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DNA polymerases and human disease

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Abstract | The human genome encodes at least 14 DNA-dependent DNA polymerases — a surprisingly large number. These include the more abundant, high-fidelity enzymes that replicate the bulk of genomic DNA, together with eight or more specialized DNA polymerases that have been discovered in the past decade. Although the roles of the newly recognized polymerases are still being defined, one of their crucial functions is to allow synthesis past DNA damage that blocks replication-fork progression. We explore the reasons that might justify the need for so many DNA polymerases, describe their function and mode of regulation, and finally consider links between mutations in DNA polymerases and human disease.

Our knowledge of human DNA polymerases has undergone a striking expansion in the past decade. Since the discovery in 1957 of an enzyme that catalyses the accurate replication of DNA, there has been a progressive accumulation of evidence for five 'classical' DNA polymerases in all mammalian cells, each functioning in DNA replication and/or repair (FIG. 1a). In an exciting and unanticipated development that has unfolded over the past decade, several 'new' DNA polymerases have been discovered[1] (FIG. 1b).

The five classical DNA polymerases are each located on a different chromosome (FIG. 1b). Their principal functions have been deduced from catalytic properties and observations of cell physiology. DNA polymerase (Pol) α catalyses initiation of chromosomal DNA replication at origins and at Okazaki fragments on the lagging strand[2,3]; Pol β participates in base-excision repair[4,5]; Pol γ catalyses mitochondrial DNA synthetic processes[6]; Pol δ participates in lagging-strand synthesis[7,8]; and Pol ε has a role in the synthesis of the leading strand of chromosomal DNA[9–11]. Looking back, we can see that the classical list did not take into account the multiplicity and diversity of DNA transactions in cells. Each human cell undergoes >50,000 DNA-damaging events per day[12], and much of this damage impedes synthesis by the classical DNA polymerases. Given that some lesions are transferred to progeny cells[13], there must be mechanisms by which DNA synthesis proceeds past sites of damage. We now know that synthesis past damage is catalysed by translesion synthesis (TLS) DNA polymerases (also known as bypass DNA polymerases) that can insert nucleotides opposite specific blocking lesions, and/or extend the nucleotides that are inserted opposite damage sites. These enzymes are error prone when copying undamaged DNA, have low catalytic efficiency and are non-processive. Eight to ten of these TLS DNA polymerases seem to be present in most human cells (FIG. 1b; TABLE 1) and are probably found in all mammalian cells. Three TLS polymerases have been identified in *Saccharomyces cerevisiae*, and two are known in *Escherichia coli*[14].

New technologies are helping to establish the roles of human DNA polymerases in cellular functions. These technologies include the creation of cells and animals containing mutant DNA polymerases, methods to control the expression of specific DNA polymerases, and techniques to quantify the frequency and types of mutations in diverse cell types. We are thus well positioned to understand the function of each human DNA polymerase in cellular processes, including roles in mutagenesis and disease. Phenotypes associated with genetic diseases can be particularly informative of physiological function; however, only a limited number of inherited diseases have thus far been associated with mutations in DNA polymerases, or with changes in the expression of specific DNA polymerases or their associated proteins. This reflects the fact that DNA polymerases were studied most extensively as enzymes that are essential for DNA replication and repair. Each of the classical DNA polymerases was found to be required for viability, posing a major impediment to assignment of function on the basis of phenotypes induced by genetic manipulation.

Excellent recent reviews have focused on DNA polymerase structure and function[15], DNA replication[16,17], the role of accessory proteins in DNA replication and repair[18,19], and specialized or TLS polymerases[20]. Here, we discuss the main unanswered questions concerning the role of DNA polymerases in maintaining genetic stability; the association of DNA polymerases

Lagging strand
One of the two DNA strands that is synthesized during DNA replication. The lagging strand is synthesized by Pol δ in short segments that are known as Okasaki fragments.

Leading strand
One of the two DNA strands that is synthesized during DNA replication. The leading strand is believed to be synthesized by Pol ε, predominantly in a single segment.

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a

Polymerase	Gene	Catalytic subunit	Accessory subunits (kDa)	3′→5′ exonuclease	Fidelity	Primary function
Pol α	*POLA1*	1462 aa	49 *PRIM1*; 58 *PRIM2A*; 70 *PRIMA2*	No	10^{-4}–10^{-5}	RNA and/or DNA primers
Pol β	*POLB*	335 aa	None	No	5×10^{-4}	Base-excision repair
Pol γ	*POLG1*	1239 aa	55 *POLG2*	Yes	10^{-5}	Mitochondrial DNA replication and repair
Pol δ	*POLD1*	1107 aa	50 *POLD2*; 68 *POLD3*; 12 *POLD4*	Yes	10^{-5}–10^{-6}	Lagging-strand synthesis DNA repair
Pol ε	*POLE*	2286 aa	59 *POLE2*; 12 *POLE4*; 17 *POLE3*	Yes	10^{-6}–10^{-7}	Leading-strand synthesis

b

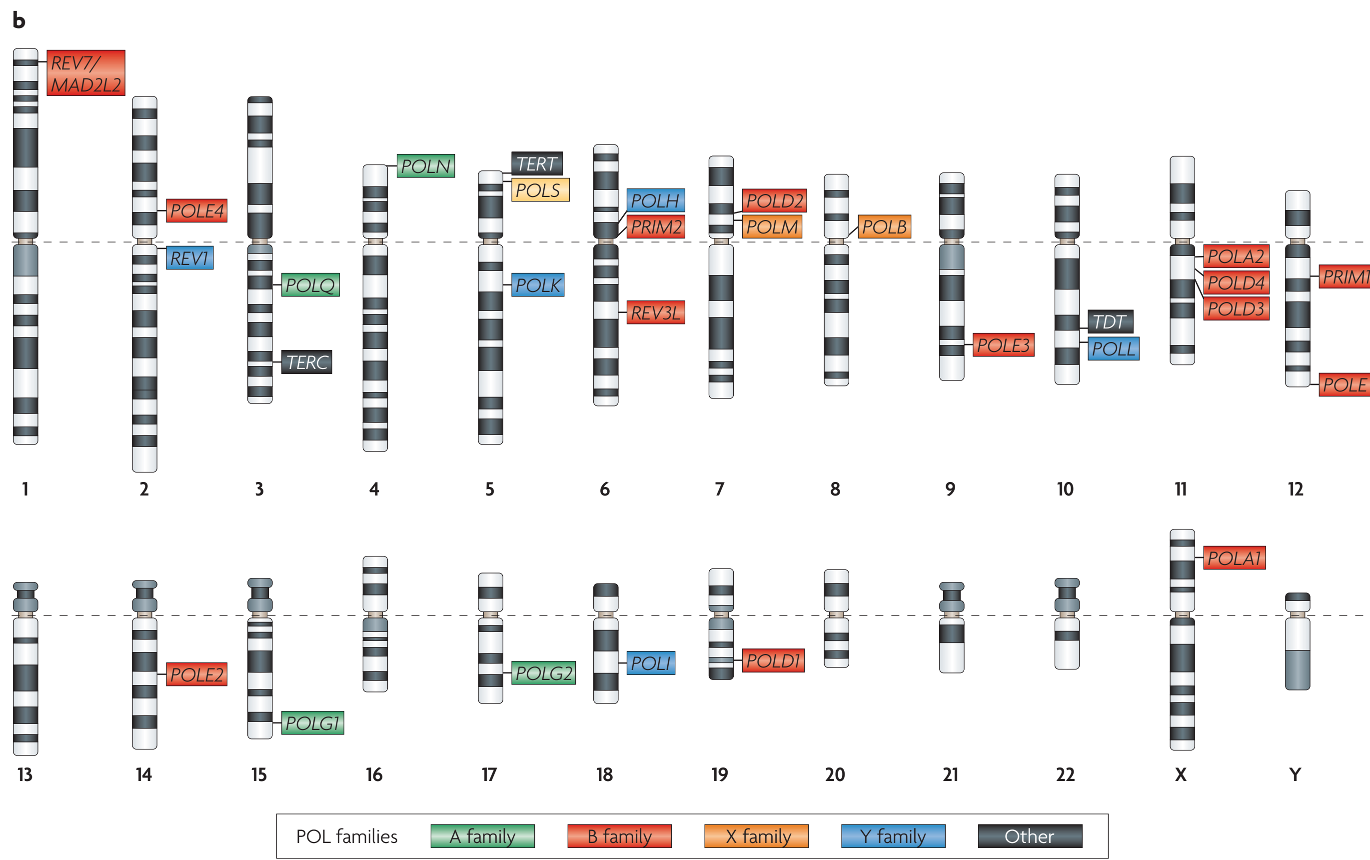

Figure 1 | **Classical DNA polymerases and associated subunits. a** | Five classical DNA polymerases and their subunits. Polymerase catalytic subunits are listed first, and are shown as polypeptides in which catalytic motifs (red lines) and exonuclease motifs (purple lines) are indicated, together with their corresponding genes and number of amino acids. These are followed by columns listing the genes for and size of any accessory subunits; whether the holoenzyme has proofreading exonuclease activity; and the fidelity of DNA synthesis given as the frequency of single-base substitutions using gapped M13 DNA as a template. Finally, the primary functions of the polymerases are listed. Data are from Kunkel and co-workers[65] or from our unpublished results. **b** | Location of DNA polymerase genes (*POL*) in the human genome. The locations of 29 genes encoding DNA polymerases and related proteins, including DNA primase genes (*PRIM*), have been plotted on a G-banded human karyotype shown at ~320 band resolution. The four human DNA polymerase gene and protein families (A, B, X and Y) are colour coded, and were originally defined on the basis of conserved sequence motifs in their catalytic subunits. Accessory subunits of multi-subunit DNA polymerases are shown in the same colour code, and are typically numbered in order of descending molecular weight after the catalytic subunit. Two other functionally related proteins are also shown: genes for the RNA (*TERC*) and protein (*TERT*) components of telomerase, and for terminal deoxynucleotidyl transferase (*TDT*). Designated gene symbols have been used throughout, with the exception of the second subunit of DNA polymerase ζ, which is listed as the widely used alias *REV7* as well as the official symbol *MAD2L2*.

Non-processive
Processivity refers to the number of nucleotide additions per binding event between DNA polymerase and a DNA template. Non-processive DNA polymerases incorporate one or a few base pairs per DNA-binding event. Processive DNA polymerases incorporate thousands of nucleotides per DNA-binding event.

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Table 1 | **Error-prone human DNA polymerases***

Polymerase	Gene	Family	Other names	Proposed function
η (eta)	*POLH*	Y	*RAD30A, XPV*	Bypass UV lesions
ι (iota)	*POLI*	Y	*RAD30B*	Bypass synthesis
κ (kappa)	*POLK*	Y	*DINB1*	Bypass synthesis
λ (lambda)	*POLL*	X	*POL4* (in *Saccharomyces cerevisiae*)	Base-excision repair, NHEJ
μ (mu)	*POLM*	X	–	NHEJ
θ (theta)	*POLQ*	A	*Mus308* (in *Drosophila melanogaster*)	DNA repair
ζ (zeta)	*POLZ*	B	*REV3*	Bypass synthesis
Rev 1	*REV1*	Y	*REV1L*	Incorporation of dC opposite abasic sites
ν (nu)	*POLN*	A	–	Unknown, but Pol ν has a unique error signature, G-dTMP mismatches[111]

*Nomenclature is according to REF. 110. For family classification of polymerase genes, see FIG. 1b. *POLQ* also encodes a helicase domain of unknown function. *Mus308*, mutagen sensitive 308; NHEJ, non-homologous end joining; UV, ultraviolet; *XPV*, xeroderma pigmentosum-variant.

with human diseases; the multiplicity, functions and redundancy of DNA polymerases; regulation of their expression; and the potential of DNA polymerases as targets for disease prevention and therapy. In addition to reviewing current knowledge, we discuss areas of research that might have practical importance but for which we have insufficient data. For example, in the case of cancer, can SNPs in DNA polymerase genes be used to stratify disease susceptibility, mutation tolerance and response to therapy? We also explore whether this concept might be extended to non-malignant disease characterized by abnormal growth, such as developmental abnormalities, atherosclerosis and psoriasis.

What do DNA polymerases do?

Structure and function. DNA polymerases are required for all DNA synthetic processes. Thus, mutations in DNA polymerases or changes in their expression could be manifested by alterations in DNA replication, in cell-cycle progression and, most prominently, in mutagenesis. DNA polymerases use a single strand of DNA as a template to assemble an exact complementary replica by polymerizing four complementary deoxynucleotides (FIG. 2a). The template dictates the sequential order of addition of the nucleoside 5′-triphosphate (dNTP) substrates onto a DNA primer bearing a 3′-OH terminus (FIG. 2b). DNA synthesis catalysed by polymerases can be followed in cells, in isolated nuclei or in single replicating molecules using a number of different assays (BOX 1). A mechanism of catalysis similar to that of DNA polymerases is exhibited by RNA replicases, RNA polymerases, reverse transcriptases and telomerase. X-ray structures of the polymerase domain of DNA polymerases have been likened to a human right hand, with three domains: a 'fingers' domain that interacts with the incoming dNTP and paired template base, and that closes at each nucleotide addition step; a 'palm' domain that catalyses the phosphoryl-transfer reaction; and a 'thumb' domain that interacts with duplex DNA (FIG. 2a). Many polymerases also encode a proofreading exonuclease as a separate domain, or have an associated exonuclease as a tightly associated subunit. These proofreading exonuclease activities remove non-complementary or altered nucleotides immediately after phosphodiester-bond formation and before the addition of another nucleotide. The picture that emerges of DNA polymerases is thus one of constant, repetitive motion with tight coordination of the polymerase and exonuclease active sites.

Understanding the molecular basis of fidelity. The fidelity of human DNA replication — approximately one error per 10^9–10^{10} nucleotides polymerized[21] — is exceptionally high and is the result of a sequential multi-step process (FIG. 3). The simplest model in which DNA polymerases 'zipper' together dNTP substrates that are prealigned on a template cannot account for the observed accuracy of these enzymes. The difference in free energy between a correct and incorrect Watson–Crick base pair in solution is less than 2.8 kcal mol^{-1}, and thus hydrogen bonding alone would generate more than one error for each one hundred nucleotides polymerized[22–24]. Because the accuracy of base selection by DNA polymerases can be as great as 10^5 (that is, 1 error per 10^5 bases incorporated), the enzyme must increase the free-energy difference between correct and incorrect base pairs. Several hypotheses have been offered to explain enhanced base selection at the catalytic site, including: multiple checkpoints that discriminate between correct and incorrect base pairs[25]; induced fit between a nucleotide and its cognate domain at each nucleotide selection step[26]; exclusion of solvent from the catalytic site[27]; and recognition of the shape of base pairs[28].

The largest unsolved problem in this area is to understand how a single active site can accommodate, differentiate and incorporate diverse substrates with similar catalytic efficiencies. Exonucleolytic proofreading increases fidelity by at least 4-fold and up to as much as 100-fold. In cells, mismatch correction provides a final fail-safe mechanism by which misincorporated nucleotides and analogues are excised post-synthetically. Multiplication of the fidelity of the individual *in vitro* reactions can account for the exceptionally high accuracy of DNA synthesis during DNA replication. Of course, this calculation is something of an artificial construct because

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the *in vitro* reactions are carried out under optimal conditions, do not take into account the effects of associated proteins or dNTP concentration, and, most importantly, are not subject to the structural constraints imposed by chromatin. Factors that are yet to be discovered are also likely to influence the fidelity of DNA replication.

Canonical DNA polymerases

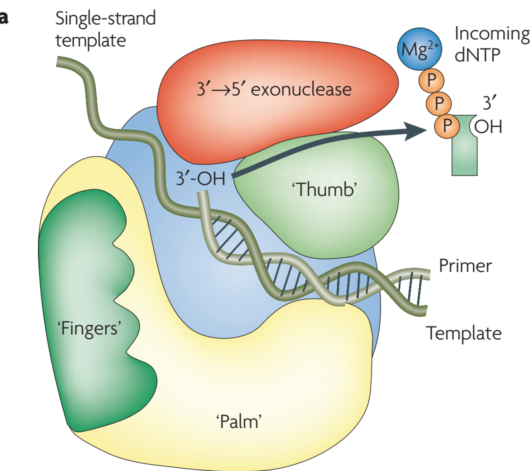
Until recently, there were five known mammalian DNA polymerases: Pol α, β, δ, ε, and γ. Evidence suggests that each is essential for viability (FIG. 1a). The chromosomal locations of the genes encoding these canonical DNA polymerases are shown in FIG. 1b.

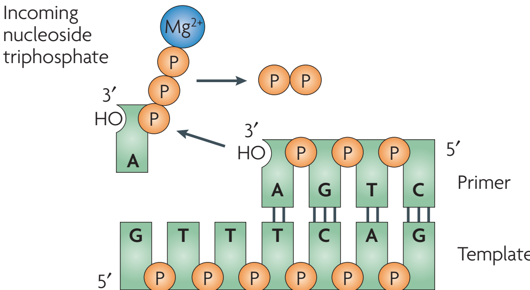
Pol α. The Pol α catalytic subunit (POLA1) is encoded as a single copy on the X chromosome (FIG. 1b). The involvement of Pol α in DNA replication was indicated in early studies by its high activity in rapidly dividing cells, its nuclear localization, and its induction when quiescent cells undergo proliferation[29]. Pol α is composed of four subunits, including a 180 kDa catalytic subunit encoded on the X chromosome and a 49 kDa primase subunit. The primase initiates DNA replication[30] by synthesizing an oligoribonucleotide primer of 8–12 nucleotides. Thereafter, the Pol α catalytic subunit extends the primer by about 20 deoxynucleotides. The suggestion that Pol α is deficient in N-syndrome, a form of X chromosome-linked mental retardation associated with chromosomal breakage and T-cell leukaemia[31], awaits definitive studies.

Pol δ and Pol ε. After initiation of synthesis by Pol α, Pol δ and Pol ε synthesize opposite strands of DNA. Studies in *S. cerevisiae* indicate that lagging-strand synthesis is catalysed by Pol δ, whereas leading-strand synthesis is catalysed by Pol ε[16]. Mutations in the exonuclease domain of Pol δ or Pol ε that abolish exonucleolytic proofreading have been shown to increase mutation rates in yeast, and the resulting misinsertions occur on opposite DNA strands, depending on which polymerase is defective[8]. Substitution of Pol ε that has a distinct mutational signature for the wild-type enzyme results in a pattern of errors indicative of involvement in leading-strand synthesis[11].

Both Pol δ and Pol ε function in multiple DNA synthetic processes. Pol δ participates in nucleotide-excision repair[32], mismatch repair[33] and recombination[34], and Pol ε has been implicated in nucleotide-excision repair and recombinational repair[32] as well as base-excision repair in the absence of Pol β. Interestingly, Pol δ and Pol ε can substitute for one another in nucleotide-excision repair[35,36].

Because Pol δ and Pol ε are essential, it has been difficult to determine whether mutations or alterations in expression are associated with human diseases. There is fragmentary evidence that somatic mutations in Pol δ are associated with human colon cancers[37]. In mice, homozygous deficiency of Pol δ exonuclease activity, but not heterozygous deficiency, results in reduced life-span owing to development of diverse tumours[38]. Mutations in the polymerase domain can be more deleterious. For example, homozygous Pol δ L604K (leucine 604





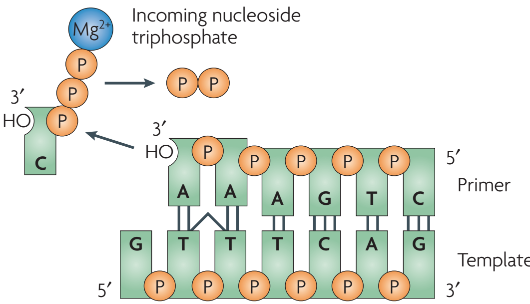


Figure 2 | **DNA polymerase catalysis and structure. a** | Model of a prototypical DNA polymerase with an associated 3′→5′ exonuclease domain that preferentially removes non-complementary nucleotides immediately after incorporation. **b** | Elongation of the primer terminus proceeds by sequential addition of 5′ deoxynucleoside monophosphates, derived from 5′ deoxynucleoside triphosphates (dNTPs), with accompanying release of pyrophosphate. A divalent cation (presumably Mg^{2+} in cells) is coordinated with the phosphate groups on the incoming dNTP[22], and the reaction proceeds by nucleophilic attack of the 3′-OH primer terminus on the α-phosphorus[105]. *In vitro* assays for DNA polymerase activity quantify the incorporation of radioactive nucleotides into an acid-insoluble product, or the elongation of a radio-labelled primer with product resolution by denaturing gel electrophoresis. **c** | Bypass synthesis of thymine dimers and other bulky adducts seems to be carried out predominantly by Y-family DNA polymerases; these contain a more spacious binding pocket at the catalytic site than do classical DNA polymerases.

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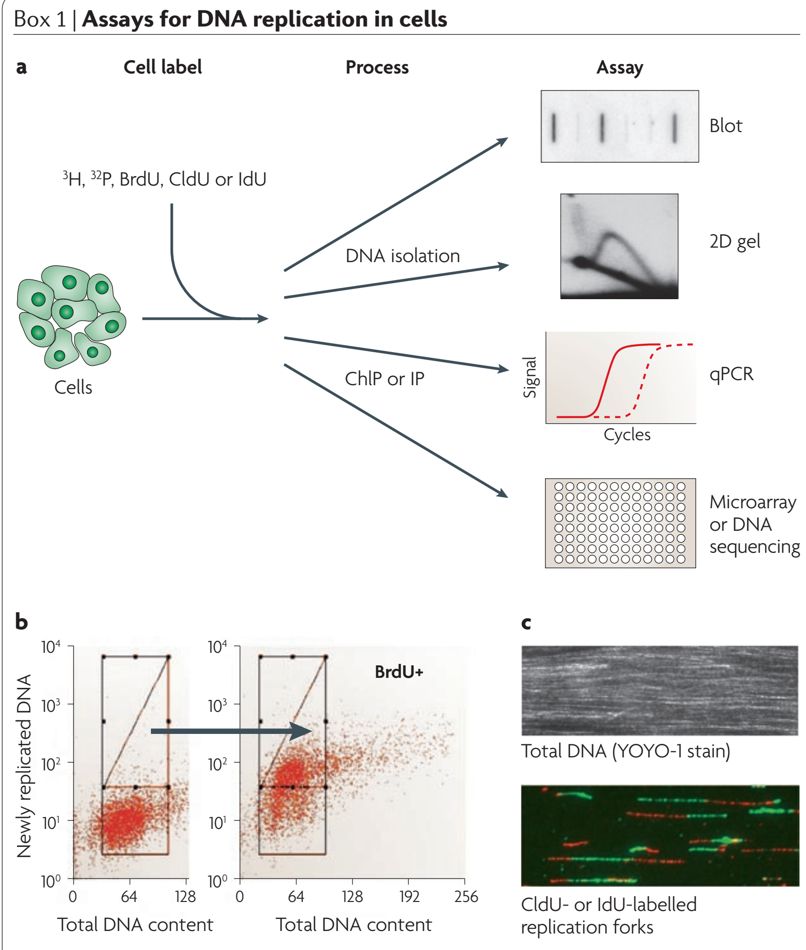
substituted by lysine) mice, which have a substitution in the polymerase domain, die *in utero*; heterozygotes exhibit reduced life-span, increased genomic instability and accelerated tumorigenesis[39]. Structure–function studies on the constituent subunits of Pol δ and Pol ε holoenzymes have been limited by difficulties in purifying sufficient quantities of the holoenzymes from baculovirus-infected insect cells[40]. The recent description of a plasmid encoding all four subunits of Pol δ in *E. coli*[41] should facilitate further biochemical and structural characterization of this polymerase.

Pol γ. Mitochondria contain a unique DNA polymerase, Polγ[42], which is encoded in the nucleus and transported into the mitochondria. Pol γ seems to be the only DNA polymerase found in mammalian mitochondria[43], and presumably functions in all mitochondrial DNA synthetic processes. Consistent with this idea is the observation that homozygous Pol γ knockout mice die *in utero*[44]. Mice that are homozygous for Pol γ exonuclease deficiency sustain a 2,000-fold increase in the frequency of single-base substitutions in mitochondrial DNA relative to wild-type mice, and have an ageing phenotype[45]. Heterozygotes exhibit a 500-fold increase in mutation frequency, but do not show an ageing phenotype; this lack of quantitative correlation suggests that single-base mutations alone are unlikely to cause ageing. By contrast, the frequency of deletion mutations has been found to correlate with the ageing phenotype[46].

Mutations in Pol γ are associated with several rare, maternally inherited human diseases[47]. Homozygous mutations in either the polymerase or exonuclease domains cause progressive external ophthalmoplegia, Alper syndrome and other neurodegenerative diseases[48]. In these diseases, skeletal muscle and nervous tissues, which have high levels of mitochondrial oxidative phosphorylation, are most frequently and/or first affected. Mutations in or altered expression of Pol γ, coupled with oxidative damage to mitochondrial DNA, might also be involved in two major human diseases: Parkinson disease[49] and Alzheimer disease[47]. Mitochondrial mutations observed in these diseases are frequently homoplasmic[47]. It is difficult to understand the origin of homoplasmic mutations, and even more perplexing are the findings that all mitochondria within some tumours have identical mutations[50]. Nevertheless, homoplasmic mutations might provide a potentially powerful marker for disease detection or diagnosis.

Pol β. The small size of Pol β (39 kDa) and the absence of accessory proteins make it particularly amenable for structure–function, mechanistic and biological studies. Pol β functions in short-patch base-excision repair, that is, repair involving resynthesis of short patches of DNA after excision of altered nucleotides. In addition to the polymerase domain, Pol β has an 8 kDa domain that is crucial for removing the terminal 5′-deoxyribose phosphate residue prior to resynthesis and for sealing of the ends by DNA ligase. Mice that are haploinsufficient for Pol β are defective in base-excision repair[51], and mice deleted for both copies are inviable. Mutations in the

Box 1 | **Assays for DNA replication in cells**



Biochemical measurement of DNA replication in cells

Assays for the measurement of DNA replication (see figure, part **a**) require labelling of newly replicated DNA with radioactive (^{3}H or ^{32}P) or halogenated nucleotide precursors (BrdU, CldU or Idu).

Blot-based hybridization assays allow newly replicated DNA from specific genomic regions to be detected and quantified. Two-dimensional (2D) gel assays separate replicating molecules on the basis of size and conformation, and thus can be used to detect and quantify replication intermediates. Replicated DNA from specific genomic regions or that associated with specific replication proteins can be detected and quantified by a combination of immunoprecipitation (IP) or chromatin immunoprecipitation (ChIP), followed by region-specific quantitative PCR (qPCR) or by genome-scale microarray or DNA sequencing.

Cytological analyses

These can be carried out by incorporating DNA precursors such as BrdU prior to immunostaining and flow cytometry (see figure, part **b**).

Assaying the replication of individual DNA molecules

The behaviour of individual replicating DNA molecules can be followed by labelling newly replicated DNA with halogenated nucleotides, such as BrdU, followed by the visualization of individual replicating DNA molecules (see figure, part **c**). Methods for stretching DNA molecules for imaging include: DNA combing, single-molecule analysis of replicated DNA (SMARD), fibre stretch and replication-track analysis. DNA molecules can be identified by staining with dyes such as YOYO-1, and newly replicated molecules can be identified by immunostaining molecules that have incorporated halogenated nucleotides. The sequential labelling of cells with two or more halogenated nucleotide precursors, such as CldU or IdU, for which there are specific antibodies, allows the identification of replication and of its direction as well as enabling the determination of the fraction of continuously replicating, newly initiated and terminated forks, and the estimation of the rate of fork movement.

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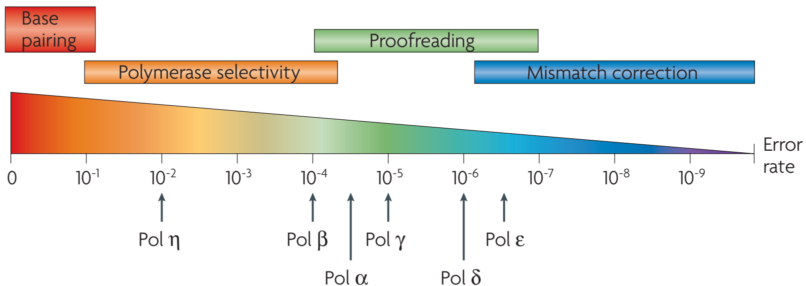

Figure 3 | **Fidelity of DNA replication.** Summarized above the line are the contributions of the different steps that enhance the fidelity of DNA replication. Below the line are representative values for the rates of single-base substitutions by different DNA polymerases. Assays for the fidelity of DNA synthesis by purified DNA polymerases have involved determining the frequency of revertants produced during copying of a single-base substitution in either primed single-stranded ϕX DNA[106] or by using M13 DNA as a template[107]. Data are from the laboratory of T.A. Kunkel[65] or our unpublished data.

Pol β gene have been reported in as many as 30% of human cancers[52]; some of these mutations enhance the error rate of Pol β and/or induce spontaneous malignant transformation in mouse cells in culture[53]. In addition, breast, colon and prostate tumours have been reported to overexpress Pol β[54], and overexpression of Pol β is associated with enhanced mutagenesis after DNA damage and increased susceptibility to hydrogen peroxide[55]. Taken together, these results indicate that Pol β can be classified as a tumour suppressor.

Specialized and TLS DNA polymerases

The canonical DNA polymerases are the main DNA synthetic enzymes in human cells and they carry out the predominant DNA polymerase activities found in cellular extracts. However, there were important hints that other DNA polymerases might be present in eukaryotic cells. First, prokaryotes were found to express damage-inducible error-prone DNA polymerases; it was therefore reasonable to assume that eukaryotes possessed enzymes with similar functions. Second, lesions that block DNA synthesis by all of the canonical DNA polymerases were maintained in successive cell divisions, implying that other enzymes might be able to copy past the lesions in a damage-tolerant pathway. Thus, it was reasonable to posit the existence of additional DNA polymerases that possessed the ability to copy past altered bases[56]. Third, it was reasonable to surmise, again from the biochemical properties of the canonical DNA polymerases, that additional or specialized DNA polymerases might be needed to ensure the replication of alternative DNA structures[57] or to promote targeted mutagenesis as part of the generation of antibody diversity[58].

The purification of the first eukaryotic TLS DNA polymerase, Pol η, was a *tour de force*. Xeroderma pigmentosum (XP) is an autosomal recessive syndrome[59] manifested by extreme sensitivity to sunlight and the development of cancer at sites of UV exposure[60]. The defect in chain elongation after UV irradiation of cells of one of the XP complementation groups, xeroderma pigmentosum-variant (XP-V), implied a deficit in a DNA polymerase. The 'new' DNA polymerase, Pol η[61], was purified based on the complementation of extracts of XP-V cells by HeLa cell nuclear extracts — these permitted copying past template cyclobutane dimers by incorporating dA residues[62]. Mice with homozygous deletions in Pol η are viable, sensitive to UV and develop skin cancer after UV exposure[63].

Pol η belongs to the Y family of DNA polymerases (FIG. 1b), which, in human cells, also includes three other members: Pol κ, Pol ι and Rev 1 (TABLE 1). These DNA polymerases can copy past bulky DNA-template adducts, have low catalytic efficiencies, are non-processive, lack proofreading activity and are error prone in copying unaltered DNA templates. Y-family polymerases have a binding pocket that is more spacious and can accommodate altered template bases, nucleotide adducts and non-complementary nucleotides[60,64,65] (FIG. 1; FIG. 2c). By contrast, the canonical replicative DNA polymerases have polymerase active sites that fit tightly with the template base and incoming nucleotide. Pol η and other members of the Y family share an ability to efficiently copy past DNA template site-specific modifications *in vitro*. However, we do not know the range and identity of their endogenous substrates.

The finding that deletion of homologues of mammalian TLS polymerases in bacteria and yeast results in a marked decrease in spontaneous mutagenesis implies that these enzymes have an important function in copying past DNA damage that is generated by normal cellular processes, including prevention of slippage of repetitive sequences during DNA replication[66]. Mutagenesis and carcinogenesis in XP patients with mutations in Pol η have been proposed to involve substitution by another error-prone DNA polymerase (TABLE 1), or stalling of replication forks and the consequent production of double-strand breaks[67]. Studies in mice using site-specific mutagens suggest that Pol κ is involved in the accurate bypass of benzopyrene-induced DNA adducts. Transient over-expression of Pol κ increases point mutations in mouse cells[68], and Pol κ mRNA is elevated in non-small-cell lung cancers[69]. These findings suggest a close association between Pol κ and mutagenesis in lung tumours. Pol ι has been implicated in both base-excision repair and in translesional bypass of UV-induced lesions[70]. Finally, Rev 1 is able to add deoxycytidine residues opposite abasic sites and other bulky DNA adducts.

In addition to the Y family of DNA polymerases, other newly discovered DNA polymerases might also be able to catalyse error-prone and/or translesion DNA synthesis. For example, Pol λ seems to be involved in base-excision repair and in non-homologous end joining[71]. Pol μ might have a role in DNA repair synthesis as part of the generation of sister chromatid exchanges[72]. Pol ζ has so far not been successfully purified from human cells, but in yeast it has been shown to be required for spontaneous mutagenesis[73]. Finally, Pol θ has an associated ATPase activity and is believed to function in interstrand DNA-crosslink repair[74].

One clear message from this brief overview is that the human genome might yet encode additional DNA

Holoenzyme
The catalytically active form of DNA polymerases, including all tightly bound associated subunits.

Homoplasmic
The presence of identical copies of mitochondrial DNA in a cell or tissue.

Haploinsufficient
Describes the situation in which half the amount of the normal gene product confers a detectable phenotype.

Nucleotide adduct
A covalent modification of a nucleotide base in DNA.

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polymerases. Several approaches might be useful in searching for these proteins. First, in addition to alignment-based homology searches that rely on conserved functional motifs, emerging structural data on the different families of DNA polymerases will facilitate structure-based search strategies. Second, fork-associated replication proteins, such as proliferating cell nuclear antigen (PCNA)[75] or damage-inducible alternative complexes such as Rad9–Hus1–Rad1 (9–1–1) or Rad17–replication factor C (RFC), could be used as 'bait' to capture additional polymerases involved in TLS or in specialized DNA synthesis. Many of these protein–protein associations depend on site-specific post-translational modification by phosphorylation, together with additional modifications by ubiquitin or small ubiquitin-like modifier (SUMO) addition[76]. Thus, it might be possible to use specifically modified peptide derivatives of polymerase catalytic or accessory subunit proteins as bait to find undiscovered polymerases or accessory proteins. A final approach — activity-based screens — is well validated and could be substantially extended by selectively depleting known polymerases using RNAi or immunodepletion prior to searching for new activities with structure- or lesion-specific templates.

Regulation of DNA polymerase gene expression

DNA polymerase expression is regulated by both genetic and epigenetic mechanisms. These mechanisms are just beginning to be delineated in human cells. The integration of new data on polymerase proteomics, structure and function, genomics, human genetic variation and expression should provide us with a much deeper view of polymerase function and the association of different DNA polymerases with human disease.

SNPs can be found in all of the human DNA polymerase genes (see, for example, the NCBI SNP database, dbSNP). We know almost nothing about the functional consequences of these polymerase gene variants, apart from the small number that are clearly deleterious and thus could lead to haploinsufficiency. Of note, aged haploinsufficient Pol $\beta^{+/-}$ mice are tumour-prone[51]. SNP variants that are not obviously deleterious might also affect polymerase gene expression (see REF. 77 for a review), and their consequences could be further amplified by the stochastic nature of gene expression[78].

Epigenetic regulation of DNA polymerase genes has a crucial function in normal development, and probably functions in disease pathogenesis. The *POLA1* gene encoding the catalytic subunit of Pol α is one well-studied example. It is located on the short arm of the X chromosome, and is subject to X inactivation early in development[79]. Inactivation of X-linked genes can vary over time, and the expression of *POLA1* and other expressed X-linked genes is transcriptionally upregulated to ensure dosage compensation is maintained[80]. The polymerase SNPs noted above should provide useful markers for determining both the levels of expression of specific polymerase alleles, and for identifying examples of extreme skewing or monoallelic expression in polymerase genes in normal cells, after DNA damage, or in specific disease states[81].

A second potentially important form of epigenetic regulation of polymerase genes might be mediated by microRNAs (miRNAs). Many human DNA polymerase genes are predicted to be targeted by one or more miRNAs (see, for example, the searchable miRNABase Targets database at the Sanger Center). These short non-coding RNAs might alter DNA polymerase expression by affecting steady-state mRNA levels or mRNA translation[82]. Well-documented recent examples include downregulation of Pol α by miR-206 during mammalian skeletal muscle differentiation[83] and downregulation of the Epstein–Barr virus (EBV) DNA polymerase by miR-BART2 (REF. 84), one of 23 miRNAs encoded by the EBV genome. Finally, miRNA expression is altered in many human tumours[85,86], although there has been no systematic effort as yet to link these miRNA expression differences to polymerase gene expression in specific tumour types.

DNA polymerase expression during development. DNA polymerases are crucial in early development, when rapid DNA replication and cell division are required. Two important transitions occur during this time (FIG. 4). First, maternally encoded proteins that are stored in the egg, including DNA polymerases, are rapidly diluted by continued cell division and are replaced by newly synthesized proteins encoded by the zygote. Variation in either polymerase gene expression or steady-state protein levels at this transition could have a substantial impact on cell division and embryogenesis. Second, the expression of polymerase genes undergoes modification as specific cell lineages, tissues and organs are specified (FIG. 4). For example, replicative DNA polymerases are downregulated in cells destined to become post-mitotic, such as brain or heart muscle. By contrast, cell types that are continuously replicating, such as epithelial cells in the skin and cells that line the gastrointestinal tract or populate the bone marrow, seem to retain the full complement of the replicative DNA polymerases.

These lineage-specific functional requirements suggest that different cell lineages might express different combinations of DNA polymerases. For example, embryonic mouse stem cells exhibit markedly lower spontaneous- and induced-mutation frequencies than do mouse fibroblasts in culture. This could be the result of expression of more accurate DNA polymerases[87,88], perhaps in conjunction with more efficient mechanisms for DNA repair[89,90]. Stem cells might be a particularly important cell type in which to search for new DNA polymerases and in which to study lineage-specific expression patterns. The ability to grow human pluripotent embryonal stem cells, and to reprogramme adult somatic cells to form induced pluripotent stem cells, should facilitate both the search for new polymerases and analyses of the role of specific polymerases such as the TLS polymerases in stem-cell and lineage-specific biology[91].

Regulation of DNA polymerase function

The association of polymerases with accessory proteins in complexes is essential for function, although a detailed understanding of even the simplest of such complexes is only now coming into view (see REF. 92 for a review).

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A central feature of replication is a requirement for the successive 'hand-off' of replication intermediates between different multi-protein complexes, in order to ensure the switch from initiation to leading- and lagging-strand synthesis, the successful replication past DNA damage, and the termination of replication with the segregation of newly replicated DNA duplexes to daughter cells.

A key protein in maintaining replication-fork structure is PCNA[75], a homotrimeric eukaryotic sliding clamp. One signal that initiates TLS is the ubiquitylation of PCNA after DNA damage. This modification is mediated by the E2 ubiquitin-conjugating enzyme Rad6 together with the E3 ubiquitin-protein ligase Rad18 at a highly conserved lysine residue, K164 (REF. 93). Monoubiquitylation of PCNA increases the affinity of PCNA for several TLS polymerases, and might facilitate recruitment or retention of TLS polymerases at the replication fork. K164 is subject to further ubiquitylation, which in human cells prevents TLS synthesis and promotes error-free synthesis through an alternative pathway[94]. Sumoylation of PCNA on K164 has also been reported and, in budding yeast, seems to favour the recruitment of a helicase, Srs2, as opposed to a TLS polymerase.

The interplay of mono- and polyubiquitylation together with sumoylation of PCNA in response to DNA damage provides a glimpse of how replication-fork structure and activity is controlled by a combination of template-DNA damage, the presence and modification status of proteins located at the fork[95], and damage-signalling pathways. The range of different outcomes that can be brought into play, including continued semi-conservative replication, TLS polymerization and recombination-dependent DNA repair, ensures continuation of DNA replication. This complex series of regulatory steps highlights many new protein and regulatory network candidates among which to search for disease-causing mutations, and identifies potential new strategies for polymerase- and replication-targeted therapeutics (see below).

New roles for polymerases in biology and disease?

Heritable SNP and structural variation, together with acquired mutations or epigenetic variation, might affect polymerase gene expression or function. These areas have been little investigated thus far despite their potential biological and medical importance, and thus might be fertile areas for translational research.

As discussed above, SNPs have been identified in each of the canonical polymerase genes and in several of the specialized polymerases[19]. The potential effects of these variants on *in vitro* catalysis could be used to identify variants with the highest potential to alter *in vivo* functions. The most interesting of these variants could then be analysed in cell culture, and in genetic association studies using large control or specific patient populations. Structural variation (that is, duplications, deletions and inversions) in the human genome is also common (see REF. 96 for an example) and could affect the copy number or expression of polymerase genes differently in individuals. For example, Pol η is contained in a segmentally duplicated region on the short arm of human chromosome 6, and thus might differ in expression between

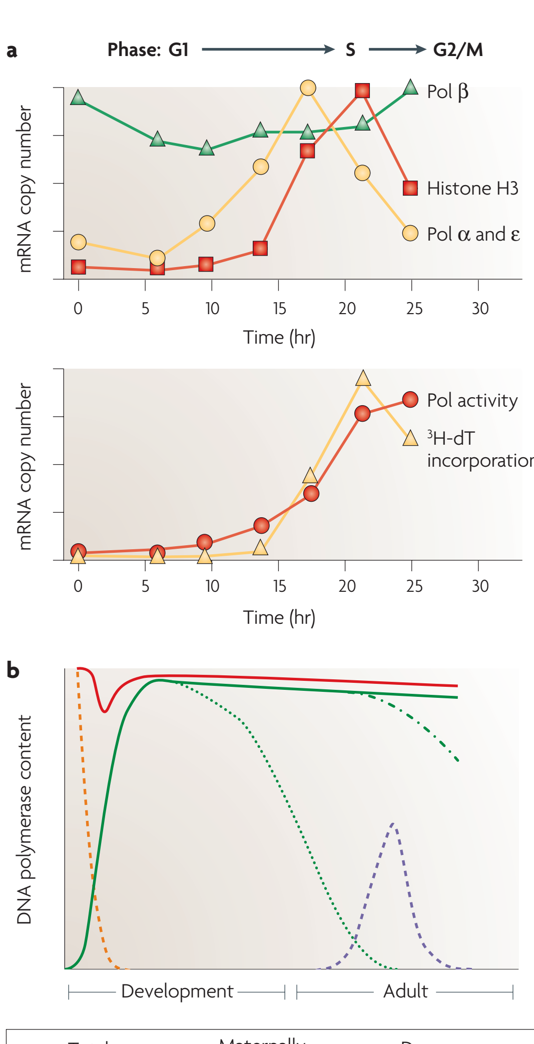

Figure 4 | **DNA polymerase expression and activity in cycling cells and whole organisms. a** | Cell-cycle changes in DNA polymerase (Pol) gene expression, DNA polymerase activity and rate of DNA synthesis. Cell-cycle phases are depicted above the graphs. The upper graph shows transcriptional upregulation of DNA Pol α and Pol ε during S phase; these are involved in replication. The expression of the core histone H3 gene that is upregulated later than replicative polymerases. By contrast, the expression of Pol β, which is involved in base-excision repair, does not change over the cell cycle. The lower graph depicts the rapid increase in two markers of the initiation of genome replication at the start of S phase: overall DNA polymerase activity, and the incorporation of the DNA precursor tritiated thymidine (^{3}H-dT). Data for the graphs are from REFS 108 & 109. **b** | DNA polymerase content as a function of time. Maternally encoded DNA polymerases are rapidly diluted with the onset of cell divisions during embryogenesis, and are replaced by newly synthesized DNA polymerase protein encoded by the zygote. The replicative polymerases can be downregulated in post-mitotic cell lineages such as neurons or cardiomyocytes. DNA polymerase content can decline with age, or can be transiently increased by the damage-dependent synthesis of specialized polymerases.

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individuals (see the Segmental Duplication Database). The existence of frequent human genomic structural variation highlights a second area in which data are lacking: is polymerase gene expression co-regulated in specific cell types or lineages, or is the expression of the different subunits of the replicative polymerases — collectively encoded by 12 different genes (FIG. 1a) — coordinated to ensure correct stoichiometry and function? Again, the tools are becoming available to simultaneously analyse genetic variation and expression of polymerase genes, and to link these data to catalytic activity alterations and disease.

Infrequent clonal somatic mutations, some clearly deleterious, have been identified in DNA polymerase genes in human tumours (see, for example, the listing contained in the Catalogue of Somatic Mutations in Cancer (COSMIC) database). However, few are well documented and consistently associated with cancer or other human diseases. Among the most convincing mutation–disease links are Pol η mutations in patients with XP-V; mutations of Pol β in adenocarcinoma of the colon[52]; and mutations of Pol γ in patients with progressive external ophthalmoplegia (reviewed in REF. 97). The number of these associations might increase with the sequencing of specific genes or cancer genomes (see, for example, the US National Cancer Institute (NCI) Cancer Genome Anatomy Project and the UK Sanger Institute Cancer Genome Project). Many of the canonical and TLS polymerase genes are contained in chromosomal regions that are frequently lost or occasionally amplified in human tumour specimens (compare FIG. 1b with the corresponding entries in the NCBI Cancer Chromosomes database). For example, Pol β is encoded by a locus on the short arm of chromosome 8 (8p11.2) that is frequently rearranged or lost in a wide variety of human tumours.

Epigenetic silencing of gene expression is common in many human tumours[98,99] and might affect DNA polymerases. A survey of DNA polymerase genes on the University of California, Santa Cruz (UCSC) Genome Browser, with the CpG-island track enabled, shows that several human DNA polymerase genes contain CpG islands, and thus might be susceptible to methylation and silencing. Because the number of known DNA polymerase genes is comparatively small (FIG. 1), it should be possible to use quantitative, methylation-specific PCR assays to comprehensively screen all of the known polymerase genes for methylation-induced changes in expression in tumour cells or in other disease states. One particularly interesting place to look for methylation-associated silencing would be in human tumours that display a CpG island methylator phenotype (CIMP tumours), for example, human colon carcinomas.

Several different human disease states might present opportunities to identify disease-associated polymerase mutations or structural variations. Mutations or structural variation that have a strong affect on function of the canonical DNA polymerases (FIG. 1) would probably lead to early embryonal or fetal lethality. These types of variation might, in consequence, be more prevalent in and contribute to the large fraction of human conceptuses that fail to reach term. By contrast, partial loss of function or haploinsufficiency might lead to either multi-system, organ-specific or cell lineage-specific developmental defects, or to the early exhaustion of continuously replicating cell lineages[100]. Thus, individuals with otherwise unexplained constitutional developmental defects, hypo or aplasia syndromes (for example, primary bone marrow failure) or genetic instability might also be good candidates in which to screen for polymerase gene mutations or dysfunction.

New targets for treatment and prevention?

Considering the essentiality of DNA polymerases in replication of infectious agents and diseased human cells, it is surprising that these enzymes have not been extensively exploited as bacteriocidal agents and cancer chemotherapeutic targets. It can be argued that their common catalytic mechanism and extensive homology at the active site(s) would mitigate against the design of drugs that can distinguish between different polymerases. However, this approach has been successful in the use of nucleotide analogues that preferentially terminate DNA synthesis by viral DNA polymerases and reverse transcriptases. These analogues include purine, pyrimidine and acyclic analogues that preferentially inhibit viral DNA polymerases and that are being used to treat infections caused by herpesvirus, varicella virus and cytomegalovirus[101] as well as those caused by HIV[102]. An extension of this strategy might be possible in the case of parasites that have unusual DNA base composition. For example, the malaria parasite *Plasmodium falciparum* has an overall AT content of 81%. This implies that the DNA polymerases of *Plasmodium* species that efficiently replicate AT-rich DNA might have distinctive mechanisms of base selection that could be exploited for drug design.

It is not clear how far this general approach — the use of nucleoside analogues and related compounds to target polymerase active sites or catalytic mechanism — can be extended to target cellular polymerases. However, DNA polymerases and associated proteins have multiple distinct sites that could serve as targets for new drug design. For example, structural information might allow the design of small molecules to disrupt protein–protein associations that are required for the function of specific polymerases. A second approach would be to use structure-based synthesis together with activity-based screening to identify nucleotide analogues with novel activities towards specific polymerases. For example, Y-family DNA polymerases have enlarged nucleotide-binding sites relative to replicative DNA polymerases. This might allow the incorporation of nucleotide analogues with the potential to either inactivate the polymerase, or to form polymerase–DNA crosslinks.

In the case of cancer treatment, an important strategy is to inhibit DNA polymerases involved in DNA repair in order to increase the efficacy of chemotherapeutic agents that damage DNA. In addition to agents that target the catalytic site, replicative DNA polymerases have other potential target sites that assist in base selection during DNA synthetic processes. For example, drugs that target

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and inhibit exonuclease proofreading or template switching between polymerase and exonuclease active sites might effectively inhibit DNA synthesis and the repair of nucleotide analogues and DNA damage by alkylating agents. Two important polymerases that would serve as immediate targets for this type of approach are Pol β and Pol λ, which function in base-excision repair. Inefficient DNA repair synthesis could increase the burden of potentially lethal repair intermediates — for example, single-strand gaps — increasing the likelihood of blocked DNA replication forks and consequent formation of lethal double-strand breaks. The recent finding that cancer cells exhibit a mutator phenotype[103] raises the possibility of a different strategy, namely treating malignancies by increasing mutagenesis. The feasibility of this strategy, initially termed 'lethal mutagenesis', has been demonstrated by the use of the mutagenic analogue 5-OH-dC to abolish HIV replication in cells in culture. Exposure to 5-OH-dC for many passages resulted in increasing numbers of mutations in the HIV genome that eventually exceeded the error threshold for viral viability[104]. This strategy might prove effective in cancer therapy, as cancer cells might have already accumulated large numbers of mutations at the time of detection, and thus a further increase in mutations might exceed the error threshold for tumour-cell viability. One practical challenge is to develop facile ways to identify both the loss of function of specific polymerases in disease, and likely compensatory, polymerase-dependent pathways that could be selectively targeted to promote cell killing or growth arrest.

Coda

Until relatively recently, the study of DNA polymerases was focused on enzymology and cellular functions. However, the DNA polymerase field has been revitalized by the discovery of a multiplicity of 'new' DNA polymerases that have unforeseen roles in biology and in human disease. The questions that these discoveries have spawned ensure that the study of human DNA polymerases will remain exciting well into the foreseeable future.

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miRNABase Targets database: http://microrna.sanger.ac.uk/targets/v5
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