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Clinical and Molecular Features of *POLG*-Related Mitochondrial Disease

Jeffrey D. Stumpf[1], Russell P. Saneto[2], and William C. Copeland[1]

[1]Laboratory of Molecular Genetics, National Institute of Environmental Health Sciences, National Institutes of Health, Research Triangle Park, North Carolina 27709

[2]Division of Pediatric Neurology, Seattle Children's Hospital/University of Washington, Seattle, Washington 98105

Correspondence: copelan1@niehs.nih.gov

The inability to replicate mitochondrial genomes (mtDNA) by the mitochondrial DNA polymerase (pol γ) leads to a subset of mitochondrial diseases. Many mutations in *POLG*, the gene that encodes pol γ, have been associated with mitochondrial diseases such as myocerebrohepatopathy spectrum (MCHS) disorders, Alpers-Huttenlocher syndrome, myoclonic epilepsy myopathy sensory ataxia (MEMSA), ataxia neuropathy spectrum (ANS), and progressive external ophthalmoplegia (PEO). This chapter explores five important topics in *POLG*-related disease: (1) clinical symptoms that identify and distinguish *POLG*-related diseases, (2) molecular characterization of defects in polymerase activity by *POLG* disease variants, (3) the importance of holoenzyme formation in disease presentation, (4) the role of pol γ exonuclease activity and mutagenesis in disease and aging, and (5) novel approaches to therapy and avoidance of toxicity based on primary research in pol γ replication.

While many polymerases are responsible for replicating billions of nucleotides in the nucleus, DNA polymerase γ replicates thousands of copies of the 16-kilobase mitochondrial genome (mtDNA) in each human cell. The holoenzyme of DNA polymerase γ (pol γ) consists of a catalytic subunit (encoded by *POLG* at chromosomal locus 15q25) and a dimeric form of its accessory subunit (encoded by *POLG2* at chromosomal locus 17q24.1) (Longley et al. 1998b; Lim et al. 1999). MtDNA encodes 13 proteins that are essential for the electron transport chain that provides most of the ATP in the cell. Therefore, mtDNA replication is essential for life as demonstrated by the embryonic lethality of *POLG* knockout mice (Hance et al. 2005). Studies over the last decade have identified over 200 mutations in *POLG* that are associated with certain mitochondrial diseases (http://tools.niehs.nih.gov/polg/) (Longley et al. 2005: Copeland 2008; Longley et al. 2010; Walter et al. 2010; Stumpf and Copeland 2011; Tang et al. 2011). *POLG*-related disorders are currently defined by at least five major phenotypes of neurodegenerative disease that include: (1) Alpers-Huttenlocher syndrome (AHS), (2) childhood myocerebrohepatopathy spectrum (MCHS), (3) myoclonic epilepsy myopathy sensory ataxia (MEMSA),

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(4) the ataxia neuropathy spectrum (ANS), and (5) progressive external ophthalmoplegia (PEO) with or without sensory ataxic neuropathy and dysarthria (SANDO) (Wong et al. 2008; Cohen et al. 2010; Saneto and Naviaux 2010; Saneto et al. 2010). These diseases associate with mutations that have been shown to decrease the activity of the polymerase, the fidelity of replication, and the formation of the holoenzyme. This work reviews the important conclusions regarding *POLG*-related diseases and discusses some strategies for disease treatment options.

CLINICAL SYMPTOMS THAT IDENTIFY AND DISTINGUISH *POLG*-RELATED DISEASES

The clinical identification of mitochondrial diseases associated with *POLG* mutations is difficult because *POLG*-related diseases evolve over time and have an overlapping range of symptoms with multiple organ system involvement and with different degrees of severity and timing of onset. However, several important clues to the diagnosis include the tempo of disease progression, the presenting organ system, the combination of symptoms (but not necessarily the sequence of presentation or severity of organ involvement), and increasing involvement of neurological signs and symptoms during illness (especially viral infections producing fevers, or other physiological stressors) (Nguyen et al. 2006; Cohen et al. 2010). While no single test identifies *POLG*-related diseases, proper diagnosis depends on the age of presentation and associated biochemical and neuroimaging findings. By definition, *POLG* mutations are necessary for *POLG*-related disease, and identification of mutations known to facilitate disease (discussed later) is necessary for use as a diagnostic tool. The correct combination of laboratory testing and clinical observations (Table 1) identifies specific diseases and allows the monitoring of the course of the disease. Although the broad clinical expression of *POLG*-related diseases can make diagnoses difficult, there are phenotypic similarities and ages of onset that reflect distinct syndromes (Table 2) (Wong et al. 2008; Saneto and Naviaux 2010).

Table 1. Evaluation of patients with *POLG* disease[a]

Organ system	Test
CNS	EEG (with possible Video-EEG required for subtle seizures)
	MRI of the brain (T1/T2/FLAIR/Diffusion)
	MRS of the brain (TE 30 and either TE 130 (1.5 T) or TE 288 (3.0 T)
	Neurological examination
Vision	Formal visual exam (including eye movements)
	Electroretinogram and visual evoked potential if clinically indicated
Hearing	Brain stem auditory evoked potentials if clinically indicated
Cardiac	EKG (may also consider ECHO if clinically indicated)
Liver	Liver biopsy (for pathological evaluation and mitochondrial DNA content)
	Liver enzyme and function studies (ALT, AST, GGT, fasting glucose, albumin, prothrombin time and INR, ammonia, bilirubin, cholesterol)
Gastrointestinal	Gastric emptying and bowel motility if clinically indicated
	Swallowing study if clinically indicated
Respiratory	Pulmonary function testing
	Sleep polysomnography to evaluate hypercapnia during sleep
Systemic	Blood acylcarnitines, total and free carnitine, plasma amino acids

[a]Adapted from Haas et al. (2007, 2008) and Cohen and Naviaux (2010).

Even with a multitude of tests for mitochondrial disease, there are many examples where the results may lead to a difficult or improper diagnosis. For instance, extracting muscle to assay electron transport chain complex activity is useful in identifying mitochondrial cytopathy, but not necessarily *POLG*-related diseases (Gonzalez-Vioque et al. 2006; de Vries et al. 2007; Saneto et al. 2010). In addition, the use of mitochondrial DNA (mtDNA) depletion assays can be misleading for two reasons: normal mtDNA values would not exclude *POLG* disease, especially early in the course of the disease, and

Table 2. Major clinical syndromes associated with *POLG* mutations[a]

Age of onset	Syndrome	References
Neonatal/infancy	Myocerebrohepatopathy spectrum (MCHS)	Ferrari et al. 2005; de Vries et al. 2007
Infancy/childhood	Alpers-Huttenlocher syndrome (AHS)	Naviaux and Nguyen 2004
Adolescent/young adult	Ataxia neuropathy spectrum (ANS)[b]	Hakonen et al. 2005
	Myoclonus, epilepsy, myopathy, sensory ataxia (MEMSA)	Van Goethem et al. 2004
	Progressive external ophthalmoplegia (PEO)[c,d]	Van Goethem et al. 2001

[a]Table data adapted from Saneto and Naviaux (2010).

[b]ANS has also been labeled as mitochondrial recessive ataxia syndrome (MIRAS), sensory ataxic neuropathy with dysarthria and ophthalmoplegia (SANDO), and spino-cerebellar ataxia epilepsy syndrome (SCAE) (Tzoulis et al. 2006).

[c]PEO is labeled as PEO+ when associated with additional symptoms that may include peripheral neuropathy, ataxia, hearing loss, depression, premature menopause, hypogonadism, Parkinsonism, cataracts, or GI dysmotility.

[d]Dominant and recessive mutations in *POLG* have been associated with PEO.

mtDNA copy number is variable among different tissues (Dimmock et al. 2010). Finally, Van Goetham et al. (2003b) described several patients with mitochondrial neurogastrointestinal encephalopathy (MNGIE)-like syndrome, but without the cardinal finding of leukoencephalopathy as described in true MNGIE (Hirano et al. 1994). The MNGIE-like patients do not have deficiency in thymidine phosphorylase activity and would not respond to allogenic stem cell transplantation. Thus, proper treatment may be delayed or incorrectly given. To assist in the proper diagnosis of *POLG* related disease, the following section lists clinical features of the five most characterized *POLG*-related disorders.

Myocerebrohepatopathy Spectrum (MCHS) Disorders

MCHS disorders present in the first two to three years of life and result in early death. The clinical features of MCHS disorders include encephalopathy/developmental delay, liver dysfunction, myopathy, and hypotonia. Like other mitochondrial disorders, gastrointestinal (GI) dysmotility and failure to thrive occurs in MCHS disorders, albeit at earlier ages. Seizures may occur in these patients, but are not a necessary or dominant feature. Liver involvement can be the most dramatic feature with early failure leading to death. For instance, one compound heterozygous MCHS patient with G848S and R1096C mutations in *POLG* presented at 5 months of age with encephalopathy, hypotonia, and liver failure (RP Saneto, unpubl.). The biopsy showed that the liver pathology was distinct from what is commonly seen in another infantile onset *POLG*-related disease, Alpers-Huttenlocher (Nguyen et al. 2006). This patient died at 11 months of age because of liver failure.

Alpers-Huttenlocher Syndrome (AHS)

Also referred to as Alpers syndrome, AHS presents most frequently during infancy (similar to MCHS) and is fatal. Epilepsy is usually the predominant feature in AHS with an early predilection of epileptiform discharges over the occipital region of the brain, and generalized discharges as the disease progresses (Tulinius and Hagne 1991; Engelsen et al. 2008; Wolf et al. 2009; Saneto et al. 2010). Early seizures have the semiology of occipital lobe seizures with vomiting, headache, and myoclonus. Most patients have multiple episodes of epilepsia partialis continua and/or convulsive status epilepticus. Early in the course of epilepsy, seizures are often controlled with conventional medications. However, as the disease progresses, the seizures become increasingly resistant to valproic acid and other medications that trigger liver failure (Bicknese et al. 1992; Engelsen et al. 2008; Uusimaa et al. 2008; Wolf et al. 2009; Saneto et al. 2010). Even without

valproic acid dosing, liver failure occurs in AHS patients, and liver pathology cannot distinguish the samples that were treated with valproic acid (Harding 1990). Characteristic features of liver pathology in AHS require at least three of the following histological findings: (1) microvesicular steatosis, (2) bile ductular proliferation, (3) hepatocyte dropout, (4) bridging fibrosis or cirrhosis, (5) collapse of liver cell plates, (6) parenchymal disarray or disorganization of normal lobular architecture, (7) regenerative nodules, and (8) oncocytic change in scattered hepatocytes not affected by steatosis (Nguyen et al. 2006).

In addition to liver pathology, other common clinical features of AHS include headaches that are migrainous in nature, cortical blindness usually in fully developed AHS, and myoclonus often in the later phases of the disease (Nguyen et al. 2006; Cohen et al. 2010). Myoclonus may become so rapid that they become indistinguishable from myoclonic seizures. The onset and tempo of cognitive impairment is variable and often triggered by infectious illness.

Myoclonic Epilepsy Myopathy Sensory Ataxia (MEMSA)

MEMSA has the dominant features of epilepsy, myopathy, and ataxia, but without ophthalmoplegia. A subclinical sensory polyneuropathy leading to ataxia is usually the first sign of the disease. Usually seen later in the disease course, myoclonic seizures are often focal, frequently involving an arm, and can become generalized over time and comorbid with a progressive encephalopathy. Two features distinguish MEMSA from other similar diseases. First, the lack of ophthalmoplegia and prominence of myopathy sets these patients apart from other *POLG* syndromes. Second, histochemical assays of the muscle reveal the absence of ragged-red fibers distinguishing this syndrome from myoclonus epilepsy with ragged-red fibers (Van Goethem et al. 2003b).

Ataxia Neuropathy Spectrum (ANS)

ANS presents from early adolescence to the late third decade, and may be the most difficult to diagnose. While the predominant features of ANS are neuropathy and ataxia without myopathy, patients may also express progressive external ophthalmoplegia (Van Goethem et al. 2004). A wide variety of symptoms have been associated with ANS, including mild cognitive impairment, involuntary movements, psychiatric symptoms, myoclonus, blindness, hearing loss, and liver involvement (Van Goethem et al. 2004; Hakonen et al. 2005; Winterthun et al. 2005; Tzoulis et al. 2006). Commonly, ANS patients also present with white matter brain lesions as shown by MRI (Van Goethem et al. 2004).

Progressive External Ophthalmoplegia (PEO)

PEO (or PEO+, which indicates multiple organ involvement) is an adult onset disorder that occurs with dominant or recessive *POLG* mutations (Van Goethem et al. 2001; Lamantea et al. 2002). Although the major clinical finding is a progressive weakness of the extraocular eye muscles resulting in ptosis and loss of eye movements in the horizontal and vertical directions (Van Goethem et al. 2001), a generalized myopathy is present in most patients. Other symptoms of PEO+ include sensory ataxia, neuropathy, dysarthria, myopathy, and restless leg syndrome (Fadic et al. 1997; Van Goethem et al. 2001; Milone et al. 2008; Aitken et al. 2009; Blok et al. 2009). Cosegregating with PEO families are features of Parkinsonism (Luoma et al. 2004), premature ovarian failure, sensory ataxia (Pagnamenta et al. 2006), and cataracts (Luoma et al. 2004; Blok et al. 2009).

MOLECULAR CHARACTERIZATION OF DEFECTS IN POLYMERASE ACTIVITY BY *POLG* DISEASE VARIANTS

Identification of *POLG* mutations is the unifying characteristic of *POLG*-related disease. Ever since the landmark paper by Van Goethem et al. (2001) identified the cosegregation of PEO with *POLG* mutations, searches for other disease-associated mutations have resulted in an exponentially increasing number of mutations found in disease patients (http://tools.niehs.nih.gov/polg). Most mutations are found in compound heterozygotes containing multiple

mutations in *cis* and in *trans*. However, some mutations do not affect enzyme function in known biochemical assays or in yeast model systems. The consideration that as few as 43 mutations have been described in multiple publications describing nonrelated families raises doubts as to whether all of the hundreds of mutations that have only shown to cosegregate with disease in a single publication are in fact causative of disease. In fact, at least 32 mutations have been identified only in *cis* with another *POLG* mutation, and the relative contribution of each mutation on the same chromosome is difficult to interpret without further analysis. Although it is useful to have a comprehensive record of *POLG*-associated mutations, it is imperative to characterize mutations that are likely to cause disease by recording adequate genotypic family histories, determining mitochondrial dysfunction in model systems, or measuring deficiency of enzymatic activity (summarized in Table 3).

The catalytic subunit of pol γ is a 140-kD protein containing DNA polymerase, 3′-5′ exonuclease and 5′ dRP lyase activities (Gray and Wong 1992; Longley et al. 1998a,b). Defects in DNA polymerase activity have been detected in many *POLG* mutations studied, suggesting that the reduced polymerase activity facilitates disease progression. Although 5′ dRP lyase is an important step in mtDNA base excision repair, the critical residues for lyase activity are unknown, making it difficult to ascertain the importance of lyase activity in maintenance of mitochondrial genomes.

Mutations in *POLG* were first identified in 2001 as a disease locus for PEO (Van Goethem et al. 2001). With the exception of one mutation, all dominant *POLG* mutations responsible for developing PEO are mapped 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 (Graziewicz et al. 2004; Stumpf et al. 2010). These enzymes retain less than 1% of the wild-type polymerase activity and display a severe decrease in processivity (Graziewicz et al. 2004), characteristics that likely cause the severe clinical presentation in R943H and Y955C heterozygotes (Graziewicz et al. 2004). In addition, in vitro assays showed that the Y955C substitution also increases nucleotide misinsertion errors 10- to 100-fold in the absence of exonucleolytic proofreading (Ponamarev et al. 2002). In vivo evidence in yeast showed that alteration of the conserved tyrosine in the yeast mitochondrial polymerase gene, *mip1*, enhanced mtDNA damage, increased mutagenesis, and resulted in cells with dysfunctional mitochondria (Baruffini et al. 2006; Stuart et al. 2006). Similar to dominance seen in human patients, heterozygous yeast that alter the conserved tyrosine show a dominant increase in mitochondrial dysfunction, suggesting that the mutant enzymes compete with the wild-type polymerases for binding at the replication fork (Stuart et al. 2006). Additionally, a mouse transgenic model with the Y955C *POLG* allele targeted to the heart resulted in cardiomyopathy, loss of mtDNA, and enlarged hearts (Lewis et al. 2007). These models strongly suggest that large reductions in pol γ polymerase activity are sufficient to cause mitochondrial dysfunction that is central to *POLG*-related disease.

Other *POLG* disease-associated mutations have been assayed for polymerase activity to determine the potential to cause disease. Polymerase active site mutations G923D and A957S are found in PEO patients and have been shown to exhibit 21% and 23% polymerase activity, respectively (Graziewicz et al. 2004). Analysis of a cluster of Alpers mutations in the thumb domain has shown a striking correlation with the severity of the defect and the degree of conservation of amino acid sequences among various eukaryotes (Kasiviswanathan et al. 2009). Mutations in the most conserved sites represented by G848S, T851A, R852C, and R853Q exhibited less than 1% WT enzyme activity (Kasiviswanathan et al. 2009). Mutations in codons for less conserved amino acids (Gln879 and Thr885) resulted in only moderate reduction in activity (Kasiviswanathan et al. 2009). Haplotype analysis of the Finnish population demonstrates a carrier frequency of 1:125 for a common polymerase domain mutation, W748S (Hakonen et al. 2005). It has been shown that the W748S mutation caused the polymerase to have a low

Table 3. Genetic and biochemical evidence for *POLG* variants causing disease

Human mutation	Yeast mutation	Human disease	Biochemical data	Genetic data	References
L244P	L211P	Alpers		Mild increase in petite frequency, 6- to 10-fold increase in mutagenesis	Stumpf et al. 2010
G268A	G244A	PEO		2.5-Fold increase in petite frequency in yeast 10-Fold increase in point mutations (haploid yeast)	Baruffini et al. 2006
G303R	G259R	Alpers		100% Petite in haploid yeast	(Baruffini et al. 2011)
L304R	L260R	PEO		40–100% Petites in haploid yeast, 7.9% in diploid	Stumpf et al. 2010
S305R	C261R	Alpers, ANS		Petite frequency 40-fold higher than the C261S "humanized" wild-type	Baruffini et al. 2011
Q308H	Q264H	PEO		100% Petites in haploid yeast	Stumpf et al. 2010
R309 L	R265 L	PEO		35% Petites in haploid yeast	Stumpf et al. 2010
A467T	I416T	Alpers syndrome, ANS, PEO	Defect in accessory subunit binding 4% Catalytic activity because of low DNA binding, low k_{cat}, high K_m	Causes mtDNA and nuclear DNA damage in yeast, 9% petites as haploid yeast	Chan et al. 2005a; Luoma et al. 2005; Stuart et al. 2006
W748S	NA	Alpers syndrome, ANS, PEO	Defect in catalysis, DNA binding, and enzyme stability		Chan et al. 2006
R807C	R607C	SANDO		100% Petites in haploid yeast	Stumpf et al. 2010
R807P	R607P	PEO		67% Petites in haploid yeast and elevated mutation frequency	Stumpf et al. 2010
G848S	G651S	Alpers, PEO	<1% Polymerase activity, reduced gap-filling activity	46% Petites in haploid yeast and elevated mutation frequency	Baruffini et al. 2007; Kasiviswanathan et al. 2009; Stumpf et al. 2010
T851A	T654A	Alpers	<1% Polymerase activity	100% Petites in haploid yeast	Kasiviswanathan et al. 2009; Stumpf et al. 2010)
R852C	R655C	Alpers	<1% Polymerase activity		Kasiviswanathan et al. 2009; Stumpf et al. 2010
R853W	R656W	PEO with ptosis		100% Petites in haploid yeast	Stumpf et al. 2010

Continued

Table 3. *Continued*

Human mutation	Yeast mutation	Human disease	Biochemical data	Genetic data	References
R853Q	R656Q	Alpers	<1% Polymerase activity	100% Petites in haploid yeast	Kasiviswanathan et al. 2009; Stumpf et al. 2010
N864S	N667S	PEO		100% Petites in haploid yeast	Stumpf et al. 2010
E873X	E676	Alpers syndrome		Stop codon leads to nonsense-mediated decay of message	Chan et al. 2005b
Q879H	NA	Alpers	50% Polymerase activity		Kasiviswanathan et al. 2009
T885S	NA	Alpers	70% Polymerase activity		Kasiviswanathan et al. 2009
G888S	G691S	Alpers		Mild increase in petite frequency and mutagenesis	Stumpf et al. 2010
A889T	A692T	PEO	Reduced gap-filling activity	17% Petite frequency in haploid yeast	Stumpf et al. 2010
G923D	G725D	PEO	21% Polymerase activity	100% Petites in haploid yeast, only 7.6% in diploid, dominant in humans	Graziewicz et al. 2004; Stuart et al. 2006; Stumpf et al. 2010
D930N	D732N	Alpers		100% Petites in haploid yeast	Baruffini et al. 2011
H932Y	H734Y	PEO, ataxia	Reduced gap-filling activity	100% Petites in haploid yeast	Stumpf et al. 2010
R943H	R745H	PEO	0.2% Polymerase activity because of high K_m(dNTP) and lower k_{cat}	100% Petites in haploid yeast, 37% in diploid yeast, loss of mtDNA in yeast, dominant in humans	Graziewicz et al. 2004; Stuart et al. 2006; Stumpf et al. 2010
K947R	K749R	PEO		100% Petites in haploid yeast	Baruffini et al. 2011
Y955C	Y757C	PEO, 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), dominant in humans	Ponamarev et al. 2002; Graziewicz et al. 2004; Baruffini et al. 2006; Stuart et al. 2006
A957S	A759S	PEO	23% Polymerase activity	35% Petites in haploid yeast, only 2.5% in diploid, dominant in humans	Graziewicz et al. 2004; Stuart et al. 2006; Stumpf et al. 2010
A957P	A759P	Alpers		100% Petites in haploid yeast	Stumpf et al. 2010

Continued

Table 3. *Continued*

Human mutation	Yeast mutation	Human disease	Biochemical data	Genetic data	References
R964C	NA	NRTI toxicity	Reduced catalytic activity, low k_{cat}	Two- to threefold increase in petite frequency upon exposure to stavudine in haploid yeast	Bailey et al. 2009a; Baruffini and Lodi 2010
G1051R	G807R	PEO, ataxia	Slightly reduced gap-filling activity	Mild increase of petites in haploid yeast	Stumpf et al. 2010
P1073 L	P829 L	Alpers		35-Fold increase in petites in haploid yeast	Baruffini et al. 2011
G1076V	G832V	PEO		100% Petites in haploid yeast	Stumpf et al. 2010
R1096C	R853C	PEO, Alpers		100% Petites in haploid yeast	Stumpf et al. 2010
R1096H	R853H	Alpers		100% Petites in haploid yeast	Stumpf et al. 2010
S1104C	S861C	PEO		100% Petites in haploid yeast	Stumpf et al. 2010
V1106I	V863I	PEO		100% Petites in haploid yeast	Stumpf et al. 2010
E1143G	E900G	Polymorphism in 4% in population	1.4-Fold more active than WT	Temperature-sensitive mutant in yeast, high petite frequency at 36°C	Chan et al. 2006; Baruffini et al. 2007
D1184N	D941N	PEO		33% Increase in petites in haploid yeast and mild mutagenesis	Stumpf et al. 2010

NA, not applicable (because there is no homolog in yeast); ANS, ataxia neuropathy syndromes.

catalytic activity and a severe DNA-binding defect (Chan et al. 2006). The W748S mutation is often found in combination with E1143G in ataxia patients (Hakonen et al. 2005; Winterthun et al. 2005) and was originally identified as an SNP in 4% of the general population (Di Fonzo et al. 2003; Van Goethem et al. 2004). However, biochemical evidence shows that the E1143G substitution partially reduces the deleterious effects of the W748S mutation, suggesting that E1143G may modulate the effects of disease mutations in *POLG* (Chan et al. 2006).

Yeast has been a useful model system to determine the functionality of *POLG* mutants by utilizing changes in residues that are in conserved stretches of amino acids (Baruffini et al. 2006, 2007, 2011; Stuart et al. 2006; Stumpf et al. 2010). Most of the mutations in conserved regions are in the polymerase domain, and many are completely unable to retain mitochondrial function because of disruption of mtDNA replication. These include PEO/SANDO-associated mutations R807C, R807P, R853W, N864S, G923D, H932Y, K947R, G1076V, R1096C, S1104C, and V1106I, and Alpers-associated mutations G848S, T851A, R853Q, D930N, A957P, P1073A, and R1096H (Stuart et al. 2006; Stumpf et al. 2010; Baruffini et al. 2011). By measuring a base substitution in mtDNA that confers erythromycin resistance, it was shown that mtDNA mutant frequency is increased by some disease-associated mutations (Baruffini et al. 2006, 2007, 2011; Stumpf et al. 2010). However, biochemical evidence of several mutants showed that the incorporation of an incorrect nucleotide is not more kinetically favorable (Kasiviswanathan et al. 2009; Szczepanowska and Foury 2010). Therefore, it is still unclear what causes the increase in mutagenesis in these mutant strains. Finally, information from the yeast studies allowed the comparison of multiple substitutions of the same residue that were associated with different diseases (Stumpf et al. 2010). The mutations associated with Alpers were more severe than those associated with PEO, which agrees with the in vitro studies that suggest that the biochemical defects correlate with the severity and age of onset found in patients (Chan and Copeland 2009).

POLG LINKER REGION IS IMPORTANT FOR BINDING TO THE ACCESSORY SUBUNIT

The linker (also called spacer) region is between the polymerase and exonuclease domain and was named prior to biochemical characterizations of pol γ that determined its functions. Structural studies identified critical contacts between the catalytic subunit of the holoenzyme and the homodimeric accessory subunit (encoded by *POLG2*) that are required for processivity (Lee et al. 2009). In particular, Glu540 and a helix comprised of amino acids 540–558 are crucial to holoenzyme assembly (Lee et al. 2009; Lee et al. 2010a). Ala467 is also important for subunit interaction, and substitution to threonine (A467T) is a common *POLG* disease mutation. Not only is the A467T mutant enzyme compromised for its interaction with the accessory subunit, it also retains only 4% polymerase activity compared to WT enzyme (Chan et al. 2005a).

The A467T mutation is estimated to occur in 36% of all alleles associated with *POLG* disease. (Ferrari et al. 2005; Horvath et al. 2006; Nguyen et al. 2006; de Vries et al. 2007; Wong et al. 2008). In the general population, the frequency of the A467T mutation has been found to exist in between 0.2% to 1% of asymptomatic European populations (Van Goethem et al. 2001; Luoma et al. 2005; Winterthun et al. 2005; Horvath et al. 2006). Ataxic patients who are homozygous for the A467T mutation present with symptoms in their early to late teens (Van Goethem et al. 2004; Winterthun et al. 2005; Tzoulis et al. 2006); however, patients have often been reported to be compound heterozygous with A467T and at least one other *POLG* disease-associated mutation. Because of the severity of the A467T mutation on processivity and catalysis (Chan et al. 2005a), it is assumed that the other mutation determines the disease progression. For instance, Naviaux et al. (1999) reported an Alpers patient with reduced electron transport chain function, dicarboxylic aciduria, fulminant hepatic failure, refractory epilepsy, and lactic acidosis, which resulted in death at 42 months. Analysis of mtDNA and pol γ activity from skeletal muscle biopsy indicated a reduction of mitochondrial DNA content to 30% of wild-type

levels and no detectable pol γ activity (Naviaux et al. 1999). Later sequencing of the *POLG* gene revealed compound heterozygosity with an A467T substitution in *trans* with a G to T nonsense mutation that converts Glu873 (GAG) to a stop codon (Naviaux and Nguyen 2004). *POLG* mRNAs containing the Glu873 nonsense mutation are degraded from the pool of mRNAs by nonsense mediated decay resulting in mono-allelic expression of *POLG* containing only the A467T mutation (Chan et al. 2005b).

Other examples of clinical presentations of compound heterozygotes include SANDO patients with A467T in one *POLG* allele and either R3P, L304R, or R627W in the other (Van Goethem et al. 2003a). One patient with ataxia-myopathy syndrome was shown to have A467T in one *POLG* allele and R627Q mutation in *cis* with the Q1236H single nucleotide polymorphism in the other *POLG* allele (Luoma et al. 2005). Other patients with ataxia were found to be heterozygous with the A467T mutation on one allele and W748S in *cis* with the E1143G mutation in the other allele (Van Goethem et al. 2004).

Very little is known about other linker domain mutations. Information about one linker domain mutation has demonstrated the difficulty of assigning disease causation to mutations, despite biochemical assays, structural data, and model systems. The G517V substitution was identified in simple and compound heterozygotes in many different disease presentations including Leigh syndrome, neuropathy, myopathy, sideroblastic anemia, and Kearns Sayre syndrome (Sarzi et al. 2007; Wong et al. 2008). However, the G517V substitution in recombinant pol γ exhibits no major changes in catalysis, DNA binding, or binding to the accessory subunit (Kasiviswanathan and Copeland 2011). There are several possibilities to explain the contradiction between the association to mitochondrial disease and the biochemical data. It is possible that other pol γ activities that are not routinely assayed, such as dRP lyase activity, are important for mtDNA replication. Alternatively, the assays may require a less minimalistic replisome and substrate to dissect the difference in activity between the enzymes, suggesting the need for proper reconstitution of mtDNA replication in vitro. More importantly, it is clear that the preponderance of evidence of association between a particular mutation and disease is neither sufficient nor a logical argument to propose a causal relationship. In the absence of genetic or biochemical data, the proper characterization of disease-associated mutations requires adequate genotypic and clinical family history.

POL γ EXONUCLEASE ACTIVITY, DISEASE, AND MTDNA MUTAGENESIS

Identification of mutations associated with mitochondrial disease in the exonuclease domain resulted in an obvious and reasonable hypothesis: exonuclease domain mutations reduce the proofreading function of pol γ, resulting in an mtDNA mutator phenotype and ultimately leading to mitochondrial dysfunction and disease. Because specific mtDNA mutations at high enough cellular concentrations (or heteroplasmy) cause maternally inherited mitochondrial disease, general mtDNA mutagenesis caused by decreased proofreading activity could be a possible contributor to human disease.

Surprisingly, mutations that have been studied in the exonuclease domain, which are most conserved from humans to yeast, have not caused increases in mutagenesis in vivo or decreases in exonuclease activity in vitro (Baruffini et al. 2006; Stumpf et al. 2010; Szczepanowska and Foury 2010). In fact, kinetic data showed that disease-associated mutations increase exonuclease activity for both correct and mismatched primer-template termini (Lee et al. 2010b; Szczepanowska and Foury 2010). Because not all disease-associated mutations have been assayed for exonuclease activity, it is still possible that lack of proofreading can cause *POLG*-related diseases. However, proofreading activity may not factor into diseases that clinicians identify with *POLG* mutations. It is unknown if exonuclease-deficient pol γ variants are found in the unaffected population, or in people affected by noncanonical *POLG*-associated diseases. It is also formally possible that mutations that eliminate exonuclease function of pol γ are embryonic lethal. This seems un-

likely considering that mice with exonuclease-deficient pol γ are viable (Trifunovic et al. 2004; Kujoth et al. 2005), and exonuclease-deficient pol γ efficiently replicates DNA in vitro.

To test the potential effects of eliminating mitochondrial proofreading function on disease, several groups used mouse models that disrupted pol γ exonuclease activity. In the first mouse model that eliminated pol γ exonuclease activity, the pol γ variant was transgenically targeted to the heart, resulting in severe cardiomyopathy accompanied by mtDNA mutations and deletions (Zhang et al. 2000). Several years later, two independent groups created mice homozygous for mutations that disrupted exonuclease function (Trifunovic et al. 2004; Kujoth et al. 2005). These mice exhibited premature aging between 6 and 9 months, characterized by graying hair, loss of hair and hearing, curvature of the spine, enlarged hearts, and decreased body weight and bone density (Trifunovic et al. 2004; Kujoth et al. 2005). These observations have not only shown that exonuclease-deficient pol γ does not cause embryonic lethality, but also have created discussion about the role of mtDNA mutations in aging and mitochondrial disease.

The degree of increase of mtDNA mutagenesis in *POLG* exonuclease-deficient mice was originally unclear. The increase in mtDNA mutagenesis reported in the mutants (three- to eightfold) is similar to the accumulation of mutations detected in 2- to 3-year-old mice (three- to 11-fold) (Trifunovic et al. 2004, 2005; Kujoth et al. 2005; Vermulst et al. 2007). However, mutation frequencies in young, wild-type mice is at or below the limit of detection using the method of PCR cloning and sequencing, which introduces mutations at 1.3×10^{-4} mutations per base pair (Kujoth et al. 2005). This limitation was alleviated by an alternative method of quantifying mutation frequencies called the "random capture method," where the frequency of mutations that cause resistance to restriction endonuclease digestion is enriched, allowing more accurate estimations of mutation frequency (7.1×10^{-7} mutations per base pair in wild-type mice and 1.6×10^{-4} mutations per base pair in young heterozygotes) (Vermulst et al. 2007). Mutation frequency in homozygous mutant mice was confirmed using next-generation sequencing technology (Williams et al. 2010). The mutation frequency of heterozygotes, which were asymptomatic, was much higher than aged wild-type mice (5.4×10^{-6} mutations per base pair) (Vermulst et al. 2007). Therefore, the increase in mutation frequency that occurs in older mice is not sufficient to cause phenotypes associated with aging. However, it is still possible that the extremely high mutation rate that occurs in homozygous *POLG* exonuclease-deficient mice is sufficient to cause or expedite the aging process. Both the relationship of mtDNA mutagenesis to disease and causes of increased mutagenesis are critical topics that need further exploration.

In addition to detecting point mutations, the random capture assay detected a 90-fold increase in mtDNA deletions in homozygous *POLG* exonuclease-deficient mice as compared to age-matched wild-type or heterozygotes (Vermulst et al. 2008). While mtDNA deletions in wild-type and heterozygotes mostly occur among direct repeats of six or more nucleotides, mtDNA deletions in homozygous mutants occur independently of direct repeats (Vermulst et al. 2008). The mechanism for deletions between direct repeats is often attributed to strand-slippage, where a mispriming event occurs downstream of the correct target, a process that appears to be prevented by the proofreading exonuclease function of a polymerase. Interestingly, the lack of increase of deletions in the heterozygote suggests that the wild-type pol γ protects against deletions that are caused by the exonuclease-deficient variant, suggesting an interplay between separate domains of both enzymes similar to the idea of extrinsic proofreading (Nick McElhinny et al. 2006). The role of mtDNA deletions in aging is controversial because there is disagreement about the absolute number of mtDNA deletions in the mitochondria. Because the random capture assay only detects relative numbers of deletions among various samples, deletion quantification has depended on Southern blots and long-range PCR techniques that are helpful when the majority of molecules have a particular deletion (Edgar et al.

2009), but not when the size and distribution of deletions are expected to be random (Vermulst et al. 2009). Next-generation sequencing techniques detected the presence of deletions or tandem duplications only in the control region (the technique cannot distinguish between the two) in homozygous mutant mice (Williams et al. 2010). Other studies have found relatively abundant levels of linear nonreplicating molecules with large mtDNA deletions, probably because of pausing at the control and OriL regions during mtDNA replication (Bailey et al. 2009b; Ameur et al. 2011). However, amplification of large sections of single mtDNA molecules extracted from 2- to 3-year-old mice showed that only 0.07%–0.2% of molecules contained mtDNA deletions, arguing against the model that mtDNA deletions drive normal aging (Guo et al. 2010). Similar to the story of mtDNA mutations, mtDNA deletions may not contribute to normal aging, and their role in premature aging in mice with exonuclease-deficient pol γ is still unclear.

An important point that has confounded the study of mtDNA mutagenesis is the complicated measurement of mtDNA mutation rates, which in nuclear DNA studies are expressed as mutations per base pair per genome sequenced. To know the number of genomes sequenced, many approximations need to be made about the number and rate of genome replications per cell and mitochondrion, the rate of mitophagy (the targeted destruction of mitochondria) per cell, the number of mtDNA genomes that are destroyed during each mitophagy event, and whether mitophagy occurs more frequently with mutant genomes. These variables make all of the previous mutation frequencies difficult to interpret. For instance, if mtDNA deletions are preferentially biased for mitophagy, then the absolute number of deleted mtDNA molecules may underrepresent the true number of deletion events. Also, if mutant mtDNA tend to segregate and be amplified in particular cells, then mutant frequency may not reflect the number of cells with suboptimal ATP production. Mutation frequencies are normally determined in a population of cells; however, determining the mtDNA dynamics of a mutation requires measurements of individual cells. For instance, in heterozygous *POLG* exonuclease-deficient 15-month-old mice, 20% of cells in the duodenum were respiratory deficient as visualized by staining for loss of cytochrome oxidase (COX) activity (Vermulst et al. 2008). Even though the vast majority of mtDNA molecules contained a point mutation in these cells (but not deletions), the heterozygotes were asymptomatic presumably because there were enough cells with mitochondrial function. In the prematurely aging homozygous mutants, there were much higher percentages of COX-negative cells in the brain, liver, and duodenum (Vermulst et al. 2008). It is still undetermined whether the COX-negative cells in the homozygous mutants contain mostly mtDNA mutations or deletions, a finding that should resolve the controversy.

GENETIC AND ENVIRONMENTAL EFFECTORS OF MITOCHONDRIAL DYSFUNCTION: SUGGESTIONS FOR THERAPEUTIC APPROACHES?

Unfortunately, treatment of *POLG*-related diseases has been limited to symptom management and supportive care. The predominance of liver involvement in the younger child has led to liver transplantation in some patients, but the outcome has been poor and is not recommended for *POLG*-related disease because of neurological involvement (Tzoulis et al. 2006; Wong et al. 2008). Even though seizures in *POLG* patients can be resistant to anti-seizure medications, the use of valproic acid should be avoided, because of induction of liver failure (Horvath et al. 2006; Tzoulis et al. 2006; Chinnery and Zeviani 2008). With a paucity of options known to treat *POLG*-related mitochondrial diseases, it is important to use basic research to understand genetic and environmental changes that can suppress the molecular phenotype related to *POLG* mutations.

Targeting Oxidative Stress for Treatment of *POLG*-Related Disease

Therapies that target oxidative stress caused by dysfunctional mitochondria show promise.

Recently, an early open-label study has suggested that a novel drug, α-tocotrienol quinone (ATQ3), may have therapeutic value in treating mitochondrial disease (Shrader et al. 2011). This drug is a strong antioxidant, which is hypothesized to block free radicals and thereby prevent oxidative injury. While this medication is currently making its way through the clinical trial pathway, the true therapeutic value needs to be validated.

Studies using basic model systems may help understand the potential effectiveness of antioxidant therapy. A yeast study with homologous disease-associated mutations in the mitochondrial polymerase showed that high frequencies of mitochondrial dysfunction could be suppressed by treatment with the antioxidants MitoQ and dihydrolipoic acid (Baruffini et al. 2006, 2011). However, the effect was specific to certain mutations for reasons that still need to be explored. In addition, the transgenic mouse model with the Y955C *POLG* allele targeted to the heart developed cardiomyopathy, loss of mtDNA, enlarged heart, and increased levels of 8-oxo-dG in its mtDNA, suggesting a role for oxidative damage in disrupting mtDNA replication and a benefit from antioxidant therapy (Lewis et al. 2007). Further support for the potential of antioxidant therapy is demonstrated by the attenuation of age-dependent cardiomyopathy in mice with mutations that inactivate pol γ exonuclease function by overexpression of catalase, which reduces oxidative damage (Dai et al. 2010).

Availability of Nucleotides Modifies Mitochondrial Dysfunction

Mitochondrial toxicity is a common and dangerous side effect of HIV patients treated with nucleotide reverse transcription inhibitors (NRTIs). While the NRTIs were designed to inhibit HIV reverse transcriptase and not cellular polymerases, NRTI incorporation inhibits pol γ replication (Kakuda 2000), and may cause mitochondrial toxicity in some cases. In support of this hypothesis, a *POLG* disease-associated mutation changing Arg964 to cysteine (R964C) was suggested to facilitate stavudine-induced toxicity (Yamanaka et al. 2007). Biochemical characterization of the R964C variant demonstrated a threefold decrease in discrimination against stavudine (Bailey et al. 2009a). A yeast system that allowed stavudine uptake showed a very mild increase in frequency of mitochondrial dysfunction in the R964C yeast homolog (Baruffini and Lodi 2010). More advanced experimental models will be required to determine whether this decrease in stavudine discrimination is sufficient to cause mitochondrial toxicity.

Nucleotide concentrations may be crucial in proper mtDNA replication as evidenced by the disease-associated mutations in the small subunit of ribonucleotide reductase that catalyzes the limiting step of dNTP production (Bourdon et al. 2007). Several disease-associated mutations show reduced binding affinities for nucleotides and would be sensitive to low nucleotide concentrations. One such example is the H932Y variant, which is able to incorporate nucleotides at near normal rates when the nucleotide concentration is raised 100-fold over the concentration needed for the wild-type enzyme (Stumpf et al. 2010). The increased frequency of mitochondrial dysfunction by the homologous mutation in yeast is suppressed by overexpressing ribonucleotide reductase, which increases nucleotide concentration (Stumpf et al. 2010). The suppression of disease-associated mutations by ribonucleotide reductase overexpression occurs for other nucleotide-binding defective mutants (Lecrenier and Foury 1995; Baruffini et al. 2006), suggesting a mechanism that could be targeted for mitochondrial therapies.

Exercise Attenuates Premature Aging in *POLG* Mutant Mice

A recent provocative study showed that premature aging in the *POLG* exonuclease-deficient mice can be at least delayed for several months by endurance exercise (Safdar et al. 2011). Not only are the mice that undergo exercise endurance phenotypically indistinguishable from wild-type, they also have similar frequency of mutant mtDNA and COX activity as wild-type. Interestingly, they exhibit about 50% higher mtDNA copy number. However, the mechanism for attenuating premature aging is unclear. It has been suggested that exercise promotes

mitochondrial biogenesis via PGC-1α facilitating a process that either dilutes mutant mtDNA or targets dysfunctional mtDNA for destruction (Safdar et al. 2011). According to this model, mitochondrial dynamics caused by fusion and fission is sufficient to offset the mutation load that is created by the lack of exonuclease activity. It is interesting to note that endurance exercise can only be undertaken by a fully developed mouse after many mitochondrial divisions and presumably the majority of mtDNA mutations have occurred. This suggests that the suppressive effects of endurance exercise may occur even if training starts later in life. Also, it would be interesting to know how temporary the effect of endurance training is on the accumulation of mtDNA mutations. This work should inspire future studies on the therapeutic effects of exercise for *POLG*-related diseases.

CONCLUSIONS

From the initial discovery of the first *POLG* disease-associated mutation a decade ago, research on *POLG* and mtDNA replication has spawned a wealth of information. Clinical reports have vastly improved and broadened the diagnoses and documented the mutations in many *POLG*-related diseases. The search for the polymerase defects in disease-associated mutants has resulted in the biochemical, genetic, and crystallographic dissection of the important activities of the polymerase. The development of the mitochondrial "mutator" mouse has inspired many labs to understand the role of mitochondrial mutations in pathology. Finally, the use of these tools allows more mechanistic approaches to therapeutic strategies. These developments in conjunction with new technology will hopefully provide new research tools that are needed to fully understand some of the mysteries of *POLG*-related diseases. For instance, improved sequencing technologies will allow broader studies of the entire spectrum of mutations in nuclear genes that affect mtDNA replication in disease patients. Also, reconstitution of mtDNA replication from tissue extracts and recombinant proteins will provide improved methods for determining mutations and environmental toxicants that cause mtDNA depletion. The better understanding of mtDNA dynamics will help determine how cells deal with mtDNA depletion and mutagenesis during the course of the disease. Finally, appropriate model systems will be developed to understand tissue specificity in mitochondrial diseases. This information will be crucial for improving both diagnosis and treatment of *POLG*-related diseases.

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Mitochondrial Evolution

Michael W. Gray