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(RESEARCH ARTICLE)

Polymerase and Proofreading Exonuclease Domains in the DNA Polymerase γ and Nuclear-encoded RNA Polymerase of the Human Mitochondria and Identification of Mitochondrial Disease Mutations in the Polymerase Active Site Region of DNA Polymerase γ

Peramachi Palanivelu *

Department of Molecular Microbiology, School of Biotechnology, Madurai Kamaraj University, Madurai – 625 021, India.

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Abstract

Recent reports have made it clear that mutations in the mitochondrial DNA (mtDNA) polymerase γ (POLG1) are a major cause of many human diseases. Mutations in the mtDNA polymerase leads to defective oxidative phosphorylation and ATP production, resulting in many mitochondrial diseases. For their mitochondrial genome replication, humans and animals use POLG1, whose catalytic site is essentially similar to the *E. coli* DNA polymerase I (DNA pol I). Multiple sequence alignment (MSA) analysis have shown that the POLG1 and *E. coli* DNA pol I use identical amino acids in their polymerase catalytic sites, viz. $^{-}{}_{943}$R^{-4}EHAKI^{1}FNYGRI$_{955}$Y^{8}G- (human DNA pol γ) as –R^{-4}RSAKA^{1}INFGLIY^{8}G- (*E. coli* DNA pol I). However, the human POLG1 shows only 31.25% identity with the *E. coli* DNA pol I, suggesting a highly divergent evolution. Mutation(s) in the POLG1 gene is one of the most common causes of many inherited mitochondrial diseases in children and adults. Depending on their location within the enzyme, mutations either lead to mtDNA depletion or accumulation of multiple mtDNA deletions leading to various mitochondrial diseases. The most common POLG1 dominant mutation, viz. Y955→H/C, which lead to a severe, early-onset of multi-systemic mitochondrial disease with bilateral sensorineural hearing loss, cataract, myopathy, and liver failure is located in the template-binding pair of the polymerase catalytic site region by MSA. Another dominant mutation, R943→H/C is observed in patients with Progressive External Ophthalmoplegia (adPEO, an autosomal, dominant, heritable mitochondrial disorder) is located in the nucleoside triphosphate (NTP) selection amino acid in the polymerase catalytic site region. The nuclear-encoded RNA polymerase (NEP) is imported from the nucleus and involves in the transcription of all the mitochondrial genes. The human mitochondrial NEP showed 39.30%, 40.12% and 26.98% identities to the NEPs of the mitochondria and chloroplasts of *Arabidopsis thaliana*, and T7 RNA polymerase, respectively, suggesting that the human and plant mitochondrial NEPs are distinctly different. Interestingly, the human NEP's catalytic core is almost completely conserved in the plant mitochondrial and chloroplast NEPs, viz. –R^{-4}KVVKQ^{1}TVMTVVY^{8}G- (Human) and –R^{-4}KLVKQ^{1}TVMTSVY^{8}G- (*A. Thaliana*). Furthermore, the mitochondrial NEP's catalytic core from human and different animal sources is remarkably conserved and is in close agreement with other NEPs of plant sources. Both the human DNA pol γ and NEP possess a typical template-binding pair (-YG-), a basic catalytic amino acid (K) to initiate catalysis and a basic nucleotide selection amino acid R at -4 from the catalytic K. The PR exonucleases of POLG1 and NEP belong to the DEDD-superfamily of exonucleases and uses a Y or H as the proton acceptor, respectively.

Keywords: Human mitochondria; Human mitochondrial diseases; DNA Polymerase γ; Nuclear-encoded RNA polymerase; Polymerase active sites; Proofreading exonucleases; Proofreading exonuclease active sites

* Corresponding author: Peramachi Palanivelu

1. Introduction

Mitochondria are found in all eukaryotes and play a crucial role in generating the much needed biological energy for the cells in the form of adenosine triphosphates (ATPs). They are semi-autonomous organelles, partly controlled by their own genome and mostly by the nuclear imports. They are double-membrane structures and harbour both the mitochondrial genome (mtDNA), which are double-stranded, circular molecules and mitochondrial plasmids. Interestingly, it is the only organelle in animals other than the nucleus, with its own DNA. Not only the mitochondrial sizes vary from 0.5 to 4 μm, but also their copy numbers and shapes vary considerably in different cell types and under different physiological conditions. Generally, about dozens of mtDNA copies are found in a single mitochondrion. As a single eukaryotic cell harbours 100s of mitochondria, the mtDNAs usually exceed > 1000 copies per cell.

The main function of mitochondria is to generate energy for cellular activities in the form of ATPs via oxidative phosphorylation (OXPHOS) by the process known as aerobic respiration and hence, they are known as the 'power house' of the cells. In addition to ATP generation, mitochondria also perform a variety of other cellular functions like forming Fe-S clusters, haem and amino acids, and Fe^{2+}/Ca^{2+} handling, regulation of signalling pathways through the release of cytochrome *c*, immune responses, inflammation, etc. Mitochondrial reactive oxygen species (mROS), generated during OXPHOS, affects the redox state of cells and has emerged as signalling molecules to communicate between cells [1]. Nemoto et al., [2] have shown that the mROS not only regulate cellular metabolism, but also is involved in tumour necrosis factor receptor signalling. Thus, the mitochondrial functions extend beyond the boundaries of the cells and influence an organism's physiology by regulating communication between cells and tissues [3]. Mitochondria also contribute to various cellular stress responses such as autophagy and apoptosis. Therefore, the mitochondrial functions are central to the physiology of humans and, consequently, ''mitochondrial dysfunction'' and has been implicated in a wide range of human diseases. In humans and most multicellular organisms, the mitochondrial mode of inheritance is strictly maternal, i.e., the mtDNA is inherited from the mother as the sperm mtDNA is actively degraded immediately after fertilization [4, 5].

1.1. mtDNA

mtDNA is organized into discrete nucleoid complexes in the mitochondrial matrix with tightly associated proteins responsible for its replication and maintenance, but with no histone(s). They are partly controlled by its own genome and mostly by nuclear imports. Replication of mitochondrial genome is independent of cell-cycle with individual mtDNA molecules being randomly selected for replication, a phenomenon referred to as relaxed replication. Human and animal mtDNAs are compact in structure and typically lack introns in all of their genes, except in 2 tRNAs (introns of ~1 kb are located between the $tRNA^{Phe}$ and $tRNA^{Pro}$ genes), whereas introns are very common in nuclear DNAs. Human mtDNA is a 16,569-base pair closed-circular molecule, encoding 13 polypeptides required for OXPHOS and 24 non-coding genes, which includes specialized tRNAs and rRNAs (12S and 16S), needed for translation within the organelle [6]. These genes are encoded asymmetrically between the two mtDNA strands, denoted heavy and light. The heavy strand encodes 2 rRNAs, 14 tRNAs and 12 mRNAs and the light strand encodes 8 tRNAs and 1 mRNA. All 13 polypeptides are in the mitochondrial respiratory chain complex (OXPHOS) and the remaining > 67 OXPHOS subunits are nuclear-encoded. In fact, a vast majority of the proteins present in the mammalian mitochondria (~1500 different types) are encoded by nuclear DNA, but the genes for some, if not most of them, are thought to have originally been of bacterial origin, and have been transferred to the nucleus during evolution. Mitochondria also exhibit heteroplasmy, i.e., the presence of different mtDNA sequences (both wild-type and mutant ones) within the same organelle in a cell. Mutations in mtDNA cause a wide variety of neurological, muscular, and tissue degenerative diseases which as many as1/2000 persons are at a life-time risk [7, 8].

1.2. Mitochondrial Diseases

Mitochondrial diseases are a group of genetic disorders that are characterized by mutations in genes in the nuclear DNA (nDNA) and/or mtDNA that encode proteins involved in mitochondrial functions. Mitochondrial diseases include: Mitochondrial myopathy, Neuropathy, Diabetes mellitus and Deafness (DAD) (this combination at an early age can be due to mitochondrial disease), Leber's Hereditary Optic Neuropathy (LHON), Leigh syndrome (subacute necrotizing encephalomyelopathy), Dementia, Neuropathy Ataxia Retinitis Pigmentosa and Ptosis (NARPP), Myoneurogenic Gastrointestinal Encephalopathy (MNGIE), progressive Myoclonic Epilepsy and "Ragged Red Fibres" (MERRF) syndrome, Mitochondrial Encephalopathy Lactic Acidosis and Stroke-like episodes (MELAS) syndrome, Mitochondrial DNA depletion syndrome (MDS) or Alper's disease. (Alpers' disease and other *POLG1* gene-related disorders are part of a larger family of mitochondrial DNA depletion disorders. Homozygous or compound heterozygous mutations cause severe deficiency of mitochondrial DNA pol γ, leading to depletion of mtDNA which results in mitochondrial dysfunction). Patients with *POLG1* mutations present PEO, a disease characterized by weakness of the ocular muscles and myopathy secondary to the depletion of mitochondria. Ataxia neuropathy spectrum (ANS) is a group of POLG1-

related disorders in which patients have difficulties with coordination along with nerve dysfunction. Alpers-Huttenlocher syndrome (AHS), which affects children is the classical form of hepatocerebral MDS, and it has been again attributed to mutations in *POLG1.* Mutations in mitochondrial tRNAs can be responsible for severe diseases like MELAS and MERRF syndromes [9]. Mutations of mtDNA can also lead to a number of other illnesses like exercise intolerance and Kearns–Sayre syndrome (KSS), which causes a person to lose full function of heart, eye, and muscle movements.

1.2.1. mtDNA-associated Diseases

Mitochondrial dysfunction is associated with a large number of common diseases, such as neurodegenerative disorders like Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD), bipolar disorder, schizophrenia, and cardiomyopathies, metabolic syndrome, obesity, diabetes, aging and senescence, anxiety disorders, sarcopenia (gradual loss of muscle mass, strength and function in elderly), chronic fatigue syndrome, etc. [3 and reference therein]. Several lines of evidence indicate that dysregulation of mitochondrial function plays an important role in cancer biology also [10, 11]. For example, data available from the International Cancer Genome Consortium (ICGC) and The Cancer Genome Atlas (TCGA), have demonstrated that ~60% of all solid tumours bear at least one mtDNA mutation. Therefore, it is clear that mtDNA mutations are of a very common occurrence across all solid tumours [12]. Moreover, large-scale sequencing efforts and clinical studies have confirmed the prevalence of mutations in mtDNA in human tumours and their potential roles in cancer progression. More specifically, mutations in the *POLG1* gene have also been reported in breast cancers [13]. Some evidence also suggests that mtDNA mutations might be a major contributor to the aging process and age-associated pathologies [14].

1.2.2. mtDNA Damage and Neurodegenerative Diseases

Point mutations, deletions, duplication or sometimes loss of entire mtDNA are implicated as the causes of aging and several neurodegenerative diseases (caused by the progressive loss of structure or function of neurons). AD, a most common form of dementia, is a brain disorder that slowly destroys memory and thinking skills, and eventually, interferes even with daily tasks. It is found that the brains of individuals with AD have elevated levels of oxidative DNA damage in both nDNA and mtDNA, but the mtDNA has approximately 10-fold higher levels of oxidative DNA damage than nDNA. This is because the mtDNA is associated with the mitochondrial inner membrane which is the site of oxidative phosphorylation and generation of large amount of mROS and H_2O_2. In AD, it has been proposed that aged mitochondria are the critical factor in the origin of neurodegeneration. Analysis of the brains of AD patients suggested an impaired function of the mtDNA repair pathway, which would cause reduced overall quality of their mtDNA [15].

HD is an autosomal, dominant genetic disorder. In HD patients, the mutant Huntingtin protein causes mitochondrial dysfunction by inhibition of mitochondrial electron transport and increased levels of mROS, leading to oxidative stress. Thus, the mutant Huntingtin protein promotes oxidative damage to mtDNA, as well as nDNA, that may contribute to the Huntington's disease pathology [16].

PD is a chronic neurologic condition named after Dr. James Parkinson, who first described the syndrome in 1817. PD is a slowly progressive disease, which causes a gradual loss of the nerve cells in the brain that produce the neurotransmitter dopamine. Dopamine carries signals to the part of the brain that control movement and coordination. Therefore, decreased dopamine levels leads to unintended or uncontrollable movements, such as shaking, stiffness, and difficulty with balancing and coordination. PD symptoms usually begin gradually and worsen over time. Again, mtDNA damage is also implicated in PD. To investigate mtDNA damage as a potential blood-based marker for PD, Qi et al., [17] have recently developed a PCR-based assay which allows accurate real-time quantification of mtDNA damage in a scalable platform.

Amyotrophic lateral sclerosis (ALS) is a rare neurodegenerative disease that results in the progressive loss of motor neurons of the brain and spinal cord that control voluntary muscles. The DNA oxidation product 8-oxoguanine (8-oxoG) is a well-established marker of oxidative DNA damage. In persons with ALS, the enzymes that normally repair 8-oxoG DNA damages in the mtDNA of spinal motor neurons are impaired leading to oxidative damage to mtDNA of motor neurons, which is suggested as a significant factor in the etiology of ALS [18].

1.3. mtDNA Replication and mtDNA Polymerase

mtDNA replication is a post-mitotic process and occurs throughout the organism's lifetime. Initiating mtDNA replication involves a process known as priming, where short RNA primers are synthesized by the pol α-associated primase (PriS/Pri1-DNA Primase small subunit), that the DNA polymerase can use as a foundation for 3'-synthesis during the

replication process. After POLG1 has finished replication of the full mitochondrial genome, the primers are removed by an RNase, and the resultant gaps are filled with dNTPs by a repair polymerase and finally ligated by a ligase [19]. The mitochondrial replication machinery consists of not only the DNA polymerase complex, but also other proteins like TWINLKE and mitochondrial single-strand binding (SSB) proteins. (TWINLKE is a helicase that unwinds short stretches of dsDNA in the 5'→3' direction and the SSB maintain the two strands separated for polymerase to proceed with DNA synthesis). All these polypeptides for replication, including the POLG1 are encoded by the nuclear genome [20].

Whereas several DNA polymerases are required to replicate nDNA, DNA pol γ (POLG1) is the sole polymerase responsible for mitochondrial DNA replication and repair. The mitochondrial DNA polymerase (EC 2.7.7.7) belongs to Family A polymerase (Family A polymerases are found both in pro-and eukaryotes and possess two exonuclease domains, (both 5′→3′ and 3′→5′ exonucleases), like bacterial DNA pol I, T7 DNA pol, pol θ and pol ν). Therefore, POLG1 is structurally homologous to the T7 DNA pol and *E. coli* DNA pol I. However, in contrast to T7 DNA pol and *E. coli* DNA pol I, the mitochondrial DNA pol γ consists of two subunits: a large subunit (POLG1) with DNA polymerase and proofreading 3′→5′ exonuclease activities, and a small subunit (POLG2) which confers high fidelity to POLG1 [21, 22]. The DNA pol γ holoenzyme is a heterotrimeric complex and comprised of two nuclear-encoded subunits, viz. the POLG1 and POLG2, where POLG2 exists as dimer). The POLG2 mediates high-affinity binding of the holoenzyme to the template DNA, primer recognition and enhances DNA pol γ processivity to several folds. Furthermore, POLG2 also confers high fidelity to POLG1, which is approximately 100-fold greater than that of nuclear polymerases.

The DNA pol γ complex, which is composed of a 140 kDa catalytic DNA polymerase subunit, encoded by the *POLG1* gene, and two 55 kDa accessory subunits, encoded by the *POLG2* gene. The POLG1 gene is 21kb in size and comprises of 4,465 bp including a 282 bp 5'-untranslated region (UTR) and a 463 bp 3'-UTR and 23 exons spanning approximately 18.5 kb. The POLG1 protein is synthesized as a precursor, containing an amino-terminal leader sequence (residues 1-25) that targets the protein to mitochondria and is cleaved-off after the import. The mature 140 kDa protein is divided into three functional domains: i) a 3'→5' exonuclease (exo) domain, ii) a linker domain, and iii) a highly conserved carboxy-terminal polymerase (pol) domain. The *POLG1* gene is located on the long arm of human chromosome 15, and it maps to 15q25, whereas the *POLG2* gene is located on Chromosome17q21 [23]. The POLG1 is an acidic protein with a pI of 6.46 and the POLG2 is highly basic with a pI of 8.64, suggesting its DNA binding property.

The mtDNA is placed in a highly oxidative environment in mitochondria. Therefore, it is vulnerable to high levels of mROS, leading to mutations, which possibly could result in cellular transformations and eventually leading to cancer. This is because the mROS can modify the structural properties of DNA bases and thus, damage the mtDNA. Upon exposure of cells to oxidative stress, a highly mutagenic damage to the DNA is done due to 7,8-dihydro-8-oxo-guanine (8-oxo-G). The 8-oxo-G is a major oxidative lesion and promutagenic. In general, the replicative DNA polymerases inaccurately bypass the lesions and tend to incorporate dATP opposite to the 8-oxo-G, leading to C:G to A:T transversion mutations. It should be noted that these mutations are among the most predominant somatic mutations in lung, breast, ovarian, gastric and colorectal cancers [24]. To circumvent this problem, the replicative polymerase, POLG1, is equipped with a 3'→5' PR exonuclease and an intrinsic deoxyribose-5-phosphate (dRP)-lyase activities. The (dRP)-lyase, as a part of the base excision repair (BER) pathway, acts as the primary and essential repair system for the removal of damaged mtDNA bases. It essentially removes the damaged bases by the oxidation by mROS in the mitochondrial genome. In this pathway, a DNA-damage-specific glycosylase removes the damaged base from the DNA by cleaving the bond between the base and the deoxyribose which creates an apurinic or apyrimidinic (AP) site. In the next step, an AP endonuclease nicks at the sugar backbone of the damaged DNA at the AP site. Thus, the AP endonuclease generates 3’-OH and 5’-dRP on the DNA strand. Now the 5’-dRP is removed by the (5′-dRP)-lyase and the gap is filled by a repair polymerase with correct dNTP and the gap is sealed by the DNA ligase I [25]. Interestingly, as POLG1 is the only DNA polymerase known to function in human and animal mitochondria [26], its efficiency in the repair system is very important to replicate the mtDNA without any mismatch. Substituting Ala for two essential acidic residues in the PR exonuclease motif, selectively eliminated the 3'→5' exonucleolytic function [27].

1.3.1. POLG1 gene Mutations and Inherited Mitochondrial Diseases

More than one hundred pathogenic mutations have been reported on this enzyme alone since it was first described [28]. Mutations are found in all three domains of POLG1 protein [29]. Such *POLG1* mutations are known to lead to mtDNA depletion, which is known as mtDNA depletion syndrome (MDS) in humans. Furthermore, mutations in the *POLG1*gene have emerged directly or indirectly, as one of the most common causes of many mitochondrial diseases in children and adults. For example, they are responsible for a heterogeneous group of mitochondrial diseases that include: 1) childhood Myocerebrohepatopathy Spectrum disorders (MCHS) which include myopathy or hypotonia, dementia, and liver dysfunction; 2) Alpers syndrome also known as Alpers progressive infantile poliodystrophy; 3) Ataxia Neuropathy Spectrum (ANS) disorders; 4) Myoclonus Epilepsy Myopathy Sensory Ataxia (MEMSA); 5) Autosomal

recessive PEO (arPEO, which includes an overlapping spectrum of disorders like sensory ataxia, neuropathy, dysarthria, and ophthalmoplegia (SANDO), and 6) adPEO, 7) Parkinsonism, 8) Dementia, 9) liver dysfunction, 10) male infertility, etc. [28]. Furthermore, a mutation on *POLG1* gene in a homozygous state is the most common cause of AHS [30].

In this communication, both the mitochondrial replicative DNA pol γ (POLG1) and the nuclear-encoded RNA polymerase (NEP) from humans and various animals are analyzed for their polymerase and proofreading exonuclease domains and to identify the mitochondrial disease mutations in *POLG1* gene, which directly affect the functioning of the POLG1. Two of the critical dominant mutations, viz. R^{943}→C/H and Y^{955}→C/H, which are involved in many mitochondrial diseases in patients with a severe form of adPEO and severe, early-onset multi-systemic mitochondrial disease with bilateral sensorineural hearing loss, cataract, myopathy, and liver failure are identified in the polymerase catalytic site amino acids.

2. Materials and Methods

The protein sequence data of the mitochondrial DNA pol γ and NEP from various animal and human mitochondria were obtained from PUBMED and SWISS-PROT databases. The advanced version of Clustal Omega was used for protein sequence analysis. The polymerase and PR active sites are arrived at by sequence similarities, site-directed mutagenesis (SDM) and X-ray crystallographic data from DNA pol γ and other DNA and RNA polymerases already reported.

3. Results and Discussion

Figure 1 shows the MSA of the mitochondrial POLG1 from human and various animal sources. (Only the regions required for the discussions are shown here). The human sequence is used as the reference and highlighted in yellow. The N-terminal domain (NTD) of ~100 amino acids are not conserved and showed many gaps in the alignment, after that, conservations are observed and a clear demarcation of the PR exonuclease and DNA polymerase domains are observed, and marked by arrows. The linker region is found between them. Large number of consecutive Qs (polyQ tract) is common in some of the POLG1 sequences in the NTD and up to 13 of them are found in the human POLG1 sequence. Several inheritable neurodegenerative diseases, (also known as the polyglutamine diseases are monogenic and are the most common genetically inherited neurodegenerative disorders), occur due to abnormal expansion of the polyglutamine tract (CAG repeats), and the important examples of polyglutamine diseases are the spinocerebellar ataxia and Huntington's disease. A unique polybasic, K rich region – ^{496}**KQKK**A**KK**V**KK**- and a **poly-E** motif (highlighted in human) are found in the linker region (Fig. 1) and its significance is not clear now.

The first completely conserved triad –D^{198}VE- of the DEDD-superfamily of exonucleases is seen after ~ 200 amino acids from the N-terminal. The PR exonuclease domain belongs to the subfamily DEDD(Y) and the active site amino acids are highlighted in light blue. The MSA analysis data are further confirmed by SDM analysis reported already [27] (Table 2). An SDM analysis of D^{198}→A, E^{200}→A and D^{274}→A of POLG1 exhibited no PR exonuclease activity [31, 32]. (Fig. 1).

The PR exonuclease domain is followed by the linker region. The linker region is implicated in the binding of the 55 kDa subunit. A large number of disease mutations are also found in the linker region. For example, Parkinsonism and ataxia, the most common movement disorders, are associated with POLG1 mutations in the highly conserved linker region (A^{467}→T, S^{511}→N, K^{512}→M and G^{517}→V) of POLG1(Fig. 1). Among them the A^{467}→T substitution is the most common. For example, the A^{467}→T mutation is associated with a wide range of mitochondrial disorders, including Alpers syndrome (AS), juvenile spinocerebellar ataxia-epilepsy syndrome, and PEO, and each with vastly different clinical presentations, tissue specificities and ages of onset. On analysis, it was found that the A^{467}→T mutant enzyme possesses only 4% of wild-type DNA polymerase activity, and the catalytic defect is manifested primarily through a 6-fold reduction in k_{cat} with minimal effect on exonuclease function [33, 34]. Very high frequencies (~36%) make the A^{467}→T, the most common disease mutation of *POLG1* gene. Therefore, it is proposed that loss of accessory subunit interaction with POLG1 and reduced DNA polymerase activity are responsible for the depletion and deletion of mtDNA, observed in patients with the above diseases. Mutations in the gene for the catalytic subunit (POLG1) have been shown to be a frequent cause of many mitochondrial disorders. Nearly 50 disease causing mutations are located in the gene of the catalytic subunit of POLG1. Alpers' syndrome is a rare, heritable, fatal neurogenetic disorder that affects the brain and liver of young children. It is an autosomal recessive disease associated with mtDNA depletion disorder, characterized by refractory seizures, neurodegeneration, and liver disease. This is found to be due to deficiency in mitochondrial POLG1 catalytic activity. In two unrelated pedigrees of Alpers' syndrome, each affected child was found to carry a homozygous mutation in exon 17 of the *POLG1* gene locus that led to a Glu^{873}→Stop mutation, just upstream of the polymerase catalytic core of the protein. In addition, each affected child was heterozygous for the mutation in exon 7 that led to an Ala^{467}→Thr substitution in the POLG1 linker region (both are highlighted in magenta) [35].

The linker region is followed by the polymerase domain which is observed after ~750 amino acids and the proposed POLG1 catalytic site amino acids are highlighted in yellow (Fig. 1). The polymerase catalytic site, -S^{942}**R**$^{-4}$EHA**K**^{947}I^{1}FNY^{951}GR^{953}I**Y**^{8}**G**^{956}A^{957}- is found to be very similar to the already reported and confirmed active site of *E. coli* DNA pol I, -Q**R**$^{-4}$RSA**K**^{758}A^{1}INFGLI**Y**^{8}**G**M- [36] and in close agreement to the active sites of the other DNA/RNA polymerases already reported and suggesting high degree of evolutionary conservation among them (Table 3) [37]. Some of the *POLG1* catalytic site mutations have been found to be associated with autosomal recessive and dominant PEO (PEO is an autosomal mitochondrial disorder associated with depletion of the mitochondrial genome and/or the accumulation of mutations and deletions within mtDNA). Interestingly, it is found that the dominant *POLG1* mutations that are known to cause PEO are located within the polymerase domain and found in the amino acid substitutions of G^{923}→D, R^{943}→H, Y^{955}→C and A^{957}→S [31]. Ponamare et al., [38] found that a point mutation (Y^{955}→C) in the DNA pol causes error-prone DNA synthesis in patients suffering from PEO. They found that this Y^{955}→C version of the enzyme retained a wild-type catalytic rate, but suffered a 45-fold decrease in apparent binding affinity for the incoming NTPs. Furthermore, the error-prone DNA synthesis observed for the mutant Y^{955}→C of POLG1 is consistent with the accumulation of mitochondrial DNA mutations in patients with PEO. Similar observation was made by van Goethem et al., [39] with 3 generations of Belgian pedigree, with an autosomal, dominant PEO, where they identified a heterozygous mutation (Y^{955}→C) in the polymerase motif B of the POLG1. Moreover, Lamantea, et al., [40] identified the heterozygous Y^{955}→C mutation in 9 unrelated families with adPEO. Among them, 4 families were Italian and 1 was from Greece and 4 were Swedish families. MSA analysis has shown that the Y^{955} is the template-binding amino acid in the -Y^{955}G- pair of the polymerase. Another mutation, viz. R^{943}→H was found in patients with a severe form of adPEO [40]. R^{943} is critically important in binding the oxygen atoms of the γ-phosphate of the incoming dNTP and helps positioning it for catalysis [39, 40]. The mutant form of R^{943}→H at this position causes again substantial loss of polymerase catalytic activity. For example, the recombinant protein expressing R^{943}→H showed only 0.2% of wild-type polymerase activity *in vitro* [41]. Furthermore, the Y^{955} and R^{943} are highly conserved from yeasts to animals and humans (Fig. 1). MSA analysis has shown that the R^{943} is placed at -4 from the catalytic K as reported and confirmed as the amino acid involved in dNTP selection with *E. coli* DNA pol I. Mutations in the *POLG1* gene are also reported in breast cancer patients. Singh et al., [42] found that the *POLG1* gene was mutated in 63% of breast cancers and identified a total of 17 mutations across the *POLG1* gene. These mutations were found in all three domains of POLG1 protein, including T^{251}→I (exonuclease domain), P^{587}→L (linker region) and E^{1143}→G (polymerase domain, highlighted in magenta).

The N- and C-terminals of the POLG1 show putative zinc-binding motifs (ZBMs) (Fig. 2). Two invariant –DxD- type metal-binding motifs are found within the polymerase domain and highlighted in green and the -**D**^{890}VD- and -HD**E**^{1136}**-** are implicated in chelating two Mg^{2+} ions [28]. In addition to the PR exonuclease activity, the POLG1 is also known to perform BER activity to remove oxidized bases and a (dRP)-lyase domain with the conserved amino acids from 353–391 is highlighted in grey. The lyase catalytic **K**^{371} is marked in red [25] and interestingly, the BER domain is also found in the PR exonuclease region. The C-terminal domain is remarkably conserved in all with a highly conserved peptide (highlighted in grey).

CLUSTAL O (1.2.4) MSA of DNA polymerase γ from human and various animal sources.

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tr|A0A6J1VAB9|A0A6J1VAB9_9SAUR      ---------------------SSSSAGLLLQEKTAPVTGAPLQEEQRMNPLGI-------      55
sp|P54099|DPOG1_MOUSE               PSQ-----------------MPSSENGQL-----------------RLNPLLI-------      59
sp|Q9QYV8|DPOG1_RAT                 QSQ-----------------MPSSENGQL-----------------RLNPLHI-------      59
tr|A0A6P6HHU9|A0A6P6HHU9_PUMCO      RAGRE--------------ARPRCTGGVAAGSWARPAGR--APGRHCGCPVGWATVRACV      104
tr|A0A2K5EPQ4|A0A2K5EPQ4_AOTNA      RQQPQ----------QQPPQVPSSEGGQL-----------------RHNPLHI-------      67
tr|F7IMR4|F7IMR4_CALJA              -QQLQ----------QQPPQVPSSEGGQL-----------------RHNPLHI-------      66
tr|A0A2K5PBZ5|A0A2K5PBZ5_CEBIM      RQQPQ----------QQPPQVPSSEGGQL-----------------RHNPLHI-------      67
tr|A0A0D9RX64|A0A0D9RX64_CHLSB      RRQQQ----------LQQPQVPSSEGGQL-----------------RHNPLHI-------      66
tr|A0A2K6AY60|A0A2K6AY60_MACNE      RQQQQ----------LQQPQVPSSEGGQL-----------------RHNPLHI-------      66
tr|A0A2K5JYU3|A0A2K5JYU3_COLAP      RQQQLQ---------QQQPQVPSSEGGQL-----------------RHNPLHI-------      67
tr|A0A2K6MGT5|A0A2K6MGT5_RHIBE      RRQQQQQ-----QQPQQQPQVPSSEDGQL-----------------RHNPLHI-------      71
tr|A0A2J8VX21|A0A2J8VX21_PONAB      RRRQQQ----QQQQQPQQPQVPSSEGGQL-----------------RHNPLHI-------      72
sp|P54098|DPOG1_HUMAN               RRQQQQQQQQQQQQQPQQPQVLSSEGGQL-----------------RHNPLDI-------      76
tr|G3R7U9|G3R7U9_GORGO              RRRQQQQQQQQPQQPQQPQVLSSEGGQL-----------------RHNPLDI-------      76
tr|A0A3Q7TNX2|A0A3Q7TNX2_VULVU      PQQQ---------QQQPPPPARSSEGGQL-----------------RHNPLHI-------      68
tr|A0A5F5XEZ4|A0A5F5XEZ4_FELCA      -PPR---------PQQ----VPSSEGGQQ-----------------RHNPLHI-------      63
tr|M3WHT6|M3WHT6_FELCA              -PPR---------PQQ----VPSSEGGQQ-----------------RHNPLHI-------      63
tr|A0A667FUI7|A0A667FUI7_LYNCA      -PPR---------PQQ----VPSSEGGQQ-----------------RHNPLHI-------      63
tr|A0A8C8YBT2|A0A8C8YBT2_PANLE      PPPR---------PQQ----VPSSEGGQQ-----------------RHNPLHI-------      64
tr|A0A7J8CKE5|A0A7J8CKE5_ROUAE      -PP------------PPQPQVPSSEGGQP-----------------RYNPLHI-------      60
tr|A0A7J8CKI1|A0A7J8CKI1_ROUAE      -PP------------PPQPQVPSSEGGQP-----------------RYNPLHI-------      60
tr|A0A5N3VIY8|A0A5N3VIY8_MUNMU      --P------------PPPPPVPSSEGGQL-----------------RHNPLHI-------      60
tr|A0A5N3X6N5|A0A5N3X6N5_MUNRE      --P------------PPPPPVPSSEGGQL-----------------RHNPLHI-------      60
tr|A0A8C2QXP3|A0A8C2QXP3_CAPHI      --P------------PPPQPVPSSVGGQL-----------------RHNPLHI-------      60
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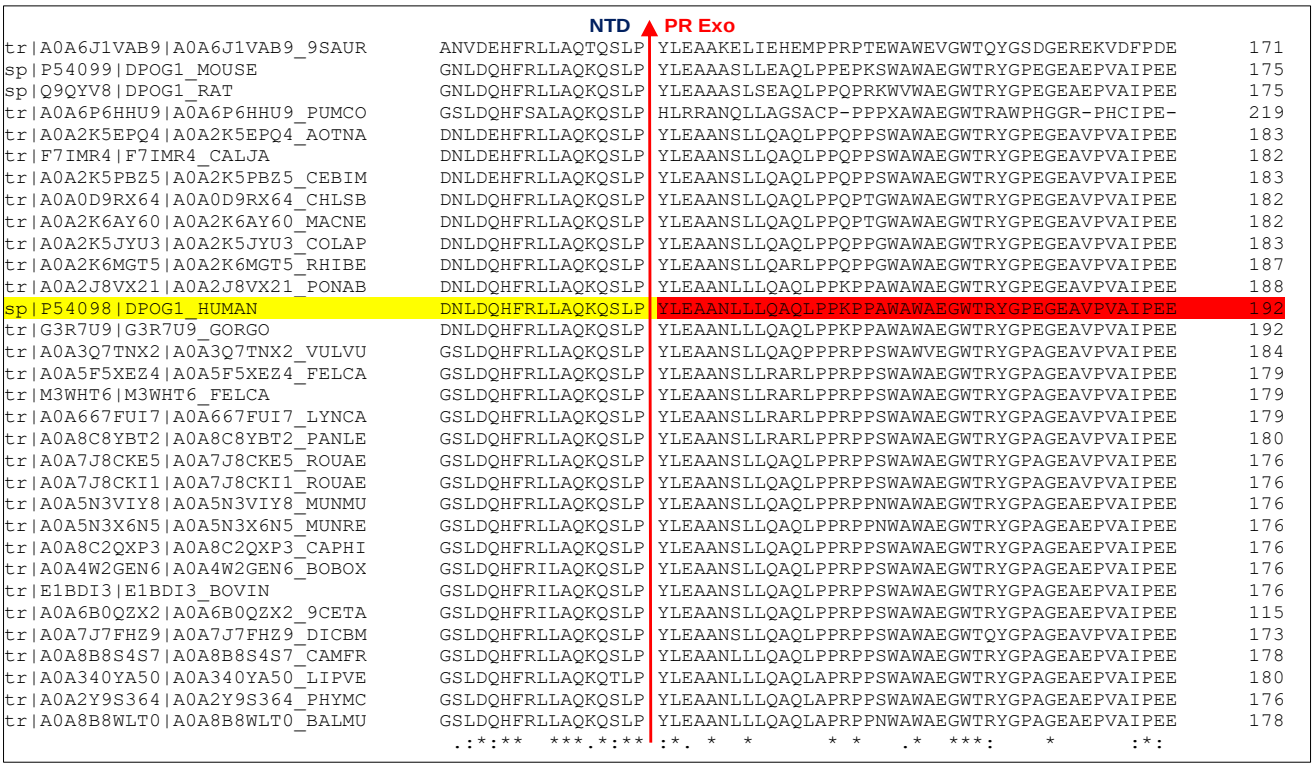
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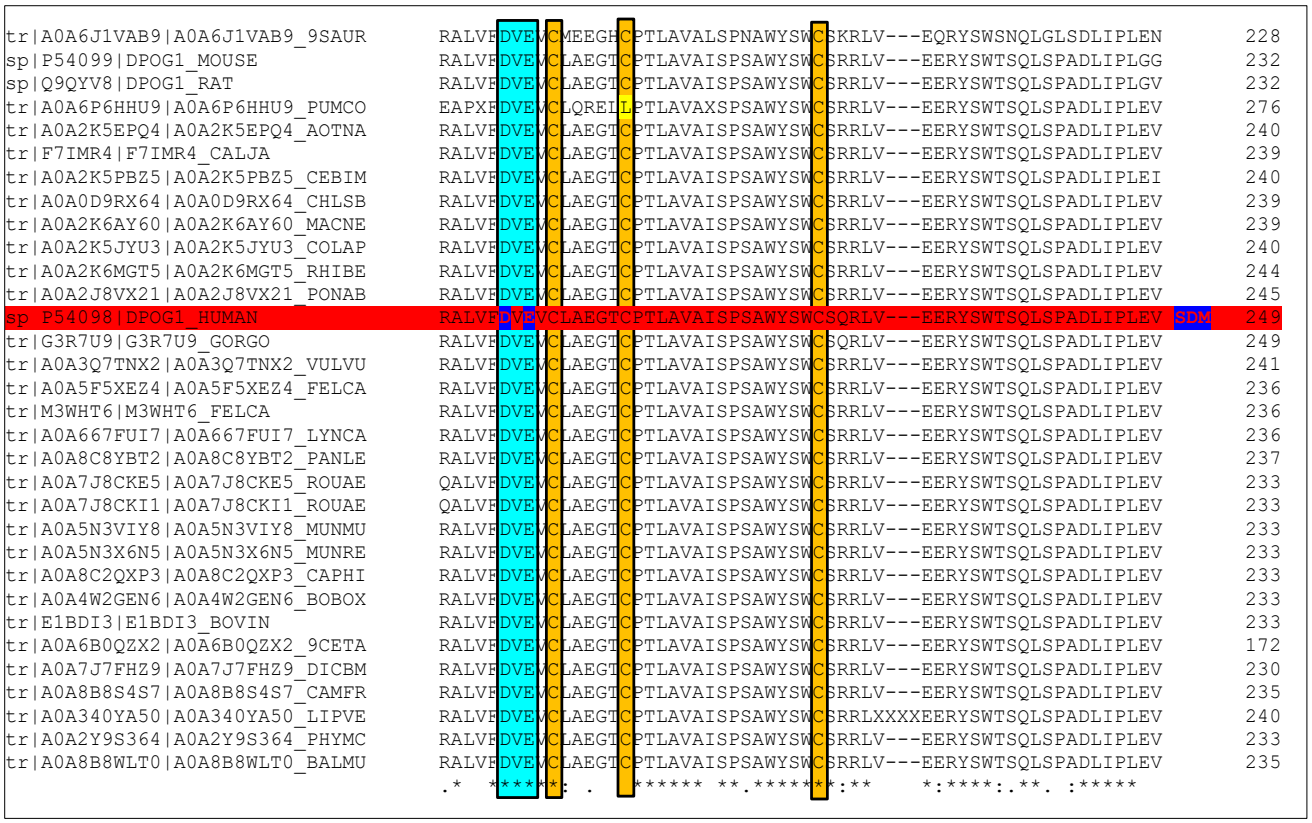
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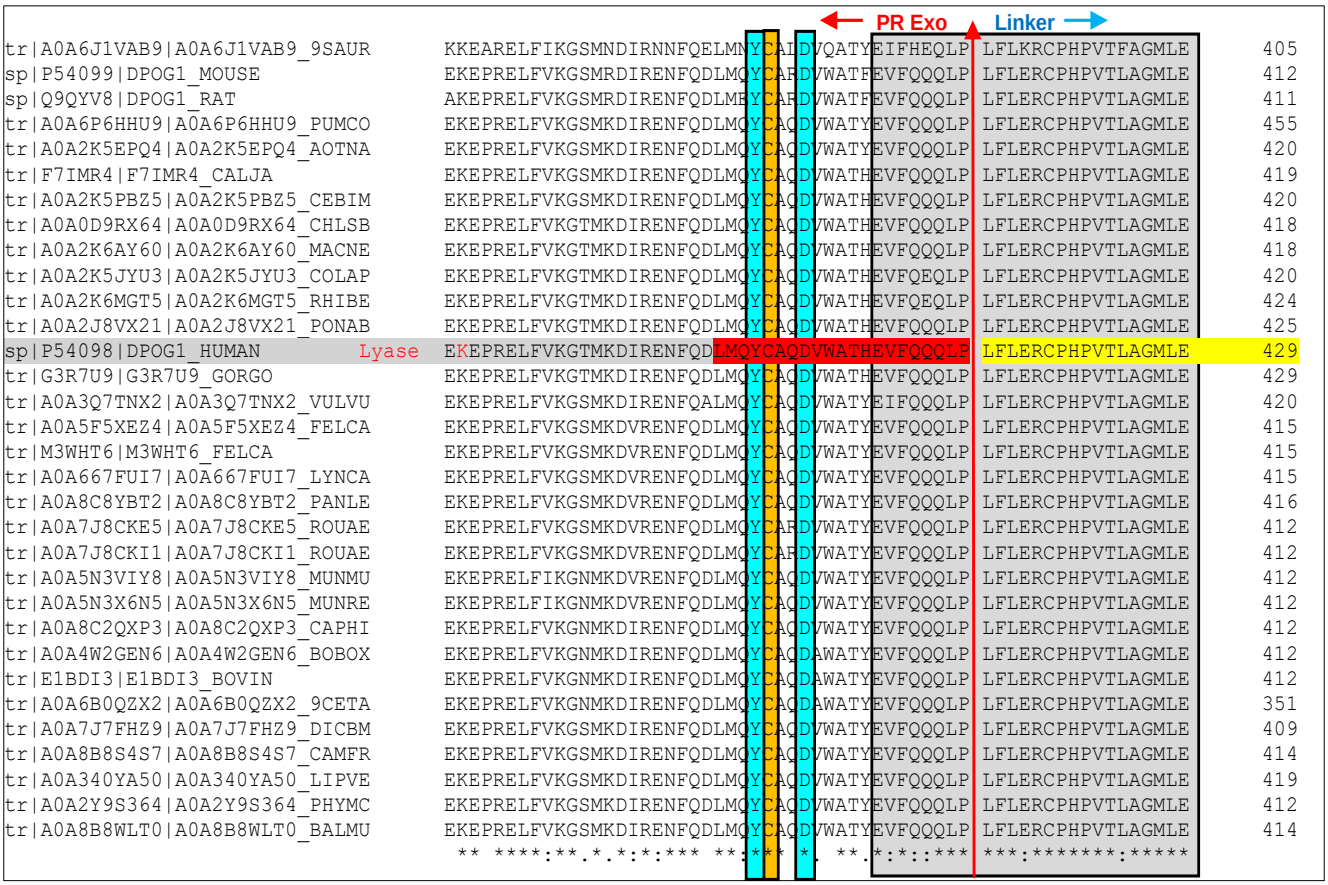
PR Exo
Linker
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sp|P54099|DPOG1_MOUSE EKEPRELFVKGSMRDIRENFQDLMQYCAFDVWATFEVFQQQLP LFLERCPHPVTLAGMLE 412
sp|Q9QYV8|DPOG1_RAT AKEPRELFVKGSMRDIRENFQDLMEYCAFDVWATFEVFQQQLP LFLERCPHPVTLAGMLE 411
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tr|A0A2K5PBZ5|A0A2K5PBZ5_CEBIM EKEPRELFVKGSMKDIRENFQDLMQYCAQDVWATHEVFQQQLP LFLERCPHPVTLAGMLE 420
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tr|A0A2J8VX21|A0A2J8VX21_PONAB EKEPRELFVKGTMKDIRENFQDLMQYCAQDVWATHEVFQQQLP LFLERCPHPVTLAGMLE 425
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tr|A0A667FUI7|A0A667FUI7_LYNCA EKEPRELFVKGSMKDVRENFQDLMQYCAQDVWATYEVFQQQLP LFLERCPHPVTLAGMLE 415
tr|A0A8C8YBT2|A0A8C8YBT2_PANLE EKEPRELFVKGSMKDVRENFQDLMQYCAQDVWATYEVFQQQLP LFLERCPHPVTLAGMLE 416
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tr|A0A8B8S4S7|A0A8B8S4S7_CAMFR EKEPRELFVKGSMKDIRENFQDLMQYCAQDVWATYEVFQQQLP LFLERCPHPVTLAGMLE 414
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tr|A0A2Y9S364|A0A2Y9S364_PHYMC EKEPRELFVKGSMKDIRENFQDLMQYCAQDVWATYEVFQQQLP LFLERCPHPVTLAGMLE 412
tr|A0A8B8WLT0|A0A8B8WLT0_BALMU EKEPRELFVKGSMKDIRENFQDLMQYCAQDVWATYEVFQQQLP LFLERCPHPVTLAGMLE 414


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tr|G3R7U9|G3R7U9_GORGO              KDTQPSYHHGNGPYNDVDIPGCWFFKLPHKDGNSCNVGSP|FAKDFLPKMEDGTLQAGPGG      785
tr|A0A3Q7TNX2|A0A3Q7TNX2_VULVU      RASQPAYHHGNGPYNDVDIPGCWFFKLPHKDGGGCNVGSP|FAKDFLPKMEDGTLQAGPGG      776
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tr|A0A667FUI7|A0A667FUI7_LYNCA      RASQPAYHHGNGPYNDVDVPGCWFFKLPHKDGSSFNVGSP|FAKDFLPKMEDGTLQAGPGG      772
tr|A0A8C8YBT2|A0A8C8YBT2_PANLE      RASQPAYHHGNGPYNDVDVPGCWFFKLPHKDGSSFNVGSP|FAKDFLPKMEDGTLQAGPGG      773
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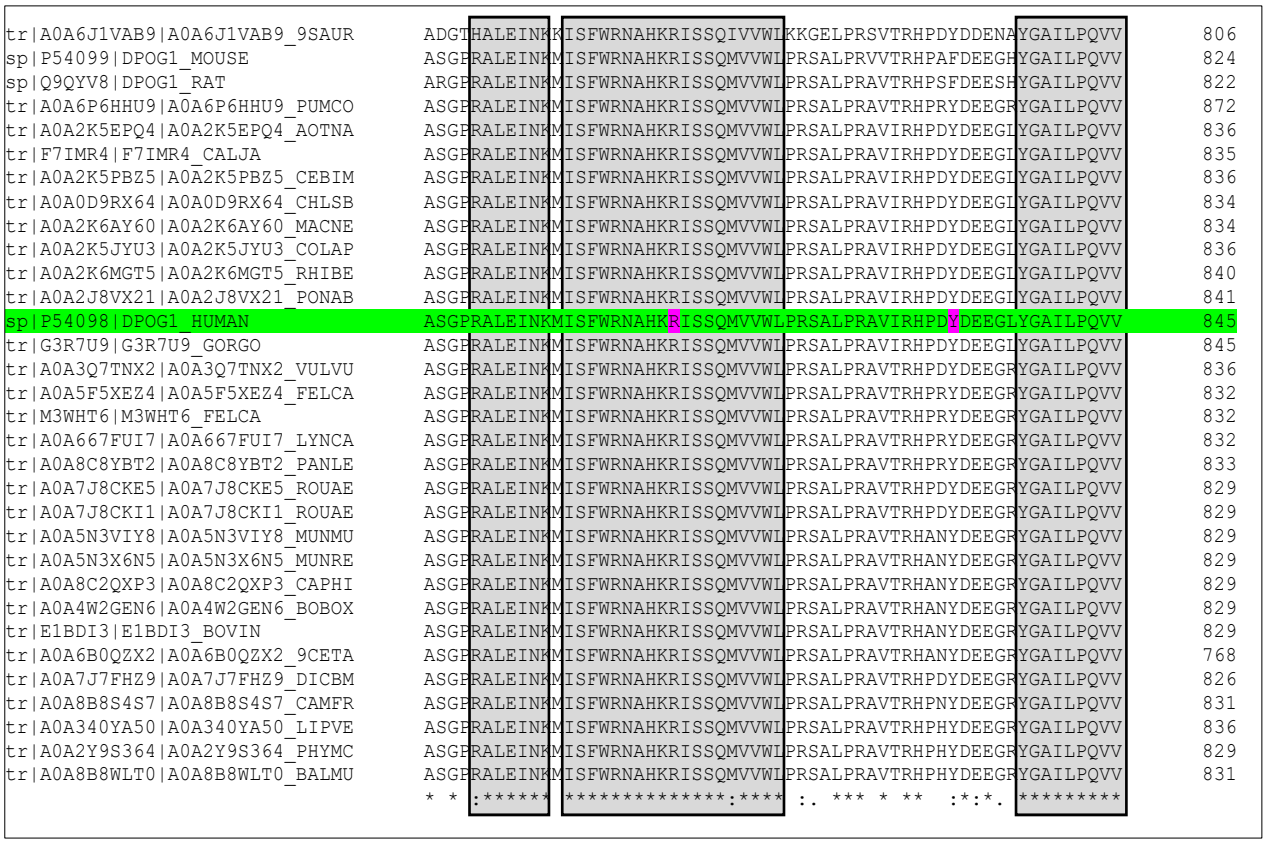
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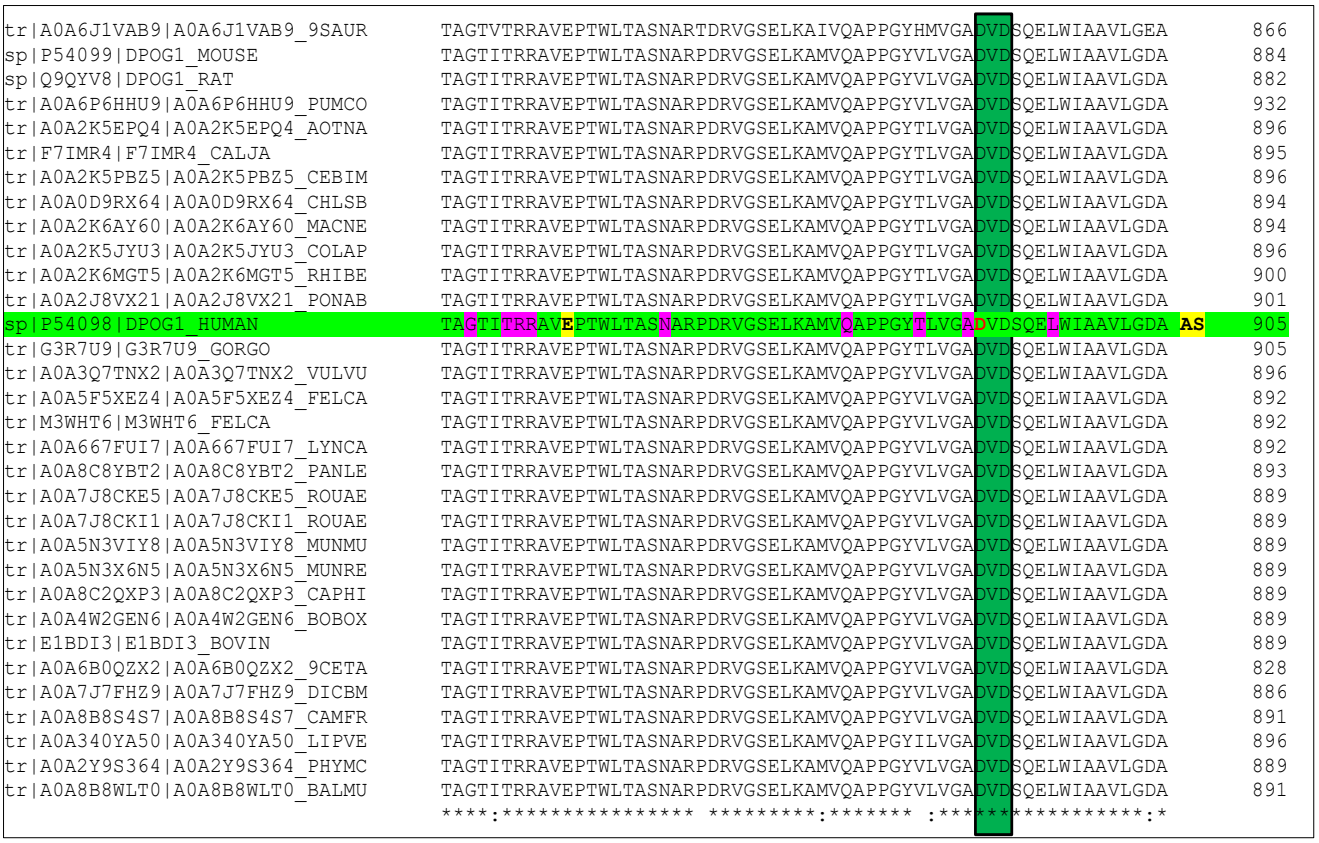
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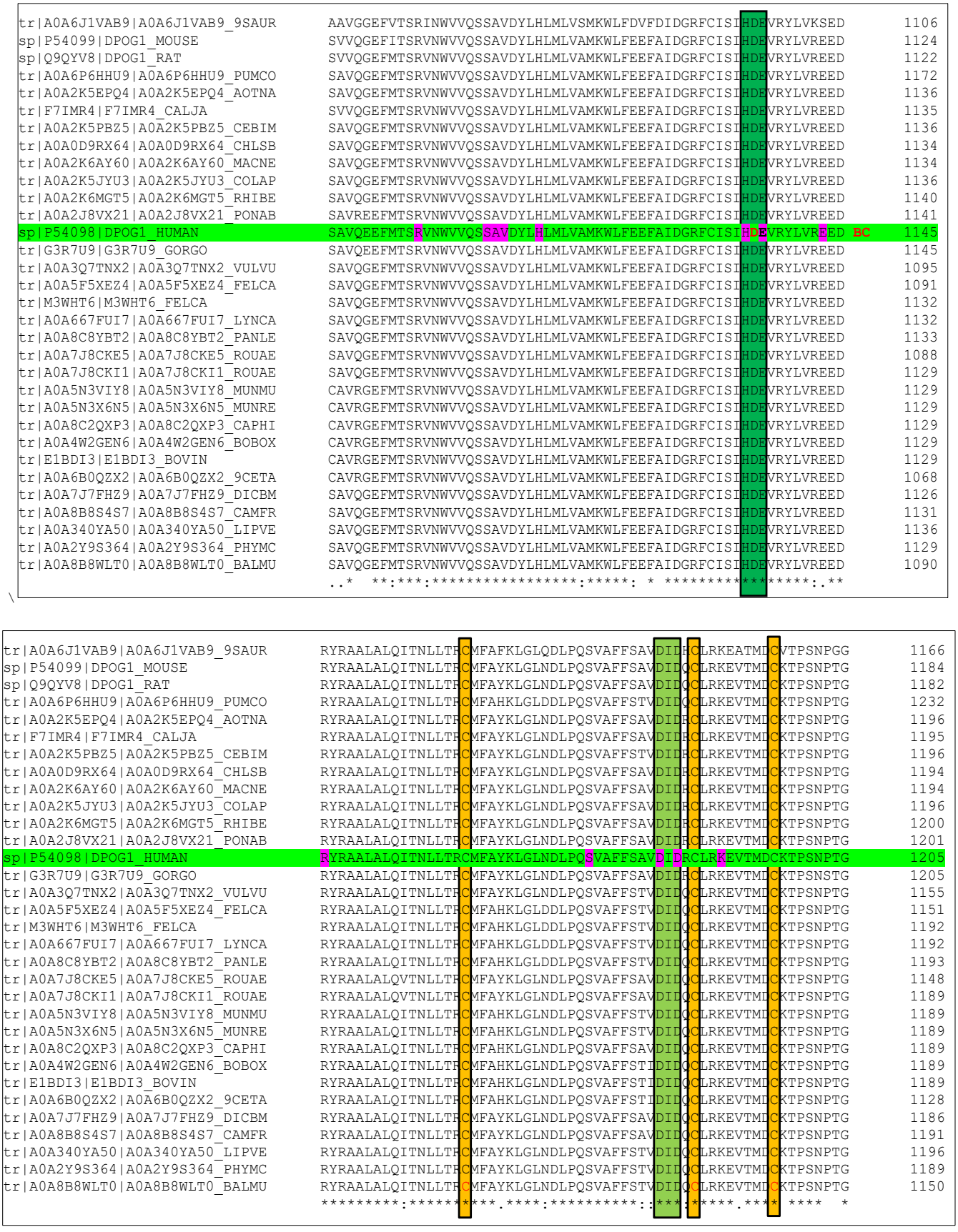
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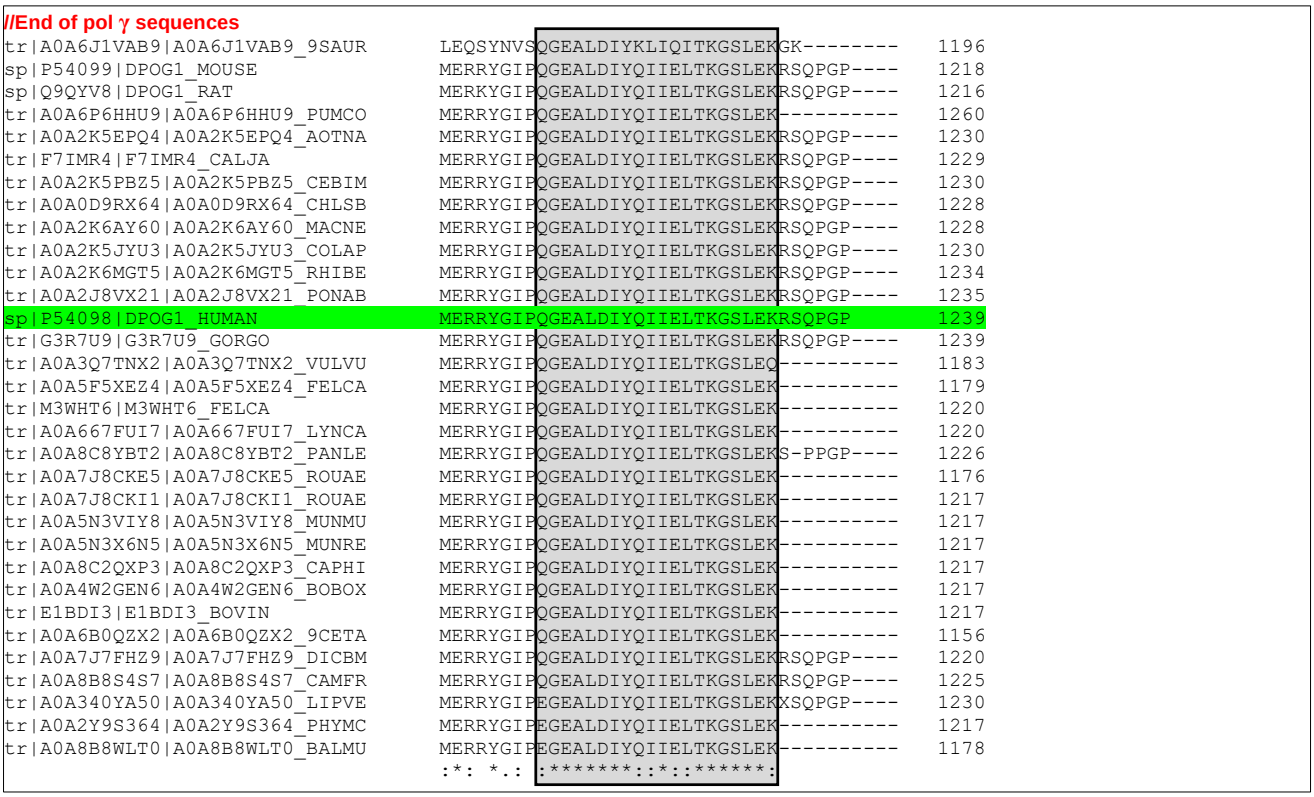


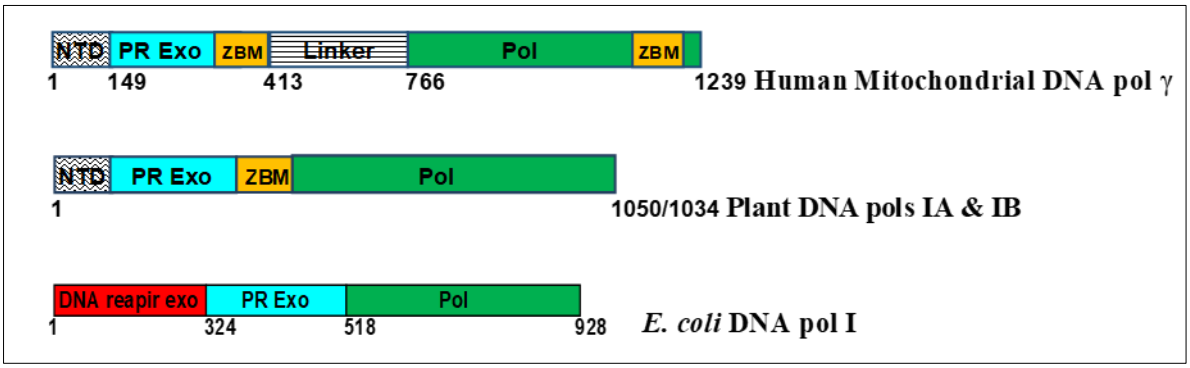
Figure 1 MSA of mitochondrial DNA polymerase γ from human and various animal sources
AS, Alper's syndrome; BC, Breast cancer; Mutations in the polymerase region are shown in magenta

A0A6J1VAB9_9SAUR *Notechis scutatus* (Snake)	P54099|DPOG1_MOUSE *Mus musculus* (Mouse)
Q9QYV8|DPOG1_RAT *Rattus norvegicus* (Rat)	A0A6P6HHU9_PUMCO *Puma concolor* (Panther)
A0A2K5EPQ4_AOTNA *Aotus nancymaae* (Monkey)	F7IMR4_CALJA *Callithrix jacchus* (Monkey)
A0A2K5PBZ5_CEBIM *Cebus imitator* (Capuchin)	A0A0D9RX64_CHLSB *Chlorocebus sabaeus* (Monkey)
A0A2K6AY60_MACNE *Macaca nemestrina* (Macaque)	A0A2K5JYU3_COLAP *Colobus angolensis palliates*
A0A2K6MGT5_RHIBE *Rhinopithecus bieti* (Monkey)	A0A2J8VX21_PONAB *Pongo abelii* (Orangutan)
P54098|DPOG1_HUMAN *Homo sapiens* (Human)	G3R7U9_GORGO *Gorilla gorilla* (Gorilla)
A0A3Q7TNX2_VULVU *Vulpes vulpes* (Red fox)	A0A5F5XEZ4_FELCA *Felis catus* (Cat)
M3WHT6_FELCA *Felis catus* (Cat)	A0A667FUI7_LYNCA *Lynx Canadensis* (Cat family)
A0A8C8YBT2_PANLE *Panthera leo* (Lion)	A0A7J8CKE5_ROUAE Rousettus aegyptiacus (Fruit bat)
A0A7J8CKI1_ROUAE Rousettus aegyptiacus (Fruit bat)	A0A5N3VIY8_MUNMU Muntiacus muntjak (Asian Deer)
A0A5N3X6N5_MUNRE *Muntiacus reevesi* (Deer family)	A0A8C2QXP3_CAPHI *Capra hircus* (Goat)
A0A4W2GEN6_BOBOX *Bos indicus/ Taurus*	E1BDI3_BOVIN *Bos Taurus* (Bovine)
A0A6B0QZX2_9CETA *Bos mutus* (Wild yak)	A0A7J7FHZ9_DICBM *Diceros bicornis* (Rhinoceros)
A0A8B8S4S7_CAMFR *Camelus ferus* (Camel)	A0A340YA50_LIPVE *Lipotes vexillifer* (River dolphin)
A0A2Y9S364_PHYMC *Physeter ma*crocephalus (Whale)	A0A8B8WLT0_BALMU *Balaenoptera musculus* (Blue whale)

In addition to the replication and repair functions, the POLG1 is also found to play an important role in embryo development. Hance et al., [43] demonstrated that POLG1 deficiency in mouse embryos caused an early developmental arrest between embryonic days 7.5 and 8.5 and was found associated with severe mtDNA depletion. Therefore, they concluded that the presence of intact POLG1 is absolutely essential for the organogenesis during mammalian embryonic development.

Figure 2 shows the proposed organization of the various domains of the human mitochondrial POLG1, plant Chloroplast/Mitochondria DNA pols IA and IB from *A. thaliana* and *E. coli* DNA pol I. Even though the N-terminal region of ~125 amino acids is not conserved in POLG1 from human and animals, the C-terminal region is highly conserved. In contrast to *E. coli* and plant enzymes, in the human and animal mitochondrial POLG1, the PR exonuclease and the polymerase domains are connected by a linker. The linker region is something special found in the human/animal mitochondrial POLG1 and it essentially interacts with the 55 kDA, the second subunit of the enzyme (other two are monomeric, SSU enzymes). The polymerase and the PR exonuclease regions of the human POLG1 show putative ZBMs

(highlighted in orange). Defects of mitochondrial polymerase POLG1 underlie wide range of human diseases like dementia, Parkinsonism, ataxia, retinopathy, cataract, cardiomyopathy, liver failure, deafness, diabetes, infantile Alpers syndrome, etc. as discussed elsewhere. It should be noted that nearly all of the adPEO mutations in human *POLG1* gene are located in the polymerase catalytic region. To date, it is found that the vast majority of dominant mutations are in the polymerase domain of *POLG1* with a few exceptions like the A^{467}→T in the linker region of the protein. The polymerase and the PR exonuclaese regions of the human POLG1 show putative ZBMs (highlighted in orange).



NTD, N-terminal domain; PR exo, 3'-5' DEDD-superfamily of exonuclease domain; ZBM, Zinc-binding motif; Pol, Polymerase domain. DNA repair exo, 5'-3' exonuclease.

Figure 2 Comparison of the three closely related pols: 1) Human Mitochondrial POLG1; 2) Mitochondrial/Chloroplast DNA pols IA & IB from *A. thaliana* and 3) *E. coli* DNA pol I

Figure 3 shows the 'Mix and Match' MSA analysis of human mitochondrial POLG1, plant mitochondrial/chloroplast DNA pols IA and IB, and *E. coli* DNA pol I (only the regions required for the discussion are shown here). The catalytic core is highly conserved in all the four DNA polymerases and the completely conserved catalytic amino acids are aligned in all the four (highlighted in yellow). Furthermore, all the four DNA polymerases possess the typical DEDD(Y)-superfamily of PR exonucleases (highlighted in light blue).

Besides, a large number of consecutive Qs is found at the NTD in the human mitochondrial POLG1 (marked in red and highlighted in yellow) and discussed elsewhere. The catalytic metal-binding Ds are marked in red and are highlighted in dark green. Putative ZBMs (highlighted in orange) are found in the human mitochondrial POLG1 and Plant mitochondrial/chloroplast DNA pols IA and IB. Even though the PR exonuclease and polymerases active site amino acids are highly conserved.

BLASTp analysis of human POLG1 with other DNA polymerases showed only 36.92% identity to the DNA pol IB and the DNA pol IB did not show much similarity and the *E. coli* DNA pol I showed only 31.25%.

CLUSTAL O (1.2.4) 'Mix and Match' MSA of human mitochondrial POLG1, Plant mitochondrial/chloroplast DNA pols IA and IB, and *E. coli* DNA pol I.

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sp|F4I6M1|POLIA_ARATH      VGNQTEVAETHQVPGTVSAWREEAN-------------KLRERNGQIARN----------   142
sp|Q84ND9|POLIB_ARATH      ------------QAGAVSAWREEVNN------------KLRGRNREYANN----------   122
                                        ::                    .    ..

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sp|P00582|DPO1_ECOLI       ATVISYDNYVTILDE-ETLKAWIAK---LEKAPVFAFDTETDSLDNISANLV----GLSF   373
sp|F4I6M1|POLIA_ARATH      ENLGKIYDKVLIVDNVQAAKDTVAKLVNQFRNHVHSCDTEVSGIEVKEETPVDHGELICF   316
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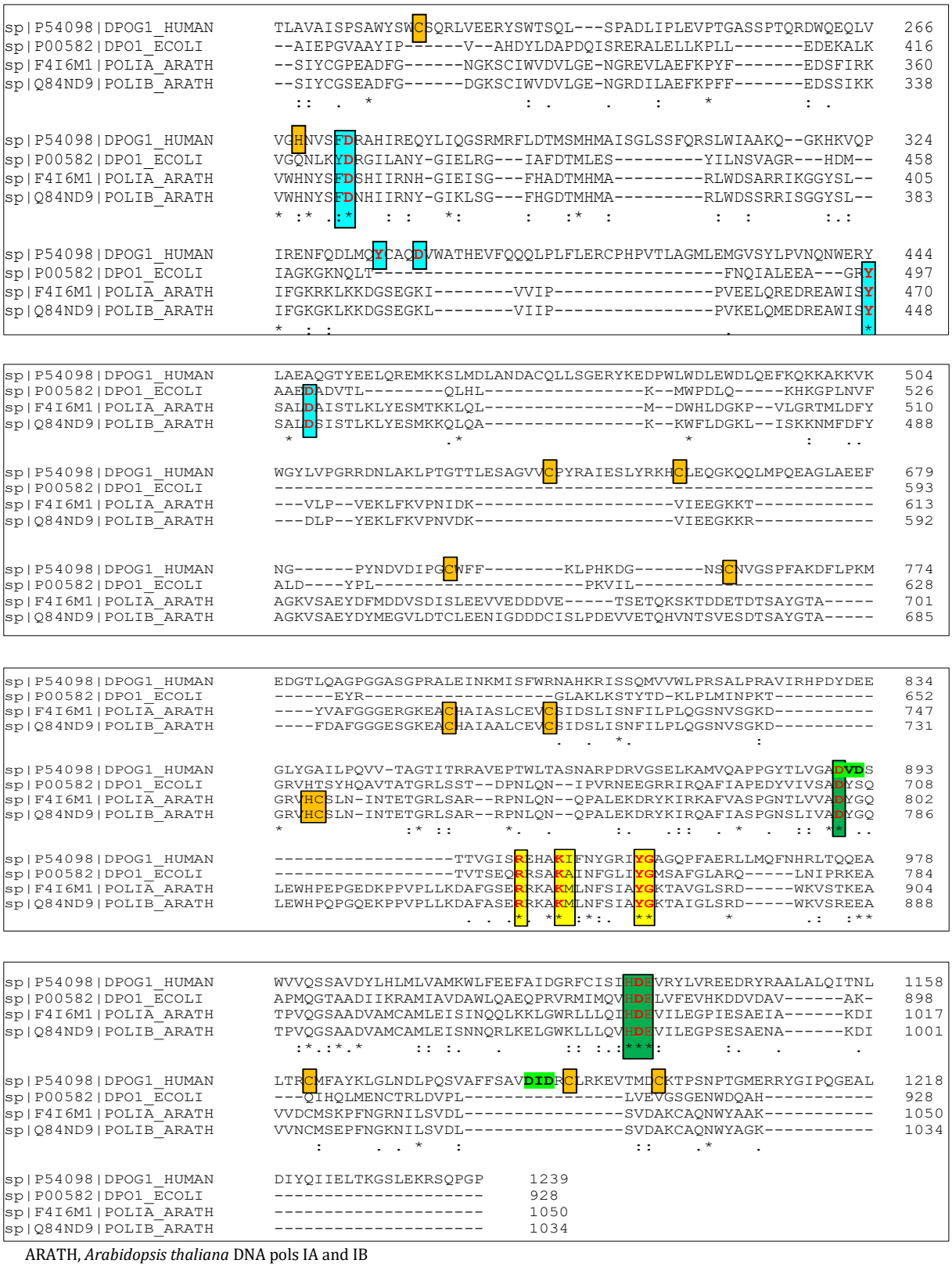

ARATH, *Arabidopsis thaliana* DNA pols IA and IB

Figure 3 'Mix and Match' MSA of human POLG1, Plant mitochondrial/chloroplast DNA pols IA and IB, and *E. coli* DNA pol I

Table 1 shows the naturally occurring clinical variants in the proposed polymerase region (766-1239) of the *POLG1* gene. The disease mutations are highlighted in magenta (Fig. 1). Interestingly, some of the disease mutations are found clustered in the proposed catalytic core, viz. -R^{943}EHAKIFNY^{951}GRIY^{955}GA^{957}- and in the metal-binding motifs, viz. -H^{1134}DE- and –D^{1184}ID- (highlighted in magenta).

Table 1 Clinical variants on the polymerase region (766-1239) of the human *POLG1* gene

Region	mt-Disease(s)*	Holoenzyme activity (p140+p55)	Reference
$A^{767}\rightarrow D$	MTDPS4A	-	PubMed:16621917
$R^{807}\rightarrow C$	SANDO	-	PubMed:16919951
$R^{807}\rightarrow P$	PEOB1(sporadic) -		PubMed:14635118
$Y^{831}\rightarrow C$	PEOA1 & MTDPS4A	-	PubMed:15534189
Thumb:			
Wild-type	Nil	100%	[44]
$G^{848}\rightarrow S$	Alpers syndrome, Leigh syndrome, MELAS, PEO with ataxia-neuropathy, PEO.	0.03%	”
$T^{851}\rightarrow A$	Alpers syndrome	0.92%	”
$R^{852}\rightarrow C$	Alpers syndrome, Ataxia-neuropathy	0.32%	”
$R^{853}\rightarrow Q$	Myocerebrohepatopathy	0.09%	”
$R^{853}\rightarrow W$	PEOB1(ar1)	-	PubMed:16401742
$N^{864}\rightarrow S$	MTDPS4B	-	PubMed:12825077
Palm:			
$Q^{879}\rightarrow H$	Alpers syndrome with valproate-induced hepatic failure	28%	[44]
$T^{885}\rightarrow S$	Alpers syndrome with valproate-induced hepatic failure	58%	”
$A^{889}\rightarrow T$	PEOB1		PubMed:12975295
$L^{896}\rightarrow R$;	Ptosis, Ophthalmoplegia, Cataracts		
$T^{914}\rightarrow P$	MTDPS4A		PubMed:16639411
$G^{923}\rightarrow D$	PEOA1	>50%	PubMed:1221079
$H^{932}\rightarrow Y$	PEOB1, SANDO (sporadic)		[45]
NTP selection amino acid:			
$R^{943}\rightarrow H$	PEOA1 dominant	0.2%	[41]
Catalytic amino acid:			
K^{947}		-	
Polymerase catalytic core:			
$Y^{951}\rightarrow H$	Ptosis, Ophthalmoplegia, Cataracts		
$R^{953}\rightarrow C$	PEOA1	-	PubMed:15351195
Template-binding pair (Y^{955}G) region:			
$Y^{955}\rightarrow C^{@}$	PEOA1 & Parkinsonism (most common, dominant) <0.1%		PubMed:11431686
$Y^{955}\rightarrow H^{\#}$	bilateral sensorineural hearing loss, cataract, myopathy, and liver failure	-	
$A^{957}\rightarrow S^{\&}$	PEOA1(dominant)		
$A^{957}\rightarrow P$	MTDPS4A		PubMed:15689359
Fingers:			
$R^{1047}\rightarrow Q$	PEOB1 (sporadic)		PubMed:12707443
$G^{1051}\rightarrow R$	SANDO		PubMed:14745080
$P^{1073}\rightarrow L$	MTDPS4A		[46]
$G^{1076}\rightarrow V$	PEOB1		PubMed:12975295
$R^{1096}\rightarrow H$	MTDPS4A		PubMed:16621917
$S^{1104}\rightarrow C$	PEOB1 (sporadic)		PubMed:12707443
$A^{1105}\rightarrow T$	PEOB1		PubMed:15351195
$V^{1106}\rightarrow I$	PEOB1		PubMed:15349879
$H^{1110}\rightarrow Y$	MTDPS4A		PubMed:18828154
$H^{1134}\rightarrow R$	MTDPS4A		PubMed:18828154
$E^{1136}\rightarrow K$	MTDPS4A		PubMed:18828154
$E^{1143}\rightarrow G$	Breast cancer		[43]
$R^{1146}\rightarrow C$	PEOB1		PubMed:16401742
$S^{1176}\rightarrow L$	PEOA1		PubMed:12210792
$D^{1184}\rightarrow N$	PEOB1		PubMed:16401742
$D^{1186}\rightarrow H$	PEOA1		PubMed:18575922
$K^{1191}\rightarrow N$	MTDPS4A		PubMed:16621917

Main source: From the Human mitochondrial genome database, www.mtdb.igp.uu.se and human mito gamma pol mutations.
*Most of them manifest in *trans* with other genetic mutations on the *POLG1* gene. **PEOA1**, Progressive external ophthalmoplegia with mitochondrial DNA deletions (Autosomal Dominant 1); **PEOB1**, autosomal recessive 1; (**AdPEO** mutations in *POLG1* are generally found in very conserved residues within the active site of the p140 DNA polymerase domain [41], while **ArPEO** mutations are spread throughout the gene). **MTDPS4A**, Mitochondrial DNA depletion syndrome, also known as Alpers syndrome, is an autosomal recessive disorder characterized by a clinical triad of psychomotor retardation, intractable epilepsy, and liver failure in infants and young children. **MTDPS4B**, Mitochondrial DNA depletion syndrome is an autosomal recessive, progressive, multisystem disorder which manifests as neurogastrointestinal encephalopathy (**MNGIE**). Patients with clinical triad of Sensory Ataxic Neuropathy, Dysarthria, and Ophthalmoparesis (**SANDO)** is a variant of autosomal recessive **PEO (ArPEO).** $^{@}$ and $^{\#}$, both mutations affect mtDNA replication and display a dominant negative effect, with the $Y^{955}\rightarrow H$ allele resulting in more severe polymerase dysfunction. $^{\&}$In functional studies, the $A^{957}\rightarrow P$ mutant showed the most striking deficiencies in the incorporation of a correct dNTP compared to the wild-type. The $A^{957}\rightarrow P$ mutant had a 2-fold order of magnitude loss of fidelity compared to the wild-type, suggesting that a buildup of mitochondrial mutations may contribute to death in infancy in those with this mutation [47].

Figure 4 shows the MSA of the NEPs from human and various animal sources (Only the regions required for the discussions are shown here). The human sequence is used as the reference and highlighted in yellow. The N-terminal region of ~470 amino acids showed many gaps and mismatches in the alignment (data not shown), after that, conservations are observed and a clear demarcation (marked with an arrow) of the PR exonuclease domain is seen (highlighted in red). The first triad, –DTE- type of the DEDD-superfamily of the PR exonucleases is not completely conserved among the NEPs from humans and various animal sources (the humans and chimpanzees use, unusually an A in the first triad, as -DAE- and in many others the third amino acid E is replaced by a Q). However, strikingly the other three active site amino acids are completely conserved (highlighted in light blue) with an invariant H as the proton acceptor in the PR exonuclease domain (Table 2).

Again, after ~830 amino acids, a second demarcation in the sequences is seen (marked by an arrow) and it contains the polymerase active site amino acids (highlighted in yellow). The polymerase region is highly conserved among them, but the catalytic core region is completely conserved in all NEPs from human and animal sources. It contains the template-binding -YG- pair, the catalytic amino acid K, and the nucleotide discriminating amino acid R at -4 from the catalytic K. The POLG1 active site, -T**R**$^{-4}$KVV**K**^{991}Q^{1}TVMT*V*V**Y**^{8}**G**V- is almost identical to the other NEPs of plant chloroplasts and mitochondria, (-D**R**$^{-4}$KLV**K**^{752}Q^{1}TVMT*S*V**Y**^{8}**G**V-), and is found to be very similar to the confirmed active site of *E. coli* DNA pol I, -Q**R**$^{-4}$RSA**K**^{758}A^{1}INFGLI**Y**^{8}**G**M- [36] and in close agreement to the active sites of the other DNA/RNA polymerases already reported [37] (Table 3). The human and animal sequences showed an additional –YG- pair (highlighted in light yellow) downstream from the proposed template-binding YG pair. The proposed catalytic metal-binding Ds in –HQ**D**^{922}- and –H**D**^{1151}C- motifs are completely conserved (highlighted in dark green). SDM data have shown that the substitutions of the Ds in –AF**D**^{537}G-and -H**D**^{812}S motifs to **D**^{537}→N and **D**^{812}→N in T7 RNAP resulted in the complete loss of activity [48, 49]. Almost all the NEPs (except the sequence from lion) invariably end in a hexapeptide -**STYFFS**- and its significance is not clear now. Unlike the self-sustained T7 RNAP, NEP needs transcription factors for promoter recognition, like TFAM and TFB2M, and also a transcription elongation factor, TEFM. (TFAM binds to the mitochondrial promoter's sequence and creates a stable protein-DNA complex. TFAM and TFB2M work synergistically to support mitochondrial transcription initiation) [50].

An unusual capping structure is found in the mt-mRNAs. In contrast to an m^{7}G cap usually found at the 5' n-mRNAs, (which is added to the nascent mRNAs by a capping complex that is associated with eukaryotic RNA polymerase II), the SSU mitochondrial RNA polymerase (mt-RNAP) caps its mRNAs with NAD^{+} and NADH. An NAD^{+} cap is added by the mt-RNAP itself during transcription initiation, which serves as a non-canonical initiating nucleotide [51]. However, like the n-mRNAS, the human mt-mRNAs are stabilized by polyadenylation which is regulated by the mitochondria-specific poly-(A) polymerase and polynucleotide phosphorylase [52]. Interestingly, inhibitors of mtDNA transcription, targeting the mt-RNAP, reduce mitochondrial metabolism and ATP levels. The mt-RNAP inhibitors, viz. IMT1 and IMT1B have been shown to reduce cancer cell growth and viability *in vitro* and induce strong antitumor responses [53].

CLUSTAL O (1.2.4) MSA of mitochondrial NEPs from human and various animal sources

```
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tr|H2QES6|H2QES6_PANTR              LYP FLCLLDEREVVRMLLQILQALPAQGESFTTLARELSARTFSRHVVQRQRVSGQVQAL      526
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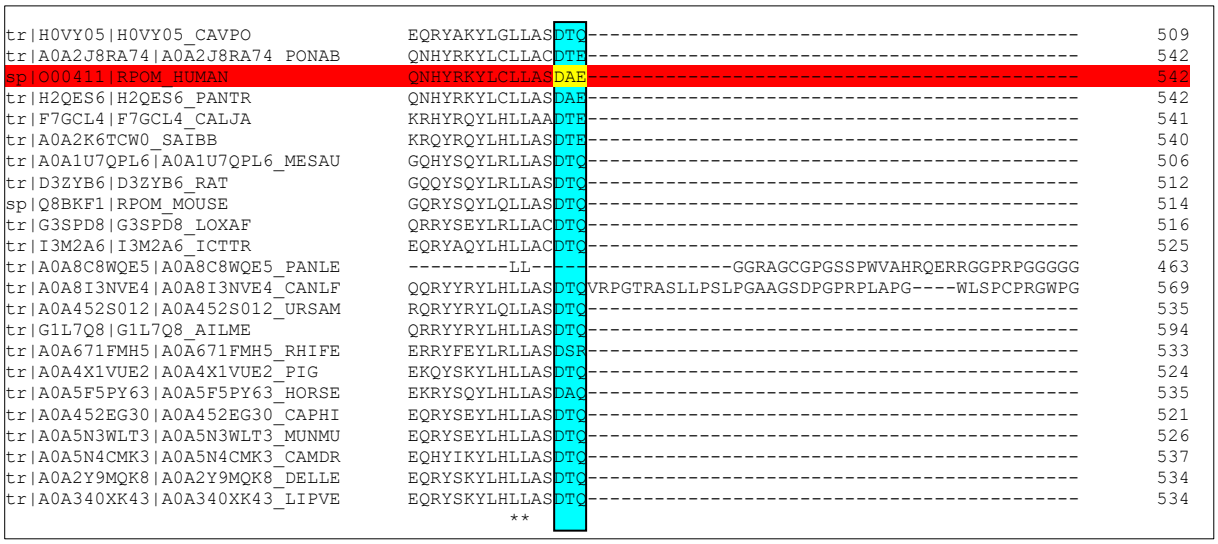
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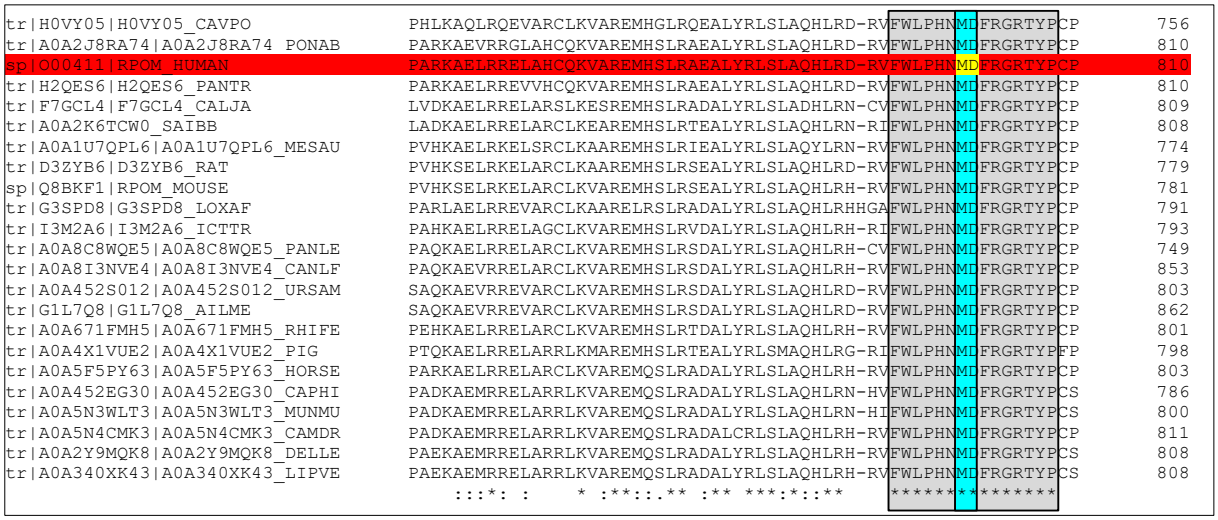
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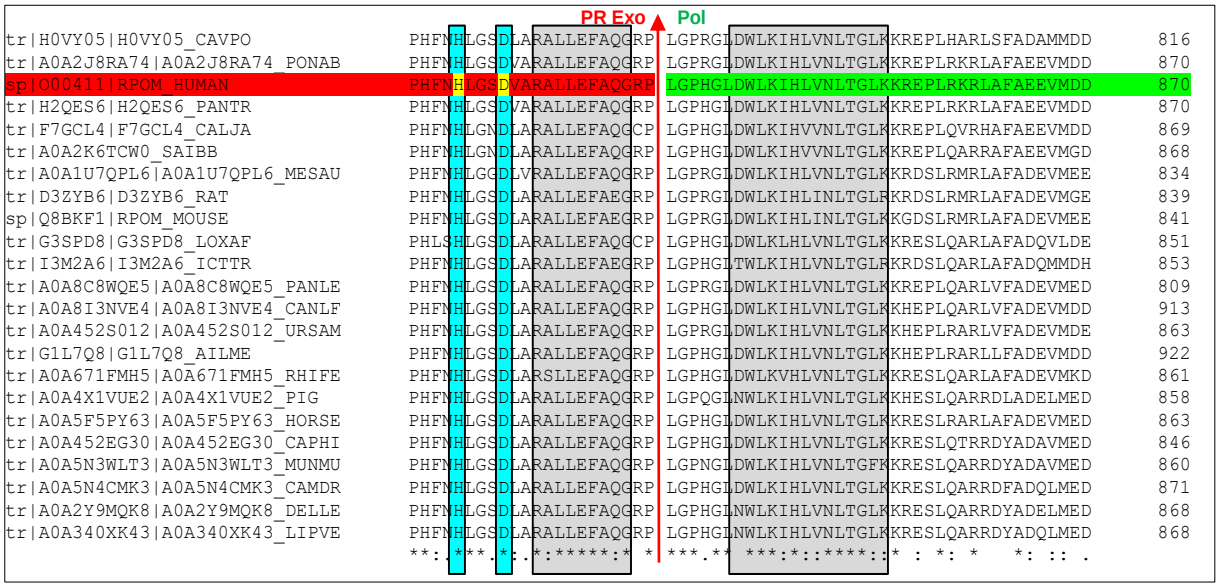
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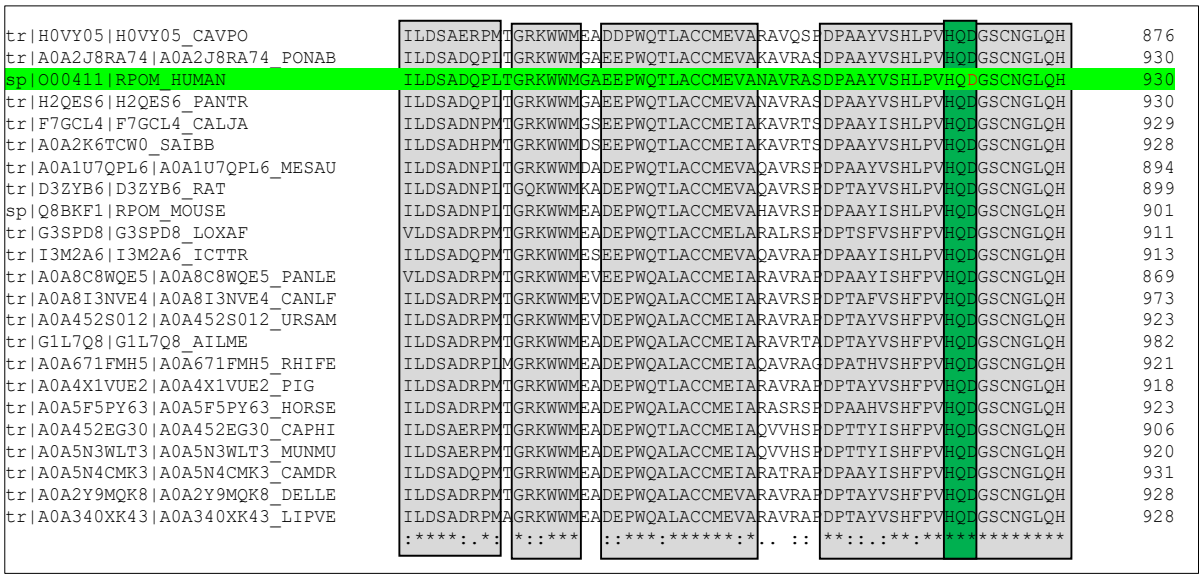
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tr|A0A5F5PY63|A0A5F5PY63_HORSE ILDSADRPMTGRKWWMEADEPWQALACCMEIARASRSRDPAAHVSHFPVHQDGSCNGLQH 923
tr|A0A452EG30|A0A452EG30_CAPHI ILDSAERPMTGRKWWMEADEPWQTLACCMEIAQVVHSRDPTTYISHFPVHQDGSCNGLQH 906
tr|A0A5N3WLT3|A0A5N3WLT3_MUNMU ILDSAERPMTGRKWWMEADEPWQALACCMEIAQVVHSRDPTTYISHFPVHQDGSCNGLQH 920
tr|A0A5N4CMK3|A0A5N4CMK3_CAMDR ILDSADQPMTGRRWWMEADEPWQALACCMEIARATRARDPAAYISHFPVHQDGSCNGLQH 931
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tr|A0A340XK43|A0A340XK43_LIPVE ILDSADRPMAGRKWWMEADEPWQALACCMEVARAVRARDPTAYVSHFPVHQDGSCNGLQH 928


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sp|O00411|RPOM_HUMAN YAALGRDSVGAASVNLEPSDVPQDVYSGVAAQVEVFRRQDAQRGMRVAQVLEGFITRKVV 990
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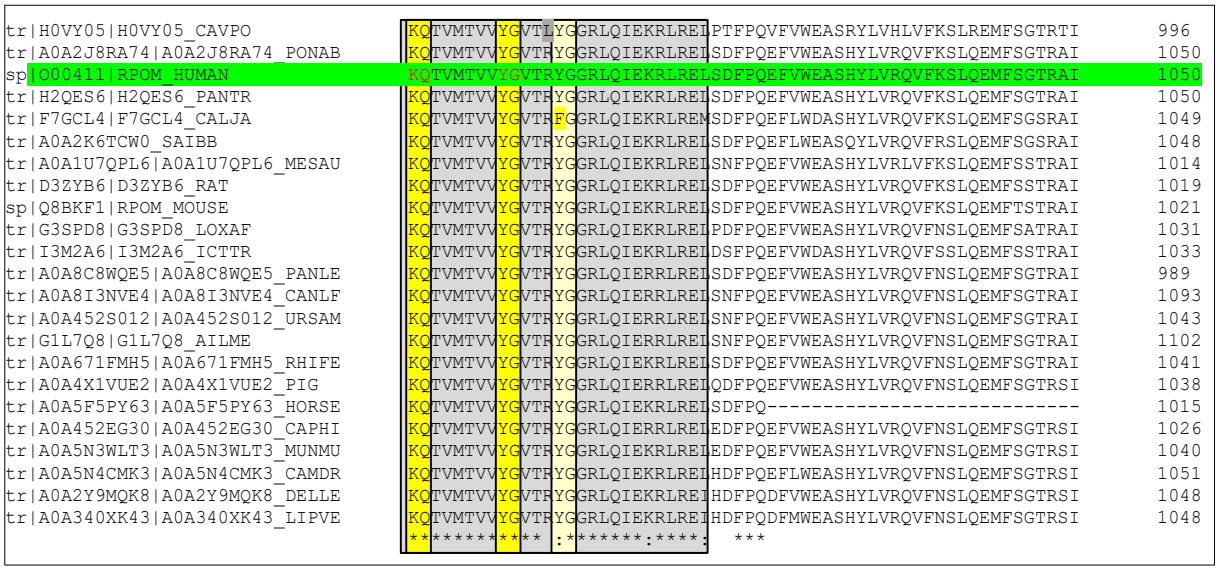
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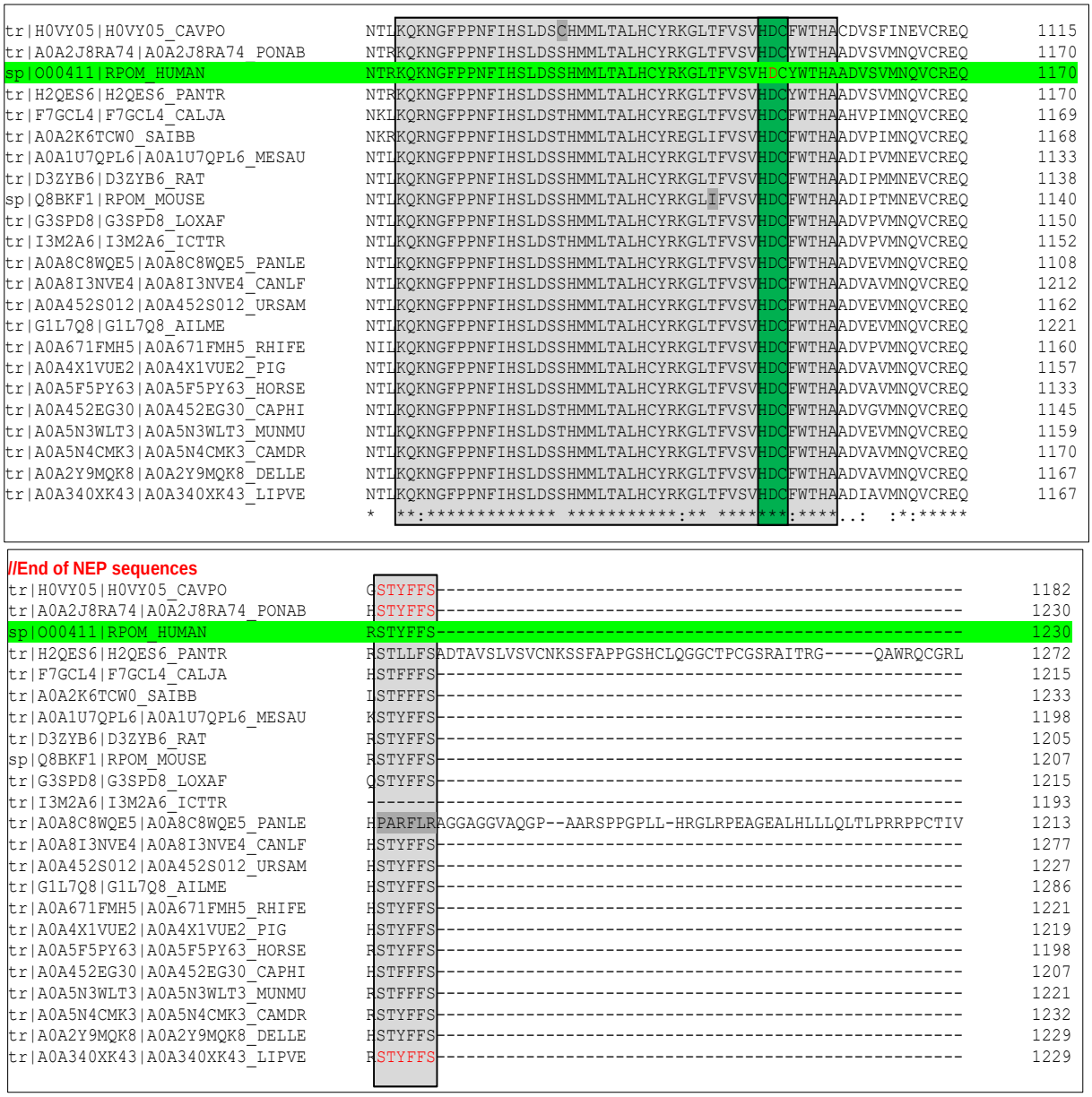

Figure 4 MSA of mitochondrial RNA polymerases (NEPs) from human and various animal sources

H0VY05_CAVPO *Cavia porcellus* (Guinea pig)
A0A2J8RA74_PONAB *Pongo abelii* (Orangutan)
O00411|RPOM_HUMAN *Homo sapiens* (Human)
H2QES6_PANTR *Pan troglodytes* (Chimpanzee)
F7GCL4_CALJA *Callithrix jacchus* (New world monkey)
A0A2K6TCW0_SAIBB *Saimiri boliviensis* (Squirrel monkey)
A0A1U7QPL6_MESAU *Mesocricetus auratus* (Hamster)
D3ZYB6_RAT *Rattus norvegicus* (Rat)
Q8BKF1|RPOM_MOUSE *Mus musculus* (Mouse)
G3SPD8_LOXAF *Loxodonta Africana* (Elephant)
I3M2A6_ICTTR *Ictidomys tridecemlineatus* (Squirrel)
A0A8C8WQE5_PANLE *Panthera leo* (Lion)
A0A8I3NVE4_CANLF *Canis lupus familiaris* (Dog)
A0A452S012_URSAM *Ursus americanus* (Bear)
G1L7Q8_AILME *Ailuropoda melanoleuca* (Panda)
A0A671FMH5_RHIFE *Rhinolophus ferrumequinum* (Bat)
A0A4X1VUE2_PIG *Sus scrofa* (Pig)
A0A5F5PY63_HORSE *Equus caballus* (Horse)
A0A452EG30_CAPHI *Capra hircus* (Goat)
A0A5N3WLT3_MUNMU *Muntiacus muntjak* (Deer)
A0A5N4CMK3_CAMDR *Camelus dromedaries* (Camel)
A0A2Y9MQK8_DELLE *Delphinapterus leucas* (Whale)
A0A340XK43_LIPVE *Lipotes vexillifer* (Dolphin)

Figure 5 shows the 'Mix and Match' MSA of all the three NEPS from the human mitochondria and from the mitochondria and chloroplasts from plants (*A. Thaliana*) (Only the regions required for the discussions are shown here). Even though the DEDD-superfamily of PR exonuclease active site amino acids are completely conserved in all the three NEPs from human and plant, the first triad does not align. However, all the other three active site amino acids align and are completely conserved in all three NEPs (highlighted in light blue) (Fig. 5). The RNA polymerase active site and metal-binding site amino acids are completely conserved in all three NEPs (highlighted in yellow and dark green, respectively). Putative ZBMs are highlighted in orange. The NEPs from plant mitochondria and chloroplasts end in –YFFN- whereas the human and animal NEP ends in –YFFS- (Figs. 4, 5).

CLUSTAL O (1.2.4) 'Mix and Match' MSA of all the three NEPs from both human and plant

Figure 5 'Mix and Match' MSA of all the three NEPs from both human and plant

Table 2 DEDD-superfamily exonuclease active site amino acids and their distance conservations.

Adapted from Palanivelu [58].

Sc, Saccharomyces cerevisiae; Hs, Homo sapiens.

*The distance between the proton acceptor (H/Y) and the last D are highly conserved to 3 to 4 amino acids.

Active site amino acids confirmed by SDM analysis are highlighted in dark blue and by X-ray are highlighted in light blue.

Similar SDM-confirmed active site amino acids are found in *E. coli* DNA pol I

Table 3 Conserved catalytic core regions of various RNA and DNA polymerases

Polymerase type	Catalytic core
Viral T7 SSU RNA pol	-620WLAY-8GVTR-4SVTKR1SVMTLAY8GS-
Viral SP6 SSU RNA Pol	-612WDSI-8GITR-4SLTKK1PVMTLPY8GS-
Mitochondrial RNA pol (*Sc*) (SSU)	-1009TR-4KVVKQ1TVMTNVY8GV-
Mitochondrial RNA pol (*Hs*) (SSU)	-986TR-4KVVKQ1TVMTVVY8GV-
E. coli DNA pol I (SSU)	-753QR-4RSAK758A1INFGLIY8GM-
Chloroplast DNA pol IA (*ARATH*) (SSU)	-873ER-4RKAK878M1LNFSIAY8GK-
Chloroplast DNA pol IB (*ARATH*) (SSU)	-857ER-4RKAK862M1LNFSIAY8GK-
Chloroplast RNA pol (NEP) (*ARATH*) (SSU)	-764DR-4KLVK769Q1TVMTSVY8GV-
Mitochondrial RNA pol (NEP) (*ARATH*) (SSU)	-747DR-4KLVK752Q1TVMTSVY8GV-
Mitochondrial RNA pol (NEP) (*Hs*) (SSU)*	-986TR-4KVVK991Q1TVMTVVY8GV-
Mitochondrial DNA pol γ, POLG1 (*Hs*)*	-942SR-4EHAK947I1FNYGRIY8GA-

Adapted from Palanivelu [58]. **Present work.*
Sc, Saccharomyces cerevisiae; ARATH, Arabidopsis thaliana. Hs, Homo sapiens;
The active site amino acids, highlighted in dark blue, are confirmed by SDM and other techniques.

4. Conclusion

Human mtDNA plays a crucial role in health and diseases [9, 59]. From the recent reports, it has become clear that mutations of *POLG1* are a major cause of many human diseases. For example, its damage is implicated in a larger number of human diseases, including neurodegenerative diseases (AD, HD, PD, ALS), cancer, diabetes, aging, etc. Many of the disease mutations are located in the DNA replicase (*POLG1*) gene. Therefore, the enzymes responsible for the mitochondrial DNA replication and transcription are analyzed for their polymerase and PR functions and their mutational consequences. The present study reveals that the mitochondrial DNA replicase, POLG1, shows a typical polymerase, DEDD(Y)-superfamily of PR exonuclease and (dRP)-lyase (BER) active site amino acids. Similarly, the mitochondrial RNA polymerase (NEP) also shows a typical polymerase, but DEDD(H)-superfamily of PR exonuclease active site amino acids. The polymerase and PR exonuclease active sites are in close agreement with the already reported RNA/DNA polymerases. The POLG1 and its mtDNA replication repair pathways are limited and could not repair all types of mutations that occur in mtDNA. As the POLG1 replicase possesses only the PR exonuclease and basic BER pathways, it is possible that the various lesions, adducts and double-stranded DNA breaks that occur on the mitochondrial genome are not repaired by these basic pathways, which could eventually lead to mtDNA damage/depletion, resulting in mitochondrial diseases. Two of the dominant mutations that cause heritable, autosomal mitochondrial diseases, viz. multi-systemic mitochondrial disease, Parkinsonism and PEO, are located within the POLG1 catalytic core region. They are the nucleotide selection (R^{943}) and template–binding (Y^{955}) amino acids of the POLG1 catalytic core.

Compliance with ethical standards

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Disclosure of conflict of interest

No conflict of interest to be disclosed.

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