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Role of Y-family Translesion DNA Polymerases in Replication Stress: Implications for New Cancer Therapeutic Targets

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Abstract

DNA replication stress, defined as the slowing or stalling of replication forks, is considered an emerging hallmark of cancer and a major contributor to genomic instability associated with tumorigenesis [1]. Recent advances have been made in attempting to target DNA repair factors involved in alleviating replication stress to potentiate genotoxic treatments. Various inhibitors of ATR and Chk1, the two major kinases involved in the intra-S-phase checkpoint, are currently in Phase I and II clinical trials [2]. In addition, currently approved inhibitors of Poly-ADP Ribose Polymerase (PARP) show synthetic lethality in cells that lack double-strand break repair such as in BRCA^{1/2} deficient tumors [3]. These drugs have also been shown to exacerbate replication stress by creating a DNA-protein crosslink, termed PARP ‘trapping’, and this is now thought to contribute to the therapeutic efficacy. Translesion synthesis (TLS) is a mechanism whereby special repair DNA polymerases accommodate and tolerate various DNA lesions to allow for damage bypass and continuation of DNA replication [4]. This class of proteins is best characterized by the Y-family, encompassing DNA polymerases (Pols) Kappa, Eta, Iota, and Rev1. While best studied for their ability to bypass physical lesions in the DNA, there is accumulating evidence for these proteins in coping with various natural replication fork barriers and alleviating replication stress. In this mini-review, we will highlight some of these recent advances, and discuss why targeting the TLS pathway may be a mechanism of enhancing cancer associated replication stress. Exacerbation of replication stress can lead to increased genome instability, which can be toxic to cancer cells and represent a therapeutic vulnerability.

Keywords

Polymerase (Pol) Kappa; Pol Eta; Translesion Synthesis (TLS); Proliferating Cell Nuclear Antigen (PCNA); Replication stress; Ubiquitin

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Conflict of interest

The authors state that there is no conflict of interest.

1. Introduction

TLS overview

The Y-family of TLS polymerases (Pol Kappa, Eta, Iota, and Rev1) have N-terminal polymerase domains with similar right-hand symmetry structure as the replicative polymerases (Pol Alpha, Delta, and Epsilon) but with some key differences: 1) they lack a 3'–5' exonuclease domain and have a more solvent exposed active site, properties which make them more error-prone on normal DNA substrates but also enables their ability to accommodate and bypass various DNA lesions [4] (Figure 1); 2) the more variable C-terminal regions include PCNA-Interacting Peptide regions (PIP) boxes which mediate relatively weak binding to PCNA, as compared to the main replicative polymerases; 3) they also possess ubiquitin-binding domains (UBDs), called Ubiquitin-binding motifs (UBMs) or Ubiquitin-binding zinc fingers (UBZs) (Pol Iota and Rev1 have UBMs and Pol Eta and Kappa have UBZs), which binds to PCNA monoubiquitinated on lysine 164 (PCNA-Ub) by the Rad6/Rad18 E2/E3 ubiquitin conjugating complex [5]. The combination of the PIP boxes and UBDs provides for specificity in the interaction between a TLS Pol and PCNA at the replication fork. An additional level of regulation is provided by Rev1 Interacting Regions (RIR) also located in the C-terminal regions of these polymerases. These allow the Y-family Pols to associate with Rev1, which behaves more like a scaffold at the replication fork, rather than as a true replicative polymerase [6]. It is still not well-understood how TLS Pols exchange with the replicative Pols during a TLS event: dynamic exchange between different TLS Pols and replicative Pols must take place to drive “insertion”, “extension”, and then switchback to the replicative polymerase when the fork encounters a DNA lesion during DNA synthesis [6].

2. TLS in difficult to replicate endogenous DNA structures

Much of the evidence over the years of the ability of different Y-family pols to bypass different exogenous and endogenous DNA lesions comes from in vitro polymerase assays. These types of assays have now included various forms of non-damaged DNA substrates that represent various non-B DNA forming structures such as G4 quadruplexes and repetitive DNA. Here, we will focus our attention primarily on endogenous DNA lesions that are likely to occur spontaneously in dividing cancerous cells.

By studying the naturally occurring G4 quadruplex structure in the β -globin locus, Rev1 was shown to be required for the complete replication of this region, in addition to known helicases such as FANCD1 [7]. Even though Rev1 is only a dCMP transferase, its catalytic activity was required for proper replication, implying that even its limited nucleotide incorporation can be enough to potentially disrupt and replicate through a G4 structure. Interestingly, the C-terminal region of Rev1 that interacts with other Y-family Pols, such as Eta and Kappa, was also critical for replication, indicating the potential requirement for other TLS Pols as well. Indeed, both Pol Kappa and Eta show ability to synthesize on oligos containing G4 repeats [8]. While Pol Eta shows enhanced activity on the actual G4 tetrads, Pol Kappa activity increased on the nucleotides approaching the G4-quadruplex, relative to the other Pols. In agreement with the in vitro assays, depletion of both Kappa and Eta in human cell lines led to sensitivity when treated with the G4 quadruplex-stabilizing

compound tetramethylsilane (TMS), further indicating the role of TLS pols in the replication of endogenous G4-quadruplexes within cells [9].

Telomeric TTAGGG repeats are a potential endogenous source of G4 quadruplexes and are known to be difficult to replicate in cells [10]. A small percentage of cancer cells that don't upregulate telomerase to maintain their telomere length utilize homologous recombination between the telomeric repeats of sister chromatids, known as Alternative Lengthening of Telomeres (ALT). Part of this repair pathway requires DNA synthesis of the recombinatory D-loops generated within the telomeric repeats. Recently, Pol Eta was shown to bind to telomeres in conjunction with TRF1, and is required for proper ALT synthesis [11]. The activity of Pol Eta for ALT was corroborated using in vitro DNA substrates containing telomeric repeats.

Common fragile sites (CFS) are other regions of the genome that comprise difficult to replicate sequences. Upon elevated levels of replication stress, often induced by low dose DNA polymerase inhibitor aphidicolin (APH), these regions are prone to breakage, which is often referred to as expression of these fragile sites [12]. Depletion of Pol Eta leads to increased fragile site expression, especially after APH treatment, and an increase of APH induced 53BP1 G1 nuclear bodies [13, 14]. While the role of the UBZ domain and PIP boxes were not tested in that context, the authors found that expression of catalytically dead Pol Eta led to an increase in phospho-Chk1 levels, and this was reduced with additional mutations in either the UBZ domain or PIP box. Though not direct, this does imply that the recruitment of Pol Eta to these sites requires PCNA-Ub in agreement with its canonical model. In another study, the recruitment of Pol Eta to replication foci required SUMOylation on lysine 163 of the polymerase [15]. Again there are increased levels of APH induced 53BP1 G1 nuclear bodies with Pol Eta loss, and the K163 residue of Pol Eta is required for proper rescue of this phenotype. Interestingly, Rad18 is required for proper Pol Eta SUMOylation, and this requirement depends on the UBZ domain of Rad18 but not its E3 ligase activity. This would suggest that PCNA-Ub is not required for this activity of Pol Eta. Depletion of Rad18 led to similar levels of APH-induced 53BP1 G1 nuclear bodies and anaphase chromosome breaks as Pol Eta depletion. While the authors show that expressing the E3 ligase-mutant (C28F) of Rad18 can rescue APH-induced anaphase chromosome breakage, they do not test if this mutant can also rescue the APH-induced 53BP1 G1 nuclear bodies. Thus, it is possible that PCNA-Ub is required for at least some of the recruitment and utilization of Pol Eta during normal DNA replication, but other regulatory mechanisms, including catalytically-independent Rad18 mediated SUMOylation of Pol Eta, also play a role. Exactly how these different modifications dictate potentially differential recruitment of Pol Eta to the replication fork needs to be further worked out.

Similarly to Pol Eta, depletion of Pol Kappa leads to an increase in expression of the FRA7H locus [16]. Here the regulation is dependent on the PIP box of Pol Kappa, as the stronger binding PIP box of p21 is necessary to prevent aberrant recruitment of Pol Kappa. Depletion of p21 results in even further FRA7H expression, which is reduced with co-depletion of Pol Kappa to its lower, but still elevated level, of fragile site expression. Thus, too little or too much Pol Kappa can lead to replication instability.

These observations are further strengthened by recent in vitro data showing both Pol Kappa and Eta enhance the ability of Pol Delta (replicative polymerase) to complete replication of the FRA16D locus [17]. This data is striking since it shows that two polymerases, with vastly different rates of DNA synthesis on normal DNA [4], can exchange with each other and lead to more efficient replication than either alone. The authors also show that this activity is not enhanced by utilizing PCNA-Ub in the reactions, and interpret this as PCNA-Ub not being required for TLS in cells. While these are impressive in vitro experiments, the lack of requirement for PCNA-Ub for these reactions does not rule out the necessity of this modification in recruiting these polymerases in the context of an intact cell with a dense nuclear environment. This dynamic switching between a TLS Pol and replicative Pol was recently shown in a study utilizing eGFP-tagged repair proteins and mCherry tagged PCNA in live-cell imaging analysis [18]. Using fluorescence recovery after photobleaching (FRAP), the authors determined that Pol Eta and Pol Delta have similar half-times of exchange on UV-damaged DNA, and both are approximately 60 times faster than that of PCNA exchange on damaged DNA. Thus, they determined that for the length of time PCNA is retained on DNA, a TLS Pol and replicative Pol could have exchanged around 60 times. How exchange between different Pols are regulated by post-translational modifications and whether perturbation of different exchange rates can lead to replication or gap repair defects will be important areas to study.

3. TLS in non-DNA damaging, replication-stalling conditions

Defined as the slowing or stalling of replication forks, replication stress is becoming an increasingly appreciated mechanism of genomic instability in cancer initiation and progression [19]. Difficulty in replicating regions of the genome containing G4 repeats or fragile sites can serve as one potential source of replication stress in cells. In addition, two tools researchers frequently use to cause replication fork stalling are aphidicolin (APH), an inhibitor of replicative DNA Pols Alpha/Delta/Epsilon, and hydroxyurea (HU), an inhibitor of ribonucleotide reductase which then causes a depletion (reduction, but not a complete loss) of the deoxynucleotide pool. While exogenous agents such as UV light, radiation, and chemotherapy can all cause direct lesions or barriers in the DNA to impede replication fork progression, using these tools has provided an avenue of studying the cell's ability to cope with more endogenous lesions present in normal DNA replication in contrast to those formed by exogenous DNA damage.

Expanding on their previous in vitro data, Barnes et al. [20] demonstrated a role for Pol Eta in broader replication stress. Treatment with APH causes similar levels of Pol Eta foci per nucleus as compared to UV treatment, a known inducer of Pol Eta recruitment to DNA damage sites [21]. Loss of Pol Eta also leads to decreased survival to a range of APH doses, as well as increased levels of S-phase checkpoint signaling in cells, which requires Pol Eta's UBZ domain. Using flow cytometry, the authors show that after 24 hours of relatively higher dose of APH treatment, an increased number of Pol Eta knockout cells were in the G2/M phase of cell cycle. Combining EdU analysis to flow cytometry, they show that these cells also have increased mitotic DNA synthesis (miDAS), indicating transmission of under-replicated DNA into mitosis [22].

Recent work from our laboratory has demonstrated a novel role for Pol Kappa in response to replication stress from high dose HU treatment [23]. Loss of Pol Kappa leads to an increase in stalled replication forks after short term HU-stalling as measured by single-molecule DNA fiber analysis. This defect leads to delays in cell cycle progression in the subsequent cell cycle phase, as well as increases in 53BP1 nuclear bodies in the subsequent G1 phase after only transient HU treatment. We showed that the recruitment of Pol Kappa requires its UBZ domains as well as ubiquitination of PCNA on lysine 164. We also used nucleoside analog-labeling techniques analyzed by DNA fibers and cell-based immunofluorescence that low level DNA synthesis that can occur under high dose HU is significantly reduced in Pol Kappa-deficient cells. Using super resolution microscopy to visualize EdU-incorporated replication tracts under high dose HU across the whole nuclei, we found that Pol Kappa depletion reduced the amount of EdU labeling within each replication factory (nuclear foci), as well as the number of EdU-positive replication factories per nuclei, suggesting a broad requirement of Pol Kappa usage across the genome for DNA synthesis during conditions of low nucleotide pools.

A recent report from Tubbs et al [24] used END-Seq, a method for genome-wide mapping of replication fork-associated double-strand breaks (DSBs) to identify break sites associated with HU-induced replication stress in a sequence-specific manner. The authors showed that after high and low dose HU treatment, the replication forks often break at early-replicating fragile sites (ERFSs), late-replicating common fragile sites (CFSs) and at the rDNA replication fork barrier. Interestingly, the DNA sequences that are enriched within these sites are long (>10) stretches of PolyA/PolyT mononucleotide repeats. They convincingly show that these stretches throughout the genome represent a natural and asymmetric fork barrier, and are a potential large contribution of endogenous replication fork breaks. Intriguingly, the authors note that a large portion of these polyT tracts mapped to HU-induced break sites contain a signature of CC/GG insertions within 10bp of the break site within the repeat. This is a noted signature of Pol Kappa usage from in vitro polymerase assays using polyT mononucleotide repeats [25]. While the authors do not perform any direct experiments to test the requirement of Pol Kappa, in agreement with observations from our recent work [23], this is indicative of the recruitment of Pol Kappa to replication forks throughout the genome to help prevent HU-mediated fork stalling events via direct error-prone DNA synthesis.

4. TLS in the protection against stalled replication fork nucleolytic resection

During replication stress, some stalled replication forks undergo fork remodeling and require protective DNA repair proteins to prevent improper nucleolytic resection of nascent DNA. The list of proteins involved in protecting stalled forks has grown from the initial discoveries of BRCA1/2 and FANCD2 [26, 27] to a large number of factors that includes other homologous recombination proteins [28, 29], replication fork remodelers [30], and epigenetic modifiers [31, 32] (collectively reviewed recently in [33] [34]). The loss of stalled fork protection has been shown to increase genomic instability and has even been implicated as a potential mechanism for chemotherapy and PARP inhibitor resistance [32].

Work from our lab has shown that Pol Kappa depletion also leads to increased nucleolytic resection of nascent DNA after HU-induced replication fork stalling [23]. This activity is dependent on the activity of the MRE11 nuclease and the fork reversal enzyme SMARCAL1, implying that the resection is occurring on reversed fork substrates. Interestingly, while the depletion of Pol Kappa was epistatic to FANCD2 loss in regard to replication fork restart after HU, these two proteins were not epistatic to stalled fork resection after HU. In a separate study, USP1 loss was synthetically lethal to loss of BRCA1, and this phenotype was due to increased stalled replication fork resection, as lethality could be rescued by inhibition of MRE11 [35]. Furthermore, depletion of Pol Kappa, which is hyper-recruited to the stalled forks due to the elevated levels of PCNA-Ub from loss of USP1 [36], also led to a rescued phenotype of stalled fork protection, implying that in this case Pol Kappa promotes nucleolytic resection. While this may seem like a discrepancy between the two studies, these authors only performed Pol Kappa depletion in the context of a BRCA1-null background, and not on a BRCA1 wild-type background as in our study. Also, the increased nucleolytic resection with USP1 depletion is only observed in the context of BRCA1 deficiency, but not in BRCA2-deficient cells. This highlights different mechanisms of stalled replication fork resection and protection compared to the classic BRCA2-loaded Rad51 stabilization model, and may explain the differences seen in regard to Pol Kappa function in fork protection. Rev1 also has a role in stalled fork protection in cooperation with FANCD2, but to replication fork stalling from camptothecin (Topoisomerase I poison) treatment [37]. Collectively, these reports highlight the new role of Y-family TLS Pols in the ability to regulate stalled replication fork protection/resection. Clearly much work needs to be done to work out the exact mechanism, and potential varying mechanisms of how TLS proteins cooperate with BRCA1, BRCA2 and the vast majority of other proteins regulating stalled fork protection/resection.

5. TLS in oncogene-induced replication stress

In pre-cancerous and cancerous lesions, a large source of replication stress and subsequent DNA damage can be caused by the activation of oncogenes [38–40]. Termed oncogene-induced replication stress, the activation of an oncogene either by mutation or overexpression drives the cell through into rapid cell cycle divisions which exacerbates multiple sources of endogenous replication stress [41]. The mechanistic sources of the replication stress are numerous, and can include dysregulated replication origin firing, increased transcription and thus replication-transcription conflicts, increased reactive oxygen species (ROS) from the increased metabolic demand of the mitochondria, and deficient levels of replication machinery requirements, which includes replication proteins such as RPA and histones as well as sufficient deoxynucleotide (dNTP) pool levels. While different oncogenes may utilize different mechanisms to promote replication stress, they all share a similar end result and consequence of increasing replication-stress associated DNA damage and genomic instability [1, 41]. Understanding how cells cope with replication stress associated DNA damage is critical for understanding cancer progression, and recent work suggests that TLS Pols may play an important role.

Both Pols Eta and Kappa have been shown to be involved in tolerance to different models of oncogene-induced replication stress. In one study by Kurashima et al [42], depletion of Pol

Eta, and to a smaller extent Pol Kappa, led to cell cycle defects and a reduction in cell growth after inducing c-Myc overexpression. Focusing on Pol Eta for the rest of the study, the authors show that in a Rad18 and PCNA-Ub dependent manner, Pol Eta localizes to replication foci caused by c-Myc overexpression. Furthermore, induction of c-Myc led to increased levels of γ H2AX signal (immunofluorescence) and DNA breaks (measured by the neutral comet assay), and both effects became further exacerbated in the absence of Pol Eta. Finally, Pol Eta depletion led to a decrease in c-Myc-induced replication fork velocity, as well as a synergistic increase in c-Myc-induced fork stalling as measured by asymmetric fork measurements via the DNA fiber technique. One main cause of replication stress attributed to c-Myc overexpression is the hyperactivation of replication origins, which interrupts the normal balance of replication machinery factors and impairs global DNA replication [43]. This final piece of data provides a potential mechanism for the cause of DNA damage observed after Pol Eta depletion: overexpression of c-Myc likely exacerbates some of the difficult to replicate sequences (potential secondary structures) in the genome that Pol Eta has been shown to help replicative Pols tolerate. Thus, without TLS activity, replication forks across the genome cannot properly negotiate these endogenous lesions and will stall more frequently, increasing the likelihood of DSBs and incomplete DNA replication.

In a separate study, Yang et al [44] show that Pol Kappa is required for tolerance to replication stress from aberrant Cyclin E/CDK2 activity. In contrast to c-Myc or Cdt1 overexpression or Ras activation, Cyclin E overexpression led to an increase in PCNA-Ub that was dependent on Rad18, and the depletion of Rad18 led to further perturbations of the cell cycle and increased phospho-RPA signals. While the protein levels of Pol Eta remained stable after Cyclin E induction, in contrast, the levels of Pol Kappa increased, and only Pol Kappa was localized to chromatin after Cyclin E induction. Pol Kappa was shown to interact with PCNA-Ub under these conditions. Induction of Cyclin E increased levels of 53BP1 nuclear bodies as well as ssDNA that accumulates in mitosis, both of which were exacerbated further by either Pol Kappa or Rad18 depletion. Depletion of Pol Kappa led to drastic decreases in replication fork velocity after Cyclin E induction, and treatment with the Wee1 inhibitor MK-1775, an analogous method of activating Cyclin E/CDK2 complexes, led to similar inhibition of fork velocity in Pol Kappa knockout MEF cells. This is another example of how oncogenes reduce replication fork velocity in the absence of a specific TLS activity. Why does activation of a specific oncogene over another appears to require Pol Kappa over Pol Eta? Since Pol Kappa depletion also showed a slower replication fork velocity during HU treatment [23], it's tempting to speculate that perhaps Cyclin E induction lowers the nucleotide pool more so than c-Myc overexpression, and this may favor Pol Kappa usage. This is supported by a study showing that oncogene-induced activation of the Rb-E2F pathway (via Cyclin E overexpression or HPV E6/E7 expression) leads to lower nucleotide pool levels, whereas c-Myc expression upregulates the nucleotide biosynthesis pathway [45]. Yang et al attempt to test the requirement of different nucleotide levels by supplementing back in nucleosides, but they only show that this doesn't reduce PCNA-Ub levels after Cyclin E induction, and don't provide any phenotypic rescue after supplementation [44]. While the mechanism of specific TLS usage during oncogene-induced

replication stress needs to be further worked out, it's clear that Y-family TLS Pols serve a crucial role in preventing genomic instability associated with oncogene induction.

In addition to oncogene activation in cancer, the hematopoietic system provides another important model of replication stress. Loss of certain DNA repair pathways such as the Fanconi Anemia pathway leads to various forms of anemia in mice and humans attributed to defects in the hematopoietic stem cell (HSC) compartment [46, 47]. Since HSCs are responsible for populating the entire hematopoietic lineage, defects in their replicative potential can be seen as inability to populate terminal blood lineages. Recent evidence suggests TLS polymerases could also play a protective role in this system. Using competitive HSC transplantation into lethally irradiated mice, Rev1 knockout HSCs showed impaired proliferation and grafting potential compared to WT controls [48]. Similar results were obtained in a mouse model with a knock-in mutation of K164R on PCNA that abrogates ubiquitination, and thus TLS recruitment [49]. These PCNA K164R HSCs not only showed proliferation defects in competitive transplantation assays but the mice themselves showed signs of increased aging as defined by much earlier increases of certain multiple potent progenitor subsets that are seen with normal aging mice. These studies highlight in an analogous model of replication stress that TLS Pols can contribute to cellular tolerance to replication stress in the absence of exogenous DNA damage.

6. TLS specificity vs redundancy in replication stress

Does the usage of either Pol Eta or Kappa merely represent a common pathway of TLS usage, or are there specific requirements for the different Pols under different circumstances? While several of the reported studies have tended to focus on the role of a specific TLS Pol, it is important to note the distinct differences between the usage and function of the two TLS Pols, which is largely dictated by the type of stress the cells are encountering. For instance, Pol Eta is required for c-Myc overexpression and after APH treatment, whereas Pol Kappa is required for Cyclin E overexpression and HU treatment. Could it be that distinct endogenous DNA lesions drive the recruitment of different Pols for DNA synthesis/bypass? This idea stems from what we previously understood concerning the preference of different TLS Pols for exogenous DNA damage; i.e. Pol Eta is recruited to sites of UV photoproducts and Pol Kappa for benzo[a] pyrene [50].

One unifying theme of these studies is the requirement of UBDs and Rad18 for the recruitment of the Y-family Pols to endogenous DNA damage sites and for DNA synthesis during replication stress conditions, thus, pointing to the requirement of the canonical PCNA-Ub pathway for TLS activity. Previous work from our lab has shown that increasing PCNA-Ub levels by depletion of USP1 led to reduced fork elongation and increased levels of micronuclei formation [36]. Only depletion of Pol Kappa, but not Pol Eta or Iota, rescues this USP1-deficient phenotype, suggesting that only hyper (aberrant)-recruitment of Pol Kappa to the replication fork is somehow deleterious to the cells during normal DNA replication. But how could PCNA-Ub dictate differential recruitment of a TLS Pol? The nature of ubiquitin modification, i.e. monoubiquitin versus polyubiquitin, is a known mechanism for recruiting different replication factors onto PCNA [51]. In yeast, PCNA-mono-Ub, catalyzed by the E2/E3 complex of Rad6/Rad18, promotes TLS for replicative

bypass and post-replication repair of DNA lesions, whereas K63-linked PCNA-poly-Ub, catalyzed by the E2 dimer Ubc13/Mms2 and E3 Rad5, mediates an error-free template switching mechanism [52]. In mammalian cells, PCNA-mono-Ub is also catalyzed by the highly conserved E2/E3 complex of Rad6/Rad18 and is thought to be the main mechanism for TLS Pol recruitment. However, the unifying picture for PCNA-poly-Ub appears to be more complicated. There are two Rad5 orthologs in mammals, HLTF and SHPRH, which can both supposedly promote K63-linked PCNA-poly-Ub [53–55]. For template switching, HLTF can catalyze fork reversal [56], and another DNA translocase ZRANB3 binds K63-linked PCNA-poly-Ub chains to mediate fork reversal [57, 58]. However, mouse cells deleted for both HLTF and SHPRH are still capable of PCNA-poly-Ub, and don't show any sensitivity to DNA-damaging agents as does Rad5 deletion in yeast [59, 60]. In recent work from our lab, we show that Pol Kappa binds to PCNA-poly-Ub in a HU-inducible manner [23]. This is distinct from the classic PCNA-poly-Ub in two ways: 1) The modifications are enriched for smaller chains (di-, tri-, and possibly tetra-ubiquitin) as opposed to the large number of chains of ubiquitin that appear as high molecular weight smears on gels. 2) The chains are K48-linked as oppose to K63-linked, as evidenced by chain cleavage analysis using linkage-specific deubiquitinases [23]. These differences of PCNA modification may dictate general TLS usage versus replication fork reversal, as well as mediate differences amongst TLS Pol recruitment. It's intriguing to speculate that Pol Kappa, which has two UBDs in contrast to Pol Eta's single UBD, can bind tighter to di- or tri-ubiquitinated PCNA, and this may enhance its preferential recruitment after HU. This may not be independent of the Rad5 homologs, as treatment with UV light or methyl methanesulfonate (MMS) was shown to differentially recruit TLS pols Eta and Kappa respectively through the different DNA damage-mediated regulation of HLTF and SHPRH [61]. Interestingly, a recent report in yeast has shown that Rad5 can actually stimulate TLS activity in addition to its known role in template switching, and that this is an important mechanism for DNA damage tolerance [62]. More work must be done on the roles of different PCNA ubiquitin modifications (i.e. mono- vs poly-Ub and K48 vs K63) and its recruitment of different TLS Pol and other replication/DNA repair factors.

Despite some clear preferences, there are also many instances of redundancy amongst the Y-family TLS Pol. Indeed even though Pol Eta depletion led to the most robust phenotype, loss of Pol Kappa also led to a noticeable proliferation defect after c-Myc overexpression, and both Pol Kappa and Rev1 depletion showed mild cell cycle defects [42]. With the loss of one TLS Pol, it is possible that another TLS pol, even if less efficient, could serve a back-up function in its place. This has been observed with well-characterized TLS preference in response to different DNA damages. Loss of Pol Eta led to much more sensitivity to UV over Pol Kappa deficiency, but loss of both led to even greater UV sensitivity [63]. Likewise loss of Pol Kappa led to greater sensitivity to (MMS) with very little sensitivity for Pol Eta deficiency, and yet loss of both led to even greater cytotoxicity [64]. Thus, targeting of the entire TLS pathway may lead to greater effects on genotoxicity than targeting one TLS Pol over another.

7. Potential for therapeutics

Recent approaches in cancer therapy have focused on exploiting genomic instability found in tumor cells by targeting various DNA repair pathways [65]. The most successful example of this approach is the synthetic lethality of PARP inhibition in tumors lacking the BRCA $\frac{1}{2}$ proteins. Multiple inhibitors have been FDA approved in ovarian and breast cancer, however resistance to these drugs continue to be an obstacle for long-lasting treatment [66]. Another approach is targeting the ATR/Chk1 damage signaling pathways to sensitize cancer cells to replication stress and genotoxic treatments[67]. As discussed in this review, it is feasible that targeting TLS Pols can be a different approach to achieve a similar goal.

One proof of principle of the concept of inhibiting a repair DNA polymerase as cancer therapy comes from a TLS polymerase of the A-family, Pol Theta. It's been shown that Pol Theta is required for an alternative form of non-homologous end-joining that relies on microhomology present at or generated at the break sites [68, 69]. Depletion of Pol Theta leads to increased DSBs in cells deficient in homologous recombination, and has been shown to be synthetically lethal when in combination to BRCA $\frac{1}{2}$ [69, 70]. Similar to PARP inhibition, cells deficient in one form of DNA repair can become reliant on a back-up form of repair, and here that can be alternative end-joining, mediated by a TLS Pol.

Further proof of principle comes from a report describing a new method of repair for abasic sites encountered at the replication fork [71]. The protein HMCES recognizes and binds to abasic sites, leading to DNA-protein crosslinks that are repaired by a distinct pathway. Without this recognition, the abasic sites could be turned into DSBs leading to lethality. HMCES knockout cells do not show increased levels of DNA damage, and iPOND analysis to determine which factors may be upregulated to compensate included Rev1 and Rev7. Indeed, co-deletion of these TLS proteins along with HMCES leads to synthetic lethality.

One potential avenue for treatment would be inhibition of TLS alongside inhibition of ATR. As TLS can be a mechanism to alleviate replication stress, combination with an ATR inhibitor can leave cells with few options of repair, which can then lead to increases in DSBs and genomic instability. Both Pol Eta and Pol Iota were hits in a siRNA screen identifying genes synthetically lethal with ATR inhibitor [72]. The increased DNA damage of Pol Eta depletion after APH treatment was exacerbated by treatment with an ATR inhibitor [20]. Targeting the TLS pathway can eliminate a distinct replication stress tolerance pathway that cancer cells can utilize in normal cancer progression and develop therapy resistance. As restoration of replication fork stability has been reported as a product of PARP and cisplatin resistance in breast cancers [32], inhibition of TLS could be a novel mechanism of counteracting fork stability.

8. Conclusion

DNA replication stress is an increasingly important hallmark of cancer. Strategies to exacerbate replication stress and exploit the genomic instability are increasingly being utilized as a cancer therapeutic. The role of TLS Pols in tolerating multiple forms of replication stress is becoming more established. They can help synthesize and repair difficult

to replicate regions in the genome, such as G4 quadruplexes and common fragile sites during normal DNA replication, and tolerate overexpression of oncogenes such as c-Myc and Cyclin E (Figure 2). As new therapies are being developed for cancer, different mechanisms of resistance are increasingly becoming a formidable challenge to overcome. Targeting translesion polymerases can be a novel mechanism of enhancing replication stress and combating therapy resistance.

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Fig. 1. Domain architecture of the four Y-family Translesion DNA Polymerases Pol Eta, Pol Iota, Pol Kappa, and Rev1. BRCT = BRCA1-C Terminal domain; PIP = PCNA Interacting Peptide; RIR = Rev1 Interacting Region; UBZ = Ubiquitin Binding Zinc finger; UBM = Ubiquitin Binding Motif.

Fig. 2.

Broad TLS requirement in normal DNA replication and replication stress. A) For normal DNA replication, TLS usage required for tolerance against difficult to replicate genomic features including fragile sites (red), mononucleotide repeats (green), and G4 quadruplexes (blue). B) Replication fork schematics showing TLS recruitment to different sources of replication stress. Pol Eta (red) is recruited to monoubiquitinated PCNA after c-Myc overexpression and aphidicolin (APH) treatment, and Pol Kappa (blue) is recruited to mono-, di-, or tri-ubiquitinated PCNA after Cyclin E overexpression and hydroxyurea (HU) treatment. Both lead to proper genome stability. C) Replication fork schematic of a stalled replication fork. A general TLS Pol can be recruited via PCNA-Ub, and ZRANB3 binds to poly-ubiquitinated PCNA for replication fork reversal, both of which help rescue and protect stalled forks.