Translesion DNA Synthesis and Mutagenesis in Eukaryotes

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The structural features that enable replicative DNA polymerases to synthesize DNA rapidly and accurately also limit their ability to copy damaged DNA. Direct replication of DNA damage is termed translesion synthesis (TLS), a mechanism conserved from bacteria to mammals and executed by an array of specialized DNA polymerases. This chapter examines how these translesion polymerases replicate damaged DNA and how they are regulated to balance their ability to replicate DNA lesions with the risk of undesirable mutagenesis. It also discusses how TLS is co-opted to increase the diversity of the immunoglobulin gene hypermutation and the contribution it makes to the mutations that sculpt the genome of cancer cells.

Genome replication is normally an extremely accurate process with an error rate as low as one in 10^{10} bases in mammalian cells. This extraordinary fidelity is the result of a combination of factors: the intrinsic ability of the replicative polymerases to select the correct nucleotide, their ability to recognize and excise misincorporated nucleotides, and the activity of postreplicative mismatch repair (Loeb and Monnat 2008; Arana and Kunkel 2010). However, the features that give the replicative polymerases their high intrinsic fidelity also mean that they are easily stalled by DNA damage. Since the DNA at the fork is unwound, any attempt to excize the damage will result in DNA breaks and replication fork collapse. To avoid this, cells replicate through the damage, restoring it to duplex DNA before attempting excision repair. This is known as damage tolerance. Damage tolerance can be achieved by recombinational mechanisms or directly by translesion synthesis (TLS).

TLS is conserved throughout evolution. It relies on specialized DNA polymerases that have active sites capable of accommodating damaged or distorted templates. However, use of these polymerases carries an increased risk of mutagenesis because damaged bases are frequently non- or misinstructional and because the structural adaptations that allow lesion replication also result in reduced fidelity when copying undamaged DNA. The principal DNA polymerases responsible for TLS during replication belong to the Y-family (Sale et al. 2012), along with the B-family enzyme Pol ζ (Gan et al. 2008). In addition, DNA polymerases of the X and A family are capable of TLS and can contribute to mutagenesis during DNA-repair reactions such as base excision repair (BER) and nonhomologous end joining (NHEJ). Whereas

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TLS plays an important role in creating mutations, the TLS polymerases possess structural features that often help them extract the correct code from diversely damaged templates. Coupled with control mechanisms that ensure their access to DNA templates is strictly regulated, this helps limit the opportunities for these polymerases to cause undesirable mutagenesis.

THE MECHANISMS OF TRANSLESION SYNTHESIS BY THE EUKARYOTIC Y-FAMILY POLYMERASES AND Pol ζ

The budding yeast *Saccharomyces cerevisiae* has three DNA polymerases specialized for TLS: RAD30 (Pol η) and Rev1, both from the Y-family, and the B-family enzyme Pol ζ. Vertebrate cells possess two further Y-family polymerases Pol κ and Pol ι (Fig. 1).

General Features of the Y-Family Polymerases

The Y-family polymerases all adopt the same overall right-handed topology as the replicative polymerases, despite little conservation of primary sequence between them (Yang and Woodgate 2007) (Fig. 2). As in replicative polymerases, the key carboxylate residues necessary for catalysis reside in the "palm" domain, but they are housed within a much larger active site pocket that can accommodate bulky or distorted templates. The fingers and thumb domains, which in the replicative polymerases are required to detect the fit of a correctly paired incoming nucleotide, are generally shorter and make fewer contacts with both the template and incoming nucleotide. This reduces the enzymes' ability to discriminate the correct incoming base and thus contributes to their diminished fidelity and processivity. An additional little-finger domain, also known as the polymerase-associated domain (PAD), helps stabilize the Y-family polymerases on DNA. Critically, the Y-family TLS polymerases also lack a 3′-5′ proofreading exonuclease domain. Together, these features result in genuinely error-prone enzymes with incorporation error rates of up to one in 10 (McCulloch and Kunkel 2008).

Pol η

Pol η, also known as RAD30 in yeast (McDonald et al. 1997), plays a key role in the accurate replication of the principal form of UV-induced DNA damage, the cyclobutane pyrimidine dimer (CPD) (Johnson et al. 1999b). On undamaged DNA, however, Pol η is significantly error prone (Washington et al. 1999; Johnson et al. 2000c; Matsuda et al. 2000; Matsuda et al. 2001). Mutations in Pol η are linked to the clinical syndrome xeroderma pigmentosum variant (XPV) (Johnson et al. 1999a; Masutani et al. 1999), characterized by hypersensitivity to UV-induced skin-pigmentation changes and an increased incidence of skin cancers (Cleaver 1972). Pol η-deficient cells are hypermutable following UV exposure (Stary et al. 2003; Choi and Pfeifer 2005; Busuttil et al. 2008), which results from inaccurate bypass of CPDs by Pol κ or Pol ι working in cooperation with Pol ζ (Wang et al. 2007; Gueranger et al. 2008; Ziv et al. 2009) and from more frequent DNA breaks and chromosome aberrations (Cleaver et al. 1999; Limoli et al. 2002a,b).

Structurally, the ability of Pol η to replicate CPDs accurately is due to its large active site, which is able to accommodate both bases of a CPD in such a manner that the linked Ts can pair correctly with incoming dA (Biertumpfel et al. 2010; Silverstein et al. 2010) (Fig. 2B). The structural distortion of the newly synthesized duplex caused by the T-T dimer is controlled by a positively charged β-strand in the little-finger domain that acts as a splint (yellow patch in Fig. 2B) that ensures the reading frame is maintained. Further DNA synthesis beyond the CPD is limited to three bases by steric clashes that likely result in the enzyme being displaced from the DNA, thereby limiting the extent of low-fidelity synthesis (Biertumpfel et al. 2010). Pol η, however, is not able to completely replicate the highly helix-distorting (6-4) pyrimidine-pyrimidone photoproduct, the second most abundant UV lesion, although Pol η can incorporate dG opposite the 3′ base of this lesion in vitro (Masutani et al. 2000; Johnson et al. 2001). In vivo, however, Pol η-deficient cells do not exhibit a significant defect in the replication of

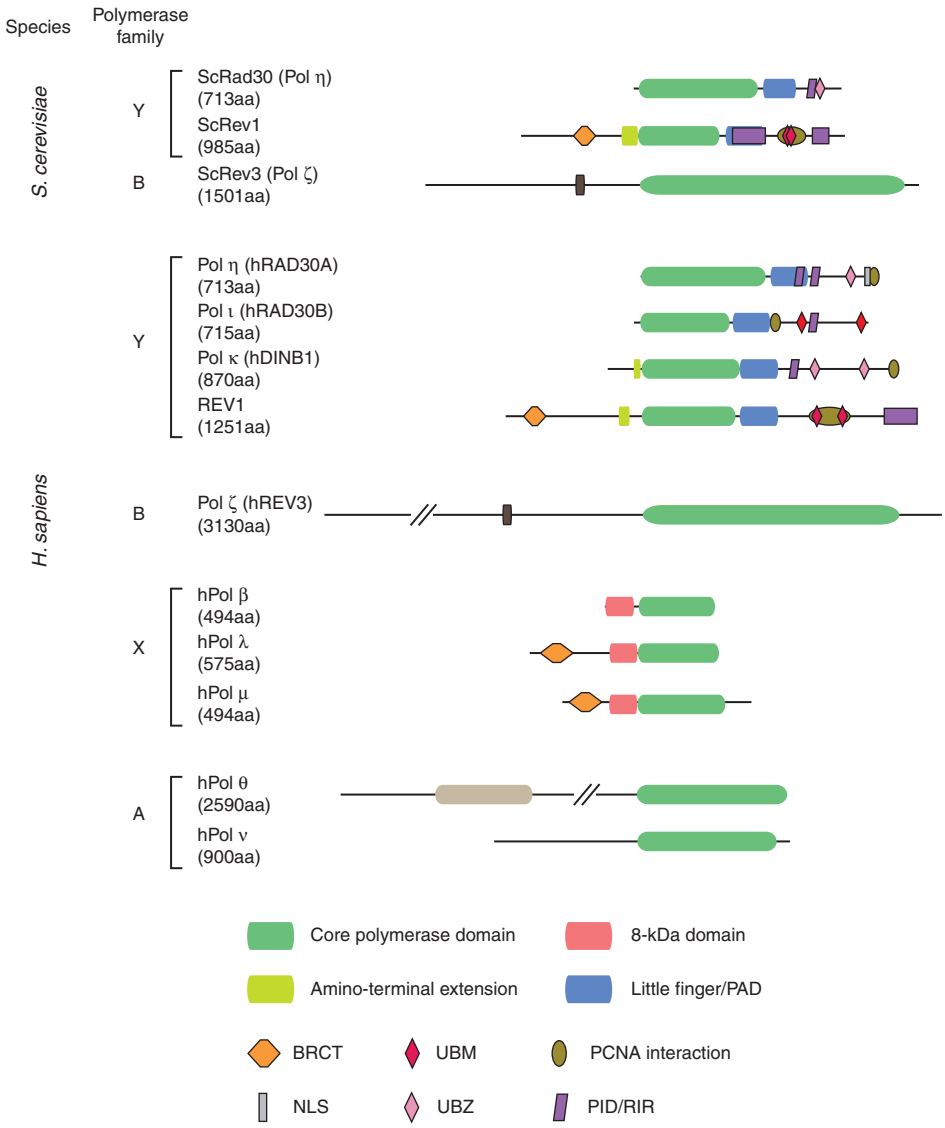

Figure 1. Domain structure of TLS polymerases in *S. cerevisiae* and *Homo sapiens*. The diagrams are to scale except that hREV3 and hPol θ are truncated, as indicated by parallel diagonal lines. The REV7 subunit of Pol ζ is not shown. PAD, polymerase-associated domain (also known as the little finger); BRCT, a domain with homology to the BRCA1 carboxyl terminus; UBM and UBZ, ubiquitin-binding domains; PID, polymerase-interacting domain (of REV1); RIR, REV1-interacting region (of other Y-family polymerases).

(6-4) photoproducts, suggesting that other polymerases, possibly Pol ζ, are normally responsible for accurate replication of this lesion (Gibbs et al. 2005; Hendel et al. 2008; Szüts et al. 2008; Yoon et al. 2010).

The ability of Pol η to accommodate a dinucleotide lesion in its active site also contributes to its ability to replicate intrastrand cross-links at G-G dinucleotides caused by cisplatin, a commonly used chemotherapeutic agent. The active site stabilizes the lesion, allowing the 3′ G to Watson–Crick pair with incoming dCTP, while still providing the rigidly inclined 5′ G space within the active site (Alt et al. 2007;

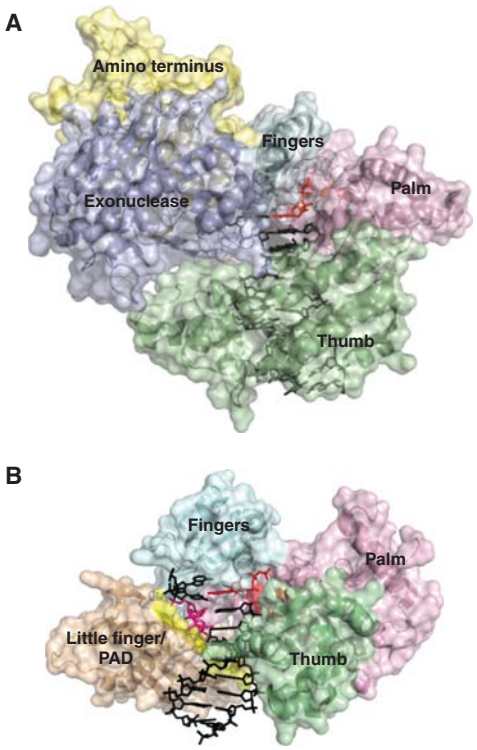

Figure 2. Comparative anatomy of a replicative polymerase and a TLS polymerase. (*A*) *S. cerevisiae* polymerase δ, PDB 3IAY (Swan et al. 2009), a replicative B-family polymerase. The domains are shaded: palm, pink; thumb, green; fingers, cyan; exonuclease, purple. The DNA is in black, and the active site in the palm and incoming nucleotide triphosphate is in red. (*B*) *H. sapiens* polymerase η, PDB 3SI8 (Biertumpfel et al. 2010), a Y-family TLS polymerase with a T-T CPD in the +2 position. The domains in common with Pol δ are shaded the same. The little-finger domain/PAD is shaded in light brown. The DNA is in black, except the CPD, which is pink. The active site and incoming nucleotide triphosphate pairing with the first base after the CPD are in red. The β-strand splint in the little finger/PAD domain that constrains the CPD is highlighted in yellow.

Ummat et al. 2012; Zhao et al. 2012). Replication of the 5′ G is much less efficient and accurate, and, in vivo, this step is catalyzed by Pol ζ (Shachar et al. 2009). Pol η also contributes to the replication of single-base lesions, notably 7,8-dihydro-8-oxoguanine (8-oxoG), the most common oxidative lesion in DNA, opposite which it incorporates the correct dC (de Padula et al. 2004). Again, however, extension requires Pol ζ (Haracska et al. 2003).

Pol ι

Pol ι evolved as a gene duplication of RAD30 (McDonald et al. 1999) and is present in most vertebrates and in some lower eukaryotes, but notably not birds or *S. cerevisiae* (Waters et al. 2009). It is remarkably error prone, exhibiting markedly different fidelities depending on the template base (Tissier et al. 2000b). This property is explained by its unusual active site, which restricts the conformation of template purines to *syn*, promoting Hoogsteen pairing with the incoming base (Nair et al. 2004). In the case of 8-oxoG, the *syn* conformation would normally result in pairing with incorrect dA. However, the narrow active site of Pol ι prevents this, favoring instead pairing with the smaller, and correct, dC (Kirouac and Ling 2011).

The in vivo roles of Pol ι have been more difficult to establish firmly. After some debate about the role of Pol ι in bypassing CPDs (Johnson et al. 2000b; Tissier et al. 2000a; Choi et al. 2006; Vaisman et al. 2006), several studies have now established that Pol ι can indeed contribute to replication of UV lesions in vivo. When both Pol ι and Pol η are disrupted, the formation of UV-induced skin tumors in mice is accelerated (Dumstorf et al. 2006; Ohkumo et al. 2006). Further, in a human Burkitt lymphoma cell line lacking both Pol ι and Pol η, mutational hotspots present in a Pol η single mutant were lost (Gueranger et al. 2008).

Pol ι is also implicated in BER. It possesses dRP lyase activity, interacts with XRCC1, and can substitute for Pol β in an in vitro short-patch BER reaction (Bebenek et al. 2001; Petta et al. 2008). However, the extent to which use of Pol ι in this context contributes to mutagenesis is not known.

Pol κ

Pol κ is the most widely conserved of the Y-family polymerases with homologs in bacteria, archaea, and eukaryotes. Interestingly, however, it is not found in *S. cerevisiae* (Ohmori et al.

2001). It is the most accurate of the Y-family polymerases on undamaged DNA (Johnson et al. 2000a) and has a less marked tendency for −1 frameshifts than its bacterial homolog dinB (Ohashi et al. 2000). Pol κ can bypass many lesions, albeit with relatively low efficiency, but is blocked by dinucleotide lesions (Bavoux et al. 2005a) as its catalytic site can only accommodate a single Watson–Crick base pair (Lone et al. 2007). It can, however, replicate some N^2-dG adducts such as N^2-furfuryl-dG (Jarosz et al. 2006); N^2-(1-carboxyethyl)-2′-dG, a byproduct of glycolysis (Yuan et al. 2008); and N^2-benzo[*a*]pyrene-diol-epoxide-dG (Zhang et al. 2000, 2002b; Rechkoblit et al. 2002) efficiently and accurately. However, N^2-dG adducts formed by the important lipid-oxidation products acrolein and 4-hydroxynonenal (γ-hydroxy-1,N^2-propano-2′-dG and *trans*-4-hydroxy-2-nonenal-dG respectively) block Pol κ. In these cases, Pol κ can operate in a two-step TLS reaction by extending from dC inserted by Pol ι (Washington et al. 2004b; Wolfle et al. 2006). Indeed, Pol κ is adept at extending a diverse range of mismatched termini (Washington et al. 2002), and this is likely to be related to the stability it gains from the manner in which it encircles DNA, largely a function of a unique domain in its amino terminus termed the N-clasp (Lone et al. 2007). However, in vivo evidence suggests that the role of extender polymerase (Fig. 3) is dominated by Pol ζ (Shachar et al. 2009), even for lesions such as N^2-benzo[*a*]pyrene-dG, that in vitro can be efficiently bypassed by Pol κ alone (Zhang et al. 2000; Avkin et al. 2004).

Pol κ also cooperates with Pol δ in the gap-filling step of nucleotide excision repair (NER), being recruited by ubiquitinated proliferating-cell-nuclear-antigen (PCNA) and the scaffold protein XRCC1 (Ogi and Lehmann 2006). The recruitment of Pol κ may facilitate repair-patch synthesis in conditions of low dNTPs, as pertains to G_1, because of the relatively low K_m of the enzyme. However, it would also be expected to contribute to mutagenesis at the rate of about one mutation per 30 repair patches (each of 30 nucleotides) (Ogi et al. 2010).

The Catalytic Activity of REV1

REV1 can insert dC opposite an abasic site (Nelson et al. 1996a) a limited number of adducts, notably N^2-adducts of guanine (Zhang et al. 2002c; Washington et al. 2004a; Nair et al. 2008), and template dG (Nelson et al. 1996a; Haracska et al. 2002). However, it does this in a most unusual way. It does not directly pair the incoming dC with the DNA template. Instead, it swings the template dG out of the helix and temporarily coordinates it with a specialized loop within the little finger. In place of the template dG, REV1 places its own Arg324, which hydrogen bonds with incoming dC (Nair et al. 2005). This mechanism thus allows the bypass of bulky dG adducts (Nair et al. 2008) and suggests how REV1 can act as a template-independent dC transferase. The catalytic activity of REV1 is employed during the bypass of abasic sites in the immunoglobulin (Ig) loci of vertebrate B cells (Jansen et al. 2006; Ross and Sale 2006; Masuda et al. 2009) (see below) and during abasic site bypass in yeast (Kim et al. 2011). REV1 has also been implicated in the replication of sequences capable of forming DNA secondary structures, notably triplet repeats (Collins et al. 2007) and G-quadruplexes (Sarkies et al. 2010). However, inactivation of the dC transferase of REV1 has no measurable effect on the sensitivity of chicken DT40 cells to a range of DNA-damaging agents (Ross et al. 2005) or on murine development (Masuda et al. 2009), but it is required for budding yeast to survive exposure to the G-adducting agent 4-nitroquinoline-1-oxide (Wiltrout and Walker 2011). Nonetheless, complete loss of REV1 results in defective TLS through lesions that are not substrates for its catalytic activity (Gibbs et al. 2000; Simpson and Sale 2003; Ross et al. 2005; Jansen et al. 2009). This is attributable to loss of a second function of REV1 in the coordination of other TLS polymerases, which is discussed further below.

Pol ζ

Pol ζ is a B-family enzyme related to the replicative polymerases Pols δ, ε, and α. It is essential

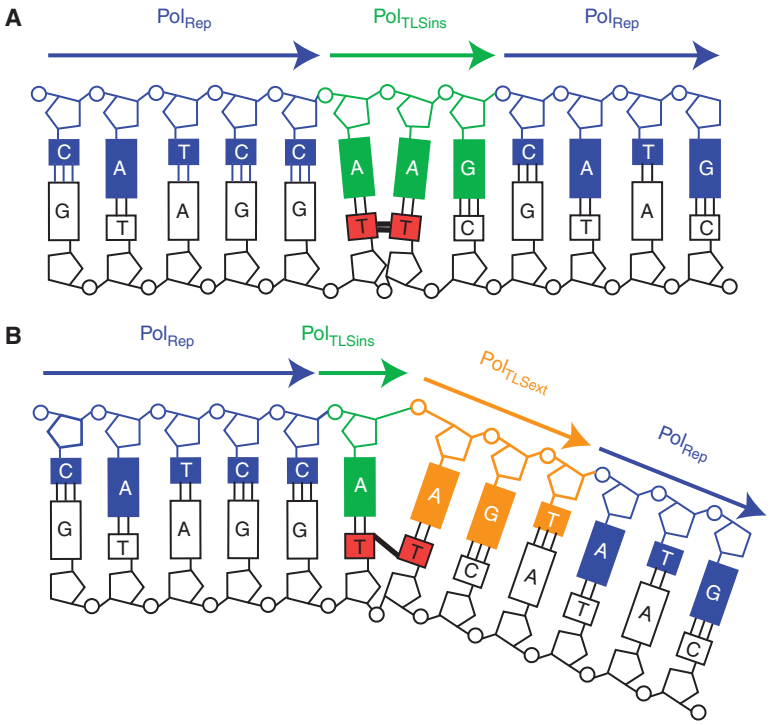

Figure 3. One-step versus two-step TLS. (*A*) The tracts of DNA synthesized during one-step bypass of a T-T CPD. Pol_{Rep}, the replicative polymerases Pol δ and Pol ε. In the case illustrated, bypass of a CPD, the inserter and extender polymerase ($Pol_{TLSins/ext}$) is Pol η, which can replicate this lesion on its own, aided by its large active site pocket and molecular split (see text). The extent of its synthesis patch is limited either by its dissociation from the DNA induced by a steric clash between the lesion and the little-finger domain as the chain elongates or by the primer at the end of a single-stranded gap. (*B*) The tracts of DNA synthesized during two-step bypass of a T-T (6-4) photoproduct. Like many lesions, the highly distorting (6-4) photoproduct is not bypassed in a single step. Instead, an inserter polymerase (Pol_{TLSins}) incorporates a base opposite the 3′T of the lesion, but cannot extend opposite the 5′T. Instead, this is carried out by an extender polymerase (Pol_{TLSext}), most commonly Pol ζ, which can deal with the highly misaligned terminus.

for practically all DNA-damage-induced and a significant proportion of spontaneous mutagenesis in yeast (Lemontt 1971; Cassier et al. 1980; Quah et al. 1980; Morrison et al. 1989; Roche et al. 1994; Harfe and Jinks-Robertson 2000), despite the relatively modest sensitivity of *pol ζ* mutants to DNA-damaging agents (Lawrence and Christensen 1976). The core of Pol ζ is made up of two subunits: Rev3, the catalytic subunit, and Rev7, an accessory subunit that stimulates the activity of Rev3 (Nelson et al. 1996b) and links Pol ζ to Rev1 (Acharya et al. 2005). Like the Pol δ catalytic subunit (Pol3), Rev3 associates with the accessory proteins Pol31 and Pol32 via its carboxyl terminus (Acharya et al. 2009; Johnson et al. 2012), which harbors an iron–sulphur cluster (Netz et al. 2012). This suggests a common element to the recruitment and regulation of the two polymerases.

Pol ζ lacks proofreading exonuclease activity, and the accuracy of the yeast enzyme is lower than Pol δ and Pol ε at about 10^{-4}, similar to Pol α (Johnson et al. 2000b). It is not significantly modified in vitro by the presence of the accessory proteins RPA, RFC, and PCNA (Zhong et al. 2006). In vitro, Pol ζ is generally inefficient at inserting bases opposite lesions but is markedly adept at extending from mismatched primer termini and those containing abnormal

structures (Johnson et al. 2000b; Haracska et al. 2003; Washington et al. 2004a). It has been proposed that this is its principal in vivo role (Prakash et al. 2005). However, genetic evidence argues that in vivo Pol ζ is also capable of significant single-step bypass of lesions such as (6-4) photoproducts (Gibbs et al. 2005; Szüts et al. 2008; Yoon et al. 2010).

Pol ζ has been notoriously hard to study in vertebrate cells. Vertebrate Pol ζ also comprises REV3 and REV7 subunits, but REV3 is considerably larger than its yeast counterpart at some 350 kDa, mostly because of the insertion of a large exon of unknown function toward its amino terminus (Gan et al. 2008). Currently, the only structural information on the complex concerns the REV7-interacting peptide from human REV3 in complex with REV7 showing how the interaction could produce an interface for the binding of REV1 (Hara et al. 2010). Despite the report of an antibody that can detect endogenous REV3 (Wang et al. 2011), the size of Pol ζ, along with its apparently very low levels of expression in cells (Ogawara et al. 2010), has significantly hampered biochemical and cell biological studies. Further, unlike its yeast counterpart, deletion of Pol ζ in mice results in embryonic lethality (Bemark et al. 2000; Esposito et al. 2000; Wittschieben et al. 2000; Kajiwara et al. 2001; O-Wang et al. 2002; Van Sloun et al. 2002) and senescence of primary fibroblasts (Lange et al. 2012). However, transformed mouse and chicken cells are able to proliferate in the absence of REV3 (Sonoda et al. 2003; Zander and Bemark 2004).

Pol ζ also plays a role in double-strand break repair. The DNA synthesis associated with recombination in *S. cerevisiae* is error prone compared with that of replication, and this mutagenesis is dependent on Pol ζ (Strathern et al. 1995; Holbeck and Strathern 1997). Further, immunoprecipitation shows that yeast Pol ζ and REV1 are associated with HO-endonuclease-induced double-strand breaks (Hirano and Sugimoto 2006). Vertebrate Pol ζ and REV1 also appear to be involved in double-strand break repair, although their precise role in this process remains poorly understood (Sonoda et al. 2003; Sharma et al. 2011).

COOPERATION BETWEEN TLS POLYMERASES IN BYPASSING LESIONS

It is evident from the examples discussed above that, in some cases, lesion bypass can be effected in a single step by a single DNA polymerase, for example, the bypass of CPDs by Pol η (Fig. 3A). However, in general, this type of TLS appears to be the exception rather than the rule. Many in vitro and structural studies have highlighted constraints that polymerases face in solving both problems of incorporation opposite a lesion and extension from the resulting mismatched terminus. Certain polymerases seem to be particularly good at the insertion step, for instance, Pol ι and REV1, whereas others are more proficient at extension, notably Pol ζ and, to a lesser extent, Pol κ. These findings led to the proposal that lesion bypass is often a two-step process (Johnson et al. 2000b) (Fig. 3B). Recent work has confirmed that two-step bypass is the norm for several important lesions and that Pol ζ plays a crucial role as an extender in vivo, even in cases where bypass can be carried out by a single polymerase in vitro (Shachar et al. 2009).

CONTROL OF TRANSLESION SYNTHESIS

The potential mutagenicity of TLS polymerases means that they must be carefully controlled. Control of TLS has been most extensively studied in relation to the Y-family polymerases and Pol ζ (Gan et al. 2008; Sale et al. 2012). The regulation of the mutagenic X- and A-family polymerases, discussed below, is much less well understood but is likely to depend on the DNA-repair mechanisms in which they participate.

Unlike in *Escherichia coli*, changes in the overall cellular levels of the translesion polymerases do not appear to play a major role in the regulation of eukaryotic TLS. The exception is Rev1 in *S. cerevisiae*, which is under profound cell-cycle control with markedly elevated levels in G_2 (Waters and Walker 2006). Rather, the preferred method seems to be to locally concentrate the enzymes in replication factories (Kannouche et al. 2001; Sabbioneda et al. 2008; Sale et al. 2012). Formation of these factories is

driven by a number of factors, including PCNA ubiquitination (discussed below), although importantly, focus formation is not strictly necessary for polymerase function (Ross et al. 2005; Gueranger et al. 2008). Nonetheless, local concentration of the Y-family polymerases in the vicinity of distressed forks may be a first step to increase the likelihood of their recruitment to stalled termini (Sabbioneda et al. 2008).

Ubiquitination of Proliferating Cell Nuclear Antigen

Following replication arrest, eukaryotic PCNA is monoubiquitinated at lysine 164 in a reaction mediated by the E3 ubiquitin ligase RAD18 working in conjunction with the E2 ubiquitin-conjugating enzyme RAD6 (Hoege et al. 2002; Stelter and Ulrich 2003; Haracska et al. 2004; Kannouche et al. 2004). In vertebrates, there are additional ubiquitin ligases capable of introducing this modification (Simpson et al. 2006; Zhang et al. 2008; Terai et al. 2010). The activity of RAD18 is stimulated by its association with RPA-coated single-stranded DNA (Davies et al. 2008), and PCNA monoubiquitation serves to recruit the Y-family polymerases to sites of replication arrest by enhancing their direct association with PCNA through an interaction between ubiquitin-PCNA and ubiquitin-binding motif (UBM) or ubiquitin-binding zinc (UBZ) domains in the carboxyl terminus of the polymerases (Bienko et al. 2005).

PCNA can also be polyubiquitinated with K63-linked chains built on the initial ubiquitin conjugated to K164 of PCNA (Hoege et al. 2002; Chiu et al. 2006). In yeast, at least, PCNA can also be SUMOylated (Papouli et al. 2005; Pfander et al. 2005). These modifications are principally linked to recombinational modes of damage bypass. Nonetheless, it is clear that there is complex cross-talk between the mechanisms that control recombination and TLS (Sale 2012). For example, the E3 ligase responsible for PCNA polyubiquitination is also required for efficient TLS in both *S. cerevisiae* (Pages et al. 2008) and *Schizosaccharomyces pombe* (Coulon et al. 2010).

S. cerevisiae lacking ubiquitinatable PCNA (*pol30-119*, which carries a K164R substitution) have a generalized defect in UV-induced mutagenesis that is indistinguishable from either a *rad6* or a *rev3* mutant (Stelter and Ulrich 2003; Haracska et al. 2004). However, both mouse and chicken cells with a *pcna*K164R mutation retain significant TLS activity (Arakawa et al. 2006; Langerak et al. 2007; Szüts et al. 2008; Hendel et al. 2011; Krijger et al. 2011). At least in DT40 cells, the majority of this PCNA ubiquitination-independent TLS is dependent on a noncatalytic function of REV1 (Arakawa et al. 2006; Edmunds et al. 2008; Szüts et al. 2008).

The Noncatalytic Role of REV1 in Translesion Synthesis

In addition to its catalytic function, REV1 plays an important part in the coordination of TLS through protein–protein interactions with other TLS polymerases, PCNA, and ubiquitin. The extreme carboxyl terminus of vertebrate REV1 interacts with the other Y-family polymerases (Guo et al. 2003; Ohashi et al. 2004; Tissier et al. 2004) and with Pol ζ via its REV7 subunit (Murakumo et al. 2001). Deletion of this domain results in the same damage-tolerance defect seen in a complete *rev1* mutant (Ross et al. 2005; Edmunds et al. 2008). The carboxyl terminus of REV1 in *S. cerevisiae* also binds to Rad30 (Pol η) and to Rev7 (Pol ζ), although the way in which it interacts with Pol η appears to be distinct from the vertebrate protein (Acharya et al. 2007; D'Souza et al. 2008; Kosarek et al. 2008). Structural studies of the mammalian REV1 carboxyl terminus have revealed an atypical four-helix bundle with distinct interaction surfaces for REV7 and the REV1-interacting regions (RIRs) of Pol η and κ (Pozhidaeva et al. 2012; Wojtaszek et al. 2012). Adjacent to this carboxy-terminal domain are two UBM domains that may facilitate the binding of REV1 to ubiquitinated PCNA (Garg and Burgers 2005; Guo et al. 2006b) or other ubiquitinated proteins in the vicinity of stalled replication forks (Edmunds et al. 2008). The region in which the UBMs are embedded also mediates a direct interaction with PCNA (Ross et al. 2005; Wood et al. 2007). This arrangement suggests that REV1 could act as an adaptor to coordinate

the interaction between PCNA, the inserter Y-family polymerases, and the extender polymerase Pol ζ during TLS.

This noncatalytic function of REV1 is required for normal replication-fork progression following DNA damage in mouse and chicken DT40 cells (Edmunds et al. 2008; Jansen et al. 2009), suggesting that REV1 plays a role at, or close to, the replication fork. In DT40 cells, this early role appears to be independent of PCNA ubiquitination (Edmunds et al. 2008). REV1 also plays a later role in filling postreplicative gaps, left when replication reprimes downstream of an arrested fork. This role is very prominent in mouse cells (Jansen et al. 2009) but much less obvious in DT40 (Edmunds et al. 2008), probably because it is masked by the very active recombinational pathways in the latter system.

The role of the amino-terminal BRCT domain of REV1 remains incompletely understood. The *rev1-1* mutant of *S. cerevisiae* carries a G193R substitution within the BRCT domain and exhibits defective mutagenic tolerance of (6-4) photoproducts despite retaining almost full catalytic activity (Nelson et al. 2000). Mouse cells lacking the BRCT domain of REV1 exhibit defective DNA damage tolerance, an increase in UVC-induced chromosome aberrations, and spontaneous intragenic deletions in the *HPRT* locus (Jansen et al. 2005). Further, there is strong evidence that the BRCT domain is required for the early role of REV1 close to replication forks (Jansen et al. 2009). The reported binding of the BRCT domain to PCNA (Guo et al. 2006a) has been challenged (de Groote et al. 2011). Instead, it is proposed to preferentially bind recessed 5′ phosphorylated primer-template junctions (de Groote et al. 2011). This raises the interesting possibility that REV1, like the 9-1-1 checkpoint complex, is recruited to postreplicative gaps by binding to the 5′ end of the gap.

TLS Polymerase Phosphorylation and the 9-1-1 Clamp

The exposure of RPA-coated single-stranded DNA at stalled replication forks not only triggers PCNA ubiquitination, but also ATR-dependent checkpoint activation (Byun et al. 2005). Both REV1 and Pol η are phosphorylated by ATR, although how this regulates TLS is still unclear (Sabbioneda et al. 2007; Pages et al. 2009; Gohler et al. 2011). Significantly, full activation of the ATR-dependent replication checkpoint requires downstream repriming and recruitment of the 9-1-1 checkpoint clamp to the 5′ phosphorylated end of the resulting RPA-coated gap (Zou and Elledge 2003; Majka et al. 2006). As well as stimulating ATR-dependent phosphorylation, this PCNA-like clamp also directly interacts with Pol κ and Pol ζ/Rev1 (Kai and Wang 2003; Sabbioneda et al. 2005). This has led to the suggestion that the apparatus needed for TLS of highly distorting lesions is actually recruited to the 5′ end of a postreplicative gap, subsequently translocating back to the arrested 3′ terminus. This mechanism would intrinsically restrict access of TLS to forks at which stalling is sufficiently persistent to give rise to downstream repriming (Jansen et al. 2007).

Ubiquitination of the TLS Polymerases

An emerging level of polymerase regulation is posttranslational modification of the polymerases themselves by ubiquitination (Bienko et al. 2005; Guo et al. 2006b; Wimmer et al. 2008; Bienko et al. 2010; Jung et al. 2010). The significance of polymerase ubiquitination is still under investigation, the best understood case being Pol η. Pol η is monoubiquitinated on its carboxyl terminus, probably by PIRH2 (Jung et al. 2010), and this inhibits its interaction with PCNA, suggesting that deubiquitination of Pol η is needed for its recruitment to stalled forks (Bienko et al. 2010).

The Fanconi Anemia Pathway

As has already been noted, TLS plays an important role in the cellular response to DNA interstrand cross-links. The replication-dependent repair of cross-links is coordinated by the Fanconi anemia (FA) complex, which is instrumental in ensuring the correct recruitment of both homologous recombination and TLS (Niedzwiedz et al. 2004; Raschle et al. 2008; Knipscheer

et al. 2009; Long et al. 2011). However, evidence is emerging that the FA pathway may also play a role in the coordination of TLS that is independent of cross-link repair, the FA pathway appearing to be particularly important for REV1-dependent TLS (Mirchandani et al. 2008; Kee and D'Andrea 2010; Kim et al. 2012).

The Role of the Lesion Itself

Despite the emergence of these refined mechanisms for polymerase recruitment, it remains largely unclear what determines polymerase selection at a particular lesion. It seems likely, based on some of the parameters discussed above, that the lesion itself plays a role in determining which polymerase is ultimately employed, even if this is simply a question of which enzyme can perform the reaction in the most straightforward manner, seen for example in the replication of CPDs by Pol η. It may also be that the context in which the lesion is found limits the options for its bypass (Sale 2012).

OTHER POTENTIALLY MUTAGENIC POLYMERASES IN VERTEBRATES

Higher eukaryotes, and particularly vertebrates, have an expanded repertoire of potentially error-prone DNA polymerases of the X and A families. These enzymes are capable of TLS and are likely to contribute to mutagenesis in vivo through the specialized support they provide to specific DNA-repair pathways.

Translesion Synthesis and Mutagenesis by Eukaryotic X-Family Polymerases

S. cerevisiae has a single X-family DNA polymerase, Pol 4, which plays a role in NHEJ (Wilson and Lieber 1999; Tseng and Tomkinson 2002) and possibly BER (Bebenek et al. 2005). It can fill in small gaps and extend primer termini that have limited homology but has a high error rate, partly because of lack of proofreading exonuclease activity (Bebenek et al. 2005). Thus, even though the patches it synthesizes are short, its deployment is likely to be mutagenic.

Mammals have four X-family polymerases: Pol β, terminal deoxynucleotidyl transferase (TdT), Pol λ, and Pol μ, of which Pols β, λ, and μ are also capable of lesion bypass. Pol β can incorporate A or C opposite 8-oxoG with roughly equal efficiency because the templating 8-oxoG can adopt both *syn* and *anti* conformations in the active site of the enzyme (Batra et al. 2012). Pol μ is closely related to TdT and, like TdT (Desiderio et al. 1984), plays a role in V(D)J recombination of Ig genes, increasing junctional diversity (Bertocci et al. 2006). However, it is more widely expressed and participates more broadly in NHEJ (Chayot et al. 2012). Pol λ is the closest relative of *S. cerevisiae* Pol 4. It possesses dRP lyase activity and can participate in BER in vitro (Garcia-Diaz et al. 2001). However, its major in vivo role also appears to be in NHEJ, both during V(D)J recombination and more generally. Pol μ and Pol λ are both capable of TLS of a range of lesions in vitro (Duvauchelle et al. 2002; Maga et al. 2002; Zhang et al. 2002a) but have a propensity for primer misalignment that leads to −1 frameshifts (Bebenek et al. 2003; Covo et al. 2004; Bebenek et al. 2008). However, Pol λ is also capable of high-fidelity bypass, including of 8-oxoG, especially in the presence of the auxiliary factors replication protein A (RPA) and PCNA (Maga et al. 2007), helped by its novel proofreading mechanism in which misincorporated dA can be removed by pyrophosphorolysis (Crespan et al. 2012). The in vivo significance of this TLS capability for repair and for mutagenesis remains to be fully explored, but it is likely to facilitate the processing of complex breaks and gaps that also contain damaged bases (Picher and Blanco 2007; Zhou et al. 2008; Skosareva et al. 2012).

Translesion Synthesis and Mutagenesis by the Vertebrate A-Family Polymerases, Pol θ and Pol ν

Pol θ (Mus308) is an unusual enzyme in that it contains both helicase and polymerase domains (Harris et al. 1996; Sharief et al. 1999; Seki et al. 2003), of which there are additional paralogs in vertebrates: Pol ν (Marini et al. 2003), which

lacks the helicase domain, and Hel308, which lacks DNA polymerase domains (Marini and Wood 2002). Unlike most A-family polymerases, such as *E. coli* Pol I, Pol θ, and Pol ν are significantly error prone (Seki et al. 2004; Takata et al. 2006). Pol θ exhibits a similar base substitution frequency to Pol κ but also has a high rate of indel mutagenesis (Arana et al. 2008, 2011), and Pol ν is unusual in incorporating T opposite template G with a frequency approaching 0.5 (Takata et al. 2006). Both enzymes are also capable of TLS. Pol θ can bypass abasic sites in a single step, incorporating A almost as readily as opposite template T (Seki et al. 2004), whereas both can bypass thymine glycol, again incorporating A. Neither Pol θ nor Pol ν can replicate the dinucleotide CPD and (6-4) photoproduct lesions completely (Seki et al. 2004; Takata et al. 2006). However, Pol θ is able to extend from mismatched primer termini including from bases incorporated opposite these UV-induced lesions (Seki and Wood 2008). Recently, Pol ν has also been shown to be capable of bypassing certain extremely large major groove adducts, including interstrand cross-links (Yamanaka et al. 2010), which is facilitated by its ability to catalyze strand-displacement DNA synthesis (Takata et al. 2006).

Evidence points to Pol θ and Pol ν being involved in a number of repair pathways, although firm clues as to their regulation are sparse. Both enzymes are processive and, given their error rates, it seems likely that their access to DNA is controlled. Mus308 was initially identified through its involvement in tolerance of interstrand cross-linking agents (Harris et al. 1996), and the role Pol θ and Pol ν play in DNA interstrand cross-link repair may reflect their ability to perform TLS or participate in certain forms of homologous recombination (Zietlow et al. 2009; Kohzaki et al. 2010; Moldovan et al. 2010; Yamanaka et al. 2010). Importantly, Pol ν both physically and functionally interacts with the FA pathway (Moldovan et al. 2010). Additionally, *Drosophila* Mus308 is involved in microhomology-mediated NHEJ (Chan et al. 2010; Yu and McVey 2010) and Pol θ in BER (Yoshimura et al. 2006; Prasad et al. 2009), which is likely facilitated by its terminal-transferase (Hogg et al. 2012) and dRP-lyase (Prasad et al. 2009) activities. As with the X-family polymerases, it seems likely that these roles are important in contexts involving complex or clustered damage.

THE ROLE OF TRANSLESION SYNTHESIS IN SOMATIC MUTATION OF IMMUNOGLOBULIN GENES

During the vertebrate immune response, TLS is co-opted to help generate an extraordinarily high level of mutagenesis in the Ig genes. Ig somatic hypermutation is driven by targeted deamination of C by activation-induced deaminase (AID) (Di Noia and Neuberger 2007). Although AID can only deaminate dC, its action is responsible for mutagenesis at all four bases. This mutagenesis can be divided into two genetically distinguishable phases, one targeted to C/G base pairs and a second targeted to A/T (Rada et al. 1998) (Fig. 4). These phases reflect the two broad mechanisms by which the translesion polymerases generate mutations; many mutations at C/G reflect error-prone replication of abasic sites, whereas mutations at A/T result from intrinsically error-prone DNA synthesis, principally by Pol η.

Mutagenesis at C/G base pairs is conceptually simple. It can result from direct replication of U generated by AID to give C/G to T/A transitions (Petersen-Mahrt et al. 2002), which account for about 60% of mutations at C/G. Alternatively, U can be excised by uracil DNA glycosylase (UNG) (Di Noia and Neuberger 2002; Rada et al. 2002; Imai et al. 2003) forming an abasic site, from which TLS can generate all possible substitutions. There is strong evidence that the deoxycytidyl transferase activity of REV1 contributes to generating C to G transversion mutations (Jansen et al. 2006; Ross and Sale 2006; Masuda et al. 2009), but it is less clear which other polymerases are involved. A role for Pol θ has been proposed (Masuda et al. 2005; Zan et al. 2005; Masuda et al. 2006), but is controversial (Martomo et al. 2008). The role played by Pol ζ is also enigmatic. Early experiments showed that lowering Pol ζ levels reduced

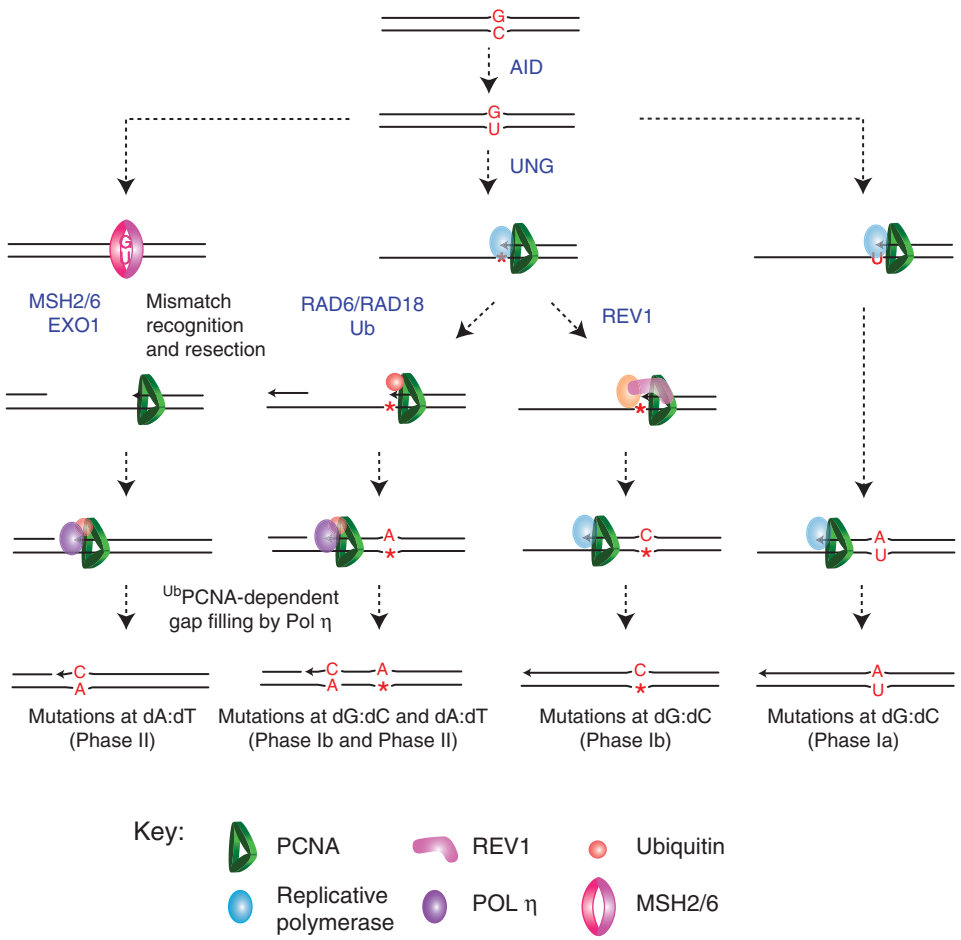

Figure 4. The role of TLS in Ig gene somatic hypermutation. Ig gene hypermutation is initiated by AID, which deaminates dC in the Ig variable-region genes to produce uracil. This triggers a number of mutagenic consequences. In the *right* column, U is replicated by Pol δ or Pol ε, resulting in C/G to T/A transitions (Phase Ia [Neuberger et al. 2003]). Alternatively, U is excised to generate an abasic site (*central* columns). This can be replicated by a polymerase capable of TLS to generate all possible mutations at dC (Phase Ib). REV1 is illustrated and is responsible for C/G to G/C transversions. Alternatively, replication of the abasic site stimulates PCNA ubiquitination, possibly by gap formation following replication arrest, and this recruits Pol η. This can result in mutations at the site of dC deamination to generate Phase Ib mutation and could also lead to Phase II mutations at A/T base pairs generated by misincorporation on undamaged DNA by Pol η. Finally, the initial U/G mismatch can be recognized as a mismatch by MSH2/6, resulting in resection by EXO1 and formation of a gap. This, in turn, stimulates PCNA ubiquitination, recruitment of Pol η, and Phase II mutagenesis at A/T base pairs.

hypermutation (Diaz et al. 2001; Zan et al. 2001), but it has been argued that this may simply be attributable to fewer cell divisions (Schenten et al. 2009). However, careful analysis of the pattern of hypermutation in Pol $\eta^{-/-}$ Pol $\zeta^{+/-}$ animals revealed a decrease in tandem base substitutions compared with Pol $\eta^{-/-}$ Pol $\zeta^{+/+}$ animals, suggesting that Pol ζ does indeed contribute (Saribasak et al. 2012). Further, genetic analysis in DT40 cells suggests that Pol ζ contributes to the extension of at least some bypass events in the Ig locus (Hirota et al. 2010).

The origin of mutations at A/T base pairs is more complex. While still dependent on AID,

A/T mutagenesis requires Pol η (Zeng et al. 2001; Delbos et al. 2005, 2007) and is likely to be the result of intrinsically error-prone synthesis by the enzyme (Pavlov et al. 2002). How is this linked to deamination of dC? A/T mutagenesis is only partially dependent on uracil excision by UNG. A second pathway, involving recognition and processing of the U/G mismatch by MSH2/6 and EXO1 is actually quantitatively more prominent (Rada et al. 2004). It seems likely that both uracil excision and mismatch recognition generate a common substrate, possibly a single-stranded gap (Sale et al. 2009), that promotes ubiquitination of PCNA and recruitment of Pol η (Langerak et al. 2007; Roa et al. 2008; Krijger et al. 2011). Under normal circumstances Pol η is the only polymerase responsible for A/T mutagenesis (Delbos et al. 2007). Pol ι does not appear to contribute to Ig diversification in mice (McDonald et al. 2003; Delbos et al. 2005; Martomo et al. 2006), whereas Pol κ can partially substitute for Pol η, resulting in an alteration in the spectrum of mutagenesis to A to C and T to G transversions (Delbos et al. 2005; Faili et al. 2009). It is thus far unclear how and why Pol η is preferentially selected.

MUTAGENESIS AND THE CANCER GENOME

Cancer is driven by the stepwise accumulation of mutations in key genes that control normal cellular behavior. The advent of massive sequencing technologies has revealed the extraordinary extent of mutagenesis in many tumors, with more than 100,000 individual point mutations per genome in some cases (Stratton 2011). The pattern of somatic mutations in cancer has been likened to an archaeological record that contains the history of all the mutagenic processes operational during the evolution of the tumor (Stratton 2011). Deciphering these patterns is going to be extremely complex and will require a detailed knowledge of how different agents damage DNA and how this damage is converted into mutations through the lesion-replication mechanisms discussed here. In some cases, it is already possible to distinguish patterns that make sense. For instance, tobacco smoke-associated lung-cancer genomes exhibit high levels of G:C to T:A transversions, characteristic of oxidative and adduct damage at G (Pleasance et al. 2010b). Likewise, the melanoma genome exhibits a high number of C to T transitions at dipyrimidine sequences characteristic of error-prone bypass of UV-induced damage (Pleasance et al. 2010a).

However, in many cases the patterns are more complex, and recent approaches have applied machine-learning algorithms to deconvolve individual mutational patterns (Nik-Zainal et al. 2012). A particularly striking example is a class of mutations detected in breast-cancer genomes, often in highly clustered mutational showers or kataegis (Nik-Zainal et al. 2012). These mutations are C to T and C to G substitutions at TpCpX trinucleotides, a signature consistent with the action of some members of the AID/APOBEC family (Beale et al. 2004). Since these enzymes act on single-stranded DNA, the clusters of mutation may reflect areas where single-stranded DNA has been exposed, for instance by replication arrest or resection during homology-directed repair (Robert et al. 2012). By analogy with Ig-gene somatic hypermutation the C to T transitions may reflect direct replication of uracil, whereas the C to G transversions may be the result of REV1-dependent abasic site replication.

TRANSLESION SYNTHESIS AND CANCER: A CAUSE AND POTENTIAL TARGET?

Although an inherited deficiency in Pol η results in a marked predisposition to skin cancer, spontaneously arising mutation of the TLS polymerases has not emerged as a common event in human cancer (Lange et al. 2011). Nonetheless, polymorphisms in the TLS polymerases (Wang et al. 2004; Sakiyama et al. 2005; He et al. 2008) and dysregulation of their expression (O-Wang et al. 2001; Yang et al. 2004; Albertella et al. 2005b; Bavoux et al. 2005b; Lee and Matsushita 2005) have been linked to cancer.

In addition to TLS potentially contributing to the development of some cancers, there is considerable interest in modulating TLS as an

adjunct to conventional cancer chemotherapy. There are two potentially important ways in which this could improve current treatments. First, downregulation of TLS sensitizes cells to commonly used chemotherapeutics, notably the widely used platinum-based DNA cross-linking agents such as cisplatin and oxaliplatin (Simpson and Sale 2003; Sonoda et al. 2003; Wu et al. 2004; Albertella et al. 2005a; Sharma et al. 2012). Proof of the potential of this approach has been provided by experiments in mice in which suppression of REV3 resulted in sensitization of an otherwise chemoresistant lung tumor to cisplatin (Doles et al. 2010). Second, many current cancer chemotherapeutics act by damaging DNA, and TLS could contribute to chemotherapy-induced mutagenesis that leads to the acquisition of resistance and, potentially, secondary tumor formation. The role of TLS in the acquisition of resistance to chemotherapeutics has been demonstrated both in cell lines (Wu et al. 2004; Okuda et al. 2005) and in mice (Xie et al. 2010). Thus, modulation of TLS holds significant promise as an adjunct to conventional chemotherapy, both as a means of increasing its effectiveness and reducing the emergence of resistance and secondary tumors. More work will be needed, however, to understand the balance between the beneficial effects of TLS inhibition and the hazardous consequences of inducing potentially more damaging genomic instability.

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