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Review Article

Modulation of Mutagenesis in Eukaryotes by DNA Replication Fork Dynamics and Quality of Nucleotide Pools

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The rate of mutations in eukaryotes depends on a plethora of factors and is not immediately derived from the fidelity of DNA polymerases (Pols). Replication of chromosomes containing the anti-parallel strands of duplex DNA occurs through the copying of leading and lagging strand templates by a trio of Pols α, δ and ε, with the assistance of Pol ζ and Y-family Pols at difficult DNA template structures or sites of DNA damage. The parameters of the synthesis at a given location are dictated by the quality and quantity of nucleotides in the pools, replication fork architecture, transcription status, regulation of Pol switches, and structure of chromatin. The result of these transactions is a subject of survey and editing by DNA repair. Environ. Mol. Mutagen. 53:699–724, 2012.

Key words: DNA polymerases; nucleotide pools; mutagenesis; Okazaki fragments

ACCURACY OF DNA REPLICATION AND MUTAGENESIS: OVERVIEW

In multi-cellular organisms, programmed variation of mutation rates and changes in transcription lead to switches of developmental programs and adaptive responses; their aberrant regulation in pathology causes cancer and other diseases [Loeb et al., 1974; Jackson and Loeb, 1998]. The proper function of the eukaryotic genome is contingent upon the accurate replication and repair of genetic material, which relies on coordinated DNA synthesis reactions by multiple DNA polymerases (Pols). The fidelity of the individual Pols determines the accuracy of synthesis of specific DNA patches by an assigned enzyme. With and without DNA damage, hereditary changes happen only when DNA adducts or replication errors are converted into new information in both strands of duplex DNA. In this sense, all mutagenesis is mediated by DNA Pols and even highly accurate enzymes take responsibility for the mutation load, participating in

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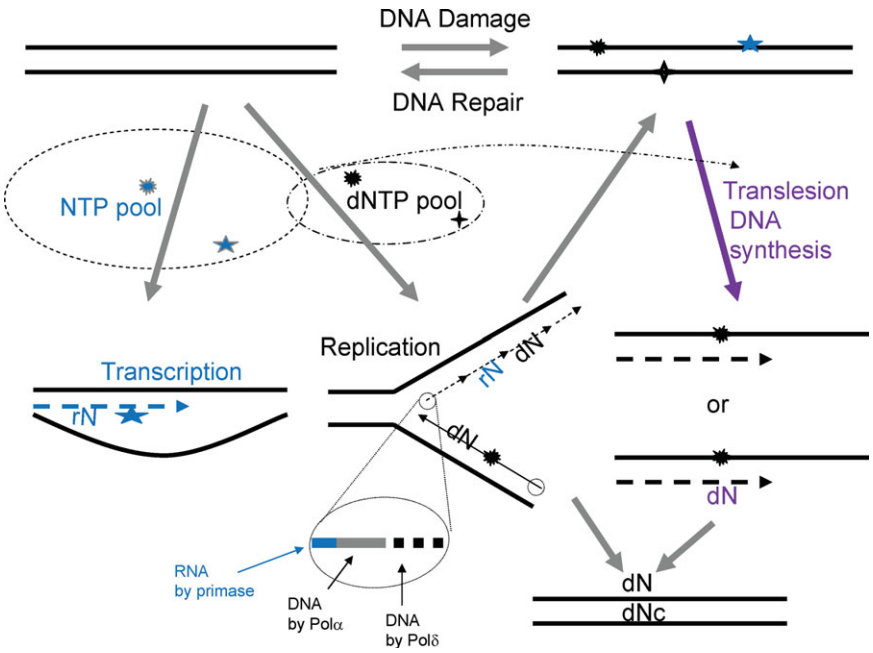

Fig. 1. Nucleic acid metabolism and the origin of mutations. New RNA and DNA are made on undamaged templates with ribo- (rN) and deoxy (dN) nucleosidetriphosphates. The rN are predominantly used by transcription apparatus, but are needed for RNA priming by primase (insert below, illustrating sequential action of primase, DNA Pol α, and Pol δ). They can also be rarely incorporated into DNA by DNA Pols. N stands for any normal but incorrect nucleotide symbolizing a change from the initial DNA sequence. Star-like shapes represent modified bases in nucleotide pools or in nucleic acid. Mutation in duplex DNA is represented by dN/dNc, where dNc is a nucleotide complementary to dN. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mutation fixation. The problem of mutagenesis, however, is bigger than a mere sum of transactions by Pols. Here we highlight the recent novel discoveries in the literature and provide original data from our laboratory illustrating how the replication environment affects the function of DNA polymerases. Due to page limitations, we will not discuss DNA Pols of Y and X families and redirect the reader to excellent reviews [Sweasy, 2003; Sweasy et al., 2006; Yang and Woodgate, 2007; Sale et al., 2012]. We will only briefly mention the enormous field of genetic instability which is caused by a massive creation of base analogs in DNA by editing deaminases [Conticello et al., 2007; Liu and Schatz, 2009].

The nucleic acid metabolism includes transcription, replication, repair and recombination (Fig. 1). All of these transactions influence mutagenesis. Transcription affects DNA structure and leads to an elevation of mutagenesis by a variety of ways [Kim and Jinks-Robertson, 2012]. Replication errors accumulate during copying of undamaged templates, due to the intrinsic limits of accuracy of DNA Pols [Kunkel, 2004; Pavlov et al., 2006b; McCulloch and Kunkel, 2008], elevated or imbalanced levels of precursors in the dNTP pools [Chabes et al., 2003; Mathews, 2006; Kumar et al., 2011], the presence of base analog nucleotides [Maki and Sekiguchi, 1992; Negishi et al., 1994; Kozmin et al., 1998; Kamiya, 2010], or the combination of these factors. For example, decreased DNA Pol fidelity may lead to elevated mutagenesis due to errors of polymerization of normal nucleotides plus elevated levels of incorporation of non-canonical precursors [Shcherbakova and Pavlov, 1996; Nick McElhinny et al., 2010; Williams et al., 2012]. DNA Pols can occasionally incorporate rNTPs from the relatively larger pool of RNA precursors (Fig. 1). This contributes to genome instability [Nick McElhinny et al., 2010; Kim et al., 2011]. Ribonucleotide incorporation by primase for the synthesis of RNA primers is a regular process during replication that happens with every initiation of Okazaki fragment (Fig. 1, insert), but this patch is thought to be completely removed during the maturation [Kao and Bambara, 2003]. Yeast mutants with a partially defective primase, however, are mutators [Longhese et al., 1993], suggesting that, under certain conditions, the change in parameters of regular rNTP incorporation could be a source of genetic instability.

Intact DNA molecules are replicated with high fidelity (up to 10^{-11} per base replicated) due to three sequential fidelity control steps: base selection by DNA polymerases, exonucleolytic proofreading and DNA mismatch repair (MMR) [Morrison et al., 1993; Schaaper, 1993; Kunkel and Bebenek, 2000]. Reduced base selectivity causes a mutator effect [Niimi et al., 2004; Li et al., 2005; Venkatesan et al., 2006; Pursell et al., 2007b; Tanaka et al., 2010]. Proofreading elevates the fidelity of DNA synthesis, and

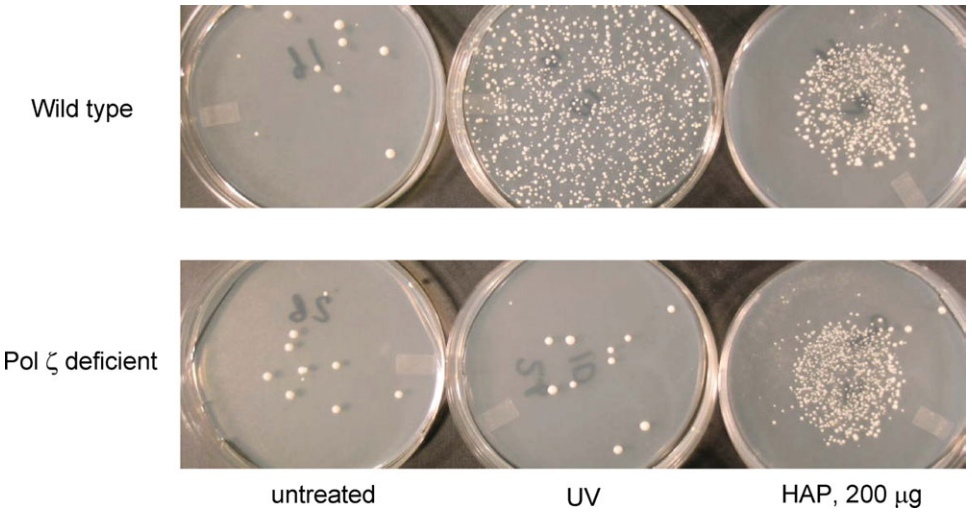

Fig. 2. Two types of mutagens: misrepair vs. misreplication. Spot-test for induction of *can1* mutants in wild-type yeast 8C-YUNI101 strain [Pavlov et al., 2001] and its *rev3::LEU2* disruption derivative. UV light is strongly mutagenic only in the wild-type strain, while HAP spotted in the center of the plates induces mutations with equal efficiency in both strains. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

mutants with defective proofreading are mutators [Morrison et al., 1991, 1993]. The DNA polymerase errors that escaped proofreading are corrected by post-replicative MMR [Modrich and Lahue, 1996; Harfe and Jinks-Robertson, 2000; Kunkel and Erie, 2005]. The failure of both proofreading and MMR leads to a synergistic increase in mutation rate, often called an ''error catastrophe,'' and death in haploid yeast [Morrison et al., 1993]. This underscores the importance of the pathways of error removal that act in series. The MMR repair system recognizes base-base and insertion/deletion mismatches in double-stranded DNA and corrects them through a precisely regulated process, involving mismatch excision followed by correct DNA synthesis [Kadyrov et al., 2006]. The DNA synthesis step in MMR is accomplished by PCNA-dependent DNA polymerase, mostly Pol δ, but the involvement of Pol ε is also possible [Longley et al., 1997; Gu et al., 1998; Tran et al., 1999; Zhang et al., 2005]. The multiplicative increase in the mutation rates of a given mutant when combined with the MMR defect indicates that the cause of the mutator effect were replication errors.

DNA molecules with errors arising during replication or damaged directly (Fig. 1, right upper corner) are the subject of DNA repair, or tolerance by so called ''error free bypass'' or by translesion DNA synthesis. In most cases, repair requires an undamaged template and, when it utilizes recombination, a homologous DNA [Friedberg, 2006]. The synthesis opposite a lesion during bypass reaction is accomplished by specialized DNA Pols with a relaxed active site, often tailored for specific lesions [Yang and Woodgate, 2007; Biertumpfel et al., 2010]. This only partially solves the problem of bypass of persisting damage, because the extension from the aberrant primer termini is not a trivial task. The final bypass often associated with mutation fixation is achieved by a switch to a specialized ''extender'' DNA Pols and then, most likely, to further synthesis by major replicative DNA Pols. The most prominent extender is Pol ζ, which belongs to the same the B-family as major replicative Pols. The whole chain of reactions leading to damage bypass uses specialized machinery and, therefore, is a form of DNA repair.

The crucial role of extender Pols is illustrated by the fact that the frequency of induced mutagenesis declines to negligible values when Pol ζ is inactivated [Lawrence et al., 2000; Gan et al., 2008]. The dependence of mutagenesis on Pol ζ allows for robust and unambiguous classification of mutagens into misreplication versus misrepair classes (Fig. 2). Classical ''misrepair'' mutagen, UV light, does not induce mutations in yeast with deletion of the *REV3*. Most mutagens act via this pathway. Quite contrary, mutagenesis by base analogs (Fig. 2), or mutagenesis caused by DNA Pol errors, such as those developed in proofreading exonuclease-deficient background, is independent of Pol ζ [Shcherbakova et al., 1996; Datta et al., 2000].

All of these transactions occur in the eukaryotic cell in the chromatin environment that has an impact on every DNA metabolism transaction [Ransom et al., 2010]. Chromatin structure is one of the important, but understudied, factors modulating mutation rates and shaping the eukaryotic genome [Sasaki et al., 2009].

Replicative DNA Pols

The four Pols, α, ε, δ, and ζ, that are critical for replication and successful cell proliferation [Lange et al.,

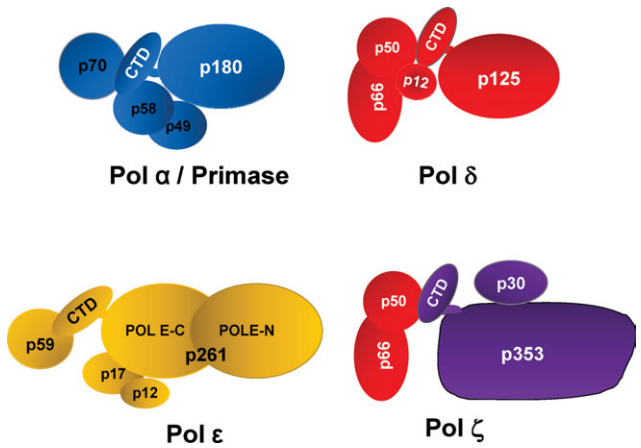

Fig. 3. Subunit structure of human DNA polymerases of the B-family. Pols are color-coded. Pol δ and Pol ζ have two common subunits. The catalytic subunits are called POLA1 or p180, POLD1 or p125, POLE or p261 and REV3L or p353 in humans. In budding yeast, the subunits are called Pol1, Pol2, Pol3, and Rev3, respectively. The B-subunits of Pol α, Pol δ, and Pol ϵ in humans and in yeast (in brackets) are POLA2 or p70 (Pol12), POLD2 or p50 (Pol31) and POLE2 or p59 (Dpb2), respectively. The second subunit of Pol ζ is MAD2L2 or hREV7 or p30 (Rev7). The third or C-subunit of Pol δ is POLD3 or p66 (Pol32). The smaller subunit of Pol δ is POLD4 or p12 (absent as a separate protein in budding yeast, called Cdm1 in *S. pombe*). Small subunits of Pol ϵ are POlE3 or p17 (Dpb3) and POLE4 or p12 (Dpb4). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

2012], belong to the B-family. In the last 5 years, there has been marked progress in the understanding of their structures and functions [Johansson and Macneill, 2010] [Tahirov, 2012] (Fig. 3). Their catalytic subunits possess the same general arrangement of essential motifs on the primary amino acid sequence [Pavlov et al., 2006b; Gan et al., 2008; Tahirov et al., 2009]. In addition to the polymerase domain, all Pols have relatively conserved sequences for the 3′→5′ exonuclease domains. Pol α and ζ, however, lack exonuclease activity, because the sequence of catalytic motifs in the Exo domains is altered and catalytic residues are wiped out. This suggests that ancestor enzymes possessed proofreading activity that was lost in evolution during specialization of Pol functions. The catalytic core of yeast Pol δ aligns with RB69 DNA Pol in the general plan of organization with unique structural features [Swan et al., 2009]. All Pols possess remnants of the uracil recognizing domain but do not sense uracil like their archael homologs [Wardle et al., 2008]. The C-terminal domain of Pols (CTD) has two cysteine-rich metal binding motifs (MBM1 and MBM2) critical for the assembly of the holoenzymes [Dua et al., 1998; Sanchez Garcia et al., 2004; Klinge et al., 2009; Tahirov et al., 2009]. Before 2012, both of these sites were thought to bind zinc. These two zinc atoms were indeed present in the structure of CTD of the yeast Pol α [Klinge et al., 2009]. The understanding of Pols changed drastically when it was found that MBM2 at the CTD of the catalytic subunits of yeast B family Pols and human Pol δ and ζ Pols coordinates the iron-sulfur cluster [Netz et al., 2012b; Baranovskiy et al., 2012]. Mutations affecting MBM2 in yeast in either Pol δ or Pol ζ lead to the absence of induced mutagenesis [Giot et al., 1997; Baranovskiy et al., 2012; Johnson et al., 2012]. The possible role of iron will be discussed in the next section.

The size of the catalytic subunits widely varies due to the presence of additional and, sometimes very long, N-terminal (in yeast Rev3, human REV3L and yeast Pol1, human POLA) or C-terminal extensions (in yeast Pol2 and human POLE). The structure and roles of these extensions are mainly unknown. It was recently found that the anomalously large catalytic subunit of Pol ε is a fusion of two distinct, active and inactive, Pols of the B family (Fig. 3), [Tahirov et al., 2009].

Functional B-family Pols in eukaryotes are multi-subunit complexes (Fig. 3). The nomenclature for names of human and yeast homologs is described in the legend to this figure. Pol α is a four-subunit complex [Muzi-Falconi et al., 2003] (Fig. 3, top left). This complex is responsible for the initiation of Okazaki fragments by concerted action of primase and DNA Polα. All four subunits are essential. The largest subunit, p180, is a catalytic polypeptide capable of relatively accurate and robust but low-processivity DNA synthesis [Takada-Takayama et al., 1990; Copeland and Wang, 1991; Mizuno et al., 1999]. The current model is partially based on the crystallography of yeast Pol α fragments, and partially based on low-resolution electron microscopy (EM) images. This suggests that one larger domain has all the structural elements required for the DNA polymerase reaction and is connected by a flexible linker to the C-terminal Zn-finger domain responsible for interactions with the other subunits [Klinge et al., 2009; Nunez-Ramirez et al., 2011]. The smallest polypeptide (p49) in the four-subunit complex is the catalytic primase subunit. It is tightly associated with the larger accessory primase subunit (p58) that, in turn, interacts with the CTD of the catalytic subunit of Pol α. The second largest primase subunit has a unique iron-sulphur domain, which is essential for the priming reaction in addition to the primase catalytic subunit and is also responsible for the association with the origin recognition complex [Uchiyama and Wang, 2004; Klinge et al., 2007; Vaithiyalingam et al., 2010].

Pol δ is a complex of four (three in budding yeast) polypeptides [MacNeill et al., 2001; McHenry, 2003; Garg and Burgers, 2005; Tahirov, 2012] (Fig. 3, top right). The largest catalytic subunit (p125) has DNA polymerase and 3′→5′ exonuclease active sites, as well as sites for protein–protein interactions [Sanchez Garcia et al., 2004; Sanchez Garcia et al., 2009, Baranovskiy, 2012 #70], including the PCNA binding motif [Garg and Burgers, 2005]. One distinguishing characteristic of Pol δ is that its processivity and robust activity is contingent

upon PCNA and accessory factors [Prelich et al., 1987].The essential second subunit (p50) serves as a stabilizer for the catalytic subunit and as a matchmaker with the third subunit (p66). In yeast, mutations that abolish interactions between the second and third subunit phenocopy the deletion of the gene encoding for the third subunit [Baranovskiy et al., 2008]. The third subunit (often called C-subunit) plays several important roles. The N-terminal part stabilizes the second subunit. We hypothesized that this domain is actually an integral part of the second subunit (B-subunits) of Pol α and Pol ε [Baranovskiy et al., 2008]. The available NMR structure of 75 amino acid N-terminal fragments of the second subunit of Pol ε with a 24 amino acid tag sequence [Nuutinen et al., 2008] is difficult to interpret in the context of the Pol complex. The proof of our hypothesis awaits the determination of the corresponding full crystal structures.

The third subunit has a conserved PCNA-binding motif and a motif that mediates interaction with Pol α [Gomes and Burgers, 2000; Gray et al., 2004; Johansson et al., 2004]. However, the corresponding gene, *POL32*, is dispensable for growth in budding yeast [Garg and Burgers, 2005]. Deletion of this gene leads to ultraviolet (UV) sensitivity and immutability, essentially the same phenotype as deletions of *REV3, REV7,* and *REV1* genes encoding for components of translesion synthesis (TLS) machinery. This suggests a role of the yeast Pol32 protein in the regulation of error-prone TLS [Lawrence, 2002; Prakash et al., 2005; Pavlov et al., 2006b]. Pol32 interacts with Rev1 and can recruit Pol ζ via this interaction, which could explain the role of this subunit in induced mutagenesis [Acharya et al., 2009; Pustovalova et al., 2012]. A new finding of sharing subunits of Pol δ and Polζ allow for additional opportunities for the explanation of these effects (see the discussion in the next section). The role of the fourth subunit (p12) is less defined. The deletion does not result in noticeable phenotypes in fission yeast [Reynolds et al., 1998], while the experiments with human enzyme suggested that it plays a regulatory role in Pol δ response to DNA damage [Zhang et al., 2007].

Pol ε is a four-subunit complex [Pospiech and Syvaoja, 2003; Pursell and Kunkel, 2008] (Fig. 3 bottom left) with an available yeast low-resolution cryo-EM structure [Asturias et al., 2006]. The largest catalytic subunit (p261) has robust processive DNA polymerase and proofreading exonuclease activity alone, and even without a C-terminal half [Morrison et al., 1990; Maki et al., 1998; Bermudez et al., 2011]. The second subunit (p59) mediates protein-protein interactions within the holoenzyme and is essential. Mutations in the corresponding gene in yeast that weaken these interactions confer a mutator phenotype [Jaszczur et al., 2009], which is increased synergistically when either proofreading or mismatch repair are not operating [Jaszczur et al., 2008]. The exact mechanism of the generation of replication errors under these conditions has to be determined. The third (p17) and fourth (p12) subunits bind directly to the catalytic subunit [Bermudez et al., 2011] and are thought to be involved in the interaction with double-stranded DNA [Tsubota et al., 2003; Asturias et al., 2006], but in yeast are not required for growth. The absence of the third and fourth subunit in yeast results in an elevation of spontaneous mutagenesis [Araki et al., 1991]. This elevation is partially dependent on the *REV3* function and independent of mismatch repair [Northam et al., 2006; Aksenova et al., 2010]. The fourth subunit is involved in chromatin remodeling [Iida and Araki, 2004]. Pol ε is additionally regulated by multiple accessory factors involved in origin recognition [Pospiech and Syvaoja, 2003; Takayama et al., 2003; Pursell and Kunkel, 2008] and chromatin structure [Li et al., 2011].

Pol ζ has been isolated from yeast in the moderately active form as a two-subunit complex [Nelson et al., 1996; Johnson et al., 2000; Zhong et al., 2006]. A heterodimer of the Rev3 protein (p353 in humans) and the second subunit encoded by the *REV7* gene (p30 in humans) has DNA polymerase activity and is uniquely proficient in the extension of mismatched primer termini [Lawrence, 2002; Prakash et al., 2005]. The activity of this form of Pol ζ is enhanced by PCNA [Garg et al., 2005]. It is interesting that the interaction with PCNA of both Rev3 and translesion Pol of Y-family Rev1 occurs via a distinct site from other Pols [Northam et al., 2006; Sharma et al., 2011]. Our recent studies suggest that human Pol ζ is actually a four subunit complex (Fig. 3, bottom right), in which the C-terminal part of the catalytic subunit interacts with two subunits of Pol δ [Baranovskiy et al., 2012]. Similar conclusions were reached for yeast Pol ζ [Johnson et al., 2012]. If this is a major form of Pol ζ, the activity, fidelity and PCNA stimulation data obtained with the two-subunit complex should be revisited to accommodate for the possible effects of the new subunits.

The genetic data suggest that the role of Pol ζ as a key player in TLS is conserved between yeast and humans [Li et al., 2002; Gan et al., 2008]. In addition, the human homolog of the second subunit of the yeast enzyme may have an additional role in regulating cell cycle progression. The human REV7 (p30) is similar to the spindle checkpoint protein MAD2 and was reported to interact with MAD2 in vitro [Murakumo et al., 2000].

The evolutionary history of the four polymerases is very complex [Koonin, 2006; Tahirov et al., 2009]. For example, in addition to the fusion of two Pols to generate Pol ε as discussed previously, the second subunits (so-called B-subunits) of Pol α, Pol δ, and Pol ε share some similarity and have homologs in Archaea [Aravind and Koonin, 1998; Makiniemi et al., 1999]. In Archaea, these polypeptides have 3′->5′ exonuclease motifs and are subunits of a so-called D family of DNA polymerases [Jokela et al., 2005]. Changes of critical catalytic residues in the phosphodiesterase motifs in the eukaryotic orthologs of

B-subunits renders them inactive as nucleases. In addition, the B subunit of human Pol δ possesses an OB-fold DNA binding domain and a surface for interaction with the other subunits [Baranovskiy et al., 2008]. The evolution of metal binding sites of catalytic subunits of all four Pols involves duplication and the use of different ancestors for catalytic subunit of Polε and the other B-family Pols [Tahirov et al., 2009].

Dynamics of Pols at the Replication Fork

The asymmetric nature of the DNA duplex poses topological problems for the replication of the antiparallel strands by a fork moving in one direction. So the replication of the two strands is inherently different [Kornberg and Baker, 1991]. In simple DNA replication systems, such as bacteriophage T4, one B-family DNA polymerase is sufficient for synthesis of leading and lagging strands [Trakselis et al., 2001]. A minimal set for eukaryotes is Pol α and Pol δ, and replication of the mammalian virus SV40 requires only these two Pols for synthesis of both strands [Waga and Stillman, 1994]. Yeast can also use only those two Pols to replicate their whole genome, but at the expense of a poor growth rate [Kesti et al., 1999] and genome instability [Ohya et al., 2002]. Under normal circumstances, robust chromosomal replication in eukaryotes requires three DNA polymerases, Pol α, Pol ε and Pol δ [Garg and Burgers, 2005; Pavlov et al., 2006b; Kunkel and Burgers, 2008]. Pol ζ assists major Pols when they experience difficulties with copying the template [Northam et al., 2006; Northam et al., 2010]. In addition, many specialized TLS polymerases assist replicative polymerases at certain conditions, e.g. DNA damage [Bebenek and Kunkel, 2004; Prakash et al., 2005; Zhong et al., 2006].

The process of replication of the eukaryotic genome is started at multiple sites by origin recognition by Orc1-6 followed by a series of steps, resulting in the assembly of the Cdc45-Mcm2-7-GINS complex, an active helicase [Araki, 2011]. Curiously, Pol ε and its accessory factors are obligatory components of the process and thus it is the first DNA Pol arriving at the emerging replication fork. Its Pol activity is useless at this time, however, because there are no primers available for the synthesis yet. Pol α-primase plays the main role in the actual start of DNA synthesis.

DNA polymerases cannot begin synthesis without primers, while RNA polymerases can. Therefore, specialized RNA polymerases called primases [Kuchta and Stengel, 2010] are indispensable for both the start of leading strand replication and the start of replication of each of the millions of Okazaki fragments on the lagging strands. Primase is a remarkable enzyme. It is able to count the length of RNA primers by itself and synthesizes in vitro 8-10 nucleotide-long unit-length RNA primers and multiples of this unit. It is inhibited by dNTPs and has a quite low fidelity of polymerizing of rNTPS [Sheaff and Kuchta, 1994]. The problem of switching from synthesis of RNA to synthesis of DNA is solved by a dedicated Pol α that works in tight complex with primase [Muzi-Falconi et al., 2003]. The reaction occurs intra-molecularly, without the dissociation of primase and Pol α after the switch inhibits primase [Copeland and Wang, 1993; Sheaff et al., 1994]. Pol α is not processive and lacks an intrinsic proofreading exonuclease. Pol α extends these RNA primers by synthesizing short stretches of DNA. It is believed that the size of these patches is around 20-40 nucleotides [Bullock et al., 1991; Tsurimoto and Stillman, 1991b; Waga and Stillman, 1998; Liu et al., 2004]. A switch then occurs to start the processive synthesis of numerous Okazaki fragments by Pol δ or, rarely, to Pol ε, when it starts the leading strand. RNA primers as well as most of the DNA synthesized by Pol α are removed by nucleases: RNase H, Dna2, and flap endonuclease FEN1 [Kao and Bambara, 2003; Burgers, 2009; Zheng and Shen, 2011; Gloor et al., 2012; Reijns et al., 2012].

It was commonly accepted that Pol α synthesizes a small amount of DNA in comparison to other replicative DNA polymerases – 1/10 of Okazaki fragment, estimated to be from 165 to 250 base pairs, [Waga and Stillman, 1998] [Pearson et al., 2005; Smith and Whitehouse, 2012]. Thus, the a priori is not expected to contribute to the accuracy of genomic DNA replication. Moreover, these patches are removed with RNA primer during Okazaki fragment maturation, or errors by Pol α are the subject of correction by Pol δ [Pavlov et al., 2006a] and by MMR [Niimi et al., 2004; Pavlov et al., 2006a]. There is also a possibility that some of these errors are corrected by flap endonuclease [Rumbaugh et al., 1999]. However, mutations reducing base selectivity of Pol α (for example, *pol1-L868M* in yeast) can lead to very strong mutator phenotypes when combined with mismatch repair defects [Liu et al., 1999; Pavlov et al., 2001; Gutierrez and Wang, 2003; Ogawa et al., 2003; Niimi et al., 2004; Pavlov et al., 2004; Tanaka et al., 2010]. Thus, Pol α plays an important role in maintaining genome stability. The nucleotide selectivity of Pol α contributes to the overall mutation rate [Niimi et al., 2004; Tanaka et al., 2010].

After a short patch of synthesis of RNA-DNA primer a switch occurs to DNA synthesis by the more accurate proofreading proficient Pols. The switch to DNA Pol δ was investigated quite thoroughly in the SV40 system that utilized only two Pols [Tsurimoto et al., 1990; Tsurimoto and Stillman, 1991a; Tsurimoto and Stillman, 1991b], while the switch to Pol ε is understudied. Pol accessory factors RFC play the main role in the switch. RFC limits the extent of synthesis by Pol α [Tsurimoto and Stillman, 1991b; Mossi et al., 2000], and along with PCNA, assists in the transfer of the primer to the Pol δ active site [Maga et al., 2000]. Structural details of this reaction have not been studied.

Pol δ then continues the synthesis of the lagging strand until it bumps to the start of another Okazaki fragment [Garg et al., 2004]. Several solid genetic lines of evidence and direct biochemical experiments suggest that Pol δ is operating on the lagging strand *in vivo* [Garg et al., 2004; Garg and Burgers, 2005; Jin et al., 2005; Nick McElhinny et al., 2008]. This Pol is well suited for this, because, contrary to Pol ε, it can idle at the nick until it is ligated. The size of Okazaki fragments in eukaryotes is an order of magnitude smaller than in prokaryotes. Earlier estimates with the SV40 system were 40-290 nucleotides with an average of 135 [Anderson and DePamphilis, 1979]. It was immediately realized that it could be connected to chromatin structure and the size of nucleosomes in eukaryotes [Herman et al., 1979]. The contemporary estimate of the Okazaki fragment in yeast agrees with those earlier results and directly links the size of the fragments to the size of the nucleosomes [Smith and Whitehouse, 2012]. The measurements performed in ligase I deficient strains revealed that there is some heterogeneity of the sizes of Okazaki fragments, but a substantial fraction of them were around 165 base pairs, which parallels the size of the nucleosomal repeat. It is possible that nucleosome assembly by itself regulates the extent of synthesis by Pol δ. Notably, mutations in the genes of post-replicative nucleosome assembly factors (CAF-1) drastically altered the length distribution. Curiously, these mutations do not exert any strong deleterious effects in vegetative yeast cells.

We proposed that the leading DNA strand in eukaryotes is replicated both by Pol ε (near the origins) and Pol δ (most of the leading strand), while the lagging DNA strand is replicated mostly by Pol δ [Pavlov and Shcherbakova, 2010]. The exact roles of these two Pols, which are equally processive with proper accessory factors [Chilkova et al., 2007; Bermudez et al., 2011], are not fully understood in biochemical terms (see reviews [Waga and Stillman, 1998; Pospiech and Syvaoja, 2003; Shcherbakova et al., 2003a; Garg and Burgers, 2005; Pavlov et al., 2006b]). One of the first models of the replication fork, that was proposed after the discovery of Pol ε, postulates that Pol ε is responsible for exclusively copying the leading strand DNA template, and Pol δ is responsible for the lagging strand replication [Morrison et al., 1990; Fukui et al., 2004; Garg and Burgers, 2005; Hiraga et al., 2005; Kunkel and Burgers, 2008]. The model is currently favored, however, there are no mechanistic insights into how the two Pols work together. The evidence is mainly based on genetic observations in yeast with the use of various Pol mutants and sensitized genetic backgrounds that may distort the replication parameters [Morrison and Sugino, 1994; Shcherbakova and Pavlov, 1996; Karthikeyan et al., 2000; Kunkel and Burgers, 2008; Miyabe et al., 2011]. We discussed the evidence for and against this broadly accepted model in-depth [Pavlov and Shcherbakova, 2010]. One of the main arguments against it is that the polymerase activity Pol ε is not absolutely required for replication and could be substituted by another Pol, which is based on the viability of mutants with deletions of the polymerization-proficient half of the catalytic subunit [Kesti et al., 1999].

Similar to the currently accepted model, Pol α synthesizes short RNA-DNA fragments at the origins and on the lagging strand in our model. Pol δ extends these fragments on the lagging strand. The principal novel feature of the model is in the mechanism of leading strand synthesis. We postulate that Pol ε is responsible for the early steps of leading strand synthesis, as well as elongation of the leading strand in the vicinity of the origin. It dissociates from the primer terminus with an increasing probability as the distance from the origin increases, and Pol δ takes over the leading strand synthesis. As a result, the majority of the genome replication involves copying of both DNA strands by Pol δ. When transactions of Pol ε can be tracked genetically, errors attributable to this Pol are found on the leading DNA strand. These traces, however, fade with an increase in distance from replication origins [Larrea et al., 2010]. The extent and nature of the sites of participation of Pol ε in leading strand replication should be a subject of further biochemical and genetics experiments.

The fourth Pol at the replication fork is Pol ζ [Pavlov and Shcherbakova, 2010]. It is critical for replication at difficult template sites or when replicative DNA Pols are compromised [Northam et al., 2010] and during DNA damage bypass [Prakash et al., 2005; Pavlov et al., 2006b; Waters et al., 2009]. Induced mutagenesis associated with bulky lesions completely depends on Pol ζ, therefore, this Pol operates on damaged sites on both leading and lagging DNA strands.

DNA Pols ζ and δ in Induced Mutagenesis

One property of mutagenesis induced by the majority of DNA damaging agents (Fig. 2) was difficult to explain. Certain mutations affecting the function of Pol δ had the same ''immutability'' phenotype as the complete absence of Pol ζ. A severe decrease of UV and MMS-induced mutagenesis in yeast was described for mutations in the three genes for the subunits of Pol δ: for the point mutation partially destroying the iron cluster binding site in MBM2 of the catalytic subunit, *pol3-13* [Giot et al., 1997], for the mutations affecting the interaction of the second with the third subunit [Baranovskiy et al., 2008], and for the deletions of the whole gene or parts of the gene for the third subunit [Gerik et al., 1998; Huang et al., 2000; Huang et al., 2002; Johansson et al., 2004]. What is the connection between the two Pols apparently playing different roles in replication?

The discovery that human Pol δ shares two accessory subunits with Pol ζ provides a simple explanation of these effects [Baranovskiy et al., 2012]. When Pol δ bumps in to the lesion, its catalytic subunit is exchanged in the complex to the heterodimer of the catalytic subunit of Pol ζ with Rev7 to accomplish the vital role of Pol ζ in mutagenesis. Bacteria are also known to exchange Pols at damaged sites. However, Pols are exchanged using a sliding clamp as a platform for docking/exchange [Furukohri et al., 2008]. In eukaryotes, the exchange appears to be more elaborate, because the docking/exchange platform is provided by the B-subunit of the Pol delta and the exchange is redox-dependent. The initiation of exchange depends on iron-sulfur clusters, which are present in both Pol δ and Pol ζ. Therefore, mutations affecting this site in catalytic subunits of either Pol lead to immutability.

How is this new idea accommodated in the existing models of DNA damage bypass [Prakash et al., 2005; Waters et al., 2009; Ulrich, 2011]? We envision the following. When Pol δ is stalled by a damaged site, PCNA is monoubiquitylated by Rad18/Rad6 [Hoege et al., 2002; Stelter and Ulrich, 2003], which signals to an effector protein to change the oxidation state of iron in the C-terminus of Pol δ. One candidate for the effector protein is Mgs1, which is known to modulate induced mutagenesis and to act downstream from PCNA modification [Saugar et al., 2012]. It is known that iron sulfur cluster function is required for the assembly of the catalytic subunit of Pol δ with accessory subunits [Netz et al., 2012b]. Then, the catalytic subunit of Pol ζ binds to the platform of p50/p66 abandoned by the catalytic subunit of Pol δ [Baranovskiy et al., 2012] and REV7 further strengthens this complex interaction with translesion Pol REV1 [Acharya et al., 2009; Pustovalova et al., 2012]. This complex extends the products of translesion DNA synthesis by Y-family DNA Pols. Subsequent oxidation of iron in the catalytic subunit of Pol ζ results in its dissociation and the binding of a reduced catalytic subunit of Pol δ back to the p50/p66 complex.

Recent biochemical evidence confirms that subunit sharing between Pol δ and Pol ζ is evolutionarily conserved and Pol31 and Pol32 subunits are part of the four-subunit Pol ζ complex in yeast [Johnson et al., 2012]. The full complex is more stable and more active.

It appears that Pol δ is central in the regulation of TLS. Mutations affecting the components of Pol δ responsible for polymerase switches abolish all induced mutagenesis, which means that this happens in both leading and lagging DNA strands. This suggests that either Pol δ is a main replicase for both DNA strands or TLS events on the leading strand should include the switch for Pol δ as an initiating event when this strand is replicated by Pol ε. The possibility of such a switch has never been investigated. If TLS is delayed and not coupled with fast replication [Daigaku et al., 2010; Diamant et al., 2012] the time should be sufficient for more complicated switches of Pols.

The studies of the exact roles and inter-dependence of the iron–sulfur clusters in DNA Pols δ and ζ and PCNA ubiquitylation in Pols switches have just begun, but it is already obvious that the cluster's role in genome stability is dramatic. The insertion of the iron–sulfur cluster into proteins requires elaborate machinery [Lill, 2009], therefore, it is likely that replication and DNA repair depend on the status of iron metabolism. One of the recent highlights is the finding that the classical ''DNA repair'' gene, *MMS19*, encodes for a protein responsible for the insertion of the iron-sulfur cluster into proteins of DNA metabolism, including DNA Pols [Gari et al., 2012; Stehling et al., 2012]. This has broad biological significance, because it opens a previously unrecognized new mechanism of regulation of DNA transactions by mitochondria and by cytosolic factors, where components of Fe/S clusters are synthesized and required for the proper assembly of iron-sulfur-containing proteins [Lill et al., 2012; Netz et al., 2012b].

As sometimes happens, the genetic proof of this concept was provided a decade ago without the realization of its significance, because many genes involved in iron–sulfur biogenesis were not described [Chanet and Heude, 2003]. The aforementioned allele *pol3-13* that encodes for MBM2-defective Pol δ and abolished UV mutagenesis was found to be synthetically lethal with mutations, which later has been found to affect the biogenesis of iron–sulfur cluster, e.g. *nbs35* [Netz et al., 2012a] and *tah18* [Netz et al., 2010]. It is noteworthy that mutations in the *RAD18* gene, which encodes for E3 ligase participating in PCNA monoubiquitylation, rescues lethal *mms19 pol3-13* double mutant combination [Chanet and Heude, 2003]. Because MMS19 is a cytosolic factor inserting an iron–sulfur cluster in Pol δ [Gari et al., 2012], this links the function of the iron cluster in DNA Pols to PCNA modification.

Quality of Nucleotide Pools and Mutagenesis

It is well known that levels of nucleotides affect mutagenesis outcomes [Kunz, 1988; Mathews and Ji, 1992; Kumar et al., 2011]. Another problem is the overlap of NTP and dNTP pools (Fig. 1). As we discussed, ribonucleotides are a regular part of nascent DNA chains (Fig. 1). If the machinery of their removal fails, the remnants of these primers can be left in DNA [Reijns et al., 2012]. In addition, under certain conditions, DNA Pols can directly incorporate ribonucleotides into DNA, causing genetic instability [Nick McElhinny et al., 2010; Miyabe et al., 2011]. Here we will focus on mutagenesis by deoxynucleoside triphosphosphates of base analogs that deceive DNA polymerases and become drivers of replication errors (Fig. 4A).

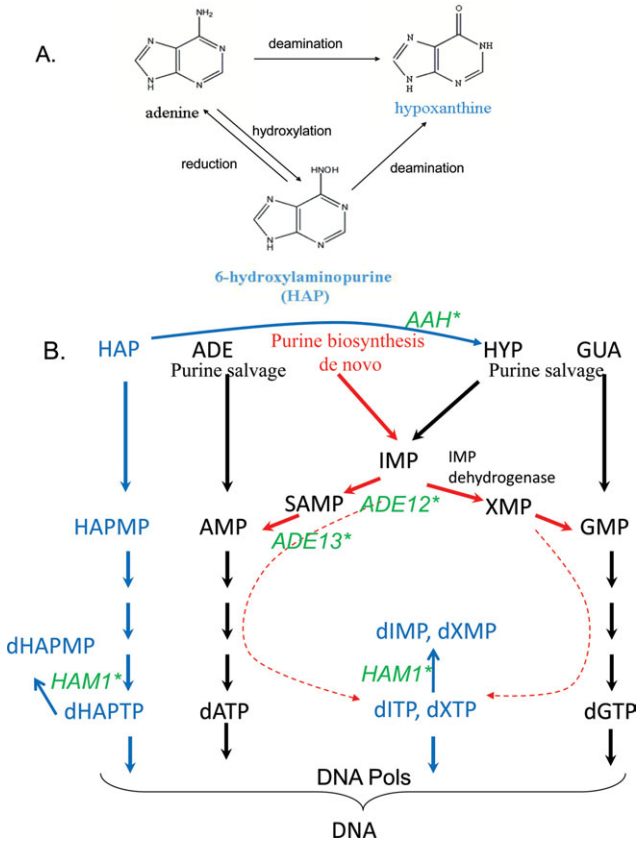

Fig. 4. Pathways of base analogs. **A:** Inter-conversion of adenine (black) and base analog hypoxanthine and HAP (blue). **B:** Salvage pathways and nucleotide biosynthesis de novo as a source of IMP-related base analogs in DNA. Base analogs and their derivatives are in blue font. ADE, adenine; HYP, hypoxanthine; GUA, guanine; SAMP, succinyl-AMP; IMP, inosine-5′-monophosphate; HAPMP, HAP-5′-monophosphate; HAPTP, HAP-5′-triphosphate; and dHAPTP, HAP-2′-deoxyriboside-5′-triphosphate. *Gene inactivation leads to an increase of HYP and xanthine in DNA or in sensitivity to the mutagenic or toxic effect of HAP in bacteria and yeast [Stepchenkova et al., 2005, 2009a; Pang et al., 2012]. *AHH1*in yeast encodes for adenine aminohydrolase; *ADE12* (*ADSSL* in humans) - for SAMP synthase; *ADE13* (*ADSL* in humans) - adenylosuccinate lyase; *HAM1* (*rdgB* in *E. coli* and *ITPA* in humans) – inosine triphosphate pyrophosphohydrolase. Biosynthesis *de novo* (red arrows) generates IMP, then the pathway splits into branches leading to AMP and GMP. A series of well studied reactions (black arrows pointing down, we do not specify participating enzymes for simplicity) lead to dATP and dGTP, respectively. IMP, AMP, and GMP could also be generated by salvage pathway by direct activation of hypoxanthine, adenine, and guanine bases, respectively, and by corresponding phosphoribosyl transferases (black arrows). The exact sequence of events leading to the conversion of IMP and XMP directly to dIMP and dXTP is not known; presumably it happens due to some promiscuity of enzymes activating AMP and GMP (red dotted arrows). HAP activation to dHAPTP (blue pathway on the left) most likely utilized the pathway of conversion of adenine [Stepchenkova et al., 2009a]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Environmental factors and biochemical reactions during oxidative stress and inflammation damage the bases of natural nucleoside triphosphates [Simandan et al., 1998; Colussi et al., 2002; Hakim et al., 2003; Polidori et al., 2003; Dedon and Tannenbaum, 2004]. Such mutagenic contaminants in dNTP pools lead to elevated mutation rates, chromosome instability [Abolhassani et al., 2010], risk of cancer [Sekiguchi and Tsuzuki, 2002], and developmental problems [Behmanesh et al., 2009]. One of the best studied base analogs in the nucleotide pool is 8-oxo deoxyguanosine triphosphate [Maki and Sekiguchi, 1992]. An accumulation of oxyguanine in DNA leads to several diseases, including cancer and age-dependent trinucleotide expansions, leading to neurodegenerative diseases [Kovtun et al., 2007; D'Errico et al., 2008; De Luca et al., 2008].

Cleansing of the precursor dNTP pool from potentially mutagenic nucleotide analogs is an important prerequisite for high fidelity DNA replication [Hochhauser and Weiss, 1978; Ames and Gold, 1991; Maki and Sekiguchi, 1992; Michaels and Miller, 1992; Grollman and Moriya, 1993]. The significance of base analog mutagens is that, in most cases, they are produced within the cells intrinsically, as a result of normal biosynthetic pathways (Fig. 4B). In addition, they are widely exploited by nature to generate genetic instability on demand, such as in the immune system [Chelico et al., 2009], or change gene expression [Guo et al., 2011]. The use of such dangerous agents, capable of changing the genetic information in the genome, demands tight regulation of base analog mutagenesis to avoid genome destruction. In this paper, we discuss the effects of purine base analogs that are generated by normal cell metabolism; hypoxanthine and xanthine are generated by spontaneous or enzymatic deamination of adenine and guanine bases, respectively, and their mimic, hydroxylaminopurine (HAP, Fig. 4A).

IMP and XMP are central intermediates in purine metabolism (Fig. 4B). Inadvertent activation of these compounds into triphosphates (dotted red arrows in Fig. 4B) will result in their incorporation into DNA and lead to clastogenic effects [Budke and Kuzminov, 2009]. When the flow of biochemical reactions in purine biosynthesis in yeast is interrupted genetically (by mutations in the genes regulating the downstream conversions of IMP–*ADE12*, or destruction of aberrant triphosphates containing base analogs–*HAM1*), there is a significant accumulation of base analogs in DNA [Pang et al., 2012] and an increase of HAP mutagenesis [Stepchenkova et al., 2009a]. Under certain conditions, the amount of ITP in the pool reaches 10% of the amount of adenine [Sakumi et al., 2010]. A related compound, HAP, can arise from hydroxylation of adenine and can be converted to adenine or hypoxanthine [Kozmin et al., 2008; Sakumi et al., 2010] (Fig. 4A). Deoxy- and ribonucleoside triphosphates XTP, ITP, and HAPTP are destroyed by the same enzyme, inosine triphosphate pyrophosphatase (ITPA). HAP incorporations are easy to track because they are highly mutagenic. Many systems protect cells from the mutagenic and inhibitory effects of HAP [Pavlov Iu, 1986; Noskov et al., 1996; Kozmin et al., 1998; Kozmin

et al., 2000; Stepchenkova et al., 2005]. This suggests that HAP might be a natural contaminant of dNTP pools [Kozmin et al., 2000]. That makes HAP a biologically relevant tool for the study of DNA replication and effects of the natural base analogs, hypoxanthine and xanthine.

Among multiple mechanisms protecting DNA from these base analogs [Kozmin et al., 1998; Bradshaw and Kuzminov, 2003; Burgis et al., 2003; Kozmin et al., 2008; Budke and Kuzminov, 2009], ITPA is one of the most important enzymes in eukaryotes (Fig. 4B) [Pavlov Iu, 1986; Noskov et al., 1996; Menezes et al., 2012]. ITPA orthologs from humans, yeast and bacteria control the levels of triphosphate forms of hypoxanthine, xanthine and HAP [Lin et al., 2001a; Burgis et al., 2003; Burgis and Cunningham, 2007]. ITPA is highly conserved among different species [Kozmin et al., 1998; Hwang et al., 1999; Lin et al., 2001b]. In *E. coli*, the *rdgB* mutation is synthetically lethal with the *recA* mutation that abolishes homologous recombination [Bradshaw and Kuzminov, 2003; Burgis et al., 2003]. The *rdgB* mutation sensitizes cells to the mutagenic and recombinogenic effects of HAP in the molybdenum-cofactor defective strain background (another system protecting from HAP [Kozmin et al., 2000; Kozmin and Schaaper, 2007; Kozmin et al., 2008]) because of a massive accumulation of breaks in DNA [Burgis et al., 2003; Lukas and Kuzminov, 2006]. DNA damage is caused by intermediates in the repair of base analogs in DNA by Endo V encoded by the *nfi* gene. This has been proven by the viability of triple *rdgB recA nfi* mutants [Burgis et al., 2003]. In most organisms, including yeast *S. pombe* and humans, there are orthologs of endonuclease V [Moe et al., 2003], [Dalhus et al., 2009], but there is no evidence that this enzyme can robustly repair hypoxanthine in DNA. It is known that human APNG can excise at least hypoxanthine [Saparbaev et al., 2000]. We propose that DNA fragmentation and genomic instability, due to the presence of base analogs in DNA in humans, are caused by the intermediates of DNA repair reactions, but the exact players in these reactions are unknown [Waisertreiger et al., 2010; Menezes et al., 2012].

It is worth mentioning that the *rgdB* mutation in *E. coli* is suppressed by an overexpression of the *purA* (involved in the conversion of IMP to AMP in de novo biosynthesis of adenine), thereby unequivocally pointing out that the main source of ITP and deoxy ITP in the pools is normal cellular metabolism [Clyman and Cunningham, 1987]. The relationship between ITPA, ADSS, and ADSL enzymes of purine biosynthesis is indicated by a systems biology approach but it was not studied in human cells experimentally.

ITPases prevent contamination of the nucleotide pool, which could lead to the incorporation of abnormal nucleotides into DNA and RNA or dilution of the ATP/GTP needed by numerous proteins. Underscoring the importance of this process, when *Itpa* knockout mice were developed, it was discovered that more than half of the mice die before birth. Those that do survive display growth retardation, heart abnormalities, ataxia, and abnormal breathing and die within two weeks [Behmanesh et al., 2009]. Furthermore, cells from *Itpa* knockout mice exhibited elevated levels of inosine in the RNA and DNA and had higher levels of chromosomal abnormalities than wild type cells until they are suppressed in later passages by the spontaneous expression of the NUDIX protein NUDT16 [Abolhassani et al., 2010]. This clearly demonstrates that if non-canonical nucleotides are allowed to accumulate, then they can interfere with normal cellular processes and can affect genome stability.

The precise cellular function of human ITPase, ITPA, is not clearly defined. ITPA is expressed in many human tissues [Lin et al., 2001b]. Several polymorphic variants have been identified in the *ITPA* gene, which result in varying degrees of ITPA deficiency. A 94C>A polymorphism, encoding for a protein with a proline to threonine change at amino acid 32 (P32T), is a clinically significant variant, because it causes ITPA deficiency, measured typically in erythrocytes [Cao and Hegele, 2002; Sumi et al., 2002]. Individuals heterozygous for this mutation have approximately 25% residual enzymatic activity, while homozygotes have essentially zero activity, which could be explained if heterodimeric ITPA/ITPA P32T leads to the same defect of ITPA as the ITPA P32T homodimer. The allelic frequency of this mutation ranges from 5-19% with the highest frequency found in the Asian population [Marsh et al., 2004]. Additional gene variants causing ITPA deficiency have been identified, but the P32T mutation leads to the most severe reduction in activity [Bierau et al., 2007].

Base Analog HAP as a Tool for Studying Replication

HAP (Fig. 4A) is a potent universal base analog mutagen with ambiguous base pairing capacity (see reviews [Pavlov et al., 1991; Kozmin et al., 1998]). In an amine state, it forms two hydrogen bonds with ''T'', and in an imine state it makes two hydrogen bonds with ''C.'' Consistent with this, dHAPTP replaces both dATP and dGTP during *in vitro* DNA synthesis by prokaryotic and eukaryotic DNA polymerases [Abdul-Masih and Bessman, 1986]. The proof that the active mutagenic form of HAP is deoxyribonucleoside triphosphate comes from the fact that mutants deficient in a pyrophosphatase specific for abnormal purine triphosphates are hypersensitive to HAP [Noskov et al., 1996; Porta et al., 2006]. Both dCTP and dTTP can be incorporated opposite HAP *in vivo* in yeast [Noskov et al., 1994]. HAP induces both G-C to A-T and A-T to G-C transitions [Shcherbakova and Pavlov, 1993; Noskov et al., 1994; Kulikov et al., 2001]. Most likely, HAP is converted to dHAPTP inside the cell and is

misincorporated into the DNA opposite template C. In the next round of DNA replication, T is incorporated opposite HAP with a high probability [Noskov et al., 1994]. Therefore, the C to T transition pathway is initiated when dHAP is misincorporated opposite the cytosine during replication. The C-containing strand defines the template for C-G to T-A mutagenic replication after HAP treatment. The initial HAP incorporation opposite T and consequent C misincorporation opposite HAP in the next replication cycle leads to an A-T to G-C transition [Shcherbakova and Pavlov, 1996]. This occurs less frequently.

HAP is a very useful tool for studying the mechanism of DNA replication fidelity because its mutagenic action does not depend on general DNA repair systems dealing with DNA damage [Pavlov et al., 1991]. HAP mutagenesis is also not dependent on mutations inactivating damage bypass/specialized DNA polymerase genes, *REV3* (Pol ζ) [Shcherbakova et al., 1996] (Fig. 2), *RAD30* (Pol η) and *REV1* (Pol Rev1) (unpublished observations). Mutants defective in proofreading have elevated levels of HAP mutagenesis, suggesting that errors produced by HAP are subject to proofreading.

In the conclusion for this section we would like to emphasize the dependence of mutagenesis of DNA Pol fidelity and the quality of nucleotide pools. In vivo, DNA Pols utilize natural pools that may have many contaminants. Therefore, the cases of correlation between the fidelity of DNA pols and their inaccurate variants in vitro, when the Pols are highly controlled by experimenters, and mutational signatures of the same Pols in vivo [Pavlov et al., 2002b; Shcherbakova et al., 2003b; Pursell et al., 2007a], could be regarded as fortunate exceptions rather than the rule.

Mutagenesis During the Synthesis of Leading and Lagging DNA Strands in the Presence of Base Analogs

In the case of spontaneous base-pair substitutions, it is difficult to determine the DNA strand where the initial error occurred. For example, the change of a G-C base pair to an A-T base pair could be initiated by a G-dTTP mispair on one strand or a dATP-C mispair on the other strand. In this case, strand assignment relies on assumptions that the ratio of these reciprocal mispairs is different [Iwaki et al., 1996; Fijalkowska et al., 1998]. Assigning a DNA strand where a mutation occurs is more reliable with the use of specific means to target DNA changes to one strand. Possible solutions are strand-specific DNA damage [Veaute and Fuchs, 1993], the use of base analogs [Shcherbakova and Pavlov, 1996], nucleotide pools imbalances [Roberts et al., 1994] or a mutator DNA polymerase with strong asymmetry in rates of dPyTP/Pu versus dPuTP/Py errors [Nick McElhinny et al., 2007; Pursell et al., 2007a]. We are advocates of the base analog approach, because HAP is not as toxic as many mutagens or pool imbalances and it is mutagenic even in wild-type strains, so no specific mutational alteration of replication parameters are required [Kozmin et al., 1998].

In earlier studies, HAP-induced errors occurred preferentially in one DNA strand [Shcherbakova and Pavlov, 1993; Shcherbakova and Pavlov, 1996; Kulikov et al., 2001] and HAP-induced errors were proofread by Pol δ and by Pol ε on opposite DNA strands [Shcherbakova and Pavlov, 1996]. To study this phenomenon in more detail, we placed a reversion reporter gene within chromosome III, in two orientations, and at multiple locations relative to early replication origins [Pavlov et al., 2002a]. The rate of G-C to A-T transitions, resulting from HAP misincorporation, differed by three- to ten-fold, depending on the reporter orientation and its distance from the flanking replication origins. Those results demonstrated that active origins establish a mutational strand bias *in vivo* that is maintained over at least 70,000 base pairs, and reflects lower mutagenesis associated with replication of the lagging strand DNA template. The mechanism of the bias is unknown. It could be connected to the intrinsic property of lagging strand replication, to the identity of Pols replicating leading and lagging DNA strands or the efficiency of MMR on different strands. We have shown that MMR is the most important contributor to strand bias for another base analog, 8-oxoguanine [Pavlov et al., 2003]. We decided to investigate this phenomenon in more depth using a forward mutation system.

Mutational System of the *URA3* Gene

A system for the selection of the *ura3* mutants with 5-fluoroorotic acid was devised in 1984 [Boeke et al., 1984]. The *URA3* is 804 bp-long, not too large for sequencing, yet not too small to limit a variety of sequence contexts where mutations could be found. The first spectrum of induced mutations in the *URA3* gene was published in 1986 [von Borstel and Lee, 1986]. Later, the *URA3* system was used for studies of induced and spontaneous mutagenesis in wild-type strains [Lee et al., 1988]. Mutation spectra were also determined in different polymerase mutants [von Borstel et al., 1993; Morrison and Sugino, 1994; Pursell et al., 2007a; Nick McElhinny et al., 2008]. Rates of mutations in the *URA3* vary about six-fold when the gene is placed artificially at different chromosomal locations. This may be connected to replication timing [Lang and Murray, 2011].

Several studies have utilized the *URA3* gene mutational system to study the effects of carcinogens, 2-acetylaminofluorene [Roy and Fuchs, 1994] and benzopyrene diol epoxide [Xie et al., 2003]. It was found that, under certain conditions, this forward mutation system provides biased estimates of error specificity due to the presence of ''jackpot'' mutation sites. We have found several hotspots for HAP-induced errors in a spectrum with a limited

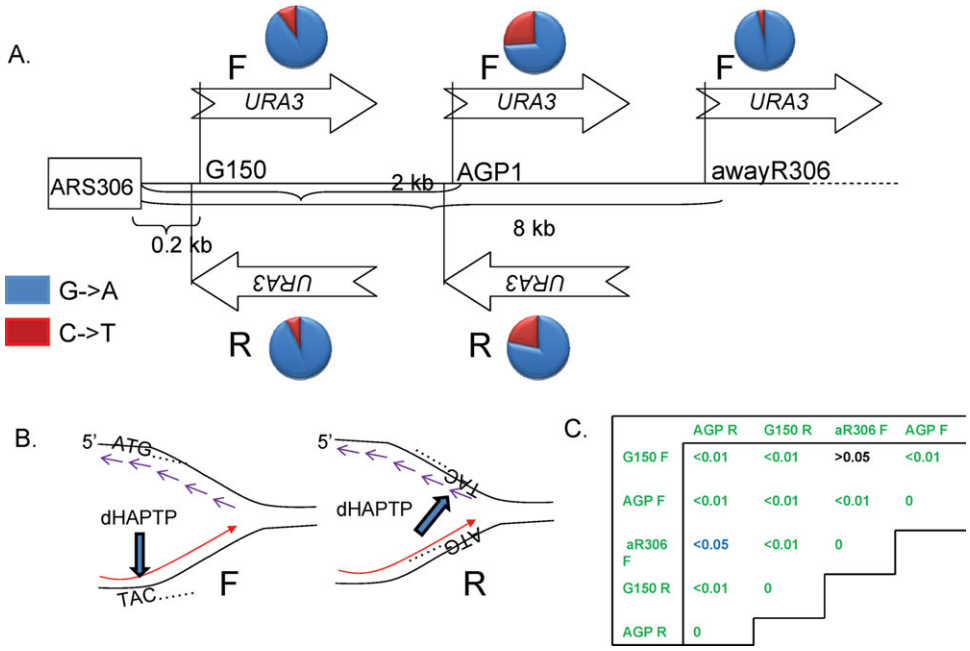

Fig. 5. Specificity of HAP mutagenesis in the *URA3* gene at different distances from the replication origin is independent from the orientation of the gene. **A:** Position and orientations of the reporter gene and the ratios of G to A versus C to T transitions (based on the sequencing of 40 mutants in each position). **B:** To generate a G to A transition, read on non-transcribed strand, dHAPTP is misincorporated opposite C in the leading strand in the forward (F) orientation of the reporter and into the lagging strand in reverse orientation (R). **C:** Statistical analysis of the differences between mutation spectra in different orientations. The analysis was performed as described in [Khromov-Borisov et al., 1999]. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

number of mutations [Shcherbakova and Pavlov, 1993]. In extreme cases, in strains with a variant of Pol ε with low base selectivity, 60% of mutations in the *URA3* target occur in one spot, which severely reduces the power of this forward mutational system [Pursell et al., 2007a].

We placed the *URA3* gene to the right of a strong replication origin ARS306 using PCR fragments of the gene region flanked by the tails homologous to the selected site as described [Pavlov et al., 2002a] in yeast strain 9A-YPOM206 [Baranovskiy et al., 2008] (Fig. 5A). One location is close to the origin, another is 2 kb away and the last one is 8 kb away from the origin. For the two locations closest to the origin, we also inserted the gene in reverse orientation. Treatment by HAP caused a 50-fold increase of mutation rates which were similar in all variants. Most of the mutations were transitions in GC pairs. Pie diagrams near the arrows representing the *URA3* gene in Figure 5A show types of mutational changes represented on the non-transcribed strand of the *URA3*. This sequence corresponds to the sequence of mRNA. Therefore, G to A transitions in forward orientation occur when HAPTP is incorporated opposite C into the leading DNA strand (Fig. 5B). In the reverse orientation, G to A transitions are caused by the incorporation of dHAPTP into the lagging DNA strand.

Mutation spectra of five variants were generally dissimilar: the change of location and the inversion of the reporter resulted in a statistically different distribution of mutations (Fig. 5C). Contrary to our expectations based on the data with the reversion system (see above), the proportion of G to A mutations was independent from the orientation of the gene, but partially dependent on the location (Fig. 5A). It appeared that initial HAP incorporations were predominant when replicating the transcribed DNA strand, irrespective of the direction of replication. The effect could be due to the dependence on transcription or a unique collection of HAP mutable sites in the transcribed strand. To consolidate the data with the previous results, we propose that there are two components of HAP-induced mutagenesis, comprising the final mutation spectra. One, minor component, which we were lucky to reveal in reversion system [Shcherbakova and Pavlov, 1996; Pavlov et al., 2002a], depends on replication direction. The major component depends on transcription or the identity of the transcribed DNA strand of the *URA3* gene, which is clearly visible in the forward spectra. Because of this mixture, the spectra in forward and reverse orientations were statistically different. It is known that HAP mutagenesis is dependent on replicative DNA Pols, therefore, the results will stimulate new experiments to reveal the additional properties of replication of the two strands of the *URA3* gene by these enzymes. The possible mechanism of transcription dependence of HAP mutagenesis is not known.

Okazaki Fragments and Mutagenesis Hotspots

The mechanisms of coordination of activities of all DNA Pols at the fork are largely unknown. The best defined, as we discussed previously, is the role of Pol α. The patches synthesized by Pol α are at the beginning of each Okazaki fragment. Therefore, hotspots of mutations attributed to inaccurate Pol α are informative for the size of Okazaki fragments in vivo. In this study we have chosen the *pol1-L868M* allele that encodes for very inaccurate Pol α [Niimi et al., 2004]. To prevent the correction of errors made by inaccurate Pol α, we inhibited MMR by cadmium (Cd^{2+}) [Jin et al., 2003], which we have shown synergistically increases mutation rates in the *pol1-L868M* mutant [Pavlov et al., 2006a].

The probability of detecting the same sites of Okazaki fragments in the genome among different cells is undoubtedly low. Therefore, it is generally impossible to find mutations that happen in the beginning of the Okazaki fragments. There is one exception. The start sites of the Okazaki fragments will be at the same site in the reporter gene very close to the origin. Such a site could be used to map mutations associated with short patches synthesized by Pol α at the 5′ ends of Okazaki fragments. Therefore, we inserted the *URA3* cassette in strain (-2)l-7B-YUNI300 (nicknamed Δ1) that we used previously to monitor strand-specific replication errors [Pavlov et al., 2002a]. We performed the analysis of the timing of the *ARS306* firing in the selected region of chromosome III by the two-dimensional gel analysis of chromosomal replication intermediates [Poloumienko et al., 2001]. The results for the wild-type strain and the strain with insertion very close to the right side of ARS306 (R306) are presented in Figure 6A.

We observed that replication intermediates started to appear 15 minutes after release from the cell cycle arrest and reached the maximum at 30 min. The placement of our reporter cassette very close to the replication origin does not affect ARS306 firing. Control experiments reveal that the *pol1-L868M* mutation or the addition of HAP to growth medium also did not change the timing of *ARS306* firing (Fig. 6A). Next, we collected FOA^r mutants induced by Cd^{2+} in the *pol1-L868M* strain with the insertion of the *URA3* cassette in the R306 location and compared this distribution with the distribution of mutations under UV or in the strain with the *RAD27* deletion (Fig. 6B).

During the initial crude analysis we divided the whole gene into 100 bp intervals and found that the distribution of mutations in the strains with the *pol1-L868M* mutation is similar to the distribution in the *rad27* strain (correlation coefficient 0.76, $P=0.02$) but different from the UV spectrum (left panels versus top right panel). It is known that a defect on the maturation of Okazaki fragments on the lagging DNA strand in *rad27* strains results in an accumulation of duplications [Tishkoff et al., 1997; Jin et al., 2001]. The mechanism of duplication involves the ligation of unrepaired 5′ flaps. Therefore, the 5′ end of duplications in the *rad27* strains marks sites where Okazaki fragments were initiated. The similarity of the two spectra, when boundaries of Okazaki fragments are affected by different mechanisms, indicates that the approach we have designed could be legitimate. It is noteworthy that the distribution of a detectable position in the *URA3* gene is quite uniform (Fig. 6B, bottom right panel), so all the observed differences reflect the peculiarities of the mutation process itself.

Next, in order to get further proof that the hotspots might be connected with patches at the 5′ ends of Okazaki fragments, we moved the reporter by small increments away from the origin. The rationale for this experiment is outlined in a hypothetical scheme in Figure 6C. We expected that the hotspots of mutations would exhibit periodicity, which would depend on the position of the reporter in respect to the origin, and the positions of the peaks of mutations inside the gene would shift, but the distance of the peaks from the origin would be maintained (Fig. 6C). To implement this idea, we inserted the *URA3* gene in two orientations at positions differing by 75 bp in respect to replication origin and sequenced *ura3* in the *pol1-L868M* strains induced by Cd^{2+} in all locations (Fig. 6D). The results show remarkable patterns of mutation distribution changes when the reporter is moved or inverted (Fig. 6D). First, the distribution of hotspots changed with the inversion of the reporter (compare three forward locations, upper half, with three reverse locations, lower half), suggesting that the mutation distribution depends on what strand the synthesis by inaccurate Pol α happens. Second, some hotspot regions are independent of the distance from the origin and are fixed for each orientation (red dotted vertical lines). It is interesting that all of these hotspots, corresponding for both orientations, were detected among 78 sequenced mutations when the reporter is located in forward orientation far from the origin (between ARS305 and ARS306, where either strand could be replicated as a leading or lagging location in the *AUT4* gene [Pavlov et al., 2002a]). These mutation hotspots may be related to starts of sequence-specific Okazaki fragments or reflect an unknown pathway of mutagenesis in strains with inaccurate Pol α. Third, we detected another class of hotspots, ‘‘moving hotspots’’ (black dotted vertical lines in Fig. 6D). When the reporter moved, they also moved, but their location is constant in respect to the distance from ARS. They were not detected in the location *URA3::AUT4* mentioned above due to the sample size limitation and likely the asynchronous starts of Okazaki fragments at this location.

We hypothesize that the moving hotspots represent mutations made by Pol α at the starts of Okazaki fragments. In a more likely mechanism, the location of the

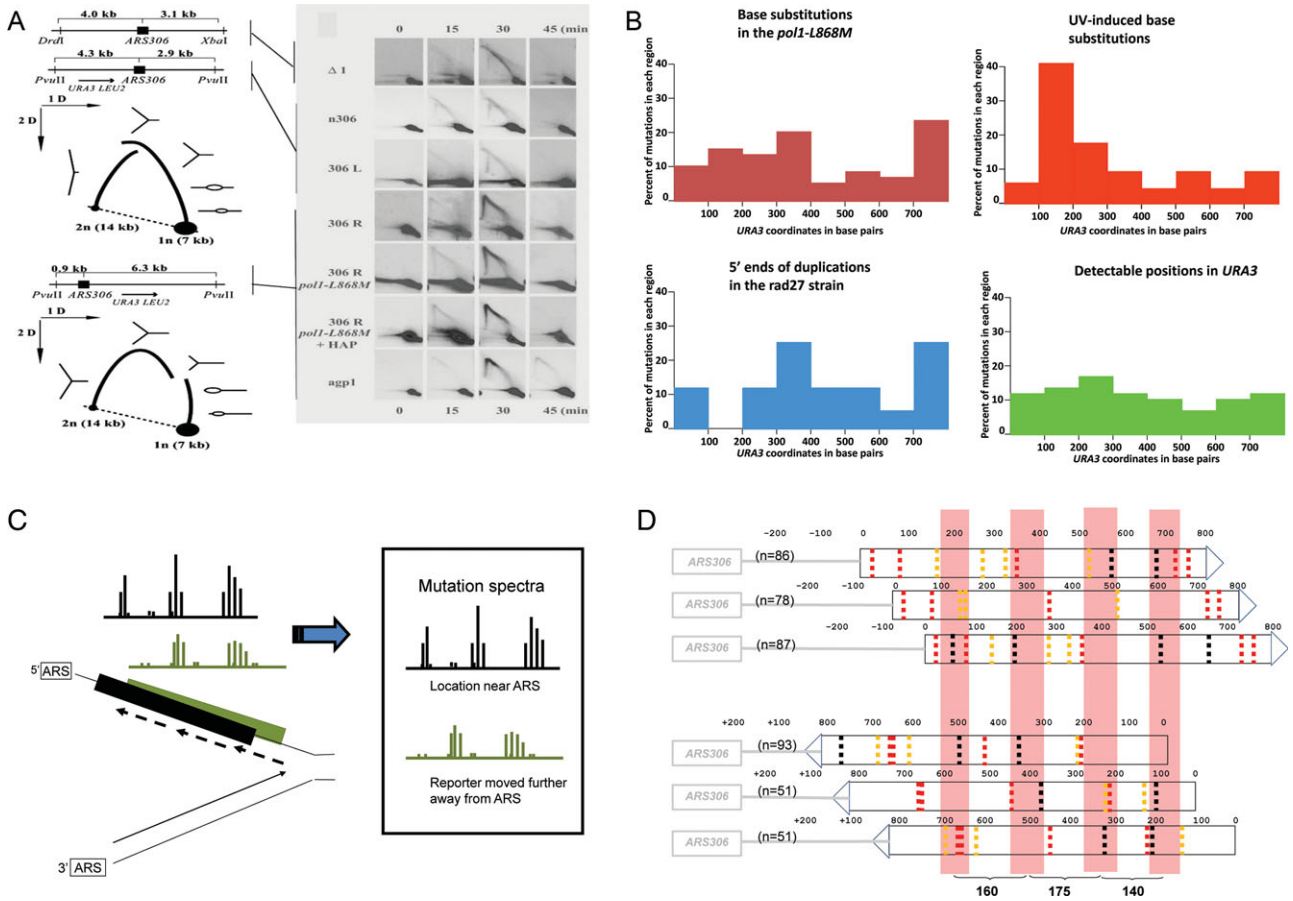

Fig. 6. Mutagenesis associated with the patches of DNA synthesized by inaccurate Pol α at the 5′ ends of Okazaki fragments. **A**: Analysis of the timing of origin activation in strains with the insertion of a reporter cassette. Left panel - Schematic presentation of 2D gel patterns anticipated for molecules with an active origin of replication. Fragments containing ARS306 of approximately the same length were analyzed: 7.140 bp *Xba*I-*Drd*I fragment for control Δ1 strain with no insertion; 7,222 bp *Pvu*II-*Pvu*II fragment for n306; 7194 bp *Pvu*II-*Pvu*II fragment for 306 L; 7220 bp *Pvu*II-*Pvu*II fragment for 306 R; and 7213 bp *Pvu*II-*Pvu*II fragment for agp1. Right panel - Images of the 2D gels for different strains. Lines connect the gel pattern to the schemes of molecule distribution. Δ1 – control strain, n306 – insertion of the URA3 LEU2 cassette to the left to the origin 2 kb from it, 306L – insertion to the left but very close to the origin, 306R – insertion very close to the right from the origin, 306R *pol1-L868M* – the same but in the *pol1-L868M* strain, 306R *pol1-L868M* + HAP – the same, but with the inclusion of HAP in the medium, and agp1 – insertion at 2 Kb to the right from the origin. Cells were released synchronously into the S phase from an α-factor block with the addition of 50 mg/ml pronase into fresh YPD media. Samples were taken at the indicated times after release and processed as described [Friedman and Brewer, 1995]. Neutral/neutral two-dimensional gel analysis was used to monitor replication. The 6-*N*-Hydroxylaminopurine (HAP) in a concentration of 0.1 mg/ml was added to YPD medium along with pronase. **B**: The distribution of mutations along the *URA3* gene caused by inaccurate Pol α is similar to the distribution in the *rad27* strains and is different from the distribution of mutations induced by UV light. The proportion of mutations falling into arbitrary chosen regions of 100 nucleotides is shown. The *URA3* is in forward orientation very close to the right side of ARS306. Left top (purple-brown bars) - mutations induced by Cd^{2+} in the *pol1-L868M* strain 2 (n = 85). Left bottom (blue bars) – 5′ ends of duplications in the *rad27* strain (n = 17). Right top (red bars) - UV light induced base pair changes (n = 25). Right bottom (green bars) – detectable position in the genes (based on the analysis of 151 mutations induced under various conditions). **C:** A schematic representation of experimental design - positions of hotspots of mutations associated with the boundaries of Okazaki fragments when the reporter gene is moved. **D:** Constant and moving hotspots of *ura3* mutations in the three locations and two orientations of the *URA3* gene in respect to replication origin. DNA fragment containing the *URA3* gene with its own promoter and terminator was PCR amplified with primers allowing for integration of the reporter at the desired locations. All sequences are available on demand. In the closest to the origin forward orientation (top diagram of the chromosome with *ARS* and the *URA3* reporter, where the numbers above the rectangle representing the *URA3* gene correspond to the distance in base pairs from the start of the gene) the ATG codon is 278 bp from the right side of *ARS306*. The next two diagrams represent the reporter moved 75 bp and 150 bp away from the initial location. In the closest to the origin reverse orientation, the TAA codon is 193 bp from the right side of *ARS306* (diagram four). Then the reporter was moved away by the same increments as before. The independent *ura3* mutants arising in the resulting strains while MMR was inhibited by the presence of cadmium (Cd^{2+}) were sequenced to build the mutation spectra. The mutation rate was almost two orders of magnitude higher in the presence of Cd^{2+} as described previously [Pavlov et al., 2006a] and similar in all strains. The numbers in brackets are the number of mutations in the each spectrum. The mutation hotspots were detected as described [Rogozin et al., 2005]. Black dotted lines represent the hotspots that are unique for a given spectrum (moving hotspots), yellow dotted lines are the hotspots that are present in two out of three spectra in the same orientation. Red dotted lines represent hotspots present in all three spectra (constant hotspots, positions 26, 98, 368, 743 and 764 in the forward orientation; positions 200-210, 434-436, 646 and 679 in the reverse orientation). Vertical pink rectangles represent areas where moving hotspots are found in at least one forward spectrum and at least one reverse spectrum. The distances between the centers of these zones are at the bottom of the figure and we hypothesize that they correspond to the sizes of the Okazaki fragments.

majority of Okazaki fragments is determined by the distance from the origin of replication. Of course, the hotspots are not in identical positions, but they are close. Some hotspots shown by dotted yellow lines (for example, at position 167 in the forward orientation, or 605 in the reverse orientation) belong to a mixed class (they are observed in a pair but not in all three locations), adding to the complexity of the analysis.

In attempt to correlate the mutation hotspots to the sizes of Okazaki fragments, we argue that the moving hotspots are the most plausible ''witnesses'' of transactions of Pol α at the beginning of the fragments. In Figure 6D we used pink rectangles to mark the areas of the reporter that have the ''moving'' hotspots at the same distance from the *ARS306* in at least one (out of three) location of the *URA3* gene simultaneously with such a hotspot in at least one reverse orientation. Note that ''moving'' hotspots sometimes coincide with constant ''red'' hotspots or ''yellow'' hotspots (Fig. 6D). We have found three such regions, separated by 140–175 bp. This indicates that the sizes of the Okazaki fragments estimated by our method in vivo fall within the size range consistent with biochemical estimates of the size of the Okazaki fragment mentioned previously and a recent estimate of the size of the Okazaki fragment in yeast [Smith and Whitehouse, 2012].

The mechanisms of mutation generation in ''constant'' and ''moving'' hotspots in a mutant with inaccurate Pol α are not apparent. If all hotspots correlate with the start of Okazaki fragments it could be predicted that the start sites of some Okazaki fragments are fixed by the sequence of the reporter, and in some, depend only on the distance from the origin. We understand that the observed spectra are the result of many factors, such as the distribution of detectable positions and mutation hotspots. This may obscure the detection of some hotspots.

Our results suggest that base pair substitution mutability of the gene could be influenced by a very slight (75 bp) change of its location in the chromosome, because mutation hotspots are influenced by the positions of Okazaki fragments. Okazaki fragments in eukaryotes are linked to nucleosomes [Smith and Whitehouse, 2012], and therefore, it is likely that chromatin remodeling factors will affect the distribution of mutations in the genome. Taken together with the critical role of these factors in mismatch repair [Kadyrova et al., 2011; Schopf et al., 2012], in forthcoming years we expect to see more interpretations of mutagenic specificity that take chromatin structure into account. Mutagenesis is much more complicated than it was thought several years ago and mechanisms regulating the genome wide distribution of mutations are just beginning to emerge [Lang and Murray, 2011]. Sequencing of cancer genomes revealed that tumor genomes are highly enriched with mutations [Loeb, 2011] and the mutations are sometimes found in clusters, named mutation storms or kataegis [Nik-Zainal et al., 2012]. The exact nature of kataegis is not known and it is likely that multiple factors are involved, including the generation of base analogs in DNA by editing deaminases, patches of DNA synthesis by inaccurate translesion Pols, collisions with transcription and others.

Mutagenesis During Specialized Replication Processes

In the cell there are several other processes beyond genome duplication requiring DNA synthesis. During the repair of DSB by homologous recombination, DNA synthesis is initiated by the invasion of the 3′ end of the broken DNA-Rad51 nucleofilament into a donor DNA sequence. It was originally proposed that the classical replication fork, with leading and lagging strands, is formed and, hence all three replicative DNA polymerases, Pol α, Pol δ, and Pol ε, were required for the completion of DSB-induced recombination [Holmes and Haber, 1999]. Later, the roles of polymerases were reevaluated and it was shown that DNA synthesis during recombinational repair involved the leading DNA strand and only redundant functions of Pol δ and Pol ε were required [Wang et al., 2004]. These replicative DNA polymerases are accurate, which is consistent with the notion that recombinational repair of DNA damage does not frequently generate mutations (hence, it is often referred to as "error-free" repair) [Friedberg, 2006]. However, the observation that the mutation rates are higher in meiosis than in mitosis led to a proposal that recombination can cause mutations [Magni and von Borstel, 1962]. It has been demonstrated that DNA synthesis associated with DSB repair in yeast is two orders of magnitude less accurate than during normal DNA replication, suggesting the involvement of a rather inaccurate DNA polymerase [Strathern et al., 1995]. The mutagenesis during recombination in their system was largely dependent on Pol ζ [Holbeck and Strathern, 1997; Rattray et al., 2002]. This suggests that Pol ζ can also be recruited to perform DNA synthesis on substrates that are generated during homologous recombination. Interestingly, the rate of recombination in the absence of Pol ζ was not changed. The unanswered questions are why and how ''mutagenic'' Pol ζ is allowed to participate in the reactions that could be performed by accurate DNA polymerases. One possibility is that Pol ζ could participate in the processing of damaged DNA recombination intermediates. Because the proportion of such abnormal intermediates is presumably small, the absence of Pol ζ would affect the frequency of mutation, but not the overall rate of recombination.

Recently, the interest for the phenomenon of mutagenesis during recombination was renewed. It was found that the synthesis of DNA during gene conversion is even more inaccurate than was thought originally and was dependent on replicative DNA Pols [Hicks et al., 2010].

Importantly, the specialized synthesis of DNA driven by double strand breaks was inaccurate over long distances (hundreds of kilobases) in break-induced replication [Deem et al., 2011]. Replication established under these conditions suffers from moderate but numerous limitations, such as elevated dNTP pools, somewhat reduced proofreading, and less effective mismatch repair. Multiplication of these mild effects results in a tremendous increase of mutation rates.

Another special condition of mutagenesis involves circumstances when single-stranded DNA persists longer than usual and results in localized mutagenesis and clustered mutations [Burch et al., 2011; Roberts et al., 2012]. The appearance of these mutations is proposed to contribute to cancer development [Nik-Zainal et al., 2012]. The hypothesis that persistent ssDNA is a substrate of editing deaminases [Roberts et al., 2012] has to be reconciled with the ability of replication protein A, that covers ssDNA, to inhibit activity and processivity of editing deaminases [Pham et al., 2008; Lada et al., 2011].

Human Diseases Caused by Replication Problems

Genome stability is compromised not only by DNA damage. Some DNA sequence contexts can impede DNA replication or repair. A classic example of genomic instability caused by problems in replicating an unusual DNA template is repeat expansions. These so-called ''dynamic mutations'' are the cause of more than 40 human disorders with a wide range of manifestations, such as mental retardation, muscular atrophy, cranial dysplasia, and increased risk of prostate cancer [Pearson et al., 2005]. The current models of triplet instability predict that the maximal size of repeat expansion depends on the size of the Okazaki fragments on the lagging strand of DNA [Pearson et al., 2005; Shishkin et al., 2009]. This size may depend on the relative activity of primase and Pol α as well as on the efficiency of chromatin remodeling [Trakselis et al., 2001; Smith and Whitehouse, 2012]. The adverse effects of the repeated DNA on replication and repair are linked to the ability of these sequences to form aberrant DNA structures, such as intra-strand hairpins, and triple- and quadruple-stranded DNA [Kovtun and McMurray, 2001; Lahue and Slater, 2003; Cleary and Pearson, 2005; Mirkin, 2005; Mirkin, 2006], which are difficult to replicate by Pols δ and ε [Abdulovic et al., 2011; Korona et al., 2011]. Mutations under these conditions are most likely generated by various switches of template DNA and the forks discussed in excellent reviews [Maki, 2002; Cleary and Pearson, 2005; Chang and Cimprich, 2009].

The perturbation of DNA replication due to depletion or imbalance of DNA precursor pools or insufficient DNA polymerase activity, leads to chromosome instability localized in the regions that are difficult to replicate [Mathews and Ji, 1992; Glover et al., 2005; Lemoine et al., 2005; Nakamura et al., 2005; Admire et al., 2006]. In the case of replicative DNA polymerases, any significant changes in their activity are lethal, but variations in their activity or fidelity may have consequences for human disease. It is intriguing that a well known cancer cell line, DLD-1, possesses an allele of the *POLD1* gene encoding for the most inaccurate variant of Pol δ ever seen [Daee et al., 2010].

Defects in the proofreading activity of replicative Pol δ and Pol ε in mice result in an increased cancer incidence [Goldsby et al., 2001; Goldsby et al., 2002; Albertson et al., 2009]. The defect of proofreading of mitochondrial Pol γ in mice leads to an accumulation of mutations in mitochondria and to premature aging [Kujoth et al., 2005]. This suggests that defective proofreading in humans will likely result in cancer predisposition and accelerated aging as well. Several mutations in the polymerase and exonuclease domains of human Pol γ or in the gene encoding the accessory subunit of Pol γ have been associated with progressive external ophthalmoplegia (PEO), a rare disease characterized by the accumulation of point mutations and large deletions in mitochondrial DNA [Van Goethem et al., 2001; Lamantea et al., 2002]. Mutations in the POLG gene have also been associated with Parkinson's disease, male infertility and ataxia-neuropatia [Graziewicz et al., 2006]. Recent work demonstrated that mice heterozygous for active site mutation leading to compromised base selection of Pol δ accumulate tumors at a higher rate than control mice, while homozygosity for such a mutation is incompatible with mouse life [Venkatesan et al., 2006].

The activity of TLS polymerases modulates the rate of genotoxicant-induced mutations. The dysfunction of these enzymes is associated with disease, too. Humans carrying mutations in the XPV (POLH/RAD30) gene that inactivate the function of Pol η suffer from Xeroderma pigmentosum, exhibiting sensitivity to sunlight and skin cancer predisposition [Johnson et al., 1999; Masutani et al., 1999]. In XPV patients that lack Pol η, cyclobutane pyrimidine dimers are processed in a manner that generates the mutations that lead to skin cancer. It is also possible that the mutagenic processing of oxidative DNA damage produced by sunlight radiation is elevated when Pol η is defective [Kozmin et al., 2005]. Interestingly, the two seemingly opposite properties of Pol η are important for human health. Relevant to protection from UV-induced carcinogenesis is the ability of Pol η to contribute to the prevention of mutations during the bypass of damage. At the same time, the ability of Pol η to generate mutations at a high rate is important for the proper function of the immune system [Vaisman et al., 2004]. In the cells from XP-V patients lacking Pol η, related Pol ι is responsible for the high frequency of UV-induced mutagenesis, and ultimately malignant transformation [Lawrence et al.,

2000]. Defects and polymorphism in the *POLI* are associated with an increased risk of lung cancer [Lee and Matsushita, 2005; Sakiyama et al., 2005] and 129/J mice, devoid of Pol ι, are prone to an elevated occurrence of UV-induced skin tumors [Dumstorf et al., 2006; Ohkumo et al., 2006].

While DNA polymerases can contribute to genome stability and cancer incidence, they are also critical for the propagation of tumors. A better understanding of their roles is important for the identification of new prognostic markers, as well as for the design of anti-cancer drugs [Wang, 2001; Madhusudan and Middleton, 2005].

Single nucleotide polymorphisms (SNPs) in the human *REV1* gene were also found to be associated with increased cancer risk [Sakiyama et al., 2005]. In addition, changes in the expression of genes encoding Pol ι and Pol κ have been found in human tumors [O-Wang et al., 2001; Yang et al., 2004; Albertella et al., 2005; Bavoux et al., 2005]. While DNA polymerases can contribute to genome stability and cancer incidence, they are also critical for the propagation of tumors. A better understanding of their roles is important for the identification of new prognostic markers, as well as for the design of anti-cancer drugs [Wang, 2001; Madhusudan and Middleton, 2005].

ITPA deficiency causing alterations of RNA and DNA precursor pools has been found to play a role in the response to several drugs. Marinaki et al. were the first to discover an association between ITPA deficiency and increased levels of adverse reactions from the drug azathioprine used in the treatment of inflammatory bowel disease [Marinaki et al., 2004]. Azathioprine and other mercaptopurine drugs are also used in the treatment of leukemia and auto immune disorders. Stocco et al. have shown that ITPA deficiency is associated with febrile neutropenia in acute lymphoblastic leukemia patients treated with mercaptopurines [Stocco et al., 2009]. On the other hand, ITPA deficiency was observed to be associated with a better response to low-dose azathioprine in the treatment of systemic lupus erythematosus [Okada et al., 2009]. ITPA deficiency was found to protect against hemolytic anemia in hepatitis C patients treated with ribavirin [Fellay et al., 2010]. The ITP which accumulates in erythrocytes compensates for ribavirin-induced decreases in ATP and GTP by substituting for GTP in the adenylosuccinate synthase reaction, which leads to ATP production [Hitomi et al., 2011]. This is additional evidence for the connection of the pathways of base analogs and purine biosynthesis (Fig. 4).

In the crystal structure, the location of P32 is away from the active site [Porta et al., 2006; Stenmark et al., 2007]. Consistent with that, ITPA P32T is almost as active as wild-type ITPA but it is temperature labile [Stepchenkova et al., 2009b; Herting et al., 2010]. The level of ITPA protein in human dermal fibroblasts with endogenous ITPA-P32T is approximately nine-fold lower than by a corresponding cell line with wild-type ITPA, thus alluding to the possibility that this mutation triggers degradation of ITPA [Stepchenkova et al., 2009b]. Taken together, these two observations help to understand why the severity of the defect of ITPA P32T is much less than anticipated by extrapolation from mouse knockout data. ITPA P32T can provide enough ITPase activity in some tissues but not enough in the other. It is known that the activity and levels of ITPA are very different in different tissues [Verhoef et al., 1980]. Although ITPA deficiency in humans is currently considered benign, it is possible that the presence of ITPA P32T creates a predisposition to cancer, in general, and a drug-induced secondary cancer, in particular.

We have found that the P32T variant of ITPA aggravates base analog induced killing and instability of genetic material [Waisertreiger et al., 2010]. In cell lines, inhibition of the *ITPA* by shRNA leads to elevated levels of DNA breaks, mutations, and apoptosis [Menezes et al., 2012]. Therefore, the investigation of ITPA goes beyond the study of the adverse reactions to purine analogs in patients with ITPA polymorphism [Maeda et al., 2005; Zelinkova et al., 2006]. Given the high allele frequency of this variant in the human population, it is clear that it represents potential risk factors for cancer and other diseases affecting a substantial proportion of the human population.

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AUTHOR CONTRIBUTIONS

YP, THT, ES and IBR wrote the manuscript. IW and VL performed experiments presented in Figures 5 and 6 and participated in writing. MM participated in writing and performed experiments related to Fig. 2. HMK and KL performed the experiments described in Fig. 6A and in writing. IBR performed the statistical analysis of the mutation spectra.

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