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# Critical Reviews and Perspectives

# Roles of trans-lesion synthesis TLS DNA polymerases in tumorigenesis and cancer therapy

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## ABSTRACT

**DNA damage tolerance and mutagenesis are hallmarks and enabling characteristics of neoplastic cells that drive tumorigenesis and allow cancer cells to resist therapy. The 'Y-family' trans-lesion synthesis (TLS) DNA polymerases enable cells to replicate damaged genomes, thereby conferring DNA damage tolerance. Moreover, Y-family DNA polymerases are inherently error-prone and cause mutations. Therefore, TLS DNA polymerases are potential mediators of important tumorigenic phenotypes. The skin cancer-propensity syndrome *xeroderma pigmentosum-variant* (XPV) results from defects in the Y-family DNA Polymerase Pol eta (Polη) and compensatory deployment of alternative inappropriate DNA polymerases. However, the extent to which dysregulated TLS contributes to the underlying etiology of other human cancers is unclear. Here we consider the broad impact of TLS polymerases on tumorigenesis and cancer therapy. We survey the ways in which TLS DNA polymerases are pathologically altered in cancer. We summarize evidence that TLS polymerases shape cancer genomes, and review studies implicating dysregulated TLS as a driver of carcinogenesis. Because many cancer treatment regimens comprise DNA-damaging agents, pharmacological inhibition of TLS is an attractive strategy for sensitizing tumors to genotoxic therapies. Therefore, we discuss the pharmacological tractability of the TLS pathway and summarize recent progress on development of TLS inhibitors for therapeutic purposes.**

## INTRODUCTION

Mutability has long been recognized as a key hallmark and enabling characteristic of cancer (1,2). Identification of trans-lesion synthesis (TLS) DNA polymerases that perform error-prone replication of damaged DNA templates was once heralded as a major breakthrough in the field of chemical carcinogenesis: TLS polymerases provided a new molecular mechanism for mutagenesis and thus were hypothesized to be potential drivers of carcinogenesis (3). The extent to which this hypothesis has been validated is discussed here.

Historically, TLS was first viewed largely as a mechanism for replicative bypass of bulky DNA adducts and was presumed to be restricted to the S-phase of the cell cycle. We now know that TLS polymerases perform diverse functions in genome maintenance far beyond mediating replicative bypass of damaged DNA templates. TLS is an important gap-filling process that eliminates single-stranded DNA (ssDNA) discontinuities in the genome. TLS DNA polymerases are not always restricted to S-phase and may also be deployed in G0, G1 and G2 (4,5,6,7,8,9,10). Moreover, TLS is closely integrated with the replicative cell cycle and other genome maintenance mechanisms. It is now appreciated that neoplastic cells experience considerable DNA damage from intrinsic and therapeutic sources. Thus, in

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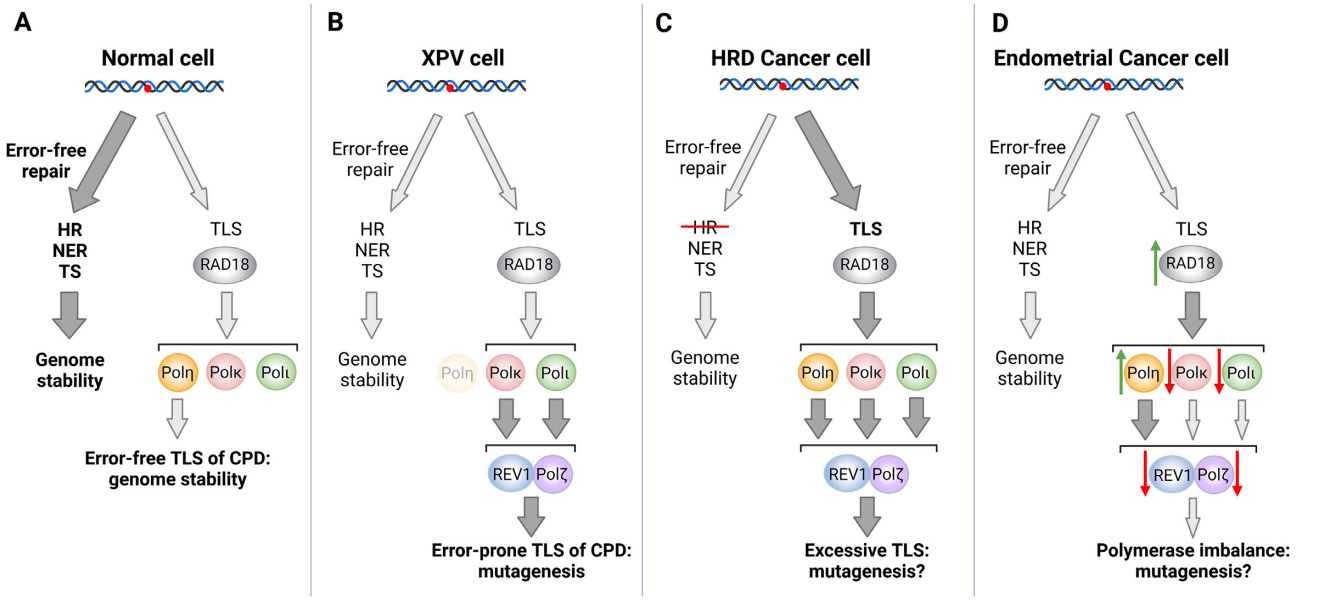



**Figure 1.** Aberrant activation of TLS affects genome stability. (**A**) In normal cells, Polη performs error-free bypass of CPD. TLS is restrained and used sparingly to minimize error-prone DNA synthesis and mutagenesis. (**B**) In XPV cells, Polη is absent. Compensatory error-prone TLS of Polη-cognate lesions by alternative Y-family inserter DNA polymerases and the B-family extender DNA polymerase Polζ leads to mutagenesis. (**C**) HR-deficient cancer (such as BRCA1/2 mutant cells) have increased dependency on TLS for ssDNA gap-filling, which may lead to increased error-prone DNA synthesis and mutagenesis. The mutagenic outcome will depend on the type of DNA damage and the choice of TLS polymerase deployed. (**D**) Cancer-associated increase (green arrow) or decrease (red arrow) in expression of TLS mediators might affect TLS polymerase selection. The upregulation of RAD18 and the TLS polymerase imbalances illustrated here represent the mRNA expression patterns observed in endometrial cancers when compared with normal adjacent tissues (Figure 3).

addition to promoting mutagenesis during carcinogenesis, the DNA damage-tolerance conferred by TLS DNA polymerases has the potential to sustain proliferation and viability of cancer cells. Accordingly, TLS is a dependency of some cancer cells and represents an appealing target pathway for therapeutic development. Small molecule inhibitors of TLS polymerases sensitize cancer cells to genotoxic therapeutic agents and preferentially inhibit the viability of neoplastic cell lines when compared with untransformed cells.

Here, we review the molecular anatomy of the TLS pathway and describe its proposed roles in genome maintenance. We emphasize potential mechanisms by which the TLS pathway activity may be dysregulated in cancer. We survey the evidence that TLS shapes the genomic landscape of cancer cells and contributes to carcinogenesis. We discuss emerging evidence that TLS is a dependency and vulnerability of cancer cells. Finally, we describe ongoing strategies and efforts to target the TLS pathway for therapeutic purposes.

## TLS PATHWAY IMBALANCE DUE TO POLη-DEFICIENCY CAUSES SKIN CANCER

Our understanding of TLS and its relationship with cancer is founded largely on the Hanaoka group's seminal discovery of the TLS DNA polymerase eta (Polη) as the mutated gene product of *xeroderma pigmentosum-Variant* (XPV) patients (11,12). XPV is a sunlight-sensitivity and skin cancer-propensity syndrome. Solar UVB (290–320 nm) exposure is causally linked to skin cancer (13). Two frequent DNA lesions induced by UVB are *cis–syn* cyclobutane pyrimidine dimers (CPD) and pyrimidine (4,5,6) pyrimidone photoproducts (14). In XPV patients nucleotide excision repair (NER) of CPD is intact yet post-replicative DNA repair (to be defined later) is compromised. Using biochemical complementation, Masutani et al. identified Polη as the factor that corrects defective replication of CPD-damaged DNA templates by XPV cell extracts (11). Those workers subsequently showed that the *POLH* gene is mutated in XPV patients and that Polη is the human homologue of yeast Rad30. How then are the clinical features of XPV explained by Polη-deficiency?

Polη has low processivity and low fidelity when replicating undamaged DNA templates. However, this enzyme is specialized to perform efficient and error-free replicative bypass of templates containing helix-distorting CPD (12). Thus, Polη is important for ongoing DNA replication of genomes harboring CPD and confers 'DNA damage tolerance'. XPV patients lacking Polη cannot replicate their UV-damaged genomes and lose viability, thereby explaining the sunlight-sensitivity phenotype. XPV patients develop carcinoma (both squamous- and basal cell carcinoma) with frequencies that are 10,000- and 2,000-fold higher, respectively, when compared with the general population (15). XPV patients are also prone to melanoma (16,17). The high skin cancer incidence of XPV patients is attributed to increased rates of UV-induced mutagenesis in those individuals. Mechanistically, when Polη is absent, compensatory, yet error-prone TLS of CPD lesions by other 'inserter' Y-family DNA polymerases, Pol kappa (Polκ) and Pol iota (Polι) working cooperatively with the B-family 'extender' DNA polymerase Polζ leads to hypermutability (18,19) (Figure 1). There is also evidence for

compensatory usage of other genome-altering DNA repair pathways in UV-irradiated XPV cells, including Homologous Recombination (HR) which leads to increased sister chromatid exchanges, or SCE (20). Therefore, XPV is a malignancy resulting from deployment of alternative, more error-prone genome maintenance mediators when an individual Y-family TLS polymerase (Polη) is absent (Figure 1). Such 'imbalance' and altered DNA repair pathway choice is a general conceptual framework for explaining mechanisms of genome instability in cancer (21). A good example of how imbalanced DNA repair pathway choice may lead to genetic change is provided by *BRCA1*-deficient ovarian cancers in which HR is defective. D'Andrea and colleagues showed that those HRD tumors rely on compensatory overexpression and deployment of the error-prone DNA polymerase POLQ for DSB repair (22). POLQ mediates error-prone microhomology-mediated end-joining (MMEJ) of DSB which helps explain the genomic landscape of *BRCA*-mutated cancers (23,24).

Each TLS polymerase is specialized to perform relatively accurate and efficient bypass of a specific type of DNA damage, referred to as their 'cognate lesion' (25,26). For example CPD are cognate lesions for Polη. Given the error-propensity of TLS polymerases on undamaged DNA templates or non-cognate lesions, their imbalanced activities in relation to each other might promote mutagenesis. XPV is a situation in which Polη activity is reduced relative to other TLS polymerases. By analogy, there are other possible ways in which imbalance and altered pathway choice may develop between the different TLS DNA polymerases (or between TLS polymerases and other DNA repair mechanisms). Figure 1 illustrates possible ways in which TLS might be altered in cancer. Later, we consider some of these possibilities and review evidence that altered TLS impacts DNA damage-sensitivity and the genomic features of cancer cells. First, we describe mechanisms for activation of the Y-family DNA polymerases and integration of TLS with the cell cycle and other elements of the DNA damage response.

## THE TLS POLYMERASE SWITCH: ROLES OF RAD18-MEDIATED UBIQUITIN SIGNALING

To minimize the risk of mutagenesis, TLS DNA polymerases must be tightly regulated and employed only when necessary to replicate damaged templates. During S-phase, encounters between leading strand DNA polymerases and bulky DNA lesions (or other hard-to-replicate helix-distorting structures) lead to DNA replication fork stalling, triggering replacement of replicative DNA polymerases with TLS polymerase(s) at the replisome. This 'polymerase switch' is stimulated by ubiquitin modification of Proliferating Cell Nuclear Antigen (PCNA, a DNA polymerase processivity factor). In response to DNA replication fork stalling, PCNA is mono-ubiquitinated at lysine residue 164 by the RAD18-RAD6 complex (comprising two molecules of the E3 ubiquitin ligase RAD18 and one molecule of the E2 ubiquitin-conjugating enzyme RAD6) (27,28).

RAD18 is recruited to sites of DNA damage through direct interactions with RPA-coated single-stranded DNA (29). During S-phase, ssDNA can arise via helicase-polymerase uncoupling at stalled replication forks (30). The kinetics of RAD18-mediated PCNA mono-ubiquitination typically coincide with ATR/CHK1 signaling which is also initiated by accumulation of RPA-ssDNA. ssDNA can also be generated outside of S-phase, (for example as an intermediate during NER or BER), explaining how both PCNA-mono-ubiquitination and CHK1 activation may occur independently of DNA replication (6,7,31,32).

The four Y-family TLS DNA polymerases (Polη, Polκ, Polι, REV1) associate preferentially with the mono-ubiquitinated form of PCNA through their Ubiquitin-Binding Zinc Finger (UBZ) and Ubiquitin-Binding Motif (UBM) domains (33). Polη, Polκ, and Polι also contain PCNA-interacting Peptide (PIP) domains that interact with PCNA directly to facilitate replisome-binding (33,34). REV1 lacks a PIP-box and instead binds PCNA via its N-terminal BRCA1 C-terminus (BRCT) domain or polymerase-associated domain (PAD) (35,36,37). PCNA-association of Polη is also facilitated by a direct interaction with RAD18 which helps to chaperone the polymerase to the vicinity of stalled DNA replication forks (38,39).

Different Y-family TLS polymerases have different preferred 'cognate lesions' that are bypassed with relative accuracy and efficiency. For example, Polη performs relatively error-free bypass of UV-damaged DNA templates but is error-prone when replicating Benzo[a]pyrene (B[a]P)-adducted DNA (40,41). Conversely, Polκ can bypass B[a]P adducts in a relatively error-free manner (42,43) yet is mutagenic when replicating DNA harboring CPD lesions. Unlike other Y-family enzymes, REV1 cannot synthesize DNA polymers, but adds single C residues to primer termini (44). The main role of REV1 in TLS is non-catalytic, as discussed below. TLS DNA polymerases all lack proofreading exonuclease activity and are viewed as DNA damage tolerance factors. Collectively, recruitment of Y-family TLS DNA Polymerases to mono-ubiquitinated PCNA helps cells to replicate damaged genomes and tolerate diverse forms of genotoxicity. Depending on the nature of DNA damage and the choice of Y-family DNA polymerase(s) deployed, TLS may be error-free or mutagenic (Figure 1).

PCNA-ubiquitination is a trigger for recruitment of all Y-family DNA polymerases to stalled DNA replication forks. It is unclear how the DNA polymerases are selectively recruited in response to their cognate DNA lesions. One possibility is a 'trial-and-error' mechanism whereby all TLS DNA polymerases are recruited to mono-ubiquitinated PCNA but only the relevant polymerase will stably engage the stalled replisome. This model implies that TLS DNA polymerases may compete for mono-ubiquitinated PCNA at stalled replication forks. If Y-family DNA polymerases compete for binding mono-ubiquitinated PCNA, altered expression of any individual DNA polymerase in cancer cells may influence DNA polymerase selection, DNA damage tolerance and mutagenesis.

TLS DNA polymerases also have different affinities for PCNA, a feature that may impact selection of TLS polymerases at stalled DNA replication forks (38,45). For exam-

ple, the Polη PIP box binds PCNA with much higher affinity than the Polκ PIP box (38). Polη is a versatile enzyme that can bypass diverse species of DNA lesions. The high-affinity PIP box may explain why Polη is generally the default TLS polymerase recruited in response to wide variety of DNA lesions.

For some adducts, a single TLS DNA polymerase can perform both nucleotide incorporation opposite the DNA lesion and the subsequent extension from the inserted nucleotide. For example, Polη alone performs both insertion and extension phases of TLS when replicating UV-induced CPD (46,47). However, for many DNA lesions, particularly those that significantly distort the DNA double helix, TLS is mediated by the sequential actions of an 'inserter' and then a separate 'extender' DNA polymerase. First, a Y-family TLS enzyme (usually Polη, Polι, or Polκ) inserts a nucleotide across a DNA lesion. In the second step, the 4-subunit B-family DNA polymerase Polζ (comprising Rev3/Rev7/PolD2/PolD3) performs extension from the initial nucleotide when inserted across from a diverse array of DNA lesions (48,49). Sometimes, Polζ can act as both the inserter and extender, for example when replicating over thymine glycol lesions generated by free-radicals (50).

The requirement for dual DNA polymerases to perform TLS necessitates a second switch from inserter to extender. The inserter/extender TLS polymerase switch is mediated by REV1. REV1 has very limited DNA polymerase enzymatic activity. Instead, the main role of REV1 in TLS is non-catalytic. Consistent with a non-enzymatic role for REV1 in TLS, a catalytically-inactive human REV1 mutant rescues DNA damage-sensitivity of Δrev1 DT40 cells (51). Moreover, human full length REV1, catalytically-inactive REV1, and REV1 lacking the BRCT correct replication fork progression defects of NQO-treated Δrev1 cells to wild-type levels (52). However, a REV1 mutant lacking the C-terminal domain is inactive for DNA damage tolerance (52). The major role of REV1 in TLS is to facilitate dynamic exchange of DNA polymerase partners with PCNA. The C-terminal of REV1 (REV1-CT) can simultaneously bind the REV7 subunit of Polζ (the extender) and REV1-interacting regions (RIRs) from Polη, Polκ and Polι (inserters) (53,54,55). The POLD3 subunit of Polζ also contains an RIR that interacts with the REV1-CT (56).

Taken together, analysis of TLS polymerase-PCNA association dynamics and structure/function studies suggest that the default inserter Polη interacts with monoubiquitinated PCNA via its UBZ domain and high-affinity PIP motif. Simultaneously REV1 associates with a different monoubiquitinated subunit of the same PCNA trimer via BRCT (57,58), PAD and UBM domains (33). If Polη fails to perform bypass (e.g. due to a non-cognate DNA lesion), a more appropriate Y-family enzyme may substitute, also associating with mono-ubiquitinated PCNA via UBZ/PIP-box interactions in a manner that is facilitated by RIR-REV1-CT interactions. Following insertion, Polζ displaces the inserter RIR/Rev1-CT interaction with the RIR motif of its POLD3 subunit. The REV1-CT interactions with REV7 and PolD3 subunits of Polζ promote an 'extender' Rev1/Polζ assembly that is tethered to mono-ubiquitinated PCNA via the BRCT, PAD and UBM domains of REV1 and the PIP motif of PolD3 (56). According to this model, non-enzymatic scaffold functions of REV1 are critically important for survival after DNA damage. Targeting the non-catalytic domains of REV1 may be an effective approach for sensitizing cancer cells to therapy-induced genotoxicity. In particular, the TLS polymerase-interacting C-terminal region represents an appealing target for small molecules that disrupt protein-protein interactions.

## RAD18-INDEPENDENT MECHANISMS OF TLS PATHWAY ACTIVATION

RAD6/RAD18 activity accounts for most of the PCNA K164 mono-ubiquitination in mammalian cells and represents the canonical mechanism of TLS activation. However, PCNA can also be monoubiquitinated at K164 by other E3 ligases that are redundant with RAD18 (59). For example, the E3 ubiquitin ligase cullin-4 in complex with the substrate recognition factor CDT2 ($CRL4^{CDT2}$, which targets multiple cell cycle-regulated proteins for degradation) mono-ubiquitinates PCNA at K164 to activate mutagenic TLS. Moreover, CDT2-depleted cells are cisplatin-sensitive, consistent with a role for $CRL4^{CDT2}$-mediated PCNA ubiquitination in DNA damage tolerance (59). In some cell lines, CDT2 is dispensable for PCNA mono-ubiquitination following UV treatment, suggesting a minor role of CDT2 in TLS when compared with RAD18 (59). The RAD5 homologue HLTF also plays a back-up role in mediating PCNA mono-ubiquitination when RAD18 is absent (60).

TLS pathway activation can also occur in the complete absence of PCNA K164 mono-ubiquitination. For example, PCNA ubiquitination does not disrupt Polδ-PCNA interactions or enhance interaction of TLS DNA polymerases with PCNA (61). It has been suggested that K164 mono-ubiquitination displaces putative inhibitors of PCNA–TLS Polymerase interactions (61). A UBZ-deficient Polη mutant retaining the PCNA-interacting Peptide ('PIP' domain) corrects UV-sensitivity defects of XPV cells, again suggesting that PCNA mono-ubiquitination is dispensable for TLS polymerase activation (62).

On the other hand, mouse embryonic fibroblasts (MEFs) harboring a $PCNA^{K164R/K164R}$ mutant show reduced colony-forming ability after UV irradiation when compared to wild-type cells, indicating that TLS is defective when PCNA cannot be mono-ubiquitinated (63). However, knockdown of *Rev3l*, *Rev1* and *Polh* in $Pcna^{K164R/K164R}$ cells results in additive UV-sensitivity, suggesting existence of a secondary PCNA-ubiquitination independent TLS pathway (63). Polκ is the main TLS polymerase responsible for bypassing DNA lesions induced by the alkylating agent methyl methanesulfonate (MMS). Double mutant $Pcna^{K164R}$ $Polk^{-/-}$ MEFs show greater sensitivity to MMS treatment when compared with MEFs harboring individual $Pcna^{K164R}$ or $Polk^{-/-}$ mutations. Therefore, Polκ functions in a PCNA-mono-ubiquitination independent pathway for tolerance of MMS-induced DNA damage (64). Double mutant $Pcna^{K164R}$ $Poln^{+/-}$ mouse pre-B cells show greater sensitivity to UV and cisplatin treatment than either single mutant alone, again suggesting a PCNA-ubiquitination independent role of Polη in TLS (65). Taken together, PCNA mono-ubiquitination-independent mechanisms contribute to TLS under some circumstances.

## REGULATED EXPRESSION AND ACTIVITY OF TLS FACTORS

Genome maintenance factors are often regulated at the level of expression, and via post-translational modifications that control subcellular distribution, stability, activity, and interactions with binding partners. Such regulatory mechanisms are often important for integrating genome maintenance with the cell cycle. For example, HR is a relatively accurate DSB repair mechanism when compared with NHEJ. However, HR must be restricted to S-phase and G2 when DNA has been replicated and an intact sister chromatid is available as template for repair. Cyclin-dependent kinases (CDKs) play an important role in the HR versus NHEJ pathway choice, particularly by controlling DSB resection, which is required for HR but not NHEJ. Many of the DNA DSB resection and repair factors involved in HR and NHEJ are subject to phosphorylation by CDKs to restrict error-prone NHEJ to G1 and promote relatively error-free HR in S/G2 (66,67,68,69,70,71,72,73).

Transcriptional regulation of resection factors also coordinates DSB repair pathway choice with the cell cycle (74). Cyclins and CDKs are highly dysregulated in neoplastic cells and likely affect DNA repair pathways that impact tumorigenesis and cancer therapy. By comparison with DSB repair factors, little is known regarding transcriptional and post-translational control of TLS proteins, although existence of such regulatory mechanisms seems likely.

Cell cycle-dependent regulation at the level of mRNA and protein expression is reported for REV1 and RAD18. In fission yeast, Rev1 protein levels peak during G1 and decrease during the G1/S transition in a proteasome- dependent manner (75). In contrast, in budding yeast *Saccharomyces cerevisiae*, Rev1 protein levels are low in G1 and early S phase, increase in late S-phase and peak during G2/M (76). *REV1* mRNA levels only change 3-fold throughout the cell cycle whereas Rev1 protein levels are 50-fold higher in G2/M than in G1, suggesting that Rev1 protein is regulated post-transcriptionally (76). DNA damage by UV exposure does not significantly alter Rev1 protein expression (76). Rev1 protein expression is relatively low in S-phase, perhaps to limit mutagenic TLS during DNA replication. Rev1 protein expression peaking during G2/M suggests that TLS plays a role outside of active DNA replication in S-phase. Expression of RAD18 is also cell cycle regulated. In synchronized and untransformed human fibroblasts, RAD18 protein levels are high in S-phase when compared with G0 (quiescent) and G1 cells (6). *RAD18* expression is under transcriptional control of E2F3 (77), possibly explaining the elevated expression of RAD18 in S-phase when RB is phosphorylated and E2F proteins are derepressed. Loss of RB and excess E2F activity are common in cancer and may lead to elevated RAD18 expression. As discussed later, *RAD18* mRNA levels are typically higher in cancer when compared with normal tissue. The RAD18 protein can also be stabilized and overexpressed due to its cancer-specific binding partner, the Cancer/Testes Antigen MAGEA4 (78). Over-production of RAD18 in cultured cells can lead to increased PCNA mono-ubiquitination and DNA damage-independent recruitment of Y-family TLS polymerases to chromatin (79,80). Therefore, high-level expression of RAD18 in cancer (via E2F activity, MAGEA4-binding or other processes) represents a potential mechanism for aberrant TLS activation and mutagenic DNA synthesis. The *POLH* promoter contains a p53-response element (81) and DNA damage-inducible expression of Polη is p53-mediated in several human cell lines (82). Importantly, p53-inducible Polη expression contributes to accelerated bypass of UV-lesions (83).

Integration of TLS factors with the cell cycle and other branches of the DDR is also achieved via phosphorylation-based mechanisms. For example, basal and DNA damage-inducible interaction between RAD18 and Polη is dependent upon RAD18 phosphorylation in the Polη-interacting domain by S-phase kinase CDC7 (84). CDC7 is both a biomarker and drug target in cancer (85,86). Therefore, it is likely that CDC7-mediated RAD18 phosphorylation contributes to TLS pathway activation in tumor cells.

The Polη-interacting domain of RAD18 is also phosphorylated in a DNA damage-inducible manner by c-Jun N-terminal kinase (JNK) to facilitate TLS (87). JNK controls many adaptive responses to both intracellular stresses and environmental exposures including UV light (88). Therefore, RAD18 phosphorylation by JNK might integrate TLS with other stress response pathways. During an unperturbed cell cycle, the RAD18-associated E2 ubiquitin-conjugating enzyme RAD6 is also phosphorylated by CDKs, leading to an increase in its catalytic activity (89). In synchronized cells, RAD6 phosphorylation peaks in G2/M. The extent to which TLS is affected by CDK-induced RAD6 phosphorylation has not been determined. However, RAD6 phosphorylation is a possible mechanism for promoting TLS after completion of S-phase.

Polη is phosphorylated by ATR and Protein Kinase C in response to UV and therapeutic agents (90). The Polη site targeted by ATR (Ser 601) is necessary for normal recovery of DNA replication and DNA damage tolerance after UV-irradiation (91). Polη is also phosphorylated at Ser 687 by CDK2 in a cell cycle-dependent manner. Ser 687 phosphorylation regulates Polη stability during the cell cycle, allowing accumulation in late S and G2 (92).

Whether Polι and Polκ are regulated via direct phosphorylation has not been determined. Nevertheless, multiple core components of the TLS pathway (RAD18, REV1, RAD6, Polη) are directly phosphorylated by kinases whose activities are altered in cancer (CDKs, CDC7, ATR, JNK). Protein phosphorylation represents a potential mechanism for creating TLS pathway imbalances that contribute to mutability and altered DNA damage tolerance of cancer cells.

## TLS OCCURS BOTH AT STALLED LEADING DNA REPLICATION FORK AND POST-REPLICATIVELY

TLS may directly alleviate replication blockades on stalled leading strands (termed TLS 'on-the-fly'), and may operate post-replicatively (i.e. behind an active leading strand) to eliminate daughter strand discontinuities.

### TLS on-the-fly

Seminal work by Lehmann and colleagues revealed that the conversion of the low molecular weight nascent DNAs syn-

thesized in UV-irradiated cells into high-molecular-weight DNA is compromised in XPV cells (93,94). The term post-replication repair (PRR) was used to describe growth of low molecular weight DNA to high molecular weight species. Hanaoka's work eventually revealed that Polη-mediated TLS accounted for PRR (11,12). Subsequent work from many groups has shown that Polη contributes to replication fork progression in cells harboring CPD and other lesions (95,96,97). Polη travels with the replication fork in unperturbed cells (98), is recruited to the vicinity of replication forks in UV-irradiated cells, and resolves helicase/polymerase uncoupling (99). In summary, for UV-induced CPD, on-the-fly direct lesion bypass by Polη is the default response. In the absence of Polη-mediated TLS, other compensatory mechanisms are deployed to sustain DNA synthesis.

### TLS at post-replicative ssDNA gaps

It has long been recognized that UV-irradiation leads to ssDNA-gapped daughter strand DNA on both leading and lagging strands (100,101). Potentially such gaps may result from persistence of Okazaki fragments on the discontinuous lagging strand, and/or due to repriming downstream of a blocked leading strand. Two excellent recent reviews provide a historical perspective on mechanisms of ssDNA gap formation and repair (102,103).

TLS is an important mechanism for filling post-replicative ssDNA gaps. Lopes *et al.* analyzed DNA replication dynamics of TLS-compromised *S. cerevisiae* mutant strains following UV-irradiation (104). Those studies showed that strains lacking rev1, rev3 and rad30 (yeast genes encoding homologues of human REV1, REV3 and Polη respectively) maintain normal rates of fork progression following UV-treatment. However, TLS mutant strains accumulate more ssDNA gaps along replicated duplexes. It was concluded that post-replicative gaps accumulate along replicated daughter strand DNA due to repriming events downstream of the lesions on both leading and lagging strands. Furthermore, TLS (together with HR) counteract gap accumulation without affecting replication fork progression (104). Karras and Jentsch showed that restricting expression of rev3, rad30 or rad18 to G2/ M-phase is sufficient to confer lesion tolerance, further supporting the notion that TLS acts on ssDNA gaps behind newly reprimed replication forks (9). In a related study, Daigaku et al. directly visualized and quantified PRR tracts after UV-irradiation in TLS-mutant yeast strains. Those workers showed that TLS is temporally and spatially separable from global genomic DNA replication (10). Similar PRR roles have been proposed for TLS in human cells (95,105,106). Notably, UVC-irradiated XPV fibroblasts accumulate longer ssDNA stretches both at the replication fork, and behind the leading strand when compared with isogenic Polη-complemented cells (99). Therefore, Polη resolves helicase/polymerase uncoupling and also prevents post-replicative gap accumulation in cells harboring CPD.

The type of DNA damage might determine whether TLS occurs directly at or behind the stalled replication fork (95). For example, repriming may be favored when TLS is absent (99), when template switching is suppressed (107) or when replication fork obstacles are too bulky to be accommodated by DNA polymerases (103).

Insight into a re-priming/restart process that allows continued elongation of nascent daughter strands copying UV-damaged templates was revealed by the discovery of Primase-Polymerase (PRIMPOL; CCDC111). PRIMPOL is an archaeal-eukaryotic primase (AEP) in eukaryotic cells that performs lesion bypass and additionally reprimes DNA replication downstream of bulky lesions and other barriers to DNA synthesis (108,109,110). PRIMPOL is recruited to ssDNA via its interaction with RPA. PRIMPOL does not interact with PCNA, yet contains two RPA-binding motifs, one of which mediates recruitment to stalled replication forks. RPA also stimulates PRIMPOL primase activity (110,111). Therefore, direct associations of PRIMPOL with RPA-coated ssDNA at stalled DNA replication forks may stimulate restart of DNA synthesis and generate gaps behind the newly-reprimed daughter strand. PRIMPOL-mediated repriming activity provides a possible explanation for the presence of post-replicative ssDNA gaps that are filled by the Y-family TLS polymerases (112). Consistent with this idea, PRIMPOL depletion impedes fork progression to a higher extent in XPV fibroblasts when compared with isogenic *POLH*-complemented cells, and suppresses formation of post-replicative ssDNA gaps (99). Interestingly, PRIMPOL primase catalytic activity is dispensable for generation of ssDNA gaps in UV-irradiated XPV cells (99). Moreover, PRIMPOL is not the sole mediator of repriming when Polη is absent (99). RAD51 may also contribute to repriming downstream of stalled replication forks, independently of its roles in recombination-based mechanisms (99).

Post-replicative ssDNA gaps can also be filled by an error-free DNA damage-avoidance mechanism termed 'template switching' (TS). TS relies on DNA replication fork reversal to generate 'chicken-foot structures' and uses the newly synthesized strand as a template to replicate across damaged DNA and suppress discontinuities (113,114,115). TS is initiated by HLTF and SHPRH, mammalian homologues of the yeast E3 ubiquitin ligase and SWI/SNF ATPase family member RAD5 (116). HLTF and SHPRH recruit the ubiquitin-conjugating enzyme UBC13 and a non-canonical UBC variant MMS2 to damaged chromatin, to form a complex that cooperates with RAD6–RAD18 to poly-ubiquitinate PCNA at K164 (27). The two RAD5 homologues poly-ubiquitinate PCNA via distinct mechanisms. SHPRH mediates extension of mono-ubiquitinated K164 (117), while HLTF forms a thiol-linked Ub chain on UBC13 that is transferred to RAD6. RAD18 then transfers the pre-conjugated Ub chain to K164 of unmodified PCNA (118). HLTF and SHPRH promote TS in a DNA damage-specific manner (60). SHPRH mediates responses to alkylating agents (e.g. MMS) whereas HLTF mediates PCNA poly-ubiquitination and confers DNA damage tolerance in response to bulky DNA lesions (including UV, 4-NQO and MMC) (119,120). Poly-ubiquitinated PCNA generated via the concerted actions of RAD18 and RAD5 provides a platform for recruiting ZRANB3 (Zn finger, RAN-binding domain containing 3, also known as Annealing Helicase 2 or AH2) (121). Once recruited to the stalled fork, ZRANB3 facilitates fork regression, replica-

tion fork restart and confers DNA damage tolerance (92–94). TS is intimately coordinated with the TLS pathway. For example, in response to UV-irradiation, HLTF promotes PCNA mono-ubiquitination and Polη recruitment, while inhibiting SHPRH function. Conversely, MMS promotes formation of a RAD18-SHPRH complex and induces HLTF degradation (60). It is unclear how cells choose between error-prone TLS and error-free TS. It is possible that TS is deployed for DNA damage that is too severe to be bypassed via TLS and results in persistence of 3′ ends at stalled DNA replication forks (122). Like TLS, the TS pathway in *S cerevisiae* is also functional when RAD5 is restricted to G2. Therefore, template switching may also play a role in ssDNA gap maintenance in G2 phase (9). Figure 2 illustrates the canonical mechanisms of PRR gap-filling via TS and TLS.

Tirman et al. showed that in cisplatin-treated U2OS cells, TLS factors are involved in temporally distinct mechanisms of gap filling: during G2 phase, ssDNA gap filling is dependent upon RAD18-mediated PCNA mono-ubiquitination and subsequent recruitment of REV1-Polζ (8). However, gap-filling during S-phase is RAD18-independent and requires RAD51, UBC13 and REV1-Polζ, (8). In avian DT40 cells, RAD18 and PCNA ubiquitination, but not REV1, are essential for post-replicative gap filling after UV exposure (52). Like cisplatin-treated U2OS cells, REV1 (but not RAD18 or mono-ubiquitinated PCNA) is required to maintain replication fork progression following DNA damage. In DT40 cells, mutation of REV1, but not RAD18 or PCNA, results in more frequent fork stalling after UV or NQO exposure (52), perhaps reflecting a role of REV1 in template-switching during S-phase. Taken together, these studies underscore the complexity of TLS and demonstrate temporally distinct mechanisms of gap-filling involving different mediators.

The specific factors involved in gap filling, and whether gap filling involves canonical TLS or TS (as may be the case for REV1/Polζ) may depend on multiple factors such as cell type, the species of DNA damage, and availability of other DDR pathways (103). In this regard, oncogenes and the intrinsic replication stresses of neoplastic cells are emerging as key causes of ssDNA gaps that require remediation via the TLS pathway. Yang et al. showed that RAD18 and Polκ are important for preventing both accumulation of ssDNA gaps and mitotic catastrophe in primary cells expressing *CCNE1* or *KRAS* (123). In a related study, Nayak et al. showed that pharmacological inhibition of REV1 interferes with DNA replication and induces ssDNA gap formation in cancer cells (124). HR-deficient cancer cells may be particularly dependent on TLS for gap-filling (125,126). For example in *BRCA1* mutant breast cancer cells, spontaneous ssDNA gaps arising from PRIMPOL-mediated repriming are filled by REV1 and Polζ-dependent TLS (127). It is important to determine precisely how global DNA repair status as well as other factors impact TLS-dependency of cancer cells. This knowledge will enable precision medicine strategies for targeting cancer cells using TLS inhibitors based on their unique genome maintenance characteristics. A more detailed discussion of mechanisms of gap formation may be found in two excellent reviews by Menck and Vindigni (102,103).

## EVIDENCE THAT TLS POLYMERASES CONTRIBUTE TO CANCER

Since mutagenesis is a hallmark of cancer cells and drives carcinogenesis, it is intuitive to hypothesize that error-prone TLS polymerases promote cancer. Since cancer cells rely on TLS for ssDNA gap suppression and tolerance of intrinsic replication stresses (123,124) it is also tempting to hypothesize that TLS sustains carcinogenesis. Here we review evidence that TLS polymerases shape cancer genomes. We describe how TLS polymerases and their regulators are altered in cancer, and we summarize experimental evidence that TLS drives carcinogenesis.

### Contributions of TLS to cancer based on analysis of mutation signatures

Each cancer cell harbors a composite 'mutational portrait' comprising many individual mutations generated by various error-prone genome maintenance processes (128). The 'mutation signature' concept provides a convenient way to analyze large cancer genome sequence data sets and annotate different classes of superimposed mutations in tumors. Studies of multiple cancer types have revealed over 30 single-base substitution (SBS) mutation signatures. Classification of SBS signatures is based upon relative frequencies of the six base substitutions (C>A, C>G, C>T, T>A, T>C and T>G) in tri-nucleotide sequence settings that consider every possible combination of flanking 5′ and 3′ nucleotides. Additionally, methods have been developed for classification of doublet-base substitutions (DBSs) and insertion/deletion mutations (indels): Indels are classified as deletions or insertions. Single-based indels are classified based upon the length of the mononucleotide repeat tract in which they occur. Longer indels are classified based on whether they reside at repeats or with overlapping microhomology at deletion boundaries, and based upon indel-size, repeat and microhomology (128). The etiologies of many mutational signatures have been attributed to specific genotoxic exposures and mutagenic processes. It is proposed that TLS polymerases mediate four of the SBS signatures, namely signatures 2, 5, 13 and 9.

Mutational signature 13, found in breast and bladder cancers, is attributed to the concerted activities of APOBEC (apolipoprotein B mRNA editing enzyme, catalytic) and REV1. APOBEC proteins deaminate Cytosine to Uracil. Subsequent excision of Uracils formed via cytosine deamination generates non-coding AP sites. REV1 is postulated to insert cytosines across the AP sites that lead to C>T or C>G mutations (129,130,131). A recent study experimentally validated the role of REV1 in APOBEC3-mediated mutagenesis. Petljak et al. annotated mutation signatures in human cancer cells that accumulate APOBEC3-associated mutations over time (131). Using REV1-knockout derivative lines, these workers demonstrated that REV1 has a critical role in the generation of signatures SBS2 and SBS13, and might also contribute to a low-fidelity TLS process underlying SBS5 (131). Therefore, there is strong evidence that REV1 contributes to mutagenesis in human tumors.

The other Y-family TLS polymerase strongly implicated in generating mutation signatures in human cancers is

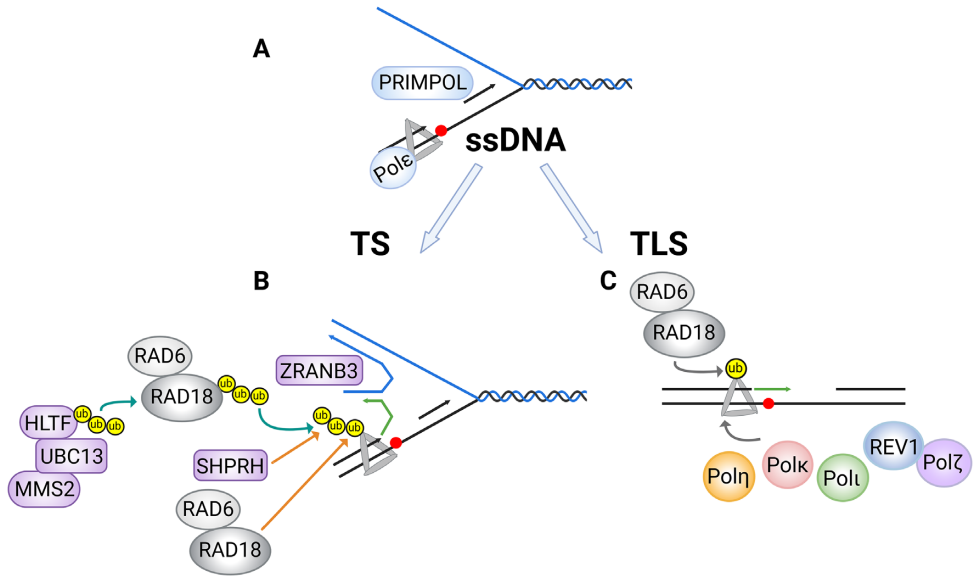



**Figure 2.** Post-Replication Repair (PRR) is mediated by TS and TLS. (**A**) Stalling of replicative DNA polymerases leads to recruitment of the primase/polymerase PRIMPOL and re-initiation of DNA synthesis downstream (3′) of the stalled DNA polymerase. PRIMPOL-mediated restart of DNA synthesis generates ssDNA gaps or discontinuities in the daughter strand. (**B**) TS is activated by PCNA poly-ubiquitination, and is dependent upon RAD5 homologues HLTF and SHPRH. (**C**) TLS is mediated by PCNA K164 mono-ubiquitination, which recruits Y-family TLS polymerases in a process termed 'polymerase switching'. The RAD6/RAD18 (E2/E3 ubiquitin ligase) complex accounts for most (∼95%) of the mono-ubiquitinated PCNA in mammalian cells.

Polη. Mutation signature 9, found in Chronic Lymphocytic Leukemias (CLL) and malignant B-cell lymphomas, is attributed to Polη-dependent somatic hypermutation (SHM) activity. Consistent with a role for Polη in mediating signature 9, *Polh*$^{-/-}$ mice have a GC-biased mutation profile demonstrating that Polη is a major contributor to A/T mutations during SHM (132). Supek and Lehner identified a prevalent mutational signature in solid tumors that is directed toward the 3′ end of active chromatin, associated with carcinogen exposure, and attributed to Polη (133). However, there has been no direct experimental validation that signature 9 is dependent upon Polη. There remains a need for experiments that test how specific TLS polymerase deficiencies impact mutational signatures of cancer genomes.

### Altered expression of TLS polymerases and their regulators in cancer

There are several reports that TLS polymerases and their regulators are altered in cancer cells or patient-derived tumors. Polymorphisms in TLS polymerase genes are documented in a small set of studies and these alterations may be associated with cancer predisposition. For example, mutations in *REV1* are associated with lung cancer (134), *POLK* mutations are linked with lung and breast cancer (134,135), and *POLI* mutations correlate with risk of adenocarcinoma and squamous cell carcinoma (136). Whether such TLS polymerase mutations necessarily affect their biochemical activities and participation in TLS, or threaten genome stability is not clear. However, some-cancer associated REV1 mutants reportedly have altered DNA polymerase and DNA-binding activities that lead to increased mutagenesis (137).

Altered expression of TLS polymerases represents another possible mechanism for TLS imbalances and mutagenesis. Potentially, over-expressed or aberrantly activated TLS polymerases might compete with replicative DNA polymerases for access to the replisome leading to mutagenesis (138). Improperly-expressed (both over-expressed and under-expressed) TLS polymerases may also compete with each other for replisome association via their PIP boxes and ubiquitin-binding motifs. Elevated expression of individual TLS polymerases may increase error-prone DNA synthesis on an undamaged template or might lead to aberrant deployment for bypass of non-cognate DNA lesions, leading to increased mutagenesis. For example, overexpression of *POLK* is reported to increase N-methyl-N-nitrosurea (MNU)-induced mutagenesis and occurrence of intestinal adenomas (139).

Pathological changes in TLS modulators can also lead to XPV-like imbalances that impact genome stability. The E3 ubiquitin ligase MDM2 which is overexpressed in many cancers degrades Polη and recapitulates hallmark TLS defects of XPV cells (140). The *NPM1* gene, which encodes the Polη-binding protein Nucleophosmin, is frequently mutated in Acute myeloid leukemia (AML). A prevalent *NPM1* gene mutation in AML patients leads to excessive Polη degradation and reduces error-free TLS activity (141).

The proximal TLS activator RAD18 is often overexpressed in cancer both at the mRNA level (Figure 3) and at the protein level, owing to its stabilization by a cancer selective binding partner Melanoma Antigen A4 (MAGEA4, a Cancer/Testes Antigen or CTA) (78). TLS polymerase activation and mutagenesis are highly sensitive to changes in RAD18 expression levels. Ectopically-expressed RAD18 induces PCNA mono-ubiquitination and recruitment of TLS polymerases to replicating DNA, even in the absence of

| | Endometrial cancer (UCEC) | Endometrioid in UCEC | Pancreatic cancer (PAAD) | Glioblastoma Multiforme (GBM) | Prostate cancer (PRAD) | Bladder cancer (BLCA) | Lung squamous cell carcinoma (LUSC) |
|---|---|---|---|---|---|---|---|
| Pol η | Overexpressed *Positively* correlated with SNVs | Positively correlated with SNVs | NS Negatively correlated with SNVs in all tumors. | Overexpressed | Downregulated | NS | Downregulated, Negatively correlated with SNVs in non-smokers. |
| Pol ι | Downregulated | Downregulated Negatively correlated with SNVs in all tumors. | NS Negatively correlated with SNVs in all tumors. | NS Positively correlated with SNVs in all tumors. | NS | NS | Downregulated |
| Pol κ | Downregulated Positively correlated with SNVs in all tumors. | Downregulated Positively correlated with SNVs in all tumors. | NS Negatively correlated with SNVs in all tumors. | NS | Downregulated | Downregulated | Downregulated |
| REV1 | Downregulated | Downregulated | NS | NS | Downregulated | Downregulated | NS |
| MAD2L2 | Overexpressed | Overexpressed | NS | Overexpressed | Overexpressed Positively correlated with SNVs in all tumors | Overexpressed | Overexpressed |
| REV3L | Downregulated | Downregulated | NS Negatively correlated with SNVs in all tumors. | NS | NS Negatively correlated with SNVs in all tumors. Downregulated in tumors. | Downregulated | Downregulated |
| RAD18 | Overexpressed in tumors. | Overexpressed Positively correlated with SNVs in all tumors. | NS Positively correlated with SNVs in all tumors. 3 of 4 normal tissues low. | NS | NS Positively correlated with SNVs in all tumors. | Overexpressed, Positively correlated with SNVs. | Overexpressed Positively correlated with SNVs in smokers. |

| | Lung Adenocarcinoma (LUAD) |
|---|---|
| Pol η | NS Negatively correlated with SNVs in smokers. |
| Pol ι | Downregulated Negatively correlated with SNVs in smokers. |
| Pol κ | Downregulated Negatively correlated with SNVs in all tumors. |
| REV1 | Downregulated Negatively correlated with SNVs in smokers. |
| MAD2L2 | Downregulated Positively correlated with SNVs in smokers. |
| REV3L | Downregulated |
| RAD18 | Overexpressed Positively correlated with SNVs in all tumors irrespective of smoking history. |

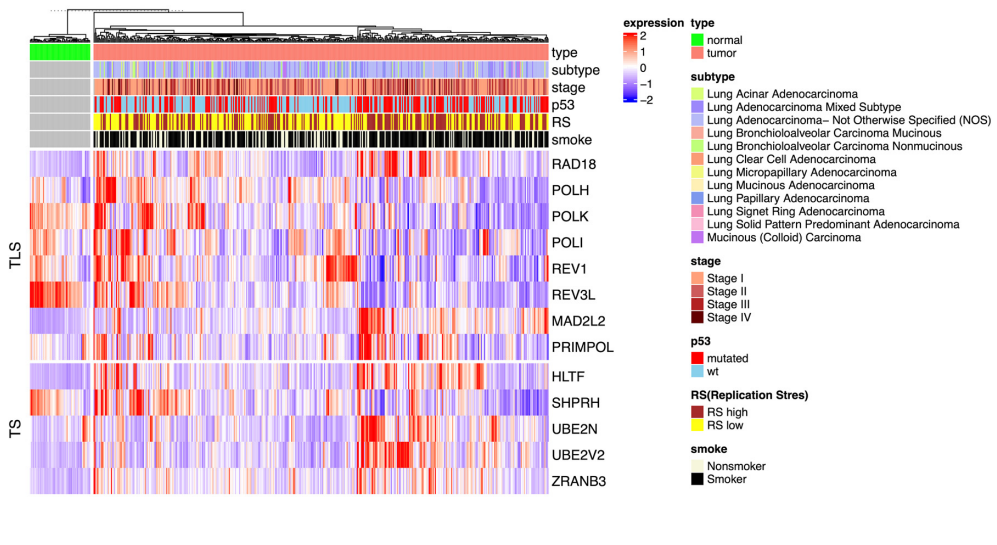



**Figure 3.** Heatmap showing expression of TLS and TS pathway genes in Lung Adenocarcinoma (LUAD). The table shows analysis of TLS pathway genes in representative tumor types from TCGA gene expression datasets. The heatmap illustrates relative expression levels of mRNAs corresponding to TLS and TS pathway genes in LUAD tumors and adjacent normal tissues. Classifiers for tumor stage, histologic subtype, TP53 mutation, Replication Stress (RS), and smoking, are shown for each tumor. Tumors harboring at least one of the following genetic alterations are classified as 'RS-high': (i) *CCNE1* amplification, *RB1* two-copy loss, *CDKN2A* two-copy loss; (ii) *KRAS* amplification, *NF1* mutations, *MYC* amplification, *MYCL1* amplification, *ERBB2* amplification (264). As an example of TLS polymerase imbalance revealed by these analyses, we considered the ratios of POLH:POLK expression in normal and cancer tissues. In UCEC, the average expression of POLH in tumors is 1.14 times the average of POLH in the adjacent normal, and the average expression of POLK in tumors is 0.57 times the average of POLK in adjacent normal. Therefore, the ratio for the fold change for POLH: POLK in UCEC is 1.14: 0.57. For LUAD, LUSC and BLCA, the ratios for fold change in POLH:POLK when comparing tumors with adjacent normal tissues are 1.06: 0.74, 0.90: 0.54 and 0.99: 0.63 respectively.

DNA damage (78,79,80). DNA damage-independent activation of TLS polymerases due to excessive RAD18 could be a source of mutagenesis. Different TLS polymerases have different affinity for PCNA (45) and may be differentially reliant on RAD18-mediated PCNA mono-ubiquitination or chaperone activity for engaging the replisome (38). Excessive RAD18-mediated chaperone activity or PCNA-mono-ubiquitination favoring any individual TLS polymerase could create a selection bias and TLS imbalance that contributes to mutagenesis in cancer cells. The E3 ubiquitin ligase RNF168 is another potential TLS modulator which is often overexpressed in cancer (142). RNF168 increases levels of ubiquitinated H2A in the vicinity of replicating DNA which recruits Polη via direct interactions (143).

However, there is no single paradigm for how TLS polymerases (or other TLS pathway components) are altered in cancer and whether such changes correlate with mutation burden or other tumorigenic properties. Therefore, we interrogated Cancer Genome Atlas (TCGA) gene expression datasets and determined the extent to which TLS pathway gene expression levels are altered in representative cancers. Additionally, we asked whether expression levels of TLS pathway genes correlate with mutation load. We analyzed expression levels of *RAD18* (upstream activator of Y-family TLS polymerases), *POLH*, *POLK*, *POLI* and *REV1* (the four Y-family TLS polymerases), and *REV3L* and *MAD2L2* (encoding the two subunits of Polζ, which mediates the extension phase of TLS).

We examined data from three different cancer types with a smoking-related etiology: Bladder cancer (BLCA), Lung Adenocarcinoma (LUAD), and Lung squamous cell carcinoma (LUSC). We reasoned that tobacco smoke genotoxicity may stimulate mutagenesis and reveal associations with TLS status for these cancers. For comparison, we also examined TCGA data from tumors whose etiology is unrelated to tobacco smoke exposure, namely prostate cancer (PRAD), glioblastoma (GBM), pancreatic cancer (PAAD) and endometrial cancer (UCEC).

Figure 3 and Supplementary Figure S1 summarize expression patterns of TLS genes in tumor samples relative to adjacent normal tissue. Also indicated are correlations between TLS gene expression and total Single Nucleotide Variation (SNV) burden (which is a direct measure of genome-wide point mutations). Several key patterns and trends are revealed by our analyses: Of the TLS genes studied here, only *RAD18* is overexpressed in most tumor types. The only tumor not significantly overexpressing *RAD18* is pancreatic cancer (for which datasets were available from only 4 normal samples, limiting our ability to accurately determine fold changes in tumor samples). Interestingly, *RAD18* expression shows a strong positive correlation with the total SNV numbers in most cancer types, both in smokers and non-smokers. The overexpression of RAD18 in most tumors is consistent with the hypothesis that excessive RAD18 stimulates error-prone TLS and increases mutation burden. *MAD2L2* (encoding REV7) is another TLS gene that is also overexpressed in most tumors. However, unlike *RAD18*, *MAD2L2* expression does not show a strong correlation with SNV burden. *REV3L* is typically expressed at low levels in tumor cells when compared with adjacent normal tissues. Moreover, in some tumors (e.g. PAAD, PRAD), *REV3L* expression is negatively correlated with SNVs. *Rev3l* loss in mice leads to spontaneous tumorigenesis (144). Therefore, it is possible that the reduced *REV3L* expression in human tumors contributes to tumorigenicity.

There are reports that the Y-family polymerases are expressed at higher levels in tumors than normal cells and tissues (145,146,147,148). Surprisingly however, our expression analyses reveal that *POLH*, *POLK*, *POLI* and *REV1* expression levels in cancers are typically equivalent to or reduced when compared with adjacent normal tissues. In LUAD, the expression levels of Y-family DNA polymerases are negatively correlated with the total SNV counts in tumors from smokers. In contrast, in PAAD, the general trend is that tumors with reduced expression of *POLH*, *POLK*, *POLI* and *REV1* have a high number of SNV counts.

Taken together, these analyses reveal remarkable complexity in expression patterns of the core TLS pathway genes and how they are altered in different cancers. With the important caveat that mRNA expression is an imperfect surrogate for protein level and activity, the expression patterns described in Figure 3 suggest many ways in which imbalance between TLS polymerases and their activators might affect DNA damage tolerance and mutability in different cancers. The impact of such TLS pathway alterations is also likely to depend on the availability of other genome maintenance mechanisms. For example, HR-compromised cells have increased reliance on TLS (127). Accordingly, altered expression of TLS genes might be far more consequential in a *BRCA*-mutant cancer cell than in a *BRCA*-sufficient background. There is a need to determine the ways in which TLS and its intersecting DNA repair pathways are rewired in all individual tumor settings, and how those changes impact mutagenesis and DNA damage tolerance. For example, it is reported that HLTF promoter methylation and loss of HLTF expression are observed in some cancers (149). Changes in relative expression or activities of RAD5 homologues could affect the selection of (error-free) TS vs (error-prone) TLS and influence mutagenic outcomes. To investigate potential TS pathway alterations in cancer, we also analyzed expression patterns of HLTF, ZRANB3, SHPRH, MMS2 and UBC13 for all the malignancies described in Figure 3. In contrast with reported findings (149), HLTF expression was not reduced in tumors. Interestingly, we observed several trends: HLTF was overexpressed in tumors and positively correlated with SNVs in most cancer types. However, SHPRH was downregulated in tumors of most cancer types.

It is important to recognize that cancer constitutes many different diseases in which tumor types and subtypes are highly divergent based on proteogenomic characteristics. It is overly-simplistic and imprecise to conclude that any specific TLS factor is universally over- or under-expressed in cancer when compared to normal tissues. The analyses and a corresponding heatmap in Figure 3 illustrate that even despite general trends, every individual tumor is unique and expresses a distinct repertoire of TLS factors (as well as other genome maintenance genes). The goal of targeting TLS for therapy may require a precision medicine approach which is contingent on understanding the unique DNA repair characteristics of every individual patient tumor. The correlations we have identified between TLS factors and SNV burden in cancer provide an essential source of hypothesis. The results of Figure 3 could help prompt future studies to model pathologically-relevant TLS polymerase imbalances and test the impact of those imbalances on mutagenesis.

### TLS is a dependency of some cancer cells

During multistep tumorigenesis, neoplastic cells acquire extensive DNA damage from intrinsic sources including oncogenes. Oncogene signaling leads to DNA damage via multiple mechanisms including: (a) dysregulated replication origin licensing and firing leading to under- or over-replicated

DNA (b) increased transcription factor activation leading to replication-transcription conflicts (RTCs) (c) altered nucleotide metabolism leading to reduced dNTP pools and (d) increased ROS production causing oxidative DNA damage (150,151,152). Oncogenes may also deregulate R-loop homeostasis which further leads to RTCs (153). Taken together, oncogene signaling impedes replication fork advancement via multiple mechanisms and causes DNA replication stress.

Accumulating evidence suggests that TLS averts formation of lethal DNA damage in cells experiencing various forms of oncogene-induced DNA replication stress. TLS can be error-prone on undamaged DNA or templates containing non-cognate lesions. Therefore, it is possible that error-prone TLS sustains tumorigenesis by promoting both DNA damage tolerance and mutagenesis. Here we discuss key evidence that supports a role of TLS in cancer cell survival.

A cancer-essential gene (also termed a cancer-dependent essential gene) is required for proliferation or survival of cancer cells (but not normal cells) (154). Several reports suggest that TLS factors fulfill criteria for being genetic dependencies of cancer cells: TLS facilitates continued replication in cells challenged by oncogene-induced replication stress (123), prevents accumulation of ssDNA gaps and promotes cell survival (124). Inhibition of TLS by a small molecule, JH-RE-06 (which targets REV1) reduces survival of several cancer cell lines even in absence of an external stress (124). JH-RE-06 also suppresses growth of A549 and H1299 lung cancer cells grown as xenografts *in vivo* (155). Further consistent with a role for TLS in sustaining tumors, RAD18 overexpression elevates colonization of esophageal cancer cells in the mice lung (156), while RAD18-deficient TNBC xenografts show reduced tumor volume (157). Taken together, these studies suggest that TLS is a cancer-dependency at least under select conditions. TLS limits fork slowing by restricting fork reversal without altering replication restart or dormant origin firing through its gap filling function (124). Recent studies suggest that the cancer cell reliance on TLS for survival is contingent on ssDNA gap suppression (124,158).

Not all cancer cells rely on TLS for viability (124). According to the cancer dependency map (DepMap, a database of loss-of-function CRISPR screens in large number of cancer cell lines), REV1 is a dependency in only few cell lines (CRISPR 16/1086; RNAi 0/597). Similarly, other TLS polymerases are rarely identified as dependencies in DepMap. It remains to be determined what factor(s) determine whether TLS is a cancer dependency. Different oncogenes might induce distinct species and levels of DNA damage that are differentially reliant on TLS for remediation. For example, ectopic expression of *CCNE1* in primary untransformed human fibroblasts induces a more robust expression of DDR markers (including mono-ubiquitinated PCNA) when compared with ectopically-expressed oncogenic RAS (123). Moreover, multiple DNA repair pathways (including HR, TMEJ, NHEJ) contribute to tolerance of oncogene-induced replication stress and DNA damage (123,152). Every cancer cell has a distinct DNA repair landscape. Therefore, the repertoire of redundant pathways available to repair intrinsic DNA damage might determine the extent to which any cancer cell depends on TLS.

Defining the factors and biomarkers that accurately predict TLS-dependency of cancer cells is critical if we are to develop precision medicine approaches based upon TLS inhibition. One factor that has been shown to create TLS-dependency in cancer cells is loss of *BRCA1*. Homologous recombination-deficient *BRCA1* mutant breast cancer cells develop spontaneous ssDNA gaps which are repaired via PRIMPOL and REV1-Polζ-dependent gap-filling mechanism required for viability (127). Tutt and colleagues showed that cancer cells mis-expressing the germ cell protein HORMAD1 (a Cancer Testes Antigen or CTA) have increased dependency on several TLS mediators including POLH, POLK, REV1, REV3L and REV7 (159). Functions of HORMAD1 in cancer cells are not well understood. It is reported that HORMAD1 can both inhibit HR (160) and activate HR (161,162) when mis-expressed in cancer cells. Nevertheless, aberrant expression of HORMAD1 could generate species of DNA damage that are repaired via TLS. More work is needed to reveal the mechanistic underpinnings of TLS-dependencies in neoplastic cells. The availability of the REV1 inhibitor JH-RE-06 has been a valuable tool for demonstrating that some cancer cell lines rely on TLS for survival. The use of orthogonal methods such as CRISPR and RNAi to ablate REV1 (and other TLS genes) will help validate the TLS pathway as a cancer dependency. It is also crucial to test the TLS-dependency of cancer cells in pathologically-relevant models (including GEMM, PDX, organoids) that better recapitulate the characteristics and dependencies of tumors in patients.

## Contributions of TLS to cancer based on mouse models

Mouse models are important for validating hypothetical roles of oncogenic drivers or tumor suppressors in carcinogenesis *in vivo*. Here, we consider the evidence that TLS polymerases and RAD18 are physiologically-relevant modifiers of spontaneous or induced carcinogenesis *in vivo*.

*Spontaneous tumorigenesis.* Deletion of the TLS activator *Rad18* in mice does not affect rates of spontaneous mutagenesis or incidence of spontaneous tumors (163,164). *Polk*-deficiency elevates mutagenesis at G:C base pairs in the mouse lung, liver and kidney (165), while *Rev1*-deficient mice have reduced weight and lifespan (166,167). However, loss of *Rad18* (164), TLS polymerases *Polh* (168), *Poli* (168,169), *Polk* (165), *Rev1* (166)) or *Rev7*/*Mad2l2* (170) does not affect spontaneous tumorigenesis in mice. Intriguingly, *Rev3l*-deficient mice suffer from embryonic lethality (171,172,173) and conditional deletion of *Rev3l* in adult epidermal or lymphoid cells leads to spontaneous squamous cell carcinomas in specialized sebaceous glands and skins, and lymphomas, respectively (144,174). Although the cancer-propensity of Rev3L-deficient mice may point to a tumor-suppressive role for Polζ, this phenotype is not seen in *Rev7*-deficient mice. Therefore, Rev3L may have additional Rev7-independent functions in tumor-suppression, possibly unrelated to TLS. Unfortunately, *in vivo* experiments assessing the tumorigenic consequences of TLS imbalance due to overexpressed TLS polymerases are

scarce. In a one-of-a-kind experiment, Sasatani et al. did not observe spontaneous tumorigenesis in the intestine of Rev1-overexpressing mice (139). Collectively, the studies described above suggest that altered expression of TLS polymerases and Rad18, under unperturbed conditions is not oncogenic in mice.

*Oncogene-induced tumorigenesis.* Although TLS is implicated in mediating tolerance of oncogene-induced DNA replication stress, there is no published information regarding the requirement for TLS in oncogene-induced tumorigenesis *in vivo*. In our unpublished study, *Rad18*-defciency did not affect $Kras^{G12D}$-driven lung carcinogenesis in either $p53^{+/+}$ or $p53^{-/-}$ C57BL6 mice. This result could indicate that TLS is dispensable for oncogenic Kras-driven lung carcinogenesis, or that Rad18-independent TLS mechanisms are sufficient to sustain $Kras^{G12D}$-driven lung tumors. *Rad18* loss also did not affect the mutation burden of *Kras*-driven lung tumors in *p53*-null mice. RAD18 is typically overexpressed (not ablated) in human lung tumors. It would also be interesting to determine whether Rad18 overexpression in the mouse lung affects rates of oncogene-induced lung carcinogenesis.

*Orthotopic tumor models.* Implanting cancer cells orthotopically in mice provides a convenient approach to study tumor growth in a physiologically relevant environment. Orthotopic injection of esophageal cancer cells overexpressing RAD18 increases colonization (156), while RAD18-deficient TNBC cell xenografts show reduced tumor growth (157). TLS inhibition by the REV1 inhibitor JH-RE-06 decreases colonization of lung cancer cells in mice (155). Therefore, studies with orthotopically-implanted cells support a role for TLS in tumor growth and as a cancer target.

*Carcinogen-induced tumorigenesis.* *Polh-/-* mice fully phenocopy the UV-induced skin cancer-propensity of XP-V patients. Heterozygous $Polh^{+/-}$ mice are also sensitive to UV-induced skin carcinogenesis, but succumb at a lower rate when compared with $Polh^{-/-}$ (175). Although $Poli^{-/-}$ mice are not susceptible to UV-induced skin carcinogenesis; $Poli^{-/-}$ $Polh^{-/-}$ double knockout mice have increased rates of UV radiation-induced skin carcinogenesis, compared with $Polh^{-/-}$ animals (168,169). Therefore, both Polη and Polι guard against UV-induced carcinogenesis. Interestingly though, UVB-treatment of $Poli^{-/-}$ deficient animals induces mesenchymal tumors that are not observed in $Polh^{-/-}$ mice (169). $Polk^{-/-}$ mice are viable, but have a shorter lifespan than $Polk^{+/-}$ and $Polk^{+/+}$ mice (165). Moreover, $Polk^{-/-}$ mice have a spontaneous mutator phenotype in the kidney, liver and lung, which is attributed to a role of Polκ for accurate lesion bypass of bulky DNA adducts generated endogenously by cholesterol and its metabolites. Therefore, Polκ protects against spontaneous mutagenesis. $Rev1^{-/-}$ mutant mice are viable on a 129/OLA background, but not on a C57BL/6 background. $Rev1^{-/-}$ mice show reduced weight, but no other gross abnormalities (166). Whether *Rev1* mutation impacts carcinogenesis has not been reported.

Although Rad18 is proximal to Y-family TLS polymerases, *Rad18*-deficient mice do not phenocopy the UV-induced skin tumor-susceptibility of XPV (176). One possible explanation is that Polη bypasses UV-induced CPD in a *Rad18*-independent manner in the mouse skin. Interestingly, $Chk2^{-/-}Rad18^{-/-}$ double knockout mice develop spontaneous lymphomagenesis whereas $Chk2^{-/-}$ or $Rad18^{-/-}$ mice do not. *Chk2*-deficiency alleviates UV-induced apoptosis of $Rad18^{-/-}$ cells but leads to increased genomic instability (176). Therefore, the genetic interaction of *Rad18* and checkpoint genes is important for maintaining genome stability and preventing carcinogenesis.

In a DMBA-ingestion tumorigenesis model, the incidence of leukemia and liver tumors is reduced in $Rad18^{-/-}$ mice when compared with $Rad18^{+/+}$ littermates (164). DMBA-induced skin tumor incidence is unaffected by *Rad18*, yet the mutation signatures of $Rad18^{+/+}$ and $Rad18^{-/-}$ skin tumor genomes are profoundly different: *Rad18*-deficiency is associated with reduced overall SNV burdens, increased levels of insertion/deletion (indels) and different contributions of COSMIC mutation signatures to the overall tumor mutational portrait (164). Therefore, Rad18 promotes error-prone TLS of DMBA-induced lesions, resulting in mutagenesis (SNV). However, in the absence of Rad18, TLS polymerases may not be recruited to sites of PAH-induced DNA damage efficiently, leading to replication stalling and fork collapse. Error-prone repair of DBSs resulting from collapsed replication forks could explain the insertions and deletions present in $Rad18^{-/-}$ tumors.

In summary, most TLS polymerase- or *Rad18*-deficiencies do not lead to spontaneous tumorigenesis in mice. However, Rad18 and TLS pol-defects lead to altered mutagenesis and carcinogenesis following genotoxic exposures (UV, DMBA, cholesterol metabolites). It is unknown whether over-production of Rad18 or TLS polymerases impacts mutagenesis and carcinogenesis *in vivo*. Whether Rad18 and TLS polymerase-deficiencies compromise growth of oncogene-driven tumors *in vivo* has not been addressed comprehensively. However, accumulating evidence supports roles for TLS in conferring resistance to cancer therapy.

## HOW DOES TLS AFFECT RESPONSE TO CANCER THERAPY?

Genotoxic cancer therapies are intended to interfere with DNA-templated processes and ultimately cause permanent replicative arrest and cell death. Many therapeutic genotoxins induce primary lesions that cause DNA replication fork stalling. Processing of stalled DNA replication forks may lead to secondary forms of DNA damage such as ssDNA and DSB (the latter often being viewed as the species of DNA damage responsible for lethality). The integrity of DNA repair and DDR signaling in both cancer cells and normal healthy cells critically impacts the overall response to therapeutic agents and patient outcomes. DDR is a protective mechanism and its deployment in cancer cells will antagonize the desired effects of therapy. Accordingly, inhibiting DNA repair processes and compromising DNA damage tolerance in cancer cells is predicted to sensitize tumors to therapy. We exemplify three classes of therapeutic agents whose mechanisms of action are highly dependent

on TLS including platinum drugs, checkpoint protein kinase inhibitors and poly(ADP-ribose) polymerase (PARP) inhibitors, as described below.

### Platinum drugs

Platinum compounds such as cisplatin and carboplatin are important genotoxic drugs used for the treatment of many cancers (177). Platinum drugs induce DNA damage (primarily intra-strand bulky DNA adducts and ∼1% inter-strand cross-links or ICL) that interfere with DNA synthesis in proliferating cancer cells. Platinating agents primarily induce formation of DNA mono-adducts that induce replication stress, mitotic catastrophe and cell death via apoptosis.

However, many cancer cells have innate resistance to platinum drugs, or acquire cisplatin-resistance during treatment and become refractory to therapy. Mechanisms of cisplatin-resistance include decreased uptake and increased efflux of the platinating agents, and increased DNA repair and increased DNA damage tolerance (178,179). Cisplatin therapy also leads to serious side effects including ototoxicity, nephrotoxicity and neurotoxicity due to cisplatin-induced ROS that cause apoptosis of neuronal and renal cells (180,181,182). Therefore, it is important to devise means to overcome the current limitations of cisplatin therapy.

TLS can help cancer cells acquire resistance to platinating agents and other genotoxic chemotherapies (183). Polη allows replication of cisplatin-damaged DNA templates and the structural basis for Polη-mediated chemoresistance to cisplatin has been elucidated (184,185,186). TLS-deficient cells lacking Polη (187,188,189) or RAD18 (190,191) fail to replicate cisplatin-damaged genomes and instead accumulate unfilled post-replicative gaps, collapsed replication forks and lethal DNA double stranded breaks (DSB). Moreover, high-level Polη expression is correlated with poor survival of platinum-treated non-small cell lung cancer and gastric adenocarcinoma patients (192,193). Polη expression and activity is elevated in cancer stem cells (CSC), the rare progenitors that both self-renew, repopulate tumors and account for chemoresistance (194). Other TLS polymerases are also implicated in mediating cisplatin-resistance. Overexpression of REV1 is strongly correlated with reduced survival probability of prostate cancer patients (195,196,197). Similarly, expression of REV1 is significantly upregulated in lung tumors compared with matched adjacent tissues, and such upregulation is associated with poor prognosis (155). Suppression of *REV1* or *REV3L* not only sensitizes drug-resistant cancers to cisplatin, but also prevents acquisition of drug resistance in murine tumor models (198,199). Inhibition of TLS through RNAi-mediated depletion of *REV1* and *REV3L* profoundly sensitizes human prostate cancer cells to a cisplatin prodrug and dramatically extends animal survival *in vivo* (200). Similarly, knockout of *Rev7* in $KRAS^{G12D}$, $TP53^{-/-}$ non-small cell lung cancer (NSCLC) renders cancer cells hypersensitive to cisplatin treatment with significant improvement of animal survival over the *REV7* proficient tumor (201).

Inhibiting TLS also represents an opportunity for overcoming toxic side effects of cisplatin therapy: high doses of cisplatin induce ROS and cause apoptosis of neuronal and renal cells leading to neurotoxicity and ototoxicity (180,181). Thus, many toxic side effects of cisplatin therapy are mediated via a DNA damage-independent mechanism that is not affected by TLS. Inhibiting TLS could lower the doses required for cisplatin therapy and reduce the toxic side effects associated with high doses of platinating agents. $Rad18^{-/-}$ and $Polh^{-/-}$ mice are viable and display no overt developmental defects. Thus, specific inhibition of TLS is unlikely to cause toxicity or be detrimental to cancer patients. In summary, there is very strong rationale to explore the TLS pathway as a therapeutic target whose inhibition will confer killing of cancer cells by cisplatin and reduce toxic side effects of cisplatin. By analogy, combining other genotoxic therapeutic agents with TLS inhibition will likely be an effective strategy to inhibit DNA damage tolerance in neoplastic cells.

### Checkpoint kinase inhibitors

Cell cycle checkpoints integrate DNA repair with cell cycle progression to promote cell viability. The ATM/CHK2 and ATR/CHK1 pathways represent two major branches of the DDR and mediate DNA damage-inducible checkpoints that arrest cells in G1, S and G2 phases (202). Protein kinase inhibitors targeting ATM, ATR, CHK1 and CHK2 provide a strategy for uncoupling DNA repair from cell cycle progression and sensitizing cancer cells to therapy-induced genotoxicity (182,202,203,204,205,206,207).

ATR/CHK1 signaling and RAD18-mediated TLS occur simultaneously in response to replication stalling. ATR and RAD18 pathways are temporally-coincident because both are activated by RPA-coated ssDNA generated via helicase-polymerase uncoupling (205,208). There is also extensive cross-talk between the ATR and TLS pathways. RAD18 and Polη activities are regulated by ATR/CHK1-dependent phosphorylation (84,87,91). Conversely, when TLS is compromised (e.g. in XPV cells), there is a compensatory increase in ATR/CHK1 signaling which helps preserve cell viability (209). The compensatory ATR response of TLS-deficient cells following genotoxin exposure explains early observations that XPV cells are not UV-sensitive unless treated with caffeine (210). It is now appreciated that caffeine inhibits ATR, and that the ATR/CHK1 and TLS pathways are partially-redundant (209). Therefore, synthetic lethality resulting from inhibiting ATR/CHK1 together with TLS might represent a therapeutic strategy for sensitizing cancer cells to intrinsic or therapy-induced DNA damage.

Pharmacological inhibition of the WEE1 protein kinase also leads to bypass of cell cycle checkpoints and lethality. WEE1 performs inhibitory phosphorylation of Y15 on Cyclin-Dependent Kinases 1 and 2, thereby restricting S-phase and G2/M progression (211). WEE1 inhibitors derepress CDK2 and CDK1 activities and promote bypass of S-phase and G2/M cell cycle checkpoints (212,213). Adavosertib is an inhibitor of WEE1 that is currently in clinical trials to treat multiple cancers and has been shown to have promising results in overcoming the resistance to platinum-based drugs, such as cisplatin (214).

In addition to promoting bypass of S/G2 checkpoints, the aberrant activation of CDK2 due to WEE1 inhibition in cancer cells causes excessive origin firing that triggers a DDR (211). WEE1 inhibitors are pharmacological equivalents of Cyclin E overexpression which is often used to experimentally model oncogene-induced DNA replication stress. In WEE1 inhibitor-treated cells, the combination of S-phase damage (due to excess CDK2 activity) and a compromised G2 checkpoint (due to excess CDK1 activity) allows S-phase cells to enter mitosis inappropriately and succumb to lethal mitotic catastrophe (211). Interestingly, TLS prevents WEE1 inhibitor-induced lethality (123,215). The role of TLS in protecting against the lethal effect of WEE1 inhibition is likely due to post-replicative gap filling since *Rad18* and *Polk*-ablated cells aberrantly accumulate ssDNA in G2 after WEE1 inhibition (123). Therefore, concurrent inhibition of WEE1 and TLS represents a potential strategy for inducing mitotic catastrophe in cancer cells.

### Poly(ADP-ribose) polymerase (PARP) inhibitors

PARP inhibition has received extensive attention as a synthetic lethal approach for killing BRCA-mutant HR-compromised tumors. PARP enzymes are DNA damage sensors and transducers that bind DNA at ssDNA breaks and synthesize negatively-charged, branched poly(ADP-ribose) (PAR) chains on target proteins in the vicinity of the damage (216). This PTM (termed PARylation) leads to the recruitment of DNA repair enzymes such as ERCC1 and remodeling of damaged DNA (217). In 2005, two groups reported the seminal observation that *BRCA2*-deficient cells, because of their deficiency in Homologous Recombination, are acutely sensitive to PARP inhibitors (218,219). Therefore, targeting PARPs is an attractive strategy for killing *BRCA2*-deficient tumors (220).

The synthetic lethal interaction between PARP inhibition and *BRCA*-deficiency was originally proposed to be due to persistent SSBs which are repaired by HR during S-phase. Thus, HR was originally proposed to avert fork collapse and lethal DSB. Subsequently it has become evident that PARPi not only block PARP-mediated SSB repair but also trap the PARP enzyme on damaged DNA. Thus, the trapped PARP protein itself is also an obstacle to replisome movement which cannot be resolved in the absence of HR (220,221,222).

Several recent studies suggest that PARPi toxicity in HR-deficient (HRD) cells is caused by DNA replication-associated single-stranded DNA (ssDNA) gaps (125,126,158,223) - a vulnerability which also suggests interesting and potential tractable roles of the TLS pathway as a therapeutic target in HRD tumors. Cantor's excellent recent review describes the various ways in which ssDNA gaps arise in BRCA1 and BRCA2-mutant cells (126). Interestingly, repriming mediated by PRIMPOL is a major source of spontaneous ssDNA gaps when HR is compromised (127). Consistent with TLS as a post-replicative gap-filling process that operates behind newly-reprimed forks, *BRCA1/2*-deficient cells are dependent upon RAD18 and Polζ -for gap suppression and viability (127). The demonstration that HR-deficiency creates a reliance on TLS suggests that patient stratification based on 'BRCA-ness' might reveal clinical contexts in which TLS inhibition has most therapeutic benefit.

## ROLE OF TLS IN THERAPY-INDUCED MUTAGENESIS

The action of TLS polymerases on non-cognate lesions could plausibly play a role in acquisition of therapy-induced mutations. Therapy-induced mutations are of great potential clinical significance because they might help drive acquired chemoresistance. A good example of a clinical setting in which therapy-induced genotoxicity is linked to acquired chemoresistance is during Temozolomide (TMZ) treatment of brain cancers.

Temozolomide (TMZ) is a DNA methylating chemotherapeutic agent used in the treatment of Glioblastomas (GBM) and is the only FDA-approved first-line chemotherapeutic drug for this disease (224). All GBM eventually become TMZ-refractory and recur (225). The extent to which TMZ- induced hypermutation causes GBM recurrence is debated, but it has been proposed that therapy-induced mutations account for the adaptations that allow GBM to resist TMZ (226,227,228). Tumors from TMZ-treated GBM patients harbor a specific mutational signature (designated Single Base Substitution 11 or SBS 11) which is characterized by a preponderance of G:C>A:T transitions at non-CpG sites (229). Moreover, the therapy-associated mutational signature of hypermutated gliomas has been recapitulated by TMZ-treatment (230). However, the underlying error-prone DNA repair mechanisms that mediate *de novo* and therapy-induced mutation patterns and hypermutability in GBM are not fully understood. GBM cells do rely on RAD18 and Polκ to tolerate TMZ treatment (231,232,233). Overexpression of *POLK* in GBM patients is associated with TMZ-resistance and is a prognostic indicator for shorter survival (234,235). However, the contribution of TLS polymerases to TMZ-induced mutagenesis is unknown. To address the contribution of TLS to TMZ-induced hypermutation, Yang et al. annotated TMZ-induced mutation signatures in genomes from TMZ-treated isogenic $RAD18^{+/+}$ and $RAD18^{-/-}$ GBM. In those experiments, analysis of mutation signatures from TMZ-treated GBM reveals a role for RAD18 in error-free bypass of $O^6$mG (the most toxic TMZ-induced lesion), and error-prone bypass of other TMZ-induced lesions. However, the RAD18-dependent TLS polymerases responsible for mutagenic bypass of TMZ-induced DNA lesions have not yet been identified.

Cisplatin-induced mutation patterns resemble the cancer mutation signatures attributed to smoking and other sources of guanine-directed base damage. TLS has been implicated in generating cisplatin-induced mutational spectra at sites of guanine-directed base damage (236). Mutagenesis of cisplatin-induced DNA lesions is most likely due to the inserter/extender properties of Polη and Polζ which cooperate to perform bypass of GG cisplatin adducts (237). Further work is needed to elucidate how TLS polymerases impact therapy-induced mutations associated with acquired chemoresistance (238). Platinum-based and other chemotherapies for solid tumors are associated with increased risk of secondary hematological neoplasms (notably myelodysplastic syndrome, MDS and acute nonlym-

phocytic leukemia, ANLL) (239,240). Presumably these therapy-induced hematological malignancies are caused by genotoxicity-induced oncogenic mutations in hematopoietic stem cells. The extent to which TLS mediates mutagenic events leading to therapy-induced secondary neoplasms has not been determined.

## TARGETING TLS FACTORS FOR THERAPY

There have been attempts to develop small molecules that target components of the TLS pathway (Table 1). The REV1 inhibitor JH-RE-06 was discovered by the Zhou and Hong laboratories in a screen for small molecule inhibitors that target the REV7-binding surface of the REV1 CTD and disrupt the REV1–REV7 interaction (241). Binding of JH-RE-06 induces REV1 dimerization, thereby blocking the REV1–REV7 interaction and preventing Polζ recruitment to the replisome. JH-RE-06 treatments induce all the hallmarks of TLS-deficiency including inhibition of mutagenic lesion bypass and enhancement of DNA damage-sensitivity in cultured human and mouse cell lines (241). Co-administration of JH-RE-06 with cisplatin suppresses the growth of human melanoma xenografts *in vivo*, thereby providing encouraging pre-clinical validation for TLS inhibitors as a novel class of chemosensitizers for cancer therapy. A different small molecule inhibitor of REV1 inhibitor that binds to the REV1-CTD and inhibits interaction with TLS insertion polymerases also sensitizes cultured murine and human cells to genotoxic exposures (242). Therefore, pharmacological disruption of associations between REV1 and its partner DNA polymerases is emerging as a feasible approach for inhibiting TLS and chemosensitizing cancer cells (243).

Given the central role of PCNA as a hub for inserter and extender polymerase engagement at the replisome, targeting the interaction between PCNA and TLS polymerases may be a feasible strategy for inhibiting TLS. Vanarotti and colleagues identified small molecules that bind to the UBM2 domain of REV1 and prevent its association with monoubiquitinated PCNA (244). The REV1–UBM2-binding compound prevents cisplatin-induced recruitment of REV1 to PCNA on chromatin, prevents UV-induced mutagenesis of the *HPRT* gene, and sensitizes cells to cyclophosphamide or cisplatin. Another study identified a PCNA-binding small molecule designated PCNA-I1S that stabilizes the PCNA trimer, and reduces levels of chromatin-associated PCNA (245). PCNA-I1S inhibits cell growth and enhances cytotoxic effects of UV radiation and cisplatin. However, it is unclear whether the cytotoxic effects of PCNA-I1S result from inhibition of TLS or other PCNA-mediated genome maintenance processes.

The most ideal therapeutic target pathways in cancer are ones that are critically required for neoplastic cell growth, yet are dispensable for viability of normal healthy cells. The TLS pathway meets this criterion in some contexts. For example, oncogene-expressing cells are more dependent on Rad18 and Polκ when compared with untransformed cells (123). Some cancer cells pathologically stabilize RAD18 via a mis-expressed Cancer/Testes Antigen (CTA), termed MAGEA4 (78). Moreover, MAGEA4-expressing cancer cells become critically dependent on MAGEA4 to maintain RAD18 expression, activate TLS, and tolerate DNA damage (78). Owing to its cancer cell-specific expression and requirement for DNA damage tolerance, the MAGEA4/RAD18 signaling axis is an appealing pathway for therapeutic inhibition. Fleming et al. identified small cyclic peptide inhibitors that bind directly to MAGEA4 and inhibit RAD18 binding with nanomolar potency (246). That study validates the chemical tractability of MAGEA4/RAD18 as a cancer-specific vulnerability and justifies further work to identify more drug-like pharmacological inhibitors of pathological TLS in cancer. RAD18 is also implicated in DSB repair (247,248,249) and ICL repair (250) independently of its roles in TLS. Inhibiting MAGEA4-dependent RAD18 activities in genome maintenance might also sensitize cancer cells to diverse classes of anti-cancer agents that induce replication fork stalling, DSB, and ICL.

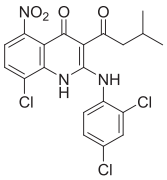
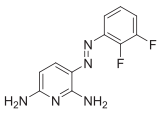
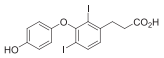
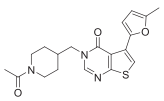
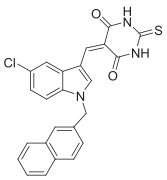
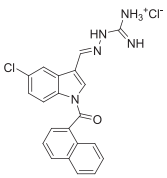
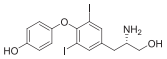
## CONCLUSIONS AND FUTURE DIRECTIONS

In the past decades our understanding of TLS as a PRR mechanism merely involving a PCNA-ubiquitination-mediated DNA polymerase switch has expanded considerably. We are now aware of multiple ubiquitination-dependent and independent TLS activation mechanisms that may operate both within and outside of S-phase, and that are intimately integrated with other components of the DDR. Future work will likely reveal additional complexity and fine-tuning mechanisms that regulate TLS polymerases and coordinate genome maintenance with other cellular processes. Matsuoka and Elledge showed that substrates of the proximal DDR mediators ATM and ATR are involved in diverse pathways including metabolism, chromatin packing and remodeling, trafficking, transcription, and translation (251). By analogy, integration of TLS with broad regulatory pathways in a cell is likely important for genome stability and DNA damage tolerance.

For many other genome maintenance mechanisms (notably DSB signaling and NER), chromatin architecture and histone modifications are critical determinants of DNA repair activity (252,253). Pharmacological modifiers of chromatin structure impact the nuclear mobility of TLS polymerases (254), yet little else is known regarding links between chromatin and TLS. More connections between chromatin modification and TLS are likely to be discovered. In the case of DSB repair, pathway choice between error-free HR and error-prone NHEJ/MMEJ is enormously consequential for genome stability (255,256). It is likely that mechanisms of pathway choice between error-prone TLS and error-free TS (or other processes) will similarly emerge as important determinants of mutagenesis and DNA damage tolerance.

Many questions remain regarding the relationship between TLS and cancer and the extent to which TLS sustains tumorigenic phenotypes such as mutagenesis and DNA damage tolerance. Whole genome sequencing is the new standard for evaluating roles of candidate genome maintenance pathways in cancer-associated mutation patterns. Genome sequencing approaches have provided evidence

**Table 1.** TLS targeting agents

| Target | Inhibitor | Chemical structure | Function | $IC_{50}$ or $K_d$ | Structure of the Inhibitor-bound complex | *In vitro* & *In vivo* |
|---|---|---|---|---|---|---|
| REV1 (REV7 interface) | JH-RE-06 |  | Blocks the REV1-REV7 interaction | $IC_{50} = 0.78$ μM (AlphaScreen)<br>$K_d = 0.42$ μM (ITC) | PDB: 6C8C (241) | *In vitro*:<br>• Sensitize multiple tumor cell lines to cisplatin (241)<br>• Selective killing of *BRCA1*-deficient breast and ovarian cancers (127)<br>*In vivo*:<br>• Synergize with cisplatin to suppress melanoma growth in mice (241);<br>• Selective suppression of *BRCA1*-deficient breast tumorigenesis in mice (127);<br>• Suppress lung tumorigenesis in mice (155). |
| REV1 (RIR interface) | Phenazopyridine (PAP) analog 1 |  | Blocks the REV1-RIR interaction | $K_d = 21$ μM (MST) | PDB: 6WS5 (265) | *In vitro & in vivo*:<br>• Synergize with Olaparib/ temozolomide and induces cell death in PDXres 1518–3 SCLC cells (258) |
| REV1 UBM2 (ubiquitin interface) | MLAF50 |  | Blocks the REV1-ubiquitin interaction | $K_d = 37$ μM (SPR) | ND | *In vitro:*<br>• Inhibit chromatin co-localization of REV1 with PCNA (259) |
| REV7 | Compound 7 |  | Binds REV7 | $IC_{50} = 78$ μM (FP) | ND | *In vitro*:<br>• Sensitize HeLa cells to cisplatin (260) |
| Pol eta/REV1 | PNR-7-02 |  | Inhibits the catalytic activity of Pol eta and REV1 with similar efficiency | $IC_{50} = 8.0$ μM (polymerase activity assay) | ND | *In vitro*:<br>• Sensitize HAP-1 cells to cisplatin (261) |
| Pol kappa | IAG-10 |  | Selectively inhibits human Pol Kappa | $IC_{50} = 0.7$–$7$ μM (polymerase activity assay) | ND | *In vitro*:<br>• Sensitize HAP-1 cells to TMZ (232) |
| PCNA | T2AA |  | Binds to PCNA in a bimolecular fashion; inhibits PNCA-interaction with Pol eta or REV1 | $IC_{50} = 1$ μM (FP) | PDB: 3WGW (262) | *In vitro*:<br>• Sensitize HeLa and U2OS cells to cisplatin treatment (262,263) |
| MAGEA4 (Rad18 interface) | Cyclic and linear MTP peptides | | Blocks MAGEA4/RAD18 interaction | $IC_{50}$: <1 nM to 10 μM (TR-FRET) | PDB: 7UOA (246) | *In vitro:*<br>• Selectively pulldown MAGEA4 (246) |

that TLS polymerases contribute to some of the cosmic mutation signatures in cancer. There is a need for more studies to model the effects of TLS-deficiencies on genome-wide mutation patterns and to determine whether TLS polymerases contribute to the mutation signatures of unknown etiology. The role of TLS in mediating therapy-induced mutations that drive disease recurrence and secondary malignancies is also unclear and must be addressed. Putative TLS-mediated mutational scars of cancer cells could provide biomarkers that are predictive or prognostic of sensitivity to specific therapeutic agents and that guide clinical decisions.

There is increasing evidence that TLS allows neoplastic cells to tolerate intrinsically-arising DNA damage. Such TLS-dependency could be a useful and actionable vulnerability that presents opportunities to target cancer cells using TLS inhibitors. It is important to define the pathological contexts in which tolerance of various types of intrinsic (and therapy-induced) DNA damage is TLS-dependent. For example ssDNA gap-suppression has been proposed as an important role of TLS in cancer cells (124), but not all cancer cells are sensitive to REV1-inhibition.

It is possible that different cancer cells harbor distinct forms of intrinsic DNA damage and also have differential TLS-dependencies based upon availability of other DDR pathways. It will also be valuable to identify synthetic lethal genetic interactions between TLS and other DNA repair pathways which could be exploited for therapy.

Genetically engineered mouse models (GEMM) have been invaluable for identifying genes that sustain multistep tumorigenesis. There exist sophisticated GEMM that recapitulate the genetics, disease progression and histopathological features of many human cancers. As yet however, there have been few studies to model impact of TLS perturbations on oncogene-driven cancers.

Therefore, GEMM would also be useful for defining the cancer settings in which TLS sustains tumor cells (and in which TLS inhibition would likely provide therapeutic benefit). GEMM would also be useful to determine whether the altered expression patterns of TLS polymerases and regulators observed in cancers (e.g. over-abundance of *RAD18* or reduced *REV1* expression compared with normal tissues) impact disease progression and shape mutation signatures. GEMM would also be extremely useful in preclinical studies to determine therapeutic efficacies of pharmacological TLS inhibitors.

For effective cancer therapy it is important to selectively discriminate between neoplastic cells and normal healthy cells. Representative untransformed cells and cancer cell lines might have different TLS-dependencies (123,257). However, cancer is a highly heterogeneous disease. Mechanisms of DNA damage tolerance and chemoresistance often differ tremendously between different patients, even those with the same cancers and cancer subtypes. Therefore, it may prove impossible to conclude that all cancers are globally dependent on TLS. Ultimately, precision medicine approaches that target individual tumors based on their unique disease characteristics (including driver oncogenes, intrinsic stresses and repertoire of available DDR pathways) might provide the best vehicle for TLS inhibitors as cancer therapies.

## METHODS

Lung adenocarcinoma (LUAD), bladder urothelial carcinoma (BLCA), lung squamous cell carcinoma (LUSC), uterine corpus endometrial carcinoma (UCEC), pancreatic adenocarcinoma (PAAD), glioblastoma (GBM), were selected for evaluation of relationships between TLS/TS gene status and genome instability in human tumors. TCGA datasets containing RNA expression, mutation, genomic alteration and clinical information for these tumors were from the TCGA data portal (https://portal.gdc.cancer.gov) and were all downloaded in April 2022. Specific datasets used in this study include: (i) HTSeq-FPKM-UQ gene expression quantification (duplicated samples for the same patients were removed), (ii) somatic mutations aggregated and masked by Mutect2 and organized as MAF files, publicly available, (iii) smoking information for most subjects in LUAD, LUSC and BLCA patients. To avoid zeros when using a log scale, log2(FPKM + 1) was used to display gene expression data obtained by RNA-seq.

### Statistical analysis and data presentation

R (version 4.1.0) was used for data analysis and presentation. Wilcoxon Rank Sum Test (wilcox.test, with option alterative = 'two sided' and paired = False) was used for comparison of gene expression between tumor and adjacent normal samples as well as the comparison of SNVs. R package ComplexHeatmap (version 2.8.0) was used to generate the heatmap illustrating relative expression levels of TLS and TS pathway genes in LUAD. Z-score transformed FPKM-UQ value was used as the gene expression value in the heatmap. Stage, p53 mutation, Replication stress (RS) and smoke annotations were derived from clinical files, Mutect2 MAF files and GISTIC2 copy number files.

## DATA AVAILABILIY

The raw data in this study including RNAexpression, clinical information, mutation, and genomic alterations can be found at TCGA data portal (https://portal.gdc.cancer.gov).

## SUPPLEMENTARY DATA

Supplementary Data are available at NAR Cancer Online.

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