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Translesion DNA synthesis in the context of cancer research

Philip A Knobel and Thomas M Marti[*]

Abstract

During cell division, replication of the genomic DNA is performed by high-fidelity DNA polymerases but these error-free enzymes can not synthesize across damaged DNA. Specialized DNA polymerases, so called DNA translesion synthesis polymerases (TLS polymerases), can replicate damaged DNA thereby avoiding replication fork breakdown and subsequent chromosomal instability.

We focus on the involvement of mammalian TLS polymerases in DNA damage tolerance mechanisms. In detail, we review the discovery of TLS polymerases and describe the molecular features of all the mammalian TLS polymerases identified so far. We give a short overview of the mechanisms that regulate the selectivity and activity of TLS polymerases. In addition, we summarize the current knowledge how different types of DNA damage, relevant either for the induction or treatment of cancer, are bypassed by TLS polymerases. Finally, we elucidate the relevance of TLS polymerases in the context of cancer therapy.
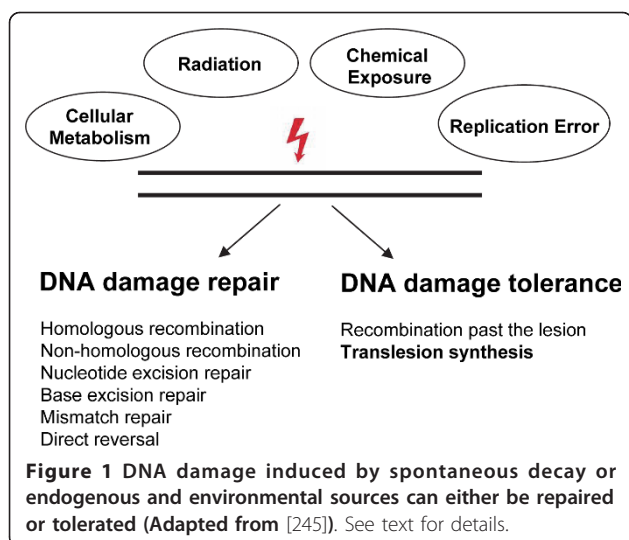
DNA damage response (DDR)

Genomic information is stored as deoxyribonucleic acid (DNA) in every living organism and needs to be protected and maintained to guarantee genomic integrity. Each of the 10^{13} cells of the human body contains 30'000-40'000 genes encoded by 3×10^9 base pairs of the DNA [1-3]. The integrity of the DNA is constantly threatened either by spontaneous decay or by damage induced by endogenous and environmental sources. In every single cell, tens of thousands of DNA lesions per day are formed due to spontaneous hydrolysis and the attack of reactive oxygen species (ROS) and other intracellular metabolites [4]. In the context of cancer research, prominent examples for environmental factors which induce DNA damage are ultraviolet (UV)-light inducing [6-4]pyrimidine-pyrimidone photoproducts ([6-4]PP) and cyclobutane pyrimidine dimers (CPDs), and cigarette smoke, which contains a variety of carcinogens, e.g. benzo(α)pyrene (BaP) [5]. Cancer treatment regimens are frequently based on DNA damage inducing agents. For instance, multimodality therapies of solid tumors are often based on cisplatin, a platinum analogue, which induces intra- and interstrand DNA crosslinks [6].

In addition, accurate DNA duplication is an essential step carried out by a complex DNA replication machinery but errors during this process can also compromise genomic integrity. For example, damaged DNA, which cannot be replicated by the high fidelity replicative DNA polymerases, can lead to stalled replication forks and subsequent replication fork breakdown results in chromosomal instability [7].

To counteract the constant loss or the modification of DNA bases, cells evolved a complex and interplaying system, the so-called DNA damage response (DDR) [8,9]. During DDR, DNA lesions are detected, leading to the activation of a signal cascade resulting either in the repair or the tolerance of the DNA damage, thereby regulating the cellular outcome after genomic insult [4] (Figure 1).

The cellular DNA repair machinery includes non-homologous end joining (NHEJ) and homologous recombination (HR) to repair double strand breaks (DSBs), base excision repair (BER) to counteract modification of the nitrogenous bases, nucleotide excision repair (NER) to excise bulky nucleotide alterations such as UV-induced [6-4]PPs, mismatch repair (MMR) to exchange mispaired nucleotides and direct damage repair for reversal of alkylated nucleotides (Figure 1) [10]. Although DNA repair processes are not as accurate as high-fidelity DNA replication, DNA repair is

* Correspondence: thomas.marti@usz.ch
Laboratory of Molecular Oncology, Clinic and Polyclinic of Oncology, University Hospital Zürich, Häldeliweg 4, CH-8044 Zürich, Switzerland

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Figure 1 DNA damage induced by spontaneous decay or endogenous and environmental sources can either be repaired or tolerated (Adapted from [245]**)**. See text for details.

considered to be error-free. In eukaryotes, DNA damage tolerance involves a error-free pathway dependent on homologous recombination and a more mutagenic pathway based on TLS polymerases [11]. In this review we focus on the contribution of TLS polymerases to DNA damage tolerance and their relevance in cancer research.

Mammalian TLS polymerases: state-of the art
History and Discovery
In 1956, the group of Arthur Kornberg discovered and described an enzyme purified from *Escherichia coli* (*E. coli*), which is able to create an appropriate copy of its DNA substrate, i.e. DNA polymerase (Pol) I [12]. DNA Pol I was shown to generate a copy of the single-stranded DNA of the small bacterial virus ΦX174. The generated DNA kept the infectious activity, thereby confirming that DNA Pol I is able to generate genetically active DNA [13,14]. DNA Pol II was discovered in 1970 [15,16] and shortly afterwards DNA Pol III was discovered as the third DNA-replicating enzyme [17]. Miroslav Radman and coworkers published in 1974 the "SOS repair" model, proposing that the UV-induced mutations of both λ phage and host *E. coli* are due to a "mutation-prone" cellular replication mechanism [18]. Also in the 1970s, a screen in *Saccharomyces cerevisiae* (*S. cerevisiae*) for reversionless (rev) mutants unable to revert an auxotropic marker after UV irradiation led to the discovery of the first eukaryotic genes encoding error-prone TLS polymerases, i.e. *REV1* (encoding the TLS Pol Rev1) and *REV3* (encoding the catalytic subunit of TLS Pol ζ) [19]. A strategy similar to the one which led earlier to the discovery of the UmuDC genes (defects in these genes render cells non-mutable) in *E. coli* [20] resulted in the discovery of and REV7, the structural subunit of TLS Pol ζ, in *S. cerevisiae* [21]. The co-

discovery of bacterial and eukaryotic TLS polymerases revealed the conservation of a cellular process that had until then been considered to be a bacterial-specific function. Subsequently, it was shown that DNA Pol II is part of the "SOS repair" [22,23]. Although the DinB [24] and the UmuDC [20] genes of *E. coli* were discovered much earlier, it was shown only in the 1990s that these gene products constitute the error-prone DNA Pol IV [25] and Pol V, respectively [26,27]. DNA Pol IV and Pol V are inducible by DNA damage and can efficiently bypass various forms of DNA lesions thereby generating most of the SOS-repair dependent mutations [28]. Other mammalian TLS polymerases such as Pol η (eta; *hRAD30A/XPV*), Pol ι (iota; *hRAD30B*), Pol κ (kappa; *DINB1*) and Pol θ (theta; *POLQ*) were identified by searches for homologues of genes of previously identified TLS polymerases [25,29-32]. The TLS Pol μ (mu) [33,34] and Pol λ (lambda) were discovered and described more recently [33,35]. The most recently described TLS Pol ν (nu) was found due to homology with *mus308* [36].

The ability of eukaryotic TLS polymerases to bypass DNA lesions, was firstly described for the yeast TLS Pol ζ, mediating the bypass of UV-induced thymine-thymine cyclobutane pyrimidine dimers (TT-CPDs) [37]. The property of Rev1 to insert deoxycytidine monophosphate (dCMPs) opposite abasic sites was first described in yeast [38]. Subsequently, the role of human TLS Pol ζ to bypass DNA lesions [39] and the function of human Rev1 as dCMP transferase opposite abasic sites [40] were proposed. The UV lesion bypass activity of human TLS Pol η was discovered by the fact that xeroderma pigmentosum variant (XPV) patients show increased susceptibility to UV-induced skin cancer [41] and hyper-mutability [42] due to a defect of TLS Pol η. The human TLS Pol ι was shown to be able to incorporate deoxynucleotides opposite the 3' T of [6-4]PPs and abasic sites [43] and opposite N2-adducted guanines [44]. Human TLS Pol κ was identified and it was first shown that TLS Pol κ protect cells against the lethal and mutagenic effects of BaP [45]. Human TLS Pol θ was identified more recently and it was shown that TLS Pol θ is implicated in somatic hypermutation (SHM) [46-48]. Similarly, TLS Pol λ and TLS Pol μ are both implicated in V(d)J recombination during the immunoglobulin (IgG) diversification process [49,50] whereas TLS Pol ν is able to bypass thymine glycols (Tg) [51]. It can not be excluded that additional TLS polymerases will be discovered in mammalian genomes.

Fidelity of TLS
TLS is defined as the incorporation of a nucleotide across DNA damage followed by extension of the potentially mispaired primer-template, which can be error-

free or error-prone [52]. The basic necessity for the presence of TLS polymerases reflects a trade-off between the maintenance of genomic integrity by avoiding replication fork breakdown and subsequent chromosomal instability and the occurrence of mutations on the nucleotide level by the TLS polymerases mediated DNA damage bypass reaction.

Although the tertiary structure consisting of palm, thumb and fingers is conserved among the different polymerase families, the thumb and fingers of the TLS polymerases are smaller. Compared to the DNA replication polymerases where the fingers tightly bind the incoming dNTPs and make a conformational change upon correct Watson-Crick base pairing, the active site of TLS polymerases is more open and less constrained to reject wrong paired base pairs. Therefore, TLS polymerases are able to mediate the bypass reaction of noncoding DNA lesions. The additional little finger of the Y family TLS polymerases supports the stabilization of the template DNA and influences fidelity and activity [53].

The error rate of DNA replication polymerases of the families A, B and C including correct incorporation of the nucleotide and the proofreading activity is between 10^{-6} and 10^{-8}. Auxiliary proteins such as proliferating cell nuclear antigen (PCNA) and replication protein A (RP-A) [54] and postreplicative MMR decrease the error rate to 10^{-8} and 10^{-10}. The error rate of the TLS polymerases ranges from 10^{-1} to 10^{-3} for replication of undamaged DNA [55,56]. Due to the characteristic low fidelity DNA synthesis and the lack of an exonuclease proofreading activity, it was initially assumed that TLS is generally a mutagenic process. Recently it became clear that the use of specialized TLS polymerases at specific lesions can be error-free. The best example is the ability of TLS Pol η to bypass TT-CPDs, the main DNA lesion induced by both UVB and UVA radiations, in a non-mutagenic manner [57,58].

In this context, it is important to address whether the accurate bypass of a particular lesion by a TLS Pol *in vitro* can be used as an indicator whether this polymerase also processes the corresponding lesion *in vivo*. In addition to the TT-CPDs *in vitro* bypass activity of TLS Pol η, it was also shown that inactivation and deletion of Pol η decreases UV survival of human [59,60] and yeast cells [29,61]. Similarly, TLS Pol θ is able to bypass oxidative DNA lesions, i.e. apurinic/apyrimidinic (AP) sites *in vitro* [62] and knockout of TLS Pol θ in the chicken DT40 B-cell line resulted in hypersensitivity to hydrogen peroxide (H_2O_2) [63]. Thus, in general, the *in vivo* sensitivity to a DNA damage inducing agent of cells deficient for a specific polymerase can be predicted by the *in vitro* ability of the Pol to bypass the induced DNA lesion. However, it became clear that the bypass reaction of most DNA adducts requires the concerted action of protein complexes containing several TLS polymerases. Inactivation or deletion of a TLS polymerase can disrupt protein-protein interactions essential for lesion bypass therefore indirectly affecting the *in vivo* sensitivity to the DNA lesion inducing agent. Thus, based on the *in vivo* sensitivity of an inactivation or deletion mutant, it can not be concluded that the induced DNA damage is processed by the modified TLS Pol activity. For example, although TLS Pol ζ is sensitive to UV-irradiation, other TLS polymerases perform the bypass reaction whereas Pol ζ mainly performs the subsequent extension step [64] (see also Table 1).

TLS polymerase families
The Y-family TLS polymerases η, ι, κ and REV1 [65] and the B-family TLS Pol ζ [66,67] perform most of the TLS in mammalian cells and are well characterized. Less is known about the member of the A-family TLS polymerases, which were more recently identified. An involvement in DNA damage tolerance was also shown for the TLS polymerase members of the X-family although their involvement in DNA repair processes might be their main cellular function (Figure 2).

Family A: TLS polymerases theta (θ) and nu (ν)
The A family polymerases consist of DNA Pol γ (gamma), which is the mitochondrial DNA replicase and the TLS Pol θ and TLS Pol ν.
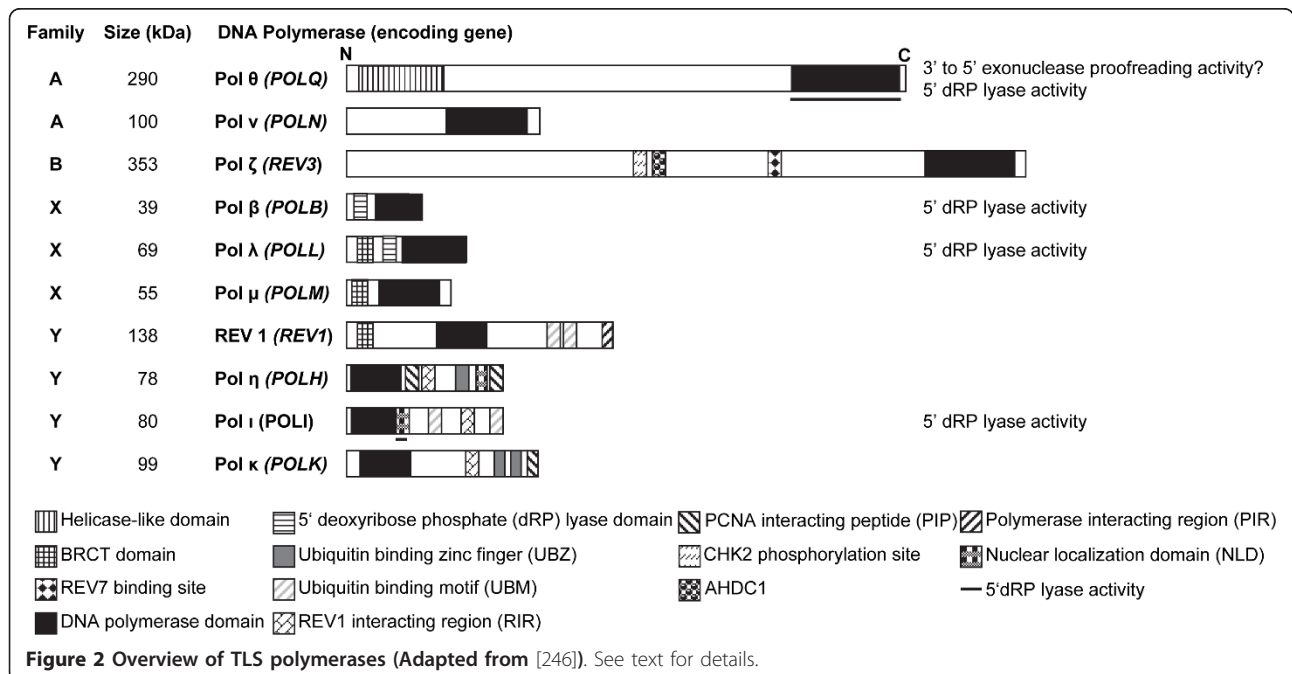
TLS Pol θ
The *POLQ* gene encoding TLS Pol θ was mapped on chromosome 3q. The C-terminal region of TLS Pol θ includes the polymerase motifs A, B and C, which are typical for A family polymerases whereas the N-terminal region contains an ATP domain. The *POLQ* gene encodes a protein of 2592 amino acids (aa). The protein sequence shares homology to the Mus308 protein of *Drosphila melanogaster* [30,68]. Further research estimated a protein of 290 kDa, with an N-terminal ATPase helicase domain although no helicase activity could be detected so far [68]. Additionally, TLS Pol θ has been shown to have 5' dRP lyase activity that is involved in short patch BER *in vitro* [69]. It was shown that TLS Pol θ is able to bypass AP sites and also thymine glycols, e.g. a DNA damage product of ionizing radiation and other oxidative mutagens. TLS Pol θ preferentially incorporates adenine (A) opposite an AP site, which allows DNA replication to continue by using the incorporated nucleotide as a primer (henceforth referred to as extension step) [62]. Additionally, TLS Pol θ can carry out the extension step from mismatches after error prone dNTP incorporation by human TLS Pol ι [70] or *S. cerevisiae* TLS Pol ζ opposite [6-4]PPs *in vitro* [43]. Due to the lacking 3' to 5' exonuclease proofreading activity [68], the fidelity of TLS Pol θ during dNTP

Table 1 TLS opposite DNA lesions by mammalian one and two-polymerase mechanisms

DNA lesion	Insertion	Extension	Outcome
Apurinic/apyrimidinic (AP) site	TLS Pol β/κ/θ/η/λ/μ/δ+PCNA	same Pol	Mutagenic [184,185,189,62,186,191] [187,188,190,191]
	REV1	(Pol η; Pol δ/PCNA)	Mutagenic [191]
	TLS Pol ι	(Pol η; Pol δ/PCNA)	Mutagenic [191]
7, 8-dihydro-8-oxoguanine (8-oxo-G)	TLS Pol ι/κ/μ	same Pol	Accurate [197]/Mutagenic [196,190]
	TLS Pol β/λ/η	ND	Mutagenic/Accurate (+PCNA and RPA) [198,199]
Thymine glycol (Tg)	TLS Pol κ	TLS Pol ζ	Accurate [201]
	TLS Pol ν	same Pol	Accurate (5S-Tg); Mutagenic (5R-Tg) [51]
	TLS Pol β/λ	same Pol	Mutagenic [200]
	TLS Pol θ	ND	Mutagenic [62]
[6-4]photoproduct ([6-4]PP)	ND	TLS Pol ζ	Accurate? [205]
	TLS Pol ι	TLS Pol θ	Mutagenic [70,205]
	TLS Pol η	ND	Mutagenic [51,205]
Cyclobutane pyrimidine dimer (CPD)	TLS Pol η	same Pol	Accurate [51,208,209]
	TLS Pol μ	TLS Pol ζ	Accurate [190]
	TLS Pol ι	TLS Pol ζ	Mutagenic [160]
	TLS Pol κ	TLS Pol ζ	Mutagenic [160]
	ND	TLS Pol ζ	Mutagenic [160]
Benzo[α]pyrene-guanine (BP-G)	TLS Pol κ/η/ND	TLS Pol ζ	Accurate/Mutagenic/Accurate? [64]
	TLS Pol κ/μ	same Pol	Mutagenic [190,211]
Intrastrand-crosslink	TLS Pol η	same Pol; Pol ζ/REV1	Accurate [186,213,64,105]
	Pol β/ζ/μ	same Pol	Mutagenic [122,212,64,214,215]
	TLS Pol κ	TLS Pol ζ	Mutagenic [64]
Interstrand-crosslink (ICL)	Recombination-independent ICL repair including NER, REV1 and TLS Pol ζ		Mutagenic [221]
	Recombination-dependent ICL repair including NER, REV1, TLS Pol ν and ζ		Mutagenic [76,104,222]

Abbreviations: One-polymerase mechanism (same Pol); not determined (ND). Note: TLS activity by a one-polymerase mechanism was in general determined by *in vitro* experiments. Therefore, it can not be excluded that the extension step *in vivo* is performed by another TLS polymerase, i.e. a two-polymerase mechanism.

Figure 2 Overview of TLS polymerases (Adapted from [246]). See text for details.

incorporation is lower than usual for A family polymerases [62]. It is proposed that TLS Pol θ has a function in SHM of IgG diversification due to misincorporation of nucleotides opposite AP sites and the low fidelity during DNA replication of undamaged DNA [46-48]. Conversely, it was suggested that human TLS Pol θ from HeLa cells nuclear extracts synthesizes DNA with a high fidelity and possesses 3' to 5' exonuclease proofreading activity [71].

TLS Pol θ mutant mice show an increase of spontaneous and radiation-induced micronuclei formation [72,73] and TLS Pol θ knockout chicken DT40 B-cell line shows hypersensitivity to hydrogen peroxide (H_2O_2) [63]. Furthermore, CH12 mouse B lymphoma cells containing a knockdown of TLS Pol θ showed elevated sensitivity to UV irradiation, the crosslinking agent mitomycin C and cisplatin, etoposide, ionizing irradiation and the alkylating agent methyl methanesulphonate (MMS) [74]. Thus, TLS Pol θ is involved in the tolerance of a broad range of DNA adducts, which indicates that TLS Pol θ mainly functions as an extender polymerase similar to TLS Pol ζ.

TLS Pol ν
The full length *POLN* gene comprises 24 exons with a length of 900 aa. The *POLN* gene is located on chromosome 4p16.2 and is deleted in approximately 50% of breast carcinomas [75]. The *POLN* gene encodes a protein with a size of 160 kDa. The C-terminal polymerase domain of TLS Pol ν consists of the typical A family polymerase motifs A, B and C and shares 29% identity with the C-terminus of TLS Pol θ. Neither a 3' to 5' nor a 5' to 3' nuclease domain were identified [36]. *In vitro* experiments showed the ability of TLS Pol ν to bypass thymine glycols [51]. Interestingly, it has been shown that TLS Pol ν is involved in cross link repair and homologous recombination. In detail, depletion of TLS Pol ν sensitizes HeLa cells to the DNA cross-linking agent mitomycin C but not to UV irradiation [76]. Depletion of TLS Pol ν in U2OS cells reduced the efficiency of homologous recombination in a GFP-based reporter assay and increased the sensitivity of HeLa cells to camptothecin-induced DSBs, i.e. a substrate for homologous recombination [77]. Contradictorily, deletion of POLN did not sensitize chicken DT40 cells to campothecin. However, the same study showed that TLS Pol ν in chicken DT40 cells has a dominant role in homologous recombination-dependent immunoglobulin gene conversion and in TLS-dependent immunoglobulin hypermutation [78].

Family B: TLS Pol zeta (ζ)
The family B includes the highly accurate DNA polymerases δ (delta), ε (epsilon), α (alpha), and the error-prone TLS Pol ζ [79,80].

TLS Pol ζ
Unlike the replicative DNA polymerases δ and ε, TLS Pol ζ lacks the 3' to 5' exonuclease proofreading activity. The human TLS Pol ζ and its yeast homologue are heterodimeric proteins consisting of the catalytic subunit REV3 and the structural subunit REV7 [37,81]. The human REV3 protein has two transcripts that have a length of 3052 and 3130 aa and the larger protein has a size of 353 kDa compared to 173 kDa of the yeast REV3. The discrepancy in size between the yeast REV3 and the human REV3 is due to the exon 13 with a length of 1388 aa. Human REV3 shows ~36% identity with the N-terminal region, ~29% identity with the central REV7 binding region and ~39% identity with the C-terminal DNA polymerase region of the yeast homologue. The C-terminal region consists of six B-family conserved DNA polymerase motifs and two zinc finger motifs [66,67,82]. Human *REV3* is located on chromosome 6q21 and its mouse equivalent on chromosome 10 [83,84]. Interestingly, the *REV3* gene is located in the chromosomal region 6q21 within the fragile site FRA6F, which is known to be commonly deleted in several types of human leukemias and solid tumors [85]. The human *REV3* contains an out-of-frame ATG in the 5' region that reduces the rate of correct transcripts. Moreover, a sequence upstream of the AUG initiator codon has the potential to form a stem-loop hairpin that lowers the rate of translation. It is suggested that the characteristic structural features in combination with the alternative splicing are responsible for the observed low *REV3* expression levels [66,83]. Indeed, the protein concentration of REV3 in *Xenopus laevis* egg extracts is much lower than those of other replication and repair proteins and does not change within the early embryonic development [86].

In contrast to viable *REV3* null yeast mutants, the disruption of *REV3* in mice causes embryonic lethality around midgestation [87-90]. It is known that during the early stages of embryogenesis checkpoints are actively silenced [91] to allow rapid cell division and it was proposed that REV3 is essential during this strict temporal program. The embryonic lethal effect could not be rescued by the absence of p53 suggesting a p53-independent pathway. However, mouse embryonic fibroblasts (MEFs) with a p53-deficient background could be generated [92,93].

An *in vitro* study showed that *REV3* expression levels are directly regulated by a p53 responding element in the *REV3* promoter region. In addition, *REV3* expression was increased after DNA damage induction in a p53-dependent manner [94]. An independent study also showed increased *REV3* mRNA level after cisplatin treatment [95]. Over-expression of *REV3* in yeast led to

an elevated rate of UV-induced mutagenesis [96]. These findings together with the embryonic lethal effect after *REV3* abrogation indicate that the level of REV3 has to be tightly regulated to maintain genomic integrity.

Within the exon 13 of *REV3*, serine 995 was shown to be phosphorylated by CHK2 [97]. In addition, REV3 shares an AT-hook domain with AHDC1, which has been proposed to be phosphorylated by either ATM and ATR upon DNA damage, indicating a putative regulation of REV3 by ATM and ATR [98].

The human TLS Pol ζ is thought to be the major contributor to the error-prone bypass of DNA lesions. Relevant for tumorigenesis, *REV3* knockout in MEFs leads to increased chromosomal instability in a p53-deficient background [92]. Moreover, in mice with a conditional deletion of REV3, thymic lymphomas occurred with decreased latency and elevated incident in a p53-deficient background [99]. Relevant for cancer therapy, REV3 down-regulation in human foreskin fibroblasts revealed decreased mutation frequency after treatment with UV or BaP-diolepoxide [100]. Similarly, MEFs derived from mice expressing *REV3* antisense revealed decreased mutagenic frequency after UV treatment [101]. Recent *in vitro* and *in vivo* studies revealed that inhibition of *REV3* expression increased the sensitivity of lymphoma to cisplatin [102]. Similarly, REV3 depletion in combination with cisplatin treatment decreased the growth rate of a p53-deficient non small cell lung cancer cell line (NSCLC) transplanted into mice and prolonged the survival of the host. The same study showed that the frequency of 6-thioguanine resistant colonies after cisplatin treatment was reduced in REV3 deficient cells compared to the control [103]. Thus, although REV3 is involved in the maintenance of genomic integrity, inhibition of REV3 expression might enhance the anti-tumor activity of DNA-damage inducing agents as discussed below.

Recent findings propose TLS Pol ζ to have a function not only in TLS synthesis but also in DNA repair, e.g. *REV3* deletion impairs HR [95] and ICL repair [104,105]. Furthermore, SHM and/or class switch recombination (CSR) of IgG were affected by TLS Pol ζ ablation [106].

Although human REV3 contains a REV7 binding region no interaction between full-length REV3 and REV7 could be demonstrated so far. However, it was shown, that a human REV3 fragment interacts with full-length REV7 and a part of human REV7 interacts with human REV3 and REV1 [107]. The human REV7 protein has a length of 211 aa and a size of 24 kDa and shares ~23% identity with the yeast REV7. The human *REV7* is located on the human chromosome 1p36. REV7 displays ~23% identity with the spindle checkpoint assembly protein MAD2 and it is therefore also known as MAD2B and MAD2L2 in higher eukaryotes. The REV7 contains a HORMA (Hop1/Rev7/Mad2) domain that is known to interact with chromatin [108]. Additionally, REV7 interacts with CDH1 and CDC20 of the anaphase-promoting complex/cyclosome (APC/C) [109] and the protein MAD2, a spindle checkpoint protein [81], indicating that REV7 is involved in the regulation of mitosis. Interestingly, the bacterial pathogen *Shigella* delivers the effector IpaB into epithelial cells to efficiently colonize the epithelium. It has been shown that IpaB interacts with MAD2L thereby inducing a cell cycle arrest [110].

Family X: DNA Pol beta (β), TLS Pol lambda (λ) and TLS Pol mu (μ)

The polymerases of the X family include DNA Pol β, terminal deoxynucleotidyl transferase (TdT), TLS Pol λ and TLS Pol μ. All the X family polymerases lack the 3' to 5' exonuclease proofreading activity.

DNA Pol β

DNA Pol β is a 39 kDa monomeric protein and the encoding gene *POLB* is located on chromosome 8 in both mice and human [111]. DNA Pol β consists of two protease resistant segments linked by a short protease sensitive segment indicating that DNA Pol β activity might be controlled by proteolytic activity. The 8 kDa N-terminal lyase domain shows a strong affinity to ssDNA [112], whereas the 31 kDa C-terminal polymerase domain specifically binds double-stranded nucleic acids [113]. The 31 kDa polymerase domain consists of three subdomains. The catalytic subdomain (palm) coordinates two metal-ions and mediates the nucleotidyl-transferase reaction [114] and the other subdomains mediate the binding of duplex DNA (thumb) and nascent base pairs (fingers) [115]. It has been shown that DNA Pol β is able to fill short gaps in double stranded DNA [116]. The 8 kDa lyase domain was shown to direct DNA Pol β to phosphorylated 5' side of a DNA gap for its bypass [117] and recently to mediate the removal of 5' dRP from the AP site via β-elimination after the incision step by the AP endonuclease [118]. Beside the role of DNA Pol β in short patch BER, a function in long patch BER was proposed. Both short- and long-patch repair are impaired after DNA Pol β ablation [119-121]. Additionally, DNA Pol β has a role in bypassing DNA lesions such as cisplatin-DNA adducts [122]. It was shown that DNA Pol β is not involved in the diversification of IgG [123]. DNA Pol β knockout mice are not viable reflecting the important role of Pol β during embryonic development [124].

Down-regulation of DNA Pol β sensitized mouse fibroblasts to cisplatin, UV-irradiation, oxidizing- and methylating agents [125-128]. Ectopic expression of

DNA Pol β leads to aneuploidy, aberrant localization of the centrosome-localized γ-tubulin protein during mitosis, checkpoints defects *in vitro* and tumour induction *in vivo* [129]. In addition, DNA Pol β expression is upregulated in chronic myelogenous leukemia (CML) patients [130]. Thus, a strict regulation of DNA Pol β activity is essential to maintain genomic integrity.

TLS Pol λ
TLS Pol λ has a size of approximately 69 kDa and its gene *POLL* is located on the chromosome 10 in human and on the chromosome 19 in mice [33,35]. The human TLS Pol λ consists of 575 aa and shares 32% residue identity with Pol β, comprising a C-terminal Pol domain including palm, thumb and fingers and the 8 kDa 5' dRP lyase domain. Additionally, TLS Pol λ contains an N-terminal BRCT (BRCA1 C-terminus) domain followed by a serine/proline rich region that is absent in Pol β [131]. Tandem BRCT domains mediate binding to phosphorylated proteins and are widely found in proteins involved in DDR [132]. TLS Pol λ shows terminal TdT activity [131], which prefers the incorporation of pyrimidine nucleotides [133]. The TLS Pol λ has been shown to be less accurate for base substitutions and much less accurate for single-base deletions [134]. Further, TLS Pol λ is unable to differentiate between matched and mismatched primer termini during the extension step, therefore suggesting TLS Pol λ as a candidate for NHEJ and as mismatch extender during TLS [134,135]. Additional *in vitro* studies showed that TLS by Pol λ requires the BRCT domain and is physically and functionally dependent on Ku during NHEJ [136]. Recently, it has been shown, that a TLS Pol λ variant containing of a single nucleotide polymorphism (SNP), a cytosine/thymine variation, leads to increased mutation frequency, chromosomal aberration and defects in NHEJ [137].

TLS Pol λ is also discussed to participate in BER. Uracil-containing DNA was efficiently repaired in an *in vitro* reconstituted BER reaction by the 5' dRP lyase activity of TLS Pol λ, in coordination with its polymerization activity [138]. TLS Pol λ null mice are viable and fertile, but shortening of the heavy chain coding joints was reported [49].

TLS Pol μ
TLS Pol μ has a size of 55 kDa and its gene *POLM* is located on the human chromosome 7. It consists of 492 aa and shares 42% identity with TdT [33,35]. TLS Pol μ, as TLS Pol λ, contains a polymerase domain and a BRTC domain. In contrast to Pol β and λ, the TLS Pol μ lacks a 5' dRP lyase activity [138]. Isolated TLS Pol μ is highly error-prone for frameshifts during DNA synthesis. Interestingly, TLS Pol μ is able to extend from

mismatches by frameshift synthesis mechanism and thereby promoting microhomology search and microhomology pairing between the primer and the DNA template [139]. Moreover, TLS Pol μ shows template-independent polymerase activity under physiological conditions (Mg^{2+} present) preferring the incorporation of pyrimidines and thereby generating terminal microhomology, which can be ligated by the XRCC4-DNA ligase IV [140]. All these findings suggest TLS Pol μ as a candidate for NHEJ of DSBs. It has been shown that TLS Pol μ, as TLS Pol λ, interacts with the Ku-DNA complex through its BRCT domain [136].

Interestingly, TLS Pol μ null mice are viable and fertile, but they show impaired V(D)J recombination due to shortening of the light chain coding ends, but not of the heavy chain coding ends [49,50].

Family Y: Rev1, TLS Pol eta (η), kappa (k) and iota (ι)
The human TLS Pol members of the Y family include REV1, TLS Pol η, TLS Pol k and TLS Pol ι [65]. All the Y family members lack the 3' to 5' exonuclease proofreading activity [56] and share a general conserved N-terminal polymerase domain for the catalytic activity and a non-conserved C-terminus, which, at least for the human TLS Pol ι [141] and Pol κ [142], is responsible for the regulation of the activity. The conserved N-terminus of the DNA polymerase domain includes five motifs (I to V) corresponding to the catalytic core complex. Motif I and II form the catalytic epicentre (palm) with its three acidic residues harbouring the two metal ions mediating the nucleotide transfer. Despite sequence differences, the palm domain with its nucleotide transfer function is widely conserved between Y-family TLS, A- and B-family replicative DNA polymerases. The motifs III and IV belong to the finger and thumb domain, respectively. They bind the triphosphate of the nascent incoming dNTP and mediate the incorporation of the nascent nucleotide whereas the additional motif V binds the primer strand. The C-terminus of the motif V resides either the so called little finger (LF), polymerase associated domain (PAD) or the wrist that supports the DNA synthesis activity and is conserved and unique among the Y-family TLS polymerases [53,143,144].

Rev1
The 1251 aa human REV1 protein has a size of 138 kDa and is encoded by the gene REV1, located on chromosome 2. Beneath the typical Y-family conserved domains, a BRCT domain is located at the N-terminus [132]. At the C-terminal end, there are two ubiquitin binding motifs (UBM) [145] followed by a polymerase interaction region [146].

The polymerase activity of Rev1 is restricted to the incorporation of C over G and DNA lesions such as AP

sites [147]. It has been proposed that the nucleotide insertion activity is not the main function of REV1 but that REV1 helps to coordinate the polymerase switch between the normal- and the substituting TLS Pol upon PCNA monoubiquitination. Murine Rev1 binds ubiquitin through its UBMs and thereby mediating its localization to DNA damage foci. UBM mutants showed increased mutational aberrations after UV irradiation and elevated sensitivity to UV irradiation and cisplatin, which was further increased in UBM and BRCT double-mutants [148]. Although it was shown that murine REV1 binds monoubiquitinated PCNA via its UBMs, it is assumed that the polymerase switch function of REV1 might also be dependent on the other protein interaction domains of REV1, e.g. the BRCT and the polymerase interaction region. In detail, the C-terminal end of human Rev1 is able to interact with several TLS polymerases including Pol η, Pol ι, Pol κ and Pol ζ, thus supporting the assumption that Rev1 acts as a scaffold protein for several TLS polymerases [107,146,149]. Additionally, murine REV1 binds PCNA through its BRCT domain and the monoubiquitination of PCNA enhances this reaction [150].

Rev1 ablation sensitizes DT40 chicken cells to various DNA damaging agents including cisplatin, UV irradiation and MMS. Additionally, Rev1 is required for the maintenance of chromosomal stability after UV irradiation [150]. Rev1 deletion in chicken DT40 cells did not affect basal and damage induced sister chromatid exchange and immunoglobulin gene conversion indicating that homologous recombination repair is likely to be intact [151]. However, the same study showed that knockout of Rev1 in chicken DT40 cells reduced the level of non-templated immunoglobulin gene mutations indicating a defect in translesion bypass of DNA replication blocking lesions. An *in vivo* mouse model showed that REV1 inhibition in B-cell lymphoma reduces cisplatin and cyclophosphamide induced mutagenesis and prolongs survival of mice upon cyclophosphamide treatment [102].

Recently, it has been shown that Rev1 silencing impairs the replication of G-quadruplex (G4) structures thereby, on one side, limiting the recycling of histones and, on the other side, favouring the incorporation of newly synthesized histones resulting in changes of the epigenetic pattern, e.g. gene silencing [152].

TLS Pol η

Loss of TLS Pol η activity in human results in a cancer-prone syndrome known as xeroderma pigmentosum variant (XPV), which is characterized by an increased incidence of skin cancers and sensitivity to sunlight [153]. Human TLS Pol η consists of 713 aa and is encoded by the *POLH* (Xeroderma pigmentosum variant, XPV)

gene, localized on chromosome 6. TLS Pol η has a size of 78 kDa. Additional to the N-terminal conserved polymerase domain, TLS Pol η consists of a Rev1-interacting region (RIR), an ubiquitin binding zinc finger (UBZ), a nuclear localization domain (NLD) and two PIPs [154]. XPV cells are sensitive to UV irradiation and show an increased mutagenic rate despite functional NER indicating that TLS Pol η bypasses specific UV lesions in a non-mutagenic manner [41,57,155]. It is proposed that in the absence of TLS Pol η, TLS Pol ι serves as the error-prone polymerase which bypasses the UV-induced lesions [156]. Also Rev1 is suggested to have a regulatory role in TLS of UV-induced lesions [157]. The PIP and the UBZ domain of TLS Pol η are essential for binding the monoubiquitinated PCNA and its TLS activity. Mutation in either the PIP or the UBZ domain increases the UV sensitivity [145].

Interestingly, it has been shown that loss of TLS Pol η in mice leads to decrease in adenine/thymine mutations during SHM of IgG indicating that TLS Pol η bypasses adenine and thymine in an error prone manner [158].

TLS Pol κ

The human TLS Pol κ is encoded by *POLK* gene and has a length of 870 aa. The TLS Pol κ has a size of 99 kDa and is located on chromosome 5. The N-terminal part consists of the conserved polymerase domain whereas the variable C-terminus consists of a RIR, two UBZ and a PIP. It has been shown that TLS Pol κ co-localizes to a lesser extend with PCNA at replication foci after UV irradiation, hydroxyurea or BaP treatment compared to TLS Pol η [159]. It has been shown that embryonic stem (ES) cells deficient in the TLS Pol κ gene are more sensitive and acquire more mutations after treatment with BaP and that TLS Pol κ bypasses BaP-G accurately and efficiently *in vivo* [45]. Additionally, XPV cells treated with siRNA against TLS Pol κ reveal increased UV sensitivity [160] indicating that TLS Pol κ is able to bypass UV-induced DNA lesions. Recent findings suggest that TLS Pol κ has a function during NER and that its activity is dependent on RAD18 and monoubiquitinated PCNA [161].

A role of TLS Pol κ in promoting tumorigenesis has been discussed since ectopic expression of Pol κ leads to DSBs, aneuploidy and tumorigenesis in nude mice [162].

TLS Pol ι

Human TLS Pol ι is encoded by the *POLI* gene consists of 715 aa. The TLS Pol ι has a size of 80 kDa and is localized on chromosome 18. TLS Pol ι shares with the other Y family members the N terminal conserved polymerase domain and at the variable C-terminal a RIR, two UBMs and the PIP. Interestingly, TLS Pol ι

possesses a 5' dRP lyase activity [163] that is located within a NLD [164].

As in the case of TLS Pol η, the PIP and the UBM domains of TLS Pol ι are important for localization to the replication fork by binding monoubiquitinated PCNA [145]. The localization and accumulation of TLS Pol ι to stalled replication forks is dependent on physical interaction with TLS Pol η [165]. Recently, it has been shown that BER activity is decreased in human fibroblasts in which TLS Pol ι is stably downregulated resulting in increased sensitivity to the oxidizing agents H_2O_2 and menadione [164]. Additionally, after H_2O_2 treatment, TLS Pol ι binds to chromatin and interacts with the BER factor XRCC1, suggesting a role of TLS Pol ι not only in TLS but also in the repair process of oxidative DNA damage [164].

Activation of TLS and polymerase switch reaction

In response to DNA damage, it is proposed that activation of the DNA damage tolerance mechanisms is mainly mediated by modifications of PCNA [166,167]. Monoubiquitination of PCNA by the RAD6-RAD18 complex triggers DNA damage tolerance by TLS. Y-family TLS polymerases can bind to monoubiquitinated PCNA through ubiquitin-binding domains such as the UBM, the UBZ and a PCNA interacting peptide box (PIP), thereby initiating TLS [145,148].

In *S. cerevisiae*, monoubiquitinated PCNA is subsequently polyubiquitinated by RAD5/Ubc13/Mms2, which triggers the error-free DNA damage tolerance response carried out by template switching including fork reversal or recombination past the lesion [168,169]. There are two Rad5 orthologs in humans, HLTF and SHPRH, which are both capable of polyubiquitinating PCNA *in vitro* [170,171]. An interplaying mechanism was proposed between the deubiquitinating enzyme USP1 and ubiquitination factors including RAD6 and RAD18. USP1 acts as a negative regulator, thus removing the ubiquitin residue from the monoubiquitinated PCNA to reduce the mutagenic effect of TLS polymerases [172,173].

Studies in yeast indicate that other factors than PCNA might be involved in the recruitment of TLS polymerases to damaged sites and the subsequent polymerase exchange reaction. Similar to PCNA, the Fanconia anaemia (FA)-ID complex might also promote the exchange of replicative DNA polymerases by TLS polymerases after replication fork blockage [174-176]. The yeast Rad9-Rad1-Hus1 (9-1-1) checkpoint clamp, which is loaded by the RAD24-replication factor C (RFC) clamp loader, physically interacts with Pol ζ indicating a putative role of the (9-1-1) checkpoint clamp in the recruitment of TLS polymerases after checkpoint activation [177].
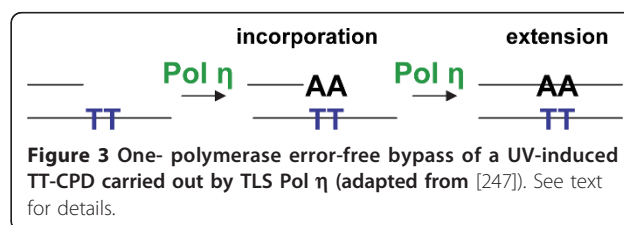
It was proposed that the major function of TLS polymerases is to allow replication to continue in the presence of DNA damage during S-phase [178]. However, subsequent studies in yeast revealed that TLS polymerases also have a function in post-replicative gap filling, the so called post replicative repair (PRR) [179]. PRR is not necessarily temporally separated from S-phase. Yeast REV1, which interacts and thereby regulates the activity of several TLS polymerases, is highly expressed in late S and early G_2 phase [180]. Additionally, studies in mammalian U2OS cells revealed that human REV3 accumulates in G_1-phase and at the G_2/M transition [97]. In addition, it was show that replication-dependent TLS across UV-adducts is not affected in REV3-deficient MEFs. However, although a significant fraction of CPDs were still bypassed, post-replicative repair of [6-4]PPs was completely abolished in REV3-deficient MEFs [181]. Recently, it was shown in chicken DT40 cells that REV1 is essential to maintain normal replication fork progression in the presence of replication-blocking DNA lesions whereas PCNA is required for post-replicative gap filling [182].

Taken together, the activation of the DNA damage tolerance pathway is regulated by the mono- or polyubiquitination of PCNA, REV1, and the FA-ID complex, and might also dependent on the stage of the cell cycle. The regulation of TLS and post-replicative repair recently got into the limelight but more studies are needed to fully elucidate how TLS is regulated during DDR.

One- and two-polymerase mechanism

Some TLS polymerases are able to autonomously replicate over a DNA lesion by both incorporating nucleotides opposite the damaged DNA and by extending from the inserted nucleotides. This so called one- polymerase mechanism has been shown to be performed by TLS Pol κ replicating over AP sites *in vitro* and by TLS Pol η bypassing UV-induced CPDs *in vivo*. Interestingly, CPDs, the main DNA lesions induced by both UVB and UVA radiation, are bypassed by Pol η in an error-free manner (Figure 3).

However, other lesions such as BaP-G or cisplatin-DNA adducts are mainly bypassed in a process requiring the continuous processing by two TLS polymerases, a so

Figure 3 One- polymerase error-free bypass of a UV-induced TT-CPD carried out by TLS Pol η (adapted from [247]). See text for details.

called two-polymerase mechanism. The first TLS polymerase incorporates the nucleotides opposite the DNA lesion and a second TLS polymerase subsequently extends from the inserted nucleotide. Depending on the type of DNA lesion, different pairs of TLS polymerases interact together to replicate the DNA lesion resulting in either an error-free or error-prone bypass (Figure 4).

DNA damage specific TLS

A variety of different DNA damages can result in the arrest of DNA replication, subsequently requiring TLS to avoid the conversion of a stalled replication fork or post-replication gap into a genotoxic DNA double strand break. Thus, TLS is essential to maintain the genomic integrity and it is therefore not surprising that the system is redundant, i.e. every kind of DNA damage can be bypassed by various combinations of one- or two TLS polymerases (Table 1).

DNA apurinic/apyrimidinic (AP) sites

Nonenzymatic hydrolysis of the base-sugar bonds in DNA and the accumulation of BER-intermediates results in the formation of an estimated 10'000 AP sites per human cell per day [4]. Ionizing radiation and bleomycin, which both are used to treat various types of cancers, not only induce cytotoxic DNA DSBs but also AP sites [183]. AP sites are processed by BER and Pol β is the primary enzyme used for gap filling DNA synthesis

Figure 4 Two-polymerase mechanisms for bypassing BaP-G (top) or cisplatin (cisPt) (bottom) DNA adducts. The insertion step is performed by one or a combination of several TLS polymerases incorporating nucleotides in an error-free or error-prone manner opposite the adduct whereas the extension step is mainly carried out by TLS Pol ζ (adapted from [247]). See text for details.

during BER. If not repaired, AP sites can be bypassed by TLS polymerases. However, TLS across an AP site is highly error-prone since the sequence information of an AP site is missing. It was shown that TLS Pol β can bypass an AP site resulting in deletions and base substituting errors [184,185]. A one-polymerase mechanism has been proposed based on an *in vitro* assay where the TLS Pol θ preferentially incorporates an adenine opposite the AP site followed by a guanine (G) and cytosine/thymine (C/T) [62]. An A opposite the AP site is also the best primer for the extension step by TLS Pol θ [62]. In addition, it has been shown *in vitro* that TLS Pol η is able to incorporate nucleotides opposite AP sites preferentially A and G and extend from the incorporated nucleotide favouring an A [186]. *In vitro* studies showed that isolated TLS Pol λ from calf thymus is able to replicate a DNA template containing an AP site *in vitro* [187]. Similarly, isolated human TLS Pol λ is able to synthesize over an AP site and this bypass is stimulated by PCNA *in vitro* [188]. TLS Pol κ is able to autonomously bypass AP sites *in vitro* [189]. Similarly, TLS Pol μ is capable to incorporate nucleotides opposite AP sites *in vitro* although deletions are frequently generated due to primer realignment [190]. Additionally, The replicative DNA Pol δ in the presence of PCNA preferentially inserted A across AP sites and is also able to extend from the lesion [191].

Alternatively, AP sites might also be bypassed by a two-polymerase mechanism. TLS Pol ι and REV1 are able to incorporate one nucleotide opposite an AP site but the extending polymerase was not identified [191].
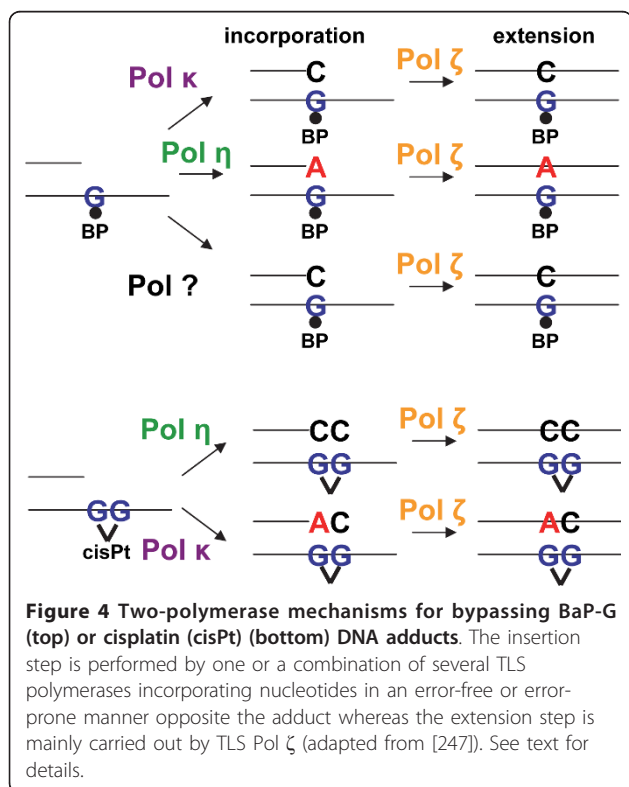
7, 8-dihydro-8-oxoguanine (8-oxo-G)

Oxidative stress can lead to the generation of reactive oxygen species (ROS), which induce base modifications such as 8-oxo-G and thymine glycol [192,193]. Studies have shown an increased level of oxidative DNA damage in cancerous tissue, e.g. a 9-fold increase of 8-oxo-G in tissue from breast cancer compared to surrounding normal tissue [194]. 8-oxo-G is generated by oxidative stress and leads to frequent misincorporation (10-75%) of adenine by human replicative DNA polymerases generating a G:C to T:A transversion [195]. In addition, TLS Pol κ also inserts mainly adenine opposite 8-oxo-G [196]. *In vitro* experiments revealed that TLS Pol ι is able to bypass 8-oxo-G with low efficiency in a generally non-mutagenic manner thereby preferring the incorporation of C over 8-oxo-G followed by A and G [197]. It has been shown that 8-oxo-G lesion *in vitro* can be bypassed by TLS Pol μ resulting in a -1 deletion due to primer realignment during TLS [190]. More recently, it has been shown *in vitro* that 8-oxo-G lesions are mainly bypassed by TLS Pol λ and TLS Pol η and that the presence of PCNA and RP-A increases the fidelity of

correct C incorporation over the incorrect A incorporation opposite the 8-oxo-G 1200-fold for TLS Pol λ and 68-fold for TLS Pol η [198]. Additionally, PCNA and RP-A inhibited error-prone TLS opposite an 8-oxoG by DNA polyerase β [199].

Thymine glycol

Thymine glycol is the most common thymine lesion induced by reactive oxygen species (ROS). *In vitro* studies have shown, that TLS Pol θ is able to incorporate nucleotides opposite both 5R- and 5S-diastereoisomers of thymine glycol with similar efficiency but fails to process the extension step [62]. TLS Pol ν is able to bypass 5S-thymine glycol in an error-free manner whereas the bypass of 5R-thymine glycol was less accurate [51]. TLS Pol β and TLS Pol λ have been shown to bypass thymine glycols in gapped DNA structures. Additionally, dependent on the size of the gap, TLS Pol λ is able to perform the extension step. The bypass fidelity of TLS Pol λ is increased by the presence of PCNA [200]. Recently, a two-polymerase mechanism for error-free thymine glycol bypass including TLS Pol κ as nucleotide inserter and TLS Pol ζ as extender was proposed [201].

[6-4]pyrimidine-pyrimidone photoproduct ([6-4]PP)

The most abundant environmental source of DNA damage is UV-light, which induces nucleotide dimerization, e.g. CPDs and [6-4]PPs at a 3:1 ratio [202]. One hour at the beach results in the induction of approximately 1×10^5 UV-adducts per exposed cell [203].

The induction of [6-4]PPs induces a bend of 44° in the DNA helix, which triggers the efficient recognition and repair of [6-4]PPs by NER [204]. In a primer extension assay, only TLS Pol η was able to autonomously insert nucleotides opposite an [6-4]PP although with a significant lower efficiency than opposite CPDs and without detectable extension [51]. Replication-dependent bypass of UV-adducts was not delayed in REV3-deficient MEFs. However, post-replicative repair of [6-4]PPs was completely dependent on REV3, i.e. TLS Pol ζ [181]. Based on a *in vivo* plasmid assay, alternative two-polymerase mechanism models have been proposed for the bypass of [6-4]PPs. In the first model, TLS Pol ι and TLS Pol η alternatively incorporate nucleotides opposite a [6-4]PP in a process, which is error-free or error-prone at the 3' thymine and 3' cytosine, respectively, and which results in the subsequent extension by a yet unknown DNA TLS polymerase. In the second model, a yet unknown polymerase incorporates nucleotides opposite a [6-4]PP in a process, which is error-free at the 3' thymine or 3'cytosine. Subsequent extension is carried out by TLS Pol ζ [205]. An additional two-step model was proposed where TLS Pol ι incorporates nucleotides opposite a [6-4]PP in an error-prone manner and TLS Pol θ carries out the subsequent extension step from the mismatched primer terminus [70].

Cyclobutane pyrimidine dimer (CPD)

CPDs are not as much DNA helix distorting (9°) as [6-4]PPs and are therefore not an ideal substrate for NER [206]. Hence, CPDs persist longer after UV-irradiation than [6-4]PPs thereby blocking DNA replication more frequently thus rendering their tolerance more dependent on functional TLS. Depletion of TLS Pol η renders human cells sensitive to UV-irradiation, especially to CPD induction [41,160]. TLS Pol η is able to replicate error-free over CPDs *in vitro* [207], with a higher error rate at the 3'T than at the 5'T [208].It has been shown *in vivo* that TLS was reduced and mutagenicity increased in cells lacking TLS Pol η using a quantitative TLS assay measuring TLS across CPDs. Also *in vivo*, most of the mutations were found opposite the 3'T of the CPD [209].

Subsequently, it has been proposed that CPDs in XPV cells, e.g. in the absence of TLS Pol η, are replicated by the two-polymerase mechanism in an error-prone manner. The two step model includes either TLS Pol ι, TLS Pol κ or a yet undefined polymerase or their combined action for the first and the second pyrimidine nucleotide incorporation opposite a CPD, followed by the extension step achieved by TLS Pol ζ and to a minor extend by TLS Pol κ [160]. Additionally, i*n vitro* experiments revealed that TLS Pol μ can autonomously bypass CPDs in a mainly error-free manner and that the subsequent extension was further enhanced by TLS Pol ζ [190].

Benzo[α]pyrene-guanine (BaP-G)

BaP is a major compound of tobacco smoke and forms, upon metabolic activation, a covalent BaP-G DNA adduct, which is associated with the development of lung cancer [210]. BaP-G frequently mispairs during DNA replication with A therefore leading to G:T transversions [210]. *In vitro* and *in vivo* studies showed efficient bypass of BaP-G by TLS Pol κ using a gapped plasmid containing a BaP-G lesion [211]. Additionally, BaP-G has been shown to be bypassed by a two-polymerase mechanism *in vivo*. TLS Pol κ inserts nucleotides opposite a BaP-G error-free whereas insertion by TLS Pol η is error-prone. Subsequent extension is performed by TLS Pol ζ. Since the combined inhibition of TLS Pol κ and TLS Pol η did not decrease TLS to a similar extend than depletion of TLS Pol ζ, it was suggested that a third unknown TLS polymerase might be involved in insertion opposite a BaP-G DNA adduct [64]. Indeed, it was shown that TLS Pol μ was able to bypass bulky DNA lesions including BaP-G DNA adducts [190].

Intrastrand-crosslinks

The chemical agent cisplatin is used for therapeutical treatment of most solid tumors including lung cancer and malignant pleural mesothelioma and forms DNA intra- and interstrand-crosslinks (ICLs), which can lead to a blockage of the DNA replication machinery [6]. Intrastrand-crosslinks are the most prevalent form of cisplatin-induced DNA adducts (> 90%) [6] and are bypassed by one- or the two-polymerase mechanisms. *In vitro* experiments revealed that Pol β can bypass various cisplatin intrastrand adducts [122,212]. It has been reported that TLS Pol η *in vitro* is able to bypass a d(GpG)-cisplatin intrastrand adduct thereby preferentially incorporating C opposite the d(GpG)-cisplatin intrastrand adduct [186,213]. TLS Pol ζ can also bypass cisplatin intrastrand adducts although with low efficiency [214]. It was subsequently shown *in vitro* that TLS Pol μ is less efficient than pol η in catalyzing translesion synthesis past platinum intrastrand adducts but appears to be significantly more efficient than Pol β or Pol ζ [215]. Recently, it has been shown *in vivo*, that either TLS Pol η or TLS Pol κ incorporate the correct or incorrect, respectively, nucleotide opposite the d(GpG)-cisplatin intrastrand adduct and TLS Pol ζ carries out the extension step [64]. Similarly, *in vivo* experiments proposed a model where RAD18/RAD6 dependent monoubiquitination of PCNA activates the bypass of the d(GpG)-cisplatin intrastrand adduct by TLS Pol η and activated TLS Pol ζ performs the subsequent extension in a REV1-dependent manner [105].

Interstrand-crosslink (ICL)

An excellent review of ICL repair and cancer has been published recently [216]. In contrast to the DNA damages described above, ICLs cannot be bypassed since, as the name implies, both DNA strands are covalently linked and therefore no template for DNA synthesis is available. It was proposed that ICLs kill cells due to 1.) Blockage of DNA replication 2.) Stalling of transcription or 3.) Distortion of the chromatin thereby preventing the access of DNA-interacting proteins (reviewed in [216]). Thus, a complex ICL repair machinery evolved and it was shown that TLS polymerases are involved in this process.

Lipid peroxidation can occur inside the body or in foods before they are eaten resulting in the production of by-products capable of crosslinking DNA, e.g. β-unsaturated aldehydes [217]. Recent biochemical and cellular studies implicated that dialdehydes formed by lipid peroxidation induce minor groove ICLs, which are repaired by a TLS Pol κ dependent mechanism [218]. In cancer therapy, cisplatin is the most widely used crosslinking drug but only approximately 10% of the total DNA adducts induced by cisplatin are ICLs [6]. XPV cells, which are deficient for TLS Pol η, are hypersensitive to cisplatin [219,220].

During the G_1 phase of the cell cycle, ICL are repaired in a recombination-independent pathway including NER, TLS Pol ζ and Rev1 [221]. Experiments with *X. laevis* egg extracts revealed that during replication-dependent ICL repair, the replicative DNA polymerase stalls about 24 nucleotides before the crosslink [222]. At this site, the replicative DNA polymerase is replaced by a TLS polymerase, most probably TLS Pol ν [76], which extends the nascent strand to within 1 base pair of the ICL. Subsequently, DNA nucleases unhook the ICL and REV1 inserts a cytosine opposite the unhooked ICL [223,224]. Several of the TLS polymerases may extend DNA synthesis beyond the ICL but only deletion of TLS Pol ζ abrogates TLS extension in *X. laevis* extracts and is was suggested that only Pol ν, REV1 and Pol ζ are essential for ICL repair (reviewed in [216]). Indeed, a recent study showed in human cells that only REV1 and TLS Pol ζ are required for the repair of ICLs whereas RAD18, TLS Pol η, REV1 and TLS Pol ζ are all necessary for replicative bypass of cisplatin intrastrand DNA crosslinks [175].

Relevance of TLS polymerases in cancer therapy

Targeting the error-prone Pol ζ by deletion of *REV3* in yeast results in a reduced spontaneous mutation rate [225]. On the other hand, selective deletion of *REV3* in mature B cells impaired proliferation and genomic stability [106]. Thus, as indicated in Figure 5, a reduction of the mutation rate by inhibition of TLS is inversely correlated with an increase in gross chromosomal instability. Since genomic instability is a hallmark of cancer, an important question is how carcinogenesis is influenced
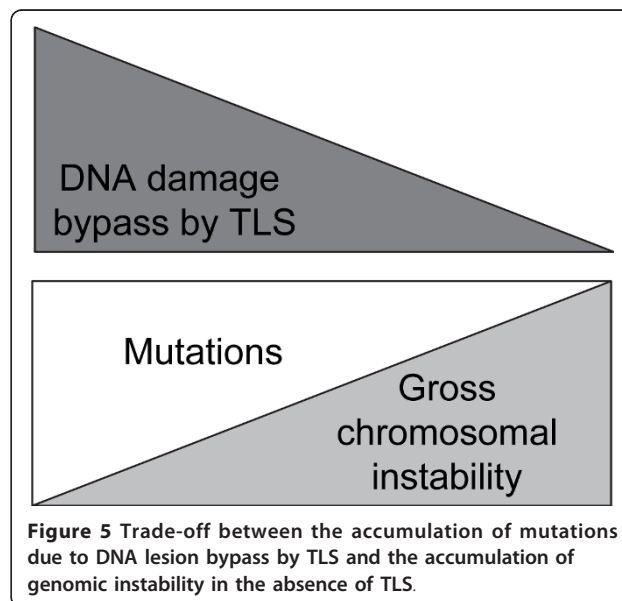

Figure 5 Trade-off between the accumulation of mutations due to DNA lesion bypass by TLS and the accumulation of genomic instability in the absence of TLS.

by changes in the expression or activity of TLS polymerases. XPV patients who are deficient for TLS Pol η activity, suffer from a very high cancer incidence since TT-CPDs, which are bypassed error-free by Pol η, are bypassed in the absence of Pol η by alternative TLS polymerases in an error-prone manner [160]. In addition, somatic mutations of Pol β were identified in adenocarcinoma of the colon [226] and mutations in the gene encoding Pol ι are associated with increased susceptibility to lung cancer in mice [10,227,228] and humans [229]. Single nucleotide polymorphisms in the human *REV1* gene are associated with increased lung cancer risk [229]. Changes in expression and mutations in the genes encoding Pol ι and Pol κ have been found in human tumors [230-232]. Gene expression of TLS Pol θ is upregulated in two cohorts of patients with untreated primary breast cancers, which correlates with poor clinical outcome [233]. In summary, there is a growing body of literature indicating that increased as well as decreased TLS activity is associated with increased and/or accelerated tumorigenesis.

However, transient inhibition of TLS activity might be beneficial for cancer treatment. In detail, the concept of "synthetic lethality" is applied as a therapeutic approach where defects in two pathways alone can be tolerated but become lethal when combined. DDR is often abrogated in cancer cells and it was proposed to develop cancer treatments taking advantage of cancer-specific DDR alterations [234]. The principle of synthetic lethality was successfully applied in cancer therapy of patients carrying mutations in *BRCA1* or *BRCA2*, a specific DNA-repair defect. Inhibition of poly(adenosine diphosphate [ADP]-ribose) polymerase (PARP) resulted in synergistic antitumor activity in the treatment of hereditary ovarian- and breast cancer of patients with *BRCA* mutations [235].

In analogy, there are indications that targeting TLS polymerases *per se*, i.e. without additional chemotherapy, might be applicable for cancer therapy. For example, based on the overlap in function of mismatch repair and DNA polymerase proofreading activity, it was recently shown that inhibition of Pol β or γ induces synthetic sickness/lethality in MSH2- or MLH1-deficient human cancer cells, respectively [236]. Similarly, a recent study from our laboratory revealed that inhibition of *REV3* expression *per se* resulted in decreased colony formation and accumulation of persistent DNA damage in cancer cell lines of different origin whereas cell growth of control cell lines was less affected [237].

In addition, it was proposed that targeting TLS should enhance the therapeutic effect and reduce the resistance formation of DNA-damaging chemotherapeutics [238]. Indeed, inhibition of *REV3* expression sensitized human fibroblasts to cisplatin and decreased the formation of cisplatin resistant cells *in vitro* [95]. Recent findings in a transplantable mouse xenograft model showed that suppression of *REV3* expression increased the sensitivity of chemoresistant adenocarcinomas to cisplatin and reduced occurrence of cisplatin-induced resistance [103]. Similarly, using a preclinical mouse model of Burkitt's lymphoma, it was shown that suppression of both *REV1* and *REV3* expression, sensitized lymphomas to cisplatin [102]. The same study also showed that *REV1* suppression in lymphoma cells inhibited resistance formation after cyclophosphamide treatment *in vitro* and improved cyclophosphamide-based chemotherapy of lymphomas *in vivo*.

Interestingly, studies in yeast showed that resistance formation after hydroxyurea treatment, which inhibits the ribonucleotide reductase and therefore reduces/imbalances the nucleotide pool, is also suppressed by *REV3* deletion [239]. Thus, suppression of TLS polymerases might also reduce resistance formation of drugs whose therapeutic effect is also based on the reduction/imbalance of the nucleotide pool, e.g. gemcitabine and pemetrexed. In summary, inhibition of the expression/activity of TLS activity may increase responsiveness to genotoxic treatments and improve the clinical outcome.

Although the effects of TLS inhibition on cancer cells are well investigated, less is known how inhibition of TLS affects normal cells. No deficiency in cell growth/survival was mentioned after antisense-based inhibition of *REV3* expression in human non-tumor cell lines [66,100,237]. In contrast, it was shown by different groups that *REV3* knockout reduced cell growth of MEFs [92,93]. Thus, it will be crucial to carefully evaluate how normal cells are affected by any cancer therapy based on TLS inhibition. To minimize the risk of malignant transformation of normal cells due to the induction of genomic instability by TLS inhibition during cancer therapy, transient inhibition would be favourable over long-term inhibition, e.g. specific small molecules inhibitors would be favourable over a lentiviral-based system constitutively delivering a TLS polymerase-targeting siRNA.

To date, no specific inhibitors for Y family TLS polymerases are available except for the pyrene nucleotide analogs oxetanocin (OXT)-GTP and -ATP, which are able to inhibit TLS Pol η [240]. Additionally, some natural inhibitors are known to have inhibitory effects such as Petasiphenol, which is a specific inhibitor of TLS Pol λ *in vitro* [241]. It has been shown that Petasiphenol has antiangiogenic activity [242,243]. Tormetic acid is another inhibitor of TLS Pol λ and β but also of replicative DNA polymerases, e.g. Pol α. Tormetic acid showed an antitumorigenic activity *in vivo* [243].

Conclusions

The existence of error-prone TLS polymerases reflects a trade-off between avoiding gross chromosomal instability due to replication fork breakdown and the occurrence of mutations on the nucleotide level (Figure 5).

It was proposed that the evolution of long lived and large animals such as vertebrates necessarily entailed the acquisition of potent tumor suppressive mechanisms [244]. Thus, compared to lower organisms such as bacteria and yeast, it can be speculated that the increased number of TLS polymerases evolved in higher organisms as a tumor suppressive adaptation. Alternatively, research carried out in the last years showed that mammalian TLS polymerases are not only involved in bypassing and repair of DNA lesions but also in the diversification processes of IgG and the maintenance of epigenetic modifications. In summary, TLS polymerases have a function beyond the maintenance of the genomic integrity and it will be interesting tofurther elucidate the involvement of TLS in the delicate balance between cancer suppression and longevity.

Authors' contributions
PAK and TMM were both involved in writing and editing the manuscript. Both authors read and approved the final manuscript.

Competing interests
The authors declare that they have no competing interests.

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