiments utilizing mitochondria isolated from human HeLa cells demonstrated there are sufficient *in vivo* levels of mtSSB to cover the displaced parental H-strand during mtDNA replication and mtSSB specifically restricts the initiation of nascent L-strand synthesis to O_L [32]. Furthermore, exploiting immunoprecipitation and DNA sequencing, mtSSB was demonstrated to bind exclusively to the H-strand and there is a gradient of high to low mtSSB occupancy from immediately downstream of O_H in the control region towards O_L, in a clockwise direction, Figure 1 [32]. This observation supports the hypothesis that mtSSB stabilizes the H-strand when displaced during replication. Before termination of daughter strand replication, the two mtDNA must segregate to avoid catenation. A recent study of human breast cancer and osteosarcoma cell lines has determined that the type IIA topoisomerase, Top2□□ is the most prevalent human mitochondrial gyrase critical for decatenation of mtDNA circles during replication and relaxation of positive supercoils introduced during transcription and mtDNA replication [33].

Another model termed the bootlace model posits that processed RNA transcripts are “threaded” onto the displaced H-strand in a 3’-5’ direction and remain hybridized until they are displaced, degraded or further processed during the replication cycle [34]. Thus, the bootlace model suggests that formation of single-stranded sections of H-strand could be prevented. Advantages of mtDNA harboring an H-strand duplexed with mtRNA include: increased genomic stability due to the ability to repair single H-strand breaks annealed to RNA, protection of the H-strand from base damage, and providing the information for mtDNA repair, as pol γ is proficient in performing single-nucleotide reverse transcription [35].

Of the two models, recent evidence using ChIP-seq mapping of the mtSSB to the displaced loop and the retention of primers at the two origins in RNaseH1 deficient cells clearly points to the strand-displacement model as the more favored model of mtDNA replication.

Disorders of *POLG*, the catalytic subunit of the human mtDNA polymerase γ

In 2001, Van Goethem *et al.* published a seminal paper describing 4 mutations in the *POLG* gene associated with progressive external ophthalmoplegia (PEO) [36]. To date, there are nearly 300 pathogenic mutations in *POLG* (http://tools.niehs.nih.gov/polg/) [6,37-40], Figure 3. *POLG* disorders are very polymorphic in regard to the timing of presentation, organ-systems affected and overall symptoms. These disorders are currently defined by at least six major phenotypes of neurodegenerative disease that include: AHS, MCHS, myoclonic epilepsy myopathy sensory ataxia (MEMSA), the ataxia neuropathy spectrum (ANS), autosomal recessive PEO (arPEO), and autosomal dominant PEO (adPEO) [7,8,41,42]. Also, alteration of the $(CAG)_{10}$ repeat in the 2nd exon of *POLG* has been implicated in male infertility, testicular cancer, and Parkinsonism [8]. The *POLG* gene is unique in regard to the number of pathogenic mutations spread out over the gene and by the variety of diseases that they cause.

PEO is a mitochondrial disorder associated with mtDNA deletions and point mutations [36,43-45]. PEO is characterized by late onset (between 18 and 40 years of age) bilateral ptosis (sometimes initially unilateral), progressive weakening of the external eye muscle (ophthalmoparesis), proximal muscle weakness and wasting, and exercise intolerance. The disease is often accompanied by cataract, hypogonadism, dysphagia, hearing loss and may, within several years, lead to development of neuromuscular problems [43,46]. Neurological problems may include depression or avoidant personality [47]. Skeletal muscles of PEO patients present ragged red fibers and lowered activity of respiratory chain enzymes. AdPEO mutations in *POLG* are generally found in very conserved residues within the active site of the

p140 DNA polymerase domain [48], while recessive PEO mutations are spread throughout the gene.

Alpers syndrome typically occurs as an autosomal recessive mtDNA depletion disorder that affects children and young adults. It is a devastating disease characterized by psychomotor retardation, hepatic failure, and intractable seizures, as well as tissue-specific mtDNA depletion. Alpers patients rarely survive past 10 years of age.

In an attempt to understand the disease progression and severity, biochemical and genetic analysis of *POLG* mutations have provided a useful understanding of the defects as well as the ability to predict the recessive or dominant nature of mutations. Structures of the pol γ trimer (3.2 Å resolution), the pol γ-DNA complex bound to 2'-3'-dideoxycytidine triphosphate (3.3 Å), and the pol γ-DNA complex bound to deoxycytidine triphosphate (3.5 Å) have been solved by Yin and coworkers, Figure 2 [14,15]. These structures reveal asymmetric binding of the dimeric processivity subunit with the catalytic subunit, providing valuable insight into our understanding of the p140-p55 subunit interface. The catalytic subunit partially extends an 'arm', known as the AID subdomain around p55 (Figure 2). In the structures of the replication complexes, p55 rotates by 22° toward the p140 polymerase domain but does not alter the interface between p140 and the proximal p55 monomer. However, the distal p55 monomer becomes 16 Å closer, resulting in a substantial enhancement of the inter-subunit contacts between p140 and the distal monomer. Collectively these differences contribute to a dynamic p140-distal p55 interface that may permit greater regulation of the DNA polymerase and 3'-5' exonuclease functions, as compared to the subunit interface in the 3.2 Å pol γ structure. Analysis of the structure-function relationship of Alpers mutations has revealed that recessive mutations cluster within five distinct functional modules in the pol γ catalytic subunit [49]. This clustering can serve as a diagnostic tool to evaluate the consequence of new *POLG* mutations.

A study of unrelated families with two mutant *POLG* alleles reported that A467T is the most common *POLG* disease mutation [40]. G848S, W748S, and T251I-P587L mutations are the second, third, and fourth most common *POLG* disease alleles, respectively. A467T is commonly associated with Alpers, PEO, and ataxia-neuropathy. Biochemical studies of the A467T p140 variant demonstrated reduced template binding, lower processivity and ~4% activity [50,51]. Furthermore, this residue results in compromised p55 interaction [52]. The A467 residue is located in a hydrophobic center of the thumb subdomain and the T467 hydroxyl group substitution may interrupt the local hydrophobicity of this region as previously suggested [49].

With a single exception, all dominant *POLG* mutations that cause PEO map to the polymerase domain of pol γ. Three of the substitutions, H932Y, R943H and Y955C, change side chains that interact directly with the incoming dNTP [48,53]. These enzymes retain less than 1% of the WT polymerase activity and display a severe decrease in processivity [48], characteristics that likely cause the severe clinical presentation in heterozygotes. In addition, the Y955C substitution increases nucleotide misinsertion errors 10- to 100-fold in the absence of exonucleolytic proofreading [54], and the Y955C pol γ displays relaxed discrimination during incorporation of 8-oxo-dGTP or translesion synthesis opposite 8-oxo-dG [55]. A mouse transgenic model with the Y955C *POLG* allele targeted to the heart resulted in cardiomyopathy, loss of mtDNA, and enlarged hearts [56]. These experiments strongly suggest that large reductions in pol γ polymerase activity are sufficient to cause mitochondrial dysfunction that is central to *POLG*-related disease.

Disorders of *POLG2*, the mtDNA polymerase γ p55 processivity subunit

The first *POLG2* mutation described (c.1352G>A; p.G451E) was identified in a late onset PEO patient with multiple mtDNA deletions in muscle and ptosis [57]. Biochemical experiments

revealed that the G451E p55 homodimer completely failed to stimulate pol γ due to an inability to bind p140 [57,58]. The second case also involved a late onset adPEO patient with mtDNA deletions and harbored a c.1207-1208ins24 mutation, causing mis-splicing and skipping of exon 7, thus impairing the C-terminal domain required for enzyme processivity [38].

Seven more novel heterozygous mutations in *POLG2* were identified in a cohort of 112 patients suspected of *POLG* involvement but lacking *POLG* mutations [58]. Recombinant homodimeric proteins harboring these alterations were assessed for stimulation of processive DNA synthesis, binding to the catalytic subunit, binding to dsDNA and self-dimerization [58,59]. In this analysis, G103S, L153V, D386E and S423Y displayed wild-type behavior, while P205R and R369G had reduced stimulations of processivity. The L475DfsX2 variant was unable to bind the p140 catalytic subunit [58,59].

Because currently identified *POLG2* patients harbor heterozygous mutations, and because monomers within the p55 homodimer do not readily dissociate, the patients should harbor a mixture of p55 molecules: 25% WT homodimers, 25% variant homodimers, and 50% heterodimers [4]. Using a tandem affinity strategy and biochemistry to study p55 heterodimers we showed that one p55 disease variant, G451E, is dominant negative and associates with a wild-type p55 monomer in pol γ to poison the enzyme's activity. These results are in agreement with previous observations, that homodimeric G451E substitutions are located in critical regions of both monomers that interact with p140 [15] and that these substitutions result in decreased processivity due to compromised p55-p140 subunit interaction [57,58].

In contrast to the WT/G451E p55 heterodimer, L475DfsX2, P205R, and R369G p55 heterodimers maintain WT levels of processivity *in vitro*. However, the P205R and L475DfsX2 p55 disease variants failed to localize to mitochondrial nucleoids in vivo when tagged with GFP. Furthermore, homogenous preparations of P205R and L475DfsX2 formed aberrant reducible multimers *in vitro*. This suggests that abnormal protein folding or aggregation or both contribute

to the pathophysiology in patients harboring these mutations. Lastly, bioenergetics analysis in HEK293 cell lines stably expressing mutant p55 proteins utilizing the Seahorse Extracellular Flux Analyzer demonstrated significant decreases in reserve respiratory capacity [4]. We predict that the various defects associated with p55 disease variants ultimately result in diminished cellular energy reserves and by extension mitochondrial disease.

While the catalytic subunit has been shown to be essential for embryo development [60], genetic data regarding the processivity subunit has been lacking in mammalian systems. To address the role of *POLG2* in vertebrates we generated heterozygous (*Polg2*$^{+/-}$) and homozygous (*Polg2*$^{-/-}$) knockout (KO) mice [61]. *Polg2*$^{+/-}$ mice are haplosufficient and developed normally with no discernable difference in mitochondrial function through 2 years of age. In contrast, *Polg2*$^{-/-}$ mice were embryonic lethal at day 8.0-8.5 p.c. with concomitant loss of mtDNA and mtDNA gene products. This finding was similar to the *POLG* KO mouse [60]. Electron microscopy demonstrated severe ultra-structural defects and loss of organized cristae in mitochondria of the *Polg2*$^{-/-}$ embryos as well as an increase in lipid accumulation compared with both WT and *Polg2*$^{+/-}$ embryos. This data indicates that p55 and p140 function is essential for mammalian embryogenesis and mtDNA replication.

Disorders of *Twinkle*, the mtDNA helicase

The mitochondrial replicative helicase, referred as the Twinkle helicase, is encoded by the *Twinkle* gene (also known as *PEO1* or *C10orf2*) and was originally identified by Spelbrink and co-workers in 2001 [62]. Electron microscopy and small angle X-ray scattering were recently utilized to examine the structure of Twinkle and revealed it forms hexamers and heptamers of variable conformation [63]. Missense mutations in *Twinkle* co-segregate with mitochondrial disorders such as adult-onset PEO, hepatocerebral syndrome with mtDNA depletion syndrome, and infantile-onset spinocerebellar ataxia. Screening of *Twinkle* in individuals with adPEO, associated with multiple mtDNA deletions, identified 11 different

mutations that co-segregated with the disorder in 12 affected families [62]. At least 23 additional missense mutations in *Twinkle* associated diseases have been reported in adPEO [64,65]. Although mutations in *Twinkle* are mainly associated with adPEO, several reports have described recessive mutations as a cause of either epileptic encephalopathy with mtDNA depletion or infantile-onset spinocerebellar ataxia [66-68].

Expression of this protein in baculovirus, purification, and characterization has verified that Twinkle functions as a 5'-3' DNA helicase and its activity is stimulated by mtSSB [69]. Furthermore, when the core replisome components are combined in an *in vitro* reaction (containing pol γ p140 + pol γ p55, Twinkle, and mtSSB) the reconstituted system efficiently utilize dsDNA mini-circle templates to synthesize ssDNA molecules greater than 15,000 nucleotides in length, about the size of human mtDNA [70]. Overexpression of dominant disease variants of the mtDNA helicase in cultured human or Schneider cells results in stalled mtDNA replication or depletion of mtDNA [71-73], which emulates the disease state. Two of five adPEO mutants exhibited a dominant negative phenotype with mtDNA depletion in Schneider cells [72]. Disease mutations in the linker region were shown to disrupt protein hexamerization and abolish DNA helicase activity [74]. Four mutations in the N-terminal domain demonstrated a dramatic decrease in ATPase activity [75].

A comprehensive study of 20 recombinant disease variants overproduced and purified from *Escherichia coli* has reveled mild to moderate defects in helicase activity and ATP hydrolysis [37]. Utilizing optimized *in vitro* conditions some of the 20 variants also displayed partial reductions in DNA binding affinity and thermal stability. Such partial defects are consistent with the delayed presentation of mitochondrial diseases associated with mutation of the *Twinkle* gene.

A mouse model of *Twinkle* deficiency has been produced by transgenic expression of a *Twinkle* cDNA with an autosomal dominant mutation found in patients [76,77]. These mice developed progressive respiratory chain deficiency at 1 year of age in skeletal muscle,

cerebellar Pukinje cells, and hippocampal neurons. The affected cells accumulated multiple mtDNA deletions. These ‘Deletor’ mice recapitulates many of the symptoms associated with PEO and provides a useful model for further study.

Disorders of ***RNASEH1***

A recent study by Reyes *et al.* examined three families with recessive inheritance patterns consistent with affected individuals harboring causative homozygous or compound-heterozygous mutations [78]. Whole-exome sequencing revealed mutations in the *RNASEH1* gene. *RNASEH1* encodes the nuclear and mitochondrial isoforms of RNaseH1 endoribonuclease, which hydrolyze RNA strands in RNA-DNA hybrids containing a stretch of at least four ribonucleotides [79]. Two in-frame methionine codons are located at the 5’-end of the gene and translation from the first produces RNaseH1 harboring a MTS that localizes it to the mitochondria while the second targets RNaseH1 to the nucleus [79,80]. All of the mitochondrial disease-associated amino acid substitutions map within the RNaseH1 catalytic domain. Recombinant disease variants harboring these substitutions had significantly reduced endoribonuclease activity relative to WT RNaseH1. Two patients from two separate families were found to harbor compound-heterozygous mutations and four other affected siblings from a third family were found to harbor identical homozygous substitutions. All affected individuals presented with chronic PEO and exercise intolerance in their twenties. As the disorder progressed they also exhibited muscle weakness, dysphagia, impaired gait coordination, dysmetria and dysarthria. Muscle biopsies revealed impaired mitochondrial respiratory chain complexes as well as ragged-red and COX-negative fibers. Presumably, virtually all damage was mitochondrial genomic alterations in these patients (and in *RNASEH1* KO mice [80]) due to a compensatory function of nuclear RNaseH2, which is not found within the mitochondrion.

Disorders of ***DNA2*****, Dna2 Helicase/nuclease**

While mutations in *POLG* are the major cause of mtDNA-deletion, disorders diagnosis is typically only achieved in about half of the cases. In a cohort of patients suffering from childhood- and adult-onset mtDNA-deletion disorders, Ronchi and co-workers identified mutations in the gene encoding the mitochondrial helicase/nuclease *DNA2* [81]. Human Dna2 localizes to both the nucleus and to mitochondria and is required for mtDNA and nuclear DNA maintenance [21]. Dna2 participates in the mtDNA long-patch BER pathway (LP-BER) and the LP-BER machinery repairs small lesions such as those induced by oxidative damage. The four patients identified in this study harbored heterozygous *DNA2* mutations associated with hallmark mtDNA-deletion disease molecular and histochemical defects, mtDNA deletions and COX-negative muscle fibers respectively [81]. Recombinant forms of these Dna2 disease variants were determined to alter enzymatic nuclease, helicase, and ATPase activities and therefore, theoretically could compromise the LP-BER machinery *in vivo*.

Disorders of *MGME1*, MGME1 RecB-type exonuclease

Homozygous nonsense mutations in the *MGME1* gene were identified in several individuals with severe, recessive multi-systemic mitochondrial disorder from two families [17]. *MGME1* encodes a mitochondrial RecB-type exonuclease of the PD-(D/E)XK nuclease superfamily. Cellular fractionation indicated mitochondrial localization and protease-resistance for the native protein, and confocal microscopy convincingly demonstrated mitochondrial localization of a GFP-tagged recombinant form. Patient samples exhibited partial deletion and depletion of mtDNA, and the postulated direct involvement of MGME1 in the maintenance of mtDNA and turnover of prematurely terminated 7S DNA replication intermediates is quite compelling. Indeed, *MGME1* null patient fibroblasts depleted of mtDNA by continuous culturing in the presence of 2', 3'-dideoxycytidine (ddC) failed to repopulate their mtDNA upon release from ddC. The accumulation of mtDNA replication intermediates in HeLa cells subjected to *MGME1* siRNA was clearly demonstrated by 2D native agarose gel electrophoresis, further

supporting a role for MGME1 in maintenance of mtDNA replication *in vivo*. Preliminary qualitative characterization revealed the recombinant enzyme cleaves DNA but not RNA, requires a free 5'-end to a nucleic acid substrate, and prefers ssDNA over dsDNA *in vitro* [17].

Conclusions

Many unresolved issues exist in our understanding of mitochondrial syndromes. *POLG* disorders are especially polymorphic and the question remains as to why some organs and tissues affected in mitochondrial disease and not others? Does mtDNA mutation, deletion, and depletion play a role in tissue specific effects? What role do mtDNA polymorphisms play in mitochondrial disease? Do environmental toxicants influence these disorders? These questions are important areas for future research endeavors and will pave the way to understand disease pathophysiology and eventually to design therapies for treatment. It is clear that nuclear genes functioning in maintenance of mtDNA are commonly altered alleles in mitochondrial disease. Disorders of mtDNA stability are found in core proteins of mtDNA replication or in genes involved in supplying the mitochondrial nucleotide precursors needed for DNA replication (Table 1). With current next generation sequencing techniques, and our awareness of current disease causing mutations in these genes, the incidence of identified variants in mitochondrial patients will continue to increase with molecular screening. As an example, the number of individuals harboring a recessive pathogenic mutation in *POLG* has been estimated to approach 2% in the population [82]. However, the varied polymorphic nature of these diseases, as well as the age of presentation due to these gene mutations, stumps our understanding and challenges clinicians and researchers. Why do individuals with certain *POLG* mutations present early with a devastating disorder, while others with the same *POLG* mutations present much later in life? Continued *in vitro* biochemistry and model systems, such as yeast, tissue culture, and mice, are essential to understanding the consequence of these

mutations and to predict the *in vivo* consequences of newly identified mutations within these genes.

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Figure Legends

Figure 1. Map of the human mitochondrial genome and the mtDNA replication fork. The outer circle represents the 16,569 bp covalently closed circular double-stranded mtDNA. Counterclockwise from the top of the circle: Grey, control region including the heavy-strand origin of replication (O_H) and the displacement-loop (D-loop); Green; 12 and 16 S rRNA; Blue, NADH dehydrogenase (ND) 1 and 2; Red, cytochrome oxidase (COX) I and II; Yellow, ATPase 8 and 6; Red, COX III; Blue, ND 3, 4L, 4, 5, 6; Purple, cytochrome b. The D-loop form of mtDNA is a triple-stranded structure that results from the template-directed termination of H-strand synthesis soon after initiation resulting in mtDNA molecules with nascent H-strand annealed to them [83]. Recent evidence supports that the loading of the Twinkle helicase at the 3'-end of the D-loop is reversible, indicating that this site is critical to regulating the switch between formation of D-loop molecules and initiation of mtDNA replication [84]. Black rectangles represent the 22 tRNA genes. The inset illustrates the replisome at an area near the light-strand origin (O_L) of replication located within the WANCY cluster of genes, which encode for tryptophan, alanine, asparagine, cysteine, and tyrosine tRNAs. Black lines represent template mtDNA while green lines represent nascent mtDNA. Main factors highlighted at the replication fork include: *1)* the 5'-3' DNA polymerase pol γ *2)* the enzyme topoisomerase (Topo) required for mtDNA unwinding ahead of the replication fork. The phospodiester backbones of both mtDNA strands are enzymatically broken and rejoined allowing relaxation of positive supercoils introduced ahead of the replisome during replication fork elongation, *3)* the hexameric replicative Twinkle mtDNA helicase required for ATP-dependent disruption of the hydrogen bonds that hold the two DNA strands together causing mtDNA duplex denaturation (strand separation), *4)* mitochondrial RNA polymerase (mtRNAP) required for mitochondrial transcription as well as for RNA primer formation to initiate DNA replication, *5)* RNaseH1 required for RNA primer removal [31,70,85], *6)* mitochondrial single-stranded DNA (ssDNA)

binding protein (mtSSB) required for ssDNA stabilization during mtDNA replication, *7)* DNA ligase III (mtLigIII) required for mtDNA break (nick) sealing, *8)* mitochondrial transcription factor A (TFAM), *9)* mitochondrial genome maintenance 5'-3' exonuclease 1 (MGME1), *10)* flap endonuclease (FEN1), and *11)* the helicase/nuclease, DNA2.

Figure 2. DNA polymerase γ ternary structure. The p140 catalytic subunit consist of: 1) an amino terminal domain (NTD, light grey), 2) an exonuclease domain (*exo*, dark grey), 3) a spacer domain comprised of an intrinsic processivity (IP) subdomain (yellow) plus the accessory-interacting determinant (AID) subdomain (orange), and 4) a DNA polymerase (*pol*) domain, which folds to resemble a "right-hand" comprised of three subdomains: the thumb (green), fingers (dark blue), and palm (red). The p55 processivity subunit dimer is comprised of the proximal monomer (purple) and the distal protomer, light blue. The DNA primer strand is colored red while the template strand is colored pink. The figure was generated using UCSF Chimera and the published 3.3 Å crystal structure PDB ID 4ZTU; Szymanski *et al.* [15].

Figure 3. Schematic diagram of *POLG*, the human DNA polymerase γ catalytic subunit gene, and the linear sequence of the p140 amino acid residues. Amino acid substitutions encoded by *POLG* disease mutations are listed on the linear map and p140 domains and subdomains are color coded as in Figure 2.

Table 1. Nuclear genes identified in mitochondrial patients that affect mtDNA stability*

Gene	Disorder	Chromosomal locus	Function
mtDNA replication and repair			
POLG	PEO / Alpers / ataxia	15q25	Pol γ catalytic subunit
POLG2	PEO	17q	Pol γ processivity subunit
Twinkle (PEO1 or C10orf2)	PEO / ataxia	10q24	MtDNA helicase
RNASEH1	PEO / ataxia	2p25	Mitochondrial and nuclear RNaseH1 [78]
DNA2	PEO	10q21.3-22.1	Mitochondrial and nuclear helicase/nuclease [81]
MGME1	PEO, MtDNA depletion	20p11.23	RecB type exonuclease
Maintaining dNTP pools			
ANT1	PEO	4q35	Adenine nucleotide translocator
rTP	MNGIE	22q13.33	Thymidine phosphorylase
DGUOK	MtDNA depletion	2p13	Deoxyguanosine kinase
TK2	MtDNA depletion	16q22-23.1	Mitochondrial thymidine kinase
SUCLA2	MtDNA depletion	13q14.2	ATP-dependent Succinate-CoA ligase
SUCLG1	MtDNA depletion	2p11.2	GTP-dependent Succinate CoA ligase
RRM2B	MtDNA depletion	8q23.1	p53-Ribonucleotide reductase, small subunit
MPV17	MtDNA depletion and deletion	2p23.3	Mitochondrial inner membrane protein
ABAT	MtDNA depletion	16p13.2	4-aminobutyrate aminotransferase [86]
Mitochondrial homeostasis and dynamics			
OPA1	Dominant optic atrophy	3q29	Dynamin related GTPase
MFN2	Recessive optic atrophy	1p36.22	Mitofusin 2 [87]
FBXL4	MtDNA depletion, Encephalopathy	6q16.1-16.3	Mitochondrial LLR F-Box protein

*Additional references for genes listed in the table can be found in the text of this article and in reference [88].

Figure 1
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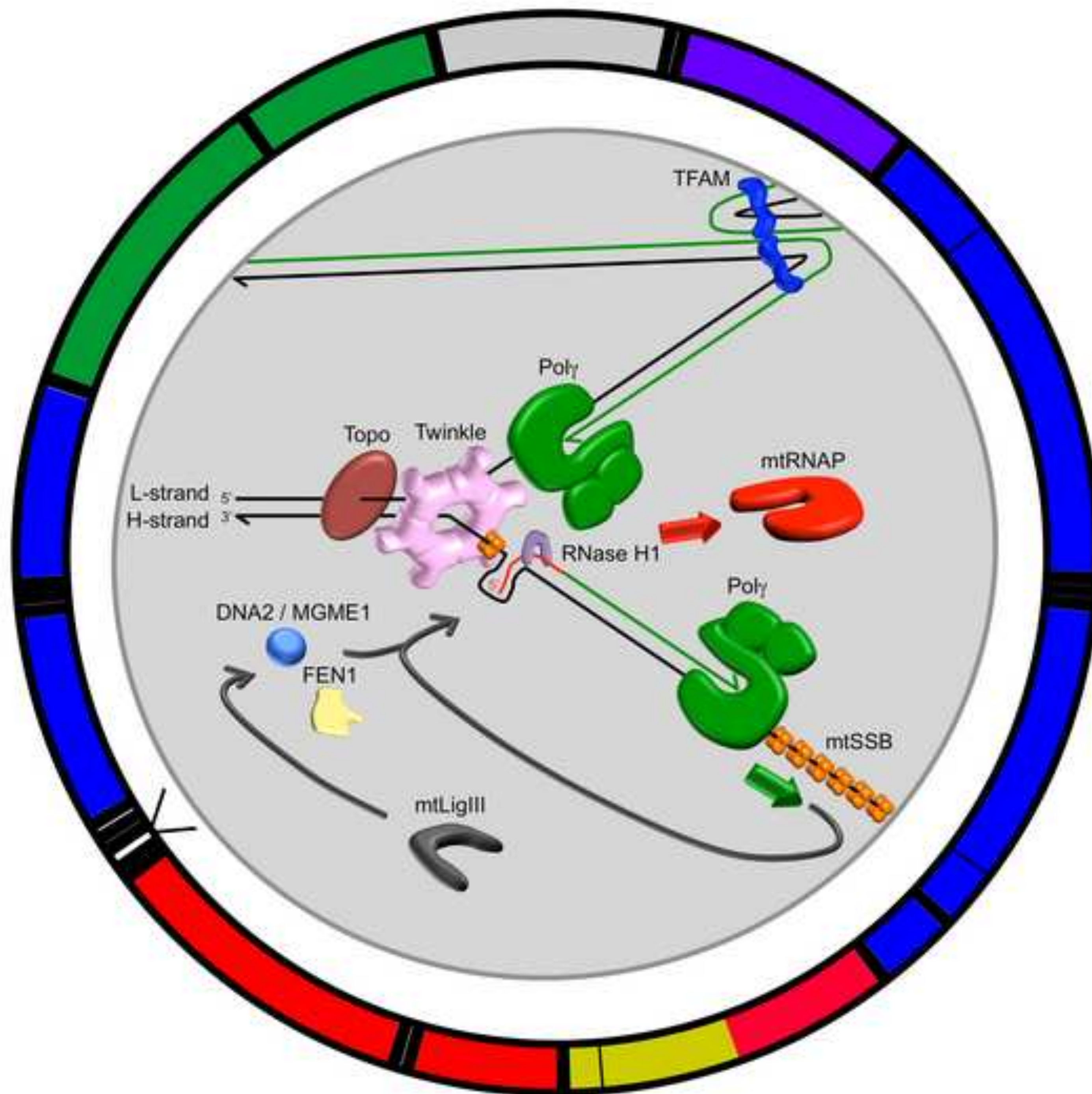

Figure 2
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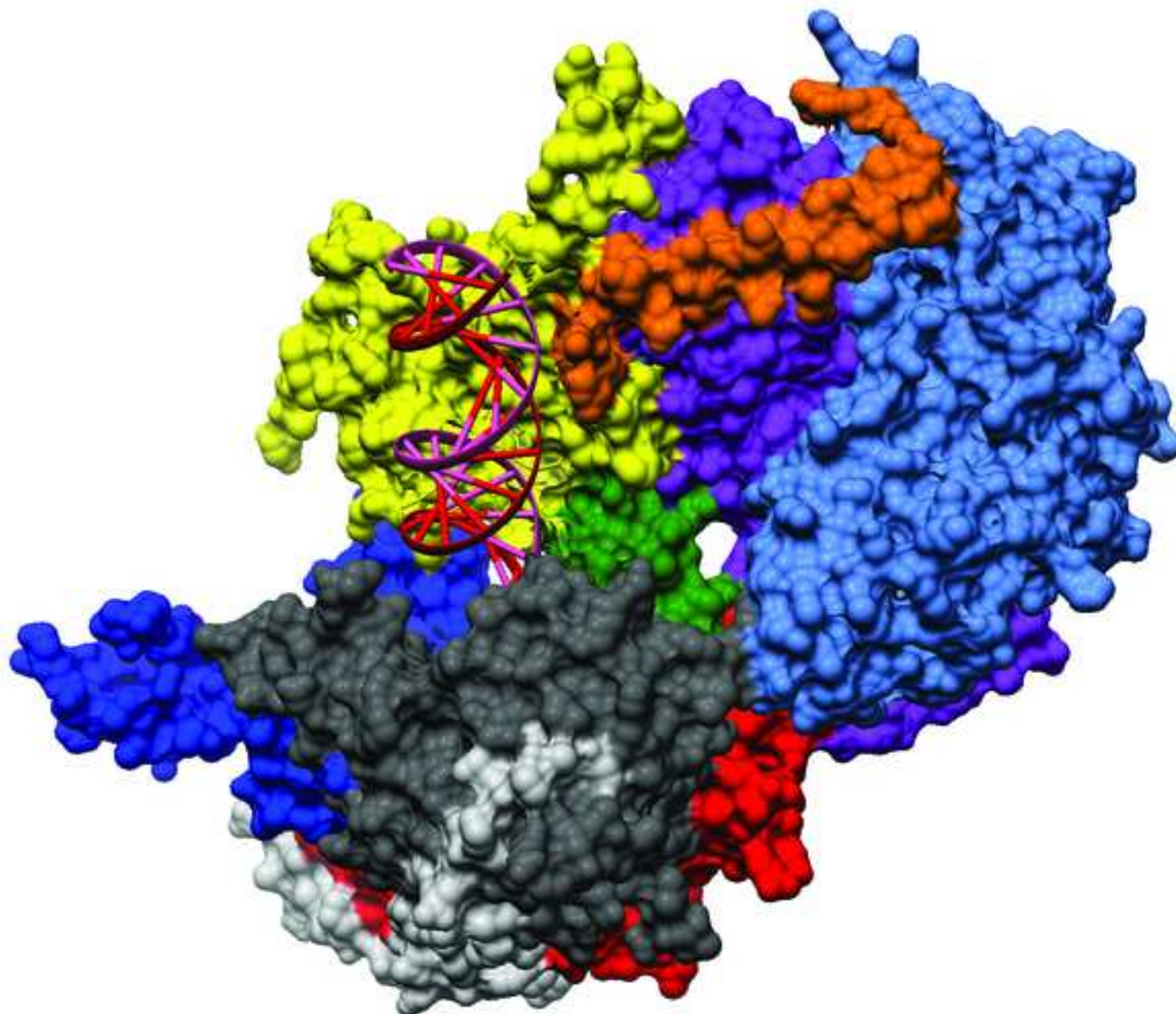

Figure 3

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