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The Rev1-Pol ζ Translesion Synthesis Mutasome: Structure, Interactions and Inhibition

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Abstract

DNA contains information that must be safeguarded, but also accessed for transcription and replication. To perform replication, eukaryotic cells use the B-family DNA polymerase enzymes Pol δ and Pol ϵ , which are optimized for accuracy, speed, and processivity. The molecular basis of these high-performance characteristics causes these replicative polymerases to fail at sites of DNA damage (lesions), which would lead to genomic instability and cell death. To avoid this, cells possess additional DNA polymerases such as the Y-family of polymerases and the B-family member Pol ζ that can replicate over sites of DNA damage in a process called translesion synthesis (TLS). While able to replicate over DNA lesions, the TLS polymerases exhibit low-fidelity on undamaged DNA and, consequently, must be prevented from replicating DNA under normal circumstances and recruited only when necessary. The replicative bypass of most types of DNA lesions requires the consecutive action of these specialized TLS polymerases assembled into a dynamic multiprotein complex called the Rev1/Pol ζ mutasome. To this end, posttranslational modifications and a network of protein-protein interactions mediated by accessory domains/subunits of the TLS polymerases control the assembly and rearrangements of the Rev1/Pol ζ mutasome and recruitment of TLS proteins to sites of DNA damage. This chapter focuses on the structures and interactions that control these processes underlying the function of the Rev1/Pol ζ mutasome, as well as the development of small molecule inhibitors of the Rev1/Pol ζ dependent TLS holding promise as a potential anticancer therapy.

Keywords

DNA damage tolerance; translesion synthesis; DNA polymerase; protein structure; protein-protein interactions; anti-cancer therapeutics

1. Introduction

Semiconservative DNA replication is a mechanism that is found in all forms of life, from viruses and bacteria to humans. During replication, DNA is unwound and the two parental strands are separated to allow DNA polymerase complexes access to read the template bases and then build the daughter strands (Figure 1A) [1]. Most DNA replication in eukaryotic cells is carried out by the two replicative B-family polymerase complexes, Pol δ and Pol ϵ ,

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which are believed to act individually on the lagging and leading strands, respectively (Figure 1A, right) [2].

Given the role of DNA as information storage, replication must be performed accurately, and through evolution, the replicative DNA polymerases are able to achieve an error rate as low as $\sim 10^{-6}$ - 10^{-8} [3]. This high fidelity, as well as processivity and speed, is achieved in part, by having an active site whose size and structure restrict the insertion of nucleotides in the daughter strand to the canonical Watson-Crick base pairs (GC & AT) [3]. As a consequence, when damaged DNA eludes repair prior to S phase, Pol δ and Pol ϵ are unable to read the chemically modified base in the template strand because it no longer fits within the active site, nor contains the expected molecular surface for Watson-Crick base-pairing (Figure 1B). This scenario can lead to fork collapse, incomplete replication of the genome, and, ultimately, chromosomal instability.

Of course, DNA can be modified by exogenous and endogenous genotoxic agents such as ultraviolet light, components of smoke, the by-products of metabolism, or other sources which form lesions that can alter the structure of an individual base or the helical superstructure. Usually, these lesions are detected and removed from DNA prior to S phase by one of the DNA repair pathways that living cells have evolved, including nucleotide excision repair (NER) and base excision repair (BER), but, given the number of lesions a cell experiences per day, some DNA damage will remain when replication begins [4–7].

In response, cells have evolved DNA damage tolerance (DDT) pathways that allow them to replicate opposite, but not repair, damaged DNA [8, 9]. First, the cell may perform low-fidelity replications opposite the damaged DNA in an error-prone process called translesion synthesis (TLS) [10–12] (Figure 1B, bottom). Most generally, TLS is carried out by a group of specialized Y-family (Rev1, Pol η , Pol ι , Pol κ) and B-family (Pol ζ) DNA polymerases (Figure 2) that assemble into a multiprotein complex called the Rev1/Pol ζ mutasome [10–17], although some DNA lesions can be efficiently bypassed by individual TLS enzymes [18–22]. These polymerases trade the ability to replicate damaged DNA in exchange for a decrease in fidelity on undamaged DNA. For the cell, this compromise is worth it, because inserting any nucleotide opposite a lesion is preferable to fork collapse and incomplete replication [8, 9]. Alternatively, the cell may utilize the template-switching mechanism for the error-free DNA lesion bypass, where the daughter strand from the sister chromatid is used as a template for DNA replication [8].

Because the TLS polymerases are low-fidelity, they must not be allowed to copy undamaged DNA. To prevent them from accessing undamaged DNA, their recruitment is regulated by posttranslational modifications and a network of protein-protein interactions mediated by accessory proteins, domains, and motifs that are specific to the TLS polymerases (Figure 2) [23–29]. Thus, switching from normal replication to TLS is regulated by monoubiquitination of the Proliferating Cell Nuclear Antigen (PCNA) at residue K164 (Figure 1B, bottom) [30–32], while subsequent polyubiquitination of PCNA via the formation of K63-linked ubiquitin chains can trigger the error-free DNA lesion bypass by the template switching mechanism [8, 33–37]. The recruitment of TLS DNA polymerases and their assembly into a multiprotein TLS complex – the Rev1/Pol ζ mutasome is governed by their interaction with

ubiquitinated PCNA through the PIP-box motifs (or BRCT domain in Rev1) and ubiquitin-binding UBM or UBZ domains found in all Y-family TLS polymerases [25, 26] (Figure 2). Additional interactions that control assembly and rearrangement of the Rev1/Pol ζ mutasome are mediated by Rev1, which plays scaffolding role in TLS by binding Y-family DNA polymerases and subunits of Pol ζ through the Rev1 C-terminal (Rev1-CT) domain [38–50] (Figure 2). Not only these protein-protein interactions control the access of TLS polymerases to DNA, but they also facilitate rearrangements of the Rev1/Pol ζ mutasome underlying polymerase switching events.

While TLS is important for the maintenance of genome stability, the error-prone TLS polymerases can also introduce mutations leading to cancer [51, 52]. Furthermore, Rev1/Pol ζ -dependent TLS is responsible for the bypass of DNA adducts formed by genotoxic chemotherapeutics, increasing survival of cancer cells following first-line therapy [51–54]. In addition, TLS increases the frequency of mutation in tumors, which can lead to the onset of drug resistance [55, 56]. Therefore, inhibition of the Rev1/Pol ζ -dependent TLS has recently emerged as a strategy to improve the efficacy of first-line chemotherapy and suppress acquired chemoresistance [56–61] (reviewed in [62–66]).

This chapter focuses on the structure and interactions of the non-catalytic accessory domains and subunits in the DNA polymerases that take part in Rev1/Pol ζ -dependent TLS. For further review of the structure and activity of the catalytic domains, we point readers to recent reviews [10, 16, 67]. The remainder of this chapter will give an overview of TLS in eukaryotes with the focus on humans and *S. cerevisiae* yeast, and then address the assembly of the Rev1/Pol ζ complex and its interaction with the remaining Y-family polymerases in greater depth, while trying to give mechanistic insights that arise from the sum of previous work. We conclude by describing targeting the Rev1/Pol ζ mutasome with small molecules for TLS inhibition, which provides a potential strategy to improve first-line anticancer chemotherapy.

2. Mechanisms of translesion synthesis

2.1. The TLS polymerases

The components of the TLS pathway show remarkable sequence and structural similarity that goes back as far as bacteria, while at the same time displaying divergence and evolution [67, 68]. In eukaryotes, TLS is carried out by members of the Y-family of DNA polymerases, which include Rev1, Pol η , Pol ι , and Pol κ , and by the B-family member Pol ζ , in humans [10–17] (Figure 2). While the mechanisms of TLS in yeast are related to humans, the *S. cerevisiae* genome does not contain Pol ι or Pol κ (Figure 2). Still, the use of yeast as a model system was critical for the development of our understanding of TLS and will likely continue to make such a contribution in the future.

The Y-family polymerases are classified distinctly from other DNA polymerases based on their catalytic domains. Structurally, the TLS polymerases resemble the replicative B-family polymerases in this region, with both containing the thumb, palm, and finger domains; however, the Y-family TLS polymerases lack the N-terminal 3'–5' exonuclease domain that performs proofreading and contain an additional C-terminal polymerase-associated domain

(PAD) (Figure 3) [14, 69–71]. While the architecture of the active site is similar, the finger and thumb domains of the TLS polymerase are smaller and have been described as “stubby” [14, 15, 72] (Figure 3). Crystal structures of the catalytic domains of the *S. cerevisiae* B-family polymerase, Pol δ , and Y-family polymerase, Pol η , in complex with DNA demonstrate the effect of this difference on the extent to which the Y-family polymerase grasps the DNA strand less fully and allows the DNA to protrude out from the active site to a greater extent (Figure 3). In short, the more spacious and flexible active sites in the Y-family polymerases compared to the B-family Pol δ or Pole allow the TLS polymerases to accommodate DNA templates with bulky modifications and perform TLS.

The tradeoff for this adaptability is a reduction in processivity and accuracy, with increased error rates on undamaged templates of 10^{-1} – 10^{-4} versus 10^{-6} – 10^{-8} for the replicative polymerases [3, 73]. Of course, the fidelity of replication opposite the lesion itself is also low, because the altered structure (with few exceptions [18–22]) usually cannot be decoded by the polymerase. To this point, the recruitment of the TLS polymerases to DNA must be subject to regulation, within the framework of DNA replication as a whole, to prevent unnecessary mutagenesis.

2.2. Recruitment of the TLS polymerases through interaction with PCNA

When damage is present during replication, the single-strand DNA at sites of DNA damage where Pol δ and Pole failed to perform are coated with Replication protein A (RPA), which in turn recruits the E2 ubiquitin-conjugating enzyme Rad6 and the E3 ubiquitin-protein ligase Rad18 [74]. This E2/E3 pair then signals for TLS by monoubiquitinating the homotrimeric sliding clamp PCNA, at residue K164 (Figure 4A, right) [30–32]. In this case, monoubiquitination refers to a single ubiquitin moiety in each chain, but each of the three equivalent K164 residues in the trimer may be ubiquitinated [30]. Because all four Y-family polymerases contain at least one ubiquitin-binding domain (UBM or UBZ) in addition to PCNA-binding PIP box motifs (Figure 2A), ubiquitination of PCNA increases the number of interaction points and, therefore, affinity between the TLS polymerases and the replication fork compared with Pol δ and Pole, which lack ubiquitin-binding domains [25, 26]. This presumably leads to a preference for TLS activity at the site of DNA damage and the switch from a replicative to TLS polymerase (Figure 4A, left).

The alternative, error-free branch of DDT is initiated by additionally polyubiquitinating PCNA at residue K164 into K63-linked chains by the E2 enzyme complex Ubc13/Mms2 and E3 enzymes HLTF or SHPRH [33–37] in humans. In yeast, the mechanism is the same, except there is a single E3, Rad5 [37]. This template-switching strategy uses a mechanism where the DNA translocase activity of HLTF or Rad5 (in addition to their E3 role) facilitates fork reversal and the formation of Holiday-like junctions that allow the daughter strand from the undamaged sister chromatid to be used as the template for accurate DNA replication [8].

2.3. Assembly of the multiprotein TLS complex

Besides the interaction with the ubiquitinated PCNA, the TLS DNA polymerases contain additional subunits, domains, and motifs that they use to interact with each other to assemble in the multiprotein TLS complex called the Rev1/Pol ζ mutasome. At the core of the Rev1/

Pol ζ mutasome, the C-terminal domain from the Y-family polymerase Rev1 (Rev1-CT) and the Rev7 subunit of Pol ζ form a complex that bridges the Y-family polymerases with the B-family polymerase Pol ζ (Figure 5A). These and other interactions that mediate assembly of the Rev1/Pol ζ mutasome are discussed in detail below.

Considering the number of possible interactions, the structure of the Rev1/Pol ζ mutasome at any point in time is difficult to discern, and to describe the complex as a static structure may be inappropriate. At the very least, based only on interactions that are confirmed by crystal structure, the basic unit of the human Rev1/Pol ζ mutasome could be a seven-subunit complex composed of Rev1, a Y-family polymerase (Pol η , ι , κ), and Pol ζ (Rev3/Rev7₂/PolD2/PolD3 [17, 75–78]) (Figure 5A) assembled on the ubiquitinated PCNA. A better understanding of whether the Rev1/Pol ζ mutasome is a stoichiometric complex or a more dynamic assembly would contribute to our understanding of the mechanisms of TLS.

2.4. Timing and spacing of TLS

Once PCNA is ubiquitinated, the TLS polymerases and PCNA localize into nuclear foci (bright spots), which are referred to as “replication factories”, and can be visualized using fluorescently-tagged proteins or immunofluorescence (Figure 5B) [23–29]. Recruitment to these damage-specific replication factories is dependent on the interaction subunits, domains, and motifs in the Y-family and B-family TLS polymerases and, as expected, when mutation or deletion of the interaction domains prevents localization to foci, *in vitro* and *in vivo* assays show reduced TLS activity [23–29]. Presumably, these foci are the sites of DNA damage where the TLS polymerases are active.

TLS activity in the cell may occur in two temporally distinct situations. In one case, the TLS DNA polymerases gain access to stalled replication forks during S phase, exchange with Pol δ and Pol ϵ , insert nucleotides opposite the lesion, and are then switched back with Pol δ and Pol ϵ “on the fly” (Figure 4B, left) [79]. In the second case, the TLS polymerases are recruited after the bulk of replication is complete to fill in single-strand gaps that are left where Pol δ or Pol ϵ failed (Figure 4B, right) [80–84]. In addition, TLS polymerases are involved in gap-filling synthesis after DNA repair (ref [65] and Chapters 5 and 6 of this volume).

2.5. Two-step Rev1/Pol ζ -dependent TLS

In the majority of cases, TLS is believed to occur by a two-step mechanism where one polymerase (Rev1, Pol η , Pol ι , or Pol κ) acts as the “insertor” polymerase to replicate opposite the lesion, while another polymerase, usually Pol ζ but potentially other TLS enzymes such as Pol κ [85, 86], then acts as the “extender” polymerase and replicates starting from the distorted primer terminus (Figure 5C) [87–90]. How the insertor polymerase is chosen is not clear, although some polymerases have demonstrated specificity for certain (cognate) lesions. For instance, Pol η is able to accurately replicate opposite UV-induced cyclobutane pyrimidine dimers and the GG intrastrand crosslink that is created by cisplatin [18–22, 91, 92]; Pol κ can efficiently bypass *N*²-dG adducts formed by a major component of tobacco smoke benzo[a]pyrene (BaP-G) [93–95]. Presumably, the selection of the insertor polymerase and the switch from insertor to extender is regulated by protein-

protein interactions involving the accessory domains and subunits in the TLS polymerase structures (Figure 2).

The proposed two-step model [87–90] is consistent with the structure of the Rev1/Pol ζ mutasome as we currently understand it. After ubiquitination of PCNA initiates Rev1/Pol ζ -dependent TLS (Figure 4A), a Y-family “insertor” (Rev1, Pol η , Pol ι , or Pol κ) and the B-family “extender” (Pol ζ) assemble into a complex that enables insertion of a nucleotide opposite the damaged base and the following extension of the distorted primer terminus (Figure 5C). The recruitment of the TLS polymerases to sites of DNA damage and assembly of the TLS complex - the Rev1/Pol ζ mutasome is regulated by a network of protein-protein interactions that are mediated by accessory modules in the TLS polymerase structures (Figure 2, Figure 5A). The central hubs for these interactions are Rev1, through its C-terminal domain (Rev1-CT) and PCNA. Through interactions with Rev1-CT, a Y-family insertor and the B-family extender Pol ζ are brought together to the site of DNA damage to perform their sequential action (Figure 5A). In the remaining sections, this chapter focuses on, where available, the structural elements of the interaction network underlying the two-step Rev1/Pol ζ -dependent TLS and the functional consequences of these protein-protein interactions.

3. Rev1

3.1. Scaffolding function of Rev1

Rev1 is a Y-family DNA polymerase that is found in all eukaryotes, including humans and *S. cerevisiae*. The gene was originally named REV1 in yeast after knockout experiments showed a reversionless phenotype for auxotrophic mutants after treatment with DNA damaging agents [96]. While possessing a catalytic domain (Figure 6A), the enzymatic activity of Rev1 is weak and most studies conclude that Rev1 is restricted to inserting only cytosine opposite a lesion or abasic sites [97]. Alternatively, Rev1 can also replicate across certain DNA structures such as G-quadruplexes [98]. The catalytic activity of Rev1 also appears to be dispensable for survival after treatment with DNA damaging agents in vertebrate cells, although in yeast the loss causes sensitization to certain agents [99–101].

Instead, the primary function of Rev1 is to act as a molecular scaffold through its modular interaction domains and form the core of the Rev1/Pol ζ mutasome (Figure 5A) [38–42]. Rev1 accomplishes this through three types of domains: BRCA1 C-terminal domain (BRCT) [24, 102], ubiquitin-binding motif-1 & 2 (UBM1 and UBM2) [103, 104], and the C-terminal domain (Rev1-CT) (Figure 5A, Figure 6A) [42–50]. Of these three domains, which are addressed in-depth below, Rev1-CT is of particular interest with its two interaction interfaces that can bind both another Y-family polymerase and the Rev7 subunit of Pol ζ simultaneously in both humans and yeast, although more is known structurally about the human proteins [42–50]. In the two-step Rev1/Pol ζ -dependent TLS (Figure 5C), both the Y-family insertor polymerase and the extender Pol ζ will be held in close proximity and exchanged, in part, by the interaction with Rev1-CT [42–50]. Essentially, through the network of Rev1-mediated interactions, the Rev1/Pol ζ mutasome puts all the tools needed in one toolbox and brings it to the job site (DNA damage). How the catalytic activity of Rev1

fits into this model is less clear. It could be an inserter or rarely act. To be sure, the catalytic role of Rev1 should not be dismissed [101].

3.2. BRCT domain – first interaction point for PCNA

BRCT domains are interaction modules that are canonically known for interacting with phosphorylated peptides, but now have a growing number of interaction partners including DNA, poly(ADP-ribose), or nonphosphorylated proteins [105]. Rev1-BRCT is one of the domains in the TLS pathway that has been characterized structurally in both humans and yeast (Figure 6B) [102, 106]. In both cases, the structures show the mixed β/α topology of the BRCT fold [102, 106], which makes this domain unique among the TLS polymerases, as no other Y-family polymerase contains a similar domain (Figure 2). This likely reflects the unique role of Rev1 in TLS as both a polymerase and interaction hub [38–42].

Rev1-BRCT is known to bind both DNA [107] and PCNA [24]. In the case of DNA, a small helix preceding the N-terminus of the BRCT fold is required for binding [107]. Based on NMR titration experiments, Rev1-BRCT/PCNA complex in yeast is moderately weak, with a K_d of 78 μ M however the complex does show a 1:1 (or 3:3) stoichiometry [102]. While the structure of this complex is not available because of the weak affinity impeding crystallization, the binding interfaces for PCNA on Rev1-BRCT and *vice versa* are known from an NMR chemical shift perturbation analysis [102]. Rev1-BRCT site for PCNA is located in the region including loops 1–3 and a part of α -helix 1 (Figure 6B) [102]. PCNA/Rev1-BRCT interaction occurs through the interdomain connecting loop (ICL) interface on PCNA, which overlaps with PCNA site for the PIP-box motifs found in most PCNA interacting proteins, but lacking in Rev1 (Figure 6C) [102].

3.3. UBM1 & UBM2 – second interaction point for PCNA

Rev1 has two ubiquitin-binding motifs (UBMs), although most studies have concluded that only the second UBM (Rev1-UBM2) is able to bind ubiquitin [103, 104]. The function of the UBMs seems obvious: when PCNA is ubiquitinated at sites of DNA damage, Rev1 is recruited through interactions between the Rev1-UBMs and ubiquitinated PCNA in combination with the Rev1-BRCT domain [24]. Before the ubiquitination event, other polymerases may be preferred to Rev1, which only has one interaction point through Rev1-BRCT. After ubiquitination of PCNA (Figure 4A), Rev1 now possesses two points of attachment and is able to bind PCNA with the increased affinity and potentially outcompete other proteins.

Structures of human Rev1-UBM2, alone and in complex with ubiquitin, are available and show the Rev1-UBM2 interacting with an interface centered around L8 on ubiquitin (Figure 6D). This is near the canonical L8/I44/V70 interaction interface on ubiquitin; however, mutation of I44 does not prevent Rev1-UBM2 from interacting with ubiquitin [104]. While most studies show that Rev1-UBM1 does not bind ubiquitin, this is likely because Rev1-UBM1 is marginally stable and only partially folded *in vitro*, based on NMR studies [104].

3.4. C-terminal domain – interaction hub for TLS

3.4.1. Mammalian Rev1-CT—Rev1-CT is a small domain (~100 amino acids) and interaction hub that can simultaneously bind the Rev7 subunit of Pol ζ and one of the three Y-family polymerases (Pol η , Pol ι , Pol κ) (Figure 7A) [42–50]. Mechanistically, the role of this domain in the two-step Rev1/Pol ζ -dependent TLS is to bring together a Y-family polymerase to perform the insertion step and the B-family polymerase Pol ζ to perform the extension step into immediate proximity (Figure 7A). Initially, structural studies on Rev1-CT were most successful using human and mouse proteins, and structures Rev1-CT were determined by NMR (Figure 7B) [46, 47]. These structures show a four-helix bundle with an N-terminal β -hairpin packed against the first two helices (Figure 7B), making this domain somewhat an anomaly as no other Y-family polymerase possesses a similar domain to Rev1-CT (Figure 2). The presence of this domain likely conveys a unique role of Rev1 as a scaffold to mediate assembly of the Rev1/Pol ζ mutasome for TLS.

The first type of interaction mediated by mammalian Rev1-CT occurs with RIR (Rev1-interacting region) motifs, which are found in the unstructured C-terminal regions of all three of the remaining Y-family polymerases, as well as PolD3 subunit of Pol ζ , in addition to other proteins such as XRCC1 and Spartan (Figure 2) [42, 50, 108]. The RIR is a peptide motif that contains a pair of phenylalanine residues in its consensus sequence to mediate the interaction (Figure 7C). Several structures of the Rev1-CT/RIR complex have been determined by NMR (Figure 7D) [46, 47, 50] and show that, during binding, the side chains of the two phenylalanine residues in the RIR motif become buried in an N-terminal pocket on Rev1-CT, while the RIR peptide folds into a small α -helix (Figure 7D, right).

Besides the RIR motif, Rev1-CT also interacts with the Rev7 subunit of Pol ζ (Figure 8A). This interaction occurs through a separate interface from the RIR motif, which allows Rev1-CT to simultaneously bind a Y-family polymerase (through the RIR) and Pol ζ (through Rev7) [44, 45, 48, 49]. X-ray crystal structures are now available for the triple and quadruple complexes of Rev7^{R124A}/Rev3^{RBM1}/Rev1-CT (human) and Rev7^{R124A}/Rev3^{RBM1}/Rev1-CT/Pol κ -RIR (mouse) [44, 45, 48, 49], giving us direct insights into the architecture of the complex that forms the core of the Rev1/Pol ζ mutasome (Figure 8B). As these structures demonstrate, through binding Rev7 and a RIR, Rev1 is able to connect the functions of Pol ζ and the Y-family polymerases.

3.4.2. Yeast Rev1-CT—At the structural level, our understanding of the role of Rev1-CT in yeast has developed less rapidly than in vertebrates. Early studies demonstrated an interaction between Rev1/Pol η and between Rev1/Rev7 and then showed a functional role for scRev1-CT in cell survival after treatment with the DNA-damaging agent methyl methanesulfonate (MMS) [109, 110]; however, a clear RIR motif has not been identified in any of the TLS polymerases in yeast except for one study. Here, the PIP box in Pol η was suggested to bind both PCNA and scRev1-CT *via* a pair of phenylalanine residues at the heart of the interaction [111]. This observation led Washington and colleagues to propose a hypothesis that the RIR motif and the PCNA-binding PIP box are the same motif with overlapping specificities [112], which is yet to be thoroughly tested.

While *bona fide* RIR motifs in yeast may be unknown, Xu *et al.* succeeded in identifying Rad5 as a binding partner for Rev1-CT in yeast, and then used the interaction to stabilize scRev1-CT for X-ray crystallography [113]. This study was both the first to determine the structure of Rev1-CT in yeast, and also to show an interaction between Rev1 and Rad5 (homologue of HLTF/SHPRH in humans), which is known for its function in the error-free template-switching branch of DTT [37]. Although the sequence is different from a canonical RIR motif (Figure 7E), the structure of scRev1-CT in complex with the Rad5 peptide, which contains the phenylalanine pair, demonstrates that Rad5 binds to the same interface on yeast Rev1-CT as the RIR motifs on mammalian Rev1-CT [113]. The relevance of the homologous interaction between HLTF or SHPRH and Rev1-CT in mammals must be explored in the future.

3.4. Functional significance of Rev1 interactions

Studies of Rev1 in cells have been carried out since the gene was first discovered. In *S. cerevisiae*, the mutation G193R was known early on to cause defects in TLS but have no effect on catalysis [38, 114]. The *rev1-1* strain of yeast bearing this mutation displays a marked decrease in mutagenesis and survival after DNA damage [96, 115]. G193 maps onto the scRev1-BRCT domain and is among the most conserved residues in the BRCT superfamily [116]. In the scRev1-BRCT structure, G193 lies in a tight turn between the first α -helix and the following β -strand and mutation of this residue to arginine would result in a side-chain pointing toward the interior of the protein leading to unfolding of the domain and eliminating its interactions [102], with the ensuing consequences on TLS [38]. In humans, deletion of Rev1-BRCT prevents Rev1 from localizing to nuclear foci in unirradiated cells and results in reduced cell viability and altered mutagenesis after treatment with DNA-damaging agents [24]. Similarly, Rev1^{-/-} mouse embryonic fibroblasts (MEFs) complemented with Rev1- BRCT exhibit defects in TLS and the Rev1- BRCT mouse model shows increased sensitivity to DNA damage [114, 117].

Rev1-UBMs are required for the fully functional TLS. For instance, in chicken DT40 cells, deletion of Rev1 results in chromosomal abnormalities that are rescued by complementation with Rev1, but not when a destabilizing mutation is present in Rev1-UBM1 [27]. At the same time, yeast with mutations to Rev1-UBM2 showed increased sensitivity to DNA damaging agents and altered mutagenesis, while mutations to Rev1-UBM1 showed only marginally worse outcomes [27]. Most recently, experiments in yeast focused on Rev1-UBM2 and demonstrated its role in TLS and UV-induced mutagenesis [104].

Considering its role as a bivalent interaction module and scaffold, many studies have focused on Rev1-CT. Overexpression of Rev1-CT in *S. cerevisiae* results in a dominant negative effect on TLS by, presumably, futility occupying binding sites [109, 118]. Disruption of Rev1-CT by mutation also affects survival after treatment with UV light and shows a hypomutable phenotype [109]. In chicken DT40 cells, complementation with human Rev1 is able to rescue hypersensitivity to cisplatin and UV light in a Rev1^{-/-} knockout, while complementation with Rev1 CT is not [99]. Presumably, this phenotype is due to a deactivation of TLS that occurs because Rev1 has lost the ability to interact with Rev7 and another Y-family polymerase through the RIR motif. In another study using

chicken DT40 cells, a Rev1 knockout line was complemented with the K1199E Rev1 mutant deficient in the interaction with Rev7 [45]. As expected, treatment with DNA damaged agents reduced survival when complemented with Rev1^{K1199} versus Rev1^{WT}. In keeping with the role of Rev1-CT as the interaction hub for TLS, Pol κ with F to A mutations in the RIR motif deficient in Rev1 binding was unable to complement the Pol κ ^{-/-} MEFs for survival and mutagenesis after DNA damage [42].

In summary, structural and functional studies highlight the role of Rev1 as a scaffold to assemble the Rev1/Pol ζ mutasome by binding Pol ζ and Y-family polymerases through its C-terminal domain and ubiquitinated PCNA through its BRCT and UBM domains (Figure 5A). These interactions, along with those between subunits of Pol ζ and between PCNA and the Y-family polymerases mediated by PIP-boxes and ubiquitin-binding domains (reviewed below) constitute the network of protein-protein interactions underlying the two-step Rev1/Pol ζ -dependent TLS.

4. Pol ζ

4.1. Architecture of Pol ζ — a multi-subunit extender TLS polymerase

Pol ζ , a B-family DNA polymerase based on the primary sequence, is found in eukaryotes from yeast to mammals and is the only B-family member which primarily functions in TLS [17, 75–78, 119]. Like the other B-family polymerases, including the primase complex Pol α and the two replicative polymerases Pol δ and Pole [1], Pol ζ is itself a protein complex comprising a catalytic subunit (Rev3) and several regulatory subunits (Figure 9A) [17, 75–78, 119]. Although belonging to the same polymerase family, the roles of Pol ζ in mutagenic TLS and Pol α , Pol δ , and Pole in accurate and processive replication of bulk DNA stand in contrast [1, 17].

Although in certain cases Pol ζ exhibits ability as an inserter polymerase [120–123], canonically it is known for its role as an extender polymerase in TLS [87, 88]. In the two-step model of Rev1/Pol ζ -dependent TLS (Figure 5C), after an inserter polymerase (typically Y-family Pol η , Pol ι , or Pol κ) replicates opposite the damaged base, Pol ζ picks up the extension from the mismatched base pair and performs low-fidelity replication to complete the process of TLS (Figure 5C, lower) [87–90]. Viewed from the temporal models of TLS (“on the fly” or postreplication gap-filling) (Figure 4B) the function of Pol ζ is either extension TLS after the insertion step, at which point the bases at the 3’ terminus of the daughter strand now form a Watson-Crick base-pair that can be extended by Pol δ /Pole (on the fly) or, alternatively, to fill in a single-strand gap that is left by Pol δ /Pole before the resulting nick can be ligated by DNA ligase I [10, 11, 67, 79–84].

Architecturally, Pol ζ was long known as a two-subunit complex of Rev3 and Rev7, with Rev3 containing the catalytic domain and Rev7 acting as an interaction module [120]. The first binding site for Rev7 on Rev3 (RBM: Rev7-binding motif; Pxxx(A/P)P) was identified in humans and a crystal structure of the complex of Rev7/Rev3-RBM1 was determined [124, 125]. Subsequently, the Wood laboratory discovered a second Rev7 binding motif in human Rev3 (RBM2) suggesting a 2:1 stoichiometry for the Rev7/Rev3 interaction [119], and the structure of the Rev7/Rev3-RBM2 complex was solved [78]. In yeast, precise Rev7-

interacting Rev3-RBM motif(s) have not yet been identified, although their location has been narrowed down to a region of ~250 amino acids [120, 126]. The structures of all other domains of Rev3, including the catalytic domain, are unknown in both humans and yeast and would be of interest to the field. Recent studies have shown that Pol ζ contains two additional subunits, PolD2 and PolD3 (Pol31 and Pol32, in yeast), that are also the accessory subunits of the replicative B-family polymerase Pol δ [17, 75–78, 127]. These subunits interact with Rev3 through the Fe-S cluster in its C-terminus (Rev3-CTD) similar to that found in Pol δ (Figure 9A) [75–77, 127]. This immediately leads to a potential mechanism for the switch between the replicative and TLS polymerases through sharing a common interaction partner – the two accessory subunits of Pol δ and Pol ζ [17, 75–77], although the finding that yeast Pol ζ may exist as a four-subunit complex through all phases of the cell cycle argue against polymerase switch by direct sharing of the subunits [127]. Overall, with identification of the two binding sites for Rev7 on human Rev3 (RBM1 and RBM2) and discovery of the two additional subunits, PolD2 and PolD3, human Pol ζ can be described as a five-protein Rev3/Rev7₂/PolD2/PolD3 complex, while stoichiometry of yeast Pol ζ is less certain.

In an attempt to study the architecture of Pol ζ , Aggarwal's group generated a low-resolution model of the four-subunit yeast Pol ζ (Rev3/Rev7/Pol31/Pol32) complex [128]. First, the subunits Rev7, Pol31, Pol32 were modeled based on homology with their human counterparts and the catalytic domain of Rev3 and Rev3-CTD were modeled using yeast Pol δ . Then, the Pol ζ components were fit together using the electron microscopy data, revealing a bilobal architecture of the Pol ζ complex in which the catalytic and accessory subunits form the two distinct modules (Figure 9D) [128]. Note that the model was built assuming that the Pol ζ complex in *S. cerevisiae* contains only one copy of Rev7, contrary to what is now known about Pol ζ in humans [78, 119]. Since this work was published, single-particle cryoEM has undergone explosive advancement, suggesting that cryoEM may be a viable strategy to study Pol ζ and other protein complexes involved in TLS in the future.

4.2. Rev3: the catalytic domain

Rev3 is found in both mammals and yeast and contains the catalytic polymerase domain of Pol ζ [120]. It was first identified and named based on genetic studies in yeast (similarly to Rev1) where deletion of Rev3 resulted in a hypomutable phenotype after treatment with UV light or chemical agents [96, 129]. These initial studies showed that Pol ζ is responsible for ~96% of UV-induced mutagenesis and ~50% of spontaneous mutagenesis in yeast [96, 129]. Thus, although Pol ζ is a B-family polymerase, which tends to work with high fidelity and processivity, its activity is mutagenic and Rev3, overall, is responsible for a significant fraction of the total mutagenesis in a cell's lifetime [96, 129]. While the sequence and architecture of the catalytic domain classify Rev3 as a B-family polymerase, little is known structurally about Rev3.

While most TLS polymerases are part of the Y-family and have active sites that are designed to accommodate bulky templates, Rev3 is a B-family polymerase based on sequence homology and shows little ability in vitro to replicate damaged DNA [1, 10, 17]. Whether the structure of the catalytic domain in Rev3 resembles the more restrictive architecture like

other B-family member or whether the active site is more malleable are unknown, because crystal structures are not available for the catalytic domain in either mammals or yeast.

One known difference with the other B-family polymerases is in the proofreading activity. An analysis of the sequence suggests that critical residues are missing in Rev3, while biochemical experiments on the catalytic domain show 3'–5' proofreading activity is not present [120]. With respect to the two-step mechanism of Rev1/Pol ζ -dependent TLS, the proofreading function of Rev3 must be inactive for proper execution of the TLS pathway because Pol ζ , by definition of its extender role, will always be performing DNA replication after a mismatched base-pair (Figure 9B). If the proofreading domain were active, the nucleotide that was just added during the insertion step of TLS would be detected as a mismatch and excised, causing TLS to enter a futile cycle of inserting and removing at the site of the lesion (Figure 9B). In short, the catalytic domain of Rev3 appears to be specially adapted to accept the 3' -OH from a distorted base pair into its active site for extension [120].

4.3. Rev3: Fe-S cluster and interaction with POLD2/POLD3 (Pol31/Pol32)

Like the other B-family polymerases, Rev3 contains a metal-binding cysteine-rich motif in its C-terminus (Rev3-CTD) [130]. Based on secondary structure prediction, this region in Rev3 most closely resembles the C-terminal domain in Pol δ compared to the other B-family polymerases (Pol α and Pol ϵ) [75]. This led to the discovery that Rev3 shares the PolD2 (Pol31 in yeast)/PolD3 (Pol32 in yeast) subunits with Pol δ through an interaction between Rev3-CTD and PolD2/PolD3 (Figure 9A) [75–77, 127]. Structurally, this C-terminal Fe-S cluster contains two metal-binding sites called CysA and CysB, which, in Rev3 and Pol δ , bind zinc and iron, respectively [130], although high-resolution structures are not available for these domains.

The subunits that bind to Rev3-CTD, PolD2 (yeast Pol31) and PolD3 (yeast Pol32), are regulatory proteins that were commonly known as the B- and C-subunits of Pol δ [1]. They interact with each other through two folded domains, and a structure of this complex is available in humans (Figure 9C) [131]. Besides N-terminal domain that binds PolD2, the PolD3 subunit also possesses an unstructured C-terminus that contains a PIP box and RIR motif (Figure 9A) to bind PCNA and Rev1-CT, respectively [50]. In yeast, the interaction between Rev7 and Pol32 has also been shown to contribute to additional stabilization of the Pol ζ complex [128]. Otherwise, neither PolD2/PolD3 nor Pol31/Pol32 possess any catalytic activity, suggesting their function is solely in facilitating protein-protein interactions.

From a mechanistic standpoint, the interactions mediated by PolD2 and PolD3 in humans or Pol31 and Pol32 in yeast may contribute to the layered regulation that governs a pathway as critical as DNA replication. For instance, the interaction between PolD2/PolD3 and Rev3 could provide a link in the switch from normal replication by Pol δ to TLS through the displacement of the PolD1 (the catalytic subunit of Pol δ) in favor of the Rev1/Pol ζ mutasome at sites of DNA damage [75–77, 127]. At the same time, the presence of an RIR motif in PolD3 in humans may point to a mechanism for the inserter to extender polymerase switching in the two-step model of Rev1/Pol ζ -dependent TLS (Figure 5C) [50]. Once the insertion step is complete, the RIR motif from PolD3 exchanges with the RIR motif from the

inserter polymerase and binds to Rev1, thereby displacing the inserter polymerase from the replication fork and allowing Pol ζ to carry out its extension step (Figure 9E) [50].

4.4. Other regions in Rev3: NTD, PCD and RBMs

Besides the catalytic domain and CTD, yeast and mammalian Rev3 also contain several conserved regions downstream of the catalytic domain, which are involved in protein interactions and/or other functions, in addition to also showing differences (Figure 2). Given the known difficulty of working with Pol ζ in vitro, the most extensive biochemical analysis of human Rev3 (and Pol ζ) was carried out by Yang's group after the discovery of the additional PolD2/PolD3 subunits [77]. In this study, sequence alignments of the two species and structural predictions were used to guide the design of a set of minimal functional units of Rev3, which were assessed by in vitro DNA polymerase assays [77]. Human Rev3 is over twice as large as Rev3 in *S. cerevisiae* (3130 vs 1504 amino acids), which was used to the researchers' advantage as they assumed that yeast Rev3 contains the minimal domains required for full activity and then identified the "extra" regions in human Rev3 that were not present in yeast.

Structurally, this study revealed which regions are indispensable for Pol ζ activity and also suggests that the additional amino acids in mammalian Rev3 are primarily unstructured, although this does not mean they are nonfunctional [77]. To maintain optimal enzymatic activity of Pol ζ , the N-terminal domain (NTD), a positively-charged domain (PCD), and the Rev7-binding motifs (RBMs), as well as C-terminal Fe-S cluster must be present in Rev3 (Figure 2), although how the Rev3-NTD and PCD contribute to the function of Rev3 and Pol ζ is not completely clear [77]. While the interaction of Rev3-RBMs with human Rev7 has been studied in the greatest depth (Figure 9A, Figure 10), further investigation into the structure and function of other domains/regions of Rev3 would strengthen our understanding of Pol ζ and TLS.

4.5. Rev7 – a universal interaction module

Rev7 is a small (~210–240 amino acids, depending on species) protein whose size belies its versatility [132, 133]. As discussed above, Rev7 functions as a subunit of Pol ζ and interaction module in TLS by binding both Rev3 and Rev1 (Figure 9A) [43–45]. In doing so, it acts as a bridge between the inserter and extender polymerases in the two-step model of Rev1/Pol ζ -dependent TLS (Figure 5C) [87–90].

Structurally, Rev7 belongs to the HORMA (Hop1, Rev7, Mad2) domain family [134] and has no other homologues in TLS or DNA replication in general. HORMA domain proteins, including Rev7, contain three helices packed against an antiparallel β -sheet at the core [125, 135] and are known to act as protein interaction modules (Figure 10A). HORMA domains are marked by a number of distinguishing features including (i) the "safety belt" binding mechanism in which the ligand binds underneath the safety belt loop (Figure 10A), (ii) a binding-induced conformational change from an "open" to "closed" state, and (iii) homodimerization and heterodimerization with other HORMA domains [133–138].

Several crystal structures of human Rev7 in complex with Rev3-RBM1 and -RBM2 are available and show the canonical HORMA fold and binding by the "safety belt" mechanism,

in which a Rev3-RBM peptide is enveloped by the Rev7 safety belt loop (Figures 10A,B) [78, 125]. While Rev7 forms a dimer like most HORMA domains [124], a crystal structure of the Rev7 dimer is unavailable because, most likely, the moderate affinity of the dimer (~2–13 μ M) prevented crystallization due to monomer-dimer equilibrium [78, 124]. These issues led Hara et al. to use a mutation, R124A, to break the dimer and determine the first structure of Rev7 in complex with the Rev3 fragment in a monomeric state (Figure 10A) [124, 125]. The binding interface for Rev1-CT is located in the two C-terminal β -strands formed in the closed conformation of Rev7 induced by binding Rev3 (Figure 10A, left). Because this site is not correctly formed in an open HORMA structure [44, 45, 135–137], one can imagine how a tighter level of regulation might be achieved if the interaction between Rev7 and Rev1 can only occur temporally after Rev7 forms a complex with Rev3 and enters the closed conformation [44]. To date, the structure of yeast Rev7, which is ~30 amino acids (~15%) larger than human Rev7, has not been determined.

As noted above, human Rev7 can form a dimer [124], and because both subunits of the Rev7 dimer are held in proximity by the two RBMs on Rev3, this dimer is effectively tethered together by Rev3 (Figure 9A) [78]. In lieu of a crystal structure, the human Rev7 dimer was modeled using a combination of crystallography, mutagenesis, small-/wide-angle X-ray scattering (SAXS/WAXS), and docking (Figures 10C,D) [78]. Perhaps unexpectedly, despite the presence of two Rev7 in Pol ζ , only a single copy of Rev1 is able to bind the Rev7 dimer based on the orientation of the binding interfaces for Rev1-CT on Rev7 - once the first copy of Rev1-CT binds, the second binding site on the Rev7 dimer is occluded by the first copy (Figure 10E) [78]. In yeast, the dimerization of Rev7 has not been studied directly; however, the electron microscopy model of yeast Pol ζ does not contain any obvious extra density that would correspond to a second copy of Rev7 in the complex (Figure 9D) [128].

4.6. Functional significance of interactions between Pol ζ subunits

Similar to REV1, mutations in REV3 and REV7 genes encoding the subunits of Pol ζ demonstrated UV sensitive nonmutable phenotype in the original screens for reversionless mutants in *S. cerevisiae* [96, 139]. The *in vitro* assays have shown that catalytic activity of Rev3 is significantly (20–30 fold, in yeast) enhanced in the presence of Rev7 [120], while assembly of the complex between Rev3/Rev7 and PolD2/PolD3 or Pol31/Pol32 and interaction with PCNA stimulated Pol ζ activity even further (2–5 fold in yeast; 30 fold in humans) [77, 127]. These results highlight the importance of protein-protein interactions that mediate assembly of the multi-subunit Pol ζ complex. As expected, most studies generally indicate that knockout of the catalytic subunit Rev3 or deletion of the domains in Rev3 responsible for protein-protein interactions lead to nonmutable DNA damage sensitive phenotype that is marked by genomic instability [10, 17]. Consistent with the role of Pol31/Pol32 as accessory subunits of yeast Pol ζ , mutations in the CysB Fe-S cluster or deletion of Rev3-CTD that abolish interaction between Rev3 and Pol31 caused decrease in DNA damage induced mutagenesis [75, 76, 127, 140]; however, Rev3 CTD strain of yeast still displayed reduced but significant mutagenesis [140], pointing to additional interactions between Rev3/Rev7 and Pol31/Pol32 subunits contributing to stabilization of the Pol ζ complex (e.g. between Rev7 and C-terminal part of Pol32 [128]).

The essential regulatory subunit of Pol ζ - Rev7 is required for DNA damage resistance and mutagenesis in both mammalian cells and yeast [120, 141]. Complementation experiments in Rev3^{-/-} MEFs [142] demonstrated that both Rev7 binding motifs in Rev3 (RBM1 and RBM2) are required to confer resistance to UV and cisplatin DNA damage, highlighting the importance of Rev3/Rev7 interaction for Pol ζ function [119]. In addition, an intact Rev7 dimerization interface is required for resistance to cisplatin in mouse cells, however, the mechanistic basis for this is not clear [78]. One explanation is that loss of the Rev7 dimer leads to inactivation of TLS. However, besides TLS, Rev7 is also involved in other pathways including mitotic checkpoint signaling by contributing to the regulation of the anaphase-promoting complex/cyclosome (APC/C) [143] and the repair of double-strand breaks [144, 145]. Therefore, Rev7 could be interacting with proteins from other pathways through its dimerization interface [78] and thus the phenotype may reflect a loss of those interactions rather than a deactivation of TLS.

5. Pol η , Pol ι , and Pol κ

5.1. Interaction domains of the inserter Y-family polymerases

The three Y-family polymerases that perform the insertion step during Rev1/Pol ζ -dependent TLS are Pol η , Pol ι , and Pol κ , although Pol ι and Pol κ are absent *S. cerevisiae*. Each of these TLS enzymes is designated to replicate over certain types of DNA lesions [146], with the most prominent example provided by Pol η accurately bypassing UV-induced T-T dimers [18–22]. However, the mechanism of lesion discrimination and selection of an appropriate inserter polymerase still remains elusive — whether it be the conformational selection of the lesion in the active site of the enzymes, signaling through protein-protein interaction domains, or other mechanisms [146]. Structurally, the inserter Y family polymerases Pol η , Pol ι and Pol κ are smaller than Rev1 (~700–900 vs 1251 amino acids, in humans) and show the same general architecture with the catalytic domain followed by a C-terminal region containing modular interaction domains and motifs [10–17] (Figure 2). Human Pol η , Pol ι and Pol κ contain at least one RIR motif to bind Rev1-CT (while RIR is lacking in *S. cerevisiae* Pol η ; see section 3.4. on Rev1-CT/RIR interaction), PIP box motif(s) to bind PCNA, and one or more ubiquitin-binding (UBM or UBZ) modules. These interaction domains presumably contribute to the regulated assembly and recruitment of the Rev1/Pol ζ mutasome (Figure 5A).

5.2. PIP box – interaction point for PCNA

A common structural element of the inserter TLS polymerases Pol η , Pol ι and Pol κ , the PIP box, is a peptide motif that interacts PCNA at the IDL region (Figure 11A, 11B) [147, 148]. This is the same site on PCNA where the Rev1-BRCT domain binds, suggesting a potential for a competitive interplay between the two domains [102], although the PCNA trimer does contain three binding sites and can potentially accommodate up to three binding partners (Figure 11B).

The canonical PIP box sequence Qxx(L/I/M)xx(F/Y)(F/Y) contains a conserved pair of aromatic residues that insert into a pocket on PCNA surface, a hydrophobic residue, and glutamine that binds to another site (Q-pocket) in the ICL region of PCNA (Figure 11A).

[147, 148]. One or several PIP box motifs were found in each of the Y-family inserter TLS polymerases that deviate from canonical sequence having amino acid substitutions at the first and/or last positions [149] (Figure 11A). Thus, three PIP box motifs were identified in human Pol η with one of the motifs, Pol η -PIP3 (residues 475–486), sharing the conserved FF pair with Pol η -RIR that mediates interaction with Rev1 (Figure 11A, Figure 7C). Human Pol ι and Pol κ were reported to have one and two PIP-box motifs, respectively. Crystal structures of human PCNA in complex with Pol η -PIP2, Pol ι -PIP, and Pol κ -PIP2 peptides have been determined, providing a rationale for the relatively weak PCNA binding affinities for noncanonical PIP-box sequences [148] (Figure 11C), while no structure has been reported to date for the PCNA/Pol η -PIP complex in yeast.

5.3. Ubiquitin-binding motifs & zinc fingers

All three inserter Y-family polymerases contain domains that bind ubiquitin. These domains are responsible for the interactions that recruit these polymerases to sites of DNA damage through binding ubiquitinated PCNA [30–32]. The C-terminus of Pol η contains one Type-III C₂H₂ UBZ (ubiquitin binding zinc-finger) domain (Figure 12A). Like most DNA-binding and ubiquitin-binding zinc fingers, Pol η -UBZ has the canonical $\beta\beta\alpha$ fold and coordinates a zinc ion in the core of the protein [25, 150–152]. Based on homology between the sequence of the helix in the Pol η -UBZ and other single-helix motifs that interact with ubiquitin such as the UIM (motif interacting with ubiquitin) or inverse-UIM and NMR chemical shift perturbation data Bomar *et al.* created a model of the human Pol η -UBZ/ubiquitin complex (Figure 12A) [151]. Like most ubiquitin-binding domains, Pol η -UBZ interacts with ubiquitin through the conserved L8/I44/V70 hydrophobic face, while on the Pol η -UBZ side, the interaction with ubiquitin occurs through a surface on the helix centered around residues D652 and A656 (Figure 12B).

Human Pol κ contains two UBZ domains (UBZ1 and UBZ2) between residues ~621–802. As of this time, no crystal or NMR structures of either UBZ is available, however based on sequence homology with the UBZ domain from Rad18, both UBZ domains in Pol κ are Type-IV UBZs, as opposed to the Type-III UBZ in Pol η (Figure 12E, Figure 12F) [151, 152]. The hallmark of the UBZ-IV domains are the hydrophobic residues in and around strand- β 1 and the first zinc-coordinating residue, whereas in Type-III UBZ domains, the analogous residues are positively charged (Figure 12E). The effect is to rotate the binding site for ubiquitin around the helical axis from the outer face of the helix in Type-III domains to the side including strand- β 1 in Type-IV (Figure 12A vs 12F). The presence of two types of UBZ domain with different binding modes of ubiquitin binding in Pol κ and Pol η may provide a potential molecular basis for selective recruitment of one inserter polymerase over another.

The C-terminus of human Pol ι contains two ubiquitin-binding motifs (UBM) (Figure 12C). The UBM is a small helix-turn-helix module that borders on the minimum number of amino acids required to autonomously fold a domain (Figure 12C) [103, 153, 154]. Structures of Pol ι -UBMs alone and in complex with ubiquitin are available and indicate that these domains also bind to the hydrophobic interface centered at L8, I44, V70 (Figure 12D) [103, 153, 154].

5.4. Functions of the interaction domains of the inserter TLS polymerases

Pol η , Pol ι and Pol κ function as inserter polymerases in the two-step model of Rev1/Pol ζ -dependent TLS. While they contain a number of modular domains in their structures to interact with PCNA, ubiquitin, and Rev1-CT, these domains are believed to contribute to polymerase recruitment and/or switching, rather than act as scaffolds like multivalent interaction modules such as Rev1-CT and Rev7 [10–17]. Most generally, PIP-box motifs and ubiquitin binding (UBM and UBZ) domains of the Y-family TLS polymerases control localization to sites of DNA damage and stimulate DNA synthesis in the presence of ubiquitinated PCNA, and thus confer resistance to DNA damaging agents [149, 155–158]. Ubiquitin-binding domains enhance the affinity of Y-family polymerases to monoubiquitinated PCNA and are thought to play a role in replicative to TLS polymerase switching [25, 26, 30–32]. On the other hand, the Rev1-interacting RIR motifs in the C-terminal parts of Pol η , Pol ι and Pol κ control association of the inserter Y-family polymerases with the Rev1/Pol ζ mutasome, and were suggested to play a role in the inserter to extender polymerase exchange during the Rev1/Pol ζ -dependent TLS [50] (reviewed in section 3.4).

6. Inhibition of TLS as a therapeutic strategy

6.1. TLS in the clinic

Normally, allowing cells to tolerate DNA damage with low-fidelity TLS has a net benefit because mutagenic replication is preferable to fork collapse, incomplete replication, or chromosomal instability. For instance, loss of proteins involved in TLS can lead to diseases that exhibit DNA instability such as Fanconi anemia or a variant of Xeroderma pigmentosum (XP-V) [18, 20, 159]. The TLS polymerases also contribute to immune function by introducing mutations in the variable regions of immunoglobulin genes (somatic hypermutation) for adaptation to new antigens [100, 160–165].

For cancer patients, the benefits of TLS become complicated because many first-line cancer treatments such as platinum-based chemotherapeutics (cisplatin, carboplatin, oxaliplatin) act by binding or modifying DNA and blocking replication, leading to apoptosis of rapidly dividing cancer cells and remission [53, 54]. As TLS permits normal cells to bypass and survive uninvited damage to DNA, TLS also allows cancer cells to survive chemotherapy [51, 52]. Furthermore, the increased mutagenesis that is introduced by widespread TLS activity is believed to contribute to tumor heterogeneity, the onset of acquired chemoresistance, and secondary malignancies [55, 56]. This mechanism would suggest that inhibition of TLS in combination with first-line genotoxic chemotherapy may improve outcomes for cancer patients by preventing DDT systems from rescuing cancer [56–58]. This hypothesis was borne out in several studies, where knock out or knock down of genes involved in Rev1/Pol ζ -dependent TLS was shown to increase the sensitivity of cancer cells to genotoxic chemotherapy and to delay the development of drug resistance [56–61]. For a more thorough treatment on the therapeutic potential of TLS inhibition, we point readers to recent reviews [62–66].

6.2. Direct inhibitors

Targeting the enzymatic activity of TLS polymerases with small molecules represents the most obvious strategy for TLS inhibition. Given the similarity of the catalytic mechanism among the DNA polymerase families, the selectivity of the inhibitors must be a primary concern. Several groups have attempted to develop catalytic inhibitors of the TLS polymerases, however, these efforts have not led to the level of selectivity that would be necessary to move into the clinic [166, 167]. Still, the specificity of individual TLS polymerases for certain types of DNA damage and the inability of the replicative B-family polymerases to accommodate DNA lesions in their active sites suggests there might be some leeway at the molecular level that will support selectivity. Certainly, the Y-family and B-family polymerases have structurally diverse active sites to some extent (Figure 3) [14, 15, 72], suggesting that the development of catalysis TLS inhibitors represents a viable strategy that should not be dismissed.

Targeting the signaling enzymes upstream of TLS provides another promising strategy for the development of TLS inhibitors. In this vein, inhibitors of the E2 enzyme Rad6 have been developed that prevent ubiquitination of PCNA and recruitment of the TLS polymerases to the site of DNA damage [168]. In a cancer cell model, these inhibitors increase sensitization to platinum-based anticancer drugs [168].

6.3. Protein-protein interaction inhibitors

As outlined above, the TLS polymerases are regulated by a network of protein-protein interactions (PPIs) that control recruitment to DNA when damage is present and mediate assembly of the multiprotein TLS complex (Figure 5A). Thus, disrupting essential PPIs between modular domains and subunits of TLS polymerases with small molecule inhibitors provides a promising strategy to deactivate TLS when undergoing chemotherapy [62–66].

Normally, targeting protein interaction interfaces is considered difficult because the surfaces tend to be flat and featureless (“undruggable”) compared to the deep pockets in enzymatic active sites, which renders PPI interfaces unable to support the kind of binding energy required for small molecule inhibition [169–171]. Fortunately, several essential PPIs within the Rev1/Pol ζ mutasome involve aromatic amino acid side-chains that insert into relatively deep pockets on their complementary surfaces that are more amenable to targeting with small molecules (Figure 13A) [172–174].

Most attempts to inhibit assembly of the Rev1/Pol ζ mutasome have focused on Rev1, given its role as a central TLS scaffold and, more specifically, on the interaction between Rev1-CT and the RIR motif (Figure 13A) [172–174]. This interaction is mediated by an FF pair on the RIR motif where one side chain inserts into a pocket on Rev1-CT in an orthogonal orientation with respect to the binding surface (Figure 13A). In a series of three papers, this interaction interface was targeted by small molecules identified by high-throughput screens, virtual screens and modeling, which block the Rev1-CT binding site for the RIR motif. These inhibitors were shown to sensitize cancer cells to cisplatin and reduce cisplatin-induced mutagenesis consistent with deactivation of TLS [172–174]. The Rev1-CT/Rev7 interface also contains druggable pockets (Figure 13B) [62], and a small molecule inhibitor

has been recently reported that binds Rev1-CT, induces its dimerization and blocks the Rev1-CT/Rev7 interaction [175].

Besides the interactions involving Rev1 and Rev7, a number of other interactions in the TLS pathway are either legitimate targets or have been targeted before. For instance, inhibitors of the PPI between Rev1-UBM and ubiquitin have been developed, and also demonstrated an anti-TLS activity in cells [176]. The interaction between PCNA and the PIP box is similarly mediated by a pair of aromatic (F or Y) side chains and which inserts into a pocket on PCNA surface (Figure 13C), and small molecule inhibitions of this PPI have been reported [177–180].

Considering the number of PPIs and interaction domains involved in TLS (Figure 2, Figure 5A), only a few have been analyzed for their potential as a therapeutic target. Given the number of recent reviews [62–66], interest in this topic is growing exponentially.

7. Concluding remarks

TLS is a contingency plan that executes when DNA damage is not repaired prior to replication. At this point, the cell tolerates the damage by replicating over it to pass on a complete genome to its progeny (Figure 4B, Figure 5C). In most cases, TLS is performed by the Rev1/Pol ζ mutasome, which is a protein complex whose catalytic subunits are optimized to perform two-step TLS over lesions in DNA (Figure 5A, Figure 5C). As a result of this ability to replicate across sites of DNA damage, the TLS polymerases exhibit low fidelity when replicating undamaged DNA and must be kept away from DNA under normal circumstances. For this purpose, the recruitment of the TLS polymerases is regulated by ubiquitination of PCNA and the resulting network of protein-protein interactions (Figure 4A, Figure 5A).

Many of these interactions have been characterized at the atomic level. For example, we now know that an interaction network exists such that the TLS polymerases can assemble into a protein complex on ubiquitinated PCNA comprising, at least, Rev1, Pol ζ , and another Y-family polymerase, and we understand the structural basis for the majority of interactions that mediate assembly of such complex (Figure 5A). From a practical standpoint, the design of anticancer therapeutics that target essential PPIs in the TLS pathway can benefit from the combination of high-resolution structural information and understanding the interactions that lead to TLS (Figure 13). As structural biology techniques advance, in particular, cryoEM, we expect that structures of more and larger complexes will become available. The greater understanding of the structures and interactions that facilitate TLS has provided new insights into TLS and DDT.

This wealth of structural information also synergizes with the revolution in CRISPR-based genetic manipulation by guiding the design of in vivo or cell-based experiments and assays that knock out a gene and then complement with a copy that contains a mutation to the binding interface, while otherwise keeping the protein folded. Such fine-grained analyses that allow narrow conclusions about specific interactions may now help uncover unknown

features of TLS or links between TLS and other pathways which may have been obscured in previous experiments that took a more ham-handed approach.

In total, the structures and interactions that lead to assembly and recruitment of the Rev1/Pol ζ mutasome have been studied in detail since the initial discovery of TLS, although surely much remains to be unearthed.

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Figure 1. Semiconservative DNA replication by Pol δ and Pol ϵ . **(A)** Canonical replication by the replicative polymerases Pol δ and Pol ϵ . **(B)** Pol δ and Pol ϵ are unable to replicate damaged DNA. Instead, a group of specialized polymerases are recruited to perform translesion synthesis (TLS).

Figure 2.
The protein subunits of the Rev1/Polζ mutasome in humans and *S. cerevisiae* yeast.
Domains with known three-dimensional structure are marked by ‘*’.

Figure 3.

Structural comparison of the *S. cerevisiae* B-family polymerase Polδ in complex with undamaged DNA (PDB: 3IAY) and the Y-family polymerase Polη in complex with damaged (PDB: 3MFH) and undamaged DNA (3MFI). The Y-family polymerases grasps the DNA (black) less closely than the B-family polymerase, which allows the Y-family polymerase to tolerate damaged DNA and perform TLS.

Figure 4.

Recruitment and mechanism of TLS polymerases. (A) RPA coats single-strand DNA at sites of DNA damage where the replicative polymerase stalled. This recruits the Rad6/Rad18 complex to ubiquitinate PCNA to signal for TLS. Subsequent polyubiquitination leads to an alternative template-switching branch of DDT. (B) Two possible mechanisms for TLS: the "on the fly" model where the TLS polymerases exchanged with the replicative polymerases during replication (left) or the postreplication gap-filling model where the TLS polymerases are recruited to single-strand gaps that were left after replication of the genome is otherwise complete (right).

Figure 5. Interactions and mechanism of TLS. (A) Model of the minimal unit of the Rev1/Polζ mutasome based on known interactions. (B) Nuclear foci containing Rev1 forms after treatment with UV light. Adapted from Guo *et al.* (2006) [24]. (C) When a replicative polymerase encounters DNA damage, it failed to perform replication (top). The TLS polymerases then perform two-step Rev1/Polζ-dependent translesion synthesis.

Figure 6. Rev1 and interactions with PCNA. (A) Domains and motifs in human Rev1. (B) Overlay of the structures of human and yeast Rev1-BRCT domain (PDB: 2EBW, 2M2I, 4ID3, 5UMV, 5VX7). (C) Structure of human PCNA (PDB: 1AXC). The interdomain connecting loop (ICL) where the Rev1-BRCT and PIP-box interact is highlighted. (D) NMR structure of the Rev1-UBM2 in complex with ubiquitin (PDB: 6ASR, 6ASD).

Figure 7.

Rev1-CT and the RIR motif. (A) Schematic of the interactions at the heart of the Rev1/Pol ζ mutasome. The arrow highlights the Rev1-CT/RIR interaction. (B) NMR structures of human and mouse Rev1-CT. (PDB: 2LSY, 2LSG) (C). Sequence alignment of RIR motifs (human unless noted; 'h' – helix forming residue, 'n' – N-capping residue). (D) NMR structure of human Rev1-CT in complex with a RIR motif from Pol η . (PDB: 2LSK). Right: close-up of the Rev1-CT/RIR binding interface. F531 and F532 form the core of the RIR motif. (E) Crystal structure of yeast Rev1-CT in complex with a motif from Rad5 (PDB: 5YRQ). Adapted from Xu et al (2016) [113].

Figure 8. Rev1-CT/Polζ/RIR complex. (A) Interactions at the heart of the Rev1/Polζ mutasome. The arrow highlights the Rev1-CT/Rev7 interaction. (B) Crystal structures of protein complex at the core of the Rev1/Polζ mutasome: Rev1-CT/Rev7/Rev3-RBM1/Polκ-RIR (PDB 4FJO) and Rev1-CT/Rev7/Rev3-RBM1 (PDB: 3VU7).

Figure 9.

Polζ. (A) The five-protein Rev3/Rev7₂/PolD2/PolD3 Polζ complex (green) bound to Rev1-CT (blue). (B) The structure of the replication fork as Polζ is beginning the extension step of TLS. If the 3'–5' proofreading activity of Polζ was active, then the nucleotide which was just added during the insertion step of TLS would be removed and TLS might enter an infinite loop of inserting and excising. (C) Crystal structure of the human PolD2/PolD3 complex (PDB: 3E0J).

(D) Electron microscopy reconstruction of yeast Polζ. Adapted from Gómez-Llorente et al. (2013) [128]. (E) Mechanism of the insert to extender TLS polymerase switch. The RIR motif in PolD3 replaces the Y-family inserter polymerase at the site of DNA damage to complete TLS by performing the extension step.

Fig. 10. Rev7. (A) Structure of the human Rev7^{R124A} monomer in complex with Rev3-RBM1 or -RBM2 motifs. The structure is colored on a continuum that ranges from blue at the N-terminus to red at the C-terminus. The box surrounds the region highlighted in (B) (PDB: 6BI7, 6EKM, 6BCD, 6BC8, 5O8K, 3ABD, 3ABE). (B) Zoomed-in view of the Rev7/Rev3 interaction interface (PDB: 3ABD). (C) Model of the Rev7 dimer (Accession: PDB-Dev_000000009). (D) Close-up of the Rev7 dimerization interface from the model in (C). (E) Despite the presence of two copies of Rev7 in the Polζ, the complex only contains a single Rev1 based on the geometry of the binding sites for Rev1-CT in the Rev7 dimer. Compare the location of the interface on the C-terminal β-strands (deep red) in (A) to their location in (C). The interfaces point inward toward each other.

Figure 11.

PIP box interaction with PCNA. (A) Sequence alignment of a canonical PIP box from p21 and the PIP box motifs from the TLS polymerases (h – hydrophobic residue L/I/M). (B) Structure of PCNA (grey) in complex with the PIP box motif (colored) (PDB: 2ZVK). The PIP box motif and Rev1-BRCT bind to the same site in the interdomain-connecting loop region of PCNA. (C) Close-up view of the PCNA/PIP box complex that highlights the FF pair in the PIP box from Pol η (PDB: 2ZVK).

Figure 12.

Interaction modules of the Y-family inserter polymerases. (A) Model of the complex between the Polη-UBZ domain (colored) and ubiquitin (grey). (B) Close-up view of the Polη-UBZ/ubiquitin complex that highlights the critical residue D652. (C) Structures of the complex between the Polι-UBM domains (1 or 2) and ubiquitin (PDB: 3KWV, 2KTF, 2KWV, 2KHW) (D) Close-up view of the Polι-UBM/ubiquitin complex (PDB: 3KWV). (E) Sequence alignment of the type-IV UBZ domain from Rad18 and the UBZ domains from Polη (type-III) and Polκ (type-IV). (F) NMR structure of the Rad18/ubiquitin complex (PDB: 2MRE).

Figure 13.

Protein interaction interfaces that may be targets for TLS inhibition. (A) The Rev1-CT/Polη-RIR complex (PDB: 2LSK) (B) The Rev1-CT/Rev7 complex (PDB: 3VU7) (C) The PCNA/PIP box complex (PDB: 2ZVK). Adapted from Korzhnev and Hadden (2016) [62].